Andreas Sing *Editor*

Zoonoses - Infections Affecting Humans and Animals

Focus on Public Health Aspects

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Preface

Zoonoses are infectious diseases caused by microorganisms passing from animals to humans and vice versa. In the last few decades most emerging and re-emerging diseases were in fact either of zoonotic origin or zoonotic potential.

The term "zoonosis" was coined by the German physician Rudolf Virchow, mainly known as father of scientific pathology, but also as an important political figure in nineteenth century Germany. Although rooted in a classical faculty-based university system, he and his Canadian disciple William Osler, also a physician by training, very early recognized the need for interdisciplinary collaboration between human and veterinary medicine and also—probably even more importantly—the public health, social and political aspects of zoonotic diseases. While the scientific basis for both of them was pathology, the rise of microbiology as a medical discipline allowed to put the focus on microorganisms as the obvious and easiest walkable bridge between human and animal infectious diseases. This is even more true since the advent of especially DNA-based typing techniques for analyzing microorganisms isolated from different species thus allowing to study their real zoonotic potential.

By incorporating life and social science subdisciplines (e.g. immunology or epidemiology) a systemic paradigm was introduced in medical science thus preparing the ground for inter- and transdisciplinary approaches both in human and veterinary medicine. A striking example for the consequences of this paradigm shift on a population level are the concepts of New Public Health.

Not at last driven by the need for global public health efforts to combat both real or anticipated releases from Pandora's box in an interconnected and globalized world the One Health concept rapidly gained momentum in the last decade after the establishment of the 2004 "Manhattan Principles".

This book is based on the One Health concept with a focus on the public health impacts of zoonoses, both medically and societally. Important aspects in understanding zoonoses are not restricted to more classical issues, e.g. their epidemiology in both humans and animals or disease symptoms in the respective two-legged, four- or more-legged, feathered or unfeathered species, but have to take into account molecularly based epidemiological data and systemic, e.g. ecological approaches.

To give an impression of the wide range of zoonotic research issues, the authors of this book were chosen from a variety of academic and professional backgrounds, from the fields of human and veterinary medicine, from universities and public health institutions, and from all continents. The underlying ideas was not to get an encyclopedic review on all known zoonotic disease entities, but to have a forum for identifying or discussing urgent issues of zoonoses under a public health perspective. Accordingly, the main target groups are the respective scientific communities, medical and veterinary practitioners, their students, public health and veterinary public health practitioners as well as decision makers in the field of public health and veterinary public health.

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Part I
Zoonoses in Food-Chain Animals with Public Health Relevance

Chapter 1
Important Public Health Zoonoses Through Cattle

Mo D. Salman and Katie Steneroden

Abstract Cattle production is a vital component of the global food chain. Animal protein, through meat or milk, is an essential dietary requirement for the majority of people across the world. Increased cattle production will attempt to meet the need for more protein with both positive and negative impacts, including the spread of diseases from livestock to people directly or indirectly through products such as milk, meat, hide or manure. The following zoonotic diseases of cattle are included in this chapter due to their potential severity in humans or cattle population and/or their wide distribution or recent emergence: anthrax, bovine spongiform encephalopathy (BSE), bovine cysticercosis, bovine tuberculosis, brucellosis, cryptosporidium, *Escherichia coli* O157:H7, leptospirosis, methicillin resistant *Staphylococcus aureus* (MRSA), Q fever, Rift Valley Fever, and *Salmonella.*

Cattle production is a vital component of the global food chain. Animal protein, through meat or milk, is an essential dietary requirement for the majority of people across the world. The need for animal protein is increasing. An estimated 50 % increase in demand is expected by the year 2030 (Delgado et al. 1999; Jones and Thornton 2009). Increased cattle production will attempt to meet the need for more protein with both positive and negative impacts, including the spread of diseases from livestock to people directly or indirectly through products such as milk, meat, hide or manure.

Threats from old and new pathogens continue to emerge, with contribution from changes in the environment, agriculture and food production systems, food processing, and the demography and connectivity of our world. At one extreme is low-intensity cattle farming, the type traditionally practiced in developing countries and rural households. The impact of disease outbreaks on the lives and livelihoods of these poor farmers is significant (Jones and Thornton 2009). In contrast, intensive farming systems in developed countries may contribute to the large scale spread of pathogens during disease outbreaks. Zoonotic diseases can have a great impact on national and international trade in addition to contribution to human illness. We are faced with a changing landscape of infectious disease that affects both humans and

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animals. This change poses significant threats to the health and food security of the global citizenry (Atkins and Robinson 2013).

The majority of human pathogens now described are linked to animals. An average of three new infections are reported approximately every 2 years with a new pathogen published every week (Gideon Informatics 2013). Nevertheless, good progress continues to be made in the control of several important livestock pathogens and mechanisms are now in place to bring together the critical scientific expertise and political will to succeed.

The following zoonotic diseases of cattle are included in this chapter due to their potential severity in humans or cattle population and/or their wide distribution or recent emergence: anthrax, bovine spongiform encephalopathy (BSE), bovine cysticercosis, bovine tuberculosis, brucellosis, cryptosporidium, *Escherichia coli* O157:H7, leptospirosis, methicillin resistant *Staphylococcus aureus* (MRSA), Q fever, Rift Valley Fever, and *Salmonella*.

Considerable challenges are presented by zoonotic pathogens to the health and wellbeing of cattle and humans. For some critically important diseases, the first line of defense will be the implementation of scientific approaches to diagnosis and control. What the future will bring with regard to zoonotic diseases is difficult to predict. A future where human and animal health practitioners work together to discover, control and prevent zoonotic diseases will surely bring surprising and meaningful results.

1.1 Anthrax

Bacillus anthracis, the causative agent of anthrax has a worldwide distribution in both animal and human populations. In developing countries anthrax is a significant problem in livestock and wildlife and among occupationally exposed individuals including veterinarians, agricultural workers and butchers (WHO 2013a). In developed countries anthrax is no longer an important disease of livestock due to appropriate control measures including prophylactic vaccination. While anthrax does occur sporadically in developed countries, its main significance lies in its potential use as an agent of bioterrorism.

Bacillus anthracis is a Gram-positive bacterium that forms spores when exposed to oxygen, which are highly resistant and long lasting in the environment. Human cases of anthrax are associated with infection in livestock or exposure to contaminated products such as carcasses, hides or wool. Animal cases of anthrax are associated with spore-contaminated pastures. The incidence of anthrax varies with the soil type, climate, animal husbandry, industrial hygiene, and disease reporting status of the country. Globally, anthrax is underreported in both humans and animal populations due to under-diagnosis and lack of internal and international reporting.

Infection can enter the body by ingestion, inhalation, or direct contact. It is generally considered that animals are infected by ingestion of contaminated food or

water. In humans, infection mainly occurs by direct contact through a break in the skin. Biting flies and other insects have the ability to transmit the disease mechanically.

In cattle, anthrax usually manifests as peracute or acute disease. The peracute form is most common at the beginning of an outbreak and animals are found dead without premonitory signs. After death, discharge of blood from the nostrils, mouth, anus and vulva are common. The acute form runs a course of about 48 h with severe depression, lethargy, abortion and fever. Necropsy findings include absence of rigor mortis and gross enlargement of the spleen with natural orifices exuding dark, tarry unclotted blood. If anthrax is suspected, the carcass should not be opened, as exposure to oxygen will cause spores to form, which may infect individuals and contaminate the environment.

In humans the three main forms of disease are cutaneous, gastrointestinal and inhalation anthrax. Cutaneous anthrax is most common and accounts for the vast majority of cases. The gastrointestinal form occurs from ingesting contaminated meat. Inhalation anthrax occurs through inhalation of the spores and is the most severe form (Decker 2003).

There are different assays for screening and diagnosis of anthrax in cattle. A stained smear of peripheral blood is usually considered as the primary screening test to determine the presence of the bacilli in the blood. Confirmation is by blood culture to identify the bacterial colonies. Fluorescent antibody techniques may also be used to confirm the infection. Animal passage assay may be necessary, if antibiotic therapy is used (Dragon et al. 1999).

Two types of vaccines are currently used in cattle. The most commonly known vaccine is the living attenuated strain of *B. anthracis* that results in long-term immunity (26 months), but there is risk of causing the disease. The second vaccine is the cell-free filtrate of a culture of *B. anthracis*—incapable of causing anthrax, but it has only a short-term immunity (3–6 months) (WHO 2013a).

Treatment in animals and humans is mainly through the application of antibiotics. In animals, penicillin, streptomycin, and oxytetracycline are used. Anti-anthrax serum may be used in animals during the early stages of disease, but severely ill animals are unlikely to recover. Human treatment is by penicillin and other antibiotics (Dragon et al. 1999; CDC 2003).

Control measures are wide range and include the use of vaccination, appropriate carcass disposal methods and decontamination, quarantine, and movement restrictions on milk and meat.

1.2 Bovine Spongiform Encephalopathy (BSE)

Bovine Spongiform Encephalopathy (BSE), also known as “mad cow disease,” is a degenerative neurological disease of cattle. BSE is caused by misfolded proteins (prions) in the host cell that build up in the central nervous system (CNS)

and eventually kill nerve cells. The nature of the transmissible agent is not well understood. The most accepted theory so far is that the agent is a modified form of a normal protein known as prion protein. For reasons that are not yet understood, the normal prion protein changes into a pathogenic (harmful) form that then damages the central nervous system.

BSE is one of several rare neurological diseases called Transmissible Spongiform Encephalopathy (TSE). The other TSE diseases include scrapie, which affects sheep and goats; transmissible mink encephalopathy; feline spongiform encephalopathy; and chronic wasting disease of deer and elk. There are six TSE diseases that affect humans: kuru, classical Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, and sporadic fatal insomnia.

Variant Creutzfeldt-Jakob disease (vCJD) is a rare human TSE that research from the United Kingdom has associated with consumption of products contaminated with CNS tissue from BSE-infected cattle. There have been about 200 cases of vCJD in the world (most of these in the United Kingdom). Human TSE's also include sporadic Creutzfeldt-Jakob disease (sCJD or CJD), which is not related to BSE. About 85 % of CJD cases are sporadic with an annual incidence of about one case per 1 million people worldwide. The new variant or variant form (vCJD) affects younger people (average age at onset is 26 years), and has different clinical features from CJD.

There is strong epidemiologic and laboratory evidence suggesting that vCJD and BSE are caused by the same infectious agent. All cases of confirmed vCJD have occurred in people who have lived in geographic areas with BSE cases; the majority occurred in the United Kingdom, which has had the largest number of cases of BSE in cattle. The specific foods, if any that may be associated with the transmission of this agent from cattle to humans are unknown. However, milk and milk products are unlikely to pose any risk for human exposure to the BSE agent.

Research indicates that the first probable infections of BSE in cows occurred during the 1970's with the first two cases of BSE being identified in 1986. BSE may have originated from feeding cattle meat-and-bone meal (MBM) that contained BSE-infected products from a spontaneously occurring case of BSE or scrapie-infected sheep products. There is strong evidence and general agreement that the outbreak was then amplified and spread throughout the United Kingdom cattle industry by feeding rendered, prion-infected, bovine meat-and-bone meal to young calves.

There is increasing evidence that there are different strains of BSE: the typical BSE strain responsible for the outbreak in the United Kingdom and two atypical strains (H and L strains). The typical BSE strain is responsible for most of the BSE cases in the world. In cattle naturally infected with BSE, the BSE agent has been found in brain tissue, in the spinal cord, and in the retina of the eye. Additional experimental studies suggest that the BSE agent may also be present in the small intestine, tonsil, bone marrow, and dorsal root ganglia (lying along the vertebral column).

In response to the BSE epidemic, several countries instituted a series of measures to minimize the risk of disease transmission among both animals and humans.

These included a ban on feeding ruminant protein to ruminants and removal of some "high risk" materials (such as brain, spinal cord and intestines) from cattle at slaughter. Following institution of these measures, the number of BSE cases has been decreased significantly (USDA-APHIS 2006, 2007).

To prevent BSE from entering the country, several countries prohibited the importation of live ruminants from countries where BSE is known to exist in native cattle. Some countries eliminated the importation of live ruminants and most ruminant products, including meat, meat-and-bone meal, offal, glands, etc. from all of Europe. The majority of these countries also prohibited the use of most mammalian protein in the manufacture of animal feeds given to ruminants. Testing for BSE under national surveillance program among slaughtered cattle was implemented in several developed countries. Due to these safeguard measures the risk of transmitting BSE agent to humans was becoming negligible (Salman et al. 2012).

1.3 Bovine Cysticercosis—Taeniasis

Although bovine cysticercosis does not in itself represent an exceptionally serious human health risk, it is a signal of much more serious food safety and public health concerns. A finding of bovine cysticercosis is a signal that the animal feed system is contaminated and that cows are consuming human feces. Aside from *Taenia saginata*, other contaminants that pose threats to bovine and human health would also be expected to be present in human feces. These contaminants include, but are not limited to drug resistant bacteria, such as *E. coli* and *Salmonella*, *Taenia solium* (the pork tapeworm), drug residues, pain killers, hormones, other prescription drugs, illicit drugs, heavy metals, solvents and other toxicants.

Taenia saginata (T. saginata) is a cestode tapeworm that causes bovine cysticercosis in cattle and taeniasis in humans. *T. saginata* is found worldwide and human disease is highly endemic in Latin America, Africa, Asia and some Mediterranean countries (Spickler 2003). Bovine cysticercosis occurs in areas where poor sanitation, poor food inspection and close contact between humans and livestock are common (Acha and Szyfres 2003).

T. saginata infection cycles between humans (primary host) and cattle (reservoir host). Humans infected with the tapeworm pass the eggs in their feces. Cattle become infected by ingesting materials contaminated with tapeworm eggs. Larvae form cysticerci in the animal's muscle tissue, humans ingest cysticerci in raw or under-cooked beef, and the cycle continues. Tapeworms cannot be passed from person to person or spread between cattle. Clinical signs of cysticercosis in cattle and humans are mild to non-existent (Acha and Szyfres 2003). The most visible sign of tapeworm infection in humans is the active passing of tapeworm segments through the anus and in the feces.

Diagnosis of bovine cysticercosis is largely done during visual inspection of the carcass at slaughter. Serological tests including ELISA have been used in epidemiological studies for individual and herd diagnosis (WHO 2005). Taeniasis in humans

is diagnosed by finding eggs or cestode segments on the human body or in the feces with peri-anal adhesive tape tests. Feces microscopy, ELISA and molecular tests such as PCR may also be utilized (WHO 2005).

Infection in humans can be prevented by proper meat inspection and handling of meat at slaughter. When disease is found in cattle the meat may be condemned or temperature treated by freezing or heating to kill the parasite. Preventing and treating disease in people will prevent disease in cattle. Tapeworm eggs can survive in the environment for many months depending on humidity and temperature. Infected people can shed hundreds of thousands of eggs each day, so it is important for people to seek treatment to break the cycle.

1.4 Bovine Tuberculosis

Bovine Tuberculosis (BTB) is a zoonotic and economically important disease of livestock. The disease was described over 2000 years ago and is responsible for devastating illness and death in both humans and animals. Bovine tuberculosis has been largely controlled in developing countries through government control programs and milk pasteurization. In developing nations where surveillance and control measures are lacking or inadequate, humans continue to become infected with BTB through animal contact and ingestion of unpasteurized dairy products. Few developing countries have BTB control programs and immune system compromising disease conditions such as HIV allow for co-infection and increased morbidity and mortality (Miller and Sweeney 2013).

Most warm-blooded vertebrates, including humans, are susceptible to the disease causing agents. Although the principle reservoir of *Mycobacterium bovis* (*M. bovis*) is cattle, this organism has a wide host range with the capacity to produce progressive disease. Ungulates differ somewhat in resistance to *M. bovis*, but have similar immune responses and pathological conditions. They all exhibit the classical lesions of tuberculosis.

The infection is caused by the bacterial genus *Mycobacterium*. Mycobacteria are acid-fast, aerobic, non-spore-forming, non-motile, gram-positive rods containing high lipid content. Some of the lipids possess virulent and immunologic properties. The possible pathogenic role and the effect on the immune response of components of the complex mycobacterial cell wall are the subject of much attention and controversy (Behr 2013).

Bovine tuberculosis occurs throughout the world. The prevalence of *M. bovis* in cattle is low in developed countries due to successful eradication programs. Other countries have experienced increases in the rate of infection due to relaxation in surveillance activities.

Risk factors for cattle include overcrowding, introduction of tuberculous animals, soil type, wild life contact in specific geographical regions (UK, Ireland: Badger, New Zealand: Possum), purpose of the cattle: dairy vs. beef; and type of management and husbandry—specifically in the type of disposal of the manure.

The most common mode of transmission of BTB is the aerogenous route. Infection can occur by ingestion and other less likely modes such as milk-borne, congenital, or sexually transmitted. Bacteria are excreted in exhaled air, sputum, feces, urine, milk, and discharges from uterus, vagina, and draining peripheral lymph nodes. Cattle can develop bovine tuberculosis through exposure to other *M. bovis* infected species such as humans, deer, and elk (Bovine TB Advisory Group 2009).

Clinical signs of disease in cattle are variable depending on the location and extent of the lesions. Even with advanced disease, visible signs are frequently absent. If superficial lymph nodes are involved, they may be visibly enlarged and can rupture and drain through the skin. Enlarged internal nodes can cause signs of obstruction. With pulmonary involvement, a chronic cough can develop due to bronchopneumonia. In advanced lung disease, dyspnea occurs with increased respiratory rate and depth. Tuberculosis mastitis causes a marked induration and hypertrophy of the udder. General findings include anorexia, dyspnea, weight loss, weakness, and low-grade fluctuating fever. Often the main sign of tuberculosis is emaciation, despite adequate nutrition and care.

A definitive diagnosis for mycobacterial infection can be made by bacterial isolation and identification, which can be difficult and time consuming. For example, in *M. bovis* cultures visible growth arises following 3–8 weeks of incubation. Conventional mycobacteriological identification procedures on culture media rely on differences in culture growth times, colony morphology, cellular morphology, antimicrobial sensitivity, and various biochemical test reactions. More recent techniques such as radiometric procedures can expedite mycobacterial detection times, whereas gas-liquid chromatography, and DNA robes can accelerate mycobacterial identification from cultures. Research on the use of the DNA probes, specifically polymerase chain reaction (PCR), is currently in progress to be used for molecular epidemiology of the disease in livestock species.

The tuberculin skin test is an in vivo diagnostic test used to evaluate the cell-mediated immune response to mycobacteria exposure. The test is unable to differentiate between disease and immunity. To determine whether or not an animal is infected with *M. bovis*, tuberculin made from either the human or bovine bacilli (the mammalian tuberculins) is injected intradermally into the animal. Reactivity to tuberculin made from either of these bacilli is similar and is normally the greatest in animals sensitized specifically to these bacilli. The inflammatory response to the injection peaks from 24 to 72 h following tuberculin injection and can linger for several weeks before diminishing. Failure of animals with observable evidence of tuberculosis to show a palpable skin response to tuberculin at the time of test reading has been defined as anergy. Anergy is indicative of deficient T lymphocyte function.

Vaccines against *M. bovis* stimulate cell-mediated immunity. BCG (Bacillus of Calmette–Guerin, the modified *M. bovis* vaccine strain named after its two developers) is an attenuated strain of *M. bovis* used in human vaccination. BCG has also been utilized extensively to vaccinate cattle in numerous countries for many years. Protection produced by BCG vaccination of cattle is poor and causes tuberculin

sensitivity in the animals, interfering with control and eradication programs based on tuberculin skin testing. By 1968, none of the national control programs for bovine tuberculosis included vaccination.

Treatment of tuberculosis in animals in general is discouraged due to possible public health hazards in retaining tuberculous animals. However, throughout the years, numerous procedures have been tried without success to treat tuberculous cattle, including injection of live or dead bacilli, specific diets, fresh air, change of climatic conditions, x-ray therapy, serotherapy, pneumothorax, and pneumoperitoneum. Chemotherapeutic drugs, including isoniazid, have been used in cattle and were found to only suppress the bacilli during the duration of drug therapy, with shedding of the organism possible after treatment.

Control measures include test and slaughter, active detection of the lesioned cattle in slaughterhouses followed by trace back systems and control of the disease in wildlife populations.

1.5 Brucellosis

Brucellosis is a zoonotic disease of major social and economic importance in most countries of the world. It is caused by several species of *Brucella* bacteria and effects several livestock species—mainly cattle, sheep, and goats. The economic importance of the disease in cattle is due to a loss of production, primarily decreased milk production, abortion, and infertility. Brucellosis is found worldwide, however in some geographical areas it is limited to a specific *Brucella* species and host species. Several countries have succeeded in the eradication of the disease from specific host species; other countries are engaged in eradication programs. The growing phenomenon of international migration and tourism renew our concern with the prevalence and persistence of human brucellosis.

The *Brucella* spp. have a wide host range, however, they are not readily transmitted from preferential to dissimilar hosts. Non-preferential hosts may harbor the bacteria, but it is considered an incidental infection. This incidental infection is usually localized and/or shows different clinical and pathological manifestations from those observed in the specific host. The host preferences of this bacterial agent are: *Brucella abortus* in cattle, *Brucella melitensis* in sheep and goats, *Brucella suis* in swine and *Brucella ovis* in sheep (Moreno et al. 2002).

The bacteria is an intracellular organism which is an important factor in its survival in the host and may explain both the transitory titers occurring in some hosts following isolated episodes of bacteremia and the disappearance of titers in hosts with latent infection. The bacteria can survive on grass for variable periods depending on environmental conditions. In temperate climates, infectivity may persist for 100 days in winter and 30 days in summer. The organism is susceptible to heat, sunlight, and standard disinfectants, but freezing is conducive to almost indefinite survival (Blasco and Molina-Flores 2011).

Risk factors associated with infection and the diseases in cattle population include: (1) Contact with infected materials—aborted fetus, placenta, semen, secretion, etc.; (2) Direct contact with infected animals—including wildlife species; (3) High population density, particularly in dairy farming systems; (4) Breeding management and husbandry such as contaminated maternity pens, unregulated breeding time; and (5) Poor hygiene/husbandry—particularly during calving seasons.

The infection in humans is nonspecific and manifests as fluctuating fever, pain in joints, sweating, and weakness. Transmission to humans occurs through contact with contaminated materials from infected animals particularly as an occupational hazard; consumption of infected milk and dairy products; non-intentional injection of live animal vaccine; and inhalation of large amounts of bacteria contaminated aerosols. Human infection with brucellosis is most serious when it results from exposure to *B. melitensis*, which is usually linked to exposure to infected goats and sheep (Corbel 2006).

The disease in animals is transmitted through ingestion of contaminated materials; penetration of intact skin and conjunctiva; and contamination of the udder during milking. Intra-herd spread occurs by both vertical and horizontal transmission. Congenital infection due to *in utero* infection does occur, but its importance has not been defined. Horizontal transmission can occur both directly and indirectly. Flies, dogs, rats, ticks, infected boots, fodder, and other inanimate objects are possible ways for indirect transmission. Preventive measures in cattle population are mainly related to early detection of infected cattle with removal of serologically positive animals (test and culling) and the application of vaccine.

No reliable vaccine is available for human use. Humans are usually treated prophylactically with antibiotics if exposure is suspected. Preventive measures for human infection include precaution in handling contaminated materials from infected animals and precautions during the use of the vaccine in animals and avoiding consumption of unpasteurized milk or dairy products.

1.5.1 Cryptosporidium parvum

Cryptosporidium parvum is a coccidian protozoan that is an important cause of diarrhea in cattle and humans worldwide. It has emerged since the 1970's as a major cause calf-hood diarrhea. It is one of the top four agents responsible for moderate to severe gastrointestinal illness in children in developing countries and can be a fatal complication of AIDS (Kotloff et al. 2013) (Mosier and Oberst 2000). Cryptosporidiosis is one of the most common causes of waterborne disease among humans in the United States (CDC 2013a).

C. *parvum* resides in the small intestine of the host where it forms oocysts, which are shed in great numbers in the feces. Transmission occurs through ingestion of food and water contaminated with fecal matter from an infected animals or humans, direct contact with infected feces or ingestion of contaminated water. Large outbreaks have been associated with drinking water, food, swimming pools and lakes.

Community-wide outbreaks of cryptosporidiosis have been linked to drinking municipal water or recreational water contaminated with *Cryptosporidium*. One large-scale outbreak occurred in Wisconsin, USA in 1993 when more than 400,000 people became ill from a malfunctioning municipal water filtration system. The total cost of outbreak-associated illness was US$ 92 million. (Corso et al. 2003) The source of the *Cryptosporidium* oocysts in this outbreak, whether from cattle, slaughterhouse run off or from human sewage, remains speculative (Mac Kenzie et al. 1994).

In healthy humans, infection is usually asymptomatic and self-limiting. In immunodeficient people disease can be severe with profuse watery diarrhea and substantial fluid loss (Acha and Szyfres 2003). Most animals can become infected with *Cryptosporidium* spp., but clinical signs of diarrhea, tenesmus, anorexia and weight loss are most commonly observed in calves less than one month old.

Cryptosporidiosis is diagnosed by examining fecal samples using acid-fast staining, direct fluorescent antibody and/or enzyme immunoassays (CDC 2013a). The oocysts are not shed continuously and repeated sampling may be necessary. Cryptosporidiosis can also be diagnosed in stained biopsy/necropsy specimens or fresh intestinal scrapings. Molecular methods, which can detect *Cryptosporidium* species, are increasingly being used in diagnostic laboratories.

There is no specific treatment available for Cryptosporidiosis; supportive therapy is usually effective. Prevention efforts focus on hand washing, especially after handling or being around animals and before eating or handling food.

1.5.2 E. coli O157:H7

Escherichia coli is in the family *Enterobacteriaceae* and is a normal component of the flora in the large intestine of humans and warm-blooded animals. *E. coli* O157:H7 is a specific pathogenic subset of *E. coli* found worldwide, that produces watery diarrhea, hemorrhagic colitis and rarely, hemolytic-uremia syndrome (HUS) in children.

Cattle are a reservoir hosts, harbor the bacteria asymptomatically and are an important source of infection for humans. Prevalence estimates vary, and it appears that while a large percentage of cattle herds may have infected animals, the actual number of individual infected animals at any one time is relatively low (USDA 2003). The costs associated with attempts to control prevalence in cattle, contaminated food recall, and human healthcare costs make the economic and social burden *E. coli* O157:H7 high (Callaway 2010).

Transmission of *E. coli* O157:H7 occurs through consumption of contaminated food or water, direct contact with infected animals, their feces or contaminated soil. Primary sources of *E. coli* O157:H7 outbreaks are raw or undercooked ground meat products, raw milk and fecal contamination of vegetables. Person-to-person spread can occur during outbreaks (Spickler 2009). Visiting farms and other venues where the general public might come into direct contact with farm animals, particularly

calves, has been identified as an important risk factor for *E. coli* O157:H7 infection (WHO 2011a). A low dose of bacteria is sufficient for infection.

E. coli O157:H7 occurs asymptomatically in cattle and is shed intermittently. In humans, illness can range from mild diarrhea to severe hemorrhagic colitis. In most cases the illness is self-limiting. Hemolytic uremic syndrome, a particularly severe complication, can occur in a small percentage of cases leading to renal failure and death in children and elderly. Selective and differential culture media have been developed to diagnose *E. coli* O157:H7 in human and bovine fecal samples.

Measures to prevent and control *E. coli* O157:H7 in cattle include management changes (biosecurity, housing, transport and stress reduction), water and feed management, including additives and probiotics; bacteriophages and vaccines (Callaway 2010). Pre-harvest strategies are important, but do not eliminate the need for good sanitation in processing plants and households. Good hygienic slaughtering practices reduce contamination of carcasses. Education on hygienic handling of foods is essential for farm workers, abattoir and food production workers to reduce contamination. Household preventive measures are similar to those recommended for other foodborne diseases (WHO 2011a).

1.6 Leptospirosis

Leptospirosis is a zoonotic disease of worldwide importance. Also a neglected tropical disease, leptospirosis largely affects vulnerable rural and semi-urban populations. Global annual incidence of endemic human leptospirosis is grossly underestimated due to lack of awareness, under diagnosis, misdiagnosis and difficulty with diagnostic testing. Efforts to determine the burden of disease are ongoing (WHO 2011b). Leptospirosis is endemic in countries with humid subtropical and tropical climates, epidemics occur often as a result of flooding. Individuals at greatest risk include farmers, ranchers, slaughterhouse workers, trappers, loggers, veterinarians, sewer workers, rice field workers and military personnel.

Leptospirosis is caused by a variety of species of *Leptospira*, a spirochete with more than 250 pathogenic serovars that are adapted to different wild or domestic reservoir hosts. The classification system for *Leptospira* changed in 1989, leading to some confusion, as pathogenic and non-pathogenic serovars are now included in the same species. Serovars vary by geographic region (Spickler 2005). Host adaptation is not a static situation as serovars are adapting to new hosts, vaccine pressures are altering serovars in different species and climate change may be altering hosts and serovars (Hartskeerl et al. 2011). These facts lead to difficulties in prediction, prevention and use of vaccines. Reservoir hosts include wild mammals (rats and rodents are the most common) as well as domestic cattle, pigs, pigs and sheep and dogs. Reservoir hosts experience asymptomatic, mild or chronic disease and can shed for months to years.

Leptospires reside in the kidneys of infected reservoir hosts and are shed in urine into the environment where they can reside for long periods of time depending on

environmental conditions. Freshwater ponds, streams, run-off and groundwater are common water sources of Leptospires. *Leptospira* spp. can also be excreted in vaginal secretions and with aborted fetuses after calving (Spickler 2005). *Leptospira* spp. can be spread directly between individuals, through skin contact with contaminated water or urine, ingested in contaminated food or water or spread via aerosol.

At least 13 serovars of *Leptospira* spp. have been isolated from cattle (Acha and Szyfres 2003). Clinical signs vary with the serovar and in acutely affected calves include fever, anorexia, conjunctivitis and diarrhea. In adult cattle clinical signs may be mild and go undetected. More serious infection may result in abortions, decreased fertility or decreased milk yields (Spickler 2005). Clinical signs are associated with kidney disease, liver disease or reproductive dysfunction; younger animals suffer more severe disease. Differential diagnosis includes brucellosis, neospirosis, bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis (IBR).

In humans, disease ranges from mild to severe depending on the serovar and immune status of the patient. Clinical signs mimic other infectious diseases including influenza, hepatitis, dengue, hantavirus, yellow fever, malaria, brucellosis, borreliosis, typhoid fever, other enteric diseases and pneumonia (Spickler 2005).

Rapid screening tests are available for presumptive diagnosis in humans, but require confirmatory diagnosis by culture, PCR or microagglutination test (MAT). The most commonly used test for diagnosis in animals is the MAT tests; ELISA tests are also used.

Human vaccines against leptospirosis are available in some countries. Animal vaccines are in use and must contain serovars present in the local environment; most of them require yearly boostering. In developed countries cattle, pigs and dogs are routinely immunized. In developing countries vaccines with locally relevant serovars are not as available (Hartskeerl et al. 2011). Prevention programs must be tailor-made and based on predominant serovar and local reservoir hosts. Public health prevention measures include reservoir control through rodent control and vaccination of livestock and dogs, improved sanitation, improvement of water sources that may be contaminated, as well as outreach and education for high-risk individuals and high-risk areas.

1.6.1 Methicillin-Resistant Staphylococcus aureus

Methicillin-resistant *Staphylococcus aureus* (MRSA) are Gram-positive bacteria that are resistant to methicillin and other beta-lactams in this large group of antibiotics that are widely used in veterinary and human medicine. MRSA is found worldwide in humans and animals.

MRSA was first isolated from cattle with mastitis in 1972, which was the first recognition of this emerging disease in animals (Devriese et al. 1972). Since that time MRSA has been found in many species of animals including pigs, horses, dogs, cats, pet birds, zoo animals and marine mammals (Spickler 2011). Most of the strains isolated from animals have been of human origin; this changed, however,

in 2003–2005 with the emergence of a new type of MRSA, CC398, isolated from humans and pigs in The Netherlands. This livestock-associated strain appears to be less host specific than other MRSA strains and has spread to other livestock including cattle (Vanderhaeghen et al. 2010). The livestock-associated MRSA can cause disease in animals and as well as in humans in close contact with them (Vanderhaeghen et al. 2010) and there is evidence of limited human-to-human spread of this strain as well (Voss et al. 2005). The data on this new type of livestock-associated MRSA is limited and the burden of CC398 in cattle is unclear (Vanderhaeghen et al. 2010).

MRSA is transmitted most commonly through direct contact with colonized or infected individuals (animals or humans) (Spickler 2011). Contaminated environments, including air in confinement operations, are other potential routes (Gibbs et al. 2006). Human and livestock-associated strains of MRSA can be found in contaminated food (Jones et al. 2002), meat (van Loo et al. 2007; de Boer et al. 2009), and raw milk products (Normanno et al. 2007).

Cattle colonized or infected with MRSA most commonly present with clinical or subclinical mastitis. MRSA colonization has been associated with veal calves (Graveland et al. 2010) and beef calves (Mooij et al. 2007). MRSA can cause a wide variety of infections in humans including skin and soft tissue infections as well as more invasive infections including pneumonia, endocarditis, septic arthritis and septicemia; MRSA is one of the most prevalent causes of nosocomial infections worldwide (Spickler 2011).

Diagnosis of infection or colonization with *S. aureus* can be accomplished through culture of the organism. Methicillin-resistant strains can be identified through antibiotic susceptibility or genetic testing. Genetic testing can identify the various human and animal associated strains.

In general, prevention and control of MRSA includes good biosecurity and infection control practices including hand washing, barrier precautions and environmental disinfection (Spickler 2011). MRSA is not particularly hardy and can be inactivated by sodium hypochlorite, alcohols and quaternary ammonium compounds (Spickler 2011). The emerging livestock-associated MRSA urgently requires more research to determine the risk factors and transmission routes (Vanderhaeghen et al. 2010).

1.7 Q Fever

Q fever is a highly contagious zoonotic disease caused by *Coxiella burnetii*, an obligate intracellular bacterium. Livestock are the major source of infection in humans worldwide. Q fever can infect a wide range of hosts including pets, wildlife, birds, reptiles and ticks. Because illness can be mild and go undetected, Q fever is under-diagnosed and under-reported globally and the true burden of disease unknown. However, a large outbreak with approximately 4000 human cases occurred in the Netherlands during 2007–2010. Dairy goat farms near densely populated

areas were the source of the outbreak, which was spread via a windborne route (Schimmer et al. 2009).

Animals that carry this organism usually do not show any signs of disease, but abortions and stillbirths can occur with great quantities of bacteria shed. Both symptomatic and asymptomatic animals shed *C. burnetii* in large quantities at parturition. The bacteria can also be shed in feces, urine, and milk. The organisms persist in the environment for long periods, are highly resistant to disinfectants and can be spread long distances by the wind (Spickler 2007).

Human infection usually occurs from inhalation of bacteria from air that is contaminated by feces of infected animals. Q fever is also rarely transmitted to humans by tick bites and through ingestion of unpasteurized milk or milk products (CDC 2013b). Most often, sporadic cases occur in people who are occupationally exposed such as biomedical research facility workers, farmers, ranch-hands, veterinarians, and slaughterhouse workers (CDC 2013b). These cases tend to result from exposure to parturient ruminants; however, cats, dogs, rabbits and other species have also been implicated. Although Q fever is usually asymptomatic or mild, a small percentage of people develop serious disease. Pneumonia or hepatitis may occur in acute cases, and chronic infections can result in endocarditis or a wide variety of other diseases (Spickler 2007).

In humans Q fever is usually diagnosed by serology or PCR. Diagnosis of Q fever in aborting animals involves testing of the fetuses and placentas. Veterinary diagnosticians typically identify the organism by the use of special stains applied to microscopic sections of these tissues, and/or PCR.

Q fever can be prevented in humans by limiting exposure to livestock during birthing, personal hygiene measures and wearing of personal protective equipment and only eating and drinking pasteurized milk and milk products. In animals prevention of Q fever is based on herd management and prevention of contact with wildlife and tick vectors. Isolating infected pregnant animals and disposing of reproductive tissues can decrease transmission (Spickler 2007). Prevention in both humans and animals can be difficult, because Q fever can be transmitted on fomites or in aerosols over great distances. Effective vaccines are available in some countries for both humans and animals.

1.8 Rift Valley Fever

Rift Valley Fever (RVF) is a zoonotic disease that primarily affects ruminants (cattle, sheep, goats and camels) and can also infect humans. Disease can be severe in both humans and animals and may cause severe economic losses as a result of livestock death and abortion. Infection with RVF is caused by a virus in the in the family Bunyaviridae and is primarily transmitted by mosquitoes. Recently, RVF has received more attention as a potential agricultural and zoonotic disease threat in Europe and North America due to the increasing numbers of competent vector species present in those regions (Salman 2013).

RVF is endemic in much of Africa with occasional spread to countries in the Arabian Peninsula. Epidemics occur sporadically when climate conditions supports breeding of mosquitoes. Rift Valley Fever virus (RVFV) was first isolated from lambs in the Rift Valley of Kenya in the 1930s. Major outbreaks have been recorded in many parts of Africa since that time and the virus was first detected outside of the African continent in Saudi Arabia and Yemen in 2000. The first report of RVF outside of Africa was attributed to the importation of cattle and small ruminants from the Horn of Africa (Pepin et al. 2010).

Transmission of infection in cattle is mainly via the bites of infected mosquitoes. As an epidemic progresses, direct contact transmission by infectious animals or contaminated tissues including aborted fetuses may occur. Transmission via infected mosquitoes is important for the dissemination of RVFV between herds over short distances and also over long distances through movement of infected animals or translocation of infected mosquitoes (Abdo et al. 2011).

Disease, especially in young animals, may be severe and includes fever, depression and anorexia. The classic clinical sign of RVF in a herd of cattle is a large number of nearly simultaneous abortions among pregnant animals, regardless of the stage of pregnancy. This abortion storm differentiates RVF from other common infectious causes of abortion in cattle such as Q fever, chlamydiosis, brucellosis, salmonellosis, listeriosis or toxoplasmosis. RVF may also cause sudden death in cattle. Aborted fetal materials and placental membranes contain large numbers of virus particles, which can either contaminate the local environment directly or infect animals or humans in close contact. The RVFV may persist for relatively long periods in the environment.

Direct contact and aerosol exposure to infected tissues or bodily fluids constitutes the main route of infection for humans. Certain groups are at increased risk due to occupation such as herders, farmers, slaughterhouse workers and veterinarians. There is evidence for shedding of the virus into milk so that consumption of unpasteurized milk has major consequences for disease transmission and public health. Most human infections are inapparent or demonstrate mild flu-like symptoms (fever, headache, and myalgia). In some cases, infection progresses with severe complications including hemorrhagic fever, encephalitis, and acute hepatitis.

RVFV can be diagnosed by several different methods including virus isolation from blood and other tissues and by using serological tests such as ELISA.

There is presently no vaccine licensed for human use, though inactivated vaccines have been in development. Both live attenuated virus vaccines and inactivated virus vaccines are available for use in livestock. The live vaccine produces better immunity and requires only one dose, but may induce abortions and birth defects in pregnant animals. Inactivated vaccines require multiple doses in order to provide protection making their use problematic in endemic areas. In endemic areas sustained animal vaccination programs can help to prevent outbreaks.

In order to slow the expansion of RVF movement restrictions of livestock may prevent disease from entering new areas. Outbreaks of RVF in animals precede outbreaks in humans, so sustained surveillance and monitoring systems in animals

can act as an early warning system to public health authorities. Raising human awareness of protective measures for mosquito bites and safe handling practices during slaughter, appropriate barrier precautions and proper pasteurization of milk to prevent spread from animals may prevent human infection. Vector control, RVF forecasting and climatic models to predict when climate conditions are favorable for RVF outbreaks can also help to guide prevention efforts.

1.8.1 Salmonella

Salmonella is a major cause of foodborne disease globally. The global burden of zoonotic disease from *Salmonella* is high. An estimated 93.8 million illnesses and 155,000 deaths result each year from non-typhoidal *Salmonella,* the vast majority of which are foodborne (Majowicz et al. 2010). In the EU alone over 100,000 human cases are reported each year with an estimated overall economic burden as high a 3 billion € a year (EFSA 2013). *Salmonella* strains that are resistant to a range of antimicrobials have emerged since the 1990s and are now a serious public health concern (WHO 2013b). Salmonellosis has a worldwide distribution, but serovars vary geographically. *Salmonella* is most prevalent where livestock are farmed intensively (Spickler 2005).

Salmonella bacteria are classified into over 2500 different serovars based on surface proteins. *Salmonella* are shed in the feces of a wide variety of infected animals including cattle, which are infected by ingestion of contaminated feed, water or grass. The bacteria are hardy and can survive for months to years in the environment (Spickler 2005).

Transmission is generally through the fecal-oral route and humans generally contract salmonellosis through consumption of contaminated food including meat, eggs, poultry and unpasteurized milk products. Less often *Salmonella* is transmitted through green vegetables contaminated by manure. Person-to-person transmission through the fecal-oral route can also occur. Human cases may also occur through contact with infected livestock, which often do not show signs of disease. Most cases of salmonellosis in humans are mild, but can result in severe disease and death depending on host facts and the strain of *Salmonella*. Humans may develop diarrhea, abdominal cramping, and fever, which can be very severe.

Salmonella is often carried asymptomatically in cattle, but young, stressed or pregnant animals are the most susceptible to infection, which may result in enteritis and septicemia (Spickler 2005). *Salmonella* infection is diagnosed by isolating the organism from feces. In cases of disseminated disease bacteria can be isolated from the blood.

To reduce the risk of foodborne transmission basic food hygiene practices and adequate cooking should be used. To prevent transmission from animals to humans, hand hygiene after touching or working with animals is critical. To reduce the risk of *Salmonella* in cattle, herd management strategies and proactive biosecurity, rodent control and *Salmonella*-free feed and water sources should be utilized. Fecal

contamination of water supplies and feed should be prevented. Vaccines are available in some countries for some serovars and can reduce the level of colonization, shedding and clinical disease (Spickler 2005).

1.9 Summary

Zoonotic diseases originating from cattle can cause mild or asymptomatic human infection or severe disease and death. A number of zoonotic diseases were not covered in this chapter, but might be considered to varying degrees depending on geographic location and local circumstances, e.g., listeriosis, rabies, ringworm, and Human African Trypanosomiasis. While some diseases are rare, the potential for serious outcomes makes it critical for veterinarians and public health practitioners to provide outreach to those individuals at greatest risk including farmers—small scale and large.

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Chapter 2
Zoonotic Diseases of Swine: Food-borne and Occupational Aspects of Infection

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Abstract Swine and their products have become a central part of food systems around the world. Global pork production has rapidly increased over the past 30 years, leading to the intensification of the swine industry: though there are fewer farms now, those farms that do persist raise ever-larger numbers of animals. This increases the transmission of pathogens both amongst animal herds, and between animals and their human caretakers. Furthermore, increased stress to animals and the potential for amplification of pathogens in the farming environment can lead to a higher burden of disease-causing organisms in and on meat products, which then make their way to consumers world-wide. As such, swine and their meat products have the potential to introduce new zoonotic diseases into populations via multiple routes of transmission. Here we discuss several examples of zoonotic diseases of swine origin, reviewing diseases with bacterial, viral, or parasitic causes.

2.1 Background and Introduction

Pork is rapidly becoming the world's source of protein. Global pork production increased more than 80% between 1985 and 2010 (Fournie et al. 2012), and this trend has led to the intensification of swine husbandry, with fewer and fewer facilities present, but each raising larger numbers of individual animals. China has been a driver of this market, accounting for approximately 50% of total global pig production (Fournie et al. 2012). As swine production has intensified, so has concern over how these modifications in husbandry may affect the transmission of disease amongst pigs as well as to human caretakers. It has been estimated that more than 60% of emerging diseases are zoonotic (Jones et al. 2008). A recent review (Fournie et al. 2012) identified 77 pathogens that had not been described in swine prior to 1985, including 39 viruses and 32 bacterial species. Not surprisingly, the top 20% of pork-producing countries accounted for 82% of these emerging pathogens. Of these 77 novel species found to infect swine, 30 (39%) are zoonotic, and

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26% of these were identified in the context of an outbreak investigation (Fournie et al. 2012). Densely populated South East Asia is the epicenter of emergence of novel zoonotic diseases due to inter-species transmission. However, outbreaks of host specific lethal zoonoses have occurred in industrialized nations as well (Davies 2012). It is plausible that a dramatic change in swine industry demographics in recent decades without adequate biosecurity may have served as a tonic for the emergence of swine zoonosis (Davies 2012). Zoonotic diseases impose significant economic burden with increased morbidity and mortality globally. A change in ecological niche, climatic change, rapid growth in human population and socio-economic factors are among the major contributing factors for the emergence of zoonoses (Jones et al. 2008).

Outbreaks of human disease related to swine-origin pathogens, including *Streptococcus suis* in China (Lun et al. 2007), Nipah virus in Malaysia (Chua 2012) and the novel H1N1 variant influenza virus have gained significant media attention in the past decade. Here we discuss several examples of zoonotic diseases of swine origin, reviewing diseases with bacterial, viral, or parasitic causes.

2.1.1 Yersinia enterocolitica

Yersinia enterocolitica is a gram negative bacterium in the family *Enterobacteriaceae*. *Y. enterocolitica* is widely distributed throughout nature, having many animal and aquatic reservoirs; however, swine are considered the main reservoir for strains that are pathogenic to humans. It is the main causative agent of yersiniosis, a disease that affects animals and humans worldwide (Holt et al. 2000).

Yersinia enterocolitica can be classified into distinct subgroups based on biochemical characteristics (biotypes) and O-antigen specificity (serotypes). There are six biotypes (1A, 1B, 2, 3, 4, and 5) and 60 serotypes, 11 of which are associated with human illness (Nesbakken et al. 2006; Bottone 1997). Biotype 1B is considered the only highly pathogenic strain, while the others are considered moderately pathogenic, except for biotype 1A, which is considered nonpathogenic although this has recently become a contentious topic due to recent reports of 1A infections (Stephan et al. 2013). Biotype 1B is mainly found in North America and Japan and is different from other biotypes in that it can be found in water and other environmental sources, and can also be carried by swine and rodents. Biotypes 2 and 4 are associated with human infections in Europe; their main reservoirs are pigs and cattle. Biotypes 3 and 5 are uncommon, but are also associated with animal reservoirs (EFSA 2009; Fredriksson-Ahomaa et al. 2006a) .

Yersiniosis is a gastrointestinal disease causing fever and watery, occasionally bloody, diarrhea. Rarely, *Y. enterocolitica* can cause septicemia, and in some cases long-term sequelae can occur. Symptoms generally occur 4–7 days after exposure and may last for up to a month (Bottone 1997; Huovinen et al. 2010). Approximately 16.5 cases per 1,000,000 persons occur each year in Europe (EFSA 2009), while in the United States, approximately 3.5 cases per 1,000,000 are seen each year

(Long et al. 2010). Children are infected more frequently than adults, and infections occur most commonly in temperate locations during colder months (Bottone 1997).

Pigs are commonly asymptomatic carriers of pathogenic strains of *Y. enterocolitica*. The bacteria typically reside in the gastrointestinal tract, especially the tonsils, lymph nodes, intestines and feces (Fredriksson-Ahomaa et al. 2007; Bhaduri et al. 2005). Cattle and goats have also been found to be carriers (Lanada et al. 2005a, 2005b), and milk products from these animals have been the source of numerous outbreaks in human populations (Black et al. 1978; Shayegani et al. 1983; Morse et al. 1984; Tacket et al. 1984; Ackers et al. 2000). Deer, rabbits, rodents (Quan et al. 1974), dogs (Byun et al. 2011), and cats (Fredriksson-Ahomaa et al. 2001) have also been found to carry as well as to be infected with *Y. enterocolitica*. In addition to livestock, water sources including wells, rivers and lakes can serve as reservoirs for the bacteria as a result of contamination by feces of carriers or leakage from latrines.

The major risk factors for developing yersiniosis include eating raw or undercooked pork (Boqvist et al. 2009; Fredriksson-Ahomaa et al. 2006b), drinking contaminated milk (Black et al. 1978; Tacket et al. 1984; Ackers et al. 2000), and consuming contaminated drinking water (Thompson and Gravel 1986; Christensen 1979). Porcine sources are frequently associated with the pathogenic serotypes O:3, O:9, and O:5,27 and sometimes with the highly virulent serotype O:8. Outbreaks in 2006 in Norway were identified as biotype 2 and 4 and indicated a processed pork product to be the likely source (Grahek-Ogden et al. 2007; Stenstad et al. 2007). In the United States, raw pork intestines were found to be the source of an outbreak among infants (Lee et al. 1990; Jones 2003).. The occurrence of pathogenic *Y. enterocolitica* in pigs and pork has been established by PCR in several studies (Korte et al. 2003; Fredriksson-Ahomaa et al. 2003). The *ail* gene located within the genome of pathogenic *Y. enterocolitica* strains is the most frequently used target of amplification for positive identification. In Switzerland, the prevalence of *ail*-positive *Y. enterocolitica* in tonsils of slaughter pigs was shown to be 88 % by PCR and 34 % by culture methods (Fredriksson-Ahomaa et al. 2007). In the USA, *ail*-positive *Y. enterocolitica* were detected in 12 % of pig feces sampled by PCR, and in 4 % of them using culture methods. Similarly, 40 % of the pig lymph nodes were positive by PCR, but none by culturing (Boyapalle et al. 2001). These results indicate that PCR based assays are the most sensitive and accurate means to detect *Y. enterocolitica* colonization.

Clinical presentations of yersiniosis are typical of enteric illness. Infants and children often present with fever, vomiting, and bloody diarrhea that can last from 3–28 days (Metchock et al. 1991; Lee et al. 1991). Adults generally have one to two weeks of fever, diarrhea, and abdominal pain that can mimic appendicitis. In more severe cases of gastroenteritis, necrotizing enterocolitis and ulceration may occur. *Y. enterocolitica* can also cause septicemia, leading to abscesses in the liver and spleen, pneumonia, septic arthritis, meningitis, cellulitis, empyema, osteomyelitis, and may evolve into endocarditis. Post-infection sequelae may also occur, particularly after infections with biotype 4, serotype O:3 (Bottone 1999). Reactive arthritis and erythema nodosum are the most common sequelae, butglomerulonephritis and myocarditis have also been reported (Bottone 1997).

Yersiniosis is diagnosed by positive identification of *Y. enterocolitica* in stool samples, although it is not routinely tested for. It can also be recovered from the throat, lymph nodes, joint fluid, urine, bile, or blood. Most cases resolve on their own, although it may take up to 3 weeks to recover. In severe cases, antibiotics such as aminoglycosides, doxycycline, trimethoprim-sulfamethoxazole, or fluoroquinolones may be prescribed. Prevention is key in avoiding infection. Raw or undercooked pork and unpasteurized milk or milk products should be avoided, as should drinking untreated water. Good hand hygiene when preparing food and after contact with animals should also be practiced to avoid infection.

2.1.2 Staphylococcus aureus

Staphylococcus aureus is a nonmotile, nonspore-forming, Gram positive coccus that occurs singly, in pairs, or in clusters. *S. aureus* produces protein A (*spa*), which is used in molecular testing for strain typing purposes, as well as several other toxins and superantigens (De Vos et al. 2009).

S. aureus is often isolated from the nasal membranes and skin of warm-blooded animals. Approximately 20–30% of the human population is colonized with *S. aureus* in the nose, throat, or both (Smith et al. 2012; Gorwitz et al. 2008; Graham et al. 2006). The most important site for colonization is the anterior nares (Wertheim et al. 2005). Colonization itself is not harmful; however, it is a risk factor for developing subsequent infections (Graham et al. 2006; Fritz et al. 2009). Both asymptomatic carriers and infected individuals may transmit the bacterium to others through close contact. *S. aureus* may also be acquired via contact with fomites contaminated with the organism, as well as with animals that are colonized or infected with *S. aureus*.

Skin infections including furuncles, carbuncles, impetigo, and scalded skin syndrome, as well as more severe infections like pneumonia, osteomyelitis, endocarditis, myocarditis, pericarditis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, cerebritis, meningitis, bacteremia, toxic shock syndrome, and abscesses of muscles, skin, and organs can occur as a result of *S. aureus* infection.. Other mammals and birds are also susceptible to infections, including mastitis, synovitis, arthritis, endometritis, furuncles, suppurative dermatitis, pyemia and septicemia (De Vos et al. 2009). Pigs are common carriers of *S. aureus*; one study in the U.S. found overall MRSA prevalence was 70% (147/209) from seven farms in the Midwest (Smith et al. 2009). In the Netherlands, surveillance for MRSA on hog farms has shown that isolates obtained from swine and their human caretakers are frequently indistinguishable, suggesting that the organism is transmitted between the two species (Smith et al. 2009; Huijsdens et al. 2006; Khanna et al. 2007).

S. aureus infections are often resistant to many antibiotics. Approximately 1.5% of the U.S. population carries methicillin-resistant S. aureus (MRSA) (Gorwitz et al. 2008). Resistance to methicillin developed within 6 months of the first clinical use and has become a major cause of morbidity and mortality around the world. In the U.S. in 2011, there were 80,461 invasive MRSA infections, an incidence rate of 25.82 cases per 100,000 persons. Many animals, including cows, goats, sheep,

rabbits, and poultry, can be infected by S. aureus, and these infections can have large economic costs (Fitzgerald 2012).

The epidemiology of MRSA has changed rapidly in the past few decades. After developing resistance in the 1960s following methicillin introduction, MRSA became a superbug that primarily affected hospitalized patients. Due to association with the healthcare environment, these infections were called healthcare-associated MRSA (HA-MRSA). More recently, cases of MRSA infection have been detected in people without prior hospitalization and with no underlying illnesses or healthcare related risk factors; these are referred to as community-associated MRSA (CA-MRSA) infections. Cases of HA-MRSA are usually resistant to several classes of antibiotics and tend to carry the methicillin-resistance gene, *mecA*, on the Staphylococcal Chromosome Cassette (SCC) of type II (SCC*mec* type II). They are often associated with *spa* type t002 and multi-locus sequence type (MLST) ST5. Contrastingly, CA-MRSA infections tend to be resistant to fewer classes of antibiotics, carry the Panton-Valentine leukocidin (PVL) encoding gene, and carry SCC*mec* type IV, *spa* type t008, and MLST ST8. A third group of infections, livestock-associated MRSA (LA-MRSA), has recently been identified (Wulf and Voss 2008) and has typically been associated with swine or cattle. LA-MRSA include strains such as ST398 and ST9, often carry SCC*mec* type V, are typically PVL negative, and (like HA-MRSA) tend to be resistant to multiple classes of antibiotics. However, both CA-MRSA and LA-MRSA have caused nosocomial infections in hospitals (Jenkins et al. 2009; Fanoy et al. 2009; van Rijen et al. 2008, van Rijen et al. 2009; Wulf et al. 2008; Kourbatova et al. 2005; Seybold et al. 2006; Tattevin et al. 2009).

Livestock-associated MRSA first came to attention in 2005 after its identification in pigs in France (Armand-Lefevre et al. 2005) and in swine farmers in the Netherlands (Wulf and Voss 2008). Dutch researchers found that swine farmers were colonized with MRSA at a rate of 760 times higher than that of the general population (Voss et al. 2005). Since then, LA-MRSA has been found in a number of countries in Europe, Asia, and the Americas (Smith and Pearson 2011; Graveland et al. 2011; Fluit 2012).

Recent reports from Germany and the Netherlands have found a high proportion of ST398 carriage in areas that have a high density of livestock (Kock et al. 2009; Kock et al. 2011; Wulf et al. 2012). While originally thought not to cause severe infections, there have been increasing reports of invasive disease caused by ST398 (Hartmeyer et al. 2010; Mammina et al. 2010; Potel et al. 2010; Aspiroz et al. 2010). Methicillin-sensitive S. aureus(MSSA) ST398 isolates have also caused invasive disease in the eastern U.S. (Mediavilla et al. 2012), Europe (Witte et al. 2007; Declercq et al. 2008; van Belkum et al. 2008), South America (Jimenez et al. 2011) and Canada (Golding et al. 2010), and at least one death in France (Laurent 2009).

While the majority of individuals colonized or infected with LA-MRSA have had contact with swine, colonization with ST398 has also occurred in individuals lacking any identified contact with a livestock reservoir (Bhat et al. 2009; Aires-de-Sousa et al. 2006). It has been suggested that one mode of transmission into the community is via contaminated food. Numerous studies in the U.S. have found MRSA in 5% of 120 meat samples (Pu et al. 2008), MSSA in 16.4% and MRSA

in 1.2% of 125 meat samples (Hanson et al. 2011), MSSA in 64.8% and MRSA in 6.6% of 256 pork samples (O'Brien et al. 2012), and multi-drug resistant *S. aureus* in 52% of 136 meat and poultry samples (Waters et al. 2011). Additionally, two studies in the Netherlands found rates of 2.5% of 79 pork and beef samples (van Loo et al. 2007) and 11.9% of 2217 meat and poultry samples, respectively (de Boer et al. 2009). However, to date there have not been any confirmed infections with ST398 caused by contaminated food.

Most MRSA skin infections appear as pustules or boils which often are red, swollen, painful, and have pus or other drainage. They often are mistaken for spider or insect bites. These skin infections commonly occur at sites of visible skin trauma, such as cuts and abrasions, and areas of the body covered by hair. Health professionals may provide antibiotics and drainage if necessary to treat such infections. More severe infections may require hospitalization and intravenous antibiotic therapy. Good hygiene is the key to prevention of MRSA infections.

2.1.3 Salmonella

Salmonella is a genus of Gram-negative, rod shaped, non-spore forming enterobacteria with peritrichous flagella. Originally classified utilizing serotyping of the somatic lipopolysaccharide (O) and flagellar protein (H) antigens, each serological variant (serovar) was considered its own species under the *Salmonella* genus (White 1926; Kauffmann 1978) as reviewed in (Beltran et al. 1988). This methodology led to misclassifications due to horizontal transfer of cell surface antigens, leading to classification of genetically distinct strains within the same serovar (Beltran et al. 1988; Selander et al. 1990).

In 2005, the Judicial Commission of the International Committee for Systematics and Prokaryotes (JICSP) decided to change the type species of the *Salmonella* genus to *enterica* with subspecies and serovars (Prokaryotes JCotICoSo 2005). The JICSP indicated *Salmonella enterica* had seven subspecies, *enterica* (type I), *salamae* (type II), *arizonae* (type IIIa), *diarizonae* (type IVb), *bongori* (type V), *houtenae* (type IV), and *indica* (type VI). Subspecies *bongori* was shortly after promoted to species status (Grimont and Weill 2007). A third *Salmonella* species was approved by the JICSP in 2005, named *Salmonella subterranea* (Shelobolina et al. 2004), but this species may not fit within the genus *Salmonella* (Grimont and Weill 2007). *S. bongori* and all subspecies of *S. enterica* besides *S. enterica* subsp. *enterica* are associated mainly with cold-blooded animals (Aleksic et al. 1996; Woodward et al. 1997), (Aleksic et al. 1996; Woodward et al. 1997), but can rarely cause human infection (CDC 2008; CDC 2012). The primary cause of human infection is *S. enterica* subsp. *enterica* (CDC 2008), as referenced in (Desai et al. 2013).

The CDC defines salmonellosis as an infection with a *Salmonella* spp. bacterium. These infections can often manifest with diarrhea (potentially bloody), fever, and abdominal cramps between 12 and 72 h post infection (CDC 2009). The illness often lasts between 4 and 7 days and is usually self-limiting. *Salmonella* infection can necessitate hospitalization in a small number of individuals (Mead et al. 1999).

Each year, *Salmonella* spp. cause roughly 1.3 billion cases of nontyphoidal salmonellosis worldwide (Chimalizeni et al. 2010). Within the United States, there were an estimated 1.4 million cases in 1999, with 95% of these estimated to be caused by foodborne exposure to *Salmonella* (Mead et al. 1999). The burden on the United States economy from these estimated 1.4 million cases was estimated to be between $ 0.5 billion and $ 2.3 billion (Frenzen et al. 2002). These estimates are likely underestimates due to the omission of secondary complications due to *Salmonella* infections. The estimates fail to include complications such as reactive arthritisor costs such as pain and suffering, or travel to obtain medical care.

The most important zoonotic reservoir for *Salmonella* are food animals, with the most important food product being eggs (Ebel and Schlosser 2000). Egg consumption has been shown to be the largest risk factor associated with *Salmonella enterica* infection (Hope et al. 2002). Pork contamination is also a possible source of human infection.. In swine, *Salmonella* infection is mainly subclinical, with rare cases manifesting as enterocolitis or septicemia (Barker and Van Dreumel), as referenced in (Fosse et al. 2009). In the United States, the percentage of farms positive for *Salmonella* are estimated to range between 38.2 and 83% with the number of positive pigs in the US from 6 to 24.6% (Oosterom and Notermans 1983; Davies et al. 1997). Transmission from pig to pig is often due to fecal shedding of the bacteria. Within swine herds, sows were observed to have an increase in *Salmonella* shedding at weaning (Nollet et al. 2005) as well as in their weaned piglets (Kranker et al. 2003). While *Salmonella* is considered primarily fecal borne, swine feed has also been shown to be a potential source of *Salmonella* infection for swine (Harris et al. 1997) with experimental data showing animals may become infected through the consumption of contaminated feed (Smith 1960). Additional risk factors for transmission between herds of swine are: contact with humans, contaminated equipment, or contaminated slurry (Langvad et al. 2006).

Individual outbreaks of *Salmonella* spp. have also been attributed to pork products. In 1989, a small northern England town experienced an outbreak where 206 individuals were infected with serovar Typhimurium (Maguire et al. 1993). Serotyping and antibiotic resistance profiles matched the infective strain to that found in cold cuts of pork purchased from a local butcher shop. In a study by Davies et al., several of the most prevalent serotypes found in swine were also among the most common causes of human infection (Davies et al. 1997).

Attempts to control *Salmonella* spp. prevalence on farms have had mixed outcomes. The use of all-in/all-out systems with multiple sites handling different stages of the rearing process have been shown to have no benefit in reducing *Salmonella* prevalence when compared to farrow-to-finish systems (Davies et al. 1997). These all-in/all-out systems may actually have a greater prevalence of *Salmonella* in finishing pigs than farrow-to-finish systems and fecal shedding of *Salmonella* was higher than observed in farrow-to-finish (Davies et al. 1997). Number of pigs per pen was also observed to be a risk factor for fecal shedding of *Salmonella* (Linton et al. 1970). Acidification or fermentation of feed is postulated to be protective against *Salmonella* contamination as dry feed and trough feeding have been shown to have an increased contamination risk (Lo Fo Wong et al. 2004; van der Wolf

et al. 1999, van der Wolf et al. 2001), but this has not been studied extensively using experimental designs.

In North America, *Salmonella* control programs have been implemented at slaughter to decrease human exposure to *Salmonella* (Funk and Gebreyes 2004). This Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) system established slaughter point performance standards for processing plants and has been shown to decrease contamination of pork products with *Salmonella* (Agriculture FSaISUDo 2004). In European Union countries, a farm-to-slaughter program has been implemented to reduce *Salmonella* (Lo Fo Wong et al. 2002). This plan calls for control measures at all production levels and focuses specifically on transportation and handling of the swine to limit the transmission between herds. In addition to prevention methods within the production system, consumer prevention is recommended by the CDC (CDC 2010). In addition to recommendations dealing with protecting infants from *Salmonella* exposure, the CDC suggests cooking meat and poultry thoroughly, washing hands, utensils, and kitchen surfaces following contact with raw meat or poultry.

2.1.4 Campylobacter

Campylobacter is a genus of gram-negative, spiral-spiral shaped bacteria that causes disease in both humans and animals (CDC 2010). *Campylobacter* is the most common cause of gastroenteritis in many developed (Nichols et al. 2012) and developing countries, causing more diarrhea than *Salmonella* globally (WHO 2011). In developing countries, infections of those under the age of two are most frequent (WHO 2011). While there are 17 species in the *Campylobacter* genus, *C. jejuni* and *C. coli* are the most frequent causes of infection (WHO 2011). Most cases are sporadic events and not part of outbreaks (CDC 2010). The main route of transmission from animals to humans is through undercooked meat and meat products, contaminated milk, or contaminated water (WHO 2011).

Disease in humans usually occurs two to five days after infection (WHO 2011) and presents with diarrhea, cramping, abdominal pain, and fever. Most infected individuals recover within five to ten days. In some severe cases, a small amount of people may develop Guillian-Barré syndrome. *Campylobacter* is thought to be responsible for between 20% (Tam et al. 2007) to 40% of cases of Guillian-Barré syndrome (CDC 2010). *Campylobacter* infections tend to be higher in males across all age groups, which suggests a higher susceptibility in males and not participation in at-risk behaviors (Nichols et al. 2012; Louis et al. 2005). In recent years, infections in those over 50 years of age have become more common, especially in men, as has infection in those between 20 and 32 years (Nichols et al. 2012). The increase in infections in those over 50 may be due to use of proton pump inhibitors (PPI's) (Nichols et al. 2012; Leonard et al. 2007). Seasonality of the infection has been noted, with the greatest impact of seasonality being in young children (Nichols et al. 2012). *Campylobacter* infections rates begin to rise in May and peak between

mid-June and mid-July (Nichols et al. 2012; Louis et al. 2005). This seasonality has been observed in many temperate countries (Nylen et al. 2002). Infection rates also tend to be higher in rural compared to urban regions (Strachan et al. 2009; Sibbald and Sharp 1985). This could be reflective of proximity to livestock or differences in access to healthcare (Nichols et al. 2012). Since 1989, there has been a steady increase in the presence of antimicrobial resistant *Campylobacter* isolates. Full and intermediate resistance to ampicillin, ciprofloxacin, nalidixic acid, tetracycline, and erythromycin has been shown (Nichols et al. 2012).

When swine are infected with *Campylobacter*, it is frequently *C. coli*, however, *C. jejuni* has been seen recently as well (Jensen et al. 2006). *Campylobacter* infections can cause diarrhea in pigs, and often colonizes the intestinal tract. Both *C. jejuni* and *C. coli* have been found in the intestinal tract of pigs and are known to be excreted in their feces (Jensen et al. 2006). *Campylobacter* has also been identified in the stomach, tonsils, liver, and carcass surfaces of swine. High colonization rates may represent an occupational health hazard, since a low dose of bacteria can cause infection (Nesbakken et al. 2003). Antimicrobial susceptibility to ciprofloxacin and nalidxic acid has been reported in swine strains. It has also been shown that *C. coli* has higher levels of quinolone resistance than *C. jejuni* in swine (von Altrock et al. 2013). However, it is unlikely that swine are a major source of foodborne *Campylobacteriosis*, as *Campylobacter* is rarely detected in retail pork, but may be a source of occupational exposure (Nesbakken et al. 2003). It has also been shown that while there is contamination of pigs in slaughter houses, *Campylobacter* spp. do not spread throughout the operation (von Altrock et al. 2013).

Campylobacter infections do not generally require treatment and are self-limiting (CDC 2010). When disease is severe, electrolyte and fluid replacement may be necessary. Antimicrobials (erythromycin, tetracycline, and quinolones) can be used to treat severe disease or to eliminate carriage (WHO 2011). Several steps can be taken to prevent *Campylobacter* infection. Proper food handling and hand hygiene can help prevent infection. All meats should be thoroughly cooked and measures should be taken to prevent cross contamination. Hands should be washed thoroughly before handling food and persons with diarrhea should wash their hands frequently to reduce the spread of infection (CDC 2010). Improved biosecurity measures and hygienic slaughtering practices will reduce the fecal contamination of carcasses (WHO 2011). Cooling meat with CO_2 has also been shown to kill the bacteria (Nesbakken et al. 2003). Adequate disposal of feces and decontamination of fecal contaminated articles will also help reduce transmission (WHO 2011).

2.1.5 Streptococcus suis

Streptococcus suis (*S. suis*) is a Gram-positive facultative anaerobe bacterium reported to colonize and cause infections primarily in the swine population worldwide (Fulde and Valentin-Weigand 2013; Wertheim et al. 2009). In conjunction with *Actinobacillus suis* and *Haemophilus parasuis*, *S. suis* completes the triad of the

"Suis-ide" disease agents given its association with a wide range of severe clinical conditions in the swine population (MacInnes and Desrosiers 1999). *S. suis* causes severe infections in pigs resulting in major economic losses to the porcine industry worldwide (Fittipaldi et al. 2012). Zoonotic infections due to *S. suis* have been reported in countries with a high density of pigs and intensive swine production (Lun et al. 2007; Wertheim et al. 2009). The increasing prevalence of infections due to *S. suis* both in swine and humans over the last few years have urged investigators to better understand the epidemiology and zoonotic potential of this primarily "pig pathogen".

S. suis isolates are verified by serotyping using slide agglutination test, capsular reaction, capillary precipitation or a co-agglutination test (Staats et al. 1997). Serotyping is based on polysaccharide capsular antigen detection. Thirty-five serotypes (1–34 and 1/2) have been identified using these tests (Lun et al. 2007; Higgins and Gottschalk 1990; Gottschalk et al. 1989, 1991a, b, 1999; Higgins et al. 1995). Serotypes 32 and 34 are observed to be closely related to *S. orisratti* (Hill et al. 2005). Serotype 2 is the most frequently reported serotype worldwide and is considered the most pathogenic both in pigs and humans. Other serotypes implicated in diseases are types 1–9 and 14 (Gottschalk et al. 2007).

Pigs colonized with *S. suis* typically harbor the organism in their tonsils and may never exhibit clinical signs or symptoms (carriers). Some carrier piglets eventually develop bacteremia, septicemia or meningitis due to dissemination of *S. suis* from tonsils and other mucosal surfaces (Fittipaldi et al. 2012; Staats et al. 1997). Disease syndromes in swine also include arthritis, pneumonia, endocarditis, encephalitis, polyserositis, abscesses and abortion (Wertheim et al. 2009). Death occurs within hours of the onset of clinical signs in pigs with peracute, i.e. very violent or acute forms of infection. Acute disease typically characterized by fever (up to 42 °C), depression, anorexia and lassitude may result in deaths, chronicity, or healthy carriers. In its chronic form, lameness and/or residual central nervous system signs may be apparent (Fulde and Valentin-Weigand 2013). Clinical manifestations of *S. suis* are observed to vary by geographical location (Wangkaew et al. 2006; Yu et al. 2006; Tang et al. 2006). There have been varying reports on the incubation period of *S. suis* ranging from 3 h to 14 days (Yu et al. 2006), and 60 h to 1 week (Mai et al. 2008). Short incubation periods are found to be consistent with direct entry of *S. suis* into the blood stream through skin wounds. There have been no consistent findings in seasonal variation of *S. suis* infection (Wangkaew et al. 2006; Mai et al. 2008; Huang et al. 2005).

S. suis infection is reported in domesticated pigs (Staats et al. 1997). In addition, the organism has been isolated from the intestinal flora of wild boars, dogs, cats, horses, deer and ruminants (Staats et al. 1997; Devriese et al. 1992; Baums et al. 2007; Devriese and Haesebrouck 1992). The rate of asymptomatic carriage in pigs is estimated to be around 80 %, representing a potential source of infection to other animals and humans (Lun et al. 2007; Staats et al. 1997; Arends et al. 1984; Ngo et al. 2011).. Pigs acquire *S. suis* via vertical and horizontal transmission as the sow is capable of harboring *S. suis* in the genital tract (Fulde and Valentin-Weigand 2013; Fittipaldi et al. 2012; Gottschalk 2011). Carrier rates are highest in

pigs between 4 and 10 weeks of age, but infection can occur at any age (Staats et al. 1997; Clifton-Hadley et al. 1984). Environmental contaminants such as feces, dust, water and feed are considered to be secondary sources of infection (Staats et al. 1997). Vectors such as houseflies (Fulde and Valentin-Weigand 2013; Staats et al. 1997; Enright et al. 1987) and mice (Fulde and Valentin-Weigand 2013; Staats et al. 1997; Williams et al. 1988) are also considered to play a role in disease transmission to pigs. Factors such as stress, crowding, poor ventilation, and concurrent disease could potentially predispose herds to an outbreak of *S. suis* infection (Fulde and Valentin-Weigand 2013; Staats et al. 1997). Morbidity rate in pigs ranges from 50%, rarely exceeding 5% (Wertheim et al. 2009). Nevertheless, research has demonstrated that morbidity due to *S. suis* is severely enhanced in the presence of other bacterial and viral infectious agents suggesting the importance of surveillance for *S. suis* (Staats et al. 1997).

Human *S. suis* infection is considered an emerging zoonosis (Lun et al. 2007; Wertheim et al. 2009). Studies observed that longer duration of exposure to pigs and pork affects *S. suis* carriage in the population (Elbers et al. 1999; Smith et al. 2008; Strangmann et al. 2002). Infection rate in individuals with high-risk exposures is estimated to be 1500 times higher than that of the general population (Lun et al. 2007; Arends and Zanen 1988). Pig farmers (Smith et al. 2008; Bartelink and van Kregten 1995; Breton et al. 1986; Sriskandan and Slater 2006; Fowler et al. 2013), abattoir-workers (Arends and Zanen 1988; Bartelink and van Kregten 1995; Breton et al. 1986), veterinarians (Elbers et al. 1999), hunters (Baums et al. 2007; Halaby et al. 2000) and meat-processing workers (Tramontana et al. 2008; Yu et al. 2005) are observed to have a higher risk of *S. suis* infection. Consumption of uncooked or partially cooked pork products is also considered a potential risk factor for *S. suis* infection (Wertheim et al. 2009; Wangsomboonsiri et al. 2008). A mortality rate of 17% was observed in the population and about 2/3 of deaths occurred in the first 24 h after admission (Wangsomboonsiri et al. 2008). Human infections are typically reported as sporadic cases with an exception of two large outbreaks resulting in 25 and 204 cases, and 14 and 38 deaths, respectively (Yu et al. 2006; Tang et al. 2006). Person-to-person transmission is unlikely to occur without very close contact such as with infected blood. Nevertheless, there is strong evidence of *S. suis* transmission from pigs to humans and a great potential for reverse zoonoses, i.e. transmission from humans to animals.

S. suis is sensitive to antibiotics such as penicillin, ampicillin, amoxicillin, ceftriaxone and cephalosporin (Lun et al. 2007). Clinical disease is known to be suppressed by fortifying feed with antibiotics at therapeutic levels (Staats et al. 1997). However, it does not eliminate carriers thus negatively impacting transmission of *S. suis*. One of the major drawbacks is the development of antimicrobial resistant *S. suis* isolated from both pigs and humans (Mai et al. 2008; Gottschalk et al. 1991c; Prieto et al. 1994; Aarestrup et al. 1998; Marie et al. 2002; Shneerson et al. 1980; Wisselink et al. 2006; Vela et al. 2005). Vaccines currently in use prevent outbreak in pig herds, but are observed to have varying efficacy (Lun et al. 2007; Haesebrouck et al. 2004). A human vaccine for *S. suis* is not available (Lun et al. 2007; Wertheim et al. 2009).

Prevention of *S. suis* transmission in both humans and pigs depends on control of contact with sick animals. Improving pig-raising and breeding conditions, and vaccination of pigs could ensure reduction in *S. suis* infection outbreaks and prevent transmission to humans (Lun et al. 2007). In addition, the potential risk of transmission via contact or consumption of contaminated pork products can be diminished by education and increasing awareness on preventative measures to eliminate this mode of transmission (Lun et al. 2007). World Health Organization (WHO) recommends cooking pork to an internal temperature of 70 °C or until juices appear clear rather than pink (Lun et al. 2007). Use of clean gloves and hand hygiene should also be encouraged when handling raw or undercooked pork products. Review of the current literature exposed a knowledge gap on differences in the virulence capacity and geographical variation of *S. suis* strains. Addition of this information to other available epidemiological data on *S. suis* is warranted to prevent further propagation and losses worldwide due to this pathogen.

2.1.6 Shiga-Toxin Producing Escherchia coli (STEC)

Escherchia coli, a short, rod shaped, Gram-negative, non-sporing, facultative anaerobic bacterium belongs to the family *Enterobacteriacae* (Sussman 1985; Mainil 2013). The gastro-intestinal tract of humans and other warm blooded animals are the primary hosts of this organism (Cheleste et al. 2002; Bell 2002). Although most *E. coli* strains are non-pathogenic, and part of normal microflora, some strains have evolved as pathogenic (Mainil 2013; Bell 2002, 2012). Pathogenic strains of *E. coli* acquire mobile virulence gene located on pathogenicity islands, integrated bacteriophages, or on plasmids (Bell 2002, 2011), and are able to cause wide spectrum of diseases in many species including pigs, cattle, rabbits and humans (Mainil 2013; Jay et al. 2007). On the basis of their virulence traits, pathogenic strains of *E.coli* are categorized into at least six groups: entero-pathogenic *E.coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), entero-haemorrhagic *E. coli* (EHEC), entero-aggregative *E. coli* (EaggEC), and diffusely adherent *E. coli* (DAEC) (Bell 2002, 2012; Catalina Lopez-Saucedo et al. 2003) .

Shiga toxin-producing *E. coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC) are a diverse group of pathogens that has become of significant health concern. These strains of *E. coli* are able to cause disease in both humans and animals. Although EHEC 0157:H7 is recognized as the most prominent STEC, over 200 non-O157:H7 STEC serotypes have been identified, and over 100 strains can cause disease in humans (Bell 2002; Josefa et al. 2005; Fratamico et al. 2004; Patricia and Griffin 1991). In the United States, most EHEC strains are serotype O157:H7 that accounts for 30–50 % of EHEC strains (Johnson and Sears 2006). Infection with non-O157:H7 serotype is more common in other nations including Australia, Argentina and many European countries, and may account for the majority of haemolytic uremic syndrome (HUS) infections in these countries (Fratamico et al. 2004). Serotypes 026, 045, 0103, 0111, 0121, and 0145 have been associated

with human disease and may account for approximately 70% of Non-O157:H7 STEC human infections in the United States (Wells et al. 2012). *E. coli* O157:H7 was first identified as pathogenic strain following two outbreaks of hemorrhagic colitis by consuming undercooked ground beef in 1982 in the United States (Josefa et al. 2005; Patricia and Griffin 1991; Pennington 2010; Beilei Ge et al. 2002; phillip Tarr and Chandler 2005). Since the discovery of *E. coli* O157:H7, large food-borne outbreaks and sporadic incidence have been documented in the United States and many parts of the world (Bell 2011; phillip Tarr and Chandler 2005; Tiiomas et al. 1995). Annually, EHEC O157:H7 and other serotypes of STEC accounts approximately 110,000 cases of illness in the United States (Cornick and Helgerson 2004).

STEC is a worldwide public health threat. Over 100 different serotypes can cause human illness (Acheson 1999). The exact global prevalence of STEC infection is unknown since there is no uniform surveillance and reporting system. Annually, an estimated 73,000 cases are caused by *E. coli* O157:H7 in the United States leading to estimated 2168 hospitalizations and 61 deaths (Josefa et al. 2005; Beilei Ge et al. 2002). Non-O157:H7 accounts for 37,740 cases and 30 deaths annually in the United States (Beilei Ge et al. 2002). Studies have indicated that STEC infection is more prevalent in the northern regions of the United States, and is more common in summer season (phillip Tarr and Chandler 2005; Tiiomas et al. 1995). *E. coli* O157:H7 can infect people of any age. However, children and elderly are more prone to develop severe illness and HUS compared to any other age groups (phillip Tarr and Chandler 2005; Bell 2011). Various studies have suggested that animals including cattle, sheep, goats and pigs are reservoirs for different STEC strains (Cheleste et al. 2002; Bell 2002; Fratamico et al. 2004). Although cattle are considered to be the primary reservoir of *E. coli* O157:H7, it is implicated in fecal shedding of other domestic livestock and wildlife (Jay et al. 2007). Evidence from epidemiological studies suggests that domestic pigs are potential reservoirs and biologically competent hosts of *E.coli* O157:H7 (Jay et al. 2007; Fratamico et al. 2004; Cornick and Helgerson 2004). In 2006, spinach associated outbreak of *E. coli* in the United States caused 205 cases of illness and six deaths. A successful isolation of the outbreak strain from feral swine living close to spinach field provides insight on swine-to-swine transmission and transmission between cattle and swine.. A study conducted by Jay et al. was able to recover related *E. coli* O157: H7 subtypes from feral swine, cattle, surface water, soil and sediment that were contaminated with spinach causing the outbreak (Jay et al. 2007). *E. coli* O157:H7-infected swine can shed the bacteria in feces for about two months thus serving as a reservoir host (Cornick and Helgerson 2004). Rios et al. isolated enterohemorrhagic STEC subgroup 026 and 0111 from the intestinal content of pigs. These strains had virulence genes (*stx1*, *stx2*) suggesting they were potential human pathogens (Fratamico et al. 2004; Maritza Rios et al. 1999). Fratamico et al. isolated STEC serogroup O2, O5, O7, O8, O9, O15, O65, O91, O101, O120, O121, O163, and several others from fecal samples of pigs (Fratamico et al. 2004). Other studies have indicated that STEC strains can be isolated from both healthy pigs and pigs with diarrhea and edema disease (Fratamico et al. 2004; Cornick and Helgerson 2004).

Various O, H, and K antigens of *E.coli* are identified (Kauffmann 1947). Virulent strains have gens for fimbriae, adhesions, and wide varieties of exotoxins that help pathogenic *E. coli* to colonize human tissues (Mainil 2013). *E. coli* O157:H7 produces a type III secretion system that injects two types of proteins which disrupt the cells metabolism and provide surface for attachment (Mainil 2013; Pennington 2010). Shiga toxin is the key virulence factor of STEC (Patricia and Griffin 1991; Werner Brunder and Helge 1997), and it causes necrosis of host cells and tissues (Pennington 2010). Although several virulence factors encoded by a 60-MDa plasmid such as a bifuctional catalase-peroxidase, secreted serine protease(EspP), α-hemolysin (EHEC-Hly), and chromosomally encoded enterotoxin EAST1 have been found, their role in pathogenicity still remains unclear (Cheleste et al. 2002; Werner Brunder and Helge 1997; Paul and Mead 1998). All *E. coli* belonging to STEC strains can produce Shiga toxin1 (*Stx1*) and/or Shiga toxin 2 (*Stx2*) or variants of *Stx1 or Stx2*. *Stx2e* variant strain of STEC cause edema disease in swine (Fratamico et al. 2004; Patricia and Griffin 1991).

The incubation period of STEC infection is 2–4 days, but may vary from 1–5 days (Acheson 1999). Many people infected with STEC remain asymptomatic (Pennington 2010); others suffer from mild to severe gastro-intestinal symptoms. STEC infection ranges from mild to life-threatening. Symptoms include watery diarrhea which can be bloody as the disease progresses), severe abdominal pain, low to mild-grade fever and nausea and vomiting. Fecal and peripheral leukocytosis is often present. Most people recover within 5–7 days of the onset of infection (Cheleste et al. 2002; Bell 2002; Patricia and Griffin 1991; Acheson 1999). Hemolytic uremic syndrome (HUS) is developed in 5–10 % of STEC cases (Acheson 1999). HUS is a serious complication characterized by hemolytic anemia, thrombocytopenia, fever, and kidney damage (Cheleste et al. 2002; Josefa et al. 2005; Acheson 1999; Frederick Koster et al. 1978). HUS often develops in children below age 5 as a complication of *E. coli* infection. HUS accounts 15 % of EHEC infection in children below 10 years old. HUS is seen as a complication in 6–9 % of overall infections (Bell 2002; phillip Tarr and Chandler 2005; Tiiomas et al. 1995). 5–10 % of HUS patient may die or develop further complications (stroke) (Cheleste et al. 2002). An estimated 50 % of HUS patients may have permanent kidney damage. Since patients with HUS are in risk of renal failure, they should be hospitalized (Cheleste et al. 2002; Acheson 1999).. The mortality of HUS is approximately 5 % (Acheson 1999), and the case fatality rate of HUS is approximately 10 % (Bell 2002).

The use of antibiotics could aid in Shiga toxin production thus exacerbating the disease; as such, this treatment is not recommended in the United States. Symptomatic treatment along with maintaining hydration is very important to prevent further complications. Prevention is the most important aspect of STEC infection (Acheson 1999; Paul and Mead 1998). Frequent hand-washing is the most effective tools to avoid person-to-person transmission. Proper handling of foods, preventing temperature abuse and cross-contamination of foods as well as maintaining a proper storage temperature is essential. Boiling water before drinking can help to stop waterborne transmission in developing countries where drinking water system is poor. The practice of using animal fecal as manure for crops used for human consumption

should be stopped. Foods should be cooked to the optimum temperature. Undercooked meat and unpasteurized milk should not be consumed (Bell 2002, 2012; Acheson 1999).

2.1.7 Japanese Encephalitis Virus (JEV)

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus*, family *Flaviviridae* (Weaver and Barrett 2004; Andrew et al. 2009; Solomon 2004). This virus was first isolated from a fatal human encephalitis case in Japan in 1935 (Weaver and Barrett 2004) and from *Culex tritaeniorhynchus* mosquitoes in 1938 (Andrew et al. 2009). This arbovirus (arthropod-transmitted) (Weaver and Barrett 2004; Igarashi 2002) is the leading cause of worldwide epidemics of viral encephalitis (Weaver and Barrett 2004; Tom Solomon et al. 2000). This single stranded positive sense RNA virus with a genome length of 11 kilobases (Weaver and Barrett 2004; Solomon 2004) consists of a spherical virion with a 30 nm core that is surrounded by a lipid envelop. The RNA genome of JEV encodes a single polypeptide that is cleaved into non-structural proteins such as NSI, 2A, 2B, 3, 4A, 4B, and 5, and structural capsid, member (M) and envelope (E) proteins (Tiroumourougane et al. 2002; Spickler 2007). The E protein plays vital antigenic role as it is important for viral attachment and entry into host cells (Solomon 2004; Mouhamadou Diagana and Dumas 2007). This virus has only one serotype and two subtypes, and is closely related to St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, and dengue fever virus (Solomon 2004; Tiroumourougane et al. 2002; Spickler 2007). On the basis of nucleotide sequencing of the viral pre-membrane (prM), JEV can be categorized into four different genotypes. Moreover, the phylogenic analysis of the viral envelop 'E' gene has classified JEV strains into five genotypes (Health WOfA2009). A wide range of host species might be infected by JEV including cattle, snakes, birds, pigs, horses and other farm animals (Weaver and Barrett 2004; Andrew et al. 2009; Spickler 2007). High heat (56 °C for 30 min), acidic environment (pH 1–3) and various chemicals and disinfectants such as iodine, phenol, and formaldehyde also inactivate the virus. JEV is quite sensitive to ultraviolet light and gamma irradiation (Health WOfA 2009).

JEV is transmitted between wild and domestic birds and pigs by *Culex* species mosquitoes (Tom Solomon et al. 2000; van-den-Hurk et al. 2008). *Culex tritaeniorhynchus* plays a major role, because many animals such as horses, swine, humans, and birds are susceptible hosts. It is also the most important vector for human infections (Weaver and Barrett 2004; Tom Solomon et al. 2000). These mosquitoes particularly breed in pools of stagnant water, especially in flooded rice fields (Tom Solomon et al. 2000; Erlanger et al. 2009). JEV has also been isolated from other species of mosquitoes (Tiroumourougane et al. 2002). Ardeid or wading birds (herons and egrets) are considered as the primary maintenance hosts (Igarshi 2002; van-den-Hurk et al. 2008; Erlanger et al. 2009) and pigs are the main amplifying hosts (Weaver and Barrett 2004; Andrew et al. 2009; Tom Solomon et al. 2000;

Spickler 2007; van-den-Hurk et al. 2008; Erlanger et al. 2009) which are necessary for pre-epizootic amplification of the virus. Pigs can act as maintenance hosts in endemic regions (Andrew et al. 2009). Pigs in close proximity to humans are the most important natural hosts for transmission of JEV to humans (Weaver and Barrett 2004; Solomon 2004; Tom Solomon et al. 2000; Tiroumourougane et al. 2002). Pigs have a prolonged and high viraemia and a high natural infection rate of 98-100 % (Andrew et al. 2009). Domestic pig rearing aids in the transmission to humans (Erlanger et al. 2009). Humans and horses are dead-end or incidental hosts (Andrew et al. 2009; Tiroumourougane et al. 2002). Human-to-human transmission of JVE has not been reported yet (Tiroumourougane et al. 2002).

JEV remains the major cause of viral encephalitis in Southeast Asia (van-den-Hurk et al. 2008), but it is widely spread in eastern and south-eastern Asian countries, the Pacific Rim, and in Northern Australia. However, related neurotropic flaviviruses are found worldwide (Tom Solomon et al. 2000; Erlanger et al. 2009). Japanese encephalitis claims about 50,000 human cases and 15,000 deaths annually (Weaver and Barrett 2004; Tom Solomon et al. 2000). Due to lack of surveillance and inadequate data collection the actual incidence rate might be a lot higher. It is estimated that 175,000 cases of Japanese encephalitis occurs annually worldwide.. 11,000 cases and more than 2000 deaths resulted from JEV outbreaks in Nepal and Northern India between 2005 and 2007 (Andrew et al. 2009). Children under 15 years of age are mainly affected by JEV in endemic areas (Tiroumourougane et al. 2002). Pediatric encephalitis is caused by this virus in many Asian countries including India, Korea and China. More than one third of world populations are at risk of infection of JEV. The epidemiological patterns of JEV involve endemic and epidemic activities in tropical regions and temperate and subtropical areas, respectively. There is no seasonal pattern in endemic areas, but epidemic activity is observed in summer and autumn months in temperate and subtropical areas. Migratory birds help the virus to travel large distances (Weaver and Barrett 2004). Japanese encephalitis is mainly a disease of rural areas. It is endemic in tropical regions and often associated with irrigated rice agriculture (Andrew et al. 2009). The annual incidence of Japanese encephalitis is between 10–100 per 100,000 population in endemic areas (Tiroumourougane et al. 2002).

The incubation period of Japanese encephalitis in man is not exactly known. It varies from 1–6 days, and can be as long as 14 days (Tiroumourougane et al. 2002). Incubation period in horses is 8–10 days (Spickler 2007). Most infections of Japanese encephalitis are asymptomatic. Clinical features are developed only in 1 in 50 to 1 in 1000 infections. The clinical manifestations range from mild flu-like illness to severe and lethal meningoencephalomyelitis (Andrew et al. 2009; Tom Solomon et al. 2000). High grade of fever with or without rigors, headache, general malaise, and vomiting are present in the prodromal stage. It is followed by the encephalitis stage which is characterized by abnormal movements, muscular rigidity, neck stiffness, convulsions, altered neurological functions and other CNS signs (Tiroumourougane et al. 2002). Convulsion often occurs and it is reported in about 85 % of children and 10 % of adults (Tom Solomon et al. 2000). The recovery stage may be accompanied by signs of CNS injury. Thick, slow speech, aphasia and paresis

are seen as residual neurological impairments. Complications include secondary bacterial infection, urinary tract infection, and statis ulcers (Tiroumourougane et al. 2002). Abnormal mental behaviours may exist in some patients leading to misdiagnosis of mental illness (Tom Solomon et al. 2000). Poliomyelitis-like acute flaccid paralysis and "fever associated seizure disorder" has also been identified in some cases (Tiroumourougane et al. 2002). Almost one third of patients admitted to hospital with Japanese encephalitis die (Tom Solomon et al. 2000), and 45 to 70 % of survivors suffer from neurological sequelae that last for months. The case fatality rate of Japanese encephalitis can be as high as 67 %. Higher fatality rates are seen in children and the elderly population (Andrew et al. 2009). The mortality rate varies in a range of 8.5 to 72 % (Tiroumourougane et al. 2002). Pigs with Japanese encephalitis exhibit reproductive disease with stillbirth as the most common symptom (Andrew et al. 2009).

JEV can be detected using reverse transcriptase polymerase chain reaction in human CSF samples. But the reliability of this test still remains unconfirmed. Serological tests such as IgM and IgG ELISA are widely used for the detection of antibodies to JEV in human and swine. However, this test requires complex equipment and it is not feasible in the rural areas of developing countries. A recent modification of this test to a simple nitrocellulose membrane based format is more useful in rural areas, since it does not require any sophisticated equipment and can be interpreted by eye vision (Tom Solomon et al. 2000). Virus neutralization and epitope blocking ELISAs can help to differentiate if cross-reactions have been occurred in serological tests due to the presence of other viruses of the JEV serogroup (Kitai et al. 2007; Jacobson et al. 2007). Various other serological tests such as haemagglutination inhibition, the complement fixation test, single radial hemolysis, and neutralisation are still in practice in some laboratories. Other antigen detection techniques in CSF include reverse passive haemagglutination, immunofluorescence, and staphylococcal coagglutination tests using polyclonal or monoclonal antibodies (Tom Solomon et al. 2000).

Treatment of Japanese encephalitis involves supportive and symptomatic care. Although isoquinolone compounds and monoclonal antibodies are effective *in vitro* and animal models, respectively, there is no specific treatment available for this disease. Controlling convulsion and raised intracranial pressure are crucial. Physical therapy and excellent nursing care are important to prevent further complications (Solomon 2004; Tom Solomon et al. 2000; Spickler 2007).

Prevention strategies should be focused on control of mosquito vectors, improvement in animal husbandry practices, changing agricultural practices, preventing amplification of the virus in pigs and birds, measures against reservoirs, and immunizing of species at risk (Andrew et al. 2009; Tom Solomon et al. 2000; Tiroumourougane et al. 2002; Spickler 2007; van-den-Hurk et al. 2008; Erlanger et al. 2009). Prevention of mosquito from biting human is essential (Tiroumourougane et al. 2002). Use of bed nets, wearing long sleeved shirts and long trousers, and application of insect repellent on exposed body surfaces, avoidance of outdoor sleeping, and decreasing outdoor activity during twilight and dawn would be beneficial (Spickler 2007; Mackenzie and Smith 2006). Relocation of piggeries away from

residential areas and making them mosquito proof could decrease the risk of JEV. Vaccine is recommended to people, especially for high-risk group such as laboratory workers with potential risk of exposure to JEV, travelers who spend more than a month in endemic areas, and residents of endemic areas. Travelers who spend less than 30 days during epidemics and carry out extensive outdoor activity should also get vaccinated. Vaccinations of pigs also yield positive results (Tiroumourougane et al. 2002). Vero cell-derived JEV vaccine is currently used in the United States (Fischer et al. 2010).

2.2 Nipah Virus

The first cases of Nipah virus (NiV) infection were identified during an outbreak in Malaysia in September 1998 and involved pig farmers who had contact with pigs on a regular basis (Luby and Gurley 2012). JEV was initially suspected due to the similar clinical presentation in humans and because it is endemic in the outbreak area in Malaysia (Chua 2012). Although known measures to control JEV were taken, such as vaccination, the number of cases did not decrease (Chua 2012). The cause of the outbreak was eventually discovered to be a novel paramyxovirus (Chua et al. 1999; Chua et al. 2000). Humans with direct contact with pigs were at an increased risk of contracting the virus (Chua 2003). During March 1999 in Singapore, there was an outbreak of NiV-associated encephalitis and/or pneumonia in abattoir workers (Paton et al. 1999) who were more likely to have exposures to the urine or feces of imported Malaysian pigs during the corresponding outbreak (Luby and Gurley 2012). An isolate obtained from a deceased abattoir worker in Singapore was identified as having the identical nucleotide sequence as the isolates collected from human and pig cases in Malaysia (Paton et al. 1999). This provides evidence that the same strain infected human and pig cases in Malaysia and that the outbreak had spread to a different country.

- Isolates collected from the lungs and respiratory secretions from infected pigs revealed the presence of NiV and this taken in conjunction with the observation that many of the NiV human cases were in contact with pigs has led to the suspicion that NiV may be transmitted from pigs to humans through infected saliva and potentially infected urine (Uppal 2000).
- When officials could no longer ignore the potential of a novel agent as the culprit, they began further diagnostic tests that identified a novel paramyxovirus (Chua et al. 1999; Chua et al. 2000; Chua et al. 2007) that was closely related to Hendra virus (Ksiazek et al. 2011). This novel virus was named Nipah virus after Kampung Sungei Nipah, the residential village of the patient from whom the first virus was isolated (Ksiazek et al. 2011). Since this initial outbreak in 1998–1999, the exact geographical distribution of NiV remains unknown, but it appears to occur in Asia Pacific and South East Asia (Young et al. 1996) with Singapore, Bangladesh, and India as countries specifically encountering NiV outbreaks (Luby and Gurley 2012). Nipah virus outbreaks have been reported

nearly every year beginning in 2001 in the western and northwestern regions of Bangladesh and in West Bengal, India (Luby and Gurley 2012).

- Through surveillance efforts, fruit bats, from the genus Pteropus, have been found to be the reservoir of NiV (Arif et al. 2012). This virus can then be spread to humans through pigs acting as an intermediate host (Arif et al. 2012). The distribution of this genus of fruit bats encompasses areas from the eastern coast of Africa to the South East of Asia, to the Philippines and Pacific Islands, and as far south as Australia (Young et al. 1996). The distribution of this genus of bats suggests that it may be possible for NiV to be spread across the regions inhabited by these fruit bats; public health officials need to be aware of the potential for NiV to spread to these susceptible areas that may not have seen this disease before. A study performed in Goalondo of Rajbari district in central Bangladesh during a 2004 outbreak found that NiV was found in 14% of blood samples contained antibodies from clinically healthy bats (Arif et al. 2012).
- It is suspected that pigs may contract the virus from ingesting fruits that have been nibbled on by NiV infected fruit bats living near pig farms (Arif et al. 2012). Moreover, the virus has been isolated from fruits that have been half eaten by bats in Malaysia (Chua et al. 2000). The majority of NiV-infected pigs develop mild illness (Parashar et al. 2000), while some pigs never develop clinical signs of disease. In infected adult pigs, the case fatality rate is less than 1–5% (Mohd et al. 2000). Some mathematical models suggest that in order for NiV to be sustained at epidemic levels in pigs, multiple spillover events are necessary for the development of a dynamic population with numbers of susceptible pigs above the needed threshold level to maintain transmission within pigs for months (Pulliam et al. 2012).

There is evidence that various husbandry practices may play a role in determining how long an epidemic will last and in influencing the characteristics of an outbreak (Luby and Gurley 2012). In large factory farms, thousands of pigs are raised together in more compact conditions and have an increased risk of interacting with pigs from other farms so longer transmission chains in pigs will be more likely to occur (Luby and Gurley 2012). However, in rural areas with fewer pigs kept on individual farms that may have limited contact with pigs from other farms, a shorter transmission chain may be seen, because the number of susceptible pigs will decrease more slowly overtime since fewer will be in contact with each other. This second type of husbandry practice has not been linked to an outbreak to date; however, human cases have been linked to animal infections with this form of husbandry (Luby and Gurley 2012).

The majority of infected pigs present with a mild illness, if an apparent illness is detected at all. Those which develop clinical symptoms present most often with fever, agitation, trembling, and twitching (Mohd et al. 2000). These symptoms appear along with labored and rapid respirations, increased drooling, and a loud, nonproductive barking cough (Mohd et al. 2000). Almost all of the pigs with symptoms are often diagnosed with acute respiratory syndrome (Kay-Sin Tan and Goh 1999; Chua 2003). Necropsies revealed that pigs with severe disease have had extensive

lung damage with giant cell pneumonia; NiV antigen can be detected in lung tissue as well as in the epithelial cells lining the upper airways, respiratory secretions, and in renal tubular epithelial cells (Chua et al. 2000; Middleton et al. 2002).

Symptoms in humans may vary from severe to mild to asymptomatic (O'Sullivan et al. 1997), may lead to debilitating chronic neurologic conditions, and can be fatal (Arif et al. 2012). The incubation period has been reported to be between four and 18 days, but occasionally clinical symptoms may not develop until an average of 8 months after an exposure (Holmes 2001). Clinical symptoms in humans usually present as severe acute encephalitis with individual symptoms that include fever, headache, vomiting, breathing difficulty, seizures, and progression into coma (Luby and Gurley 2012; Arif et al. 2012). Patients may also develop pneumonia from an accumulation of respiratory secretions in the lungs which has been noted to occur in up to 25 % of cases (Chua et al. 1999; Chadha et al. 2006).

Cases with NiV infection may also develop chronic illness leading to severe neurologic conditions later in life, even if the case did not present with acute symptoms shortly after exposure (Luby and Gurley 2012; Tan et al. 2002). Patients who had developed acute encephalitis and appeared to have recovered may also develop neurologic conditions and relapses of encephalitis months to years after their initial infection, with NiV antigen being found in the neurons of those who died after a case of late-onset encephalitis (Tan et al. 2002).

- In many situations, patients are more likely to be in direct contact with pigs that appeared to be sick when compared to farm controls (Parashar et al. 2000). Direct contact with pigs involves activities such as feeding pigs, processing piglets, aiding in breeding and birthing, injecting and medicating pigs, and handling dead pigs (Parashar et al. 2000). The case fatality rate may be high for humans. During the 1998–99 Malaysia outbreak, of the 283 reported cases, 39 % or 109 cases died (Chua 2003). Although contact with pigs increases the risk of contracting NiV, there have been reported cases of disease in humans without pig contact or without direct pig contact such as cleaning a crate that was used to house infected pigs (Parashar et al. 2000; Kay-Sin Tan and Goh 1999). This suggests that it may be possible for pig secretions or excretions to be infectious for hours, if not days (Kay-Sin Tan and Goh 1999).
- Human-to-human transmission has been reported, but the rates vary geographically (Luby and Gurley 2012). Numerous outbreaks involving human-to-human transmission have been recognized in Bangladesh and India, while only very few reports on human-to-human transmission exist from Malaysia (Luby and Gurley 2012). In a 2004 Bangladesh outbreak, a chain of NiV transmission was sustained through five generations, which is the longest reported chain of human-to-human transmission (Gurley et al. 2007). Through reviewing human NiV cases from 2001–2007 in Bangladesh, Luby et al. (Luby et al. 2009) found that 51 %, or 63 out of 122 cases, contracted NiV after being in close contact with a patient. Various investigations in Bangladesh suggest that the primary mode of transmission between humans is through respiratory secretions and that those who did have difficulty breathing as one of their symptoms were more likely to spread

the virus than those who did not (12% compared to 0%, p = 0.03) (Luby et al. 2009). During the 2004 Bangladesh outbreak referred to earlier, people caring for human cases who shared utensils, ate the patients' leftover food, slept in the same bed with a coughing patient, and fed and/or hugged dying patients were more likely to contract NiV (Blum et al. 2009).

Since direct contact with pigs has shown to increase the risk of contracting NiV, the use of personal protective equipment (PPE) is imperative in reducing the risk of contracting the virus (Uppal 2000). General safety measures that should be taken when contact with suspected NiV infected pigs occurs is changing needles between every pig, using soap or detergent to wash the hands and body, disinfecting abattoir and veterinary equipment, spraying disinfectant on trucks every time they leave a farm, disinfecting dead pigs before burying them, and avoiding contact with blood, urine, and feces (Uppal 2000). Recommendations for PPE to protect individuals are goggles and face masks for eye protection, masks that cover the nose and mouth, rubber gloves, a long sleeved shirt, a long apron, long pants, and rubber boots (Uppal 2000). Measures in addition to PPE use have been implemented to control the spread of the disease. In Malaysia, when PPE was employed to those who are in direct contact with pigs, especially sick pigs, along with the implementation of livestock transportation restrictions, and culling of a large number of pigs (over 900 thousand), the amount of human cases drastically declined (Uppal 2000). The importance of control must be stressed because during an outbreak there is a large social impact due to the potential closure of schools, loss of human life leading to fewer community members able to work and earn money, loss of pig populations (especially when culling is implemented) which minimizes farmer's wages (even if marginal compensation occurs for their lost revenue), and decreases a community's moral while increasing a sense of panic (Uppal 2000).

2.3 Swine Influenza

Influenza is an acute respiratory disease caused by viruses of the family *Orthomyxoviridae* (Capua and Munoz 2013; Thacker and Janke 2008). The virus was first isolated in 1930 in the United States (Kothalawala et al. 2006). Estimated 36,000 human deaths and 200,000 hospitalizations are due to influenza virus infections in the United States per year (Ramirez et al. 2006). The genome of the virus is composed of eight negative-strand RNA segments. This allows both for a high mutation rate (as an RNA virus) leading to antigenic drift and for mixing of genomic segments via recombination causing antigenic shift of the virus. Two major surface glycoprotein, haemagglutin (HA) and neuraminidase (N) are important tools for subtype classification, and determination of antigenicity and pathogenicity (Thacker and Janke 2008; Kothalawala et al. 2006). With the recent discovery of a new influenza viral subtype in a bat (Tong et al. 2012), influenza viruses are now grouped according to the expression of 17 HA (H1–H17) and 10 NA (N1–N10) subtypes (Capua and Munoz 2013).

Influenza viruses may be subject to antigenic drift and antigenic shift which impose major challenges in vaccine development. Antigenic drift is a minor genetic variation within subtypes due to series of point mutations. Antigenic shift is caused by reassortment of genes from two different viruses that result in a new combination of H or N segments (Kothalawala et al. 2006). For example: the 2009 pandemic H1N1 virus (H1N1pdm09) was the result of a virus reassortment event in swine (Skowronski et al. 2013). The H1N1 virus contains segments from North American-like triple reassortant swine H1 viruses (6 genes) and Eurasian avian-like swine viruses (neuraminidase and matrix genes) (Pascua et al. 2012).

The ultimate host of all influenza viruses appears to be wild birds, specifically waterfowl (Thacker and Janke 2008), where the viruses replicate in the respiratory tract and intestine typically without any signs and symptoms of disease. Although mammalian species are all derived from birds, studies have shown interspecies transmission of influenza virus (e.g. H3N8 from horses to dogs) (Kothalawala et al. 2006; Pascua et al. 2012; Myers et al. 2006). Interspecies transmission of influenza virus A is the principle mechanism of emergence of novel strain and pigs likely to play important role in such transmission (Myers et al. 2006; Bowman et al. 2012).

Viral attachment on host cell was thought to depend on the specificity of binding to particular sialic acids (SAs). Prior research suggested that avian influenza viruses bind to α2–3 SAs, while influenza viruses infecting mammals preferentially attach to α2–6 SAs. However, recent research (reviewed in (Capua and Munoz 2013)) suggests that it is more complicated than that. Nevertheless, the cells of pigs can bind both “avian” and “human” types of influenza viruses suggesting that they may be important in the generation of novel variants that could go on to infect humans and spread zoonotically (Scholtissek 1990).

Influenza viruses become a pandemic threat when they become capable of being transmitted efficiently from human to human and if limited protective immunity exists in the human population. There are three major interfaces for human-to-pig contact: commercial swine production, abattoirs, and agricultural fairs (in the United States). Agricultural fairs provide common ground for the transmission of influenza viruses between humans and pigs. Fairs are unique because “they facilitate prolonged comingling of pigs from numerous sources raised under varied management programs with millions of persons who have widely disparate histories of exposure to various influenza viruses” (Bowman et al. 2012).

Prior to the 2009 pandemic, a review article identified thirty-seven civilian and 13 military cases of swine influenza in humans in the literature from 1958–2005 (Myers et al. 2007), of which 19 cases were reported in the United States, 6 in Czechoslovakia, 4 in the Netherlands, 3 in Russia, 3 in Switzerland, 1 in Canada, and 1 in Hong Kong. The majority of the cases had some kind of exposure to live swine. There were no unique clinical features to distinguish swine from human influenza; healthy people and those with underlying conditions were both at risk (Myers et al. 2007). In a 2006 study of farmers, meat processing workers, veterinarians, and individuals lacking swine exposure, elevated titers to swine viruses were found in individuals with occupational exposure (Myers et al. 2006). Thus, it appears that even prior to 2009, transmission of swine influenza viruses to humans was not a rare

event. In one paper examining the 2009 pandemic, evidence was found of at least 49 discrete introductions of H1N1pdm09 influenza virus from humans into swine in 2009 (Nelson et al. 2012), suggesting bidirectional spread of these viruses from swine to humans and backwards.

"Classic" swine influenza A (H1N1) virus (cH1N1) was the predominant subtype of swine influenza viruses in North America for nearly 80 years. The entry of the H3N2 virus in 1998 that was composed of avian, human, and swine influenza genes into the US swine population resulted in the emergence of multiple reassortment influenza viruses (Thacker and Janke 2008). The true incidence of swine-to-human transmission of influenza virus A is unknown. During the period of December 2005 to April 2012, 36 human infections with variant influenza virus A were reported by the Centers for Disease Control and Prevention in the United States. There might be far more cases than what is actually reported. The US national swine influenza virus surveillance program is passive, and focuses on swine showing signs of influenza-like illness and on reacting to reports of variant influenza A cases in humans. Thus, subclinical infections are unlikely to be reported. Surveillance should instead be carried out in both healthy and sick animals (Bowman et al. 2012), but this is a costly proposal.

2.4 H1N1 2009 (Influenza A H1N1pdm09)

The earliest reports of H1N1 swine infections in the US occurred at state fairs in Minnesota and South Dakota. An estimated 150 million people attend at fairs in North America (Bowman et al. 2012) which can be a conduit to introduce influenza viruses into swine herds or vice versa (Bowman et al. 2012; Gray et al. 2012). Eleven of fifty-seven (19%) of swine tested in Minnesota in 2009 were found to be influenza positive by rRT-PCR; 4 of them harbored influenza viruses similar to H1N1pdm09. This occurred during the second wave of the 2009 pandemic. It is possible that these show pigs were infected by their owners or others prior to arrival at the fair. Notably, all pigs found to be positive exhibited infections which were subclinical in nature (Gray et al. 2012), again emphasizing the need for surveillance even of animals which appear to be healthy.

2.5 H3N2 Variant

H3N2 variant viruses contain the matrix gene from the 2009 H1N1 pandemic virus. These triple-reassortment swine viruses (A/Sw/OH/511445/2007, A/Ohio/01/2007, and A/Ohio/02/2007) were found both in pigs and humans in a 2007 fair in Ohio (Killian et al. 2013). In 2011, 12 cases of H3N2v were found in Indiana, Iowa, Maine, Pennsylvania, and West Virginia. In 2012, 309 cases H3N2v infections were found across 12 states. This virus had been seen in human since July 2011 and in

swine in “many states”. Fifteen of the sixteen cases had recent contact with pigs at a fair, and person-to-person spread was seen in at least 3 cases. Most cases were mild (CDC 2013a; b). Another outbreak of H3N2v occurred at a Pennsylvania fair in August of 2011. In this outbreak, one confirmed infection in a child was identified; serological studies determined that 82 additional suspected, 4 probable, and 3 confirmed cases also had attended the fair. The highest risk of transmission was in those who touched swine during their fair visit. Some reports of symptomatic pigs were noted, but all of these animals were sold or slaughtered before they could be tested (Wong et al. 2012). In 2012, 11 patients of H3N2v were hospitalized in Ohio. Of the reported 11 cases hospitalized, 10 had direct or indirect contact with pigs. One patient, a 61-year-old woman with type 2 diabetes, died as a result of H3N2v infection (Centers for Disease C Prevention 2012).

A recent study using a ferret model found that the zoonotic potential of four representative triple-reassortant swine influenza viruses caused mild disease and were inefficiently transferred via air, but one (an H1N2 virus) replicated well the upper and lower respiratory tract of ferrets, was efficiently transmitted via respiratory droplets and showed high lethality. As such, some field isolates from swine may show zoonotic potential (Pascua et al. 2012). Although there have been multiple outbreaks of H3N2v in recent years, a recent analysis suggests that the current pandemic potential of H3N2v is low (Skowronski et al. 2013).

Individuals working with swine or visiting swine fairs are urged to practice good hygiene and the use of proper personal protective equipment (PPE) while working with pig or pig facilities. Isolation of sick or infected pigs, partial depopulation and segregation of early weaned piglets would help to prevent the transmission of swine influenza. Inactivated, whole virus and subunit influenza virus vaccines are available for humans, horses, birds and pigs (Kothalawala et al. 2006). However, due to ongoing mutations in the HA and NA genes via antigenic drift, these vaccines need to be revised annually. Due to its ability to elicit both humoral and cellular immune responses, a live attenuated influenza virus vaccine is considered relatively better compared with an inactivated vaccine; it is currently licensed in the United Sates (Schnitzler and Schnitzler 2009). Researchers have been studying DNA vaccines as a novel alternative to the conventional vaccines using chicken, mouse, ferret, and primate models. Nonetheless, so far it has not been successful in pigs (Thacker and Janke 2008; Kothalawala et al. 2006).

2.6 Swine Parasitic Zoonoses: *Trichinella* and *Taenia*

Parasites with zoonotic potential such as *Trichinella* spp. and *Taenia* spp. are public health hazards affecting both animals and humans. Global eradication of these pathogens is a challenge due to limitations in implementation of strict regulations and policies, methodological issues in early diagnosis and treatment and above all varying cultural and social practices that govern the propagation of these parasites.

Trichinella spp. are nematode worms that are one of the most widespread food-borne zoonotic pathogens in the world (Pozio 2007). Strains of *Trichinella* have been isolated from domestic and wild animals in 66 countries (Pozio 2007). This parasite was first discovered in 1835, but was linked to food-consumption and disease only in 1860 (Dupouy-Camet 2000). The main source of human infection is pork and pork products, game meat and horse meat. Nevertheless, *Trichinella* spp. has been reported to infect other animals such as wild boars, rats, cats, dogs, bear, walrus, jackals, raccoon, foxes, warthogs, crocodiles, lizards and even birds (Pozio et al. 2009). Due to its vast host range and difficulties in identification of the pathogen or establishing an early diagnosis *Trichinella* is one of the most resilient and persistent zoonotic parasites.

Taeniasis caused by *Taenia* spp. (tapeworm) is a food-borne infection commonly observed in developing or less developed countries and adds to the global public health burden of parasitic infections. To meet the scope of this chapter, we will discuss only *Taenia solium* (*T. solium*), since this parasite is commonly observed in pigs. *T. solium* (pork tapeworm) infections are commonly referred to as the taeniasis/cysticercosis complex (Garcia et al. 2003a). Cysticercosis is endemic in Central and South America, sub-Saharan Africa, most of Asia and parts of Oceania (Garcia et al. 2003b). Neurocysticercosis is the most common cause of late adult-onset seizures in the developing world (Epilepsy CoTDotILA 1994). More than 1000 new cases of neurocysticercosis (Hawk et al. 2005) are diagnosed in the United States each year and it is the most prevalent infection of the brain, worldwide (Garcia et al. 2003b; Shandera et al. 1994).

2.6.1 Trichinella

The genus *Trichinella* has a broad range of host species. Nevertheless, clinical infection is apparent only in humans (Gottstein et al. 2009). *Trichinella* spp. are found in animals all over the world, except Antarctica possibly due to absence of surveillance (Pozio 2007). Parasites in the genus *Trichinella* are classified by the presence of a collagen capsule: encapsulated and nonencapsulated. Within the genus, *T. spiralis* is the species most adapted to domestic and wild pigs and is also most commonly isolated from human infections (Pozio and Darwin Murrell 2006). Other *Trichinella* spp. found in pigs are *T. britovi* (wild boar), *T. nelsoni* (bush pigs), *T. pseudospiralis* (domestic and wild pigs). Horses fattened with pork scraps are known to be infected with *Trichinella*.

Prevalence estimates depend on the geographical region and the detection methods used: *Trichinella* antibodies were found in 0.35% of pigs in the Netherlands (van der Giessen et al. 2007), 0.2% of wild boar muscle samples contained *Trichinella* in Spain (Boadella et al. 2012), a 0.37% prevalence was estimated in pigs in the northeastern United States (Gamble et al. 1999), in China prevalence data ranged from 0.01 to 29.95% by serological testing to 0–5.75% in pigs slaughtered at abattoirs, respectively (Cui and Wang 2011), 19.9% Vietnam pigs tested positive

by E/S ELISA (Vu Thi et al. 2010), antibody prevalence was 0.0002–0.0003 % in wild boars in France (Pozio et al. 1996), a prevalence of 1.3 % was established for wild boars in Finland (Oivanen et al. 2002) and of 11.4 % wild boars in Argentina by artificial digestion (Cohen et al. 2010). Despite a broad spectrum of hosts, *Trichinella* spp. are most common in porcine omnivores, mainly in domestic pigs, different races of wild pigs, wild boars, bush pigs, and warthogs (Pozio 2005).

Human trichinellosis has been documented in 55 countries and is most often linked to established food-consumption behaviors including consumption of raw or undercooked pork or pork products. A 2011 study observed about 261 reports of trichinellosis outbreaks worldwide (Murrell and Pozio 2011). This study estimated 65,818 cases and 42 deaths reported from 41 countries between 1989 and 2009 (Murrell and Pozio 2011). About 87 % of the cases were reported from the WHO-European region, of these 50 % were reported from Romania alone (Murrell and Pozio 2011).

Trichinellosis is the infection caused by *Trichinella* spp. in humans. Severity of trichinellosis depends on the load of ingested parasite, frequency of consumption of infected meat, method of cooking and treating meat before consumption, species involved in reproduction of larvae, and the amount of alcohol consumed at the time of meat consumption, with alcohol acting as a protective factor (Murrell and Pozio 2011; Xu et al. 1995). The estimated minimum dose necessary for causing symptomatic trichinellosis ranges from 70 to 150 larvae (Murrell and Bruschi 1994; Dupouy-Camet et al. 2002). The major signs of classical trichinellosis are myalgia, diarrhea, fever, periorbital and facial edema, and headaches as per an algorithm used to diagnose acute trichinellosis (Murrell and Pozio 2011). Complications usually develop within 2 weeks of infection (Ancelle et al. 2005; Lachkar et al. 2008; Dupouy-Camet and Bruschi 2007; Bessoudo et al. 1981; Compton et al. 1993; Ellrodt et al. 1987; Fourestie et al. 1993). Mortality due to trichinellosis is very rare and is estimated to be around 0.4 % (Murrell and Pozio 2011; Dupouy-Camet and Bruschi 2007; Kociecka 2000; Ancelle et al. 1988). Humans are observed to be asymptomatic while being chronically infected with the larvae (Dupouy-Camet et al. 2002). Children are found to be more resilient to *Trichinella* infection, while it may result in abortion or premature delivery in pregnant women (Dupouy-Camet et al. 2002).

Globalization and migration of population with culturally unique food practices and illegal introduction of *Trichinella* infected meat from endemic to non-endemic countries are some of the risk factors for human trichinellosis particularly in countries with low-incidence (Pozio and Marucci 2003b; Gallardo et al. 2007; Nockler et al. 2007; Stensvold et al. 2007). Impact of food-consumption practices are reflected by the low incidence of trichinellosis in the Muslim population worldwide (Pozio 2007; Haim et al. 1997; Marva et al. 2005). International travelers and hunters are two high-risk groups observed to acquire *Trichinella* infection from endemic countries and exposure to *Trichinella* infected animals, respectively (Ancelle et al. 2005; Moller et al. 2005a; Wang et al. 2006; Shiota et al. 1999; Nakamura et al. 2003; Centers for Disease C Prevention 2004). This population also plays a major role in propagation of *Trichinella* to a naïve population.

Diagnosis of *Trichinella* infection in animals is conducted by direct methods such as meat inspection using conventional trichinoscopy (Epizooties 2004; Kapel 2005; Nockler and Kapel 2007). Other methods used in animal detection are artificial digestion using the magnetic stirrer method (Kapel et al. 2005), multiplex PCR (Pozio and La Rosa 2003a), and serological tests for IgG antibodies (Nockler et al. 2005). Enzyme-linked immunosorbent assay (ELISA) using metabolic E/S antigens or tyvelose ELISA are the most commonly used methods for the detection and confirmation of *Trichinella* infection (Moller et al. 2005a; Gamble and Graham 1984; Gamble et al. 1983). Diagnosis in humans is based on clinical, epidemiological and laboratory criteria, as outlined by the European Center for Disease Control (Dupouy-Camet 2007).

Initiation of anthelminthic therapy such as albendazole and mebendazole early in the infection is recommended and observed to be beneficial for the cure of trichinellosis (Dupouy-Camet et al. 2002). Other treatment options are available based on the severity of infection (Gottstein et al. 2009; Dupouy-Camet et al. 2002, Dupouy-Camet and Bruschi 2007). Prognosis is reported to be poor for severe cases with cardiac or cerebral complications and fatality was found to be about 5 % in severe infections despite therapy (Gottstein et al. 2009).

2.7 Taenia (*T. solium*)

The life cycle of *T. solium* is divided between two hosts. Humans are the definitive host for the adult *T. solium*, while both pigs and humans may harbor the larvae or cysticerci with pigs being the typical intermediate host. Human infection due to *T. solium* occurs when larvae are consumed by eating poorly cooked or raw pork products. Larvae attach to the mucosa of the human small intestine using their scolex (head) and grow into adult tapeworms (Flisser 1994). Eggs from these adult worms are shed in human feces. Contamination of pig feed with such human feces results in ingestion of eggs by the pigs. Ingested eggs develop into a larval stage, travel through the intestinal wall, enter the bloodstream and lodge in various pig tissues eventually forming cysts (porcine cysticercosis) (Garcia et al. 2003b). Ingestion of such infected pig or pork products by humans result in intestinal infection or taeniasis. Humans can also ingest *T. solium* eggs through the fecal-oral route or by auto-infection (Hawk et al. 2005). Autoinfection is the retrograde transmission of taenia segments from human intestine back into the stomach, followed by release of eggs into the gut (Garcia et al. 2003a; Hawk et al. 2005; Flisser et al. 2006). Fecal-oral contamination occurs in infected food handlers who do not practice good hand hygiene. Even vegetarians who do not consume pork may acquire cysticercosis via wind, water, flies and other indirect means of transmission (Martinez et al. 2000).

Clinical manifestation of cysticercosis depends on the organ affected (Garcia et al. 2003a). The most common clinical manifestation is neurocysticercosis that develops once the viable cysticerci enter the central nervous system. Epileptic seizures are the most common presentation of neurocysticercosis. Neurocysticercosis and

ocular cysticercosis are associated with significant morbidity (Garcia et al. 2005). Other types of extraneural cysticercosis are subcutaneous, muscular, cardiac, and—in rare cases—limb enlargement due to massive parasite burdens (muscular pseudohypertrophy) (Garcia et al. 2003).

T. solium is one of the major public health hazards and causes of economic problems in pig husbandry (Flisser et al. 2005). Transmission of adult tapeworms and larvae are associated with poor hygiene and sanitation, low living standards, lack of meat inspection and control, and lack of education and awareness (Flisser et al. 2006). In addition, immigrants, overseas domestic workers, international travelers, and transport of infected pigs have spread the disease to non-endemic areas (Hira et al. 2004; Rajshekhar et al. 2003). *T. solium* is widely prevalent in regions where pigs are reared in free ranging systems and raw or undercooked pork is consumed. In most endemic villages more than 10% of the population is observed to be seropositive for *T. solium* with an observed maximum of 25% (Garcia et al. 2003a). Studies have found that up to 6% of the general population in endemic countries may harbor adult tapeworms (Allan et al. 1996b). A recent meta-analysis on the prevalence of neurocysticercosis in people with epilepsy including studies from Latin America, India, and sub-Saharan Africa found that neurocysticercosis was the cause of epilepsy in 30% of the population with epilepsy (Ndimubanzi et al. 2010). Cases of intestinal taeniasis are consistently observed to cluster in families possibly due to food consumption habits (Allan et al. 1996a). Rate of porcine infection varies and seropositivity has been observed in 30–60% pigs (Garcia et al. 1999; Gonzalez et al. 1990).

Diagnosis of cysticercosis is based on a set of criteria developed using clinical, radiologic, histologic and epidemiologic findings (Garcia and Del Brutto 2003; Kraft 2007). Neurocysticercosis is diagnosed using a computed tomography (CT) scan and performing a cerebrospinal fluid (CSF) examination (Garcia et al. 2005; Garcia and Del Brutto 2003; Kraft 2007). The MRI is a better tool in detecting certain pathological changes (Garcia et al. 2003b). Antibodies against *T. solium* generally persist even after the cyst dies, hence serology should be used as a confirmatory test secondary to clinical signs and imaging studies (Garcia et al. 2005). Frequency of stool examination for *T. solium* eggs vary among patients and may be related to the severity of infection (Garcia and Del Brutto 1999; Gilman et al. 2000). In addition, ELISA and PCR tests for coproantigen detection may be useful in screening for *T. solium* carriers in endemic regions (Mahanty and Garcia 2010).

Antiparasitic treatment such as albendazole and praziquantel in conjunction with steroids are the treatment of choice in most types of cysticercosis (Baranwal et al. 1998, 2001; Carpio et al. 1995; Padma et al. 1994; Padma et al. 1995; Corona et al. 1996; Pretell et al. 2001). Studies have shown 100% effectiveness using the TSOL18 vaccine developed against *T. solium* in pigs (Gonzalez et al. 2005; Flisser et al. 2004; Lightowlers 2004). The vaccine requires at least two doses to be effective.

Some preventative strategies for parasitic infections such as *Trichinella* and *T. solium* are improved living conditions by better sanitation, education and commercial pig production, regular monitoring and mandatory reporting for the pathogen

(Nockler and Kapel 2007; Community E 2005), strict regulations in pig-farms and slaughterhouses (Gottstein et al. 2009), adequate vaccination of pigs, health education and human mass chemotherapy (Gottstein et al. 2009), pig corralling, and regulations for processing pork and pork products (Garcia et al. 2007; Hill et al. 2010). However, these preventative practices are not implemented in high-risk countries such as Eastern Europe, Asia, parts of South America and in the African subcontinent. These regions have the potential for exponential increase in transmission of pathogens due to the dense pig population, interaction between humans and pigs, and the vast reservoir for *Trichinella* in regional wildlife. Human cultural and social practices are one of the greatest challenges in prevention of transmission of food-borne parasites such as *Trichinella* and *T. solium* on a global basis.

2.8 Discussion

In recent decades, the global emergence of infectious diseases in human, domestic animals and wildlife have attracted a greater attention of researchers and agencies. As a result, studies have now demonstrated zoonotic pathogens by quantitative analysis as risk factors for emergence in humans (Cleaveland et al. 2001). Rapid growth of human population and globalization of trade are considered to be main factors responsible for emergence of zoonotic diseases. However, several other direct and indirect factors such as ecological disruption, increasing movement of animal species, uncultivatable organisms, and terrorism play potential role in disease expansion (Brown 2004). A study by Jones et al. found a significant correlation between emerging infectious disease origin and socio-economic, environmental and ecological factors (Jones et al. 2008).

Zoonotic diseases account for 75% of all emerging diseases affecting the human population in the last two decades (Brown 2004). Almost two-thirds (61%) of human infectious diseases are zoonotic in nature. A database of disease-causing pathogens of humans and domestic mammals constructed by Cleaveland et al. showed that 61.6% of 1415 pathogens able to cause human diseases have an animal origin. A high prevalence of multi-host pathogens were observed in both human and domestic mammal pathogens, suggesting cross-species transmission (Cleaveland et al. 2001).

Recent decades have witnessed the rapid growth of pork industry (Pappas 2013). In fact, pork is the most widely eaten meat in the world, leading to the production of 1.3 billion pigs each year globally (Health CfFSaP 2013). Global increase in pork production and predicted increase in annual pork demand have reached 80 and 7%, respectively (Pappas 2013). However, the projected human population growth of 7.7 billion by 2020 still outcompetes the meat supply. To meet the demands for food, it has been suggested that it will be necessary to double the global livestock production in 2020 when compared to 2000 (Brown 2004). This increase in demand of pork production has led to intensification and industrialization of production systems. Production modifications with larger herds in a contained area have the

potential to make pigs and their caretakers increasingly vulnerable to inter-species pathogen transmission (Pappas 2013).

A recent review of emerging pig pathogens revealed at least 77 novel emerging species, of which 35 were pig-specific zoonotic (Pappas 2013) suggesting an urgent need of considering pigs as a potential vehicle for zoonotic disease transmission. The pig may also serve as an amplifying host, as is the case for both Japanese encephalitis virus and Nipah viruses discussed above (Weaver and Barrett 2004; Pappas 2013).

The economic burden imposed by zoonotic diseases is paramount. It has been estimated that the zoonotic epidemics between the period of 1995 and 2008 caused more than 120 billion dollars in economic loss. Major recent infectious disease outbreaks have been zoonotic, leading to unprecedented human morbidity and mortality causing greater human productivity loss (Cascio et al. 2011). For example, cysticercosis alone, a parasitic infection that is caused by uncooked or undercooked pork, imposes an estimated economic burden between US $ 18.6 million and US $ 34.2 million in the eastern cape of South Africa (Carabin et al. 2006).

As zoonotic infections go beyond the individual and affect the household, especially in agricultural settings, the economic impact is even more pronounced. Animal loss due to disease or mandatory regulations adds more to the economic burden of zoonoses. For example, the slaughter of more than one million pigs in Malaysia in 1999 on the wake of Nipah virus outbreak caused destruction of the local swine industry. It is predicted that the majority of future infectious disease outbreaks will be zoonotic in origin (Cascio et al. 2011), potentially amplifying this cycle.

2.9 Conclusions/Recommendations

Zoonotic diseases directly impact human morbidity and mortality. An indirect impact of zoonotic diseases is via disruption of the food chain, thus causing grave economic loss. The globe is under the threat of emergence of new species of zoonotic pathogens and their pandemic potential, such as the H1N1 pandemic of 2009. Public health intervention programs to tackle the rapid emergence of zoonoses are essential, but underfunded and understaffed. Public health interventions should be designed in the light of evidence-based practices to address the zoonotic diseases that are often neglected and associated with poverty such as zoonotic helminthes, protozoan, viral and bacterial infections. Disease-specific research priorities to support zoonotic disease control are crucial to combat the epidemics and pandemics of zoonoses (WHO 2012). What was effective in the mitigation of zoonotic diseases epidemics or pandemics a decade ago could be antediluvian in the current era largely due to rapid human growth, transportation, movement, and human migration. Early detection, rapid and enhanced surveillance and ongoing research for the discovery of new knowledge are warranted for the effective mitigation of emerging zoonotic infectious diseases. A long-term socio-political and economic commitment by government and private agencies across the globe is essential to combat

the global threat of zoonotic diseases. Policy-makers should consider prioritizing for improved surveillance, multi-sectorial interactions between public health, livestock, agriculture, natural resource and wildlife, and a measure to precisely assess the burden of zoonoses (WHO 2012).

Practice of good personal hygiene such as proper hand washing after working with animal and animal products, use of proper personal protective equipment (PPE) while working with animal or animal facilities, isolation of sick or infected animal, and good practice of cleaning and disinfection would help to prevent the zoonotic disease infection and transmission. High-risk-group should get vaccinated if one is available.

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Chapter 3
Small Ruminants and Zoonotic Infections: Live or Dead—Direct or Indirect

Snorre Stuen

Abstract Sheep and goat can be infected with several pathogens. While some may have a great impact on the small ruminant industry, others are more important as zoonotic agents. Human infections can occur from contact with both live and dead animals, animal products and wastes. Several microorganisms may cause severe infection if transmitted to vulnerable people, such as pregnant and immune-comprised persons. The present chapter will briefly deal with a selected number of zoonotic microorganisms where small ruminants play an important role as hosts for human infection.

3.1 Introduction

Sheep and goats which were domesticated by humans as early as 9000 years ago are distributed worldwide and their products, such as milk, meat, wool and skin are used extensively. Microorganisms have therefore been shared between humans and small ruminants for a long period of time. In this context, small ruminants may carry zoonotic pathogens, either transiently or permanently. The risk of transmission to humans varies considerably due to geographical area, seasons, climatic conditions and management systems. The impact on human health will also depend on pathogen species/subspecies, virulence of the pathogen, level of exposure, presence of co-infections, susceptibility of the host, the host immune status and the transmission route.

In general, microorganisms can be transmitted from small ruminants to humans by direct contact, aerosols, milk, meat, or indirectly by manure, feces, urine and wool. For instance, the development of zoonotic infections through exposure to manure, either directly or indirectly, constitutes a real and significant risk of human health. Contamination of ground, irrigation or drinking water provides not only a source of infection, but also a mean to spread the pathogens (Milinovich and Klieve 2011).

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Water and food-borne zoonotic pathogens, such as *Camplylobacter jejuni*, *Cryptosporidium parvum*, *Escherichia coli* (EHEC), *Giardia duodenalis* and *Salmonella* spp. are widespread and have a wide host range. *Salmonella* spp. for instance, are ubiquitous in nature and have been isolated from a wide variety of vertebrate hosts. *Salmonella* is also commonly found in farm animals, their environments and is one of the most important food-borne zoonotic microorganisms (Milinovich and Klieve 2011). Infections from goat products due to consumption of raw or uncooked goat meat, milk and cheese have been documented. Severe gastroenteritis and even fatalities may occur (Desenclos et al. 1996; Espié and Vaillant 2005). However, human salmonellosis may not normally arise as a result of contact with small ruminants, since this transmission pathway seems to be less frequent than that from other animals such as cattle and poultry (Kirby 1985; Rabinowitz and Conti 2010). Similarly, all the above mentioned pathogens are important zoonotic agents, but small ruminants have normally a less important role as reservoir for human infection compared to other species (Palmer et al. 1998). These microorganisms will therefore not be described further in this condensed review.

Some microorganisms commonly found in small ruminants have a limited zoonotic potential. For instance, sheep on *Ixodes ricinus* tick-infested pasture in northern Europe are often infected with *Anaplasma phagocytophilum*, which may cause serious and fatal infection. However, phylogentic studies show that strains/variants of *A. phagocytophilum* isolated from sheep differ from isolates normally affecting humans, indicating that sheep are rarely reservoir hosts for human infection (Scharf et al. 2011).

Other links between diseases in humans and animals are still debated. For instance, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causing Johne's disease in ruminants has been isolated from some humans with Crohn's disease, a chronic granulomatous infection of the human intestine (Sharp 2007; Smith and Sherman 2009). The association between this bacterium and the disease, however, is still unclear. The presence of MAP in a percentage of Crohn's disease patients is either associated with the pathogenesis of the disease or these patients may be more likely to be colonized by this organisms. The unanswered question raises the issue of meat, milk and water contamination by MAP and human health (West et al. 2009; Singh and Gopinath 2011). It is also still questioned if Borna disease virus, where sheep is one of the principle species affected, could cause infection in humans (Chalmers et al. 2005; Dürrwald et al. 2007). These issues will not be discussed further in this chapter.

3.2 Specific Infections

Zoonotic pathogens detected in small ruminants of which several could cause severe infection in humans, are listed in Tables 3.1, 3.2, and 3.3. These lists, however, are not complete. Microorganisms may for instance be transmitted to humans due to the lack of normal hygiene procedures when handling infectious material. Common pathogens, such as *Staphylococus aureus* and *Trueperella pyogenes*, which regularly cause infections in small ruminants, are not covered by this chapter. In this short

Table 3.1 Zoonotic bacteria, *Rickettsia* and *Chlamydia* detected in small ruminants

Pathogen	Host	Distribution	Transmission	Clinical symptoms (small ruminants)	Clinical symptoms (human)	References
Anaplasma phagocytophilum (several variants)	Several mammals	Northern hemisphere (Ixodes-tick)	Ticks	Fever, abortion (secondary infections)	Flu-like	Woldehiwet 2010
Bacillus anthracis	Several mammals	Worldwide	Aerosols, cutaneous, oral (spores)	Found dead	Variable, cutaneous, pulmonary and intestinal form	Turnbull 1998
Borrelia burgdorferi sensu lato	Several, incl. small rodents, birds	Northern Hemisphere (Ixodes-tick)	Ticks	Subclinical, arthritis	Variable acute subacute -chronic form	Stanek et al. 2002
Brucella melitensis (*B.abortus*)	Several, mainly small ruminants	Widespread, especially Mediterranean, Middle East	Oral (aerosols, cutaneous)	Abortion, arthritis	Variable, undulating fever chronic	See text
Burkholderia pseudomallei	Several	Widespread, mainly tropical areas	Oral, insects, vertical transmission	Abscesses, weight loss, polyarthititis meningoencephalitis	Pneumonia, septis, genitourinary infection, abscesses, suppurative parotitis, encephalomyelitis	Cheng and Currie 2005 Smith and Sherman 2009
Camphylobacter jejuni	Several, esp. poultry	Widespread	Oral	Abortion, watery diarrhoea	Flu-like, diarrhoea	Skirrow 1998
Chlamydopila abortus	Several, mainly small ruminants	Widespread	Aerosols	Abortion	Abortion, stillbirth, puerperal sepsis, renal failure, hepatic dysfunction, DIC	See text
Corynebacterium pseudotuberculosis	Several, incl. domestic animals	Widespread	Cutaneous, oral	Caseous lymphadenitis	Suppurative granulomatous lymphadenitis	Thomas 1998 Smith and Sherman 2009
Coxiella burnetii	Several, incl. livestock	Widespread	Aerosols, oral (cutaneous, ticks)	Abortion, stillbirth, weak offspring	Flu-like, pneumonia, endocarditis, hepatitis	See text

Table 3.1 (continued)

Pathogen	Host	Distribution	Transmission	Clinical symptoms (small ruminants)	Clinical symptoms (human)	References
Dermatophilus congolensis	Several species	Worldwide	Cutaneous	Dermatitis (exudate)	Dermatitis	Stewart 1972a, b Hyslop 1980
Escherichia coli (EHEC)	Several	Worldwide	Oral	Enteritis, diarrhoea, septicaemia, mastitis	Variable diarrhoea, haemorrahagic colitis HUS	Nelson et al. 1998 Smith and Sherman 2009
Francisella tularensis	Several hosts, esp. rodents	Worldwide	Aerosols, oral, cutaneous, ticks	Sepsis	Variable bubonic—intestinal -pneumonic form	Pearson 1998
Leptospira spp. serovar Pomona (serovar Hardjo-bovis)	Several mammals, incl. cattle, pig	Unknown	Oral, cutaneous	Fever, depression, dyspnea, weakness, anaemia, icterus, haemoglobinuria	Flu-like, encephalitis	See text
Listeria monocytogenes (*L. ivanovii*)	Several	Worldwide	Oral, cutaneous	Abortion, encephalitis, septicaemia, mastitis, diarrhoea, ocular disease	Meningitis, encephalitis, septicaemia	See text
Mycobacterium avium subspecies *paratuberculosis* (John's disease)	Several, esp. ruminants and rabbits	Widespread	Oral	Progressive weight loss, diarrhoea, intermandibular oedema	Chronic bronchitis, cervical lymphadenopathy, disseminated disease (Crohn's disease?)	Gallagher and Jenkins 1998 Smith and Sherman 2009
Salmonella spp.	Several	Worldwide	Oral	Gastroenteritis, septicaemia, abortion	Enteric fever, gastroenteritis, diarrhoea, septocaemia	Humphrey et al. 1998 Mearns 2007 Smith and Sherman 2009
Yersinia enterocolitica, Y. paratuberculosis	Several	Widespread	Oral	Enteritis, mastitis, abortion, ill-thrift	Enterocolitis, polyarthritis, erythema nodosum, exudative pharyngitis, sepsis	Butler 1998 Smith and Sherman 2009

Table 3.2 Zoonotic virus detected in small ruminants

Pathogen	Host	Distribution	Transmission	Clinical symptoms (small ruminants)	Clinical symptoms (human)	References
Louping-ill (genus *Flavivirus*)	Several, especially sheep and grouse	Scandinavia, United Kingdom	Ticks, (*Ixodes ricinus*) (Aerosols, oral, cutaneous)	Variable, subclinical—incoordination—paralysis	CNS-symptoms	Reid and Chianini 2007
Nairobi sheep disease (genus *Nairovirus*)	Small ruminants	Africa	Ticks (mainly *Rhipicephalus appendiculatus*)	Fever, diarrhoea, gastroenteritis, death	Rare benign illness	Swanepoel 1998 Smith and Sherman 2009
Orf (genus *Parapoxvirus*)	Several, mainly small ruminants	Worldwide	Cutaneous (oral)	Wart-like outgrows	Skin lesions	See text
Rabies (genus *Lyssavirus*)	Several	Widespread	Bite, (Aerosols, oral)	Behaviour changes, paralysis, paralytic/furious condition, death	Non-specific (prodromal period) Paralysis, aggression, unconsciousness, paralysis	King 1998
Rift Valley Fever (genus *Phlebovirus*)	Ungulates	Africa, Arabian Peninsula	Mosquitoes (*Aedes* spp.). Other insects	Abortion, fever, listlessness, recumbency	Flu-like Menigoencephalitis, haemorrhagic fever, photophobia, retinitis	See text
Wesselsbron disease (genus *Flavivirus*)	Several, incl. domestic ruminants and rodents	South Africa	Mosquitos (Aedes), (Aerosols, oral)	Abortion, sudden death	Fever, rash, arthralgia	Leake 1998 Smith and Sherman 2009

Table 3.3 Zoonotic parasites and fungus detected in small ruminants

Pathogen	Host	Distribution	Transmission	Clinical symptoms (small ruminants)	Clinical symptoms (human)	References
Cryptosporidium parvum	Several, esp. cattle	Worldwide	Oral	Subclinical (adults) Watery diarrhoea (young animals)	Diarrhoea	Wright and Coop 2007 Smith and Sherman 2009
Dicrocoelium dendriticum	Several, esp. domestic ruminants	Widespread	Oral	Subclinical Weight loss, Anaemia	Subclinical Constipation/diarrhoea, hepatomegaly	Smith and Sherman 2009
Echinococcus granulosus	Several (intermediate hosts) Canids (end host)	Widespread	Oral	Mainly subclinical	Variable (localisation/size) Shock/pulmonary oedema	See text
Eurytrema pancreaticum	Several domestic animals, esp. ruminants	Asia, South America	Oral	Subclinical, ill-thrift, weight loss emaciation	Non-specific	Lloyd and Soulsby 1998 Taylor et al. 2007 Smith and Sherman 2009
Fasciola hepatica	Several ruminant Snail: (intermediate hosts)	Worldwide	Oral	Acute, subacute, chronic (anaemia, icterus, submandibular oedema, death)	Variable Acute (hepatic) -chronic (biliary) phase	Mas-Coma 2005 Smith & Sherman 2009 Fried and Abruzzi 2010
Giardia duodenalis	Several species	Worldwide	Oral	Subclinical, enteritis, diarrhoea	Variable, diarrhoea, chronic syndrome	Thompson 1998 Taylor et al. 2007
Oestrus ovis	Small ruminants	Unknown	Flies	Nasal discharge, sneezing, rubbing (noses) (unthriftiness/ incoordination)	Catarrhal conjunctivitis, stomatitis	Beesley 1998 Taylor et al. 2007

Table 3.3 (continued)

Pathogen	Host	Distribution	Transmission	Clinical symptoms (small ruminants)	Clinical symptoms (human)	References
Schistosoma spp.	Several	Widespread, esp.tropics, subtropics)	Cutaneous	Rhinitis, enteritis, hepatitis, pneumonia	Multisystemic Non-specific Dermatitis	Taylor 1998 Smith and Sherman 2009
Taenia multiceps (*Coenurosis cerebralis*)	Sheep	Unknown,	Oral	Depression, blindness, convulsions	Variable, (localisation)	Lloyd 1998
Toxoplama gondii	Multiple intermediate hosts, Felid family (end host)	Worldwide	Oral (tissue cysts)	Abortion, stillborn, weak offspring	Mild—transient—serious abortion, congenital lesions	See text
Tricophyton verrucosum	Several, mainly cattle	Worldwide	Cutaneous	Alopecia, scaling, crusting, folliculitis	Dermatophytosis	Sparkes 1998

review, the focus is on the distribution, hosts, disease manifestations, transmission, diagnosis, treatment and control measures on a selected number of zoonotic micro-organisms where small ruminants may play an important role as reservoir hosts for human infection. Focus will be on the following pathogens: *Brucella melitensis*, *Chlamydia abortus*, *Coxiella burnetti*, *Echinococcus granulosus*, *Leptospira interrogans*, *Listeria monocytogenes*, Orf-virus, Rift Valley fever virus and *Toxoplasma gondii*.

3.2.1 Brucellosis

3.2.1.1 The Pathogen

Bacteria of the genus *Brucella* are Gram-negative coccobacilli. There are four important species that may cause infection in humans, whereas *B. melitensis* is considered as the most invasive producing the most severe disease in humans (Godfroid et al. 2005; El-Koumi et al. 2013). *B. melitensis* is associated with small ruminants, although *B. abortus* may occasionally cause infection in sheep and goats.

3.2.1.2 Occurrence

Worldwide, particularly in the Mediterranean region, Middle East, parts of Asia and Africa and Central and South America (Corbel 1997; Castrucci 2007).

3.2.1.3 Hosts

B. melitensis is primary found in sheep, goats and camels, but cattle, dogs and rats can also acquire the infection (Castrucci 2007).

3.2.1.4 Disease in Small Ruminants

B. melitensis may cause abortion, and occasionally orchitis and arthritis. Usually, abortion occurs from mid to late pregnancy. The infection may persist in the udder to the following pregnancies. Excretion of bacteria may last for 2 months in vaginal discharges and up to 180 days in milk after delivery or abortion (Castrucci 2007; Scott 2007; Smith and Sherman 2009).

3.2.1.5 Disease in Humans

It has been estimated that around 500,000 cases of human brucellosis occur annually (Franco et al. 2007). The incubation period varies from 1 week to several months. Human brucellosis can be both an acute and a chronic febrile illness with a variety

of clinical manifestations. The patient may show fever, chills, headache, muscle and joint pains, malaise, nausea, night sweats and lack of appetite for 3–6 weeks. The condition may also show a variety of non-specific haematological changes, such as anaemia and leucopenia (Plommet et al. 1998; El-Koumi et al. 2013).

3.2.1.6 Transmission (Small Ruminants-Human)

The main route of entry is via the nasopharynx, although a cutaneous route of infection does also exist. Material from abortions represents the main source of transmission in ruminants, with the excretion of enormous numbers of bacteria in the placenta, fetal fluids, and fetus (Castrucci 2007). Humans, however, are mainly infected through ingestion of fresh (unpasteurized) milk, cheese and meat, but also through direct contact with infected animals, semen, vaginal fluids or infectious aerosols (Castrucci 2007; Smith and Sherman 2009). The environmental resistance of the pathogens varies; the organisms can for instance survive in dust for 3–44 days, in tap water for 30 days, on pasture between 15 and 35 days, and in liquid manure at 15 °C or below for up to 8 months (Plommet et al. 1998; Castrucci 2007).

3.2.1.7 Diagnosis in Small Ruminants

If abortion occurs, *B. meliensis* infection can be confirmed by bacteriological methods (aborted fetus and placenta) or serology (aborted ewe/doe). The diagnosis in the chronic stage of the infection is difficult, since the infection may become non-apparent. In non-pregnant ewes, the bacterium is not excreted from the vagina. However, during pregnancy, excretion starts at the time of delivery or abortion and could continue for several months (Castrucci 2007). There are several serological tests available, such as the standard agglutination test (SAT), Rose Bengal test, compliment fixation test and ELISA.

Although all organs may be infected, microscopic examination should focus on material with suspected large amounts of bacteria, such as placenta, fetus, and vaginal discharges in case of abortion. Stained tissue smears, bacterial culture or PCR can be used for identification (Pommet et al. 1998; Redkar et al. 2001).

3.2.1.8 Treatment and Control

Brucellosis has been controlled in many countries however it remains an important health issue in many developing countries. *B. melitensis* is considered as a important food safety concern in human, because it may be present in dairy products made from milk of infected small ruminants. The bacteria survive for days in fresh milk, weeks in ice cream and months in butter, although the bacteria are killed by pasteurization and are sensitive to common disinfectants (Godfroid et al. 2005).

Chemotherapy is not 100% effective, so little is accomplished with the control and eradication of brucellosis in small ruminants. The best scheme is to identify

and cull the infected animals (Castrucci 2007). Vaccination of sheep and goats with an attenuated strain of *B. melitensis* is considered to be the main control strategy. Vaccination prevents abortion and reduces pathogen shedding from immunized animals, although the vaccine may retain some degree of virulence which may result in abortion and excretion in milk. The vaccine may also interfere with serological testing (Godfroid et al. 2005). In addition, vaccination of replacement animals is not sufficient to control the disease, especially in countries with high prevalence, uncontrolled animal movements, nomadic and low socioeconomic conditions, and illegal import of animals (Ebrahimi et al. 2012).

Surveillance, testing and massive immunization of animals, and national brucellosis control are necessary to eradicate the disease (El-Koumi et al. 2013). For human consumption, unpasteurized milk and milk products should be avoided. No human vaccine exists, however recent results are promising in developing a recombinant vaccine against *B. melitensis* (Gomez et al. 2013).

3.2.2 Chlamydiosis (Ovine Enzootic Abortion (OEA))

3.2.2.1 The Pathogen

Ovine enzootic abortion (OEA) is caused by the obligate intracellular Gram-negative bacterium *Chlamydia abortus*. The organism belongs to the family *Chlamydiaceae* and genus *Chlamydia*, which comprise two distinct developmental forms, a small extracellular infectious elementary body (EB) and a larger intracellular non-infectious, metabolically active reticulate body (Longbottom and Coulter 2003).

3.2.2.2 Occurrence

C. abortus is recognised as a major cause of reproductive loss in sheep and goats worldwide, although the disease does not appear to be a problem in either Australia or New Zealand (Aitken and Longbottom 2007).

3.2.2.3 Hosts

Main hosts are small ruminants, but the organism can also infect cattle, pigs, horses and deer, although such infections are thought to be less common (Aitken and Longbottom 2007).

3.2.2.4 Disease in Small Ruminants

Infection in animals is usually asymptomatic, except abortion, although some behavioural changes or a vaginal discharge may be observed. Ewes/does may deliver

stillborn or weakly offspring that fail to survive. The majority of infected placentas will have thickened red intercotyledonary membranes, dark red cotyledons and have a creamy-yellow coloured exudate on the surface. An infectious vaginal discharge may be observed for several days following abortion, but otherwise the ewes/does are clinically normal and are considered immune to further disease (Longbottom and Coulter 2003; Aitken and Longbottom 2007; Smith and Sherman 2009).

3.2.2.5 Disease in Humans

Although rare, the greatest threat of human infection is to pregnant women, where the outcome of infection in the first trimester of pregnancy is likely spontaneous abortion, while later infection causes stillbirths or preterm labour (Hyde and Benirschke 1997). Several cases of abortion, puerperal sepsis and shock, including renal failure, hepatic dysfunction and disseminated intravascular coagulation, as well as death have been reported (Buxton 1986; Bloodworth et al. 1987).

3.2.2.6 Transmission (Small Ruminants-Human)

Most cases in humans are associated with direct exposure to infected sheep or goats via aerosols. The major sources of infection are contact with placental membranes, dead fetuses, live lambs/kids born to infected mothers and vaginal discharges (Aitken and Longbottom 2007; Smith and Sherman 2009).

3.2.2.7 Diagnosis in Small Ruminants

A presumptive diagnosis of infection can be made based on abortion in the last 2–3 weeks of gestation and examination of the placenta. Pathological changes involve both the intercotyledonary membranes and the cotyledons. This is usually confirmed by the identification of large numbers of EBs in stained smears prepared from the placental membranes and cotyledons using for instance a modified Ziehl-Nielsen procedure. Other methods of antigen detection include immunohistochemical staining of tissue sections, immunoassays, DNA amplification methods, and isolation in cell-culture. Serological testing is normally performed by the complement fixation test on paired blood samples. However, none of the current serological tests have been proven to be suitable for detecting infection prior to abortion and are not able to differentiate vaccinated animals from those infected with wild-type strains (Longbottom 2008; Sachse et al. 2009).

3.2.2.8 Treatment and Control

If OEA is suspected to be present in a flock or herd, the administration of long-acting oxytetracyclines will reduce the severity of infection and losses resulting from

abortion. Although such treatment will reduce losses and limit the shedding of infectious organisms, it does not eliminate the infection nor reverse any pathological placental damage already done; thus abortions or the delivery of stillborn or weakly lambs can still occur and the shed organisms are a source of infection for naïve animals (Longbottom and Coulter 2003; Aitken and Longbottom 2007). Animals that have aborted are considered immune to further disease. Ewes, however, may become persistently infected carriers and continue to excrete infectious organisms at the next oestrus (Papp et al. 1994; Papp and Shewen 1996).

In humans, early therapeutic intervention is important, whereas tetracycline, erythromycin and clarithromycin should be used. Severely ill patients require supportive therapy (Sillis and Longbottom 2010).

During an OEA outbreak the primary aim is to limit the spread of infection to other naïve animals. Affected animals should be identified and isolated as quickly as possible. All dead fetuses, placental membranes and bedding should be carefully destroyed and lambing pens cleaned and disinfected. Pregnant women and immune-compromised individuals are advised not to work with sheep, particularly during the lambing period and should avoid all contact with possible sources of infection. Basic hygiene procedures, including thorough washing of hands and the use of disposable gloves are essential when handling potentially infected materials (Winter and Charnley. 1999; Longbottom and Coulter 2003).

Live-attenuated vaccines based on a temperature-sensitive mutant *C. abortus* strain have been used for several years. These vaccines must be administered at least 4 weeks prior to mating and cannot be used in combination with antibiotic treatment. Good protection from abortion is obtained, but does not completely eradicate the shedding of infectious organisms at parturition. Moreover, some vaccinated animals still abort as a result of wild-type infections. Vaccine development to produce the next generation OEA vaccine continues to progress. This is likely to be a subunit vaccine based on protective recombinant antigens identified through comparative genomic and proteomic approaches (Longbottom et al. 2013; Entrican et al. 2012).

3.2.3 Contagious Ecthyma (orf)

3.2.3.1 The Pathogen

Contagious ecthyma is caused by orf-virus, a DNA- and poxvirus belonging to the genus *Parapoxvirus*.

3.2.3.2 Occurrence

Orf-virus is distributed worldwide.

3.2.3.3 Hosts

Several ruminants may be affected by orf-virus, especially small ruminants.

3.2.3.4 Disease in Small Ruminants

Orf-virus affects the skin primarily around the mouth and udder. There is considerable heterogenicity between virus isolates, but it is still not confirmed if different virulence exists. Genetic differences in orf virus strains seem to be due to geographic locations and animals hosts involved (Reid and Rodger 2007; Li et al. 2012).

The clinical manifestation is variable. Symptoms are seen most frequently in young lambs, normally in two peaks, first in spring shortly after lambing and then 3–4 months later. Morbidity usually approaches 100 %, while in most outbreaks the mortality is low. However, occasionally up to 80 % mortality has been recorded. Severity of outbreaks seems to be attributed to environmental factors (Reid and Rodger 2007).

The lesions usually develop at sites where the skin or the mucous membranes are traumatized. The first clinical signs are local erythema, followed by formation of papules, vesicles, and pustules ending in scab formation. Without secondary infections the lesions resolve within approximately 4 weeks. In natural cases, proliferation often gives rise to wart-like outgrows which may develop into extensive cauliflower-like structures that persist for a long period. Lesions are normally found around the mouth and nostrils, but may also develop on the buccal cavity, eosophagus, ears, axilla, poll, lower limbs and coronet. The infection can also spread to the udder thus increasing the risk of mastitis (Reid and Rodger 2007; Smith and Sherman 2009; Li et al. 2012).

3.2.3.5 Disease in Humans

In humans, after an incubation period of 3–7 days, a macropustular reaction occurs, most commonly found on one finger. As in small ruminants, the development stages comprise erythema, papules, vesicles, pustules and scabs. Several lesions may be present on hand and arm, but single lesions are more common. These are usually raised, circular or oval and about 0.5–1.5 cm in diameter, often with central vesiculation and pustulation. The lesions will normally heal and detach after 6–8 weeks without leaving a scar. However, secondary bacterial infection can cause complications, especially lymphangitis and lympadenitis of the draining lymph nodes which may be associated with flu-like symptoms. Infection may in some cases develop into a generalized reaction, including widespread maculopapular eruption and erythema multiforme. Extensive lesions have especially been seen in immunosuppressed people (Martin 1991b; Reid and Rodger 2007).

3.2.3.6 Transmission (Small Ruminants-Human)

Humans are mainly infected by direct contact with lesions from live animals. Infection can also be transmitted by fomites. Persons directly handling infected animals, particular when bottle-feeding lambs, shearing and slaughtering sheep are especially at risk (Reid and Rodger 2007).

3.2.3.7 Diagnosis in Small Ruminants

Diagnosis is mainly based on clinical signs, such as papillomatous lesions around the lips and nostrils. However, the clinical picture may be atypical and laboratory confirmation is necessary. Electron microscopy has earlier been used to verify the diagnosis, but PCR-methods are now available (Reid and Rodger 2007).

3.2.3.8 Treatment and Control

Outbreaks spread rapidly in a flock, with most animals becoming affected within a few weeks. Such outbreaks will last for 6–8 weeks. No specific treatment is available. The main treatment is to avoid secondary infections. A live vaccine is available in some countries. If vaccination during an outbreak is considered necessary, an autogenous vaccine can also be prepared (Reid and Rodger 2007). Vaccine development using a DNA-vaccine has recently showed promising results (Zhao et al. 2011).

Persistently infected animals with no clinical symptoms have been described. The importance of these animals in the epidemiology of the infection is unknown. The virus may survive in buildings and handling facilities between epidemics. Orfvirus is known to survive in dry scabs for a long period, up to 23 years at 7 °C, but the infectivity is lost more rapidly at higher temperature and at more moist conditions. Disinfection of the actual pens should be performed. Infection in humans can normally be avoided through good hygienic procedures. Protective gloves should be used when handling infectious animals or infective material (Reid and Rodger 2007; Smith and Sherman 2009).

3.2.4 Echinococcosis (Hydatidosis)

3.2.4.1 The Pathogen

E. granulosus is a tapeworm that belongs to the class *Cestoda* and the family *Taeniidae*. Several species of genus *Echinococcus* exist, but it is mainly *E. granulosus* that involves small ruminants as intermediate hosts. Ten genetic types (G1–G10) of *E. granulosus* have been characterized, of which two (G1 and G2) are "sheep" strains (Moro and Schantz 2009). G1 and G6 ("camel" strain) affect goats (Smith and Sherman 2009).

3.2.4.2 Occurrence

E. granulosus is widespread in areas where sheep are reared (Brunetti and White 2012).

3.2.4.3 Hosts

The definite host are domestic dogs and some wild canids. There are several intermediate hosts such as sheep, goats, cattle, swine, camelids, cervids, lagomorphs and humans. The sheep strain G1 is most commonly associated with human infection (Moro and Schantz 2009).

3.2.4.4 Disease in Small Ruminants

Cestode eggs which contain oncospheres must be ingested in order to continue the life cycle of the parasite. After ingestion, the larval stage will develop to cysts (hydatid cyst) in different organs, most commonly in liver and lungs. No definite clinical symptoms have been observed in small ruminants, even in cases with multiple cysts in either liver or lungs (Taylor et al. 2007).

3.2.4.5 Disease in Humans

E. granulosus cysts in humans may take years to develop and produce clinical symptoms. Many cysts remain asymptomatic throughout life and are only discovered by accident. However, the infection can result in respiratory distress and abdominal enlargement depending on which organ is affected. Clinical symptoms depend on the location and size of the *E. granulosus* cyst, and are mainly due to the pressure on the actual organ and on surrounding tissues. In man, the hydatid cysts may be 5–10 cm in diameter or even larger (Martin 1991a). The most common localization is the liver (70%), followed by the lungs. Rupture of the cyst is often fatal, due to anaphylactic shock or pulmonary oedema (Moro and Schantz 2009; Brunetti and White 2012).

3.2.4.6 Transmission (Small Ruminants-Human)

The dog-sheep-dog cycle is the most important cycle in several endemic areas. Small ruminants normally contract *E. granulococus* by gracing on pasture contaminated by dog faeces containing cestode eggs. The dogs are again infected by ingestion of viscera with fertile cysts (Moro and Schantz 2009).

Man can be infected by direct contact to dogs or indirectly through contaminated food, water and infected objects. Dogs may carry eggs on the body surface and a

person can become infected by touching the animal. Close contact with dogs and lack of hygiene are important factors for transmission. Another important source of human infection is through vegetables and water contaminated with eggs. Ingestion of infected flies may also transmit the infection (Lawson and Gemmell 1990). However, direct transmission from small ruminants to man has not been observed (Moro and Schantz 2009; Smith and Sherman 2009).

3.2.4.7 Diagnosis in Small Ruminants

Numerous tests have been developed for the diagnosis in humans although few reliable serological tests are available for small ruminants. Various imaging techniques can be used to identify the hydatid cysts, but post mortem examination is still the most reliable method for diagnosis in intermediate hosts (Moro and Schantz 2009; Smith and Sherman 2009).

3.2.4.8 Treatment and Control

The main control measurement is to interrupt the transmission cycle from the intermediate to the definite host. The infection cycle would be halted if dogs lack access to the viscera of intermediate hosts. In addition, the number of dogs might be reduced or treated with efficient anthelmintics. Treatment of infected sheep/goat in order to stop the infectivity of the cysts is not yet possible. Recombinant vaccines have been developed both for sheep and dogs with promising results (Lightowlers et al. 1999; Zhang and McManus 2008).

Oncospheres have little resistance to desiccation and high temperature, however they may survive in water/damp sand for 225 days at 6 °C (Lawson and Gemmell 1983). Hygiene is important to prevent human infection, as eggs may be swallowed with uncooked vegetables contaminated with dog faeces or from fingers contaminated from soil or the fur of an infected dog. Close contact with possibly infected dogs should therefore be avoided. Early diagnosis in human is important to avoid complications and rupture of the cysts. Surgery was earlier the traditional approach for treatment in humans, but anthelmintics, percutaneous procedures and a watch-and-wait approach are now more commonly used (Brunetti and White 2012).

3.2.5 Leptospirosis

3.2.5.1 The Pathogen

Leptospirosis is caused by helical Gram-negative organisms of the family *Leptospiracea* and the genus *Leptospira*. More than 250 serovariants have been detected (Cerqueira and Pichardeau 2009). The main serovariants infecting small ruminants are *L. borgpetersenii* serovar Hardjo-bovis and *L. interrogans* serovar Pomona.

However, the importance of sheep as a maintenance host of serovar Hardjo-bovis has yet to be unravelled (West et al. 2009).

3.2.5.2 Occurrence

Leptospira involving small ruminants have a worldwide distribution.

3.2.5.3 Hosts

Several hosts are involved, including cattle and swine.

3.2.5.4 Disease in Small Ruminants

There are several serovariants of *L. interrogans* serovar Pomona. Virulence of the strains varies, whereas the majority of leptospiral infections in small ruminants are subclinical. However, septicaemia, depression, anorexia, and in some cases haematuria may occur. Severe illness is characterized by jaundice, haematuria and haemoglobinuria, which may develop to a fatal outcome. Abortion may also occur (Smith and Sherman 2009; West et al. 2009).

Sheep could be infected with *L.* Hardjo-bovis, but are usually asymptomatic and studies indicate that sheep are only transiently infected with this serovar (West et al. 2009). In addition, *L. grippotyphosa*, *L. icterohemorrhagiae* and *L. serjoe* have been involved in clinical leptospirosis in goats (Smith and Sherman 2009).

3.2.5.5 Disease in Humans

Human disease varies widely according to the serovar of *Leptospira* involved. The incubation period varies from 2 to 30 days. In the acute febrile stage, the clinical symptoms are related to a generalized vasculitis, such as severe headache, muscle pain, conjunctival suffusion, rash, and photophobia. Intrauterine infection and fetal death may occur in pregnant women. The infection may proceed to aseptic meningitis and renal failure (Ellis 1998).

3.2.5.6 Transmission (Small Ruminants-Human)

Leptospires persist in the kidney and genital tracks of carrier animals and are excreted in urine and genital fluids. Survival outside the host is favoured by warm and moist conditions. Transmission is mainly due to direct or indirect contact with persistently infected animals and occurs through contact with infected urine, products of abortion, handling of infected kidneys, and ingestion of infected milk. Leptospires gain access to the host mainly through mucous membranes, and abraded and water-softened skin (Ellis 1998).

3.2.5.7 Diagnosis in Small Ruminants

The diagnosis is based on laboratory confirmation, such as PCR analyses of blood, CSF or tissue biopsy, and serology (such as Microscopic agglutionation test (MAT) and ELISA). Leptospires in the urine is, however, not a common feature of serovar Pomona infection in sheep (West et al. 2009). No reliable method exists for detection of carrier animals.

3.2.5.8 Treatment and Control

Leptospira are important pathogens in developing countries, where poor work and living conditions increase the opportunity for transmission from animals to man. The infection often occurs after heavy rainfall, when surface water accumulates in the paddocks. Clinical cases should be treated with antibiotics (West et al. 2009). Vaccines based on killed whole leptospiral cells have been available for several years. Recent vaccine developments based on recombinant proteins showed promising results (Yan et al. 2010; Félix et al. 2011).

In order to avoid the spread of the infection, infected animals should be identified and contact with carrier animals should be minimized. Prevention should be based on environmental control, such as rodent control, elimination of standing water, and avoidance of damp bedding. In addition, contact with infected herds and import of infected animals should be avoided. In order to prevent the human infections, common water sources or potentially contaminated water supplies should be restricted. Farmers, milkers, slaughterhouse and meat-processing workers as well as veterinarians have an increased risk for exposure (Dorjee et al. 2008; Smith and Sherman 2009).

3.2.6 Listeriosis

3.2.6.1 The Pathogen

Listeria monocytogenes is a Gram-positive coccobacillus within the genus *Listeria*. At least 16 serotypes with numerous subtypes of *L. monocytogenes* exist. *L. ivanovii* may occasionally cause abortion in small ruminants, but this bacterium has not yet been associated with human disease (Smith and Sherman 2009).

3.2.6.2 Occurrence

L. monocytogenes is ubiquitious in the environment.

3.2.6.3 Hosts

Several animals including small ruminants can be infected with *L. monocytogenes*. The natural reservoir appears to be the mammalian gastrointestinal tract. Grazing animals will ingest the bacteria and further contaminate vegetation and soil (Scott 2007).

3.2.6.4 Disease in Small Ruminants

There are mainly six manifestations of the disease: abortion, septicaemia, encephalitis, diarrhoea, mastitis and ocular infections. Clinical manifestations vary according to the route of infection. *L. monocytogenes* often affects the pregnant uterus and the central nervous system. During pregnancy, infection spreads to the fetus, which will either be born severely ill or die *in utero* (Scott 2007).

Listeriosis is one of the most common neurological diseases in adult sheep. Sheep aged 18–24 months are often affected due to molar teeth eruption, which may facilitate infection. Lesions are normally localized in the brainstem and clinical signs indicate unilateral dysfunction of the third to seventh cranial nerves. Facial nerve paralysis with dropping ear, muzzle pulled to one side, and lowered upper eyelids are typical symptoms. Profuse salivation and retained food material in the cheek is also typical. Keratoconjunctivitis and iritis may occur, in addition to partial paralysis of the pharynx. The clinical course in sheep and goats is often rapid, and death may occur 4–48 hours after onset of clinical symptoms (Scott 2007; Smith and Sherman 2009).

3.2.6.5 Disease in Humans

Systemic *L. monocytogenes* infection is a serious, but usually sporadic, invasive disease that primarily affects pregnant women, neonates, and immune-compromised persons (Cork and Checkley 2011). Infections can be treated successfully with antibiotics, but 20–40% of human cases are fatal (McLauchlin and Van der Mee-Marquit 1998).

The infective dose of *L. monocytogenes* is not known. The incubation period from food-borne infection varies widely from 3 up to 70 days, with a medium incubation period estimated to be around 3 weeks. There may be strain variation in pathogenicity, but this has to be unravelled more closely. Outbreaks of listeriosis are usually spread via the faecal-oral route, resulting in a self-limiting gastroenteritis in healthy persons. However, cutaneous infection has also been observed in people during deliveries of listeria-infected animals. During pregnancy, infection spreads to the fetus. In non-pregnant human, listeriosis usually presents as meningitis, encephalitis, or septicaemia in the immune-compromised and elderly (McLauchlin and Van der Mee-Marquit 1998; Swaminathan and Gerner-Smidt 2007; Cork 2011).

3.2.6.6 Transmission (Small Ruminants-Human)

Food-borne transmission of *L. monocytogenes* is the main route of infection, whereas unpasteurized dairy products are the main source of human infection. Other sources include uncooked food of animal origin and contaminated raw vegetables. *L. monocytogenes* may also be transmitted by direct contact with infected animals or animal products. In such cases, the disease occurs principally as papular or cutaneous lesions, usually on the arms or the wrist 1–4 days after attending a listeria-abortion. This manifestation, however, has mainly been seen after contact with cattle (McLauchlin and Van der Mee-Marquit 1998; Smith and Sherman 2009).

3.2.6.7 Diagnosis in Small Ruminants

Unilateral cranial nerve paralysis affecting the eye, eyelid, ear and lips with ataxia are typical for listeriosis. Samples from cerebrospinal fluid can support the diagnosis. At post mortem examination, histological lesions such as microabscesses and perivascular cuffing in the brainstem and medulla are pathognomonic of listeriosis. Aborted fetuses due to *L. monocytogenes* are usually autolytic with miliary necrotic foci scattered throughout the liver and spleen, while listeria-septicaemia is often accomplished by focal hepatic necrosis. Listeriosis, however, can only be confirmed by isolation or identification of *L. monocytogenes* (Low and Donachie 1991; Scott 2007).

3.2.6.8 Treatment and Control

Infection can be treated with antibiotics. The drug of choice is high-dosed penicillin. Supportive therapy including fluids and electrolytes are required for animals having difficulty eating and drinking (Scott 2007).

In an outbreak, affected animals should be segregated. In silage-fed ruminants, listeriosis is mainly a winter-spring disease and is normally seen in animals fed with poorly conserved silage. Outbreaks may occur within 10 days of feeding poor silage. Use of the particular roughage should be discontinued. However, due to an incubation period of 1–3 weeks, most of the *Listeria*-infected silage may not be longer available. Animal to animal transmission may occur via the faecal-oral route. A live attenuated vaccine for use in sheep has been developed, but the results from field trial vaccinations are equivocal (Scott 2007). However, new vaccine technologies seem promising in developing a protective immune response against *L. monocytogenes* (Carrasco-Martín et al. 2012; Kim et al. 2012; Lou and Cai 2012; Mohamed et al. 2012).

To avoid infection in humans, hygiene during food preparation and storage as well as avoidance of unpasteurized dairy products are preventive measures. However, *L. monocytogenes* can survive in soil or silage for more than 2 years. It is also found in excreta from apparently healthy animals, although carriage in the gut is

likely to be transitory. Control measures should be focused on avoiding *Listeria*-contaminated food, especially since the bacteria maintain to grow during refrigeration. Pregnant women and immune-comprised individuals are at increased risk for developing listeriosis (McLauchlin and Van der Mee-Marquet 1998).

3.2.7 Q-Fever (Coxiella Burnetii)

3.2.7.1 The Pathogen

Q-fever is caused by the intracellular organism *Coxiella burnetii* within the genus *Coxiella* and the order *Legionellales* (Seshadri et al. 2003). The organism exists in two different antigenic phases. In nature, *C. burnetii* exists in phase I form, which is virulent. However, when cultivated in non-immunocompetent cell cultures or hen eggs the organism mutates irreversibly to the phase II form which is less virulent (Quevedo Diaz and Lukacova 1998). *C. burnetii* has mainly two different morphologic forms, a large and a small form. In addition, an endospore-like structure is observed in the large form, which is highly resistant to environmental degradation, such as high temperatures, ultraviolet light and osmotic shock (Mearns 2007).

3.2.7.2 Occurrence

Q fever is a worldwide zoonosis that occurs in all geographic and climate zones, with the exception of Antarctica and possibly New Zealand (Hilbink et al. 1993; West et al. 2009). However, Q fever is not a reportable disease in many countries, so it is difficult to know exactly where it occurs.

3.2.7.3 Hosts

C. burnetii is able to infect many animal species including mammals, birds and several arthropods. However, cattle, sheep and goat seem to be the primary animal reservoirs for human infection (Maurin and Raoult 1999).

3.2.7.4 Disease in Small Ruminants

In animals, *C. burnetii* infections are generally asymptomatic, except for abortion, stillbirth and the delivery of weak offspring. However, *C. burnetii* may induce pneumonia, conjunctivitis and hepatitis (Arricau-Bouvery and Rodolakis 2005). High abortion rates are rarely observed, although abortion storms in some caprine herds have been described (Sanford et al. 1994). Aborted fetuses appear normal, but infected placentas exhibit intercotyledonary fibrous thickening and discoloured exudates that may be mineralized (Moore et al. 1991).

3.2.7.5 Disease in Humans

In humans, acute Q fever is rarely diagnosed, because of non-specific initial clinical signs, such as fever, pneumonia, headache and weakness. However, chronic infection may result in severe granulomatous hepatitis, osteomyelitis and valvular endocarditis. Chronic infection can manifest itself within a few months or even years after the acute infection (Fournier et al. 1998).

3.2.7.6 Transmission (Small Ruminants-Human)

Contaminated aerosols generated from desiccation of infected placentas, body fluids or dust from contaminated manure are the main sources of both animal and human infection, and the control of fecal excretion and placental bacterial discharge is essential (Arricau-Bouvery and Rodolakis 2005). Grazing contaminated pasture and tick bites are other modes of transmission. The organism is highly infectious, with an infective dose of 1–10 bacteria (Tigertt et al. 1961). Because *C. burnetii* is extremely resistant to desiccation and to physical and chemical agents, it survives in the environment for long periods. The endospore-like form survives in dust for 120 days, in tick faeces for 568 days and in wool for 12–16 months at 4–6 °C (Mearns 2007).

3.2.7.7 Diagnosis in Small Ruminants

Current alternatives to diagnose *C. burnetii* infection in ruminants include serological analysis, isolation by cell culture, live animal inoculation, immunohistochemical and PCR-based detection. In the acute phase of the infection, *C. burnetti* can be detected in lungs, spleen, liver and blood (Fournier et al. 1998; Maurin and Raoult 1999).

Placental smear or impression of placentas could be stained for instance by using a modified Ziehl-Nielsen procedure (Mearns 2007). Several serologic tests are available, such as complement fixation test, ELISA, and a fluorescent antibody test. However, carrier animals may also have an antibody titre increase in late pregnancy (Kovacova et al. 1998; Smith and Sherman 2009). For Q fever diagnostis, it has been recommended to use PCR and immunofluorescence tests of *Coxiella* on parturition products and vaginal secretions at abortion (Arricau Bouvery et al. 2003).

3.2.7.8 Treatment and Control

If Q fever is suspected, aborting animals and other animals in late pregnancy should be treated with tetracycline, although this treatment does not totally suppress the abortions and shedding of *C. burnetii* at lambing (Berri et al. 2005). Placentas and aborted fetuses should be destroyed properly and aborted animals should be

isolated. In addition, materials such as bedding and straw contaminated with birth fluids and other secretions from affected animals should be destroyed (Smith and Sherman 2009).

The spread of *C. burnetii* infection in domestic animals depends on many factors, such as population density of animals, the system of rearing and management at parturition. Because the environment can remain infected for a long time and many species can be carriers, test and cull strategies are not appropriate for infected herds (Smith and Sherman 2009). However, during the recent outbreak of Q fever in humans in the Netherlands, the Dutch Government decided to cull more than 50,000 pregnant ewes and goats in order to halter the worst outbreak of Q fever ever known where more than 4000 human cases have been recorded from 2007–2010. The reason for this strategy was that dairy goats were believed to be the main source of human infection (van der Hoek et al. 2012).

In animals, the uterus and mammary gland of females are sites for persistent *C. burnetii* infection. Reactivation of the bacterium during pregnancy results in shedding of a great amount of infectious agent into the environment during abortion or via birth fluids, placenta and fetal membranes (Sawyer et al. 1987). Over 10^9 bacteria per gram of placenta may be released at the time of delivery (Babudieri 1959). Studies indicate that ewes shed the bacterium mostly in feces and vaginal mucus, while in goats shedding in milk seems to be the most frequent route (Rodolakis et al. 2007; Rodolakis 2009).

In animals, the most effective vaccines are those composed of inactivated whole phase I bacteria. Bacterial shedding in placentas and milk was strongly reduced in experimental infection or in natural Q fever infection in ewes vaccinated with phase I vaccines (Sampere et al. 2003). Since phase I vaccine are dangerous to produce, a subunit vaccine is now being investigated (Arricau-Bouvery and Rodolakis 2005).

To prevent human infection, drinking raw milk or consumption of raw milk products should be restricted. For inactivation, pasteurization of milk at 62.8 °C for 30 min or at 71.7 °C for 15 s is required (Kazar 1999). Q fever often occurs as an occupational disease. Persons at particular risk are livestock handlers, processors of animal products, abattoir workers, those in contact with dairy products, veterinarians and laboratory personnel working with *C. burnetii*-infected animals (Maurin and Raoult 1999). In addition, it is necessary to inform vulnerable persons such as immunosuppressed patients or those suffering from cardiac valvopathy and pregnant women that they must avoid contact with animals during lambing and kidding (Arricau-Bouvery and Rodolakis 2005).

3.2.8 Rift Valley Fever (RVF)

3.2.8.1 The Pathogen

RVFV (Rift Valley fever virus) is a single-stranded RNA-virus in the genus *Phlebovirus* of the family *Bunyaviridae*.

3.2.8.2 Distribution

RVFV is mainly distributed in sub-Saharan Africa, but has also been identified in Northern Africa and on the Arabian Peninsula (Bath 2007).

3.2.8.3 Hosts

RVFV may cause infection in several ungulate species, although their importance as reservoir host has to be unravelled. Mosquito vectors, such as in the genus *Aedes*, may maintain the virus in endemic areas by transovarial transmission. Other insects, such as *Culex* species, may also be involved in epidemics (Bath 2007).

3.2.8.4 Disease in Small Ruminants

RVFV can infect a wide variety of tissues, such as liver, lymphoid and nervous tissue. The incubation period is short, as little as 12 h in young lambs and up to 72 h in adult sheep. High fever, anorexia, listlessness, and recumbence are common in young lambs. However, clinical signs are not always observed, since young animals may die rapidly. Mortality rate may exceed 90 % in lambs under 2 weeks old. Abortion is a common sign in adult animals, and this may occur at any time during pregnancy and reach up to 100 %. Infection in older animals is often subclinical (Bath 2007).

3.2.8.5 Disease in Humans

The largest recorded outbreak in humans was in 1997–98 in East Africa where approximately 89,000 human cases and 478 fatalities were recorded (CDCP 1998). Typical symptoms in humans are flu-like illness after a short incubation period of 2–6 days. Other symptoms are photophobia, retinitis, meningoencephalitis and haemorrhagic fever. The symptoms may be severe in patients with a pre-existing liver disease. Sequelae may include widespread haemorrhages, jaundice, shock, liver and kidney failure and death. Fatality rate is normally less than 1 %, but the death toll can mount to several hundreds in severe outbreaks (Swanepoel 1998; Bath 2007).

3.2.8.6 Transmission (Small Ruminants-Human)

The route of transmission in animals is via different mosquitoes. The virus has been isolated from more than 30 mosquito species. In addition, RVFV has also been isolated from flies and midges (*Culicoides*). Both biological and mechanical vector

transmission may occur. In Sub-Saharian areas the main vector seems to be mosquitoes within the genus *Aedes* (Bath 2007; Smith and Sherman 2009).

The main transmission route in humans is via direct or indirect exposure to infected blood, tissues or body fluids of infected animals, for instance in connection with slaughtering, butchering, obstetrical procedures or treatment of infected animals. Infection may also occur via vectors, aerosols, and consumption of unpasteurized milk. Direct person-to-person transmission has not been reported. Persons at risk are veterinarians, farmers, shepherds and abattoir workers (Swanepoel 1998; Smith and Sherman 2009).

3.2.8.7 Diagnosis in Small Ruminants

The diagnosis is based on clinical symptoms and post mortem examination. In young lambs, widespread haemorrhages and liver necrosis is often recorded. Disseminated intravascular coagulopathy may occur in several internal organs. Samples from spleen, liver and brain should be used for histological examination. The diagnosis, however, has to be verified by PCR, virus isolation, and serological investigations (such as ELISA and hemagglutination-inhibition test) (Bath 2007).

3.2.8.8 Treatment and Control

Outbreak of RVF occurs at irregular intervals. The virus may persist in a vector/natural host cycle and low level of virus activity is found between outbreaks. Infected eggs from *Aedes* species may survive in the soil for years. The single most important responsible factor for an outbreak of RVF is heavy rainfall and widespread flooding which favours multiplication of the vectors. Movement of infected animals or winds that transport infected mosquitoes over long distances may spread the disease to non-endemic areas (Sellers 1980). The development of early warning systems and surveillance in and around endemic areas in order to recognize animal and human cases as early as possible, are crucial in order to control the infection.

There is no treatment available for infected animals, since the disease is usually very acute and the lesions too severe. Control measures rely on the use of efficient vaccines. A live attenuated vaccine is available for non-pregnant animals, while an inactivated whole virus vaccine can be used for pregnant animals. The last vaccine requires a booster and annual revaccination. A recombinant vaccine has recently been developed, but it must be tested in appropriate animal models before used as a livestock and human vaccine (Indran and Ikegami 2012; Morrill et al. 2013). When handling suspicious animals, wearing of eye protection, protective clothing, gloves and masks should be mandatory (Swanepoel 1998).

3.2.9 Toxoplasmosis

3.2.9.1 The Pathogen

Toxoplasma gondii is a protozooan parasite within the family *Sarcocystiidae* and genus *Toxoplasma*. The lifecycle can be divided in two parts, a sexual cycle, restricted to enteroepithelial cells in cats and the production of oocysts, and an asexual cycle (forming tissue cysts) which occurs in a wide range of warm-blooded intermediate hosts. Six major clades of *T. gondii* have been characterized (Buxton and Rodger 2007; Su et al. 2012).

3.2.9.2 Occurrence

T. gondii has a worldwide distribution.

3.2.9.3 Hosts

Multiple intermediate hosts seem to exist, but the most important domestic hosts are pigs, sheep and goats. The final host is in the felid family.

3.2.9.4 Disease in Small Ruminants

Clinical toxoplasmosis causes abortion and neonatal mortality in small ruminants, especially in sheep. Mummified fetuses, stillborn or weak offspring are common features. However, infection in early pregnancy (<55 days) may result in death or expulsion of a small fetus. Clinical signs in aborting animals are usually not observed. Abortion is associated with primary infection during pregnancy in non-immune animals, and is most commonly seen in young animals. A long-lasting immunity develops following primary exposure and animals are unlikely to abort again due to toxoplasmosis (Buxton and Rodger 2007; Smith and Sherman 2009).

3.2.9.5 Disease in Humans

In most cases, toxoplasmosis in human is a disease with relatively mild and transient symptoms. However, primary infection during pregnancy may lead to intrauterine infection, and result in abortion or congenital lesions in the fetus. In addition, in patients with impaired immunity, *T. gondii* may lead to serious and even fatal infection (Dubey and Beattie 1988).

3.2.9.6 Transmission (Small Ruminants-Human)

The proportion of the human population infected with *T. gondii* depends on the age, area and environment. Most human infection appears to result either from exposure to oocysts from a contaminated environment or from ingestion of raw or lightly cooked meat containing tissue cysts. The most common way for infection from small ruminants to humans is by ingestion of tissue cysts. In addition, human infection through drinking of unpasteurized goat milk has been reported. A low risk may also apply when assisting infected animals at lambing or kidding. However, both these last modes of transmission are probably of low significance (Dubey and Beattie 1988; Smith 1991).

3.2.9.7 Diagnosis in Small Ruminants

Abortion due to *T. gondii* occurs mainly in young animals. Typical clinical signs of abortion result following infection in mid-gestation, with ewes and does producing stillborn and/or weekly offspring often accompanied by a mummified fetus. Cotyledons will also show characteristic lesions, such as white foci of necrosis 2–3 mm in diameter which may become mineralized. Diagnosis may include serology (such as Sabin-Feldman dye test, IFAT, MAT and ELISA), histology, immune-histochemistry and PCR methods (Buxton and Rodger 2007; Taylor et al. 2007; Smith and Sherman 2009).

3.2.9.8 Treatment and Control

Susceptible animal get infected by ingestion feed or water contaminated with oocysts. The oocysts are highly resistant and survive for a long period (>500 days) at room temperature in moist conditions. The main source of *Toxoplasma* infection in small ruminants are oocysts excreted from cats. Susceptible cats become infected with *T. gondii* after ingestion of tissue cysts from for instance small rodents and may excrete a large numbers of oocysts, which then sporulate and become infective within a few days and remain so for several months. Infected faeces will then contaminate beddings, stores of hay, concentrates, water supplies and pasture. It has been estimated that although <1 % of the cat population may excrete oocysts at any time, contamination of the environment is readily maintained (Dubey and Beattie 1988; Buxton and Rodger 2007).

During an outbreak of toxoplasma-abortion little can be done. Infected placentas and dead lambs or kids should be buried or disposed to prevent their ingestion by other animals. Animal to animal transmission during lambing or kidding does not appear to occur to any significant extent. More direct preventive measures include chemoprophylaxis, chemotherapy and vaccination. A live vaccine base on an attenuated strain of *T. gondii* has been developed for sheep (Buxton and Rodger 2007; Smith and Sherman 2009).

In humans, as already mentioned, the most common way for infection from small ruminants is by ingestion of raw or lightly cooked meat. Tissue cysts may be viable for the lifetime of infected sheep (Dubey and Beattie 1988). Treatment of meat by curing, smoking, freezing at −20 °C is usually sufficient to kill the encysted *T. gondii*. However, cysts can survive insufficient microwave cooking (Lundén and Uggla 1988). In a recent study, treatment of tissue cysts in infected sheep to prevent human exposure to meat-borne toxoplasmosis have shown promising results (Kul et al. 2013). Shepherds, veterinary surgeons, slaughterhouse staff and butchers are especially at risk for contracting infection from small ruminants.

3.3 Concluding Remarks

Only a limited number of topics are covered by this brief review and important issues such as differential diagnoses are not included or discussed. A correct and swift diagnosis is a prerequisite for proper treatment and control. This may not always be available due to long incubation periods, unspecific clinical symptoms and imprecise diagnostic tests. Some pathogens may survive unnoticed in animals or animal products for a long period of time. Anthrax in humans, for instance, has occurred when handling imported goat skins for drum making, skins contaminated with spores of *B. anthracis* (Anaraki et al. 2008).

Microbial transmission will always occur between species, but the risk of transmission can be reduced with proper hygiene, management, husbandry and prophylactic treatment. In this context, recent vaccine development against several zoonotic pathogens through genomic and proteomic approaches is promising.

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Chapter 4
Zoonoses with Public Health Relevance in Poultry

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Abstract The two most important and most frequently occurring diseases transmitted from poultry to humans are *Salmonellosis* and *Campylobacteriosis*. Both are food borne diseases. They rarely cause disease in infected poultry and but frequently diarrhoea in infected humans. *Avian Influenza* and *Newcastle Disease* can cause severe disease in poultry flocks. Transmission to humans by direct contact is possible. Avian influenza viruses can cause respiratory disease in humans, which may be lethal, while Newcastle Disease Virus usually only causes conjunctivitis. *Chlamydiosis* is a systemic disease characterized by respiratory symptoms. Via inhalation of aerosols it can spread to humans, where the disease is called psittacosis or ornithosis. Main symptoms are respiratory signs. *Erysipelas* is a septicemic disease, mostly in turkeys, but also in ducks and laying hens. If transmitted to humans via skin injuries it causes a localized swelling and purple discolouration at the infection site. *Avian tuberculosis* may be transmitted from pet birds to immunocompromised humans. Transmission from poultry is rare, but cases diagnosed in poultry usually concern the owners.

4.1 Introduction

Most poultry in developed countries is kept in large flocks on specialized farms. These husbandry conditions offer a high level of biosecurity and only few humans or other animals have direct contacts with the birds. Thus the main transmission route from poultry to humans is via contaminated food, namely meat and eggs. In regard of the incidence of infected humans, Salmonella and Campylobacter are the two most important zoonotic agents in poultry causing food borne infections. In spite of significant improvements in technology and hygienic practice at all stages of the poultry production these pathogens remain a persistent threat to human health.

In developing countries, where smaller backyard flocks are more common and where many of the birds raised for meat production are traded live to the consumer, direct contact between poultry and humans is more frequent. Thus diseases are

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transmitted more often directly from poultry to humans. The prime example, which despite a relatively low number of infected humans has caused major attention in the media, is Avian Influenza. So far cases of Avian Influenza in humans have been mainly restricted to various developing countries. Other pathogens transmitted from poultry to humans by direct contact include Newcastle Disease and Chlamydiosis.

Erysipelas has an exceptional position, since it mainly infects humans through skin injuries. Thus besides persons handling infected birds personnel at slaughter houses have an elevated risk of infection.

Avian tuberculosis caused by *Mycobacterium avium* can spread occasionally from captive exotic birds or more often from swine to humans with impaired immune system. The spread from poultry to humans seems to be very rare. However, the disease was included in this chapter, because it is occasionally found in backyard or hobby poultry flocks, and these cases usually are unsettling for the owners.

Further zoonotic diseases, which have lesser importance either, because they occur only infrequently or because they have a low virulence for humans, are listed in Tables 4.1, 4.2, and 4.3.

4.2 Salmonella

4.2.1 Occurrence

Salmonellosis and salmonella infections in poultry occur worldwide. Of the more than 2500 serovars of *Salmonella enterica* ssp. *enterica* approximately 10–20 serovars are able to cause an invasive infection in poultry and may be capable of infecting humans. Currently, the most important serovars are *S. Enteritidis, S. Typhimurium,* and *S. Hadar*. Some serovars may be predominant for a number of years in a region or country, before they disappear and are replaced by another serovar. In the past the dominant type worldwide of salmonella food poisoning was *S. Typhimurium*, but since the early 1980s *S. Enteritidis* has become more important.

The prevalence of salmonella infected flocks and the serovars involved varies widely between different countries. Current data obtained by monitoring programmes in the European Union are published in regular intervals.

Within an infected flock the prevalence of infected birds can be well below 10 %.

4.2.2 Disease in Animals

The course of the infection of salmonellosis in poultry depends on a number of factors such as involved salmonella serovar, age of birds, infectious dose and route of infection. The incubation periods range between 2 and 5 days. Mortality in young birds varies from negligible to 10–20 % and in severe outbreaks may reach 80 % or higher.

Table 4.1 Zoonotic bacteria, rickettsia and chlamydia detected in poultry

Pathogen	Host	Distribution	Transmission	Clinical symptoms (poultry)	Clinical symptoms (human)	References
Campylobacter jejuni, C. coli	Several birds and mammals	Worldwide	Oral	None	Diarrhoea	Wassenaar et al. (2009)
Chlamydia psittaci	Several birds and mammals	Worldwide	Aerosol, also oral	Respiratory, conjunctivitis, anorexia, cachexia	Respiratory	Beeckman and Vanrompay (2009)
Erysipelothrix rhusiopathiae	Several vertebrates	Worldwide	Skin injuries, also oral	Septicaemia, skin lesions	Skin lesions	Bricker and Saif (2008)
E. coli O157:H7	Several birds and mammals	Worldwide	Oral	Septicaemia or asymptomatic	Diarrhoea	Hafez et al. (2001)
Mycobacterium avium subsp. avium	Several birds and mammals	Worldwide	Oral	Emaciation, Tubercles in organs	Tuberculosis like pneumonia	Fulton and Sanchez (2008)
Salmonella enterica	Several birds, mammals, reptiles	Worldwide	Oral	Septicaemia in young birds, in older asymptomatic	Diarrhoea	Hafez (2013)

Table 4.2 Zoonotic viruses detected in poultry

Pathogen	Host	Distribution	Transmission	Clinical symptoms (poultry)	Clinical symptoms (human)	References
Crimean Congo Haemorrhagic Fever Virus	Ostriches, several mammals	Asia, Africa	Ticks	Asymptomatic	Haemorrhagic fever	Swanepoel et al. (1998)
Eastern Equine Encephalitis Virus	Turkeys, wild birds and several mammals	North and South America	Ticks	Nervous	Nervous	Komar et al. (1999)
Influenza A Viruses	Several birds	Worldwide	Aerosol	Respiratory, high mortality	Flu	Abdelwhab and Hafez (2011)
Newcastle Disease Virus	Several birds	Worldwide	Aerosol	Respiratory, nervous, high mortality	Respiratory, conjunctivitis	Anon (2012)
West Nile Virus	Several birds and mammals	Africa, India, North America, rarely Europe	Ticks	Asymptomatic or nervous	Flu like, Nervous	Fitzgerald (2013)

Table 4.3 Zoonotic parasites and fungus detected in poultry

Pathogen	Host	Distribution	Transmission	Clinical symptoms (poultry)	Clinical symptoms (human)	References
Dermanyssus gallinae	Birds	Worldwide	Contact	Skin irritations	Skin irritations	Sparagano (2009)
Cryptococcus neoformans	Some birds, mammals	Worldwide	Oral	Asymptomatic	Nervous	Staib (1961)
Cryptosporidum meleagridis	Turkeys, other birds, humans	Worldwide	Oral	Diarrhoea, respiratory	Diarrhoea	Anon (2008)
Histoplasma capsulatum	Some birds, mammals	Worldwide, especially North America	Aerosol, oral	Asymptomatic?	Respiratory	Bilgi (1980)

Symptoms include an increased number of unpipped and pipped hatching eggs with dead embryos, if infection was egg transmitted or occurred in the hatchers. Signs usually seen in young birds are somnolence, weakness, drooping wings, ruffled feathers, and huddling together near heat sources. Many birds that survive for several days will become emaciated, and the feathers around the vent will be pasty with faecal material (Pasty Vent). Furthermore respiratory distress as well as lameness as a result of arthritis may be present. Adult birds serve mostly as intestinal or internal organ carriers over longer periods with little or no clinical signs.

Birds that die in an acute phase of the disease have shown a persistent or inflammed yolk sac, catarrhal and haemorrhagic enteritis, necrotic foci in liver, spleen heart muscle, and granuloma in the lungs. Furthermore, congestion of the liver, kidney, gall bladder, and heart muscle are the most constant post-mortem findings. Caeca may contain a caseous core and sometimes are filled with blood. In adult birds lesions most frequently found in chronic carrier hens are misshapen, pedunculated, discoloured cystic ova. The involved ova usually contain oily and caseous material enclosed in a thickened capsule. Ovarian and oviduct dysfunction may lead to abdominal ovulation or impassable oviduct, which in turn bring about extensive peritonitis and adhesions of the abdominal viscera. In male birds, the testes may be atrophied with thickening of tunica albuginea and multiple abscesses.

After recovery salmonellae can persist in the intestines, especially in the caeca and the caecal tonsils, and birds continue to excrete salmonellae intermittently in their faeces.

4.2.3 Disease in Humans

Infections with *S. enterica* serovars may cause intestinal inflammation with mucopurulent or bloody diarrhea, accompanied by fever, vomiting, and abdominal cramps for several days. Incubation time is between less than one day and three days. If high amounts of water and electrolytes are lost, a hypovolemic shock can result. In severe cases, especially in infants and immunocompromised persons, sepsis and spread to other organs may occur, leading to a septic shock.

4.2.4 Transmission

Lateral spread of infection takes place through contaminated feed, water, equipment, and the rearing environment. Significant reservoirs for these microorganisms are asymptomatically infected chickens, other avian species including pigeons and wild birds, other farm animals, and pets. Rodents are potential reservoirs transmitting infection between houses and contaminating stored feeding stuffs. In addition, insects are a potential source of salmonella infection in chickens.

Some invasive salmonellae can be transmitted vertically in the eggs (true transovarian). Further, salmonellae can enter eggs contaminated with faeces through the pores of the shell after laying.

During the slaughter process carcasses can become contaminated with salmonellae, if the equipment is contaminated from the same or a previous flock. Humans can contract the disease if they consume eggs or meat, which are not thoroughly cooked.

4.2.5 Diagnosis

Samples for the detection of *Salmonella* spp. in poultry flocks are organs, yolk sac, crop, caeca, liver or spleen from diseased birds. Samples for monitoring healthy flocks include composite samples of faeces, boot swabs, or dust. In many countries the sampling protocols for Salmonella monitoring are regulated by legislation. The laboratory procedure to detect *Salmonella enterica* serovars is described in ISO 6579.

4.2.6 Treatment and Control

Treatment of salmonellosis in poultry with antibiotics may reduce clinical signs, but does not clear the birds from the infection and stop the intermittent shedding of the bacteria. For this reason antimicrobials shall not be used as a specific method to control salmonellae in poultry in the European Union.

The main strategy to control salmonellae in poultry include cleaning the production pyramid from the top by culling infected breeder flocks, hatching egg sanitation, and limiting introduction and spread at the farm level through Good Animal Husbandry Practices (GAHPs). To achieve GAHPs, effective hygiene measures should be applied to poultry houses, their environment and the feed. In addition, reducing salmonellae colonization by using feed additives, competitive exclusion, or vaccines are further possibilities. Eggs and meat derived from *Salmonella* infected flocks must be heat treated to eliminate salmonellae to render it safe for human consumption.

All this measures should be, and in many countries are, regulated and accompanied by governmental supervisions and legislations.

4.3 Campylobacteriosis

4.3.1 Occurrence

Thermophilic campylobacter have been found worldwide in poultry flocks. In most countries prevalence in broiler, layer, and turkey flocks is higher than 50 %. *C. jejuni* is the predominant species in poultry, while *C. coli* is less common, and *C. lari* is rare. Flocks younger than 3 weeks are rarely affected. Additionally, there is a seasonal variation, since infection rates are higher in spring and fall than in winter and summer. Current data for the countries of the European Union have been published recently (EFSA 2012).

4.3.2 Host Range

In poultry the highest prevalence of thermophilic Campylobacter has been detected in gallinaceous birds, but wild and commercial aquatic birds are also frequently affected. Various mammalian species including cattle, sheep, pigs, pets, or rodents can also be carriers.

4.3.3 Disease in Animals

Virtually all infections of poultry with thermophilic Campylobacter are without clinical signs or pathological lesions. Only chickens infected with virulent isolates at the time of hatch may develop enteritis with accumulation of mucus and fluid or focal hepatic focal necrosis and some mortality.

Avian vibrionic hepatitis was a disease which occurred in laying hens in the 1950s and 1960s. The hepatitis was characterized by small greyish-white focal lesions. *Campylobacter* spp. were regarded as the causative organisms, but for unknown reasons the disease has not been observed in recent times.

4.3.4 Disease in Humans

After an incubation period of one to seven days the most common clinical symptom of infections of humans with thermophilic Campylobacter is diarrhoea. Other symptoms may be abdominal cramps, nausea, vomiting, fever or headache.

Some post-infectious complications may be associated with Campylobacter infections, the most important of which is Guillain–Barré syndrome. Guillain–Barré syndrome is a rapidly evolving paralysis without fever or other systemic symptoms, and apparent causes. The symptoms are the consequence of an acute inflammatory demyelinating polyneuropathy. It is thought that antibodies against gangliosides are involved in the pathogenesis, and that these auto-antibodies are originally formed against *C. jejuni* strains possessing ganglioside-like epitopes or other pathogens.

4.3.5 Transmission

It is not fully understood, how thermophilic *Campylobacter* spp. are introduced into flocks. Due to their low tenacity they probably depend strongly on living avian or mammalian vectors. Furthermore, various insects can serve as vectors for *Campylobacter* spp.

However, water supply sources, farm equipment such as trucks, forklifts, pallets, crates, and footwear have also been identified as potential sources of Campylobacter infection of poultry. Outside personnel like thinning crews also pose a major risk for the introduction of Campylobacter into flocks. Further, biofilms in water pipes offer Campylobacter an opportunity to survive for several weeks. The possibility of vertical transmission of *Campylobacter* spp. is very controversial.

Infection of humans generally occurs by the consumption of raw or undercooked meat, which originated from infected flock and/or was contaminated during the slaughter process.

4.3.6 Diagnosis

Campylobacter spp. will grow on Columbia blood agar, but other bacteria will easily overgrow them. So for the detection of thermophilic Campylobacter selective media like Karmali agar or Preston broth are used. A standardized procedure for the isolation of Campylobacter from food and feed has been published in ISO 10272-1:2006. This method may be adapted for the investigation of clinical samples.

Species differentiation by biochemical traits is considered unreliable and nowadays commonly replaced by molecular biological tools for pheno- and genotyping. Several PCR-protocols for direct detection of Campylobacter in samples without previous isolation also have been published.

4.3.7 Treatment and Control

Due to the unclear mechanisms how Campylobacter are introduced into poultry flocks, specific methods to prevent infections of flocks pre-harvest are difficult to define. Generally the same measures for biosecurity as described for *Salmonella* are suggested. However, enhanced biosecurity cannot eliminate the possibility of introduction of infection. Chlorination of water or acidifying of the litter may be helpful to reduce the spread of Campylobacter within an infected flock. Treatment with antibiotics does not remove Campylobacter from an infected flock, and commercial vaccines are not available.

Further emphasis is on reducing the level of (cross) contamination of carcasses at slaughter houses post-harvest. Measures for post-harvest control include withdrawal of feed and addition of lactic acid to the drinking water 8–12 h before slaughter and chlorination of scalding or cooling water and thoroughly cleaning the equipment between slaughtered flocks. Some of the above mentioned measures, however, seem to be not as effective against Campylobacter as they are against other bacteria and are partially prohibited in the European Union.

4.4 Avian Influenza

4.4.1 Occurrence

Avian influenza (AI) caused by influenza A viruses occurs worldwide. Regions with high infection rates include Mexico, Egypt, and South East Asia. In Europe, North America, except Mexico, and Australia infections of domestic poultry with Avian Influenza are comparatively rare.

4.4.2 Host Range

Influenza viruses can infect virtually all bird species. Wild aquatic birds like duck and geese have the highest prevalence of infection. Prevalence of infection in poultry varies widely between countries.

4.5 Disease in Poultry

The severity of clinical signs, course of the disease, and mortality in poultry after infection with AI are extremely variable from highly acute to a very mild or even inapparent form with few or no clinical signs. The most virulent form of AI in poultry is designated as "Highly Pathogenic Avian Influenza" (HPAI). Currently, only viruses of H5 and H7 subtype have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses cause HPAI.

Clinical signs may include high mortality, ruffled feathers, depression, diarrhoea, sudden drop in egg production, cyanosis of comb and wattles, oedema and swelling of head, blood-tinged discharge from nostrils, respiratory distress, incoordination and pin-point haemorrhages mostly seen on the feet and shanks.

Lesions at post mortem may include swelling of the face. Removing skin from the carcass will show a clear straw-coloured fluid in the subcutaneous tissues. Blood vessels are usually engorged. Haemorrhage may be seen in the trachea, proventriculus, and throughout the intestines. Young broilers may show signs of severe dehydration with other lesions less pronounced or entirely absent.

4.5.1 Disease in Humans

Infection of humans with AI causes disease of the lower respiratory tract, leading to cough, sore throat, breathing problems, and pneumonia. Other flu-like symptoms that may be caused by AI include fever and muscle aches. In atypical cases respiratory symptoms may be absent, and diarrhoea or neurologic signs have been reported in infected humans.

Lethality in confirmed cases is well above 50%, however especially in developing countries many non-fatal cases of the disease with mild symptoms may be undiagnosed.

4.5.2 Transmission

The disease can be transmitted directly through contact with infected birds or indirectly through contaminated equipment. The infection can easily be spread on contaminated shoes, clothing, egg crates, egg flats, and egg cases vehicles. The major way of the further spread of avian influenza viruses seems to be mechanical transfer of infective faeces through movement of man and contaminated equipment. Rodents and insects may mechanically carry the virus from infected to susceptible poultry. A distinct relation between the proximity of poultry rearing areas and migratory waterfowl routes as well as the method of rearing were observed.

There is little or no evidence of vertical transmission. However, eggshell surfaces can be contaminated with the virus.

Transmission from birds to humans occurs only after close contact with infected live birds. This happens most frequently in rural South East Asia and in Egypt, where poultry is kept close to living quarters. Food borne infections of humans have not been reported. However this possibility should not be ruled out completely, since some felids like tigers and cats got infected this way. AIV has been transmitted only in rare cases between humans.

4.5.3 Diagnosis

Influenza virus may be isolated in embryonated chicken eggs followed by typing by haemagglutination inhibition assay and classifying as highly or low pathogenic in infection studies. Alternatively, molecular biological detection and characterization can be done. Methods are described in detail by the OIE and are part of the legislation in many countries.

4.5.4 Treatment and Control

Control of AI is regulated at the national and international level, but most of the legislation is motivated by the disastrous consequences of the disease for infected poultry rather than by its public health significance. In countries with a low prevalence of AI infected flocks usually are destroyed, while countries with a high prevalence have adopted vaccination strategies.

In the European Union meat obtained from poultry infected with HPAIV has to be traced and destroyed. The same applies to table eggs laid during the presumed incubation period, unless they have been properly disinfected.

4.6 Newcastle Disease

4.6.1 Occurrence

Newcastle disease (ND) is ranked as the major virus disease of poultry in many countries worldwide. Due to widespread vaccination it is extremely difficult to assess the prevalence of ND in the world. In developed countries with established poultry industries, outbreaks with very virulent (velogenic) ND are comparatively rare or neglected.

4.6.2 Host Range

Infections with NDV have been reported worldwide and in at least 241 bird species from 27 different orders. It has been suggested that virtually all birds are susceptible to infection with NDV. Infections have also been reported in some non-avian species.

4.6.3 Disease in Animals

The course of the disease is mainly influenced by the virulence of the involved strain. Age and immune status of the birds as well as general health and environmental conditions also play a role. Infection with velogenic strains is mostly accompanied with a high mortality rate ranging between 30 and 50 %. Clinical signs may include ruffled feathers, depression, diarrhoea as well as respiratory signs in form of nasal discharge, coughing, rales, and dyspnea. These symptoms are mostly accompanied by nervous signs such as tremors, twisting of the head and neck, abnormal movement (circling, rearing, somersaulting), paresis, and paralysis. Gross lesions may be absent or include haemorrhagic lesions on the heart, in the proventriculus, in the intestine and in cecal tonsils. In addition, tracheitis and airsacculitis were observed.

4.6.4 Disease in Humans

The most common symptom of NDV infection of humans is conjunctivitis, which does not affect the cornea and is characterized by swollen and reddened eye lids and lacrimation. In rare cases a generalized infection with headaches and fever may occur.

4.6.5 Transmission

The infection can be transmitted primarily through direct contact between healthy and infected birds. The disease can also be spread by mechanical means and by vaccination and debeaking crews, manure haulers, rendering-truck drivers, or feed delivery personnel.

Transmission to humans requires close contact to infected birds that allows the virus to come into contact with the eye. Furthermore, spray vaccination poses a risk if no appropriate eye protection is worn, since lesions in humans can also be caused by vaccine strains. Additionally laboratory accidents when working with isolated NDV have been reported.

4.6.6 Diagnosis

The virus can be isolated in embryonated chicken eggs and identified by haemagglutination inhibition assay. Pathotyping can be done in infection studies in order to discriminate lentogenic or mesogenic field or vaccine strains from velogenic strains. However, virulence, or lack thereof, for poultry is not connected to virulence for humans. The classical methods can be replaced by molecular biological detection and characterization. The methods are described in detail by the OIE.

4.6.7 Treatment and Control

Measures against ND are similar to that against AI. However, vaccination against ND is used in more countries.

4.7 Chlamydiosis

4.7.1 Occurrence

Infections of birds with *Chlamydia psittaci* occur worldwide, but incidence and distribution vary widely. Eight serotypes, namely A–F as well as M56 and WC are known. The latter two serotypes are associated with mammals and attempts to infect birds with them usually result in aborted or asymptomatic infections. The other six serotypes are found in birds, but can also infect some mammals including mice and men.

4.7.2 Host Range

Infections with *C. psittaci* have been described in at least 465 bird species. Host species are mainly infected with one certain serotype and each serotype seems to have a main host, but these connections are not absolute.

In poultry, the infection is especially prevalent among turkey flocks, where outbreaks are mostly caused by isolates of serotype B or D. Outbreaks in turkeys usually involve several flocks, free ranging flocks are at considerably greater risk.

Occasional outbreaks in ducks as well as in geese have been caused by isolates of serotype C, and ducks and geese are considered the main host of serotype C. Outbreaks of Chlamydiosis in commercially reared ducks in North America are rare, but can pose a problem in Europe. Serotypes A and F are mostly associated with psittacines and serotype E with pigeons.

4.7.3 Disease in Animals

Incubation time, clinical signs, and post mortem lesions in infected birds vary considerably between inapparent and fulminant. The course of the disease is influenced mainly by the chlamydial isolate and the host species. The infectious dose and the age of the birds are less important.

Clinical disease in chickens is rare, young chickens seem more susceptible than older ones. However, there are several reports about inapparent infections in broiler flocks.

Clinically apparent and inapparent chlamydiosis in turkeys are more common. Generally, infections caused by isolates of serotype B have a longer incubation time of up to several weeks and are less severe than infections with isolates of serotype D, which have an incubation time of less than ten days and cause more severe disease. Additionally, there are differences between different isolates of the same serovar.

Clinical symptoms of infected turkeys include severe respiratory disease with nasal and ocular discharges, conjunctivitis, and green droppings. Diseased birds are lethargic and anorectic and may become cachectic. Egg production in breeder hens is reduced. Morbidity may be up to 80 % and mortality up to 30 %. Gross lesions at post mortem are enlarged spleen, liver, and heart due to vascular congestions, congested and inflamed lungs as well as fibrinous airsacculitis, pericarditis, and peritonitis.

Clinical signs and post mortem lesions in ducks are similar. Additionally, nervous signs can be observed. As in turkeys morbidity may be up to 80 % and mortality up to 30 %.

4.7.4 Disease in Humans

Historically in humans the disease was called either psittacosis or ornithosis depending on the source of the infection, and it was assumed that ornithosis was a less severe disease. Nowadays, this differentiation is no longer considered to be correct, and the disease is generally called chlamydiosis.

Incubation time usually is between one and two weeks. The disease may be inapparent or characterized by pneumonia accompanied by fever, headache, and myalgia. Symptoms may continue for several weeks. The disease is rarely fatal if treated correctly.

4.7.5 Transmission

C. psittaci is shed in large numbers in respiratory exudate, especially in the nasal secretions, and in the faeces. The primary route of infection is inhalation of aerosols containing the bacterium, but infection by ingestion can also occur. Arthropods have been implicated as vectors, but indirect transmission does not seem to play an important role. The most important way *C. psittaci* is introduced into flocks seems to be by wild birds. Vertical transmission may happen at a low frequency in turkeys, chickens, and ducks.

Humans can get infected by inhalation of infectious aerosols. Persons handling infected birds at the farm and personnel at slaughter houses are at risk. Infections by consumption of contaminated meat or eggs are not known. Spread among humans is rare.

4.7.6 Diagnosis

For a quick diagnosis organ smears or smears of swabs from conjunctiva, oropharynx or cloaca can be stained with Giemsa or related methods and investigated for red or purple chlamydial elementary bodies within infected cells. Staining of fixed tissues or immunohistochemistry are other possibilities.

For isolation samples should be placed in special transport medium if they cannot be processed immediately. A variety of cell lines including McCoy and HeLa support chlamydial growth. Furthermore, embryonated chicken eggs can be inoculated into the yolk sac. Identification is done by staining cell monolayers, yolk sacs or yolk sac smears as described above.

At present, tests for the detection of chlamydial antigens lack sensitivity as well as specificity and are not recommended, while detection of chlamydial genes by

PCR is the method of choice for routine diagnosis. Several protocols for different genes have been published.

Detection of antibodies against *C. psittaci* may identify inapparently infected birds and flocks. This can be done by complement fixation test, indirect immunofluorescence or ELISA.

4.7.7 Treatment and Control

Antibiotics recommended against *C. psittaci* are chlortetracycline and enrofloxacine. They should be given for a prolonged period of time. They will not clear the birds from the infection, but reduce shedding and clinical symptoms. Efforts to develop a vaccine have been not successful so far. For prevention of infection contact between wild birds and poultry flocks has to be avoided.

In case of a confirmed outbreak in a flock of food producing birds the responsible authorities should be informed according to legislation effective in the area. Personnel handling the birds and at the slaughter house have to wear appropriate personal protective equipment.

4.8 Erysipelas

4.8.1 Occurrence

Erysipelas is an acute infection caused by *Erysipelothrix rhusiopathiae*. The bacterium was identified more than 100 year ago as the etiologic agent of swine erysipelas. Infections of poultry with *E. rhusiopathiae* occur sporadically worldwide. In some regions the disease is considered endemic. At risk are mostly free range flocks.

The genus *Erysipelothrix* is classified into two species: *E. rhusiopathiae* and *E. tonsillarum*. Among them, 26 different serotypes are recognized. Some serotypes of *E. rhusiopathiae* are pathogenic for poultry, whereas *E. tonsillarum* strains are nonpathogenic. In poultry, serotypes 1, 2, and 5 are most prevalent; all of them belong to *E. rhusiopathiae*.

4.8.2 Host Range

The host spectrum of *E. rhusiopathiae* is extremely broad, comprising various species of mammals, birds, reptiles, and fish. All species of domestic poultry are susceptible to infection, even though the susceptibility differs between them.

The course of the disease is especially fulminant in turkeys, regardless of age and sex. However, outbreaks with high mortality have also occurred in chickens (especially layer flocks), ducks, and geese. In mammals, swine and sheep seem to be the most susceptible species.

4.8.3 Disease in Animals

Incubation time depends on the infection dose and on the infection route. In animal studies it is shorter after subcutaneous than after oral infection. In turkeys first signs may be observed two or three days after oral infection.

Infected birds show unspecific signs like moderate general depression or decreased egg production in laying hens. Further diarrhoea or cutaneous lesions, especially swollen, purple snoods in turkeys, can be observed. Unvaccinated birds showing clinical signs usually die, and some infected turkeys may suddenly die without previous clinical signs. Mortality in turkeys may be up to 50 % over several weeks.

The most prominent gross lesion at post mortem is generalised congestion of internal organs with haemorrhage in several organs. Liver, spleen and kidneys are enlarged and may have areas of necrosis. Other lesions may be enteritis, endocarditis, or fibrinopurulent exudate in joints and pericardial sac. Histopathology shows vascular congestion in all organs with intravascular aggregations of bacteria and fibrin thrombi. Parenchymal cells may be damaged in liver, spleen, and kidney.

4.8.4 Disease in Humans

E. rhusiopathiae infections in humans most often are local infections of the skin at the infection site characterized by swelling and purple discolouration. Usually the hands are affected. Rarely infections become septicemic. In these cases symptoms and lesions may be very diverse and include polyarthralgia, septic arthritis, renal failure, endocarditis, encephalitis, and peritonitis.

4.8.5 Transmission

The natural route(s) of infection are not entirely certain. It is assumed that the bacterium enters the host through injuries of the skin or mucous membranes. Experimental infections are done either subcutaneously or orally. *E. rhusiopathiae* can survive several weeks in soil and this may be the main source of infection.

Turkey hens can become infected through lesions caused by artificial insemination.

Furthermore, the disease can be introduced into flocks by contact with infected rodents, pigs, or sheep. Arthropods, such as the poultry red mite, have been incriminated as potential mechanical vectors for *E. rhusiopathiae* infections in birds. Contaminated fish meal has been implicated as a source of infection. Asymptomatically infected birds as carriers are very rare.

Humans get infected through skin injuries. Particularly at risk are animal caretakers, veterinarians, and butchers. Infection by consumption of contaminated food has not been reported.

4.8.6 Diagnosis

Presumptive diagnosis can be done by detection of Gram-positive rod shaped bacteria in smears of infected organs. *E. rhusiopathiae* can readily be isolated from heart, liver, spleen, and bone marrow of birds which succumbed to the disease. Isolation from euthanized birds is not reliable. Agar containing 5 % blood will support growth. Further selective media containing sodium azide-crystal violet or kanamycin, neomycin, vancomycin, and novobiocin to suppress other bacteria have been described.

Incubation should be done under microaerophilic conditions for two to three days. Important tests for biochemical identification are H_2S production, presence of catalase and oxidase and lack of motility. PCR assays have been described.

Detection of antibodies against *E. rhusiopathiae* is not done in routine practice.

4.8.7 Treatment and Control

Infected flocks should be treated with penicillin to reduce mortality. Therapy should continue until shortly before slaughter and in some cases can be accompanied by vaccination to prevent relapses of the disease. Inactivated vaccines are used with success in turkeys and in free range laying hens. When choosing a vaccine it should be taken into account that protection by inactivated vaccines is serotype specific. For this reason autogenous vaccines might be used.

Carcasses of dead birds should be removed from infected flocks as quickly as possible to prevent the spread of the disease by cannibalism.

4.9 Avian Tuberculosis

4.9.1 Occurrence

Avian tuberculosis has been found on all continents, but seems to show the highest incidence in the North Temperate Zone. It has been described in a large number of bird species.

4.9.2 Host Range

Birds in captivity are more often affected than wild birds. In poultry the disease occurs sporadically in backyard flocks, while outbreaks in commercial poultry facilities are rare.

Twenty-eight serovars of *Mycobacterium avium* are known and designated as *Mycobacterium avium* complex (MAC). Serovars 1, 2, and 3 together with other *Mycobacterium* spp. like *M. intracellulare, M. genavense, M. fortuitum* and rarely *M. tuberculosis* are the causative agents of avian tuberculosis. Most infections in poultry are due to infections with *M. avium* serovar 1 and 2. Infections with serovar 3 occur rarely in wild birds, the other serovars mostly infect mammals, including humans and especially swine. However, infections of AIDS patients with serovar 1 are common.

4.9.3 Disease in Animals

Avian tuberculosis is mostly observed in older birds, because the lesions take time to develop. Clinical signs are unspecific and include depression and weight loss in spite of unaltered feed intake. Sometimes lameness occurs. The disease progresses slowly, but leads inevitably to death. Sudden deaths due to ruptured inner organs can happen.

Gross lesions at post mortem consist of white, yellow, or grayish nodules of varying size in various organs. Most frequently liver, spleen, intestines, or bone marrow are affected. The nodules are encapsulated and can easily be separated from the surrounding tissue. They are rarely mineralized. Histopathology shows typical tubercles with acid fast bacilli.

In most mammals lesions due to *M. avium* are localized, but disseminated lesions have been found in rabbits and swine.

4.9.4 Disease in Humans

Disease caused by *M. avium* subspecies *avium* can affect a wide range of hosts including humans. The course of infection depends on several factors. Besides the infection dose the immune status plays a very important role. Infection in humans can be accompanied by pneumonia resembling tuberculosis. Lesions may be found in lungs, lymph nodes, and various other tissues. The role of *M. avium* subspecies *paratuberculosis* in the pathogenesis of Morbus Crohn in humans is controversially discussed in the literature.

4.9.5 Transmission

M. avium is excreted in great numbers in the faeces and to a lesser extent via the respiratory tract. In soil and litter the bacterium can survive for several years and

infect other birds. Holdings contaminated with *M. avium* pose the greatest risk of transmission, but dissemination may also occur on contaminated equipment or by infected wild birds. Infection is by oral uptake of *M. avium*. Vertical transmission has not been shown.

It is thought that most humans acquire infections with *M. avium* more likely from other humans or swine than from contact with birds.

4.9.6 Diagnosis

Most often avian tuberculosis is diagnosed at post mortem by demonstration of acid-fast bacilli in smears or histologic sections of tubercles. For differentiation of species and serovar, isolation of *M. avium* or one of the other mycobacteria causing avian tuberculosis can be done on special selective media; some strains require mycobactin as a growth factor. Growth of visible colonies may take up to three weeks. Serotyping is done by agglutination. Molecular biological tools are also available for detection of the microorganism.

Diagnosis in living birds can be done by the tuberculin test. The test antigen contains purified tuberculin derived from *M. avium* and 0.03–0.05 ml are injected into the wattle. A rapid agglutination test and an ELISA to detect antibodies against *M. avium* have been described, but both are less specific than the tuberculin test.

4.9.7 Treatment and Control

Treatment of backyard poultry with antituberculosis drugs is not an option, since the use of these drugs in feed producing animals would involve the risk to induce and spread resistance against these drugs. Affected flocks should be destroyed and the premises should not be used for keeping poultry for several years.

Vaccination is not available.

4.10 Concluding Remarks

Poultry shares a number of infectious diseases with humans, and most of the zoonotic diseases in poultry have additional reservoirs in other mammals than humans complicating their control.

Three groups of zoonoses that humans can acquire from poultry can be differentiated. The first group includes food borne diseases. The second group comprises diseases that are transmitted by direct contact between birds and humans. Diseases belonging to these two groups have been discussed in detail in this chapter.

The third group comprises diseases transmitted by insects, especially ticks from mammals and birds, including poultry, to humans. Examples for these pathogens are

the West Nile Virus, which is spreading rapidly in North America and is occasionally detected in migratory birds in Europe, as well as Eastern and Western Equine Encephalitis and others. Until now the importance of poultry in the epidemiology of these diseases is low, but the situation may change and needs to be monitored.

Another aspect of zoonotic pathogens in poultry, which could not be addressed here, is the development of antibiotic resistance in bacteria. This can happen in poultry given excessive antibiotics, and resistance may be passed on to bacteria infecting humans. This is also a continuous public health hazard which needs to be faced.

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Chapter 5
Bacterial Pathogens Associated with Aquaculture Products

Iddya Karunasagar

Abstract According to FAO statistics, aquaculture is contributing to nearly half of the global food fish production. Fish contributes to both nutritional and food security in many developing economies. Fish is also one of the most extensively traded food commodities; most of global aquaculture production takes place in developing countries and the major markets are in the developed world. European Union, Japan and United States together account for about 70 % of global fish imports. Generally, fish and fishery products have a very good safety record. But there are some bacterial hazards associated with aquaculture products. The chapter discusses the bacterial pathogens that may be associated with products of aquaculture, pathways of contamination and risk management measures reported for these bacterial hazards. In terms of antibiotic usage, there is limited data from developing countries, and a number of studies have looked at antimicrobial resistance in bacterial pathogens associated with fish and fishery products. Aspects related to antimicrobial resistance in aquaculture products are also presented in this chapter.

5.1 Introduction

Fish constitute a highly nutritious food providing proteins, polyunsaturated fatty acids (PUFA), micronutrients and vitamins. Although it is commonly believed that mostly marine fish like salmon are the major source of PUFA, particularly omega-3 fatty acids, it has been found that even fresh water farmed fish like carps that are widely produced and consumed in Asia provide much more omega-3 fatty acids than poultry or beef. Globally, fish contribute about 16.5 % of animal protein intake of human population and this proportion is even higher (24.2 %) in low income food deficit countries (FAO 2012). During 2011, the global fish production was 148.5 million t of which 59.9 million t came from aquaculture (FAO 2012). Global fish production by capture has been stagnating for the last two decades and most of the fish stocks are either fully exploited or even over-exploited. Hence, to meet the

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increasing demand for fish as food, it is important to increase fish production by aquaculture. Diverse species of fin fish and shellfish are produced by aquaculture. Fact sheets of sixty two species of fish, crustaceans and molluscs that are cultured in different parts of the world are available from the FAO website (FAO 2013). Asia accounted for 89% of global aquaculture production by volume in 2010, and this was dominated by the contribution of China, which accounted for more than 60% of global aquaculture production by volume. Freshwater fish dominate global aquaculture production (56.4%), followed by molluscs (23.6%), crustaceans (9.6%), diadromous fishes (6.0%), marine fishes (3.1%) and other aquatic animals (1.4%).

In general, fish is considered a safe food and there are very few epidemiological records regarding illness associated with farmed fish. In United States, during the last decade (2001–2010), there were 657 outbreaks of illness involving 5603 cases associated with fish and fishery products, while poultry was associated with 458 outbreaks involving 11,338 cases, fresh produce with 696 outbreaks involving 25,222 cases, beef with 363 outbreaks involving 7528 cases (CSPI 2013). Most of the fish associated illnesses were due to scrombotoxin (histamine and other biogenic amines caused by improper handling of certain species of marine fish) and ciguatoxin (toxin derived from certain microalgae found in some parts of the world). Both these problems are not associated with farmed fish. In the EU, during 2011, 78.9% of the 71 outbreaks associated with fin fish were due to scrombotoxin and 4.2% due to ciguatoxin, respectively. *Salmonella* accounted for 4.2% of infections. In case of shellfish (crustaceans, molluscs), 40.5% of the 42 outbreaks in 2011 were due to calciviruses (noroviruses) and 16.7% due to algal biotoxins (EFSA and ECDC 2013). *Salmonella* and *Escherichia coli* each accounted for 4.8% of disease episodes. In some parts of Asia, where there is the practice of consuming raw fish, a large number of parasitic diseases caused by the fish-borne trematodes have been reported to be prevalent. For example, about 1.5 million people in Korea, 6 million people in China, and over 5 million in Thailand are reported to be infected with the liver flukes, either *Clonorchis sinensis*, *Opisthorchis viverrini* or *O. felineus* (Chai et al. 2005). However, this chapter deals only with pathogenic bacteria that may be associated with farmed aquatic species.

5.2 Bacterial Hazards Associated with Aquaculture Products

Farmed fish live in an environment, where there are significant levels of aquatic bacteria; the microflora associated with fish are greatly influenced by the microflora in the surrounding environment. When fish are alive, aquatic bacteria may be associated with the skin surface, in the gill surface and in gut. Most of the bacteria that are associated naturally with an aquatic environment are generally not pathogenic to humans with the exception of a few *Vibrio* species that are discussed in later sections. Once the fish are harvested, they are handled, transported and processed and during this period, they will come in contact with human hands and various surfaces

(containers, equipments), ice and water which could influence the microflora associated with fishery products that reach the consumers. Bacteria of zoonotic potential that may be associated with fish and fishery products are discussed in the following sections.

5.2.1 Vibrio Species

Vibrio spp. are autochthonous inhabitants of the aquatic environment and of over 80 species included in the genus *Vibrio*, at least 12 are capable of causing human infections (Oliver and Kaper 2007). Most of the pathogenic species have environmental non-pathogenic strains. *Vibrio* spp. are commonly isolated from estuarine, coastal marine environments (some species like *Vibrio cholerae* are found in fresh waters) all over the world and seafoodborne illnesses are primarily caused by *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* (FAO/WHO 2003). Of these, *V. parahaemolyticus* and *V. cholerae* cause gastrointestinal disease, while *V. vulnificus* causes septicaemia. There are very few pathogens of fish that can also affect humans. For example, some strains of *V. vulnificus* cause infection in eels and can also infect humans. Most of the other *Vibrio* spp pathogenic to aquaculture species, e.g. *V. harveyi* (causing disease in shrimp) or *V. anguillarum* (pathogenic to marine fish) are not human pathogens.

V. cholerae. *V. cholerae* is a heterogeneous species consisting of over 220 serotypes, of which only serotypes O1 and O139 are known to cause the disease cholera and these are generally referred to as choleragenic *V.cholerae* (FAO/WHO 2005). Strains belonging to non O1/non-O139 serotypes of *V. cholerae* are widely distributed in the aquatic environment including aquaculture systems (Gopal et al. 2005) and they are mostly not pathogenic to humans, although occasionally, they may be associated with sporadic cases of gastroenteritis (Oliver and Kaper 2007). None of the *V. cholerae* serotypes are known to be pathogens of farmed aquatic animals. The O1 serovar is known to possess three antigenic forms: Inaba, Ogawa and Hikojima. Based on their phenotypic characteristics, *V. cholerae* O1 strains are classified into two biotypes, Classical and El Tor (Kaper et al. 1995). Since the seventh pandemic of cholera, most outbreaks have been caused by El Tor strains and the Classical biotype strains are rarely isolated from any part of the world (Sack et al. 2003). The choleragenic El Tor biotype strains of *V. cholerae* are grouped in four major clonal groups: (i) the seventh pandemic, (ii) the U.S. Gulf Coast, (iii) Australia; and (iv) Latin America, which seem to reflect broad demographic and epidemiological associations (Wachsmuth et al. 1994). When the O139 emerged in early 1990s and almost replaced O1 serotype in southeast Asia, it was thought that it might represent a new pandemic, but this strain did not spread beyond southeast Asia and even there, the cases due to O139 serotype have declined and O1 serotype has become dominant again (Oliver and Kaper 2007). The disease cholera is characterised by the passage of voluminous stools of rice water consistency leading to dehydration, hypovolemic shock, acidosis, and if appropriate treatment is not initiated, death.

However, it has been estimated that only 2% of those infected with El Tor biotype and 11% of those infected with the classical biotype develop severe disease. 5% of El Tor infections and 15% of classical infections may result in moderate illness that can be managed in outpatient clinics (Kaper et al. 1995). Infected individuals shed the pathogen in their faeces for 7–14 days. Symptoms due to O1 and O139 serotypes appear to be identical. About 80% of patients can be treated adequately through oral rehydration salts (ORS) or intravenous fluids depending on severity of symptoms. Antibiotic treatment can reduce the volume of diarrhea in patients with severe symptoms and reduce the period of fecal shedding. Doxycycline, tetracycline, trimethoprim-sulphamethaxazole and erythromycin are some of the antibiotics used. Antibiotic resistance in *V. cholerae* O1 El Tor has been reported from a number of countries (Kitaoka et al. 2011), but there are no such documented outbreaks associated with consumption of aquaculture products.

For differentiation of choleragenic *V. cholerae* from non-choleragenic types, serotyping has been commonly used, but some environmental stains could cross reactwith O1 or O139 antisera (FAO/WHO 2005). The most important virulence factor associated with *V. cholerae* O1 and O139 is the cholera toxin, which has two subunits, A and B. The *ctx* genes (*ctx*A and *ctx*B) encoding the production of the cholera toxin are present in a filamentous bacteriophage that is integrated into the genome of *V. cholerae* O1 and O139 (Faruque et al. 1998). Loss of the bacteriophage may explain the presence of non-toxigenic O1 *V. cholerae* in the environment. Molecular identification methods based on probes or PCR primers binding to the *ctx* gene have been widely used to detect toxigenic O1 or O139 *V. cholerae* in the environment and in foods (FAO/WHO 2005). FDA Bacteriological Analytical Manual recommends *ctx*-based PCR for determining toxigenicity of *V. cholerae* (Kaysner and De Paola 2004). Detection of choleragenic *V. cholerae* in fish homogenates containing less than 10 cells/ml was possible when PCR was performed after 6 h enrichment in alkaline peptone water (Karunasagar et al. 1995).

Toxigenic *V. cholerae* may persist in the environment for long periods of time in the absence of clinical cases and this explains the presence of *V. cholerae* O1 in waters in areas where cholera is not an endemic disease, e.g. the US Gulf coast and in Australia (Oliver and Kaper 2007). Adhesion to chitin has been shown to influence strongly the ecology of *V. cholerae* and strong association between levels of zooplankton like copepods and incidence of *V. cholerae* has been observed in the aquatic environment. Choleragenic *V. cholerae* has also been reported to attach to the hindgut of crabs; interestingly, it is noted that the hindgut of crustaceans is an extension of the exoskeleton and is lined with chitin (FAO/WHO 2005). However, there are very few records of isolation of *V. cholerae* O1 and O139 from aquaculture ponds and with shrimp. This could be because shrimp are benthic organisms. Studies from South-east Asia indicate the absence of *V. cholerae* O1 from raw shrimp (Karunasagar et al. 1990; Fonseka 1990, Rattagool et al. 1990; Karunasagar et al. 1992). Several studies on shrimp farms in India indicated an absence of choleragenic *V. cholerae* in shrimp culture ponds (Otta et al. 1999; Gopal et al. 2005). Dalsgaard et al. (1995a) found that *V. choleare* O1 was present in 2% (2/107) of water, sediment and shrimp samples collected from a major shrimp culture area in

South-East Asia. However, subsequent testing of the isolates indicated the absence of the *ctx* genes in both the O1 strains (Dalsgaard et al. 1995b). Ravi Kiran (1992) and Dalsgaard et al. (1995a) analyzed shrimp gut content for the presence of potential human pathogens and noted the absence of *V. cholerae* O1.

Farmed fish could be contaminated with choleragenic *V. cholerae* due to improper hygiene during postharvest handling. Saravanan et al. (2007) noted absence of choleragenic *V. cholerae* in shrimp processed under HACCP (Hazard Analysis Critical Control Point) conditions in India, but detected the organism in one shrimp sample in a domestic market. Chen et al. (2004) isolated *V. cholerae* O1 and O139 from shrimp in domestic market in Malaysia which might be due to postharvest contamination. During the Peruvian cholera epidemic, high levels of contamination (100%) were observed in a small number of raw seafood samples from street vendors, but only one out of 1011 seafood samples intended for export and processed under HACCP conditions was positive (De Paola et al. 1993). FAO/WHO risk assessment for choleragenic *V. cholerae* in warm water shrimp in international trade looked at the data from testing laboratories in shrimp importing countries during the period 1995–2000. Of a total of 21,857 samples tested, only two samples originating from an Asian country in 1995 (early days of HACCP) were positive for choleragenic *V. cholerae* (FAO/WHO 2005).

Epidemiological data indicates that a variety of fish and fishery products have been involved in outbreaks of cholera in different parts of the world (FAO/WHO 2005). Transmission of *V. cholerae* by seafood can be acute where fish and shellfish are consumed raw (DePaola 1981). Seventy five of 336 passengers in an airline were affected in the Americas in 1992 in which cold seafood salad was implicated (Eberhart-Phillips et al. 1996). The shellfish most often associated with cholera cases are molluscan shellfish (oysters) and crabs. Oysters are consumed raw in many countries, but crabs are generally cooked. However, studies (Blake et al. 1980) have shown that even after boiling crabs for up to 10 min or steaming for up to 30 min, *V. cholerae* O1 may still retain viability due to issues related to heat penetration. There are very few outbreaks linked to crustacean shellfish. In an outbreak linked to the consumption of raw shrimp in the United States in 1986, the source was found to be domestic. Contaminated crab salad served in an airplane flying from Peru to California caused 75 cases. An outbreak in Japan in 1978 was associated with lobsters imported from Indonesia and another outbreak was linked to the consumption of raw shrimp in the Philippines in 1962. However, in most cases it is not possible to assess whether *V. cholerae* O1 was naturally present or cross contaminated after harvest (FAO/WHO 2005).

There is very little data on the level of *V. cholerae* associated with aquatic animals and most studies reporting isolations were done following enrichment. Hence, it is expected that levels are generally very low. The dose response model developed in FAO/WHO risk assessment (FAO/WHO 2005) indicates that 10^6 choleragenic *V. cholerae* are required to produce disease. This suggests that if aquaculture products are contaminated with *V. cholerae*, multiplication of the organism has to occur before an infective dose is reached (Table 5.1). In raw shrimp, *V. cholerae* has to compete with other organisms for growth. The optimum temperature for growth of

Table 5.1 Bacterial pathogens that may be associated with products of aquaculture. (For references, please see text)

Pathogen	Natural habitat	Factors affecting levels in aquaculture products	Infective dose	Factors that contribute to increasing risk
Vibrio parahaemolyticus	Brackish water, coastal and estuarine environments	Could vary with environmental factors like temperature and salinity	Low risk (0.001 %) with 10^4 cells and high risk (50 %) when 10^8 cells of *tdh*$^+$ strains are consumed	Warm environmental conditions, postharvest exposure to temperatures facilitating growth
Vibrio vulnificus	Brackish water, coastal and estuarine environments	Could vary with environmental factors like temperature and salinity	10^6, but epidemiological data shows that a lower dose may cause infections	Warm environmental conditions, postharvest exposure to temperatures facilitating growth
Vibrio cholerae Non O1/139	Fresh water, brackish water, coastal and estuarine environments	Not much data available	Not much data available	Not much data available
Choleragenic *Vibrio cholerae*	Fresh water, brackish water, coastal and estuarine environments contaminated with human sewage	Extent of fecal/sewage contamination	10^6	Postharvest exposure to temperatures facilitating growth
Salmonella spp.	Fresh water, brackish water, coastal and estuarine environments	Rain, flooding, animal wastes like bird droppings, presence of animals like frogs, turtles	$>10^5$	Postharvest exposure to temperatures facilitating growth
Listeria monocytogenes	Fresh water, brackish water, coastal and estuarine environments	Contamination from decaying vegetation, sewage	$>10^{10}$, lower doses of certain strains could cause infection	Factors facilitating growth

V. cholerae is 37 °C with a range of 10–43 °C (ICMSF 1996). Kolvin and Roberts (1982) measured growth of *V. cholerae* O1 in raw and cooked seafood. No growth was observed in raw prawns, mussels and oysters, but growth occurred in cooked shellfish. Levels of 10^{10} cells/g were reported in cooked prawns and mussels stored at 37 °C. At 22 °C, there was a lag phase of 8 h for classical biotype and 4 h for the El Tor biotype. The organism is sensitive to desiccation and to heat with a D value of 2.65 min at 60 °C (ICMSF 1996). *V. cholerae* survives refrigeration, though some decline in numbers is seen. Viable cells could be recovered from raw shrimp spiked

with 10^5 cells/g *V. cholerae* O1 after 4–9 days at 5–10 °C (ICMSF 1996). Similarly, although freezing causes initial decline in numbers, the organism may survive over 180 days in fish (ICMSF 1996).

FAO/WHO Risk assessment of choleragenic *V. cholerae* in warm water shrimp in international trade indicated that the risk of cholera transmission through this commodity is very low (FAO/WHO 2005). Use of a spread sheet based risk assessment tool (Ross and Sumner 2002) for quantitative risk assessment predicted the likelihood of illness to be 1–2 cases in a decade in Japan and USA, considering the volumes of warm water shrimp imported and consumed, and 1 case in 25 years in other shrimp importing countries (FAO/WHO 2005). Quantitative approach using model based on import to consumption pathway (prevalence estimated based on data from testing laboratories in importing countries- 2 samples in 1995 positive out of 21,857 samples tested between 1995–2000) predicted the illness to be 1–5 cases every 5 years, based on the assumption that 10 % shrimp are consumed raw and 90 % after cooking (FAO/WHO 2005). Thus quantitative risk assessment indicates a very low risk and there are no epidemiological records of illness linked to imported warmwater shrimp supporting the very low risk predicted.

V. parahaemolyticus *Vibrio parahaemolyticus* is found in the estuarine and coastal environments in the tropical to temperate zones (Joseph et al. 1982) where it is considered to be part of the autochthonous microflora. There is no correlation between the presence of this organism and faecal contamination of the environments (Joseph et al. 1982; Oliver and Kaper 2007). *V. parahaemolyticus* has been isolated from seawater, sediment, marine animals, plankton, various fish and shellfish species (Joseph et al. 1982). Thus, *V. parahaemolyticus* is naturally present in shellfish (shrimp and molluscan shellfish) growing and harvesting areas. The level of this organism in various fish and shellfish may vary. Certain areas may have more favourable environmental conditions that support establishment, survival and growth of the organism such as temperature, salinity, zooplankton, tidal flushing and dissolved oxygen (Garay et al. 1985; Kaneko and Colwell 1977; Venkateswaran et al. 1990). In temperate waters, the ecology is strongly influenced by temperature and salinity. In these environments, *V. parahaemolyticus* is often detected in warmer months and the organism has been reported to survive in the sediment during winter (Kaneko and Colwell 1977, De Paola et al. 2003); however, in tropical waters, *V. parahaemolyticus* can be detected throughout the year (Natarajan et al. 1980; Deepanjali et al. 2005). Salinity may influence the levels in tropical waters, low counts being recording during post-monsoon period (Deepanjali et al. 2005). *V. parahaemolyticus* can grow in sodium chloride concentrations ranging from 0.5 to 10 % with optimum levels between 1 and 3 % (Colwell et al. 1984). Adsorption of *V. parahaemolyticus* on plankton or chitin containing materials occurs with higher efficiency under conditions of estuarine salinity (Kaneko and Colwell 1977). In tropical shrimp culture environments, *V. parahaemolyticus* is often present. This organism accounted for 0–27 % of the flora in water and sediment of shrimp ponds in India (Otta et al. 1999; Gopal et al. 2005). The level of *V. parahaemolyticus* in seafood may vary depending on the type of seafood and geographical location. In US Gulf coast oysters, during warm months, levels such as 1.1×10^4/100 g has

been reported, but in Pacific oysters that are at lower temperatures, the levels were 2.1×10^3/100 g (Drake et al. 2007). In Indian oysters, the levels range from 10^2–10^4/g (Deepanjali et al. 2005). In shrimp, the levels range from undetectable to 10^4/g, high counts being rare (Cann et al. 1981; Karunasagar et al. 1984) and in fin fish levels of ~88/g have been reported (Chan et al. 1989).

Most of the environmental strains may not be pathogenic to man. Early studies in Japan showed that 96% of clinical strains produce a thermostable direct hemolysin (TDH), while only 1% of the environmental strains produce this hemolysin (Joseph et al. 1982). Low prevalence of TDH positive strains in the environment has been confirmed from different geographical regions. In the Gulf coast in US, the percentage has been generally less than 1%, but in the Pacific northwest up to 3.2% strains could be TDH positive (FAO/WHO 2011). 6–10% of oysters from India were positive for *V. parahaemolyticus* carrying the *tdh* gene (Deepanjali et al. 2005, Raghunath et al. 2008). Some TDH negative strains from clinical cases were found to produce a TDH-related hemolysin, TRH (Honda et al. 1988). Presently, strains producing TDH and TRH are considered pathogenic to man. But there may be strain variations. There are five sequence variants of the *tdh* gene (*tdh*1– *tdh*5) and two sequence variants of the *trh* gene (*trh*1 –*trh*2) (Nishibuchi and Kaper 1990; Nishibuchi and Kaper 1995). Some strains carry both *tdh* and *trh* genes, while most clinical strains carry the *tdh*-2 gene. Diverse serotypes may be associated with human infections, but recently strains belonging to the O3:K6 serotype and its variants have been found to be the causative agents of several outbreaks in different countries (Nair et al. 2007). Although several publications refer to these strains as "pandemic", Nair et al. (2007) pointed out that this is misleading in the epidemiological sense, because outbreaks have not affected exceptionally high proportion of the population. Nevertheless, strains belonging to this group show clonality in molecular typing methods like arbitrarily primed (AP) PCR, ribotyping or pulsefield gel electrophoresis (PFGE) and are characterised by presence of only the *tdh* gene (and not *trh* gene), some mismatches in nucleotides in the *tox*RS gene and an open reading frame ORF8 derived from a filamentous bacteriophage f237 (Nair et al. 2007).

Based on data from human volunteer studies and using beta-Poisson model, a dose response relationship has been established in FAO/WHO risk assessment of *V. parahaemolyticus* in seafood (FAO/WHO 2011). This suggests that there is a low risk (0.001%) of illness following consumption of 10^4 *tdh*$^+$ *V. parahaemolyticus* and high risk (50%) when 10^8 cells are consumed. Since the levels of this pathogen found in freshly harvested or frozen seafood are generally low, growth of the organism due to mishandling at temperatures permitting growth would be necessary before the organism reaches an infective dose (Table 5.1). *V. parahaemolyticus* can grow at a temperature range of 5–43 °C and optimum temperature for growth is 37 °C (ICMSF 1996). At optimum temperature, the doubling time in shrimp was 9–10 min and at 18.3 °C, it was 144 min (Katoh 1965). At 20 °C, the doubling time was 34 min in raw shrimp and 28 min in cooked shrimp (Liston 1974). Growth rates in a range of seafoods and tryptic soy broth with 2.5% salt (NaCl) have been recorded and these data indicate that moderate populations of 10^2–10^3 organisms/g on seafood can increase to $>10^5$ organisms/g in 2–3 hours at ambient temperatures

between 20 and 35 °C (ICMSF 1996). A number of studies indicate that *V. parahaemolyticus* dies when exposed to temperatures < 5–7 °C, with highest mortality rate being in the range 0–5 °C (ICMSF 1996). A 1–2 $\log_{10}$ drop in numbers occur during freezing, but the organism can persist in frozen seafood for long periods of time (ICMSF 1996). Both pathogenic and non-pathogenic strains have been observed to respond similarly to freezing (FDA 2005). *V. parahaemolyticus* is very sensitive to heat with a D value of < 1.0 min at 65 °C in crab homogenate with an initial inoculum of 10^6 cells (ICMSF 1996), hence cooking would greatly reduce the hazard due to this pathogen.

Symptoms of *V. parahaemolyticus* infection include watery diarrhea, nausea, vomiting, abdominal cramps and less frequently headache, fever and chills (FAO/WHO 2011). Generally, the gastroenteritis is self-limiting and severe cases requiring hospitalisation are rare. Depending on seafood consumption habits, the source of infection could vary, but mostly involving consumption of raw products and cooked products subjected to postprocess contamination. Oysters are the most common source in outbreaks in US and South America, but there have been reports of involvement of other types of seafood including clams, shrimp, lobster, crayfish, scallops, crabs and fin fish (Daniels et al. 2000; Oliver and Kaper 2007). In Japan (Anonymous 2000), implicated foods include *sashimi*, pieces of raw fish fillet (responsible for 26 % of outbreaks), followed by *sushi,* vinegary rice ball with pieces of raw fish fillet (23 %), shellfish (16 %) and cooked seafood (12 %). A large outbreak linked to shrimp occurred in Lousiana in 1978 in which 1133 of the 1700 persons attending a dinner were affected and this appears to have been caused by cross contamination between raw and boiled shrimp. Shrimp boiled in the morning were kept in the same wooden seafood box used to transport raw shrimp and transported 40 miles in an unrefrigerated truck and held additional 7–8 h before serving for dinner (Oliver and Kaper 2007). In Japan, *V. parahaemolyticus* is one of the most common causes of gastroenteritis and annually 500–800 outbreaks affecting 10 000 people are reported annually (FAO/WHO 2011). This organism is the leading cause of food-borne illness in Taiwan causing 197 outbreaks during 1986–1995 (Pan et al. 1997) and accounted for 69 % of the food-borne cases between 1981–2003 (Su and Liu 2007). *V. parahaemolyticus* accounted for 31.1 % of 5770 food-borne outbreaks that occurred in China from 1991- 2001 (Liu et al. 2004).

FDA (2005) carried out quantitative risk assessment of *Vibrio parahaemolyticus* in raw oysters in which a model for predicting *V. parahaemolyticus* levels in oyster based on water temperature was developed. The postharvest oyster handling practices in the US and the effect of these practices on levels of *V. parahaemolyticus* were modelled. Data from two regions in the US (Pacific Northwest and Gulf coast) were used to estimate the proportion of strains that are pathogenic. It was estimated that about 50 % of oysters are consumed raw and each serving would be about 200 g. The risk assessment suggested that in the absence of subsequent post-harvest mitigations, "at-harvest" guidance levels of 10^5, 10^3, 10^2 total *V. parahaemolyticus* per g could potentially reduce the illness rate by 1.6, 68 and 98 % with corresponding impact of 0.25, 21 and 66 % of the harvest, respectively. If the control is applied on the basis of *V. parahaemolyticus* levels at retail, a standard to 10^4/g would reduce

illness by 99 % and 43 % of the harvest would have to be diverted from the raw market. A 5000/g standard could almost eliminate 100 % of illness, with 70 % of the harvest having to be diverted from the raw market (FDA 2005).

The FAO/WHO risk assessment of *V. parahaemolyticus* in raw oysters used a similar approach to estimate the risk of illness in Australia, New Zealand, Canada and Japan (FAO/WHO 2011). Local data on water and air temperature, local harvest practices and prevalence of *V. parahaemolyticus* in oysters in these countries was used. US data on the proportion of pathogenic *V. parahaemolyticus*, multiplication of *V. parahaemolyticus* in oysters, consumption patterns and under reporting of illness was used. The risk assessment also looked at the impact of applying microbiological criteria, e.g. 100/g, 1000/g and 10,000/g. The data showed that a criterion of 100/g would lead to a 99 % reduction in illness in Australia, but this would lead to rejection of 67 % of products currently going to the market. Considering that epidemiological records of illness are very rare in Australia, risk management based on microbiological criterion would not be a reasonable approach there. Noting wide variations in the occurrence of *V. parahaemolyticus* in different geographical regions, adopting a global microbiological criterion could not be recommended. This led the Codex Alimentarius Commission to develop a Code of Practice (CAC/GL 73-2010) for minimising the risk rather than adopt a microbiological criterion.

Since the disease caused by *V. parahaemolyticus* is generally mild, it does not require antibiotic treatment. In severe or prolonged cases, tetracycline, ampicillin or ciprofloxacin may be used. Though antimicrobial resistance has been detected in environmental strains (Baker-Austin et al. 2008), there is very little evidence that this is an issue with clinical strains. A study of *V. parahaemolyticus* strains isolated from outbreaks of illness in Chile during 2005 and 2007 showed that the they were sensitive to tetracycline, sulphamethaxazole-trimethoprim and ciprofloxacin, but were resistant to ampicillin (Dauros et al. 2011).

Vibrio vulnificus. *Vibrio vulnificus* is a common inhabitant of warm water estuarine environments all over the world. The organism has been isolated from coastal marine and estuarine waters, sediment, plankton, various shellfish (both molluscan and crustacean) and fin fish species in areas where the temperatures range from 9–31 °C. *V. vulnificus* proliferates in waters, where temperature exceeds 18 °C (Kasper and Tamplin 1993; Strom and Paranjpaye 2000, FAO/WHO 2005a; Drake et al. 2007). The abundance varies considerably and is greatly influenced by temperature and salinity. In North America, higher densities are observed in mid-Atlantic, Chesapeake Bay and Gulf coast waters, where temperatures are warmer throughout the year, while densities are lower in Pacific, Canadian and North Atlantic waters (FAO/WHO 2005a). The lowest temperature at which *V. vulnificus* has been isolated varies geographically, being 8 °C at Chesapeake Bay (Wright et al. 1996) and < 12.5 °C in the Gulf coast (Simonson and Siebeling 1986); the organism survives in sediment during winter. In tropical waters, where temperature does not go below 18 °C, abundance of *V. vulnificus* is influenced by salinity (Parvathi et al. 2004). In south India, highest *V. vulnificus* levels were found during monsoon season when the salinities were less than 5 ppt; at salinities exceeding 25 ppt these

organisms were not detectable (Parvathi et al. 2004). Salinity has a significant effect on the abundance of the organism even in temperate waters. In the waters of the US, numbers of *V. vulnificus* were high at salinity between 5–25 ppt, but dropped by 58–88 % at salinities over 30 ppt (FAO/WHO 2005a). *V. vulnificus* produces chitinase, which might help the organism to colonise zooplankton (Strom and Paranjpaye 2000) and can colonise plankton and fish gut (FAO/WHO 2005a). Through fish, the organism even reaches the gut of birds since Miyasaka et al. (2006) found 14.1 % aquatic birds in Japan to be positive for *V. vulnificus*.

Presently three biotypes are recognised based on a combination of phenotypic, serologic and host range characters (Drake et al. 2007). Biotype 1 strains are indole positive, serologically diverse and are associated with human infections. Biotype 2 strains are indole negative and considered mainly as eel pathogens, but may also be opportunistic human pathogens, being associated with infections in eel handlers. This biotype has three serotypes and strains associated with eel and human infections belong to serotype E (Sanjuan and Amaro 2004). Biotype 3 has five atypical biochemical reactions, is genetically clonal and has been isolated from 62 Israeli patients with wound infection or septicaemia. This biotype has not been associated with food-borne infections (Drake et al. 2007). The virulence of this organism seems to be related to multiple factors such as presence of a polysaccharide capsule, ability to obtain iron from transferrin, ability to produce extracellular enzymes and exotoxin (Drake et al. 2007). Most of the virulence associated factors are present in over 95 % of environmental strains. Rosche et al. (2005) using nucleotide sequence analysis showed that Biotype 1 strains can be distinguished into two types that strongly correlate with clinical (C) or environmental (E) origin. C- genotypes showed greater resistance to human serum than E-genotypes and had lower LD_{50} suggesting that C-genotype strains may be more virulent (Rosche et al. 2010). While similar levels of C and E-genotypes were found in estuarine waters, oysters had 85 % E-genotypes (Warner and Oliver 2008).

V. vulnificus can cause primary septicaemia and wound infections. The disease rarely (< 5 %) occurs in healthy individuals and risk factors for *V. vulnificus* infection include liver disease, cirrhosis due to alcohol consumption, diabetes, gastrointestinal disorders (ulcer, surgery), haematological conditions, and immunocompromised condition associated with cancer and therapy with immunosuppressive drugs. Epidemiological data suggests that men are more susceptible than women to *V. vulnificus* infection. The fatality rate (about 50 %) is the highest among food-borne pathogens (FAO/WHO 2005a) while, the attack rate is low with one illness occurring per 10,000 meals of raw United States Gulf coast oysters (containing *V. vulnificus*) served to the highest risk population, i.e. people with liver diseases (FAO/WHO 2005a). The incubation period ranges from 7 h to 10 days, with symptoms appearing in 36 h in most cases (Oliver and Kaper 2007). The symptoms include sudden onset of fever and chills, generally accompanied by nausea, vomiting, abdominal pain, hypotension (systolic pressure < 85 mm) and in over 60 % cases; secondary lesions appear, mostly on the legs that often develop necrotising fasciitis or vasculitis that may require surgical debrediment or amputation (Strom and Paranjapaye 2000; Oliver and Kaper 2007). *V. vulnificus* can be isolated from blood

and cutaneous lesions. Rare cases of atypical infections have been reported and these include septic arthritis, meningoencephalitis and ocular infection following consumption of raw oysters or raw fish. 69% of wound infections were associated with occupational exposures among oyster shuckers and commercial fishermen (Strom and Paranjapye 2000). Wound infections may progress to echymoses, cellulitis, bullae and necrotising fasciitis, but the mortality rate (25%) is much lower than in cases of primary septicaemia, though 50% of cases may require surgical debridement or amputation (Jones and Oliver 2009).

Antibiotic therapy is important for both wound infections and septicaemia. While tetracycline has been the most effective drug, in some cases, this has been used in combination with third generation cephalosporin or gentamycin or chloramphenicol (Strom and Paranjapye 2000, Liu et al. 2006). Roig et al. (2009) noted that the *V. vulnificus* biotype 2 serovar E eel pathogenic strains can develop resistance to quinolones by spontaneous mutation of the *gyr*A gene and suggested avoiding quinolones for treatment of vibriosis in eel farms. Baker-Austin et al. (2009) screened 151 environmental isolates and 10 primary septicaemia isolates for antimicrobial susceptibility. Several isolates showed resistance to antibiotics routinely prescribed for *V. vulnificus* infections such as tetracycline, doxycycline, aminoglycosides and cephalosporins. The resistance was seen at similar frequencies in C-type and E-type strains. Among environmental isolates, there was no consistent difference in the frequency of resistance between strains from pristine and anthropologically impacted areas suggesting natural rather than human derived source of resistance traits.

Epidemiological data suggest that about 100 cases of primary septicaemia due to *V. vulnificus* occur per year in the US (Drake et al. 2007). The Korean Centers for Disease Control estimates 40–70 confirmed cases per year and this high rate is suspected to be due to consumption of raw seafood or higher prevalence of predisposing factors (Drake et al. 2007). However, in Japan, Inoue et al. (2008) estimated 12–24 cases per year and in Taiwan, there was a peak occurrence in 2000 with 26 cases per million population (Hsueh et al. 2004). While in US, oysters are the main source, this is not the case in Japan since raw oysters are eaten only in winter and most infections occur during June-November with a peak in July. A mud shrimp, *Upogebia major,* was the common agent associated with *V. vulnificus* infections (Inoue et al. 2008). 72.3% of infections had septicaemia and mortality rate was 75%. Most patients (86.5%) had liver function impairment with 56.9% having liver cirrhosis and 10.1% liver cancer (Inoue et al. 2008). In Europe, *V. vulnificus* infections are rare and mostly wound infections (Baker-Austin et al. 2010). Rare cases of septicaemia have been reported from Thailand (Thamlikitkul 1990) and India (Saraswathi et al. 1989).

V. vulnificus is a natural inhabitant of the estuarine environment and hence faecal coliforms/*Escherichia coli* cannot be used as indicator organism for this pathogen. Since molluscan shellfish are filter feeding organisms, when environmental conditions are favourable, they may harbour high levels of *V. vulnificus* with levels in oysters being 100 times higher than in water surrounding them. On the United States Gulf coast, the levels in oysters may reach 10^4 cfu/g during summer months (Drake et al. 2007) and in tropical waters of India, similar levels were reached in oysters

when salinities were less than 10 ppt (Parvathi et al. 2004). *V. vulnificus* counts exceeding 10^6/g have been reported from the intestines of benthic fish inhabiting oyster reefs (De Paola et al. 1994). If the temperature of oysters is not controlled immediately after harvest, growth of *V. vulnificus* could occur. Cook (1997) demonstrated that *V. vulnificus* levels in oyster shell stocks hold without refrigeration for 3.5, 7, 10.5 and 14 h increased 0.75, 1.3, 1.74 and 1.94 log units, respectively. It has also been reported that *V. vulnificus* levels in retail oysters originating from the Gulf of Mexico were 1–2 log units greater than at harvest (Cook et al., 2002). The organism does not grow in oysters at temperatures below 13 °C and prolonged refrigeration could lead to reduction in numbers and the levels could become non-detectable (<3/g) in 14–21 days (Cook 1994; Cook and Ruple 1992). However, Kaysner et al. (1989) observed survival in artificially contaminated oysters for 14 days at 2 °C, suggesting that refrigeration cannot be relied upon for elimination of this pathogen in oysters. The rate of decline in refrigerated oyster shell stocks has been estimated to be 0041 log unit per day (Cook et al. 2002). It has been estimated that commercial cooling of oyster stocks could take an average of 5.5 h (FDA 2005) and therefore the time, that shell stock is unrefrigerated on boat decks, is an issue in control plans.

Freezing could reduce levels of *V. vulnificus* in oysters, but this cannot eliminate the organisms completely. Four to five $\log_{10}$ reductions in numbers of natural *V. vulnificus* population in oysters occur when frozen to −40 °C and stored for 3 weeks (Cook and Ruple 1992). A combination of vacuum packaging and freezing can bring down *V. vulnificus* counts by 3–4 $\log_{10}$ units in 7 days but complete elimination cannot be achieved (Parker et al. 1994). *V. vulnificus* is sensitive to heat with 6 $\log_{10}$ reduction in numbers occurring when subjected to 50 °C for 5 min in shucked oyster meat (Cook and Ruple 1992). Natural populations of *V. vulnificus* (4.3×10^3 cfu/g) could be reduced to non-detectable levels by exposing them to 50 °C for 10 min (Cook and Ruple 1992). In North and South Carolina, commercial shell stock is subjected to heat shock by submerging about 70 chilled oysters in wire baskets into a heat-shock tank containing about 850 L of potable water at a temperature of 67 °C for about 5 min depending on oyster size and condition. This process has been shown to reduce *V. vulnificus* levels by 2–4 $\log_{10}$ units (Drake et al. 2007). *V. vulnificus* cells are acid sensitive and can be inactivated at pH 2.0 (Koo et al. 2000). *V. vulnificus* is sensitive to ionising radiation and irradiation doses of 1.0 kGy applied on whole shell oysters can reduce the cell numbers from 10^7 cfu/g to undetectable levels (Andrews et al. 2003). Hydrostatic pressure of 250 Mpa for 120 s reduced *V. vulnificus* > 5 $\log_{10}$ units in oyster (Cook 2003).

A joint FAO/WHO Expert Meeting on Microbiological Risk Assessment (JEMRA) carried out a quantitative risk assessment for *V. vulnificus* in raw oysters (FAO/WHO 2005a) and this study modified the FDA *V. parahaemolyticus* risk assessment model to assess the risk of *V. vulnificus* primary septicaemia in the US. The geographical coverage was limited, because quantitative data for *V. vulnificus* levels both in oysters and at the point of consumption as well as data for the susceptible population were available only for the US (FAO/WHO 2005a). The risk assessment model used the data on *V. vulnificus* levels in oysters from four Gulf States and assumed that all strains were equally virulent. Harvest and postharvest module

used for exposure assessment were based on postharvest practices (duration oysters in harvest vessel in water, time to first refrigeration, cool down time) and based on surveys conducted in Gulf Coast. *V. vulnificus* growth in oysters, survival during refrigeration and levels at consumption were estimated based on data from studies along the US Gulf Coast (FAO/WHO 2005a). The model predicted that the mean *V. vulnificus* levels in oysters would be 5.7×10^4/g in summer and 8.0×10^1/g in winter. At a serving size of 196 g, the ingested dose would be 1.1 X 10^7 *V. vulnificus* in summer and 1.6×10^4 in winter. FDA data on the prevalence of risk factors in the US population and oyster consumption data from surveys was used in the model (FAO/WHO 2005a). The dose response relationship was modelled by estimating the exposure per eating occasion and number of eating occasions for oyster associated *V. vulnificus* cases reported to the US Centers for Disease Control and Prevention (CDC) during 1995–2001. The risk assessment also predicted the reductions in illness that could be achieved by postharvest treatments to reduce *V. vulnificus* levels to target values such as 3/g or 30/g or 300/g. In the US, there are three validated methods to achieve end-point criterion of < 3 MPN/g *V. vulnificus* and these comprise mild heat treatment (50 °C), freezing with extended frozen storage and high hydrostatic pressure. If all oysters are treated to achieve a target level of 3/g, the model predicted that the number of cases could be reduced from current 32 reported cases per year to one case every 6 years. If the target is shifted to 30 or 300/g, then the predicted cases would increase to 1.2 and 7.7 cases per year, respectively (FAO/WHO 2005a).

The FAO/WHO risk assessment model suggested that immediate cooling of oysters alone is not adequate to achieve substantial reduction in the number of *V. vulnificus* infections. The effect of delay in refrigeration of oysters after harvest on *V. vulnificus* levels and estimated cases of illness was studied. The predicted illness ranged from 17.7 to 59.3 at a time to refrigeration range of 0–20 h. Since *V. vulnificus* levels in oysters harvested from waters with a salinity of > 30 ppt is greatly reduced, it is predicted that if all oysters are harvested from waters at salinity of > 30 ppt, irrespective of the water temperature, *V. vulnificus* disease would be < 1 case per year (FAO/WHO 2005a). Relaying oysters to high salinity waters (> 32 ppt) has been shown to reduce *V. vulnificus* levels by 3–4 log units (< 10/g) within 2 weeks. Based on FAO/WHO risk assessment, the Codex Committee on Food Hygiene developed the Code of Hygienic practice for control of *Vibrio* spp. in seafood with an annex on control measures for *V. parahaemolyticus* and *V. vulnificus* in bivalve molluscs. This Code recommends assessment of the need for control measures based on (a) number of sporadic illness associated with bivalve molluscs in a certain area (b) water temperature at harvest, air temperature and harvest and post-harvest practices (c) water salinity at harvest. Since there is wide geographical variation in prevalence and levels of *V. vulnificus* in bivalves, control measures that have been validated and appropriate for the region may be adopted by the competent authority having jurisdiction and then implemented under HACCP system. Validation of control measures should be carried out in accordance with the Codex Guidelines for the validation of food safety control measures (CAC/GL 69-2008).

V. vulnificus resides inside various tissues of oysters, hence depuration is ineffective in elimination of this pathogen. However, relaying oysters in high salinity (>30 ppt) waters for 17–49 days caused a decrease in population from 10^3 cfu/g to <10 MPN/g (Motes and De Paola 1996). The United States National Shellfish Sanitation Programme (NSSP) guide (2011) includes following strategies for minimising the risk due to *V. vulnificus* in molluscan shellfish in states reporting two or more cases of *V. vulnificus* illness per year: (a) increased educational efforts targeted towards the population at risk to improve their awareness of the risks of eating raw molluscan shellfish and to change their eating behaviour to reduce or stop eating raw or untreated molluscan shellfish, (b) limited harvest restrictions on areas incriminated in outbreaks, (c) requirement for the temperature of shell stock to be brought down to 10 °C or less by using ice, mechanical refrigeration or other means within specified period (12 h when water temperature is >27 °C; 18 h when water temperature is between 15–27 °C; 24 h when water temperature is 10–15 °C and 36 h when water temperature is <10–18 °C), and (d) phased-in postharvest treatment requirements or other controls.

5.2.2 Salmonella

Currently, two species are recognised in the genus *Salmonella,* a member of the family Enterobacteriaceae (Tindall et al. 2005): *Salmonella enterica* and *Salmonella bongori*. Six subspecies are recognised in *S. enterica* subsp. *enterica*, subsp. *salamae*, subsp. *arizonae*, subsp. *diarizonae,* subsp. *houtenae*, subsp. *indica*. More than 2500 serotypes have been recorded, of which majority (59%) belong to *S. enterica* subsp. *enterica*, which are also responsible for 99% of *Salmonella* infections in humans and warm blooded animals (Brenner et al. 2000). Other subspecies may also be associated with coldblooded animals and present in the environment, but isolates from both species and all subspecies have occurred in humans (Brenner et al. 2000).

The clinical outcomes of *Salmonella* can be considered as two separate groups: (a) Typhoid fever (enteric fever) caused by *Salmonella* Typhi/Paratyphi strains is a serious systemic illness. Incubation period ranges from 7 to 28 days. Symptoms include malaise, headache, fever, cough, nausea, vomiting, constipation, abdominal pain, chills, rose spots, and bloody stools. Typhoid fever is generally transmitted through water. (b) Non-typhoid *Salmonella* caused by other strains and characterised by gastroenteritis in humans. Incubation period ranges from 8 to 72 h. The symptoms include abdominal pain, diarrhoea, chills, fever, nausea, vomiting, and malaise. Systemic infection such as septicaemia may occur especially in susceptible patients such as the very young, very old and immunocompromised. The available data measuring illness as the endpoint suggests that no response is observed until a dose of 10^6 is reached (Coleman and Marks 1998). However, outbreak investigations show that lower number of bacteria can cause infection depending upon the food matrix. There is no data with sea food matrix alone, but in an outbreak of *S. Enteritidis* associated with scallop and egg yolk a 56% attack rate was observed

at a dose of 6.3 log CFU (FAO/WHO 2002). Severe dehydration due to diarrhoea can on occasion require medical intervention through the administration of intravenous fluids and antibiotic treatment. However, occasionally some serovars of this pathogen may cause sepsis after entering the blood stream from the intestine and require intense medical intervention. Mortality is rare, if the patient is promptly hydrated and treated with antibiotics when appropriate.

Although the normal habitat of *S. enterica* subspecies *enterica* is the gut of warm blooded animals, very few serovars are host-adapted and others may be found in the environment for long periods of time. The habitats for other subspecies are coldblooded animals and the environment. *Salmonella* has been isolated from several aquatic environments in different parts of the world (FAO 2010). Water bodies contaminated with faecal matter from humans, animals including birds and aquatic mammals may contain this pathogen. *Salmonella* can survive in human waste for 10–15 days in septic system and through seepage from septic tanks, sewage or storm runoff, and reach surface waters. It can survive and even multiply in aquatic environment where it may adhere to soil particles and survive and multiply in this ecosystem for at least one year (Winfield and Groisman 2003). In Tech River (France), 574 isolates of *Salmonella* belonging to 41 serotypes were obtained during 1996–1997, some serotypes being specific to flood events (Baudart et al. 2000). In a 4 year study of coastal waters of Galecia, North western Spain, a prevalence of 2.4% in molluscs and seawater was found with *S*. Senftenberg being the most predominant (42%) among 20 different serotypes (Martinez-Urtaza et al. 2004a). The presence of S. Senftenberg could not be correlated with environmental parameters, while presence of other serotypes was associated with wind and rainfall events. *S*. Senftenberg has been very rarely reported in human infections and is halotolerant, since it has been isolated from brines with a salt concentration of 30% (Martinez-Urtaza et al. 2004b). *S.* Senftenberg has been one of the predominant serovars detected in the coastal waters of Portugal (Catalao Dionisio et al. 2000). This serovar has been isolated from crustaceans from India (Hatha and Lakshmanaperumalsamy 1997), seafood imported into the United States especially from tropical countries (Heinitz et al. 2000), and from environmental samples in France and Brazil (Baudart et al. 2000; Tavechio et al. 2002). Detection of *Salmonella* in 16% shrimp and 22.1% in mud/water in Southeast Asia led Reilly and Twiddy (1992) to suggest that *Salmonella* are part of the normal aquatic flora in tropical environments. Recent reports of detection of *Salmonella* in fish gut in natural river system in Texas (Gaertner et al. 2008), however, suggest that *Salmonella* are more widely present in aquatic systems than earlier thought. 17–33% of fish sampled in San Marcos River, Texas were positive for *Salmonella* and presence in fish gut has been attributed to ingestion of *Salmonella* present in detritus. A 3 year study performed during 2005–2007 by Byappanahally et al. (2009) showed that the filamentous alga *Cladospora* in Lake Michigan is a reservoir for *Salmonella* with a presence of these bacteria in 23–72% of samples at densities ranging from a 0.16 to 89.46 most probable number (MPN) per gram. This alga can be found in fresh and marine waters. Therefore present evidence suggests that *Salmonella* are widely distributed in the aquatic environment and could be part of the normal flora in aquaculture systems (Table 5.1).

Specific seasonal patterns or climatic characteristics have been reported to affect the dynamics of contamination of *Salmonella* in natural environments. The presence of *Salmonella* in the environment in both temperate and tropical regions has been linked to periods of rain, and more specifically, after the days of the first heavy rain signalling the washing effect of torrential rains as one of the principal environmental drivers of *Salmonella* contamination in coastal areas (FAO 2010). There may also be other sources in the marine environment. For example, *Salmonella* may colonise marine mammals like killer whales, bottlenose dolphins, seals, sea lions, elephant seals and porpoises (Higgins 2000; Old et al. 2001; Fenwick et al. 2004; Stoddard et al. 2005) and the organisms shed by these mammals may contaminate other marine fish. 21.7 % of harbour porpoises in England and Wales were positive for *Salmonella* during 1990–2002. In San Miguel Island, California, 33 % of fur seal pups and 40 % of sea lion pups were positive for *Salmonella* (Higgins 2000).

Most of the studies looking at the presence of *Salmonella* in aquatic environments have shown two main observations: only a small, but constant number of serovars have been found in these environments and, in most cases, these do not coincide with the main zoonotic serovars identified in the surrounding areas (FAO 2010). In spite of the variability in sampling size ($n = 37–251$), in most of these studies the maximum number of serotypes identified has been around 20 (FAO 2010). Among the clinically important serovars, S. Typhimurium has been shown to be the most common but mostly this accounted for only a small percentage of serotyped strains (FAO 2010); nevertheless, this attests to their capacity of adaptation to and survival in external environments (Baudart et al. 2000). *S.* Weltevreden has been identified in recent years as one of the prevailing serovars in seafood products from Asian countries. *S.* Weltevreden has been detected as the dominant *Salmonella* serotype in fish and shrimps samples collected in India (Shabarinath et al. 2007) and in other Asian countries (Reilly and Twiddy 1992; Koonse et al. 2005); moreover, this serovar has been involved in several clinical cases in Asia (Bangtrakulnonth et al. 2004; Phan et al. 2005).

Salmonella has been isolated from aquaculture systems in both developing and developed countries and the prevalence rates reported vary depending on the methodology used for detection. In aquaculture systems of Southeast Asia, 16.1 % of shrimp and 22.2 % of water/mud samples were positive for *Salmonella* (Reilly and Twiddy 1992). In US fresh water catfish ponds, Wyatt et al. (1979) reported a prevalence of 5 % while a relatively high percentage of 33 % in US catfish and 50 % in Vietnamese catfish were reported by Pal and Marshall (2009) and this may be due to the methodology used for isolation. From eel culture ponds in Japan, a prevalence of 21 % (Saheki et al. 1989) has been documented. *Salmonella* has also been isolated from pond water in a trout farm in Spain (Cesar-Javier et al. 1999). Long term persistence of *Salmonella* in fish feed plants in Norway has been reported (Nesse et al. 2003). During 2000–2004, 3.78 % of environmental samples from Norwegian fish feed production facilities were positive for *Salmonella*. The serovars recovered were mostly *S.* Senftenberg and *S.* Montevideo that account for 2 % of human cases in Norway (Lunestad et al. 2007). Thus fish feed could be source of *Salmonella* in aquaculture systems. These studies provide evidence for the rather common

prevalence of *Salmonella* in aquaculture systems across the globe. Contamination of aquaculture systems with *Salmonella* could involve multiple pathways such as runoff of organic matter into ponds during rainfall events, animal wastes introduced directly through bird droppings, frogs living in ponds or indirectly through runoff, fertilisation using no-composted manures, integrated aquaculture systems, where animals such as poultry are housed directly over aquaculture ponds, toilets discharging into ponds, contaminated source water through wildlife runoff, untreated domestic sewage, discharge from animal farms, contaminated feed or unhygienic handling practices in farm (FAO 2010).

Salmonella are often detected in seafood in markets and the prevalence rates reported vary widely. In Malaysia, 25% of raw prawns in markets were positive, the serovars found being S. Blockley, *S.* Weltevreden and *S.* Agona (Armugaswamy et al. 1995). In India, 1% of the 500 market prawns tested were positive for the serovars *S.* Newport and *S.* Infantis (Prasad and Pandurangarao 1995). *Salmonella* present in seafood at the market level could be a result of postharvest contamination. *Salmonella* including serovar Weltevreden can form biofilms on food contact surfaces and resist sanitizer treatment in biofilms (Joseph et al. 2001). In a study on 353 imported seafood items in Japan, 2/47 black tiger shrimp were positive, both with *S.* Weltevreden, and contamination levels in seafoods were < 30–40 MPN/100 g (Asai et al. 2008). Analysis of 11,312 imported and 768 domestic seafood products in the US during 1990–1998 revealed that 10% of imported and 2.8% of domestic raw seafood were positive for *Salmonella* and the overall incidence was 7.2% for imported and 1.3% for domestic seafood (Heinitz et al. 2000). The most frequent serotypes in imported seafood were *S.* Weltevreden, *S.* Senftenberg, *S.* Lexington and *S.* Paratyphi B. These most common serotypes were rarely (< 0.5%) observed in human illness in US (Helfrick et al. 1997). *S.* Enteritidis ranked 5th and *S.* Typhimurium ranked 12th (Heinitz et al. 2000). *S.* Weltevreden was also the most common serotype isolated from imported food including seafood in the US in 2000 (24/187) followed by *S.* Thompson (13/187), *S.* Lexington (12/187) and a number of other serotypes (Zhao et al. 2003). Although S. Weltevreden is rarely associated with human cases in the US, it is frequently isolated from human cases in Thailand (Bangtrakulnonth et al. 2004).

Most studies on *Salmonella* in foods including seafood have been carried out using enrichment procedure and quantitative estimation of the concentration of *Salmonella* in foods has rarely been reported. One study of imported seafood (353 samples of 29 types of seafood) in Japan found two samples of black tiger shrimp at levels of < 30–40/100 g estimated by Most Probable Number (MPN) (Asai et al. 2008). Considering that $> 10^5$ bacteria are required to cause infection (FAO/WHO 2002), it can be suggested that multiplication in fish would be necessary before the food is consumed (Table 5.1). *Salmonella* being a mesophilic organism, the growth rate of this organism is markedly reduced at temperatures < 15 °C while the growth of most strains is prevented at < 7 °C (ICMSF 1996). In raw seafoods containing a variety of bacteria, *Salmonella*, if present has to compete with other flora for growth. *S.* Heidelberg had a generation time at 8 °C of 28 and 31 h in the fish English sole and in sterile crabs, respectively (ICMSF 1996). Ingham et al. (1990)

reported proliferation of *Salmonella* in cooked crabs inoculated with *Salmonella* and stored at 8–11 °C under modified atmospheres containing low levels of CO_2 (20–50 %). The optimum pH for the proliferation of *Salmonella* is 7.0–7.5, although the organism can grow at pH values ranging from 3.8 to 9.5 (ICMSF 1996). The minimum water activity for growth is 0.94 (ICMSF 1996) and the growth is generally inhibited at 3–4 % NaCl, but salt tolerance increases with increasing temperature in the range 10–30 °C (D'Aoust and Maurer 2007). Although the resistance of *Salmonella* to drying varies, this organism may survive for months or even years in dried products and has been frequently isolated from fish meal, meat and bone meal, maize and soy products (Lunestad et al. 2007). *Salmonella* is sensitive to lower temperatures causing decrease in numbers during freezing and frozen storage, but this process does not guarantee elimination of salmonellae in foods (ICMSF 1996). *Salmonella* are heat sensitive with typical D-values (decimal reduction time—time required to bring about reduction of levels by one log unit) reported to be 0.176 min in chicken at 70 °C, and 0.36 min in ground beef at 63 °C (FAO/WHO 2002). Some strains of *Salmonella* like *S.* Senftenberg 775 W may show higher heat resistance (ICMSF 1996). Interestingly, *S.* Senftenberg is the serovar most often isolated from fish feed (Lunestad et al. 2007). D-values are influenced by the water activity, nature of the solutes and the pH of the suspending medium (ICMSF 1996).

Although *Salmonella* has been isolated from seafoods at both farm and retail level, seafood accounts for only a small proportion of salmonellosis outbreaks. Greig and Ravel (2009) analysed food borne outbreaks reported in the international literature between 1988 and 2007, for which a source could be identified ($n = 4093$). This review indicated that 46.9 % of outbreaks were due to *Salmonella* of which seafood accounted for 1.7 % compared to 14 % associated with eggs. In the US over a three decade period (1973–2006), *Salmonella* accounted for 18 of a total of 188 outbreaks involving seafood. 374 cases were associated with *Salmonella* in seafood out of 4020 seafood associated disease episodes (Iwamoto et al. 2010). In the EU, during 2011, there were 1501 foodborne outbreaks of salmonellosis, and these were grouped as 283 outbreaks with strong evidence and 1218 outbreaks with weak evidence (EFSA and ECDC 2013). Of the outbreaks with strong evidence, 17 (6 %) were due to seafood.

Considering that *Salmonella* in aquatic systems are derived from human or animal source, antibiotic resistance in seafood associated would be reflective of the situation in other sectors. Antimicrobial resistance was detected in 9 % of the total of strains isolated from environmental sources and shellfish over different studies in Spain (Martinez-Urtaza et al. 2004b; Martinez-Urtaza and Liebana 2005). On the other hand, the presence of antimicrobial resistant strains among strains isolated from the marine environment in Morocco reached 49.1 % of the strains (Setti et al. 2009), whereas in Mexico 50.4 % of the strains recovered from water samples showed resistance to antimicrobials (FAO 2010). In a study carried out in Cochin, India, 82 % of the strains isolated from seafood products showed antimicrobial resistance (Kumar et al. 2009), whereas in Vietnam antimicrobial resistance was observed in 11.1 % of strains (Van et al. 2007). It should, however, be noted that the methodology and the spectrum of antibiotics used by different investigators vary

and this may contribute to the high degree of variation observed. Khan et al. (2006) recorded varying antibiotic resistance in *Salmonella* strains belonging to several serovars isolated from imported seafood in US, but the frequency of resistance in serovar Weltvreden was low (Ponce et al. 2008).

Biosecurity and control measures to minimise the risk of *Salmonella* contamination of aquaculture products have been elaborated by FAO (2010). A number of aspects covering farm design, layout, source of water, hygiene of equipment, personnel, feed as well as good hygienic practices during harvesting, transport and processing would be important.

5.2.3 Listeria monocytogenes

The genus *Listeria* has seven species, of which only *L. monocytogenes* is considered as a pathogen to humans and *L. ivanovii* to animals (Swaminathan et al. 2007). *L. monocytogenes* is a common inhabitant of moist environments like decaying vegetation or soil and can be isolated from various aquatic environments. Therefore, the organism is commonly associated with aquaculture environments and in freshly harvest fish (Reilly and Kaferstein, 1997) and fish in retail markets (Dhanashree et al. 2003; Parihar et al. 2008). In the US catfish industry, *L. monocytogenes* has been found at a frequency of 76.7 % in chilled fresh catfish fillets and in 43.3 % of unchilled fillets. The organism was also detected in fish contact surfaces such as deheading machines, trimmingboards, chiller water and conveyor belts at different stages (Chen et al. 2010). In some studies, raw fish have been found to be the source of contamination of fish processing plants (Gudmundsdottir et al. 2005). Prevalence of *L. monocytogenes* in fish processing environments has been a particular problem, particularly for the fish smoking industry. To understand the source of contamination of the final product, various molecular techniques such as Pulse Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST) have been used. Chen et al. (2010) noted that in the US catfish industry, chiller water and processing tables are important sources of contamination for the final fillets rather than the raw material. Ciccio *et al.* (2012) noted that although raw fish could be the source of contamination of the processing environment, certain strains can persist longer than others and the strains found in the final product are generally these persistent strains. *L. monocytogenes* has been reported to form biofilms and to survive in fish processing environments for years (Wilks et al. 2006). PFGE studies on isolates provide evidence for the persistence of strains for 11 years (Vongkanjan et al. 2013).

Unlike most other foodborne pathogens, *L. monocytogenes* is psychrotrophic and capable of growing at refrigerator temperatures. The temperature range at which growth can occur is between 0–4 5 °C with an optimum temperature of 37 °C. The organism is resistant to environmental conditions such as high salinity or acidity, which enables its survival for long periods in the environment. Possibly, due to its psychrotrophic nature, the organism seems to be more prevalent in seafood

from temperate environments compared to that from tropical regions. For example, in India, the reported prevalence ranges from absence to 8.6% (Karunasagar and Karunasagar 2000; Parihar et al. 2008), while in cold water shrimp the prevalence could be 20.9% (Gudmundsdottir et al. 2006).

Despite the wide prevalence of *Listeria monocytogenes* in foods, natural and food processing environments and its asymptomatic carriage in 5–10% of humans and domestic animals, listeriosis is a rare disease. The incidence is typically in the range 0.1 to 11 cases per million people per year (FAO/WHO 2004). The severe form of listeriosis is characterised by an invasive infection often leading to septicaemia with or without infections of the central nervous system such as meningitis, meningoencephalitis, rhomboencephalitis or brain abscess. In the case of pregnancy, while the mother will often experience mild flu-like symptoms, her foetus may be stillborn, aborted or be born with generalized infections. Less common symptoms include localised infections including endocarditis, peritonitis, and arthritis. Skin infections may also occur in some patients. The incubation period is very variable ranging from 3 to 70 days and, since most people do not remember their food consumption from months earlier, it is often difficult to trace the source of infection. The median incubation period is approximately 3 weeks. If diagnosed, the disease can usually be treated effectively with a range of common antibiotics. The severe form of listeriosis occurs mostly in susceptible populations including the elderly, pregnant women, people with underlying diseases (i.e. chronic conditions such as cardiovascular disease, congestive heart failure, diabetes, cirrhosis and alcoholism) and immunocompromised individuals. Fatality rates of 20–30% are common in the severe form of listeriosis (FAO/WHO 2004).

L. monocytogenes may also cause a non-invasive febrile gastroenteritis in healthy individuals. An outbreak of gastrointestinal illness from a tuna and corn salad, affecting >1 500 schoolchildren and adults in Italy provided conclusive evidence for the existence of a febrile gastroenteritis form of listeriosis (Drevets and Bronze 2008; Allerberger and Wagner 2009). The incubation period for this form of the disease ranges from 6 to 50 h, and symptoms usually resolve without treatment after 1–2 days. Symptoms are described as "mild flu-like", including diarrhoea, abdominal pain, fever, muscle pain and headaches.

Most outbreaks of listeriosis are associated with ready-to-eat foods that can support the growth of *L. monocytogenes,* have a long refrigerated shelf life and are consumed without further listericidal treatment. Even in products that receive listericidal treatment, post-process contamination as well as cross contamination at the distribution and the home level are important issues (FAO/WHO 2004). This applies to a range of seafood products, including marinated fish and mussels, prawns, pasteurised crustacea, and smoked fish products. Cold-smoked products have received particular attention in this regard due to a high prevalence of *L. monocytogenes* in such products and the persistence of contamination in fish processing plants. Contamination rates range from 0.4 to 78.7% but more typically in the range of 4–30%. Studies by Jorgensen and Huss (1998) suggest that at the point of production, 34% of samples are positive with 28% having <10 CFU/g, 5% having 10–100 CFU/g and 1% having >100 CFU/g. After 14–20 days of storage at 5 °C,

40% were positive and the level exceeded 100 CFU/g in 10.5% of samples with levels between 10–100 CFU/g in 20% cases. Epidemiological evidence suggests that listeriosis has been associated with consumption of shrimps, smoked mussels, "gravad" trout, and smoked trout (FAO/WHO 2004). Many of these outbreaks, however, involved the gastrointestinal form of the disease and, despite the interest in RTE (ready to eat) smoked fish as a source of listeriosis, there are very few documented cases of systemic listeriosis due to seafood.

Based on somatic (O) and flagellar (H) antigens *L. monocytogenes* has been differentiated into 13 serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 6a, 6b). Most isolates involved in human disease belong to three serotypes, 1/2a, 1/2b, 1/2c and 4b. Dose-response models for *L. monocytogenes* inferred from epidemiological data and estimates of total food-borne exposure (FAO/WHO 2004) have generated ID_{50} estimates for immunocompromised people of $>10^{10}$ cells. Since the levels of *L. monocytogenes* found in seafood are generally low, multiplication of the organism is supposed to have occurred in products involved in outbreaks. Therefore, prevention of growth in seafood products would be an important risk management strategy. A number of hurdles are employed to increase the shelf life of lightly preserved seafoods, often in combination, including refrigeration, salt, phenolic (smoke) compounds, acidification with organic acids including lactate, acetate, sorbate, benzoate, citrate, or addition of salts of organic acids, addition of nitrite and modified atmosphere packaging including CO_2. Models to predict the effect of these hurdles on the growth of *L. monocytogenes* have been developed and evaluated (Mejlholm et al. 2010).

When *L. monocytogenes* was identified as a foodborne pathogen, the first response of regulatory agencies was to establish a "zero tolerance" policy, i.e. the organism should be absent in a 25 g sample of the product. This might cause trade disruptions and the issue came up before the Codex Alimentarius Commission which asked FAO/WHO to perform a risk assessment for *L. monocytogenes* in foods and specifically to "estimate the risk of serious illness from *L. monocytogenes* in food when the number of organisms ranges from absence in 25 g to 1000 colony forming units (CFU) per gram or millilitre, or does not exceed specified levels at the point of consumption". The risk assessment report (FAO/WHO 2004) noted that in areas where a regulatory level of "absence in 25g" or 0.04 CFU/g is applied and all products in market comply with the requirement, the listeriosis cases would be less than 1 case per year. In the US, however, where such a regulatory limit is applied, cases were still seen indicating that a portion of ready-to-eat food contained a substantially higher number of the pathogen than the regulatory limit. Thus the public health impact of *L. monocytogenes* is almost exclusively a function of the food products that greatly exceed the "absence in 25g" limit. The report also examined a "what if scenario" using two often discussed regulatory limits: 0.04 and 100 CFU/g. The risk assessment model indicated that at 100% compliance, the number of predicted cases is low for both limits, with an approximate 10-fold difference between them, i.e. 0.5 cases versus 5.7 cases. However, as the number of "defectives" (proportion of products not meeting the criteria) increased, the difference narrowed. For example at a presumed defective level of 0.0001%, the predicted number of cases

would be 12.3 and 17.4. The model predicted that if a microbiological limit of 0.04 CFU/g with a 0.018 % defect rate (2133 cases) was replaced with a 100 CFU/g limit and a 0.001 % defect rate (124 cases), the predicted result based on the scenario is an approximate 95 % reduction in foodborne listeriosis (FAO/WHO 2004). In view of the widespread occurrence of *L. monocytogenes* in fish processing environments, there would be a significant percentage of "defectives", if "absence in 25g" is used as criterion. Considering these findings, the Codex Alimentaruis Commission agreed to have a criterion of 100 CFU/g for ready-to-eat products that do not support the growth of *L. monocytogenes* and absence in 25 g for productsthat support the growth of the organism.

Ampicillin is the antibiotic of choice for treatment of listeriosis. Many investigators have screened clinical as well as food isolates of *L. monocytogenes* for antimicrobial susceptibility and generally the reported prevalence of resistance is very low (Walsh et al. 2001; Hansen et al. 2005). Lungu et al. (2011) compiled data regarding reported antibiotic resistance in *L. monocytogenes* isolated from food. Resistance to a variety of antimicrobials including tetracyclines, cephalosporins, β-lactams, aminoglycosides and quinolones has been noted in many foodborne strains. Interestingly, there were no such strains reported from fish or seafood.

5.3 Antimicrobial Resistance in Microorganisms Associated with Products of Aquaculture

The importance of antimicrobial agents in the protection of animal health has been widely acknowledged, but the negative impacts of the use of these agents in animals raised for food have been a cause of concern. The use of antimicrobials in agriculture, animal husbandry and aquaculture in many developing countries is often unregulated and there is very little data on their usage. In the US aquaculture industry (catfish, salmon and trout), the usage has been estimated to be 204,000–433,000 pounds (92,500 to 196,400 kg) annually (Benbrook 2002) and this is about 2 % of non-medical use in meat and companion animals. In the United Kingdom, 2 metric t of antimicrobials (mainly tetracyclines and potentiated sulphonamides) were used in aquaculture (salmon and trout) during 2000 (Rodgers and Furones 2009). In Chile, 385,600 kg antibiotics were used in 2007, while in Canada, 21,330 kg antibiotics were used in 2007 (Burridge et al. 2010). For production of the same aquaculture species, different quantities of antibiotics may be used. For example, in Chile during 2007 and 2008, 385.6 and 325.6 t of antibiotics were used to produce 300,000 and 400,000 t of salmon, while during the same period in Norway, less than a metric ton of antibiotic was used to produce 820,000 t of salmon (Burridge et al. 2010). There is no reliable data on antibiotic usage in aquaculture in Asia, which accounts for nearly 90 % of world aquaculture production (FAO 2012).

The current risk management strategy for antimicrobial residues in aquaculture products is based on the precautionary principle and there are no epidemiological records of illnesses in fish consumers due to residues. The FAO/OIE/WHO

consultation on scientific issues related to non-human usage of antimicrobials held in Geneva in December 2003, concluded that residues of antimicrobials in foods, under present regulatory regimes, represent a significantly less important human health risk than the risk related to antimicrobial resistant bacteria in food. Resistance of bacteria to antimicrobial agents is a complex issue. Some bacteria have intrinsic resistance to certain antibiotics, e.g. most Gram-negative bacteria have intrinsic resistance against penicillin G, due to the nature of cell wall in this group of bacteria. Resistance to antimicrobial compounds is a very ancient trait in environmental bacteria. Genes conferring resistance to antibiotics that are critical for human medicine today have been found in bacteria billions of years before antimicrobial usage (D'Costa et al. 2011). Bacteria resistant to β-lactams, aminoglycosides, and macrolides as well as newer drugs such as daptomycin, linezolid, telithromycin and tigecycline have been isolated from the Lechuguilla caves in New Mexico that were totally isolated for >4 million years (Bhullar et al. 2012). Recent molecular biological studies on antibiotic resistance genes provide very interesting insights into the evolution and ecology of antibiotic resistance genes. It is estimated that Class A β-lactamases evolved approximately 2.4 billion years ago and were horizontally transferred into the Gram-positive bacteria about 800 million years ago. The family of genes, including the progenitors of CTX-Ms (cefotaxime resistance genes), diverged 200–300 million years ago.In addition to being involved in hydrolysis of the β-lactam ring, metallo-β-lactamases are involved in various basic cellular processes such as hydrolysis, DNA repair, RNA processing and these enzymes can be found in all the three domains of life, i.e. Bacteria, Archaea and Eukarya (Garau et al. 2005). Ribosomal protection proteins (RPP) that mediate resistance to tetracyclines were derived through duplication and divergence of GTPase, before the divergence of the three superkingdoms: Bacteria, Archaea and Eukarya (Kobayashi et al. 2007).

There is evidence to show that clinical bacteria have acquired resistance genes from environmental bacteria. The *qnr* gene responsible for resistance to quinolones is widely distributed in aquatic bacteria such as members of *Vibrionaceae*, *Aeromonas* and *Shewanella* species (Poirel et al. 2012). The bla_{CTX-M} gene coding for resistance to extended spectrum β-lactamases (ESBL) have originated from environmental *Kluyvera* species (Canton et al. 2012).

Although antibiotic resistance genes may emerge as a process of natural genetic changes occurring in bacteria, presence of antibiotics would exert selective pressure favouring resistant bacteria and their spread. The widespread use of antibiotics in different sectors such as animal husbandry, agriculture and human medicine has contributed to selection and spread of antibiotic-resistant bacteria in the environment. Since the aquatic environment receives effluents from hospitals and animal farms, resistance traits that have developed in other sectors may end up in the aquatic environment and reach aquaculture farms (Fig. 5.1). Antibiotic resistance genes can spread among unrelated bacteria without any phylogenetic, ecological or geographical barriers. The Joint FAO/OIE/WHO Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance (FAO/OIE/WHO 2006) identified two types of hazard with respect to antimicrobial resistance:

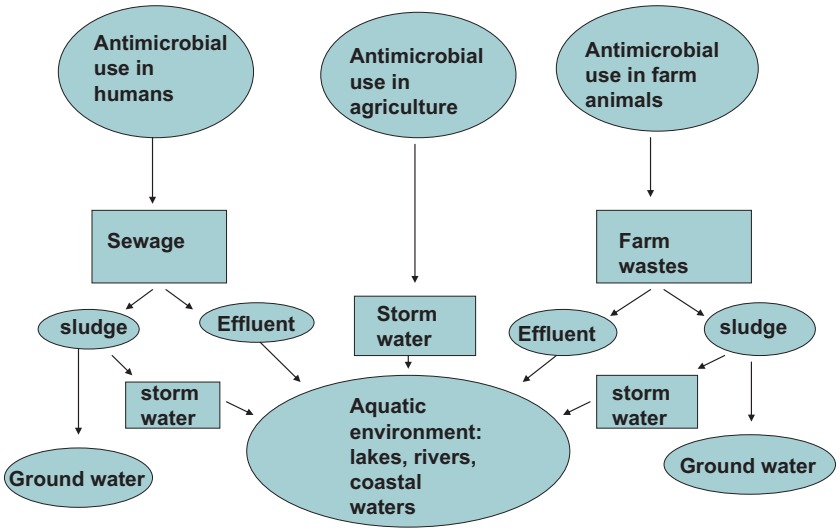

Fig. 5.1 Pathways for spread of antimicrobial residues and resistant bacteria into the aquatic environment

a. Development of acquired resistance in bacteria in aquatic environments that can infect humans. This can be regarded as a direct spread of resistance from aquatic environments to humans; and
b. Development of acquired resistance in bacteria in aquatic environments, whereby such resistant bacteria can act as a reservoir of resistance genes from which the genes can be further disseminated and ultimately end up in human pathogens. This can be viewed as an indirect spread of resistance from aquatic environments to humans caused by horizontal gene transfer.

The consequences of antimicrobial resistance in bacteria causing human infections could include increased severity of infections and increased frequency of treatment failures (FAO/OIE/WHO 2006). Antibiotic resistance in human pathogens associated with an aquaculture environment has been discussed in earlier sections. However, there are no recorded cases of human infections caused by antibiotic-resistant bacteria from aquaculture products.

Indirect spread of antibiotic resistance from aquatic bacteria and human pathogens has been considered as a possible hazard. A number of investigators have reported an increased prevalence of bacteria carrying antibiotic resistance genes in fish/shrimp ponds and in water and sediments surrounding aquaculture sites in Japan (Kim et al. 2004), Europe (Kerry et al. 1996; Schmidt et al. 2000), the US (Herwig et al. 1997), South America (Miranda and Zemelman 2002), China (Dang et al. 2009) and South-East Asia (Karunasagar et. al. 1984; Lee et al. 2005). Although the experimental transfer of antibiotic resistance from bacteria from fish- pathogenic bacteria to human gut associated *E. coli* has been demonstrated (Kruse and Sorum 1994), a possible link between antibiotic resistance in aquatic bacteria and human

pathogens in nature has not yet been clearly established. Often, similarity in genetic elements is taken as evidence of transfer, but one cannot be sure in which direction the gene flow has occurred considering that hospital effluents also discharge antibiotic resistant bacteria into the aquatic environment. Conclusions based on similarity of genetic determinants found in aquatic bacteria and human pathogens need to be evaluated carefully due to the fact that the aquatic environment receives effluents from various sectors of antimicrobial use, e.g. human medicine (hospital effluents), agricultural use, animal husbandry and aquaculture (fish farm effluents). Thus the water source used in aquaculture may be contaminated with antibiotic residues or antibiotic-resistant bacteria derived from different sectors (Fig. 5.1). FAO/OIE/WHO (2008) noted that a risk analysis of the release of human and animal effluents into aquatic environments serving as water sources for aquaculture needs to be performed, particularly with respect to the antimicrobials identified as critically important by WHO and OIE. Such a risk analysis would determine the appropriate management options through which improved effluent management measures should be implemented (e.g. measures dealing with hospital effluents). Thus the issue of antimicrobial resistance cannot be addressed for one sector (e.g. aquaculture) alone, but requires a comprehensive approach involving all sectors of antimicrobial usage.

5.4 Summary and Conclusions

Some natural aquatic organisms like *Vibrio* spp. may be pathogenic to humans and possess zoonotic potential, but generally, these are present in products of aquaculture in low numbers. Cases of human infections are generally due to products in which the pathogenic *Vibrio* spp. have multiplied and reached infectious levels. Risk management mainly involves preventing growth of these organisms. Some pathogenic *Vibrio* spp. such as *V. vulnificus* affect certain categories of consumers, especially those with underlying liver disorders or immunocompromising conditions. Enteric bacteria such as *Salmonella* may reach aquatic environment through waste water or storm water, birds, aquatic amphibians or other sources, but the outbreaks of salmonellosis are very rarely associated with products of aquaculture. Environmental bacteria such as *L. monocytogenes* may colonise fish processing environments and may be present in low numbers in some ready-to-eat fishery products like smoked fish. Although antibiotic resistance has been detected in several pathogens that can be transmitted through aquaculture products, there are no epidemiological records of illnesses caused by such resistant pathogens. Data on antibiotic resistance in aquatic bacteria needs to be evaluated carefully against the background that several environmental bacteria that are not involved in human infections naturally carry resistance determinants and could be a source of such genes for clinically important bacteria. Moreover, the aquatic environment receives resistant bacteria from hospital effluents or animal farms and these may be transmitted though aquaculture products. The problem of antimicrobial resistance needs to be addressed considering the use of antimicrobials in all sectors including human, animal, agricultural and aquaculture sectors.

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Chapter 6
Campylobacter: Animal Reservoirs, Human Infections, and Options for Control

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Abstract Campylobacteriosis is a frequently diagnosed disease in humans. Most infections are considered food-borne and are caused by *Campylobacter jejuni* and *C. coli*. The animal reservoirs of these *Campylobacter,* and the sources and routes of transmission, are described and discussed. Most warm-blooded animals can be colonized by *Campylobacter,* but avians, and in particular poultry, are preferred hosts. Much of the world's poultry production is colonized by *Campylobacter.* Source attribution studies estimate that 20–40 % of cases are attributed to the handling and consumption of chicken meat, while up to 80 % of cases are due to *Campylobacter* found in the chicken reservoir. The difference suggests that routes other than through the food chain, i.e. environmental contamination, are important. Thus the most effective interventions would be targeted to primary production. To date, only improved biosecurity is available. If effectively implemented strict biosecurity can reduce the number of *Campylobacter*-positive flocks, but implementation to this level has proved difficult for the poultry industry. Available interventions in chicken processing plants can substantially reduce *Campylobacter* numbers on carcasses and consequently reduce the risk to humans. Public health strategies

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therefore utilize control programs, which aim at reducing the level of *Campylobacter* by measures along the food chain. It is now recognized that commercially acceptable complementary interventions for primary production, such as vaccines, bacteriophages, feed additives, are urgently needed. Once *Campylobacter* in poultry is controlled then other minor sources of *Campylobacter* including contaminated drinking water, direct contact with (pet) animals, and other food items (e.g. red meat and milk), can be addressed.

6.1 Campylobacteriosis: The Disease and Its Burden in Humans

Human campylobacteriosis is primarily caused by *Campylobacter jejuni* and to a much lesser extent by its close relative *C. coli.* Human infection with either pathogen largely presents as gastrointestinal illness (Gillespie et al. 2002). *C. jejuni* and *C. coli* together account for more than 90% of all cases of human campylobacteriosis. Infections with other *Campylobacter* species may also occur, but they occur in either specific risk groups, for example people with impaired immunity (e.g. *C. fetus*) (Wagenaar et al. 2014), or are very rare (e.g. *C. lari*), or cluster in specific geographical areas (e.g. *C. upsaliensis*) (Man 2011). As these non-*C. jejuni*/*coli* infections represent only a small fraction of all human *Campylobacter* infections, this chapter will focus on *C. jejuni* and *C. coli*, and hereafter *Campylobacter* refers to these two species only. Similarly, hereafter campylobacteriosis refers to the human disease caused by *C. jejuni* and *C. coli*.

Campylobacter is the most commonly reported cause of bacterial infectious intestinal disease (IID). However, systematic disease surveillance programmes, which include campylobacteriosis, are largely limited to industrialized countries, such as the United States (US) and Member States of the European Union (EU) (EFSA and ECDC 2014; Scallan et al. 2011). To date, data from non-industrialized countries are scarce and fragmented, but suggest that campylobacteriosis generally has a lower incidence. In industrialized countries, *Campylobacter* is isolated 3–4 times more frequently from patients with IID than *Salmonella* or *Escherichia coli*. However, it is well recognized that under-reporting of such diseases is frequent. Adjusting for this, the true prevalence of campylobacteriosis was estimated to be 9.2 million in the EU in 2009 (Havelaar et al. 2013) and 1.3 million in the US in 2011 (Scallan et al. 2011). Nevertheless, serological evidence suggests that exposure to this pathogen, leading to asymptomatic infection, is substantially more frequent (Teunis et al. 2013), such that most individuals have been exposed to the organism by 20 years of age (Ang et al. 2011). Such exposure can lead to protective immunity, which might affect the outcome and impact on disease incidence and could explain the low reported prevalence of disease in developing countries despite obvious regular exposure (Havelaar et al. 2009).

There are some additional interesting epidemiological features of campylobacteriosis, many of which have yet to be fully explained. These include a seasonal peak,

which varies between countries and seems to be inconsistent with seasonal peaks observed in potential sources. There are also differences in disease incidence with age, with peaks in children under 2 years of age and in young adults, and between rural and urban areas, especially in children. Also, interestingly, there is some evidence that the incidence of disease in individuals in later life is increasing.

In the past campylobacteriosis was largely considered a mild illness, but the severity of this disease is clearly reflected in the relatively high rate of *Campylobacter*-infected individuals seeking medical attention. Surveys show that 1 in 4 cases in the Netherlands and 1 in 7 cases in the United Kingdom (UK) visit a general practitioner and approximately 1 % of these individuals are hospitalized (Havelaar et al. 2012; Tam et al. 2012). In the acute phase, campylobacteriosis is primarily characterized by gastrointestinal symptoms, such as watery (sometimes bloody) diarrhoea, abdominal cramps, nausea, vomiting and fever. The disease is usually self-limiting, lasting a week or less. Antimicrobial treatment is only indicated in severe cases (e.g. bloody diarrhoea or systemic infection). However, *Campylobacter* infection can also have serious sequelae, including Guillain-Barré and Miller-Fisher syndromes, reactive arthritis and functional gastrointestinal disorders (Doorduyn et al. 2008; Haagsma et al. 2010; Helms et al. 2006).

Recently the burden of campylobacteriosis has been quantified in term of disability-adjusted life-years (DALYs), which is a metric of health loss caused by the disease comprising years of life lost by the population due to disability and premature death. The different manifestations of campylobacteriosis were estimated to cause an average disease burden of 2060 DALYs per year in the Netherlands, calculated for the period 2005–2007. Sequelae accounted for 82 % of this burden (Mangen et al. 2013). In 2009, the DALY estimate increased by approximately 37 % to 3250 DALYs, mainly due to the higher disease incidence (Havelaar et al. 2012). Among foodborne pathogens investigated in the Netherlands, this DALY estimate was second only to *Toxoplasma gondii*. Similar studies in the US in 2011 showed *Campylobacter* to cause a burden second only to *Salmonella*, with a cost of illness of $ 1.7 billion annually (Hoffmann et al. 2012).

Despite the relative importance of campylobacteriosis, unlike for salmonellosis, there have been no effective intervention programmes implemented, with the exception of Iceland and New Zealand where very specific conditions prevailed (Sears et al. 2011; Stern et al. 2003). This is all the more surprising given that the incidence of human campylobacteriosis increased significantly during the 1980s–1990s, stabilized around the start of this century, and has tended to increase again in more recent years (CDC 2012; EFSA and ECDC 2014). The reasons for this lack of intervention are debatable, but include the complexity of foodborne and environmental sources and transmission routes; the financial imbalance accruing from interventions where the cost is to the poultry industry while the benefit is to the public health sector; and lack of consumer/political acceptance of effective measures like irradiation or chemical decontamination. In addition, there is a general lack of public interest, which is in part due to the scarcity of major outbreaks.

6.2 Characteristics of *Campylobacter*

Campylobacter comprises a genus of Gram-negative, motile, non-spore forming, mostly microaerophilic, spiral bacteria (diameter 0.2–0.5 μm, length 0.5–8 μm). These bacteria were first described in 1886 by Dr. Theodor Escherich in infants, who died of "cholera infantum". The pathogen was referred to as "Vibrio like organisms" until 1963, when Sebald and Véron (1963) named the genus as *Campylobacter*. Because of their fastidious nature, which causes difficulty in recovery and culture, these bacteria were subsequently neglected by the scientific community. However, in 1972 the first isolation from human faeces was reported (Dekeyser et al. 1972). This finding was an early "One Health" achievement, with Dr. Dekeyser as a veterinarian and Dr. Butzler as a medical doctor noticing the same morphological type of bacteria in chicken and human faeces. This report was quickly followed by improved isolation techniques and the recognition of *Campylobacter* as common agents of acute enteritis in humans (Skirrow 1977).

To date, the genus *Campylobacter* includes 24 species (www.bacterio.cict.fr/c/campylobacter.html) and with the use of molecular approaches, this number is rapidly expanding. Both *C. jejuni* and *C. coli* are thermophilic, showing optimal growth at 42 °C. For the purposes of isolation this thermotolerance, especially in combination with resistance to cephalosporin, is often used to reduce contaminating flora and improve recovery, particularly from faecal material.

Campylobacter readily generate resistance against an increasing number of classes of antimicrobials. Although antimicrobials are infrequently prescribed for campylobacteriosis, such resistance can have clinical consequences. For example, resistance to fluoroquinolones and tetracyclines is high in many regions of the world, but resistance to erythromycin and gentamicin remains generally low (Ge et al. 2013). Certainly in the UK, travelling abroad is one of the main risk factors for acquiring an infection with a fluoroquinolone-resistant *Campylobacter* strain (CSSSC 2002), suggesting that the indiscriminate use of such antimicrobials in some other countries is significant. An association between the licensed use of fluoroquinolones in poultry and increased fluoroquinolone resistance in strains isolated from humans was noticed in the 1980s (Endtz et al. 1990). This association was strengthened by a low fluoroquinolone resistance in *C. jejuni* isolates from humans in Australia, a country where fluoroquinolones were never licensed for use in production animals (Cheng et al. 2012).

Campylobacter is sensitive to many environmental stresses, including desiccation, heat, ultra-violet radiation, atmospheric oxygen and high salinity. As a consequence *Campylobacter* are unable to grow naturally outside a host and are considered generally fragile compared with, for example, *Salmonella*. Nevertheless, *Campylobacter* can survive in the environment for prolonged periods, especially in moist conditions. Survival has been recorded for up to 3 months in slurries and water contaminated with organic materials (Nicholson et al. 2005) and up to 10 months in manure compost (Inglis et al. 2010).

The fastidious nature of the organism is reflected in its demanding requirements at culture. Diagnosis of infection is usually based on isolation from faecal samples using selective media, containing appropriate antimicrobials, and incubated under reduced oxygen tension, at 42 °C for 72 h. However, the isolation technique and media constituents may vary depending on the matrix under investigation. Interestingly, such variations may affect both the efficacy of recovery and the species and/or strain types recovered (Newell et al. 2001).

The typing of *Campylobacter* has proved extremely challenging. The organisms demonstrate considerable variation at both the phenotypic and genotypic levels and many attempts have been used to exploit this variation to characterize *Campylobacter* for epidemiological studies. Initial typing methods included serotyping and phage typing. However, molecular techniques, such as *fla*-typing, ribotyping, Pulsed Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) proved more useful. More recently Multi Locus Sequence Typing (MLST) has become the preferred method for studying the relationships between strains (Dingle et al. 2001). It is now anticipated that whole genome sequencing, with subsequent data processing, will replace MLST (Didelot et al. 2012).

6.3 The Disease and Carriage in Animals

The primary habitat of *Campylobacter*, and its main amplification site, is the intestinal tract of warm-blooded animals. Both *C. jejuni* and *C. coli* are normal inhabitants of the guts of healthy livestock, pets and wild animals. There appears to be some host preference with *C. jejuni* more commonly isolated from most animals, like cattle, dogs and cats, while pigs predominantly carry *C. coli*. The reason for this is unclear. The prevalence of livestock carriage varies with factors like age, husbandry, country, etc. Certainly, a significant proportion of livestock animals is colonized. For example, in a national survey of livestock at slaughter in the UK in 2003 C. *jejuni/C. coli* were isolated from 54.6% of cattle, 43.8% of sheep and 69.3% of pigs (Milnes et al. 2008). Similarly, up to 45% of dogs are colonized (Marks et al. 2011).

The role of *C. jejuni/C. coli* as pathogens in these animals is considered of relatively minor importance. They can cause abortion in cattle and sheep, but are usually less frequently isolated from aborted foetuses than *C. fetus*. A recent exception is the spread of a single tetracycline-resistant *C. jejuni* clone causing abortion in sheep throughout the US (Wu et al. 2014), but not yet reported in other countries. Interestingly this clone has also been recovered from diarrhoeic humans in the US, but the route of transmission has not yet been identified. The role of *Campylobacter* as a pathogen in dogs remains debatable (Marks et al. 2011; Burch 2005). The high level of asymptomatic carriage (Marks et al., 2011) suggests that any association with disease is coincidental rather than causative. Nevertheless, there is certainly

evidence of such companion animals as a source for human infections (Mughini Gras et al. 2013).

Poultry in particular and (wild) avian species in general, are the preferred hosts for these organisms. This is a reflection of the bacterium's thermophilic character as 41–42 °C is the body temperature of a bird. Colonization occurs throughout the gut, but primarily in the caecum of a broiler, where levels of up to 10^9 colony forming units per gram have been reported. All the evidence indicates that *Campylobacter* act as a commensal in the avian gut, although this is occasionally disputed. The prevalence of *Campylobacter*-positive broiler flocks varies considerably, for example with age, season of the year, latitude, extensive or intensive rearing, etc. In an EU-wide survey of broiler flocks undertaken in 2008, the prevalence of *C. jejuni/C. coli* colonization varied between 5 and 100 % among Member States (EFSA 2010). The prevalence is particularly high if the flocks are free-ranging (Thonart et al. 2010). The organism is highly infectious and in each colonised flock up to 100 % of birds can be *Campylobacter*-positive. Thus overall, it is reasonable to assume that a significant proportion of broilers produced worldwide are colonized with these organisms.

6.4 Sources and Transmission Pathways of Human Campylobacteriosis

Although *Campylobacter* is considered mainly a foodborne pathogen, there is evidence for other transmission pathways, including contact with colonized animals and environments contaminated by their waste products, as well as, rarely, infected people in conditions of poor hygiene (Mughini Gras et al. 2012, 2013, 2014). It is well recognised that *Campylobacter*-containing gut contents can enter the food chain by contaminating various food products of animal origin, including meats and dairy products. Cross-contamination during food preparation at home is also an important transmission route (de Jong et al. 2008). Alternative routes with animals as sources include exposure to environments contaminated by primary production (e.g. run-off from livestock in farms and at pasture, water used for cleaning animal-containment areas, stockpiled sewage, etc.). *Campylobacter* survives for long periods in surface waters, so such contamination might pose a risk to humans through the drinking of untreated water, recreational activities, or the consumption of fresh produce irrigated or washed with manure-contaminated water.

6.4.1 Campylobacter Source Attribution

A general framework for the source attribution of campylobacteriosis has been designed by Nigel French, describing the available information, sources and modelling approaches (Wagenaar et al. 2013a; WHO 2013). Based on this framework,

animals (e.g. cattle, sheep, poultry, pets, wildlife, etc.) are defined as *reservoirs* or *amplifying hosts*; the environment and water sources, the food chain and direct contact with animals are given as examples of *pathways*; drinking water, meat, milk, occupation are given as examples of *exposure*; and examples of *risk factors* include swimming in rivers, eating chicken meat, beef, etc. In a typical example; cattle (reservoir) may contaminate the food chain (pathway) resulting in an hazard in the milk supply (exposure), which manifests itself as an increased risk associated with the consumption of unpasteurized milk (risk factor) (example adapted from Wagenaar et al. 2013a).

Source attribution models provide an estimate of the relative contribution of the different known reservoirs to the burden of human illness. They can be used to inform decision makers in order to target the most effective intervention strategies and are, therefore, an important tool for risk management. Specifically, source attribution may be used to prioritize and measure the impact of targeted interventions in the food chain, as well as to identify the most appropriate points at which such interventions should be focussed (Pires et al. 2009) to achieve significant reductions in human exposure (EFSA 2008).

Several approaches can be used for source attribution, including microbiological (microbial subtyping and comparative exposure assessment) and epidemiological (outbreak summary data and case-control studies) approaches and intervention studies (Pires et al. 2009). Structured expert opinions and comparative exposure assessment can also be used for source attribution, but will not be considered here.

6.4.1.1 Source Attribution Based on Outbreak Data

The attribution of sources based on outbreak data is generally considered of limited value for human campylobacteriosis because of the rarity of reported outbreaks (Pires et al. 2010). This is in marked contrast to *Salmonella* infections, which often present as outbreaks (Wagenaar et al. 2013b). *Campylobacter* outbreaks may of course occur more frequently, but are unreported due to the generally intermittent typing of human *Campylobacter* isolates and the lack of internationally accepted harmonized typing methods. Nevertheless, in Europe, campylobacteriosis outbreak data is collected annually and has recently been used to estimate the causative vehicles for the years 2005–2006 (Pires et al. 2010). Putative sources rank differently depending on whether the data was analysed in terms of either the proportion of outbreaks or the proportion of infected individuals reported. The majority (~64%) of outbreaks had no identified source, while ~12% were attributed to meat products as a whole and ~10% specifically to chicken. In contrast, in terms of ill individuals, the majority (~44%) was attributed to travel, ~17% to putatively contaminated drinking water, 10% each to meat and chicken and 36% were of unknown source. Although the ranking of source importance seems different, chicken remains an important source regardless of the approach taken. In fact the authors report that "among illnesses that could be attributed to a source, 29% of campylobacteriosis cases were attributed to chicken." (Pires et al. 2010).

6.4.1.2 Source Attribution Based on Case-Control Studies

Case-control studies have been used in several countries to identify those risk factors associated with sporadic *Campylobacter* infections. Overall these studies indicate that the handling and consumption of chicken meat is a very important risk factor for sporadic human campylobacteriosis (Domingues et al. 2012; Doorduyn et al. 2010; Kapperud et al. 2003; Neimann et al. 2003; Stafford et al. 2007; Studahl and Andersson 2000). Other frequently identified risk factors include, the consumption of unpasteurized milk (Friedman et al. 2004; Neimann et al. 2003; Studahl and Andersson 2000), eating in restaurants (Danis et al. 2009; Eberhart-Phillips et al. 1997; Friedman et al. 2004; Gallay et al. 2008), contact with pet dogs (especially puppies) (Carrique-Mas et al. 2005; Doorduyn et al. 2010; Eberhart-Phillips et al. 1997; Friedman et al. 2004; Mughini Gras et al. 2013; Neal and Slack 1997; Stafford et al. 2007; Tenkate and Stafford 2001), contact with livestock (Danis et al. 2009; Eberhart-Phillips et al. 1997; Friedman et al. 2004; Mughini Gras et al. 2012; Potter et al. 2003; Stafford et al. 2007; Studahl and Andersson 2000; Tenkate and Stafford 2001) and foreign travel (Eberhart-Phillips et al. 1997; Friedman et al. 2004; Gallay et al. 2008; Neal and Slack 1997; Neimann et al. 2003; Stafford et al. 2007).

The calculations of the attributable fractions for each risk factor also indicate that, like the outbreak data, chicken consumption accounts for approximately 28% of sporadic cases (Doorduyn et al. 2010). In contrast the contribution of dog ownership to human *Campylobacter* infections is around 4% (Doorduyn et al. 2010). Of course many factors can influence source attribution studies using case-control data. Recently, individuals taking proton-pump inhibitors or having a chronic gastrointestinal disease have been shown to have an increased risk of campylobacteriosis (Doorduyn et al. 2010; Mughini Gras et al. 2012; Neal and Slack 1997; Tam et al. 2009), probably as a consequence of reduced gastric acidity allowing the survival of *Campylobacter* during passage through the stomach and/or disturbed gut function facilitating intestinal infection.

Specific immunity against *Campylobacter,* acquired as a result of prior exposure, is another very important confounder of case-control studies. Certainly, repeated exposure to pathogens, such as *Campylobacter*, may lead to sufficient immunity to provide protection against severe clinical illness (Swift and Hunter 2004). Such immunity can lead to individuals being protected from disease, even when colonized (Havelaar et al. 2009), and this has been proposed as an explanation of why, in some instances, the regular consumption of poultry meat (at home) is identified as a protective, rather than a risk factor (Friedman et al. 2004).

6.4.1.3 Source Attribution Based on Microbial Subtyping

As previously indicated *Campylobacter* are highly phenotypically and genotypically variable. This variability has been exploited to develop subtyping strategies with the aim of determining sources of human infection. However, for various reasons including the high plasticity of the *Campylobacter* genome, the lateral transfer

of genetic material among strains, the time delay to diagnosis and the poor recovery from putative sources, the direct tracking of strains from source to human has not been feasible. However, the development and widespread application of MLST, a genetic technique for investigating bacterial population structures, has recently informed source attribution studies. In its basic form MLST involves the sequencing of seven target housekeeping genes, but additional gene sequences, such as the *fla* gene, are often added. Analysis of the sequences produces a sequence pattern, based on allelic differences, for each strain. This pattern is then assigned to a sequence type (ST). Similar STs, sharing the same alleles at different loci, are considered to be evolutionarily related (i.e. share a common ancestor). Such STs are combined into clonal complexes (Dingle et al. 2001). Early studies of the evolutionary relationships within populations reported that some STs are preferentially associated with certain hosts, such as cattle or poultry. Thus, using complex statistical methods, the probable source attributions can be estimated by comparison of the distribution of STs recovered from diseased humans with those recovered from a range of animal, food and environmental sources (McCarthy et al. 2007; Mughini Gras et al. 2012; Mullner et al. 2009; Wilson et al. 2008; Sheppard et al. 2009; Smid et al. 2013; Strachan et al. 2009).

These MLST studies have provided the most convincing source attribution evidence, for campylobacteriosis, to date. Overall the data estimates that the majority (50–80%) of strains infecting humans come from the chicken reservoir, 20–30% from cattle, and the remainder from other reservoirs (sheep, pigs, and wild animals) (EFSA BIOHAZ 2010).

There is an apparent conflict between the importance of poultry as a source from case control studies (20–40%) and from MLST studies (50–80%). However, case-control studies only trace human cases back to the level of exposure (e.g. food items consumed, contact with animals, etc.), while MLST indicates the original host reservoir. It has been hypothesised that the difference reflects that *Campylobacter* strains may reach humans through pathways other than food, for example through environmental exposure (EFSA BIOHAZ 2010).

6.4.1.4 Intervention Studies

On the presumption that poultry is the major source of sporadic campylobacteriosis, there have been several incidents that have acted as "natural experiments", which have been investigated to determine the effect of reduced population exposure to *Campylobacter* in the food chain. For example, in 1999, contamination of animal feed with dioxin in Belgium resulted in a nation-wide withdrawal of broiler meat from the market, which was concomitant with a 40% decrease in campylobacteriosis, country-wide (Vellinga and van Loock 2002). Similarly, in 2003 in the Netherlands, an avian influenza outbreak led to a massive poultry cull, which was associated with a subsequent 30% decrease overall in campylobacteriosis (Friesema et al. 2012). This disease reduction varied between regions from 10 to 70%, with the largest fall reported in those laboratories serving areas where the flocks were

actually culled. This observation supports the hypothesis that there were important transmission routes other than the handling and consumption poultry meat (EFSA BIOHAZ 2010; Friesema et al. 2012). As yet, the transmission routes of such alternative pathways are unclear.

Recently there have been opportunities to study the outcomes of interventions targeted at the poultry production sector and/or to the poultry meat consumer, which resulted in reduced exposure to national populations in Iceland and New Zealand. Following these interventions, the number of reported campylobacteriosis cases fell by 72% in Iceland (Stern et al. 2003) and by 54% in New Zealand (Sears et al. 2011). Furthermore, in New Zealand there was a concurrent 74% reduction in the proportion of poultry-associated campylobacteriosis cases as determined by source attribution using MLST (Sears et al. 2011) and 13% decline in hospitalizations for Guillain-Barré syndrome (Baker et al. 2012).

6.4.1.5 Summary of Sources and Transmission Routes

Overall, the conclusion from the source attribution studies described above indicates that chickens are a major reservoir of those *Campylobacter* infecting humans. The importance of broilers as a source of infection is a reflection of the huge numbers of chicken produced and eaten worldwide, the level of colonization of these birds and the production processes involved. As a consequence, most public health effort to reduce campylobacteriosis has focussed on the control and prevention of *Campylobacter* in the poultry meat food chain (see Sect. 6.5). Nevertheless, the handling and consumption of chicken meat is only a part of the human exposure risk, and environmental exposure, through routes as yet unknown, is also important. However, all warm-blooded animals can act as host reservoirs of this infection and exposure to pets and livestock, and their products, can also provide a risk of human disease, although to a lesser extent than poultry. The complexity in exposure routes can generate overall confusing data. For example, children aged less than five years, living in urban areas, seem to be largely exposed to *Campylobacter* strains from chicken, while those living in rural areas are largely exposure to strains from cattle (Mughini Gras et al. 2012; Mullner et al. 2010b; Strachan et al. 2009).

Of course specific risk groups may exist, for example dog (and particularly puppy) owners are at increased risk of *Campylobacter* infection and isolation of identical *Campylobacter* strains in humans and their pets occurs significantly more often than expected by chance (Mughini Gras et al. 2013). However, the direction of any transmission route is indeterminable. Moreover, the association may reflect a common source of infection rather than a direct zoonosis.

Foreign travel is often described as a major risk factor (Eberhart-Phillips et al. 1997; Friedman et al. 2004; Gallay et al. 2008; Neal and Slack 1997; Neimann et al. 2003; Stafford et al. 2007). This increased risk is likely to reflect poorer hygiene in the preparation of food as well as the possible presence of "exotic" *Campylobacter* strains to which travellers had not been previously exposed (Havelaar et al. 2009; Mughini Gras et al. 2014). Moreover, such "exotic" strains, introduced by returning

travellers, might subsequently spread in to the domestic population, through limited person-to-person transmission (Mughini Gras et al. 2014).

6.5 *Campylobacter* in Poultry and Intervention in Primary Production

Given that the majority of the infecting strains in humans come from chicken, targeting *Campylobacter* in poultry production has become the preferred public health measure (EFSA BIOHAZ 2011). The poultry meat chain can be viewed as two distinct stages: chicken rearing and production (largely on-farm to entry to the slaughter house) and poultry meat processing (largely lairage to retail). Theoretically control measures focussed at the production stage will prevent up to 80 % of human cases, by preventing or reducing *Campylobacter* entering the food chain and the environment, while those measures targeted at the processing stage, can prevent only an estimated 42 % of cases (Mughini Gras et al. 2012). Control of *Campylobacter* in poultry, however, has proved to be very difficult.

Campylobacter colonization occurs in all types of commercially-produced poultry (e.g. broilers, turkeys, ducks) (Wagenaar et al. 2006), but clearly the focus for intervention is broilers as these provide the highest risk to humans. The prevention of *Campylobacter* in poultry is solely targeted at meat-producing birds. This is because vertical transmission is extremely rare, if at all (Callicott et al. 2006; Cox et al. 2012). Thus each new broiler production cycle starts with *Campylobacter*-free chicken. In "all-in/all-out" production systems, poultry houses are cleaned, disinfected and dried before the arrival of a new flock. Such preparation seems to be largely effective at preventing the carry-over of *Campylobacter* from previous flocks (Newell et al. 2011), nevertheless, birds subsequently become colonized with the bacteria. Experimental studies indicate that the ingestion of as few as 40 organisms can cause colonization (Cawthraw et al. 1996). Once the first bird has been colonized, then it sheds large numbers of bacteria in its faeces (up to 10^7 cfu per gram), and most, if not all, the other birds in the flock become colonized within a few days. Thus preventing the first bird becoming colonized seems to be a prerequisite for a "*Campylobacter*-negative" flock.

Broiler flocks are frequently exposed to the *Campylobacter* from their external environment throughout their limited lifespan (approximately 42 days for intensively-reared birds) (Newell et al. 2011). However, colonization does not usually become detectable until 2–3 weeks of age of the flock. This so-called "lag-phase" appears to be due to an inherent resistance in young chickens (Kalupahana et al. 2013) which is, at least in part, a result of maternal immunity (Cawthraw and Newell 2010).

By comparing *Campylobacter*-negative and -positive flocks, many risk factors have been identified, which increase the chance of flock positivity (Newell et al. 2011; Newell and Fearnley 2003; Katsma et al. 2007). One major risk factor is the age of broilers at slaughter, which is most likely associated with exposure to

external contamination over time and is a measure of the effectiveness of biosecurity. Other biosecurity-associated risk factors, such as multiple broilers houses on the farm, the presence of other livestock, partial depopulation (thinning), pets on the farm, etc., are also important. Nevertheless, no one biosecurity-related factor seems to predominate. Moreover, although improved biosecurity can decrease the risk of a flock becoming *Campylobacter*-positive, it seems that even strict biosecurity cannot guarantee a *Campylobacter*-free flock at the time of slaughter (Newell et al. 2011). In many countries the biosecurity challenge seems even more difficult in the summer months, when the prevalence of *Campylobacter*-positive flocks increases significantly in response to some temperature-related factors (Jore et al. 2010). Some of this seasonal increase may be associated with transmission by flies. In Denmark, this risk has been significantly reduced by the application of fly-screens around broiler house ventilation systems (Bahrndorff et al. 2013). This strategy is currently being investigated in other countries (http://www.camcon-eu.net/), but efficacy may be country dependent, i.e. related to weather conditions, as well as dependent on the biosecurity level already applied.

In Europe, improved biosecurity has been strongly recommended as the only currently available intervention measure to reduce flock positivity (EFSA BIOHAZ 2011). However, the appropriate targeting of biosecurity measures has proved very frustrating for the poultry industry. Anecdotal evidence suggests the compliance of farmers with general biosecurity measures is essential and such compliance would be even more important in summer months (EFSA BIOHAZ 2011). The challenge is likely to become even greater in the future given consumer-driven concerns for animal welfare leading to an increasing trend towards the production of slower-growing animals with a longer lifespan and with outdoor access. Under such conditions good biosecurity is impractical (Kalupahana et al. 2013).

It is widely recognized that biosecurity alone cannot produce *Campylobacter*-negative flocks and that complementary measures will be required to increase the resistance to, or reduce the colonization of, birds with the bacterium (EFSA BIOHAZ 2011). Despite several years of research, vaccination against *Campylobacter* is not yet reliably effective (de Zoete et al. 2007). Neither is it yet possible to influence the intestinal flora to generate a *Campylobacter*-resistant avian gut (Schneitz 2005). The use of bacteriophages and bacteriocins looks promising, but research to solve key issues in safety, efficacy and sustainability, is still needed (Lin 2009). The use of medium chain fatty acids has been reported to have at least some effect on *Campylobacter* colonization (Hermans et al. 2012; van Gerwe et al. 2010), but the results require validation in the field.

Thus it currently seems that improved biosecurity is the only credible measure available to decrease the prevalence of *Campylobacter*-positive flocks. However, as indicated above, the identification of specific and effective biosecurity approaches has proved very difficult. Thus, a wide range of high level biosecurity measures need to be consistently maintained throughout the life of intensively-reared flocks. This is often impractical, especially when *Campylobacter* colonization is asymptomatic, and therefore with no consequent economic loss to providing an incentive for the poultry farmer.

6.5.1 Post-Harvest Control Measures in Poultry

When *Campylobacter* colonization cannot be prevented at the farm level, post-harvest treatment becomes very important. Such treatments include the prevention of cross-contamination and the application of chemical or physical methods of decontamination in the slaughterhouse. The availability and effectiveness of such methods, with particular relevance to Europe, have been reviewed previously (EFSA BIOHAZ 2011).

Cross-contamination can be a significant problem associated with the huge through-put of carcasses (circa 13,000 per hour in many processing plants), slaughter line automation and the high concentrations of *Campylobacter* in caecal contents. Any leakage of faecal material, or rupture of the gut during evisceration, can lead to surface contamination of the meat. Interestingly, there are statistically significant differences, in the level of carcass contamination between slaughterhouses (EFSA 2010), suggesting that some processing plants are better than others at controlling this problem. However, the basis of these differences has yet to be determined (EFSA BIOHAZ 2011).

The decontamination of carcasses with chemicals is allowed in the US and currently practised using several chemicals, such as organic acids, quaternary ammonium compounds, acidified sodium chlorite and trisodium phosphate. Although the decontamination of carcasses with chemicals is allowed in the EU, specific approval is required and currently no chemic decontaminants have been approved for use on chicken carcasses.

Some physical treatments (e.g. ultraviolet, ultrasound, etc.) have been specifically applied to reduce *Campylobacter* on chicken carcasses, but their effectiveness is usually limited to a reduction of only 1–2 $\log_{10}$. Highly effective irradiation procedures are poorly accepted by consumers and difficult to implement under high through-put conditions. The freezing of carcasses from positive flocks can reduce *Campylobacter* concentrations by 2–3 $\log_{10}$ and this strategy has been effectively used in Iceland as part of a programme to reduce human campylobacteriosis (Stern et al. 2003). However, from both the logistic and the economic (i.e. the preference of consumers for fresh meat) view points, such a strategy would be difficult to implement, especially in those countries with high prevalence of *Campylobacter*-positive flocks (Havelaar et al. 2007).

6.6 Interventions and Public Health Impact

The potential public health impact of intervention measures in the poultry production chain are clearly demonstrated in two successful examples from Iceland and New Zealand (see Sect. 4.1.3).

In Iceland, multiple-level measures were implemented (including producer and consumer education, enhanced biosecurity, changes in poultry processing and the

identification and freezing of products from *Campylobacter*-positive flocks) in response to a sharp increase in campylobacteriosis in 1999 (Tustin et al. 2011). As mentioned before, this spectrum of measures resulted in a 72% reduction in the incidence of campylobacteriosis (Stern et al. 2003). Of all these measures, the freezing of contaminated products is considered the most important (Tustin et al. 2011). In New Zealand, a 54% reduction in the incidence of campylobacteriosis was similarly achieved as a consequence of the introduction of a range of voluntary and regulatory measures (Baker et al. 2012; Mullner et al. 2010a; Sears et al. 2011). Despite this success, New Zealand still has the highest incidence of campylobacteriosis among reporting countries worldwide.

Given these successes, it is tempting to extrapolate those approaches implemented in New Zealand and Iceland to other countries. However, in both cases specific conditions prevailed and, therefore, success in disease reduction in other countries may not be predictable.

6.7 *Campylobacter* in Poultry—The Future

Given that *Campylobacter* is a part of the normal gut flora of birds (and is a highly successful coloniser of that site), the increasing consumer demand worldwide for low cost chicken meat (while expecting higher animal welfare during production) and the steady reduction in human populations with acquired immunity (either due to lack of natural exposure or to increased susceptibility through age, disease or medication), campylobacteriosis will remain a major foodborne pathogen in most countries (Bouwknegt et al. 2013; Swart et al. 2012). At the moment the reliable production of *Campylobacter*-negative flocks, through best-practice biosecurity alone, seems unlikely. In the future, effective vaccines and/or other complementary measures should be achievable outcomes of current research. Although, such measures may not totally eliminate colonization, significant reductions in colonization levels may be feasible. In this case risk assessment studies show that a significant reduction in public health risk can still be achieved (Nauta and Havelaar 2008). Once chicken is no longer a major source of *Campylobacter* then the importance of other animal reservoirs and transmission routes can be identified and tackled.

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Chapter 7
The zoonotic agent *Salmonella*

Wolfgang Rabsch, Angelika Fruth, Sandra Simon, Istvan Szabo and Burkhard Malorny

Abstract Salmonellosis is one of the most common foodborne diseases and is caused by different serovars of the species Salmonella enterica. More than 2600 different Salmonella serovars have been identified to date. Salmonella is an ubiquitous and quite tenacious bacterium that can survive for years in dry environments by transition into a dormant state or biofilm formation. S. enterica serovars are often associated with animal reservoirs and outbreak investigations reveal that infections in humans constantly result from animal to human transmission. The food vehicles most commonly involved are chicken and poultry, pork, beef, eggs, and seafood. Meat or egg products may contain bacteria either because animals had been infected or because fecal contamination occurred during processing. In the last years, Salmonella infections have been increasingly linked to the consumption of vegetables and fruits. Plant products can be contaminated with Salmonella in the field through application of manure, fertilizers or contaminated water. Also reptiles, rodents and insects can contaminate the herbs and spices in the country of origin before their worldwide distribution. The serovars S. Enteritidis and S. Typhimurium are the two most important serovars transmitted from animals to humans in Germany. The classical discrimination by phage typing combined with different molecular methods, e. g. ribotyping or macrorestriction analysis help us to link patients isolates with a common source and therefore to identify and trace back an outbreak back to its origin. This chapter focuses mainly on food-borne Salmonella infections. However, since "Reptile-Exotic-Pet-Associated-Salmonellosis" (REPAS) is of increasing concern we summarize our current knowledge of REPAS.

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7.1 The Zoonotic Agent *Salmonella*

Salmonella (*S.*) *enterica* serovars form a group of pathogens that differ widely in their host range within mammals, birds and reptiles. They can differ substantially in clinical manifestations, ranging from an asymptomatic state to severe illness (Jones et al. 2008). Serovars can be host-restricted (e.g *S.* Typhi in humans), host-adapted (e.g. *S. choleraesuis* in pigs and infrequently in humans) and broad range infecting diverse avian and mammalian hosts with a wide range of diseases. Currently, the traditional *Salmonella* serotyping scheme according to White-Kauffmann-Le Minor is accepted worldwide as the "gold standard" for the classification of salmonellae below the subspecies level and is widely used in surveillance of the pathogen (Grimont and Weill 2007). The use of serotyping within *Salmonella* as a typing method is so widely accepted that governmental agencies have formulated guidelines intended to reduce human salmonellosis by identifying the common serovars Typhimurium and Enteritidis (Achtman et al. 2012; Grimont and Weill 2007). The most common vehicles of transmission are meat, meat products, eggs and egg products that contain *Salmonella* serovars because animals are infected or because fecal contamination occurs during processing (ECDC 2013a). The majority of human cases are caused by only a few non-typhoidal serovars. In 2012, approximately 20,000 cases of non-typhoidal salmonellosis are reported in Germany (http://www3.rki.de//survstat). In 1995 the dominance of only a few serovars is even more pronounced in Germany, where *S.* Enteritidis (61 %) and *S.* Typhimurium (23 %) accounted for more than 80 % of human isolates reported to the NRC at the Robert Koch Institute (Rabsch et al. 2001). In 2012 this percentage for both serovars were reduced to 62 %, each approx. 31 %. Other serovars of different vehicles were found in outbreaks and also serovar analysis showed that the spectrum of single cases in children changed. In this article we focus on the prevalence of *Salmonella enterica* in animal food and humans and its change of serovars and subtypes over up to two decades.

7.2 *Salmonella* in Animal and Food

Often, animals are asymptomatic carriers of *Salmonella* and carry the bacteria in the intestine. They can shed relatively large numbers of salmonellae in the faeces over a long period. Consequently, the control of *Salmonella* in food producing animals is imperative to avoid the transmission along the food chain to humans. Usually, in developed countries livestock is regularly monitored in order to estimate the prevalence of the pathogen and serovars and to perform control measures. Livestock can be infected with *Salmonella* by vertical transmission through infected parents or by horizontal transmission between animals, faeces, animal feed, or vehicles such as rodents or birds (Davies et al. 2004). They are also widely distributed in cold-blood animals, especially in reptiles. Studies on crocodile meat have documented the common presence of salmonellae in both fresh chilled and frozen meat.

Furthermore, consumption of raw blood, viscera and raw meat as well as cooked meat of the soft-shelled terrapin or consumption of dried rattlesnake meat can cause human salmonellosis (Magnino et al. 2009; Vandeplas et al. 2010).

The incidence of *Salmonella* in livestock depends on several factors such as the conditions for intensive animal husbandry, hygiene measures and climate conditions. The incidence of *Salmonella* in Northern countries is often lower than in those located in warmer climate zones. Furthermore, *Salmonella* cases are subject to a seasonal variation with higher rates in the summer and autumn, and a decline in the winter months (ECDC 2011). Contaminated animal feed and wild animals (e.g. birds) have been recognized as important entry sites into the food chain in farm livestock (Skov et al. 2008). Another source of contamination is the slaughter process of the animals (Bolton et al. 2003).

7.2.1 Salmonella *in Poultry and Poultry Products*

Since the mid-1980s *S.* Enteritidis has established itself as the most frequently isolated serovar from poultry, especially chickens, in many parts of the world possibly by filling the ecological niche vacated by *S.* Gallinarum and *S.* Pullorum (Baumler et al. 2000); Ward et al. 2000). In consequence, *S.* Enteritidis is attaining major public health significance because it is dominantly infecting humans, transmitted by food products (ECDC 2012). In Europe, baseline surveys on the prevalence of *Salmonella* showed that 30.7% of commercial large-scale laying hen holdings and 23.7% of the broiler flocks of *Gallus gallus* were *Salmonella* positive (Efsa 2007a). *S.* Enteritidis (37.4%) was most frequently isolated followed by *S.* Infantis (20.4%). However, the variation in prevalence and predominant serovars was very large between European countries. For example, the prevalence of *S* Enteritidis in laying hen holdings ranged from 0 to 79.5%. In turkey a baseline survey performed in 2006/2007 showed 13.6% *Salmonella* positive breeding flocks and 30.7% positive fattening flocks in Europe. Control programmes in poultry populations became mandatory in the EU with aim to reduce the prevalence of certain serotypes in breeding flocks of *Gallus gallus* (Anonymous 2005), in laying hens of *Gallus gallus* (Anonymous 2006, 2011a) and in turkeys (Anonymous 2006, 2008, 2011a, 2012). Since then the rates dropped in the EU to 4.2% in 2011 for laying hen flocks and 1.9% for breeding hens flocks (ECDC 2013a). Broiler flocks were 3.2% *Salmonella* positive. Similar baseline studies and interventions were performed in the US broiler chickens with prevalence of 24% in 1996 decreasing to 6.5% in 2011 (Service USDOaFSI 2012).

Eggs and egg products have been reported frequently implicated in human salmonellosis (Hald et al. 2004). However, the finding of *Salmonella* in eggs in the EU decreased continuously. In 2011 fresh eggs, raw egg at processing, and at retail were in average 0.1% positive with highest level in Spain (1.8%) (ECDC 2013a). Since 2007, when the *Salmonella* control programmes for flocks of laying hens and broilers were implemented, this represents a reduction by half. *S.* Enteritidis was almost always isolated from table eggs.

Salmonella prevalence in fresh broiler meat at different levels of production was 5.9 % on average in the EU in 2011. This was a slightly increase compared to 2010 (4.8 %), 2009 (5.3 %) and 2008 (4.8 %). *S.* Infantis and *S.* Enteritidis were by far the most frequently isolated serovars followed by *S.* Typhimurium and *S.* Mbandaka (ECDC 2013a). However, the number of *S.* Enteritidis isolations decline over the past years. 30.9 % of ground chicken (minced meat) and 12.3 % of ground turkey were *Salmonella* positive in the US in 2011 (Service USDOaFSI 2012).

7.2.2 Salmonella *in Pork and Pork Products*

In pigs, the host-adapted serovar Choleraesuis was predominant in the 1950s and 1960s in Europe (Sojka et al. 1977). After that it decreased dramatically and is today very rarely isolated in Europe. In the United States this serovar is still among the top three of clinical cases in pigs (Foley et al. 2008). Meanwhile *S.* Typhimurium is one of the most common serovars isolated from pigs, in both Europe and the United States (ECDC 2013; Foley et al. 2008). Two European baseline studies in 2006/2007 and 2008 showed that *S.* Typhimurium is most commonly isolated in slaughter pigs and secondly most commonly in breeding pigs (Efsa 2009; 2008b). The overall *Salmonella* prevalence was with 31.8 % significantly higher in breeding pigs than in slaughter pigs (10.3 %). The second predominant serovar in pigs is *S.* Derby. In slaughter pigs and pork it ranks in the top two in Europe (ECDC 2013). Similarly, *S.* Derby was most frequently isolated in non-clinical cases and secondly most frequently isolated in clinical cases of pigs in the United States (Foley et al. 2008). In the last decade a monophasic variant of *S.* Typhimurium with seroformula 4,[5],12:i:—has been established and disseminated worldwide especially in pigs and pork (Hauser et al. 2010; Hopkins et al. 2010; Switt et al. 2009). The monophasic serovar started to emerge in Europe in the mid-1990s beginning from Spain (Echeita et al. 1999). In the EU *S.* Typhimurium 4,[5],12:i:—is meanwhile the third most frequently isolated serovar from pigs and pork.

Pork has been identified as another important source for human salmonellosis (Efsa 2008a; Pires 2011). In Europe the rate of *Salmonella* positive tested samples in fresh pig meat in 2011 was 0.7 %, ranging between 0 and 6.8 % in Belgium (ECDC 2013a). The serovar distribution is similar to that in pigs at primary production, reflecting the transmission of the serovars to humans by pork.

7.2.3 Salmonella *in Cattle and Bovine Products*

In cattle, the prevalence of *Salmonella* is generally significantly lower than in poultry or pigs. A rate of 0.9 % on animal level was detected (ECDC 2012). Bovine salmonellosis is associated primarily with *S.* Dublin and *S.* Typhimurium (Wallis and Barrow 2005). According to the low prevalence in cattle *Salmonella* in beef is rarely

found in the EU (ECDC 2013a). The average prevalence in 2010 and 2009 was 0.2%. Cattle in the Unites States was in 2011 0.8% *Salmonella* positive, slightly higher than in 2010 (0.5% positive). Ground beef was analysed 2.4% positive for *Salmonella*.

7.2.4 Salmonella *in Fresh Produce, Herbs, Spices*

Salmonella infections have been increasingly linked to the consumption of vegetables and fruit (Lynch et al. 2009; Olaimat 2012). Produce can be contaminated with *Salmonella* in the field through the application of manure, fertilizers, pesticides and irrigation with contaminated water. Insects and animals may be another source of *Salmonella* entry. Post-harvest cross-contamination can occur during washing, packaging and transport (Beuchat 2002; Park et al. 2012). In monitoring samples *Salmonella* is detected in vegetables, fruits, spices and herbs at very low level (0.6% in 2010) in the EU (ECDC 2012). Different studies on the prevalence of *Salmonella* in spices and herbs ranged from 0 to 5.6% worldwide (Zweifel and Stephan 2012). However, in the past decade *Salmonella* outbreaks due to fresh produce and spices have been increasingly reported (Table 7.1). Lettuce, tomatoes, alfalfa sprouts, cantaloupes, orange juice, and pepper were repeatedly identified as potential infection sources for humans. Worldwide trade, large-scale productions and new consumption behaviour (ready-to-eat products, increasing demand of fresh produce) contribute to the rising infection risk. Epidemic outbreaks of foodborne infections are not only a threat to public health but also erode consumer confidence in the causal food product and thus, impact the economic viability of the industry.

Mainly fruits and vegetables are contaminated on the surface possibly leading to the colonization of the plant but it has been shown that *Salmonella* can also invade plant tissues through stomata (Park et al. 2012). Consequently, cleaning and disinfection can be ineffective for removal the pathogen before consumption. The identification of routes of plant contamination by *Salmonella* is crucial to the design of intervention strategies to prevent contamination from taking place (Brandl and Sundin 2013). The study by Poza-Carrion et al. (Poza-Carrion et al. 2013) reveals by quantitative analysis of epifluorescence micrographs that established epiphytic bacteria such as *Pseudomonas fluorescens* and *Erwinia herbicola* (*Pantoea agglomerans*) influence the ability of *Salmonella enterica* to persist on plants. These bacterial species promoted the survival to desiccation conditions of *S. enterica* cells immigrating in their close vicinity on lettuce and cilantro leaves, potentially by modulating the immediate physicochemical environment at microsites. Resistance of *Salmonella* against desiccation promotes extended period of time survival in dried products such as spices because of its low water activity (Keller et al. 2013). Large outbreaks caused by spices and herbs were reported in the past years caused by black and red pepper, white pepper, fresh basil and aniseed-containing herbal tea (Zweifel and Stephan 2012).

Table 7.1 *Salmonella* outbreaks related to consumption of contaminated vegetables, fruits or seeds since 1999

Year	Serovar	Country	Vegetable/fruit/seeds	Reference
1999	*S.* Muenchen	Wisconsin, USA	Alfalfa sprout	Proctor et al. (2001)
1999	*S.* Mbandaka	USA	Alfalfa sprout	Gill et al. (2003)
2000	*S.* Typhimurium DT204b	Iceland, U.K., Netherlands, Germany	Lettuce	Crook et al. (2003)
2000	*S.* Typhimurium DT104	U.K.	Lettuce	Horby et al. (2003)
2000	*S.* Enteritidis PT4b	Netherlands	Bean sprouts	Van Duynhoven et al. (2002)
2001	*S.* Enteritidis	Canada	Mung bean sprouts	Honish and Nguyen (2001)
2001	*S.* Baildon	USA	Tomatoes	De Jong et al. (2005)
2001	*S.* Kottbus	USA, California	Sprout seeds	Winthrop et al. (2003)
2002/2003	*S.* Agona PT2	Germany	Anisseed tea	Koch et al. (2005); Rabsch et al. (2005)
2003	*S.* Newport	USA	Mangoes	Sivapalasingam et al. (2003)
2004	*S.* Thompson	Norway	Ruccola	Nygard et al. (2008)
2004	*S.* Newport	England, Scotland, Isle of Man, Ireland	Lettuce	Irvine et al. (2009)
2004	*S.* Senftenberg	Serbia	Fennel seed tea	Ilic et al. (2010)
2004	*S.* Braenderup	USA	Tomatoes	Gupta et al. (2007)
2004	*S.* Braenderup, *S.* Javiana	USA	Tomatoes	Cdc (2005)
2005	*S.* Newport	USA	Tomatoes	Greene et al. (2008)
2005	*S.* Enteritidis	Canada	Mung bean sprouts	Rohekar et al. (2008)
2005	*S.* Typhimurium, *S.* Saintpaul	USA	Orange juice	Jain et al. (2009)
2006	*S.* Enteritidis NST3	Sweden	Almonds	Ledet Muller et al. (2007)
2006	*S.* Saintpaul	Australia	Cantaloupe	Munnoch et al. (2009)
2006	*S.* Typhimurium	USA	Tomatoes	Behravesh et al. (2012)
2007	*S.* Senftenberg	U.K., Denmark, Netherlands, USA	Basil	Pezzoli et al. (2008)
2007	*S.* Weltvreden	Norway, Denmark, Finland	Alfalfa sprouts	Emberland et al. (2007)
2007	*S.* Stanley	Sweden	Alfalfa sprouts	Werner et al. (2007)
2007	*S.* Paratyphi B var. Java	Sweden, U.K., Denmark	Spinach	Denny et al. (2007)
2008	*S.* Newport, *S.* Reading	Finland	Iceberg lettuce	Lienemann et al. (2011)

Table 7.1 (continued)

Year	Serovar	Country	Vegetable/fruit/seeds	Reference
2008	*S.* Panama	Netherlands	Fresh fruit juice	Noel et al. (2010)
2009	*S.* Bovismorbificans	Finland	Alfalfa sprouts	Rimhanen-Finne et al. (2011)
2009/2010	*S.* Montevideo	USA, 44 states	Black and red pepper	Gieraltowski et al. (2013)
2010	*S.* Bareilly	U.K.	Bean sprouts	Cleary et al. (2010)
2010	*S.* Paratyphi B var. Java PT3b var 9	U.K.	Salad vegetables	Gobin et al. (2011)
2010	*S.* Montevideo	Germany	Food supplement	Stocker et al. (2011)
2011	S. Strathcona	Denmark, Germany, Austria, Italy	Datterino-tomatoes	Anonymous (2011b)
2011	*S.* Newport	England, Wales, Northern Ireland, Germany, Scotland, Ireland	Watermelon	Byrne et al. (2014)
2011	*S.* Newport	Germany, Netherlands	Mung bean sprouts	Bayer et al. (2014)

7.2.5 Salmonella *in Seafood*

Salmonella in fish and shellfish is rarely detected in the EU and only reported from single Member States. However, in other countries, especially India and African countries it may reach levels up to 10–15 % (Ia 2012). Outbreaks occur occasionally and were reported from the United States and Japan (Li et al. 2013). Shrimp can be a major source of *Salmonella*. Prevalence of *Salmonella* in fresh shrimp is at processing plant 10–14 % and was in the United States in 2001 the seafood product with most violations for *Salmonella* (Wan Norhana et al. 2010). Mostly *S.* Weltevreden, *S.* Typhimurium and *S.* Senftenberg were isolated from shrimp (Wan Norhana et al. 2010). The occurrence of *Salmonella* in fish and shellfish is often a sign of low control measurement at primary production level and poor standards of hygiene and sanitation during processing, handling, and transport (Ia 2012).

7.3 Human Salmonellosis

7.3.1 Disease Symptoms, Pathogenesis and Burden of Disease

The gram-negative bacterium *Salmonella enterica* causes a wide range of gastrointestinal and systemic diseases. While non-typhoidal strains usually remain restricted to the gastro-intestinal tract, typhoidal *Salmonella* serovars then disseminate from

the gastro-intestinal tract to mesenteric lymph nodes and colonize systemic sites, like the liver and spleen (Agbor and McCormick 2011; Wain et al. 2013). Often host-adapted *Salmonella enterica* serovars are associated with severe systemic diseases (e.g. *S*. Typhi in man). Non-typhoidal *Salmonella enterica* serovars with the most common clinical isolates of *S*. Typhimurium and *S*. Enteritidis are one of the major causes of food-borne diseases. These serovars commonly cause a self-limiting gastroenteritis characterized by diarrhea, abdominal pain and fever (Velge et al. 2012). Dysfunction of the mucosal barrier can generate life threating situations, especially in immunocompromised patients and in persons at risk such as small children and elderly (Santos et al. 2009). Severe infections, like bacteremia, meningitis, osteomyelitis and broncho-pulmonary salmonellosis have been described.

Infections with *Salmonella* lead to an acute intestinal inflammation in human and animal hosts. The two main components in *Salmonella* infections in the intestine are adherence and subsequent invasion. Adhesins and fimbriae are necessary to mediate attachment to epithelial cells in the gut. Crucial to *Salmonella* virulence is its ability to invade and break through the intestinal mucosal barrier.

Salmonella species have evolved ingenious virulence mechanisms to manipulate host cell functions to their own benefit (Agbor and McCormick 2011).Two type III secretion systems (T3SS) encoded within pathogenicity islands SPI-1 (Mills et al. 1995) and SPI-2 (Shea et al. 1996) are responsible for the delivery of a series of bacterial effectors into host cells with the intention to reprogram eukaryotic cell functions. While the T3SS apparatus is highly conserved, the translocated effectors are unique proteins with very specialized functions critical to virulence. Moreover, considerable evidence indicates that individual effectors secreted by the T3SS are modular proteins composed of functionally distinct domains that may act in different stages of the infection process (Agbor and McCormick 2011).

SPI1 plays a fundamental role in the early stages of mammalian infection through triggering Cdc42- and Rac1-mediated remodelling of the actin cytoskeleton of the host cell, and leading to internalization of the bacteria and subsequent penetration of the ileal mucosal lining SPI1 also has a pro-inflammatory potential through its ability to activate JNK- and p38-dependent nuclear responses and the release of IL-1b. Furthermore, selected SPI1-associated effector proteins cause a very strong pro-apoptotic effect in monocytic cells (Hautefort et al. 2008; Kaiser and Hardt 2011).

However, it is unclear whether this system stimulates inflammatory responsesdirectly, or whether it works in conjunction with surface (Bruno et al. 2009).

Assessing the burden of foodborne disease is complex because many different pathogens can be transmitted by food, leading to different health outcomes (Havelaar et al. 2012). Burden-of-illness estimates combining indicators of mortality and morbidity into a single measure are increasingly used to inform public health politicians (Haagsma et al. 2008; Majowicz et al. 2010).

Salmonellosis is representing one fifth of all food-borne infections in human. Worldwide it has a high economical and public health burden of disease. In Germany salmonellosis ranked fourth among six major enteric pathogens considering to YPLL (years of potential life lost) with the highest mortality rate observed, reasoned by no reported death in the group of children (Murray and Lopez 1996; Tauxe et al. 2010; Werber et al. 2013).

Surveillance programs that detect *Salmonella* contaminations in a timely manner in the entire food chain (animal feed, living animals, slaughterhouses, retail sector, and restaurants) together with sanitary measures are essential for detecting and preventing human *Salmonella* infections (Newell et al. 2010; Wattiau et al. 2011).

7.3.2 Salmonellosis Worldwide

The number of salmonellosis cases in humans in 2011 in the European Union decreased by 5.4 % compared with 2010 and by 37.9 % compared with 2007. A statistically significant decreasing trend was observed over the period from 2008 to 2011. In total, 95,548 confirmed human cases were reported by the 27 European Member States in 2011 with notification rate of 20.7 cases per 100,000 population (ECDC 2013). It is assumed that the observed reduction in salmonellosis cases is mainly a result of the successful *Salmonella* control programmes in poultry populations. Most Member States met their *Salmonella* reduction targets for poultry, and *Salmonella* is declining in these animal populations. As in previous years, *S.* Enteritidis and *S.* Typhimurium were the most frequently reported serovars from human cases, 44.4 % and 24.9 %, respectively. As a result of the harmonized reporting and also several large outbreaks, monophasic *S.* Typhimurium 1,4,[5],12:i:—was with a rate of 4.7 % the third most commonly reported serovar in the EU (ECDC 2013). Human *S.* Enteritidis cases are most commonly associated with the consumption of contaminated eggs and poultry meat, while *S.* Typhimurium cases are mostly associated with the consumption of contaminated pork.

Data collected between 2001 and 2007 from the World Health Organization Global Foodborne Infections Network including 37 countries showed that *S.* Enteritidis (43.5 %) and *S.* Typhimurium (17.1 %) were also the most common serovars isolated from humans worldwide (Hendriksen et al. 2011). In developing countries, the proportion of *S.* Enteritidis decreased from 73.9 % in 2001 to 55 % in 2007 and in developed countries the proportion of *S.* Typhimurium decreased from 26.4 to 18.8 %. *S.* Newport (3.5 %), *S.* Infantis (1.8 %), *S.* Virchow (1.5 %), *S.* Hadar (1.5 %) and *S.* Agona (0.8 %) were also frequently isolated (Hendriksen et al. 2011). However, regional differences in the prevalence of *Salmonella* serovars have been observed. For example, *S.* Heidelberg was much more frequently reported in North America (top 4) than in Europe (top 9) and Latin America (top 19) and did not occur in the African or Asian region among the 20 most common serovars.

7.3.3 S. Typhimurium *and S.* Enteritidis *in Change of Time in Germany*

Salmonella epidemiology has changed fundamentally since underdeveloped countries have entered international trade and export food-supplies that in the 1950s, due to less stringent controls, are already contaminated either in the countries of origin

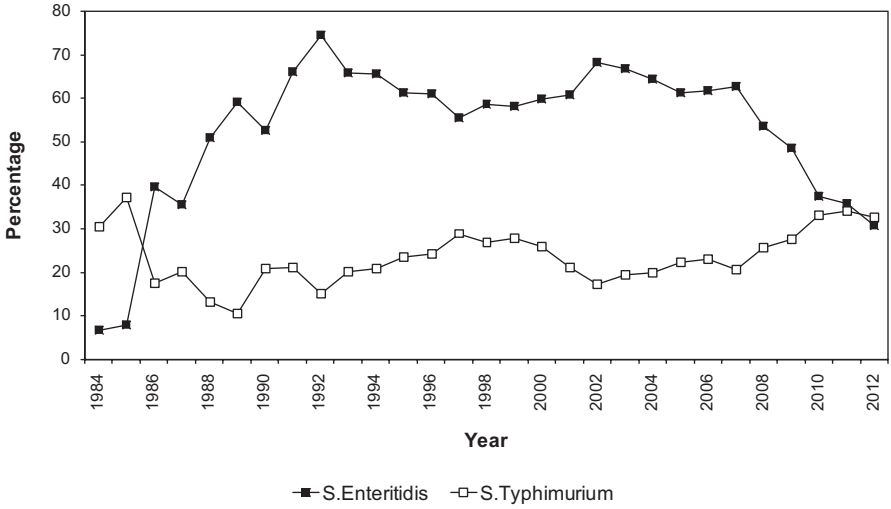

Fig. 7.1 Occurrence of *S.* Enteritidis und *S.* Typhimurium from human in Germany 1984–2012

or in transit. This is shown by numerous case histories of food-poisoning. During this period *Salmonella* spp. other than *S.* Typhimurium and *S.* Enteritidis, e.g. *S.* Bareilly, caused outbreaks (June 1953 with 10,000 cases) and were frequently isolated (Winkle and Rohde 1979). In the 1960s and 1970s *S.* Typhimurium had been the most important serovar in humans in both German states. During the mid-1980s it had been replaced from this top position by *S.* Enteritidis (Rabsch et al. 2013). A case-control study performed in Lower Saxony from 2001 to 2011 describes the successive decrease of *S.* Enteritidis and both serovars, Enteritidis and Typhimurium reaching equal levels in 2009 (Ziehm et al. 2013). In 2012 the serovar *S.* Typhimurium including its monophasic variant became dominant throughout Germany again, as it was before 1985. The decreasing percentage of *S.* Enteritidis and the increasing part of *S.* Typhimurium in the Twenty-first century is described in Fig. 7.1.

7.3.4 Microevolution in S. Typhimurium

Pathogen transformation in terms of *Salmonella* describes alterations within a serovar. Like other enterobacterial species, *Salmonella* can exchange genetic material via horizontal gene transfer. The acquisition or loss of plasmids, prophages and other genetic elements might result in changes of phage type, resistance pattern or virulence properties (Rabsch et al. 2011). Deletions of phase-two flagella related genes are responsible for the inability to express the second flagellar (H2-) antigen.

Alterations within the dominant serovar Typhimurium have been reported regularly since the 1960s. Under selective pressure caused by the intensive use of an-

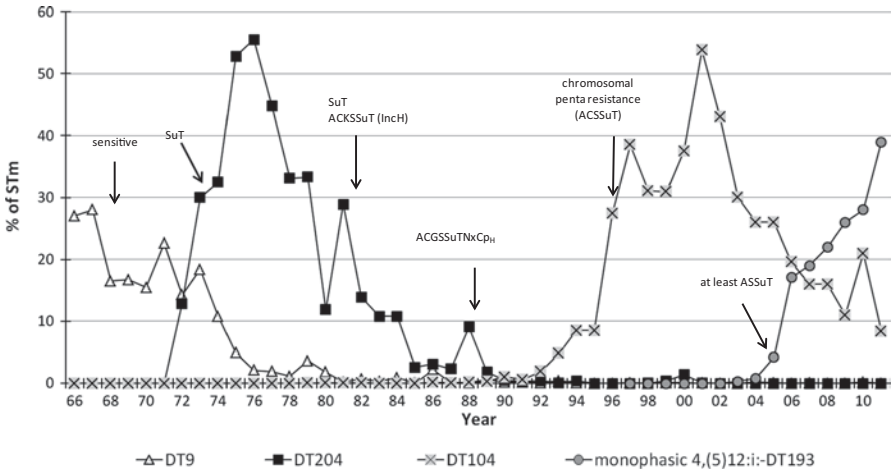

Fig. 7.2 Dominance of *S.* Typhimurium phage types DT9, DT204, DT104 and monophasic DT193 strains from human (Germany 1966–2009, NRC)

tibiotics in veterinary and human medicine multidrug-resistant strains emerged and spread rapidly. Notably, the dominant phage type of *S.* Typhimurium seems to change periodically about every fifteen years in Germany, when a new type arises and the so far dominant one almost disappears (Fig. 7.2). Sensitive strains of phage type DT9 were most important until 1972, and then sulphonamide and tetracycline resistant (later multiresistant) strains of DT204 emerged and were dominant until the late 1980s. Afterwards the phage type DT204 variant was replaced by penta-resistant phage type DT104 (Rabsch et al. 2001). Phage type DT104 strains accumulated resistance to nalidixic acid (Nx) and were responsible for an epidemiological increase in 2010 (Fig. 7.2). Since 2006 a monophasic variant of *S.* Typhimurium lacking a region encoding the phase-two related flagella genes is disseminating rapidly in Europe and Germany. The vast majority of these strains belong to phage type DT193 and exhibit at least tetra drug-resistance (see Sect. 2.5). Recently, ESBL producing mono- and biphasic *S.* Typhimurium have been described for Germany (Eller et al. 2013).

7.3.5 An Emerging Monophasic Variant of S. Typhimurium

Like most *Salmonella enterica*, *S.* Typhimurium (seroformula 4,[5],12:i:1,2) is able to express two flagella antigens encoded by the *fliC* (encoding H:I flagellin) and *fljB* (encoding H:1,2 flagellin) genes, respectively. Monophasic isolates expressing either H:i or H:1,2 have always been sporadically detected but never attracted constant attention. During the last decade, however, a monophasic *Salmonella* Typhimurium variant (seroformula 4,[5],12:i:-) not expressing the second phase flagella antigen has become one of the predominant agents causing foodborne infections in humans

in Europe. The first reports of emerging 4,[5],12:i:- strains came from Spain (Echeita et al. 1999) and the United States (Agasan et al. 2002). In Germany this serovar attracted attention in 2006 due to its rapid increase (Trupschuch et al. 2010). As shown in Fig. 7.2 monophasic variant is obviously replacing the biphasic serovar. The major infection source is pork (Hauser et al. 2010). In Germany the dominant phage type within the monophasic variant is DT193 (ca. 75%), followed by DT120 (ca. 15%). Notably, only 0.8% of the monophasic isolates from human origin were susceptible. The dominant pattern comprises tetra-resistance including ampicillin, streptomycin, sulfamerazine and tetracycline (ASSuT). The emergence and spread of monophasic *S.* Typhimurium has been also reported from various European countries (Boneet al. 2010; Dionisi et al. 2009; Hopkins et al. 2012; Lucarelli et al. 2010; Mossong et al. 2007; Wasyl and Hoszowski 2012). As a result of the rapid increase in the prevalence of human infections in Europe over a short period of time (Hopkins et al. 2010) and of the fast expansion in livestock populations, such as pigs (Hauser et al. 2010), the monophasic variant is currently considered as an emerging epidemic serovar (Barco et al. 2012). Based on phage typing, PFGE, MLST and MLVA analyses a number of variants were reported among different countries although they appear less diverse than in *S.* Typhimurium (Barco et al. 2012; Hopkins et al. 2010). Based on the extent of deleted genes encoding the phase-two structural flagellin antigen (*fljAB*, *hin*) and surrounding genes two clonal lineages were reported: one from Spain (hin^-, $iroB^-$) and one from the US (hin^+, $iroB^+$) (Garaizar et al. 2002; Soyer et al. 2009). Yet another lineage is currently characterized by hin^- but $iroB^+$ (Garcia et al. 2013). Bugarel et al. described a tenfold increase of isolations of monophasic *S.* Typhimurium in France from 2005 to 2010.They even found eight different patterns among the monophasic strains with respect to the presence or absence of seven marker genes including the *fljAB* operon (but not *iroB*) supporting the evolvement of several independent lineages (Bugarel et al. 2012). Characteristic for the European (DT193) clone defined by Garcia et al. (2013) is a novel 18.4 kb genomic island located adjacent to the *thrW* tRNA locus. Homologous sequences for several parts of the island were found in *E. coli* and *Shigella* genomes indicating a stepwise acquisition of the element. Alignment analyses revealed mainly phage-related gene products (Trupschuch et al. 2010). Although the relevance of the novel island for bacterial fitness or pathogenicity in the emerging monophasic variant of *S.* Typhimurium is not yet understood it seems to improve the resistance towards lytic phages. Moreover, it had been shown that the island is able to form a circular intermediate and can be conjugationally transferred to appropriate recipient strains, what might be an explanation for its occasional detection in biphasic *S.* Typhimurium strains (Simon 2013).

7.3.6 Salmonella Infections in Reptiles Including Reptile Exotic Pet Associated Salmonellosis (REPAS)

Although the main focus of this chapter is on food-borne *Salmonella* infections, it should not be forgotten that each year infections are also acquired through direct or indirect animal contact in homes, veterinary clinics, zoological gardens, farm

environments or other public, professional or private settings. Clinically affected animals may exhibit a higher prevalence of shedding than apparently healthy animals, but both can shed *Salmonella* over long periods of time. The public health risk varies by mammals, birds and reptile species, age group, husbandry practice and health status (Hoelzer 2011). Numerous reports exist on the prevalence of *Salmonella* sp. in reptiles, and many different serovars have been described, usually rarely isolated from humans or livestock. However, studies on the prevalence of *Salmonella* sp. in captive reptiles do not provide a uniform impression. In contrast to early reports, more recent publications demonstrate a higher prevalence in lizards in comparison to tortoises and turtles: in a study in captive lizards, *Salmonella* spp. were isolated from 76 % of all cloacal swabs, including 44 serovars (Pasmans et al. 2005). A study conducted in captive reptiles in New Zealand indicated a prevalence of 11.4 % positive by cloacal swabs, with agamid lizards being most frequently affected (41 %, 74 % in bearded dragons) (*Pogona vitticeps*) (Kikillus et al. 2011). Also Geue and Löschner found, that captive snakes and lizards were significantly higher affected (Geue and Loschner 2002). They demonstrated a significantly higher prevalence for *Salmonella* in collections of purchased reptiles and reptiles bought in pet shops (89 %). This finding is supported by a study in Japanese pet shops with a 74 % prevalence of *Salmonella* spp. and 112 identified isolates (Nakadai et al. 2005). Examining 504 reptiles in Australia, Scheelings et al. found a significant difference in the prevalence of *Salmonella*, with 47 % of all captive reptiles, but only 14 % of the wild reptiles being positive (Scheelings et al. 2011). In reptiles, a natural interaction seems to exist between *Salmonella* and the reptile host, with these bacteria being a normal part of microbial flora (Franco et al. 2011). Therefore clinical disease due to salmonellosis is rare but may occur including dermatitis, osteomyelitis, septicemia, granulomatous disease or salpingitis (Pasmans et al. 2008). In a seven year study, 235 lizards and 195 snakes were tested for the occurrence of *Salmonella enterica.* Systemic *Salmonella* infection was detected in 56.1 % of the *Salmonella*-positive lizards and snakes carcasses, 67.4 % of these were found to have pathophysiological changes of varying severity in the affected organs. There was a highly statistically significant relationship between systemic *Salmonella* infection and pathohistological changes (Sting et al. 2013). The number of *Salmonella* isolates from reptiles sent to the National Reference Laboratory for the Analysis and Testing of Zoonoses (Salmonella), NRL-Salm in Berlin, Germany increased also in Germany since 2007 (Fig. 7.3). This might be due to the fact that the popularity of reptiles as mainstream pets increased in recent years immensely, followed by more and more veterinary practices specialized in exotic animals, such as reptiles. Thus, the number of diagnostic samples for bacteriological tests from living, but also perished reptiles increased automatically.

In humans, although most reports of reptile-associated salmonellosis are from babies and children, there are also reports from adults, especially immunocompromised hosts, and furthermore in patients with impaired gastric acid production (Stam et al. 2003). Clinical signs in humans include gastroenteritis, but also more serious such as septicemia, meningitis, and subdural empyema (Chiodini and Sundberg 1981) might occur (Tabarani et al. 2010; Van Meervenne et al. 2009). Fatal outcomes following reptile-associated salmonellosis in babies have been reported.

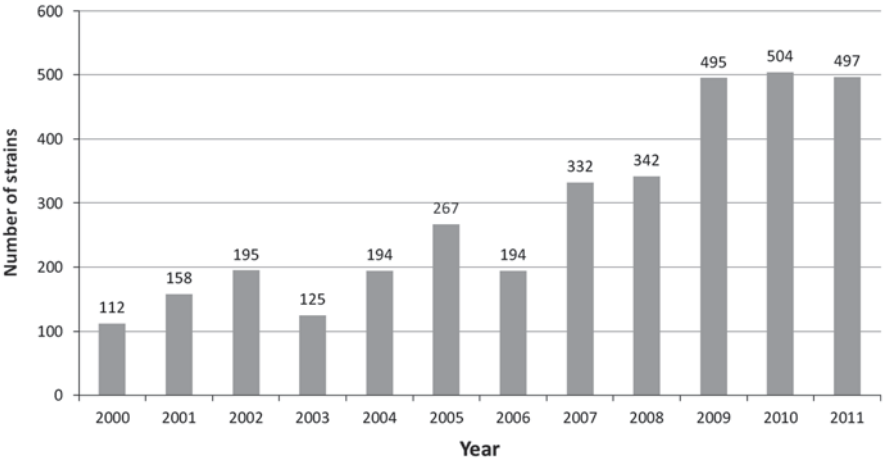

Fig. 7.3 Number of reptile isolates sent to NRL-Salm

Reptiles are suspected to be an often unrecognized source for salmonellosis in humans, and estimates state that reptile-associated salmonellosis is responsible for 3–11 % of all human salmonellosis cases in Canada and the US (Mermin et al. 2004; Warwick et al. 2001; Woodward et al. 1997). Newer studies and surveys indicate that other reptile species, especially lizards, may play a more important role today. Within the last years, various case reports on salmonellosis in infants related to reptiles, including snakes, turtles and lizards, as well as other exotic pets have been published in multiple countries (Cdc 2005; Hernandez et al. 2012; Kaibu et al. 2006; Weiss et al. 2011; Willis et al. 2002). Compared to the average values of the years 1997–2005, the proportion of reptiles-associated serovars in children under two years has tripled since 2006 (Robert Koch-Institut 2013). According to the European Surveillance System (TESSy) database, the number of infections affecting children younger than three years with serovars associated to reptiles also increased in the last years (Fig. 7.4, Johanna Takkinen, ECDC, pers. Communication, May 29, 2012).

These reports demonstrate that today reptile-associated *Salmonella* infections in humans are a world-wide problem. Based on the examination of different serovars from reptiles in captivity, Pedersen et al. indicated the need for further investigation to determine the role of pet reptiles as source of human salmonellosis (Pedersen et al. 2009). Thomas et al. concluded that the potential of captive and pet wildlife to transmit *Salmonella* sp. to humans should not be underestimated, and epidemiological studies on sources for human salmonellosis should simultaneously investigate both, the human cases and the wild and domestic animals in contact with them (Thomas et al. 2001). A study about the evidence for the transmission of *Salmonella* from reptile to children in Germany showed almost 50 % of the households answered that they kept at least one reptile. 68 % of the examined reptiles were bearded dragons (*Pogona vitticeps*). Altogether 319 *Salmonella* isolates were investigated and 44 different serovars identified. In 79 % of the households, in at least

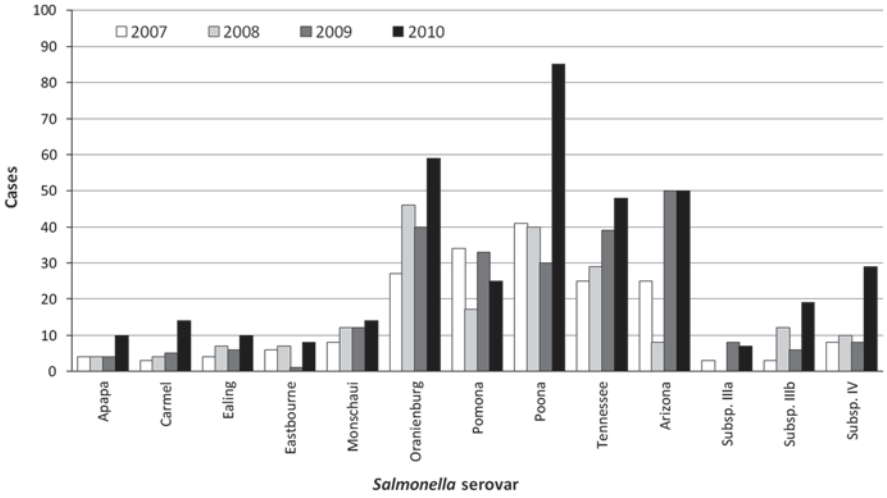

Fig. 7.4 Occurrence of REPAS-serovars in children <3 Jahren (2007–2010). (The European Surveillance System (TESSy) May, 29 2012)

one reptile the identical serovar was found and confirmed by PFGE. In 84 % of all bearded dragons examined, the identical serovar was confirmed (Pees et al. 2013).

From the epidemiological point of view (Robert Koch-Institut 2013), and in addition to a recommendation already made before ("Reptile-Associated Salmonellosis", RAS, (De Jong et al. 2005)) we propose to call this special kind of epidemic "Reptile-Exotic-Pet-Associated-Salmonellosis" (REPAS). The main argument is that within the past years, trading habits of reptiles have changed considerably. The main risk of *Salmonella* transmission from reptiles to humans is not due to European wild species, but as the results of this study also demonstrate today mainly due to "exotic" imported reptile species. Furthermore, following recent examinations *Salmonella* shedding is higher in reptiles kept in captivity in comparison to wild reptiles (Geue and Loschner 2002; Scheelings et al. 2011) and "pet" reptiles are obviously in closer contact to humans. These arguments justify the inclusion of "exotic pet" into the term describing the problem. The risk to human health connected to the reptile pet market has been highlighted recently (Arena Pc and Warwick 2012) and the accurate description of the problem using REPAS might be important to convey the problem in education and assist the European Commission to give recommendations to harmonise animal welfare and public health.

7.4 *Salmonella* Outbreaks

7.4.1 Molecular Tools for Outbreak Investigations

The ability to distinguish strains or clonal lineages of a bacterial pathogen is essential for addressing many questions in food microbiology, epidemiology, infection prevention and control. Traditional typing systems are based on phenotypes, such as serotype, phage type, or antibiotic resistance profiles. Classical phage typing has not only a long tradition in outbreak investigations but also for retrospective or long term studies to assess microevolution and virulence changes (see Sect. 2.4.). There are some prominent phage-genes, called morons in *S.* Typhimurium like *sopE* or *gtr* genes which interfere with virulence. Morons help the bacteria to survive by uptake of novel genes that make the bacteria more competitive with its peers in an old niche, or giving the bacteria the ability to exploit a new niche. So morons demonstrate the co-evolution of bacteria and their phages over time (Table 7.2). The 39 *S.* Typhimurium typing phages (2012 a new update of the Anderson scheme by E. de Pinna, PHLS, London) interfere with the prophages and/or cryptic phages and so the complex genetic microevolution can be demonstrated. This is one reason for the successful application of phage typing in *Salmonella* epidemiology since the 1950s (Rabsch et al. 2002).

However, genotyping methods have been successfully established within the past two decades to characterize a subset of defined strains. They can provide better discriminatory power to differentiate closely related *Salmonella* strains and give more information with respect to the genetic relatedness within the population (Wattiau et al. 2011). The choice of the method depends on the epidemiological question that needs to be answered. To study the phylogeny or global epidemiology of *Salmonella* a set of distantly related isolates must be selected and approaches that reflect the variation in those strains that accumulate relatively slowly need to be applied. Sequence variation within housekeeping genes is ideal for such studies because they are considered to be neutral in evolution and generally their function is well understood. In the past, multilocus enzyme electrophoresis assessed the allelic variation in the genes in a strain by determination of electromorphs (allozymes) of an enzyme (Selander et al. 1996). Distinctive allele profiles (multilocus enzyme genotypes) were designated as electrophoretic types. Nowadays, with easier and cheaper DNA sequencing techniques, the concept was revised by multilocus sequence typing (MLST). MLST generates allelic types from nucleotide sequences of housekeeping genes and not from electrophoretic mobilities of the enzymes they encode (Maiden et al. 1998). Usually nucleotide sequences of a set of seven housekeeping genes are used to determine the genetic relatedness on *Salmonella* serovar level (Achtman et al. 2012). Because MLST recognizes multilocus changes at DNA level it can detect phylogenetic lineages that are assigned to individual serovars. MLST has shown that serovars can originate from more than one common ancestor (termed as polyphyletic serovar).

Methods for outbreak studies and for tracing a contamination within the food chain, where it is important to differentiate *Salmonella* below the se-

Table 7.2 *Salmonella* prophage genes that modify phenotypes in host infection. (Virulence is in some cases confirmed. (Source: Bossi and Figueroa-Bossi (2005; Rabsch et al. (2011)

Locus (gene region)	Phage	Link to virulence or modification	References
STM2584-STM2637, *gogA* STM2614	Gifsy-1	Similar to *pipA* gene of SPI-5	Bossi and Figueroa-Bossi (2005)
gipA STM2599		Needed for growth in Peyer's patches	
gogD		Similar to *pagJ* and *pagK*	
gogB STM 2584		Similar to type III secreted proteins of LRR family	
STM1005-STM1066, *gtgA* STM1026	Gifsy-2	Similar to *pipA*	
grvA		Antivirulence gene	
ailT STM1043		Similar to attachment-invasion locus (ail) and to serum resistance proteins	
sodC1 STM1044		Periplasmic [Cu, Zn] superoxidase dismutase; protects against macrophage oxidative burst	
sseI (*srfH, gtgB*) STM1051		Type III translocated protein under SPI-2 control	
gtgE STM1055		Needed for mouse virulence	
gtgF STM1056		Similar to macrophage survival gene (*msgA*)	
pagJ	Gifsy-3	PhoP/PhoQ-activated locus	
SspH1		Type III translocated protein; downregulates interleukin-8	
StM0893-STM0929, *sodC3* STM0924	Fels-1	Periplasmic [Cu, Zn] superoxide dismutase; similar to *sodC1*	
nanH STM0928		Neuraminidase; involved in nutrient scavenging, host cell adhesion, and toxin action	
sopE (strain SL1344; gene SL2674)	SopEϕ	Type III translocated G nucleotide exchange factor; promotes epithelial cell invasion	

Table 7.2 (continued)

Locus (gene region)	Phage	Link to virulence or modification	References
STM0554-STM0559	Def1	Adjacent argU gtrABC cluster SPI-16	Casjens (2011)
STM1855–STM1871 *sopE2*(STM1855)	Def2	Type III translocated G nucleotide exchange factor; promotes epithelial cell invasion	
STM2230–STM2245 *oafA*(STM2232) *sspH2*(STM2241)	Def3	sspH2, oafA (acetylation of abequose, O5 antigen)	
STM4196–STM4219 gtrABCcluster (STM4204–4206)	Def4	glycosyltransferase, glucose translocase long term murine systemic infection SL1344 (Lawley et al. 2006)	
Genes 17–20	ES18	unknown, gene19 homolog adjacent to Pasteurella multicida phage carried toxin gene	Casjens et al. (2005)
SDT2667(*artA*) and SDT2666(*artB*), DT104	ST64B	pertussis like toxin	Saitoh et al. (2005); Pelludat et al. (2005)
gtrA, gtrB gtrC	ε 34, P22, ST64T, ST104	linked glucose translocase (flipase) antigen conversion 3,15(E2group) to 3,15,34 (E3group), O1 antigen B group, phage mediated alteration of LPS	Villafane et al. (2008); Brussow et al. (2004)
unknown	P14	LPS elongation in C group	Nnalue et al. (1990)
astA	Gifsy-2 AO	Heat stable toxin *S. abortusovis* acquisition of IS1414 (EAST1)	Bacciu et al. (2004)

rovar level, should be highly discriminative. This is commonly achieved by looking for DNA restriction sites within the test strain, e.g. using pulsed-field gel electrophoresis (PFGE), fluorescent amplified fragment length polymorphism (fAFLP) or ribotyping. PFGE is widely used and currently the method of choice for molecular subtyping *Salmonella* serovars. It has been proven to be a useful discriminatory method and was standardized by the PulseNet Consortium to share molecular epidemiologic information in real time (Gerner-Smidt and Scheutz 2006; Swaminathan et al. 2006). A forthcoming new molecular high resolution approach is the multilocus variable-number tandem-repeat analysis (MLVA) which is based on the determination of repetitive tandem DNA units within various loci. Repeating units occur of approx. 1–100 base pairs in length. The presence of repeated sequences is a fundamental feature of all genomes (Kolpakov et al. 2003). By the slipped strand misparing mechanism the number of tandem repeats can change with each generation. The more differences in tandem repeat units within a set of loci analysed are detected, the more distantly related the strains are interpreted to be. MLVA is meanwhile widely used (Lindstedt et al. 2013) and applicable for some epidemiologically important serovars including *Salmonella spp.* such as *S.* Typhimurium (Lindstedt et al. 2004), *S.* Enteritidis (Boxrudet al. 2007; Malorny et al. (2008), *S.* Infantis (Ross and Heuzenroeder 2008), *S.* Typhi (Ramisse et al. 2004), *S.* Newport (Davis et al. 2009) and *S.* Heidelberg (Young et al. 2012). MLVA has been proven as a valuable tracing tool in outbreak studies. Standardisation is in progress to make data valid and easily comparable between laboratories (Heck 2009; Hopkins et al. 2011; Sintchenko et al. 2012). Fabre et al. showed that a new family of DNA repeats named CRISPR (clustered regularly interspaced short palindromic repeats) is highly polymorphic in *Salmonella* (Fabre et al. 2012). They found that CRISPR polymorphism was strongly correlated with both serovar and multilocus sequence type. CRISPR strain characterization is an excellent potential alternative to both serotyping and PFGE, the current gold standard methods (Fabre et al. 2012).

Recently, a number of studies have used a whole-genome single nucleotide polymorphism-based approach to identify the source of outbreaks and to clarify the epidemiology of an outbreak (Grad et al. 2012; Mellmann et al. 2011). Next-generation sequencing (NGS) will play an important role in outbreak investigation in future. It provides a comprehensive tool for studying short-term evolution including aspects of antimicrobial resistance and virulence of epidemic clones. For *Salmonella*, tracing of outbreak strains by whole-genome mapping was reported for *S.* Montevideo (Bakker et al. 2011), *S.* Newport (Fey et al. 2012) and *S.* Heidelberg (Hoffmann et al. 2013). The introduction of new bioinformatic tools for rapid comparison of single-nucleotide polymorphic sites and open-access NGS databases will simplify and speed up the outbreak investigation and short-term evolution of epidemic strains in future.

7.4.2 *S. Enteritidis Outbreaks in the Last Decades in Germany*

In the last three decades *S.* Enteritidis has caused a growing worldwide pandemic. This problem was registered as a major health problem after 1986, when a large multistate outbreak occurred in the USA and when *S.* Enteritidis became the dominant serovar in England, Wales and other European countries including both parts of Germany (Cogan and Humphrey 2003; Rabsch et al. 2005; Rodrigue et al. 1990; Wall 1999). During this *S.* Enteritidis pandemic, the majority of outbreaks in Europe and the United States were traced back to foods containing raw or undercooked chicken eggs or contaminated chicken meat (Henzler et al. 1994; St Louis et al. 1988). A retrospective epidemiological analysis at an early stage of the pandemic in East Germany found the presence of different PT4/6 ribotypes in diverse farms. The circulation of different phage types or PT4/6 ribotypes at different farms of laying hens suggested that in each case the *S.* Enteritidis strains present in the environment (i.e. in rodent or vertebrates populations) were able to enter chicken flocks (Rabsch et al. 2007).

Phage typing has become a first-line method for the demonstration of epidemiological associations between strains of *Salmonella* Enteritidis and most reference laboratories now use the phage typing scheme (Ward et al. 1987), allowing the comparison of results in different countries (ECDC 2013b). Since 1993, the National Reference Center for Salmonella and other Enteric Pathogens in Wernigerode identifies *S.* Enteritidis outbreaks in Germany by phage typing with two phage typing schemes of Ward/Lalko and Laszlo, e.g. PT 4/6: phage type 4 according to Ward and 6 according to Lalko and Laszlo (Laszlo et al. 1985; Ward et al. 1987). PFGE the "golden standard" for the most serovars is not very helpful for *S.* Enteritidis. It should be pointed out, that most of the strains from the three countries which belonged to phage types 1, 4, and 6, showed the same XbaI PFGE combination profile, independently of the origin of the sample or date of isolation (Laconcha et al. 2000). For a better discrimination phage typing and *Pst*I/*Sph*I ribotyping are used in combination since 2004 (Liebana et al. 2002). In Table 7.3 the phage types of *S.* Enteritidis outbreaks from the last 17 years are described. The most frequently reported phage type of *S.* Enteritidis was PT4/6 till 2010. Other phage types like 6a/3a or 6a/7a are also involved in mainly raw egg-associated outbreaks. During the last 2 years the PT 21/1 and 8/7 were much more observed than PT4/6. But when PT4/6 was dominant in outbreaks the additional ribotype differentiation was very helpful. The distribution of PT4/6 ribotypes by the Federal States from 2004 to 2009 characterized the outbreaks very well (Table 7.4). The investigations of the food strains describe the heterogeneity of the raw egg-associated infection sources. The heterogeneity of different PT4/6 ribotypes from Table 7.4 is illustrated in Fig 7.5.

Together, these approaches allow the clear distinction of outbreak events from each other and from single infections as well as the definite identification of the infection source.

Table 7.3 Phage types of *S.* Enteritidis outbreaks from 1996 to 2012 in Germany (17 years)

Phage type	2012	2011	2010	2009	2008	2007	2006	2005	2004	2003	2002	2001	2000	1999	1998	1997	1996
1/1	1	1	3		1	2	3	3	1	3	5	2	8		2	2	1
1b/1			1								1	1					1
12/6												1	1	2			
13/7	1					3						1					
13a/7				1	2												
13a/7a				6													
14b/n.c.		1	3	1	1	1	2	1	1	2			1	2		2	1
2/1						2											
2/1b			1	4			2										
2/3															1		
20a/n.c.															1		
21/1b	7	3	4	6	9	9	7	8	6	9	15	4	9	6	1		
21c/1b							2										
25/17						1		1						1	1		1
28/7							1					1					
3/n.c.		1			1												
3a/17					1												
34/17																	1
36/6											2						1
4/6	2	1	11	18	37	54	44	26	27	32	51	51	101	52	40	24	32
4b/6							1						3				
4b/6a													1				
5/6c							2										
5c/17					1												
5c/n.c.							1										
6/6								3	1	3	2		4	4	2	3	3
6/6b	1	1		1	1	6	1										
6a/3a					1			1									
6a/7a										1		2		1	1		

Table 7.3 (continued)

Phage type	2012	2011	2010	2009	2008	2007	2006	2005	2004	2003	2002	2001	2000	1999	1998	1997	1996
7a/7					1	2											
7a/n.c.						1											
8/7	6	7	10	6	14	25	11	6	13	9	13	8	17	6	1	1	1
n.c./6											1		1		1		
n.c./7				1					1								
n.c./17						1											
n.c./n.c.											1			1			
ut/17						1											
Sum	*18*	*15*	*33*	*44*	*70*	*108*	*77*	*49*	*50*	*59*	*91*	*71*	*146*	*75*	*51*	*32*	*42*

Table 7.4 Ribotypes of 170 *S.* Enteritidis phage type 4/6 outbreaks from 2004 to 2009 in Germany[a]

Ribotype	Federal states	Number of outbreaks	Infection source
1	Baden-Württemberg	2	Cottage cheese
	Bavaria	4	Noodles
	Berlin	1	Dessert tiramisu
	Brandenburg	1	Mayonnaise
	Hesse	1	Salad
	Mecklenburg-Western Pomerania	2	Egg
	Lower Saxony	8	Sauce bolognaise with egg
	North Rhine-Westphalia	12	Potato salad
	Saxony-Anhalt	6	Cake
	Saxony	5	Poultry sausage
	Schleswig-Holstein	2	
	Thuringia	11	
1c	Baden-Württemberg	3	
	Bavaria	2	Dumplings
	Berlin	1	Bee sting cake
	Hesse	1	Potato salad
	Mecklenburg-Western Pomerania	2	Custard
	Lower Saxony	5	Egg
	North Rhine-Westphalia	5	Tiramisu
	Rhineland-Palatinate	2	Yoghurt drink
	Saxony-Anhalt	2	
	Saxony	1	
	Thuringia	2	
2	Bavaria	1	Cake (Black Forest gateau
	Thuringia	1	
3	Baden-Württemberg	1	Cake with egg
	Bavaria	3	Cream cake
	Mecklenburg-Western Pomerania	13	Crab cocktail
	Lower Saxony	7	Chocolate pudding
	North Rhine-Westphalia	1	Semolina pudding
	Rhineland-Palatinate	1	Tiramisu
	Saxony-Anhalt	5	Egg salad
	Saxony	1	
	Thuringia	4	
5	Mecklenburg-Western Pomerania	1	Lemon dessert
	Brandenburg	1	Chocolate icing
	Saxony-Anhalt	1	
	Lower Saxony	1	
6	Bavaria	2	Meat salad
	North Rhine-Westphalia	1	Cake
	Berlin	2	
	Brandenburg	1	
	Schleswig-Holstein	1	
	Lower Saxony	1	
7	Bavaria	3	Sauce bolognaise
	North Rhine-Westphalia	1	Chocolate mousse

Table 7.4 (continued)

Ribotype	Federal states	Number of outbreaks	Infection source
	Saxony	3	Chicken ragout
	Mecklenburg-Western Pomerania	1	
8	Lower Saxony	1	Red fruit jelly with custard
	Saarland	1	
9	Lower Saxony	1	Cake
	Hesse	1	Tiramisu
	Saxony	2	
12	North Rhine-Westphalia	5	Dessert
	Thuringia	3	Tiramisu
	Bavaria	1	
12a	Baden-Württemberg	1	
16	North Rhine-Westphalia	1	Rice cake
	Lower Saxony	1	
	Saxony-Anhalt	1	
17	Mecklenburg-Western Pomerania	2	Potato salad
18	Mecklenburg-Western Pomerania	2	Dessert tiramisu
	North Rhine-Westphalia	2	
	Saxony-Anhalt	1	
19	Lower Saxony	1	
20	Baden-Württemberg	1	
23	North Rhine-Westphalia	1	
26	Lower Saxony	1	
50	Saxony	(1	

[a] From 2004 and 2005 only 23 outbreaks were investigated by ribotyping

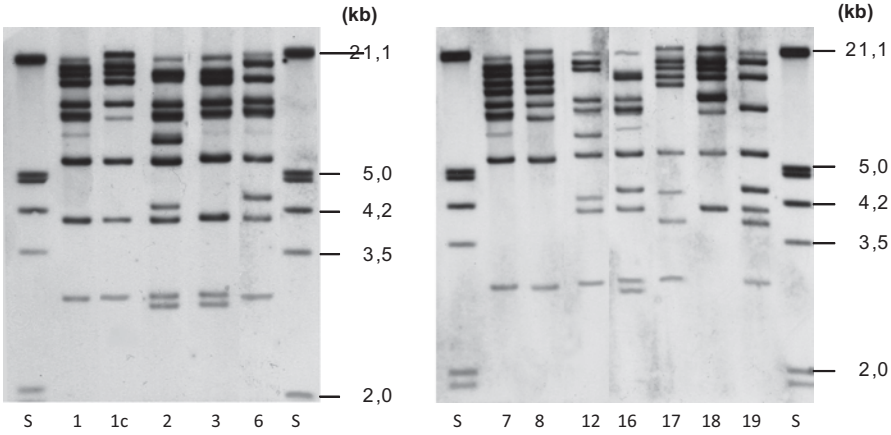

Fig. 7.5 *Pst*I-*Sph*I ribotypes (PS types) in *S.* Enteritidis isolates of phage type 4/6. (R. Prager; selected types)

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Chapter 8
Enteropathogenic *Yersinia* spp.

Maria Fredriksson-Ahomaa

Abstract *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* can cause enteric yersiniosis among humans and animals. The infection is typically acquired through contaminated food but it can also be transmitted directly through human or animal contact. Common symptoms are diarrhoea, abdominal pain and fever but sometimes sequelae as joint pain and skin rash occur. The severity of the infection depends on the age and immunity of the infected person, the virulence of the strain and the infection dose. *Y. enterocolitica* and *Y. pseudotuberculosis* differ clearly from each other both pheno- and genotypically. *Y. enterocolitica* species consists of a very heterogeneous group of bacteria and not all *Y. enterocolitica* strains are pathogenic. *Y. pseudotuberculosis* strains show little variation in biochemical reactions and they are all considered to be pathogenic. Both plasmid and chromosomal encoded virulence factors are needed for *Yersinia* pathogenicity and all pathogenic strains carry a virulence plasmid which essential for the bacteria to multiply and disseminate in the host. *Yersinia* strains can grow at low temperatures and under both aerobic and anaerobic conditions but they are heat sensitive. The isolation and identification of enteropathogenic *Yersinia* is difficult and time consuming, especially when food and environmental samples are studied. More accurate methods should be designed for isolation and identification of *Yersinia* spp. and more information is needed about transmission routes and sources of enteropathogenic *Yersinia* strains.

8.1 Introduction

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are the two species belonging to the enteropathogenic *Yersinia* spp. They are causing enteric yersiniosis, the third most frequently reported food-borne bacterial enteritis in the EU (EFSA and ECDC 2013). Both species have animal reservoirs and a faecal-oral transmission route (Laukkanen-Ninios and Fredriksson-Ahomaa 2012). The infection is usually acquired through contaminated food, especially raw or undercooked pork or

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vegetables. It can also be acquired through contact with infected humans or animals. Common symptoms are diarrhoea, abdominal pain and fever. Sometimes sequelae as joint pain and skin rash occur. Isolation and identification of *Yersinia* spp. is challenging and time-consuming (Fredriksson-Ahomaa 2009; Fredriksson-Ahomaa and Korkeala 2003). The pathogenicity of *Y. enterocolitica* strains varies from non-pathogenic to highly pathogenic, thus detection of virulence markers is also needed to determine the clinical significance of the isolated strains.

8.2 *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

8.2.1 Characteristics

The Genus *Yersinia* Includes Two Enteropathogenic *Yersinia* spp. The genus *Yersinia* belongs to the family *Enterobacteriaceae*; a group of gram-negative, oxidase-negative and facultative anaerobic rod-shaped bacteria that do not possess a capsule or spores (Bottone et al. 2005). *Y. enterocolitica* and *Y. pseudotuberculosis* are the two *Yersinia* species that can cause an enteric infection in humans and animals. They are zoonotic bacterial pathogens that can be transmitted from animals to humans on a faecal-oral route and may cause enteritis. *Y. enterocolitica* and *Y. pseudotuberculosis* differ clearly from each other both pheno- and genotypically (Wren 2003). *Y. pseudotuberculosis* is genetically closely related to the highly virulent agent of plague, *Yersinia pestis,* which is transmitted from animals to humans by fleas or in aerosols.

***Y. enterocolitica* Species Consists of a Very Heterogeneous Group of Bacteria** *Y. enterocolitica* comprises over 30 serotypes based on the variation of O antigen, the cell wall lipopolysaccharide (Wauters et al. 1991). Furthermore, *Y. enterocolitica* strains can be divided into 6 biotypes: 1A, 1B, and 2–5 (Tauxe et al. 1987). Biotype 1A is the most heterogeneous group of *Y. enterocolitica* strains including a wide range of serotypes (Bhagat and Virdi 2011). This group of bacteria shows also a high genetic diversity whereas the strains belonging to biotypes 1B and 2–5 are relatively well conserved (Bhagat and Virdi 2011).

Not all *Y. enterocolitica* Strains Are Considered Pathogenic Certain biotype and serotype combinations have been associated with human and animal infections. Bioserotype 4/O:3, which has a worldwide distribution, is the most common type associated with human disease followed by bioserotypes 2/O:9 and 2/O:5,27 (Fredriksson-Ahomaa et al. 2010). Infections due to bioserotype 3/O:3 have frequently been reported, especially in China (Wang et al. 2012). Bioserotype 1B/O:8, which is the most highly pathogenic type, is geographically limited to Northern America, although it has sporadically been found in Europe and Japan. Recently, *Y. enterocolitica* 1B/O:8 human infections have frequently been reported in Poland. The *Y. enterocolitica* 1B/O:8 strains have been clonal and were isolated from patients in different regions of the country (Rastawicki et al. 2013, in press).

Biotype 1B strain has been associated with high pathogenicity and biotypes 2–5 strains with moderate or low pathogenicity (Carniel 2001). Strains belonging to biotype 1A are usually regarded as non-pathogenic. However, discussions have been raised of a potential pathogenicity of some strains of the biotype 1A (Bhagat and Virdi 2011). Recently, biotype 1A strains isolated from faeces of humans with and without diarrhoea in Switzerland were characterised (Stephan et al. 2013). In this study, no clear difference between *Y. enterocolitica* 1A strains isolated from humans with and without diarrhoea could be demonstrated.

***Y. pseudotuberculosis* Strains Show Little Variation in Biochemical Reactions** The genetic diversity of *Y. pseudotuberculosis* strains is limited (Laukkanen-Ninios et al. 2011). There is also only little variation in biochemical reactions except variation in the sugars melibiose, rhamnose and raffinose (Tsubokura and Aleksić 1995). The strains can be divided into 4 biotypes using melibiose, raffinose and citrate; however, biotyping is not widely used. *Y. pseudotuberculosis* strains can be classified at the moment into 21 serotypes according to their varying lipopolysaccharide O-antigen structure: O:1-O:15 and 3 subtypes (a-c) in O:1 and O:2, and 2 subtypes (a and b) in O:4 and O:5. The most common serotypes associated with human disease are serotypes O:1 to O:5. Serotypes O:1 and O:3 are widely distributed and dominate in Europe, whereas O:4 and O:5 are commonly found in the Far East (Laukkanen-Ninios and Fredriksson-Ahomaa 2012). Serotypes O:6 to O:15 have so far been isolated only in non-human sources. All *Y. pseudotuberculosis* strains are considered to be pathogenic; however, they can be divided into high and low-pathogenicity strains (Carniel 2001). Strains of high pathogenicity cause severe systemic infections in humans and are lethal for mice at low doses, while strains of low pathogenicity cause more frequently only intestinal infections in humans and are non-lethal to mice at low doses.

Enteropathogenic *Yersinia* Strains can Grow at Low Temperatures *Yersinia* bacteria are able to grow at temperatures ranging from 4–43 °C; however, slow growth has been reported to occur even below 4 °C (Fredriksson-Ahomaa et al. 2010). The optimal temperature for growth and metabolic activity is around 28 °C. The ability to grow at low temperatures allows *Y. enterocolitica* and *Y. pseudotuberculosis* to multiply in refrigerated foods. *Yersinia* strains typically tolerate freezing for a prolonged time and even repeated cycles of freezing and thawing. However, yersiniae are heat sensitive and thus can easily be destroyed by heat treatment. Pasteurisation at 72 °C for 15–20 s kills *Yersinia* bacteria. As a facultative anaerobic bacterium, *Yersinia* can multiply under both aerobic and anaerobic conditions, and under modified atmosphere. *Y. enterocolitica* has recently been shown to grow well on pig cheek meat in modified atmosphere with high oxygen (70 % O_2) and carbon dioxide (30 % CO_2) concentrations during cold storage at 6 °C even in the presence of high numbers of lactic acid bacteria (Fredriksson-Ahomaa et al. 2012b). *Yersinia* bacteria are also able to grow over a wide pH range (pH 4–10). Alkalotolerance of *Yersinia* has been used to separate *Yersinia* strains from background organisms by treatment of enrichment broth with potassium hydroxide (0.25–0.5 %) before streaking onto agar plates (ISO 2003).

Table 8.1 Virulence factors found in *Y. enterocolitica* and *Y. pseudotuberculosis* strains

Species		Presence of								
		pYV	Chromosomal							
			Inv	Ail	YstA	YstB	HPI	MyfA	pH6	YPM
Y. enterocolitica	1A	−	+	−	−	+	−	−	−	−
	1B	+	+	+	+	−	+	+	−	−
	2–5	+	+	+	+	−	−	+	−	−
Y. pseudotuberculosis		+	+	+	−	−	+[a]	−	+	+[b]

[a] Serotypes O:1 (complete HPI) and O:3 (truncated HPI)
[b] Found frequently in Far Eastern strains

8.2.2 Virulence

Several Plasmid and Chromosomal Encoded Virulence Factors Are Needed for *Yersinia* Pathogenicity Several virulence factors have been identified among *Y. enterocolitica* and *Y. pseudotuberculosis* strains and some of them are common in both species (Table 8.1). The most important is the presence of an approximately 70-kb plasmid which is termed pYV (plasmid for *Yersinia* virulence) in all pathogenic *Yersinia* strains (Heesemann et al. 2006). This virulence plasmid is present in all three human pathogenic *Yersinia* species: *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis*. However, *Y. enterocolitica* strains belonging to biotype 1A are not carrying the pYV (Table 8.1), and thus are considered non-pathogenic. This virulence plasmid is essential for bacterial replication in host tissue. In addition to pYV-encoded virulence factors, also chromosomal encoded factors are needed, for pathogenicity.

Virulence Factors Encoded by the pYV Plasmid Are Essential for the Pathogenicity The pYV plasmid encodes a type III secretion system (T3SS) with its effectors (Yersinia outer proteins [Yops]), structural and regulatory proteins (Heesemann et al. 2006). The T3SS is a molecular syringe (injectisome) that delivers cytotoxic effector proteins (Yops) into the host cell (Dewoody et al. 2013, in press). Expression of T3SS genes are controlled primarily by temperature and calcium concentration. With this system, *Yersinia* can replicate extracellularly in lymphatic tissue and encounter the immune defences of the host. Additionally, pYV encodes the non-fimbrial, outer-membrane protein YadA (*Yersinia* adhesin A), This protein is a multifunctional protein that promotes the attachment of bacteria to the intestinal brush border and confers resistance to serum complement lysis. YadA is optimally expressed at 37 °C. The pYV-encoded proteins virF of *Y. enterocolitica* and lcrF of *Y. pseudotuberculosis*, respectively, are important thermo activated transcriptional regulators which activate the production of Yops (Cornelis et al. 1998). Both *yad*A and *vir*F genes are frequently used as targets to detect and identify pathogenic *Yersinia* strains (Fredriksson-Ahomaa et al. 2010).

Chromosomally Encoded Virulence Factors Are also Needed Two chromosomal genes, *inv* and *ail*, are important for mammalian cell invasion (Trcek et al. 2011). The *inv* gene codes for an outer-membrane invasion protein (Inv) which plays an important role in promoting the entry into epithelial M cells of the ileum during initial

stages of infection. Epithelial cell penetration of *Y. enterocolitica* is also enhanced by the outer membrane protein Ail encoded by the *ail* (attachment invasion locus) gene. Ail promotes serum resistance of both *Y. enterocolitica* and *Y. pseudotuberculosis*. Some strains of biotype 1A have been shown to carry *ail* gene which is thought to be restricted to strains belonging to biotypes 1B and 2–5 (Sihvonen et al. 2011; Kraushaar et al. 2011). The *yst* gene in the chromosome of *Y. enterocolitica* encodes a heat stable enterotoxin Yst (*Yersinia* stable toxin). Yersiniabactin is a siderophore which is synthesised by *Y. enterocolitica* strains belonging to biotype 1B and by *Y. pseudotuberculosis* O:1 and O:3 strains. The genes for yersiniabactin synthesis, uptake and regulation are clustered within a region of the chromosome referred to as high-pathogenicity island (HPI) (Carniel 2001). The HPI is completely present in serotype O:1 strains and truncated in serotype O:3 strains. Some *Y. pseudotuberculosis* strains can also synthesise a superantigen toxin YPM (*Y. pseudotuberculosis* -derived mitogen) which plays an important role in systemic infections, especially in the Far East. Additionally, mucoid *Yersinia* factor (Myf) of *Y. enterocolitica* and pH6 antigen of *Y. pseudotuberculosis* are surface (fimbrial) structures with high sequence similarity (Trcek et al. 2011). The pH6 antigen mediates agglutination of erythrocytes and adhesion to cultured mammal cells. MyfA, which has mostly been found in clinical *Y. enterocolitica* strains, enhances binding to the intestinal mucosa.

8.3 Yersiniosis—The Disease

8.3.1 Pathogenesis

***Yersinia* Strains Require the Virulence Plasmid (pYV) for Survival Within Tissues** Yersiniosis is usually acquired through oral ingestion of contaminated food or water. Enteropathogenic *Yersinia* strains can survive in gastric acid by producing urease which increases the pH (Gripenberg-Lerche et al. 2000). Yersiniae bind to the mucus layer that covers the epithelial cells, preferably in the terminal ileum. They attach to M cells which overlay the Peyer's patches and are specialised in the uptake of intestinal antigens. Subsequently, the yersiniae penetrate into the tissue (Grassl et al. 2003). Attachment and invasion of M cells are mediated by chromosomal determinants, e.g. Inv and Ail proteins, and the pYV-encoded YadA. After penetration of the intestinal epithelium, yersiniae colonize the Peyer's patches and local lymphoid follicles, and may spread via the lymphatic system or the blood to other tissues, typically to mesenteric lymph nodes, spleen and liver where they form microabscesses (Trcek et al. 2011). The ability to survive within these tissues is dependent on the presence of pYV. The multiplication of *Yersinia* in Peyer's patches may cause severe abdominal pain which might be confused with appendicitis. Most infections are usually localised and self-limiting due the host's inflammatory response which finally leads to elimination of the pathogen.

Some patients develop post-infective reactive arthritis (ReA). In *Yersinia*-triggered ReA, the primary infection is in the gut and the arthritis develops soon after the infection. The synovial fluid from affected joints of patients is usually

culture-negative, but contains bacterial antigens in the joint (Granfors et al. 1989). Most individuals with post-infective ReA are positive for human leukocyte antigen HLA-B27 (Hannu et al. 2006). It seems that HLA-B27-positive individuals are more prone to get severe symptoms and to show a more prolonged disease course than HLA-B27 negative patients. The factors determining the progression of acute ReA to a chronic course are not completely known. In patients with chronic *Yersinia* -triggered ReA, *Yersinia* antigens have been shown to persist in the intestinal mucosa or in lymph nodes.

8.3.2 Yersiniosis in Humans

Yersiniosis due to *Y. enterocolitica* and *Y. pseudotuberculosis* is mostly an uncomplicated enteric disease. Also asymptomatic carriage of these pathogens has been reported. The incubation period ranges from 1 day to about 1 week and the symptoms typically persist for 1–2 weeks (Cover and Aber 1989). The minimal infectious dose is unknown. The severity of the infection depends on the age and immunity of the infected person, the virulence of the strain and the infection dose. Acute diarrhoea, which may be bloody, and high fever occur most frequently in infants and children under 5 years (Rosner et al. 2010). Abdominal pain in the right lower quadrant due to mesenteric lymphadenitis and terminal ileitis is a common symptom in older children and adolescents. The abdominal pain resembles the symptoms of appendicitis and may lead to unnecessary surgery (Nuorti et al. 2004). The symptoms may persist for several days.

In some cases, *Yersinia* infections may lead to extra-intestinal complications like joint pain (reactive arthritis) and skin rash (erythema nodosum) which are common especially among adults (Rosner et al. 2010; Hannu et al. 2003; Jalava et al. 2006). Reactive arthritis, which typically develops 1 week to 1 month after primary infection, commonly affects knees, ankles and wrists. Skin rash appears usually 2 weeks after the infection and more commonly in females than in males. The symptoms of extra-intestinal infections may last for several months.

Sepsis cases caused by *Y. enterocolitica*-contaminated blood have been reported (Guinet et al. 2011). Symptoms like explosive diarrhoea and fever usually develop very quickly. If the transfused blood is heavily contaminated, large amounts of endotoxins are introduced into the circulatory system causing severe sepsis and septic shock with a fatality rate of about 50 %.

Yersiniosis is usually self-limiting and no treatment with antimicrobials is needed. Only in severe cases, like in systemic infections and bacteraemia, treatment is warranted (Guinet et al. 2011). Antimicrobials should also be considered for patients who are immune-compromised and for patients with iron overload. All *Y. enterocolitica* strains possess β-lactamase activity, and are thus resistant to penicillins. However, *Yersinia* strains are sensitive to numerous antimicrobial agents including aminoglycosides, third generation cephalosporins, co-trimoxazole, tetracyclines, chloramphenicol and fluoroquinolones (Bonke et al. 2011). In contrast to *Y. enterocolitica*, *Y. pseudotuberculosis* strains lack β-lactamase activity and are therefore usually susceptible to penicillins.

8.3.3 Yersiniosis in Animals

***Y. enterocolitica* Infections Are Rare Among Animals** Descriptions of observed clinical manifestations or patho-anatomical changes in animals due to yersiniosis are sparse. Piglets are usually capable to restrict the *Y. enterocolitica* colonisation to the tonsils and the intestinal tract without development of serious illness (Schiemann 1988). *Y. enterocolitica* has occasionally been recovered from dogs, especially puppies, and cats with diarrhoea (Fredriksson-Ahomaa et al. 2001b). Yersiniosis (pseudotuberculosis) due to *Y. enterocolitica* and *Y. pseudotuberculosis* is one of the most important causes of death in hares (Frölich et al. 2003). The signs vary from mild to severe enteritis, including enlargement of the spleen and various lymph nodes and micro-abscesses in several organs. The bioserotype 5/O:2,3 is the most common *Y. enterocolitica* type isolated from hares, and thus called hare type. The bioserotype 3/O:1,2,3, which is also called chinchilla type, can cause yersiniosis among chinchillas with typical lesions associated with yersiniosis (Wuthe and Aleksić 1992). In Australia, sheep and goats less than 1 year old have been reported to be commonly infected by *Y. enterocolitica* biotypes 3 and 5, serotype O:2,3, and sometimes sudden death due to severe diarrhoea and emaciation (Slee and Skilbeck 1992; Slee and Button 1990). Some *Y. enterocolitica* O:8 outbreaks among breeding monkeys in Japan have been reported (Nakamura et al. 2010). Fredriksson-Ahomaa et al. reported two fatal cases of *Y. enterocolitica* 4/O:3 infection in marmosets fed with *Y. enterocolitica* 4/O:3 contaminated pork (Fredriksson-Ahomaa et al. 2007a). This pathogen was isolated from the liver with disseminated miliary necrosis of both animals.

Outbreaks Due to *Y. pseudotuberculosis* Infections in Zoo Animals Are Common *Y. pseudotuberculosis* infections are most commonly latent in animals; however, several yersiniosis outbreaks, especially due to *Y. pseudotuberculosis*, have been reported in captive non-human primates and in bats (Kageyama et al. 2002; Nakamura et al. 2009, 2012, 2013). Typically, necrotizing enteritis with enlarged Peyer's patches and mesenterial lymph nodes can be found in monkeys at necropsy. In both monkeys and bats, multiple yellow-white nodules in spleen and liver were detected. *Y. pseudotuberculosis* was also isolated from lung, heart, kidney, liver, spleen and intestine samples of a free-living dead bat in Germany (Mühldorfer et al. 2010). Outbreaks due to *Y. pseudotuberculosis* infections with diarrhoea and sudden death have also been reported in farmed deer and sheep in Australia (Slee and Skilbeck 1992; Jerrett et al. 1990) and farmed deer in Canada (Sanford 1995).

8.4 Detection and Typing

8.4.1 Detection Methods

So far, there is no sensitive isolation method available for detection of enteropathogenic *Yersinia* in non-human samples, thus several PCR methods have been designed for a more sensitive detection of both *Y. enterocolitica* and *Y. pseudotuberculosis*

in different samples including animal reservoirs and food (Fredriksson-Ahomaa 2012b). In recent studies, real-time PCR methods based on SYBRGreen dye or specific dual-labelled probes have been used to screen the presence of enteropathogenic *Yersinia* in animals and food samples (Fredriksson-Ahomaa et al. 2007b, 2009; Bucher et al. 2008; Messelhäusser et al. 2011). The detection rates were shown to be clearly higher for PCR compared to culturing. PCR methods are rapid and have a superior sensitivity compared to culture methods; however, they might also detect non-viable bacteria or bacterial DNA, and do not yield bacterial isolates that are essential for further epidemiological studies. Therefore, isolation methods should also be used.

The isolation of enteropathogenic *Yersinia* is difficult and time consuming (Fredriksson-Ahomaa 2012a). Isolation methods are based on (1) direct culturing on selective agar media, (2) a short selective enrichment step before culturing on agar plates and/or (3) cold enrichment at 4 °C for 2–3 weeks (Laukkanen et al. 2010). Direct culturing without any pre-enrichment is useful for samples containing a high number of *Yersinia* such as faeces and tissue samples of humans and animals with acute yersiniosis. Direct culturing has also been shown to be very suitable for tonsil samples of fattening pigs at slaughter (Fredriksson-Ahomaa et al. 2009; Van Damme et al. 2010). An enrichment step is usually needed to increase the number of yersiniae when samples from human and animal carriers are studied. For food and environmental samples, selective enrichment is recommended prior to plating on selective agar plates, because these samples are usually heavily contaminated with other bacteria (Fredriksson-Ahomaa and Korkeala 2003). Two to four days of incubation at 25 °C in selective enrichment broth, e.g. irgasan-ticarcillin-potassium chlorate (ITC) or modified Rappaport broth (MRB), are frequently used for food and environmental samples (ISO 2003; NCFA 1996). These selective enrichment broths have been designed for *Y. enterocolitica* bioserotypes 4/O:3 and 2/O:9, and thus they are not optimal for other *Y. enterocolitica* bioserotypes and *Y. pseudotuberculosis* (Fredriksson-Ahomaa 2012a). Cold enrichment in non- or low-selective enrichment broth is widely used for isolation of both *Y. enterocolitica* and *Y. pseudotuberculosis*. Phosphate buffered saline (PBS) or PBS supplemented with peptone, sugars and bile salts are most frequently used in the cold enrichment.

Yersiniae grow well on most agar media designed for gram-negative enteric bacteria.

Several selective agar plates have been designed for isolation of *Y. enterocolitca* (Fredriksson-Ahomaa 2012a). Cefsulodin-irgasan-novobiocin (CIN) and *Salmonella-Shigella* deoxycholate calcium chloride (SSDC) agar plates have been widely used for isolation of *Y. enterocolitica* bioserotype 4/O:3 from food samples (ISO 2003; NCFA 1996). They are both commercially available. CIN agar is the most frequently used agar for naturally contaminated samples because of the high confirmation rate of presumptive isolates and its relatively high selectivity (Laukkanen et al. 2010; Savin et al. 2012). Moreover, two selective chromogenic media have recently been designed for isolation of potentially pathogenic *Y. enterocolitica* strains (Weagant 2008; Renaud et al. 2013).

The isolation of *Y. pseudotuberculosis* is mostly very difficult, because this pathogen grows slowly and thus gets easily overgrown by other bacterial species present in the sample (Fredriksson-Ahomaa 2009). No high-selective enrichment broth has so far been designed for *Y. pseudotuberculosis*. Cold enrichment for 2–3 weeks in low-selective phosphate-buffered saline broth supplemented with 1 % mannitol and 0.15 % bile salts (PMB) has widely been used for the isolation of *Y. pseudotuberculosis* from non-human sources (Ortiz Martínez et al. 2010). No selective agar plates have been created for isolation of *Y. pseudotuberculosis* and therefore CIN agar has also been widely used for this species (Savin et al. 2012). However, the growth of some *Y. pseudotuberculosis* strains is inhibited on this medium, thus MacConkey agar has been used along with CIN.

Y. enterocolitica and *Y. pseudotuberculosis* are mostly identified biochemically with commercial identification kits like API 20E and Enterotube. Non-pathogenic *Yersinia* spp. isolates are, however, usually misidentified as *Y. enterocolitica* (Hallanvuo et al. 2006; Sihvonen et al. 2009). Furthermore, using phenotypic methods, sucrose-negative pathogenic *Y. enterocolitica* is easily misidentified as non-pathogenic *Y. kristensenii*, if the pathogenicity of the strain is not assessed (Fredriksson-Ahomaa et al. 2002). *Y. pseudotuberculosis,* which should always be considered pathogenic is difficult to differentiate from the non-pathogenic species *Y. similis* and *Y. pekkanenii* (Laukkanen-Ninios et al. 2011; Niskanen et al. 2009; Murros-Kontiainen et al. 2011). Therefore, for correct identification of enteropathogenic *Yersinia*, detection of species-specific virulence genes is usually needed. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has recently been shown to be a valuable method for rapid identification of large number of *Yersinia* isolates (Stephan et al. 2011; Stamm et al. 2013).

Serological analysis could be used as an alternative method for monitoring of yersiniosis in animal reservoirs and for the estimation of the prevalence of *Y. enterocolitica* in livestock at herd level, since it is not as expensive and time-consuming as traditional culture methods (Von Altrock et al. 2011; Virtanen et al. 2012). However, serological diagnosis is not equivalent to classical microbiological detection of the organism, as the serological response is delayed with respect to the time of infection. Recently, a flow-through chemiluminescence immunochip method based on Yops encoded by the pYV of pathogenic *Yersinia* spp. was developed (Wutz et al. 2013, in press). The immunochip detects anti-*Yersinia* IgG using the recombinant YopD antigen.

8.4.2 Typing Methods

Biotyping Is Recommended to Assess the Potential Pathogenicity of the *Y. enterocolitica* Isolates Phenotyping methods based on biotyping and serotyping are widely used for characterisation of *Y. enterocolitica* isolates (Fredriksson-Ahomaa 2012b). The potentially pathogenic *Y. enterocolitica* isolates can be identified by the pyrazinamidase test, which is one of the key tests included in the biotyping

scheme. Since all correctly identified *Y. pseudotuberculosis* isolates are considered pathogenic, virulence testing is usually not necessary. However, if the suspected isolate cannot be serotyped, it is recommended to test the virulence, because it may be a non-pathogenic *Y. pseudotuberculosis*-like strain (Niskanen et al. 2009). The information of biotype and/or serotype alone often lacks the discriminatory power needed to differentiate among *Yersinia* isolates that belong to the same bioserotype. Therefore, in addition to bio- and serotyping, more discriminatory typing methods are needed.

Genotyping is Needed for Epidemiological Studies Several typing methods have been used for tracking the distribution, infection source and transmission routes (Fredriksson-Ahomaa et al. 2006a; Virdi and Sachdeva 2005). These techniques rely upon three main mechanisms of discrimination: (1) restriction analysis of the bacterial DNA; (2) PCR amplification of particular genetic targets; and (3) identification of DNA sequence polymorphisms at specific loci in the genome (Fredriksson-Ahomaa 2012b). So far, PFGE, which is based on restriction analysis, has been the most widely used method in epidemiological studies for both *Y. enterocolitica* and *Y. pseudotuberculosis*. PFGE allows subtyping of isolates belonging to the same bioserotype. Disadvantage is that the method is time-consuming, laborious, and the comparison of complex profiles is difficult. Multi-locus variable-number tandem-repeat analysis (MLVA), which is a PCR-based method, is increasingly used for typing of enteropathogenic *Yersinia* strains. This method is easy to use and the discriminatory power is very good (Wang et al. 2012). At the moment, MLVA seems to be the best method for local epidemiological investigations and for screening potential outbreaks.

8.5 Epidemiology

8.5.1 Incidence in Humans

Sporadic *Y. enterocolitica* Infections Are Common Among Humans Human yersiniosis was the fourth most common reported bacterial enteric infection after campylobacteriosis, salmonellosis and STEC infections in Europe in 2011 (EFSA and ECDC 2013). In 2011, 7017 confirmed human yersiniosis cases were reported in Europe. For the first time since 2006, a slight increase (3.5 %) in the notification rate was reported. *Y. enterocolitica* was the most common species (98 %) of the confirmed cases with species information and *Y. pseudotuberculosis* represented only 1 % of the cases. The highest country-specific notification rates were reported in Lithuania and Finland (11 and 10 cases per 100,000 inhabitants, respectively). Due to the inconsistency of surveillance systems in different countries, comparison of the incidence of yersiniosis between different countries is only suggestive. Furthermore, the incidence is probably largely underestimated, especially for *Y. pseudotuberculosis*, due to the insensitivity of the available isolation methods (Savin

et al. 2012). In Europe, the incidence is highest among children under 5 years. The majority of yersiniosis cases are sporadic and domestically acquired and no seasonal distribution has been reported (EFSA and ECDC 2013).

8.5.2 Food-Borne Outbreaks

***Y. pseudotuberculosis* Infections Have Frequently Been Associated with Food-Borne Outbreaks** *Y. pseudotuberculosis* outbreaks have mainly been notified in Finland, Japan and Russia (Fredriksson-Ahomaa 2009). During the last decade, several *Y. pseudotuberculosis* outbreaks due to vegetables have been reported (Table 8.2). In Russia, *Y. pseudotuberculosis* outbreaks due to raw vegetables (cabbage, onions, carrots) have been registered mostly in schools and day-care centres (kindergarten) (www.promedmail.org; archive numbers: 20080718.2184, 20071001.3240, 20051216.3617, 20050427.1169, 20050202.0359). The number of children hospitalised has been high. The vegetables have probably been contaminated by rodents. Incidences are highest in winter possibly due the need to store vegetables for a prolonged period of time with a subsequent higher risk to become contaminated by rodents. In 2007, one outbreak in Russia affected 121 people, mostly school children, with 38 of them admitted to hospital (www.promedmail.org; archive number: 20071001.3240). Scarlet fever symptoms (acute rise in temperature, a rash, and stomach and joint pains) were reported among some of the 24 children falling ill with *Y. pseudotuberculosis* in 2005 in Novgorod. Recurring outbreaks due to *Y. pseudotuberculosis* O:1 infections through carrots served at schools have been reported in Finland during 2003 and 2008 (http://www.thl.fi/thl-client/pdfs/d6d63c66-9690-4f4d-9ee1-319bb5648eaf). In spring 2003, an extensive *Y. pseudotuberculosis* O:1 outbreak among school children occurred in Finland with about 800 cases (Jalava et al. 2006). The investigation revealed that domestic carrots were the infection source. It was the first time that the same strain (genotype and serotype) was isolated both from human cases and the vehicle, i.e. carrots which could be traced to a farm.

Recently, Some Outbreaks due to *Y. enterocolitica* O:9 Infections Have Been Notified In 2011, an outbreak due to *Y. enterocolitica* O:9 in Norway was associated with a ready-to-eat salad mix (MacDonald et al. 2012). The outbreak was detected rapidly through systematic characterisation of *Y. enterocolitica* isolates from humans using genotyping (MLVA). However, this type could not be isolated from the salad. Furthermore, a ready-to-eat pork product (brawn) has been associated with a *Y. enterocolitica* O:9 outbreak in Norway (Grahek-Ogden et al. 2007). Brawn is made from precooked head meat and is a traditional food at Christmas. Correct cooking should eliminate *Y. enterocolitica*, but bacteria may survive in the core of the product because of its high fat content which can enhance the survival of yersiniae. Furthermore, cross-contamination before slicing and packaging could have occurred and the ability of yersiniae to grow at refrigeration temperatures

Table 8.2 Reported outbreaks due to enteropathogenic *Yersinia* during the last decade

Year of outbreak	Country	Serotype of causing agent		Infected persons	Suspected sources
		YE[a]	YP[b]		
2011	Norway	O:9		6	Lettuce
2011	US (Pennsylvania)	NR[c]		16	Milk, ice cream
2011	Russia (Krasnoyarsk)		NR	9	Vegetables
2010	Finland	O:9		42	Salad, carrots
2010	Japan	O:9		3	Unknown
2008	Finland		O:1	50	Carrots
2008	Russia (Krasnoyarsk)		NR	141[d]	Unknown
2007	New Zealand	NR		6	Sausages
2007	Russia (Yamalo-Nenetsky)		NR	121	Vegetables
2007	Russia (Yagra)		NR	11[d]	Unknown
2006	Finland		O:1	402	Carrots
2006	Finland		O:1	42	Carrots
2005–2006	Norway	O:9		11	Brawn
2005	Russia (Far East)		NR	33	Cabbage
2005	Russia (Khakassia)		NR	13	Vegetables
2005	Russia (Novogord)		NR	24	Vegetables
2005	Russia (Siberia)		NR	59[e]	Vegetables
2004	Japan	O:8		42	Salad
2004	Finland		O:1	58	Carrots
2003	Finland		O:1	111	Carrots
2003	Finland	O:3		20	Unknown
2001	Finland		O:1, O:3	123	Lettuce
2001–2002	US (Chicago, Illinois)	O:3		12	Chitterlings

[a] *Y. enterocolitica*
[b] *Y. pseudotuberculosis*
[c] Not reported
[d] Number of hospitalised cases
[e] Number of patients from three outbreaks

could further increase the problem. In 2003, an outbreak due to *Y. enterocolitica* O:3 infections with more than 20 persons was reported in Finland (www.thl.fi/thl-client/pdfs/d6d63c66-9690-4f4d-9ee1-319bb5648eaf). The outbreak was revealed when unnecessary appendectomies were performed in three patients with severe abdominal pain. The outbreak was traced to a cafeteria, the infection source, however, remained unclear. In the US, an outbreak due to *Y. enterocolitica* O:3 acquired through contaminated chitterlings (pig intestine), a traditional winter holiday food in some areas in the US, has been reported in infants (Jones et al. 2003). *Y. enterocolitica* was probably transferred from raw chitterlings to infants through contact with the hands of food preparers. Recently, a *Y. enterocolitica* outbreak through bottled pasteurised milk and ice cream was notified in the US (www.promedmail.org; archive number: 20110828.2637). The contamination route of the bottled pasteurized milk and ice cream was not clear, but it was likely to be post-pasteurisation. The contaminated products originated from the same dairy farm.

8.5.3 Prevalence in Animals

Non-pathogenic *Y. enterocolitica* Are Common in Animals Numerous works have been carried out to study the presence of enteropathogenic *Yersinia* in a variety of animals including farm, pet, wild, and zoo animals (Fredriksson-Ahomaa et al. 2010). *Y. enterocolitica* strains have frequently been isolated from animal sources; however, these strains mostly differ both biochemically and serologically from strains isolated from humans with yersiniosis (Milnes et al. 2008; Söderqvist et al. 2012). Additionally, the important virulence genes are usually missing in animal strains. Human pathogenic strains of *Y. enterocolitica* typically have been isolated from slaughtered fattening pigs (Fredriksson-Ahomaa et al. 2007b; Ortiz Martínez et al. 2010; Vanantwerpen et al. 2013; Liang et al. 2012). Recently, it was shown that there can be a large variation in the within-batch prevalence among pig farms (Vanantwerpen et al. 2013).

***Y. enterocolitica* of Bioserotype 4/O:3 Is Frequently Isolated from Pigs** Fattening pigs are mostly asymptomatic carriers of human pathogenic strains, in particular strains of bioserotype 4/O:3, the most common type associated with human disease (Fredriksson-Ahomaa et al. 2006b). Strains of bioserotype 4/O:3 have frequently been isolated from the oral cavity of pigs at slaughter in Europe, especially from the tonsils, but also from submaxillar lymph nodes and intestinal content (Gürtler et al. 2005). This pathogen has also been isolated by swabbing from the surface of pig offal at the slaughterhouse (Fredriksson-Ahomaa et al. 2001a). In UK, the diversity of the bioserotypes found in pigs at slaughter has shown to be broad (Ortiz Martínez et al. 2010). Pathogenic *Y. enterocolitica* have also been detected on freshly slaughtered pig carcasses (Lindblad et al. 2007; Van Damme et al. 2013).

Prevalence of *Y. enterocolitica* O:9 Is on the Rise in Ruminants Human pathogenic *Y. enterocolitica* strains have sporadically also been detected in other animal sources like ruminants, e.g. cattle, sheep and goats (Lanada et al. 2005; McNally

et al. 2004; Gourdon et al. 1999). *Y. enterocolitica* O:9 is the second most frequently reported serotype among human *Yersinia* infections in Europe (EFSA and ECDC 2013; Fredriksson-Ahomaa et al. 2012b) and it seems that the prevalence of this type is on the rise in ruminants. It has regularly been isolated from cattle during the last decade in the EU. Recently, *Y. enterocolitica* bioserotype 2/O:9 was isolated for the first time from faeces of asymptomatic sheep (Chenais et al. 2012, in press). These *Yersinia*-positive sheep had falsely been tested positive for *Brucella* by serology. *Y. enterocolitica* O:9, however, can give a false-positive result in serological tests for *Brucella* due to their almost identical O-antigen structure with *Brucella* spp.

Dogs and Cats Are a Source of Human Pathogenic *Y. enterocolitica* Strains Dogs and cats have been shown to sporadically excrete *Y. enterocolitica* O:3 and O:5,27 strains in the faeces (Fredriksson-Ahomaa et al. 2001b; Fukushima et al. 1985). In China, strains of bioserotype 3/O:3 are a common finding in dogs (Wang et al. 2010). Recently, Stamm et al. isolated *Y. enterocolitica* in 4.6 and 0.3 % of dogs and cats, respectively (Stamm et al. 2013). They isolated strains of different bioserotypes, the most common type being 4/O:3 followed by 2/O:9 and 2/O:5,27, which are the common types associated with human disease. One reason for the relatively low rate of strains belonging to non-pathogenic biotype 1A might be that the faeces samples originated from animals with diarrhoea. Only non-pathogenic *Y. enterocolitica* 1A strains were isolated from asymptomatic stray dogs in Ireland (Murphy et al. 2010).

Pathogenic *Y. enterocolitica* Strains Have Sporadically Been Detected in Wildlife There is only a limited number of studies on the prevalence of *Y. enterocolitica* in wild animals in Europe. In Switzerland, hunted wild boars have been shown to sporadically carry typical human pathogenic *Y. enterocolitica* strains (bioserotypes 4/O:3, 2/O:5,27 and 2/O:9) in the tonsils (Fredriksson-Ahomaa et al. 2011) and recently, a pYV-positive *Y. enterocolitica* O:3 strain was isolated in the faeces of a hunted alpine ibex (Joutsen et al. 2013, in press). Recently, mallards and pheasants were studied in Poland, but only non-pathogenic *Y. enterocolitica* 1A strains were detected (Bancerz-Kisiel et al. 2012). A high seroprevalence (55 %) of yersiniosis has been detected in hunted hares in Germany (Frölich et al. 2003). In Japan, wild rodents have shown to play an important role as a reservoir of pYV-positive *Y. enterocolitica* O:8 strains (Hayashidani et al. 1995).

***Y. pseudotuberculosis* Is a Common Finding in Wildlife** *Y. pseudotuberculosis* strains have been isolated from diverse animal sources; the isolation rates, however, have been low (Fredriksson-Ahomaa 2009). *Y. pseudotuberculosis* has mostly been isolated from wildlife, especially from birds, rodents and their predators which are therefore considered as the most important reservoirs of this pathogen (Fredriksson-Ahomaa et al. 2009; Backhans et al. 2011; Vincent et al. 2008; Niskanen et al. 2003; Fukushima and Gomyoda 1991). The animals are usually asymptomatic carriers, but due to stress they can excrete a high number of *Y. pseudotuberculosis* in the faeces and thereby contaminate the environment. *Y. pseudotuberculosis* has also sporadically been isolated from pigs at slaughter and farmed game, especially from young animals (Slee and Skilbeck 1992; Ortiz Martínez et al. 2010).

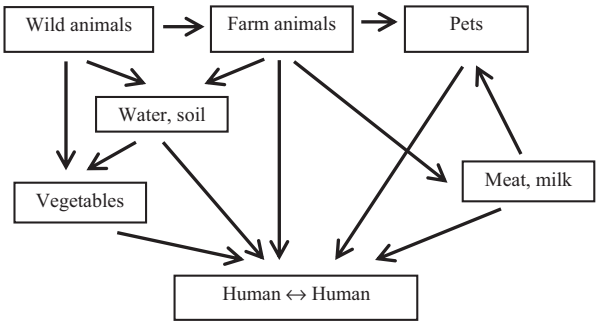

Fig. 8.1 Different transmission routes of enteropathogenic *Yersinia*

8.5.4 Prevalence in Food and Water

A High Prevalence of *Y. enterocolitica* Has Been Detected in Pork Products by PCR Enteropathogenic *Yersinia*, especially *Y. pseudotuberculosis,* have very seldomly been detected in food samples. However, pathogenic *Y. enterocolitica* have frequently been detected by PCR on pork tongues, in minced pork and pork sausages, but also sporadically in chicken and lettuce (Laukkanen-Ninios and Fredriksson-Ahomaa 2012; Fredriksson-Ahomaa et al. 2012b). When using culture methods, the detection rate for pathogenic *Y. enterocolitica* is much lower than rates obtained by using the much more sensitive PCR. Recently, *Y. enterocolitica* strains belonging to biotypes 2, 3 or 4 were isolated from raw milk in Mexico City (Bernardino-Varo et al. 2013). *Y. pseudotuberculosis* has sporadically been isolated from vegetables associated with outbreaks.

***Y. pseudotuberculosis* Has Been Isolated from Surface Water** Sporadic *Y. enterocolitica* infections in Norway have been associated with drinking of untreated water using a case-control study (Ostroff et al. 1994); however, this pathogen has seldomly been isolated from water (Laukkanen-Ninios and Fredriksson-Ahomaa 2012). Unlike *Y. enterocolitica*, *Y. pseudotuberculosis* has been isolated from the environment, especially from water (ponds, rivers, wells and spring water) (Fukushima et al. 1995; Kim et al. 2004). Surface water can easily be contaminated by faeces of infected wild animals (Fukushima et al. 1994; Tsubokura et al. 1989).

8.5.5 Transmission Routes

Both *Y. enterocolitica* and *Y. pseudotuberculosis* are primarily transmitted faecal-orally from an animal reservoir to humans by contaminated food and water, but direct animal contact is also a possible transmission route (Fig. 8.1). Transmission of enteropathogenic *Yersinia* may also occur from human to human directly or indirectly by blood transfusion.

***Y. enterocolitica* 4/O:3 is Primarily Transmitted via Contaminated Pork** For sporadic *Y. enterocolitica* 4/O:3 infections, contaminated pork and pork products

have been implicated as a major infection source (Tauxe et al. 1987; Fredriksson-Ahomaa et al. 2006a). Consumption of raw pork may play an important role in countries like Belgium, Germany and the Netherlands, where raw minced pork with pepper and onion is a delicacy that can be purchased as ready-to-eat food from butcher shops. However, transmission is more likely to occur via cross-contamination of cooked pork or foods not normally harbouring *Y. enterocolitica*.

***Y. pseudotuberculosis* Is Transmitted by Contaminated Vegetables** Both fresh produce and untreated surface water have shown to be important infection sources for *Y. pseudotuberculosis* infections. Wildlife faeces may have contaminated the vegetables during the storage or the vegetables have already been contaminated at the farm by contaminated irrigation water or soil. Untreated drinking water from wells, springs and stream contaminated with faeces of wild life has been associated with *Y. pseudotuberculosis* infections (Tsubokura et al. 1989; Fukushima et al. 1998; Han et al. 2003).

Enteropathogenic *Yersinia* can be Transmitted to Humans via Pets Companion animals like dogs and cats have been suspected as sources of human yersiniosis through close contact, especially for young children (Boqvist et al. 2009). Pathogenic *Y. enterocolitica* 4/O:3 are probably transmitted to dogs and cats via contaminated pork and offal (Fredriksson-Ahomaa et al. 2001a). Also *Y. pseudotuberculosis* has been transmitted from infected pets to humans (Fukushima et al. 1994). Direct contact with pigs, a common risk for pig farmers and slaughterhouse workers, may also be a transmission route (Seuri and Granfors 1992). Elevated serum antibody titers against *Yersinia* have been reported in pig farmers, butchers and slaughterhouse employees.

Person-to-person Transmission Occurs via Contaminated Hands These pathogens can also be transmitted from human to human either by direct contact or indirectly through blood transfusion. Direct transmission may occur when basic hygienic and hand-washing habits are inadequate (Okwori et al. 2009). Lee et al. (1990) reported *Y. enterocolitica* O:3 infections among infants who were probably exposed to infection by their caretakers. Indirect transmission may occur by transfusion of contaminated blood products (Sen 2000). In these cases, the most likely sources of *Yersinia* have been blood donors with subclinical bacteremia.

8.6 Conclusions

Y. enterocolitica and *Y. pseudotuberculosis* are important pathogens which cause enteral yersiniosis in humans and animals. These pathogens differ phenotypically and genotypically from each other, but cause a disease with similar symptoms; the animal reservoirs and transmission routes, however, may be different. For epidemiological studies, sensitive detection and discriminatory typing methods are needed. PCR is a useful method to be used in parallel with culture methods to screen

enteropathogenic *Yersinia* in animal reservoirs, food and environmental samples. Efforts should be made to develop better and standardised isolation methods for these pathogens. More accurate methods should also be designed for identification of *Yersinia* spp. and more information is needed about potential virulence of *Y. enterocolitica*- and *Y. pseudotuberculosis* -like strains.

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Chapter 9
Enterohemorrhagic *E. coli* (EHEC): Environmental-Vehicle-Human Interface

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Abstract Enterohemorrhagic *Escherichia coli* (EHEC) are a pathogenic subgroup of Shiga toxin-producing *E. coli* (STEC), and have demonstrated ability to cause severe intestinal disease and the hemolytic uremic syndrome (HUS). Cattle are the major reservoir of EHEC, where the bacteria can persist asymptomatically for years. Of particular concern are a small percentage of animals in herds that shed extremely high numbers of EHEC, termed 'supershedders', and are responsible for the majority of EHEC spread and contamination. Another transmission route is through the environment where EHEC can survive for weeks to many months, remaining viable in bovine feces, soil and water. EHEC contamination of meat during slaughter or processing, or contamination of plants via EHEC-containing water or manure are major routes of entry into the food chain. Several hundred outbreaks caused by EHEC O157 as well as non-O157 strains have been identified in industrialized countries worldwide. Current and future research efforts are focused on rapid outbreak identification, development of therapeutics, and implementation of preventative measures.

9.1 Introduction

Most members of the species *E. coli* are part of the physiological flora in the gastrointestinal tracts of humans and animals. In addition to these commensal bacteria, there are pathogenic *E. coli* that cause extraintestinal and intestinal disease. Intestinal pathogenic *E. coli* presently include seven pathogroups: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), adherent invasive *E. coli* (AIEC), diffusely adherent *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC) (Croxen et al. 2013). Each pathotype is associated with unique epidemiology and specific pathological diseases

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that cause significant morbidity and mortality. Zoonotic *E. coli*, of which EHEC are the prototype, pose many challenges to the food industry and public health and are intensively studied in human and veterinary medicine. Ongoing investigations are concerned with both ecology of EHEC in animals and persistence and survival in the environment, and how these factors affect entry into or dissemination along the food chain. Other areas of research are the epidemiology of EHEC infections in humans, diagnostics, pathogenic mechanisms of these bacteria and treatment as there is currently no specific therapy.

EHEC can cause a broad clinical spectrum of disease including watery or bloody diarrhea, and the hemolytic uremic syndrome (HUS), which is an important cause of acute renal failure in children (Tarr et al. 2005). Since the first isolation of an EHEC serotype O157:H7 outbreak strain in the USA in 1982 (Riley et al. 1983), and subsequent identification of involvement of this pathogen in outbreaks of hemorrhagic colitis and HUS (Wells et al. 1983), EHEC has emerged as an important public health concern worldwide. The large EHEC O104:H4 outbreak in Germany in 2011 with 3842 cases, 855 HUS patients and 53 deaths demonstrates the significant impact of an EHEC outbreak on human health (RKI 2011).

9.2 Expression of Shiga Toxins in EHEC

A key characteristic of the EHEC pathotype is the presence of Shiga toxins (Stx). Stx, also known as verocytotoxins (VTs), are members of a large family of cytotoxins that are characterized by a high degree of sequence diversity. The Stx family is divided into two major branches, Stx1 and Stx2, and many toxin subtypes and variants have been described in both branches (Karch et al. 2009; Bergan et al. 2012; Scheutz et al. 2012). Classification of Stx subtypes is used not only for taxonomic purposes, but also serves as an important predictor for the various clinically relevant Stxs found in strains associated with HUS versus other Stx subtypes that are carried by strains causing a milder course of disease (Scheutz et al. 2012). A sequence-based protocol for characterization of the Stx genes has been recently described (Scheutz et al. 2012), and includes three levels of classification: Types, subtypes and variants (see Table 9.1).

1. Types
   The two major branches Stx1 and Stx2 share structure and function but are not cross neutralized with heterologous antibodies. The terms Stx1 and Stx2 should only be used when the subtype is unknown.
2. Subtypes
   Currently the antigenically related members of Stx1 (Stx1a, Stx1c, and Stx1d) and Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) are distinguished.
3. Variants
   Variants include the subtype-specific prototypic toxins or related toxins within a subtype (that differ by one or more amino acids from the prototype). The

Table 9.1 Types, subtypes and variants of Shiga toxins according to Scheutz et al. (2012)

Types	Subtypes	Variants (examples)
Stx1	Stx1a	Stx1a-O157-EDL933
	Stx1c	Stx1c-O174-DG131-3
	Stx1d	Stx1d-ONT-MHI813
Stx2	Stx2a	Stx2a-O104-G5506
	Stx2b	Stx2b-O111-S-3
	Stx2c	Stx2c-O157-A75
	Stx2d	Stx2d-O91-B2F1
	Stx2e	Stx2e-O26-R107
	Stx2f	Stx2f-O128-T4-97
	Stx2g	Stx2g-O2-S86

variants are designated by toxin subtype, O-antigen group of the host E. coli strain, followed by the strain name or number from which that toxin was described, e.g. Stx1a-O157-EDL933 or Stx2a-O104-G5506 (Scheutz et al. 2012, see Table 9.1). Nucleotide variants within a given Stx subtype are italicized.

All Stx consist of a single A and five B subunits. The A subunit represents the enzymatically active component. The Stx B pentamer binds to the high and less effective cellular ligand glycosphingolipids (GSLs), globotriaosylceramide (Gb3Cer) and globotetraosylceramide (Gb4Cer), respectively (Müthing et al. 2009). Stx1 and Stx2 share identical binding specificity (Müthing et al. 2009). After binding to the cell surface, the AB_5-Gb3Cer complex is internalized by various endocytic mechanisms and routed from the early endosomes through the *trans*-Golgi-network and the Golgi stacks to the endoplasmic reticulum (Sandvig et al. 2010; Bauwens et al. 2013). Moreover, evidence suggests that Stxs (like other ribosome-inactivating proteins) remove adenine moieties not only from rRNA, but also efficiently depurinate DNA. Stx genes are found within the genomes of temperate bacteriophages, which are mobile elements that can easily integrate at specific sites in the bacterial chromosome. In vitro and in vivo studies have demonstrated that most EHEC can lose the Stx-encoding gene by bacteriophage excision during infection, isolation, or subculture, resulting in *stx*-negative isolates (Mellmann et al. 2009).

9.3 Epidemiology of EHEC in Animals

Several studies have demonstrated that cattle are the main reservoir of human pathogenic EHEC O157:H7, in addition to many pathogenic non-O157 EHEC serotypes (Naylor et al. 2005a). These bacteria have adapted to an oral-fecal cycle in cattle, where EHEC colonization begins with ingestion and subsequent entrance to the rumen and gastrointestinal tract, but they generally do not have a pathogenic effect on adult animals. EHEC has been reported to cause disease in young calves, however, in particular certain non-O157 serogroups (O26, O111, O118) (Naylor et al. 2005a). Prevalence among cattle varies widely, and may be due to several

circumstances including the geographical region, animal age, or the specific farm conditions (Ferens and Hovde 2011). Published prevalence rates vary dramatically, from 0 to 36 % among animals studied in different countries and farm types (Naylor et al. 2005a). Studies have also shown that EHEC prevalence is related to the type of farm (e.g. beef, dairy) and may be influenced by factors such as cattle movement, hygiene management, diet, and husbandry (Menrath et al. 2010; Cobbaut et al. 2009; Ferens and Hovde 2011). While cattle are the major known reservoir of EHEC, other minor reservoirs include sheep, goats, pigs, horses, dogs, poultry, and deer (Naylor et al. 2005a).

The persistence of EHEC O157:H7 in cattle may be due to its ability to colonize a particular niche within the lower gastrointestinal tract (Grauke et al. 2002). Tissue tropism for the colon has been demonstrated by immunofluorescent detection of microcolonies at the lymphoid follicle-dense mucosa at the terminal rectum within 3–5 cm proximal to the rectoanal junction (Grauke et al. 2002; Naylor et al. 2003, 2005b). This rectoanal junction colonization is hypothesized to be responsible for a highlevel of EHEC O157:H7 shedding (10^4 CFU/g of feces) in a minor subset of cattle which are termed ‘supershedders’ and are thought to be responsible for most of the pathogen spread in a farm environment (Menrath et al. 2010). In support of this theory, an association between rectoanal junction colonization and supershedding status has been described (Cobbold et al. 2007; Low 2005). Furthermore, EHEC O157 and non-O157 strains express several fimbrial and afimbrial proteins that likely play a role in ruminant reservoir persistence (Farfan and Torres 2012). In studies that used bovine terminal rectal primary epithelial cells, the H7 flagellum was demonstrated to act as an adhesin to bovine intestinal epithelium, supporting its involvement in the initiating step for colonization of the cattle reservoir (Mahajan 2009). Stx may also play a role in colonization and persistence by blocking the activation of bovine lymphocytes and thus supressing the bovine host’s immune response to the intestinal colonization (Moussay et al. 2006).

9.4 EHEC in the Environment

EHEC can survive in bovine feces long-term, making this a likely vehicle for transmission to cattle, food and the environment. Survival in feces can range from 1 to 18 weeks depending on the temperature (5, 15 and 25 °C were tested) (Fukushima et al.1999). Entry of EHEC to the environment may occur through direct deposit of feces onto land or through drainage runoff of fecal material in soil, especially after heavy rainfalls (Thurston-Enriquez et al. 2005). Moreover, under experimental conditions, EHEC can survive for more than 1 year in various manure-amended soils at different temperatures (Fremaux et al. 2008). Long-term survival of EHEC in lake water (13 weeks) and in cold river water has also been demonstrated (Wang and Doyle 1998; Maule 2000). This extended persistence in the environment likely plays a significant role in the colonization of cattle and subsequent human infection (Fremaux et al. 2008).

EHEC O157:H7 is also able to colonize various types of plants and fruits. For example, EHEC O157:H7 has been shown to form bacterial aggregates on apples (Janes et al. 2005) as well as on the surface of lettuce leaves (Seo and Frank 1999; Auty et al. 2005). Furthermore, studies have found EHEC in the internal inner tissues of plants, including radishes, carrots and lettuce (Itoh et al. 1998; Solomon et al. 2002). These subsurface localizations may be protective to the bacteria as they are inaccessible to other competitive bacteria as well as surface treatments and washing.

9.5 EHEC Infections in Humans

After ingestion of EHEC, a 3–12 day incubation period is typically followed by development of watery diarrhea accompanied with abdominal cramping and pain. Most patients will subsequently suffer from bloody diarrhea. About 1 week after the initial onset of diarrhea, HUS develops in a variable proportion of cases, depending on the serotype of the causative EHEC strain and the Stx subtype (Tarr et al. 2005). HUS patients present with widespread thrombotic microvascular lesions in the kidneys, the gastrointestinal tract, and other organs (Richardson et al. 1988). Since EHEC infections are rarely bacteremic, i.e. bacteria do not penetrate the circulatory system and are not found in patient blood cultures (Bielaszewska and Karch 2005), it is hypothesized that HUS results from vascular endothelial injury by circulating Stx. According to the generally accepted model of HUS pathogenesis, Stx is released by EHEC in the intestine, absorbed across the gut epithelium into the circulation (Hurley et al. 2001; Müthing et al. 2009), and transported to small vessel endothelial cells.

HUS is the most common cause of acute renal failure in children. The mortality rate can be up to 3% (Karch et al. 2005). While 70% of EHEC-infected patients were fully recovered within 5 years after diagnosis, the remaining 30% still experienced persistent hypertension (9%), neurological symptoms (4%), decreased glomerular filtration rate (7%), and/or proteinuria (18%) (Rosales et al. 2012). There is currently no effective causative therapy, and antibiotic treatment appears to be ineffective if not harmful (Wong et al. 2000; Davis et al. 2013). In contrast to cattle, EHEC O157:H7 colonizes humans only for a limited time of about 4 weeks (Fig. 9.1; Karch et al. 1995). Moreover, whereas in cattle many different EHEC O157:H7 PFGE subtypes can co-exist in a single animal (Jacob et al. 2011), human patients are infected mostly by a distinct O157:H7 PFGE subtype.

EHEC O157:H7 is the most prevalent EHEC serotype identified as a cause of sporadic HUS cases (Tarr et al. 2005; Karch et al. 2005). Still, non-O157:H7 EHEC (especially O26:H11, O103:H2, O111:H8, O145:H28/H25 and sorbitol-fermenting (SF) O157:H$^-$) represent a significant portion of EHEC infections leading to HUS complications (Karch et al. 2005; Mellmann et al. 2008; Bielaszewska et al. 2013).

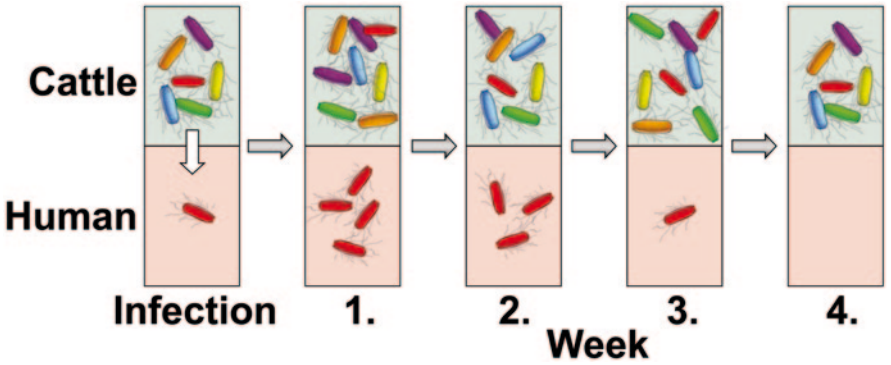

Fig. 9.1 Schematic illustration of EHEC O157:H7 infection in cattle and humans. In contrast to cattle, EHEC O157:H7 colonizes humans only for a limited time of about 4 weeks. Moreover, whereas in cattle many different EHEC O157:H7 PFGE subtypes can co-exist in a single animal, human patients are infected mostly by a distinct O157:H7 PFGE subtype. Different EHEC O157:H7 PFGE subtypes are indicated by different colors

Though EHEC strains are often considered as a pathogroup, there may be important differences between serotypes.

SF EHEC O157:H$^-$ represent a significant serotype in Europe which has not yet been detected in North America. These strains are characterized by a specific combination of their phenotypic and virulence characteristics that differentiates them from classical non-SF EHEC O157:H7 (Karch and Bielaszewska 2001). This combination includes the ability to ferment sorbitol overnight and to produce β-D-glucuronidase. A gene cluster termed *sfp*, which encodes fimbriae and mediates mannose-resistant hemagglutination, has been identified on the large plasmid of SF STEC O157:H$^-$ (Brunder et al. 2001). Notably, Sfp-encoding genes are absent in EHEC O157:H7.

The minimum infectious dose of EHEC in humans is extremely low, with approximately 10–50 bacteria needed for colonization (Teunis et al. 2004). In meat implicated as an outbreak source in the USA in 1993 there were less than 700 EHEC O157:H7 bacterial cells per hamburger patty prior to cooking (Tuttle et al. 1999). Moreover, a high degree of tolerance to acid and drying enables EHEC to survive in food items, the consumption of which had been previously considered safe with respect to the ability to cause foodborne illness (e.g., apple cider, semi-dry fermented sausage). Three principal routes of transmission of EHEC infection have been identified: (1) contaminated food and contaminated water used for drinking or swimming, (2) person-to-person transmission, and (3) animal contact, for example in petting zoos housing domesticated sheep, goats and other small animals or (occupational) farm exposure (Crump et al. 2002; Karch et al. 2005).

Table 9.2 Example of outbreaks caused by EHEC O157:H7

Year	Country	Cases/HUS/deaths[a]	Source	Reference
1982	USA	47/0/0	Hamburger[b]	Riley et al. 1983
1992–1993	USA	501/45/3	Hamburger[b]	Bell et al. 1994
1996	Scotland	345/34/16	Meat[b]	Dundas et al. 2001
1996	Japan	>6000/n.a./2	Radish sprouts	Watanabe et al. 1996
2000	Canada	~2300/28/7	Drinking water[b]	Hrudey et al. 2003
2005	Sweden	135/11/0	Lettuce	Söderström et al. 2008
2006	USA	199/31/3	Spinach[b]	CDC 2006
2006	USA	77/7/0	Iceberg lettuce	Sodha et al. 2011
2011	USA	15/4/2	Strawberries[b]	Laidler et al. 2013

n.a. Not available

[a] number of persons involved in the outbreak/number of HUS cases/number of deaths

[b] Strain isolated from the source

9.6 EHEC Outbreaks

EHEC is the cause of hundreds of outbreaks worldwide (Griffin et al. 1988; Michino et al. 1999; Karch et al. 1999). Examples of large outbreaks, including clinical impact and source, caused by EHEC O157:H7 and non-O157 are described in Tables 9.2 and 9.3, respectively. Consumption of raw or undercooked food items of bovine origin, particularly ground beef (hamburger), are common modes of EHEC O157:H7 transmission (Table 9.2). Moreover, contaminated radish sprouts, lettuce, spinach, strawberries, and contaminated water have been implicated in transmitting EHEC O157:H7 (Table 9.2).

One of the largest outbreaks to date occurred in Japan, in Sakai City, in 1996 (Watanabe et al. 1996; Michino et al. 1999), where thousands were affected, mostly school children. White radish sprouts served during school lunches were the most probable vehicle of the infection. In the winter 1992–1993, the largest outbreak of EHEC O157:H7 infection in the United States affected 501 persons in four western states including Washington, Idaho, Nevada and California (Bell et al. 1994) where 45 persons, mostly children, developed HUS and three children died. Hamburgers from a single fast-food restaurant chain were identified as the vehicle of the infection (Bell et al. 1994). The largest outbreak caused by contaminated drinking water occurred in Canada in 2000. Approximately 2300 people became seriously ill and seven died from exposure to drinking water contaminated with EHEC O157:H7. In Europe, a large EHEC O157:H7 outbreak occurred in Central Scotland in 1996; 345 people contracted an infection after consuming meat from a single butcher's shop, and 16 died (Dundas et al. 2001).

Table 9.3 describes several examples of large outbreaks caused by non-O157 EHEC strains. These include a wide range of serotypes, with the largest non-O157 outbreak occurring in Germany in 2011 associated with the contamination of fenugreek sprouts by EHEC O104:H4 (RKI 2011; Karch et al. 2012).

Table 9.3 Examples of outbreaks caused by non-O157 EHEC

Year	Serotype	Country	Cases/HUS/Deaths[a]	Source	Reference
1994	O104:H21	USA	18/0/0	Past. cow milk	CDC 1994
1995	O111:H$^-$	Australia	n.a./20/1	Sausage[b,c]	Paton et al. 1996
1999	O111:H8	USA	55/2/0	Salad bar	Brooks et al. 2004
2001	O26:H11	Germany	11/0/0	Beef	Werber et al. 2002
2004	O111:H$^-$	USA	27/0/0	Apple cider	Schaffzin et al. 2012
2006	O103:H25	Norway	17/10/1	Mutton sausage[b]	Schimmer et al. 2008
2007	O26:H11	Denmark	20/0/0	Beef sausage[b]	Ethelberg et al. 2009
2007	O145 + O26	Belgium	12/5/0	Ice cream[b]	De Schrijver et al. 2008
2009	O145:H28	Norway	16/0/0	Sheep	Wahl et al. 2011
2010	O145:H$^-$	USA	33/3/0	Romanian lettuce[b]	CDC 2010
2011	O104:H4	Germany	3842/855/53	Fenugreek sprouts	RKI 2011

n.a. not available

[a] number of persons involved in the outbreak/number of HUS cases/number of deaths

[b] Strain isolated from the source

[c] "Mettwurst", German sausage made from raw minced pork

9.7 Future Strategies and Unresolved Issues

Advances in rapid alert systems for the early detection of EHEC outbreaks have created greater awareness for both the public as well as the clinical community. Moreover, an increasing number of clinical microbiological laboratories routinely screen for EHEC by detection of Stx genes and/or toxin production. Diagnosed cases are now legally required to be reported in nearly every country. New high resolution techniques including next generation sequencing (NGS) are becoming more accessible and widely used, which enable the rapid identification of outbreaks at the earliest stages (Mellmann et al. 2011). In the future, databases and nationwide reporting systems could be in place to facilitate outbreak prevention and public health. The value of such strain linkage analysis is obvious. Common sources of infection can be identified accurately and rapidly. This is especially important considering the emerging epidemiology of foodborne infections. In particular, foodborne outbreaks nowadays less frequently follow the "church picnic" model, in which small isolated clusters of illness can easily be identified with case interviews. Instead, current outbreaks now more frequently result from the dissemination of vehicles that are contaminated by relatively low levels of pathogens. Such outbreaks can occur across state lines and international borders.

Another area where considerable efforts are being expended to bring improvement are the farming practices and environmental factors that affect infection of animals with EHEC. EHEC transmits readily between ruminants in the farm setting and wild animals can represent important vectors. For many years, the cattle industry and researchers have focused on improving the safety of meat products after slaughter. Postslaughter antimicrobial treatments of carcasses and HACCP policies in slaughter plants have been shown to significantly reduce meat contamination (Elder et al. 2000).

Due to the widespread distribution of EHEC O157 and non-O157 in farm cattle, its control will require intervention at the individual farm level. Recently, two vaccines against EHEC O157:H7 that are designed for use in cattle have been developed. While use of these vaccines could reduce the risk of EHEC in cattle by 50 %, which translates to approximately 85 % reduction in human cases, these vaccines have not yet been widely accepted by farmers due to several factors including burden of responsibility and economic factors (Matthews et al. 2013). An alternative route for the control of EHEC in cattle may be the feeding of probiotic bacteria, which can compete and interfere with pathogenic strains by producing metabolites that are inhibitory to EHEC. Still, more research is needed to develop viable strategies targeting the different levels (cattle, food, person-to-person spread, etc.) to control EHEC.

Further research is also needed to address effective therapies for humans after EHEC infection. Ongoing investigations are focused on topics such as toxin binders and Stx neutralizing immunoglobulin preparations.

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Chapter 10
Listeriosis: The Dark Side of Refrigeration and Ensiling

Franz Allerberger, Zoltán Bagó, Steliana Huhulescu and Ariane Pietzka

Abstract In contrast to most pathogenic bacteria, *Listeria monocytogenes* is *psychrotrophic*, capable of multiplying at low temperatures. In an era when food production and food storage heavily rely on refrigeration, this ability to grow (albeit slowly) in a cold environment has opened a new ecological niche for *L. monocytogenes*. Because of the severity of certain clinical manifestations (infections of the central nervous system, septicemia, and abortion), the high case-fatality rate (up to 30% of cases), and the long incubation time, human listeriosis is now a zoonosis of major public health concern. *L. monocytogenes* causes invasive illness mainly in certain well-defined high-risk groups, including immunocompromised persons, pregnant women, neonates, and the elderly. However, listeriosis can occur in otherwise healthy individuals, particularly during an outbreak. The evolvement of silage as a dominant feed for ruminants constitutes another key factor, responsible for the emergence of listeriosis as a relevant animal disease. *L. monocytogenes* has been isolated from numerous species of mammals, birds, fish, crustaceans, and insects. Nevertheless, the primary habitats of *L. monocytogenes* are considered to be the soil and decaying vegetable matter, in which it survives and grows saprophytically.

10.1 Introduction

The genus *Listeria* is presently composed of ten species: *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. rocourtiae, L. marthii, L. fleischmannii, L. weihenstephaniensis* and *L. ivanovii,* the latter comprising *L. ivanovii* subsp. *ivanovii and L. ivanovii* subsp. *londoniensis* (Leclercq et al. 2010; Bertsch et al. 2012). Although *Listeria* spp. are basically environmental bacteria, two species—*L. monocytogenes* and *L. ivanovii*—are pathogenic for animals and humans. In the genus, *L. monocytogenes* is the most commonly isolated member responsible

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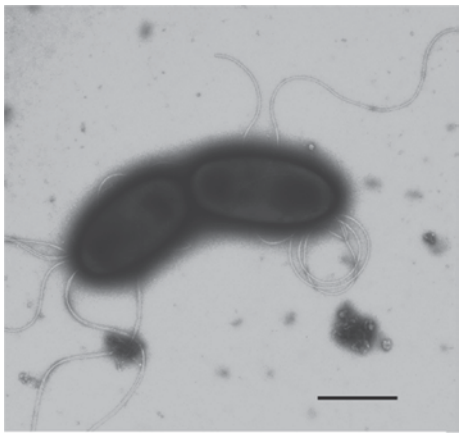

Fig. 10.1 Transmission electron microscopy image of *L. monocytogenes* grown in liquid culture at 20 °C showing flagellated bacteria. The bar is 1 μm in size. (Image gratuity: S. Richter)

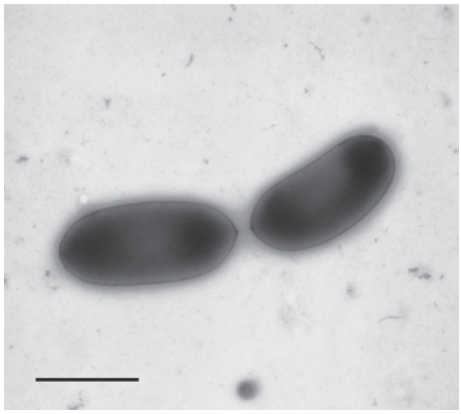

Fig. 10.2 Transmission electron microscopy image of *L. monocytogenes* grown in liquid culture at 36 °C showing bacteria without flagellae. The bar is 1 μm in size. (Image gratuity: S. Richter)

for listeriosis in humans and animals. Occasional human infections are also due to *L. ivanovii*, which is mainly responsible for abortion in sheep. In contrast to most pathogenic bacteria, listeria are *psychrotrophic*, capable of multiplying at low temperatures as applied in refrigeration. In an era when food production and food storage heavily rely on refrigeration, this ability to grow (albeit slowly) at low temperatures has opened a new ecological niche to a pathogen that previously had mediocre relevance only. Industrialized food manufacturing also constitutes an ecological niche due to the ability of *L. monocytogenes* to form biofilms for colonization of surfaces (Jemmi and Stephan 2006). The evolvement of silage as a dominant feed for ruminants during the mid-twentieth century constitutes another key factor, responsible for the emergence of listeriosis as a relevant animal disease. H.P.R. Seeliger even dubbed this zoonosis a "man-made disease" (Allerberger et al. 1997).

Listeria monocytogenes is a facultative anaerobic, rod-shaped Gram-positive bacterium that is motile when cultured at 20 °C and immotile when grown at 36 °C (Figs. 10.1 and 10.2). It is able to produce severe sepsis, meningoencephalitis, and a wide variety of focal infections in animals and in humans. *L. monocytogenes*, the

causative agent of listeriosis, was discovered in 1927 by Murray and Pirie, working independently of each other on outbreaks among laboratory rabbits and guinea pigs (Rocourt 1999). The first-documented case on human listeriosis involved a soldier who suffered from meningitis at the end of World War I (McLauchlin 1997). *L. monocytogenes* was not considered a significant animal pathogen until the late 1970s and early 1980s when it was recognized as a major foodborne pathogen (Paoli et al. 2005). Because of the severity of certain clinical manifestations (infections of the central nervous system, septicemia, and abortion), the high case-fatality rate (up to 30 % of cases), and the long incubation time, human listeriosis is now a zoonosis of major public health concern (LeMonnier and Natas 2012). Outbreaks in humans associated with contaminated coleslaw, soft cheese, ready-to-eat meat products like jellied pork or cold cuts, chocolate milk, rice salad, corn salad, sprouts, precut celery and melons have been reported (Allerberger 2007; Pichler et al. 2011). It is now recognized that most cases of listeriosis, both sporadic cases and common-source outbreak cases, are caused by *L. monocytogenes*-contaminated food or feed. Although rare, infection can also be transmitted directly from infected animals to humans as well as between humans and between animals.

10.2 Pathogenicity

Although Murray recognized the oral route of infection in his original isolation of *L. monocytogenes* in the 1930s, the key to recognizing the organism as a foodborne pathogen came nearly 60 years later, when an outbreak of listeriosis was epidemiologically linked to the consumption of contaminated coleslaw (Paoli et al. 2005). After ingestion of *L. monocytogenes*-contaminated food or feed, bacteria pass through the stomach and cross the intestinal barrier, presumably via M-cells. Entry into mammalian cells is mediated by surface invasion proteins such as internalin A (InlA), internalin B (InlB) and internalin C (InlC) (Lee et al. 2012b). The listerial protein internalin A (InlA) mediates bacterial adhesion and invasion of enterocytes in the human intestine through specific interaction with its host cell receptor E-cadherin, an adhesion molecule located at adherens junctions between epithelial cells (Lecuit et al. 2001). E-cadherin was identified as InlA receptor in 1996 (Mengaud et al. 1996). The importance of InlA for the entry of *L. monocytogenes* into non-phagocytic cells was demonstrated in 1991, when *L. monocytogenes* InlA was shown to confer to *L. innocua* the ability to enter human Caco-2 cells, cells originating from a human epithelial colorectal adenocarcinoma (Cossart et al. 2003). Internalin B (InlB) is another surface protein of *L. monocytogenes*. It contributes to invasion into a wider range of cell types such as endothelial cells, hepatocytes and fibroblasts owing to the ubiquitous nature of its receptor, the hepatocyte growth factor receptor Met (Lee et al. 2012b). InlB is responsible for internalization into Vero cells (originating from African green monkey kidneys), HeLA cells (originating from a human cervical adenocarcinoma), and CHO cells (originating from Chinese hamster ovary) (Lecuit et al. 1997). Internalin C (InlC) contributes to cell-to-cell

dissemination between polarized epithelial cells by decreasing cortical tension at apical junctions (Rajabian et al. 2009).

The intestine is the primary port of entry for *L. monocytogenes*, but questions about the exact mechanisms by which *L. monocytogenes* transgresses the intestinal barrier remain and clear differences among host species exist (Hoelzer et al. 2012). In host species deficient of functional E-cadherin such as mice, *L. monocytogenes* is thought to translocate through the intestinal wall by gaining access into M-cells, phagocytic cells in the Peyer's patches of the ileum. In species such as humans or guinea pigs that possess functional E-cadherin, *L. monocytogenes* is thought primarily to invade the epithelium of the intestinal villi, followed by bacterial replication in the underlying lamina propria. *L. monocytogenes* then rapidly translocates across the intestinal barrier, without a need for bacterial replication in the intestinal wall (Hoelzer et al. 2012).

After crossing the intestinal barrier, *Listeria* spp. are—within minutes of oral inoculation—transported by lymph or blood to the mesenteric lymph nodes, the spleen, and the liver. *L. monocytogenes* and *L. ivanovii* are facultative intracellular pathogens, which are able to replicate in macrophages and a variety of nonphagocytic cells, such as epithelial and endothelial cells, and in hepatocytes. After entering these cells, listeria escape early from the phagocytic vacuole, multiply in the host cell cytosol, and then move through the cell by induction of actin polymerization. The bacteria then protrude into cytoplasmic evaginations, and these pseudopod-like structures are phagocytosed by the neighboring cells (Schmid and Hensel 2004). When listeria enter cells, they not only trigger actin and membrane rearrangements but they also use clathrin (Lebreton et al. 2011).

All major virulence factors of *L. monocytogenes* and *L. ivanovii* are involved in a single process: the cell-to-cell spread. By this function, the pathogen can avoid extracellular environments and can escape humoral efforts of the immune system during their dissemination in the host. In *Listeria* species three virulence gene clusters have been identified to date and termed *Listeria* pathogenicity island 1, 2 and 3 (LIPI-1, LIPI-2 and LIPI 3). *Listeria* pathogenicity island 1 (LIPI-1) refers to a genomic region flanked by *prs* and *ldh* and harbouring several well-known virulence genes (*prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*) in a 9-kb gene cluster (Vazquez-Boland et al. 2001). LIPI-1 was identified in *L. monocytogenes*, *L. seeligeri* and *L. ivanovii*. The *hly* gene encodes the pore-forming listeriolysin O (LLO), a thiol-activated hemolysin, which is able to lyse erythrocytes and other cells in a cholesterol-dependent manner. LLO is an essential virulence factor of *L. monocytogenes*, and its inactivation leads to avirulence. The action of LLO is needed to disrupt the phagocytic vacuole for release of bacteria into the cytoplasm. Ribet et al. recently showed that *L. monocytogenes* is able to dampen the host response by decreasing the SUMOylation level of proteins critical for infection (Ribet et al. 2010). Also this event is triggered by the bacterial virulence factor LLO. A second island of 22 kb was termed LIPI-2. LIPI-2 is specific for *L. ivanovii* and may play a role in the tropism of this pathogen for ruminants (Gonzalez-Zorn et al. 2000). LIPI-3 is the most recently identified pathogenicity island; it contains the eight gene cluster (*llsA*, *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, *llsD*, and *llsP*) encoding listeriolysin S (LLS), a bacteriocin-like modified peptide exhibiting haemolytic and cytotoxic activities

(Clayton et al. 2011). In contrast to LIPI-1 which is found in all strains of *L. monocytogenes* as well as in other *Listeria* species, LIPI-3 is possessed only by a subset of *L. monocytogenes* lineage I (Lee et al. 2012b).

Studies on the genetic diversity of strains associated with outbreaks showed the presence of a homogeneous genetic background (Herd and Kocks 2001; Tran and Kathariou 2002; Wagner and Allerberger 2003). Outbreak-associated strains of a particular genetic background are called epidemic clones (ECs) and monitoring their presence in foodstuffs is of particular concern for veterinary and human public health. The recurrent association of epidemic clones with outbreaks can reflect enhanced virulence, but could also be due to high prevalence of these strains in food processing environments and foods. ECs are defined as *L. monocytogenes* isolates of a presumably common ancestor that are genetically related and involved in different temporally and geographically unrelated outbreaks (Cheng et al. 2008). Presently, there are seven identified ECs of *L. monocytogenes*, named ECI till ECVII (Lomonaco et al. 2013). However, the variability in *L. monocytogenes* with respect to traits of virulence and pathogenicity is far from being understood. A 50-kb listeria genomic island 1 (LGI1), which was described in *L. monocytogenes* isolates from a listeriosis outbreak in Canada in 2008 as a main factor for pathogenicity and bacterial persistence (Gilmour et al. 2010), was not detected in any of 383 *L. monocytogenes* isolates from Switzerland tested in 2012 (Bertsch et al. 2014).

10.3 Epidemiology of Listeriosis in Animals

L. monocytogenes has been isolated from numerous species of mammals, birds, fish, crustaceans, and insects. Nevertheless, the primary habitats of *L. monocytogenes* are considered to be the soil and decaying vegetable matter, in which it survives and grows saprophytically (Bille 2007).

Animal models have played fundamental roles in elucidating the pathophysiology and immunology of human listeriosis (Lecuit 2007). Such tests include intraperitoneal inoculation of mice, inoculation of the chorioallantoic membranes of embryonated eggs, and inoculation of the conjunctivae of rabbits (Anton test). Data derived from animal studies helped to characterize the importance of cell-mediated immunity in controlling infection, allowed evaluation of antimicrobial treatments for listeriosis, and contributed to quantitative assessments of the public health risk associated with *L. monocytogenes* contaminated food commodities (Hoelzer et al. 2012). However, data about species-specific differences have raised severe concern about the validity of most traditional animal models of listeriosis (Disson et al. 2008).

Even though *L. monocytogenes* can infect a wide variety of animal species, listeriosis is primarily a clinical disease of ruminants. Sheep appear to be particularly susceptible to infection, but listeriosis is also common in a variety of other polygastric species and *L. monocytogenes* has for instance been isolated from cattle, goats, llamas, alpacas, deer, reindeer, antelopes, water buffalos and moose (Hoelzer et al. 2012). Listeriosis represents one of the most common etiologies for encephalitis

among adult ruminants. Ruminants affected by encephalitis generally show marked neurological symptoms including ataxia, circling, opisthotonus, and paralysis of cranial nerves, combined with hyperthermia, anorexia and depression. Large epidemics of third trimester abortions, typically manifested as stillbirth, as well as atypical manifestations such as conjunctivitis have also repeatedly been described (Ryser and Marth 2007). With the exception of neonates and young ruminants, septicemia is unusual, but can result in mastitis, gastroenteritis, hepatitis, or pneumonitis. Notably, in a given affected herd listeriosis generally exhibits a single clinical manifestation (Ryser and Marth 2007).

Clinical listeriosis is relatively rare in most monogastric mammals such as dogs, cats, horses and pigs, but appears more common in rodents and lagomorpha. Listeriosis in monogastric mammals is predominantly manifested as septicemia. Abortion, meningoencephalitis and other manifestations such as conjunctivitis are also possible, but their relative frequency differs by animal species. Large outbreaks of listeriosis have been reported among colonies of captive rodents and lagomorpha, including chinchillas, rabbits, rats and guinea pigs (Ryser and Marth 2007). Contaminated feed such as silage or sugar beets was implicated as the outbreak vehicle in many of these outbreaks, and coprophagy may have contributed to some of these events (Ryser and Marth 2007). In a pregnant primate model, oral administration of *L. monocytogenes* resulted in stillbirth with isolation of the bacterium from placental and fetal tissues (Smith et al. 2003).

Among ruminants, listeriosis occurs seasonally with the highest incidence in winter and spring, and appears strongly associated with ingestion of spoiled silage, although cases do occur where silage feeding has not been used (Ryser and Marth 2007; Sanaa et al. 1993). Silage is high-moisture fodder that can be fed to ruminants. It is fermented and stored in a process called ensiling, and is usually made from grass crops, using the entire green plant (not just the grain) [http://en.wikipedia.org/wiki/Silage]. The ensiled product retains a much larger proportion of its nutrients than if the crop had been dried and stored as hay. Silage undergoes anaerobic fermentation, which starts about 48 h after the silo is filled. While properly produced silage is largely free of listeria, spoiled silage, often at the end of the silage-feeding period, can harbor high numbers of *L. monocytogenes*. Poor quality is often due to insufficient herbage quality or to contamination by soil or feces. The change to production of silage in polythene bales (“big bales”) corresponded to increases in ovine listeriosis in the UK (McLauchlin 2011). According to McLauchlin, the “big bale” method is more prone to spoilage and growth of *L. monocytogenes*: high numbers are often associated with sites where the damage to the bags has occurred or at the tied end. Figure 10.3 presents the number of German listeriosis reports in humans, sheep and cattle, based on data from 2007 to 2012 stratified by month of reporting, i.e. mean number of cases reported during the 6 year period for the respective month (data provided by Prof. Dr. Franz J. Conraths, Friedrich-Loeffler-Institut, Wusterhausen). Figure 10.4 gives the number of listeriosis-cases diagnosed in humans and in farm ruminants submitted for necropsy to the Institutes for Veterinary Disease Control of the Austrian Agency for Health and Food Safety, based on data from 2007 to 2012 by month of reporting, again showing strong seasonality for animal-listeriosis. The peak in the numbers of animal listeriosis in winter (goats)

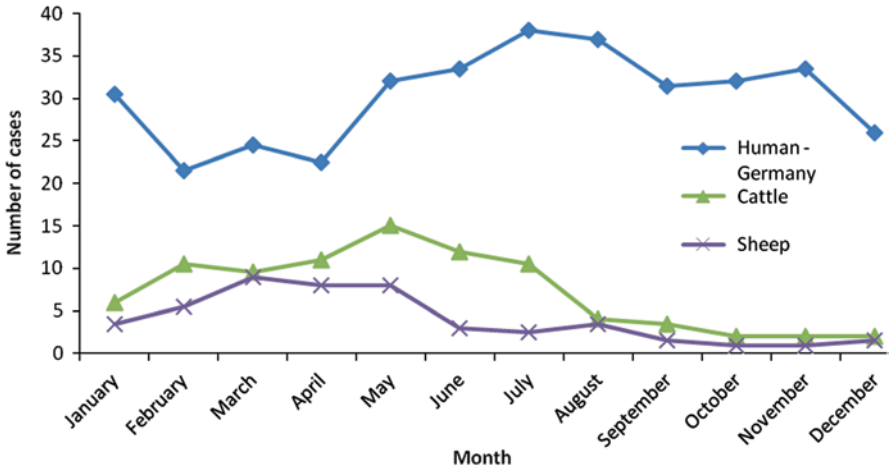

Fig. 10.3 Mean number of listeriosis reports in Germany in humans, cattle, and sheep, based on data from 2007 till 2012 stratified by month of reporting. In contrast to strong winter-spring-seasonality in ruminants, occurrence of human listeriosis shows only minor seasonal variation, with a peak in summer/autumn

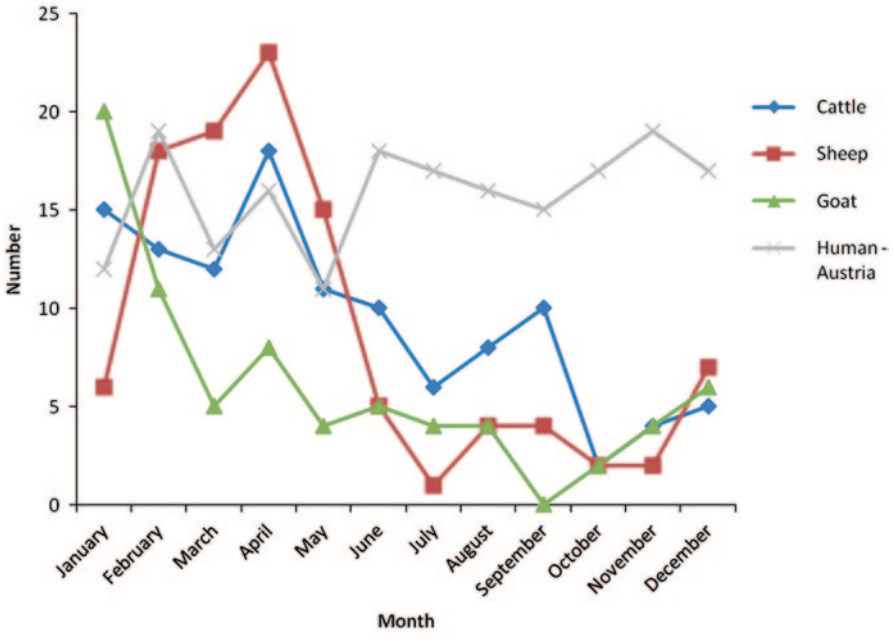

Fig. 10.4 Number of listeriosis-cases in humans ($n=190$) (according to date of receipt of the respective initial isolate at the national reference centre) and in farm ruminants ($n=293$), diagnosed by bacterial isolation or morphologically at the Austrian Agency for Health and Food Safety from 2007 till 2012 stratified by month of reporting. In contrast to strong seasonality in ruminants, occurrence of human listeriosis lacks significant seasonal variation

and in spring (cattle and sheep) may reflect a seasonal decrease in the quality of silage used for feed.

Animals may also be asymptomatic intestinal carriers and can shed the organism in significant numbers, contaminating the environment (Ho et al. 2007). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010 lists the following rates of fecal carriage of *L. monocytogenes*: cattle 5.5 %; pigs 0.0 %; sheep 7.0 %; goats 10.5 %; fowl (*Gallus gallus*) 0.4 %; water buffalo 3.7 %; and wild rodents 5.3 % (EFSA 2012). As observed in 2009, the highest proportions of positive findings were found in decreasing order in goats, sheep and cattle. In comparison to 76 cattle- and 39 sheep-isolates of *L. monocytogenes, L. ivanovii* was cultured from only one cattle and two sheep (Allerberger 2007). The 2001 report also reported rates of fecal carriage of *L. monocytogenes* for horses (fecal carriage rate: 2.1 %), red deer (8.3 %); farmed rabbit (0.7 %), cats (0.7 %), and dogs (0.0 %) (Allerberger 2007).

In most countries listeriosis in animals is not a notifiable disease. In Europe, listeriosis in animals is notifiable only in Germany, Finland, Sweden, and Norway. Usually, surveillance in animals is based on clinico-pathological observations.

10.4 Epidemiology of Listeriosis in Humans

The large majority of listeriosis cases (sporadic and outbreak-related) are caused by foodborne transmission, which—according to Scallan et al. (2011)—accounts for 99 % of human cases. In neonatal infections, *L. monocytogenes* can be transmitted from mother to child in utero or during passage through the infected birth canal. There are rare reports of nosocomial transmission in the nursery attributed to contaminated material or patient-to-patient transmission via healthcare workers (Hof and Lampidis 2001; Roberts et al. 1994). Hospital cross-infection between newborn infants occurs, usually originating form an infant born with congenital listeriosis. There is little evidence for cross-infection or person-to-person transmission outside the neonatal period.

Rarely listeriosis may be transmitted by direct contact with infected animals or animal material. Usually such local infections present as cutaneous lesions on the upper arms or wrists of farmers or veterinarian 1–4 days after attending bovine abortions but not in association with sheep (McLauchlin 2011).

Invasive illness mainly mainfests in certain well-defined high-risk groups, including immunocompromised persons, pregnant women, neonates and the elderly. However, listeriosis can occur in otherwise healthy individuals, particularly during an outbreak.

As stated above (Figs. 10.3 and 10.4), occurrence of human listeriosis lacks significant seasonal variation. The number of human cases of listeriosis exhibits only slight seasonal peak during the late summer into the fall (Wagner and McLauchlin 2008, McLauchlin 2011; Feng et al. 2013).

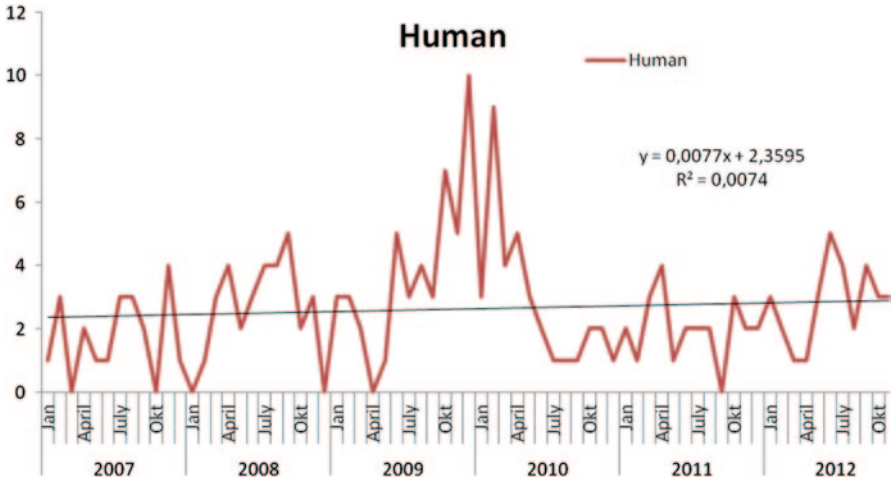

Fig. 10.5 Number of *L. monocytogenes* isolates from invasive cases registered at the Austrian National Reference Centre, based on data from the years 2007 to 2012 (by month of receipt of isolate). Two major outbreaks were documented during this period: one in August/September 2008 due to jellied pork and one from August 2009 till February 2010 due to sour milk curd cheese. The peak in June 2012 was due to isolates of serovar 1/2a (×2), 1/2b (×2) and 4b (×1), with PFGE-pattern clearly distinguishable from each other. ("pseudo-outbreak")

Investigation of several outbreaks has demonstrated that all epidemic listeriosis was caused by foodborne transmission of *L. monocytogenes*. Outbreaks of listeriosis have been associated with the ingestion of raw milk, soft cheeses, contaminated vegetables and ready-to-eat meat products such as paté. Also the sporadic cases of listeriosis mostly result from foodborne transmission. In several cases, by tracing a strain of *L. monocytogenes* isolated from a patient to a food item in the patient's refrigerator, and then to the retail source, public health officials were even able to provide microbiological confirmation of foodborne transmission of sporadic listeriosis (Pinner et al. 1992). Eating soft cheeses or food purchased from store delicatessen counters and eating undercooked chicken have been shown to increase the risk of sporadic listeriosis (Schuchat et al. 1992; Pinner et al. 1992).

According to the European Union Summary Report, the age distribution of listeriosis cases in 2010 was similar to that observed in previous years (EFSA 2012). The notification rate was highest in those aged over 65 (1.21 cases per 100,000 population), covering 60.2 % of all reported cases. Out of 1595 confirmed reported cases, the age group over 65 accounted for most of the cases (60.3 %), while 6.7 % of cases were detected in the age group 0–4 years and the majority of these cases (96.3 %, $N = 108$) were in infants (age < 1 year) (EFSA 2012).

Figure 10.5 presents the number of human invasive *L. monocytogenes* isolates registered at the Austrian National Reference Centre from 2007 to 2012. Since 2000, the incidence of listeriosis has increased in at least eight European countries (Goulet et al. 2008; Allerberger and Wagner 2010). Increases have occurred mainly among persons > or = 60 years of age and appear to be most pronounced for persons > or

=70 years of age. The cause of this selective increased incidence is unknown. In 2010, 26 member states reported 1601 confirmed human cases of listeriosis (EFSA 2012). The overall EU notification rate was 0.35 cases per 100,000 population, with the highest country-specific notification rates observed in Finland (1.33 cases per 100,000 population) followed by Denmark and Spain (1.12 cases per 100,000 population). Within each reporting member state, statistically significant increasing trends in listeriosis notification rates from 2006 to 2010 were noted in Austria, Latvia and Spain. The reason for the increased incidence and the upsurge in septicaemia cases remain unknown. It has been hypothesized, however, that the higher incidence of listeriosis might be related to higher exposure to *L. monocytogenes* (Gillespie et al. 2006; Goulet et al. 2008). Increasing use of acid inhibitors (H_2 receptor antagonists) was also postulated to contribute to increased vulnerability to *L. monocytognes* infection (Gillespie et al. 2009). Statistically significant decreasing trends were noted in Belgium, the Czech Republic, Luxembourg, and Slovakia (EFSA 2012). In the United States in 2008, the incidence of all cases was 0.3 cases per 100,000 population; the United States plan to reduce the overall incidence of invasive listeriosis to below 0.2 cases per 100,000 population by 2020 (Cartwright et al. 2013).

Sisó et al. studied the incidence of listeriosis during pregnancy over a 25 year period based on data compiled in a tertiary referral hospital in Spain. Whereas between 1985 and 2000 the incidence remained almost constant at 0.24‰, an increasing incidence was observed from then on, reaching 0.86‰ during the last years until 2010 (Sisó et al. 2012). According to their findings, a four-fold increase in listeriosis rate during pregnancy has occurred in recent years in Spain. No such increase has been reported for pregnancy-associated cases from other countries. In Austria in 2012, listeriosis occurred in four of approximately 80,000 births (0.05‰).

In the European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010, the transmission route was identified for 132 (8.26%) confirmed cases (EFSA 2012). Of those, 87 cases were infected with *L. monocytogenes* via suspected food, and 43 cases were pregnancy-associated. One case was reported as transmission by contact with animals and one as other (not specified) transmission. Of the cases infected via consumption of contaminated food, cheese was mentioned as the suspected vehicle for 13 cases, milk and fish for one case, while for the remaining cases no information on the food source was provided. In total, 98% of confirmed *L. monocytogenes* cases with known importation status (reported for 83% of cases) were of domestic origin. The validity of such data originating from statutory reporting can be critically questioned.

Despite the high contamination rates of certain food, listeriosis is a relatively rare disease compared to other common foodborne illnesses such as campylobacteriosis or salmonellosis. Because of its high case fatality rate of approximately 20%, human listeriosis ranks among the most frequent causes of foodborne death (Allerberger and Wagner 2010). Therefore, besides the economic consequences, listeriosis remains a great public health concern. The substantial burden of listeriosis in Europe is anticipated to increase in line with the projected growth of the elderly

population. The proportion of people aged at least 65 is expected to reach 20% by 2020 and almost 30% by 2050 (European Commission 2012).

Control of listeriosis requires action from public health agencies and from the food industry. Important control strategies from public health agencies include developing and maintaining timely and effective disease surveillance programmes, as well as promptly investigating clusters of listeriosis cases. Routine characterization of human, animal, food, and environmental isolates, and utilization of large-scale subtype-databases will facilitate Europe-wide outbreak detection and control in the near future (Allerberger and Wagner 2010). Outbreak investigations provide a unique source of information to improve our understanding of transmission of listeria and to identify gaps in industry and regulatory measures to safeguard against contamination of the food and feed supply. In this respect, the importance of isolating the pathogen as a prerequisite for an accurate epidemiological investigation and ultimately stopping transmission cannot be overemphasised (Cartwright et al. 2013).

10.5 Epidemiology of *Listeria monocytogenes* in Food

Listeria monocytogenes is a particularly important cause of illness, mainly found in foods that are packaged and prepared commercially, rather than those prepared in the home (Carpentier and Cerf 2011). During the last decades, consumer lifestyles have changed with less time for food preparation and more ready-to-eat (RTE) and take-away foods. Changes in food production and technology have led to the production of foods with a long shelf life that are typical "listeria risk foods," because the bacteria have time to multiply, and the food does not undergo a listericidal process such as cooking before consumption. Swaminathan named the high degree of centralization and consolidation of food production and processing, the increased use of refrigerators as the primary means of preserving food, and the above-mentioned changes in food consumption habits (increased consumer demand for convenient food) as main factors propagating the incidence of listeriosis (Swaminathan et al. 2007). Gillespie et al. studied the food exposures of listeriosis cases aged ≥60 years reported in England from 2005 to 2008 and compared them to those of market research panel members representing the same population (i.e. residents of England aged ≥60 years) and time period (Gillespie et al. 2010a, b). Cases were more likely than panel members to report the consumption of cooked meats (beef and ham/pork, but not poultry), cooked fish (specifically smoked salmon) and shellfish (prawns), dairy products (most noticeably milk, but also certain cheeses) and mixed salads. They were less likely to report the consumption of other forms of seafood, dairy spreads, other dairy products, sandwiches and fresh vegetables. The diversity of high-risk food exposures reflects the ubiquity of the microorganism in the environment and the susceptibility of those at risk, and suggests that a wide variety of foods can give rise to listeriosis. In the United States, two case-control studies on risk factors for sporadic listeriosis found that cases were most likely to have eaten melons,

hummus prepared in a commercial establishment, and soft cheeses or food purchases from store delicatessen counters (Schuchat et al. 1992; Varma et al. 2007).

L. monocytogenes is widespread in nature and has been isolated from soil, dust, food products for humans (both of animal and vegetable origin), feed, water, and sewage, and it can be carried by almost any animal species, including asymptomatic humans. The principal reservoir of the organism is said to be in soil, forage, water, mud, and silage (Heymann 2008). Due to this environmental ubiquity, listeria strains are also frequently detected in food products. In addition, growth and survival of these psychrotrophic bacteria are favored particularly due to the above-mentioned increasing use of refrigeration in food production, food distribution, and food storage. Unlike most other foodborne pathogens, *L. monocytogenes* tends to multiply in refrigerated foods that are contaminated. To understand why *L. monocytogenes* may persist in food industry equipment and premises, notably at low temperature, scientific studies have so far focused on adhesion potential, biofilm forming ability, resistance to desiccation, acid and heat, tolerance to increased sublethal concentration of disinfectants or resistance to lethal concentrations. Carpentier and Cerf recently postulated that the main factor associated with the presence of *L. monocytogenes* in production plants is growth promotion (Carpentier and Cerf 2011). Good growth conditions can be found in so-called harborage sites, i.e. shelters due to unhygienic design of equipment and premises or unhygienic or damaged materials. These sites are hard to eliminate. Carpentier and Cerf stipulated that there are no strains of *L. monocytogenes* with unique properties that lead to persistence, but harborage sites in food industry premises and equipment where *L. monocytogenes* can persist (Carpentier and Cerf 2011). In the European Union, foods that contain less than 100 colony-forming-units (cfu)/g are considered to pose a negligible risk for a healthy human population (EFSA 2012). EU legislation defines different criteria for three categories of foods (25 g samples) (European Commission 2005). In Europe, only food products for vulnerable populations (e.g. infants) have to be entirely free from *L. monocytogenes*. For food products enabling growth of *L. monocytogenes*, total absence is required for products sampled at the production plant and a level of 100 cfu *L. monocytogenes* per gram food is tolerated at the consumption stage. For food products unable to support the growth of *L. monocytogenes* a limit of 100 cfu *L. monocytogenes* per gram food is accepted, when sampled on the market during their shelf life. Foods were defined as unable to support the growth of *L. monocytogenes* by $pH \leq 4.4$; $a_w \leq 0.92$; $pH \leq 5.0$ and $a_w \leq 0.94$; shelf life less than 5 days.

In the United States, regulations require food companies to guarantee zero *L. monocytogenes* levels in all ready-to-eat products. The achievement of this objective is probably impractical, and it is clearly unattainable for raw foods or those which have not undergone a listericidal process (McLauchlin 2011).

Microbiological surveys have documented that *L. monocytogenes* may be present in a wide range of retail foods (Schuchat et al. 1992; Pinner et al. 1992). Wagner et al. studied samples of ready-to-eat (RTE) foodstuffs in Vienna, Austria, in 2007. They found 4.8% of 946 samples collected from 103 supermarkets positive for *L. monocytogenes*, with five smoked fish samples exceeding the tolerated limit of 100 cfu/g food (Wagner et al. 2007). Products showing the highest contamination rates were fish and seafood (19.4%), followed by raw meat sausages (6.3%), soft

cheese (5.5%) and cooked meat products or patés (4.5%). Pulsed field gel electrophoresis typing of the collected *L. monocytogenes* isolates revealed a high degree of diversity between the isolates. Also evidence from EU-wide routine food safety investigations indicates that a substantial proportion of RTE products is contaminated by *L. monocytogenes* (EFSA 2012). *L. monocytogenes* was detected in 1.5% of RTE meat products and meat preparations of beef tested in 2010 (in none with >100 cfu/g food), in 2.0% of RTE products and meat preparations of pork (in 0.5% with >100 cfu/g), in 1.5% of RTE products and meat preparations of poultry meat (in 0.2% with >100 cfu/g), in 0.3% of soft and semi-soft cheeses made from raw or low heat treated milk from cows (in 0.3% with >100 cfu/g), in 0.8% of soft and semi-soft cheeses made from raw or low heat treated milk from sheep and goats (in 4.5% with >100 cfu/g), and in 6.0% of RTE fish products (in 1.3% with >100 cfu/g). No major changes compared with previous years were detected in the proportions of RTE foods not in compliance with the EU microbiological criteria. Once again the highest proportions of units exceeding the limit of 100 cfu/g food were observed in RTE fish products and RTE meat products, at levels of 1.3 and 0.4%, respectively. Of the tested RTE salads 0.7% contained *L. monocytogenes* at a level above the 100 cfu/g limit (EFSA 2012).

Painter et al. studied the attribution of deaths from foodborne diseases to food commodities by using outbreak data (United States 1998–2008) and found that more deaths were attributed to poultry (19%) than to any other commodity, and that most poultry-associated deaths were caused by listeria (Painter et al. 2013).

From 1998 to 2002, three large listeriosis outbreaks were linked to turkey delicatessen meat contaminated in the processing plant after cooking (Gottlieb et al. 2006; Mead et al. 2006; Olsen et al. 2005). However, pork paté ("rillettes"), a beef meat dish and horse minced meat, were also involved in outbreaks (Goulet et al. 1998, 2013; Smith et al. 2011). A risk-ranking model for listeriosis among RTE foods identified delicatessen meat as the highest risk food (Food and Drug Administration 2003).

Soft cheeses, especially soft cheeses made with unpasteurized milk (Mexican-style), red smeared cheeses, brie, camembert and sour milk curd cheese 'Quargel', were responsible for large outbreaks in Europe and in the United States (Pichler et al. 2011; Goulet et al. 1995; Johnson et al. 2010). In Australia, 29 cases (including three fatal cases) were linked to brie, blue cheese and camembert from one company in 2013 (Carey 2013).

Produce is increasingly identified as a source of listeriosis-outbreaks: In the United States, sprouts caused an outbreak in 2009, and precut celery caused an outbreak in 2010 (CDC 2012). A total of 147 persons infected with any of five subtypes of *L. monocytogenes* were reported to the Centers for Disease Control and Prevention from 28 states in connection within an outbreak associated with cantaloupes (melons) in 2011, including 33 outbreak-associated deaths (CDC 2012).

Although fish and seafood are very often found to be contaminated with *L. monocytogenes*, this food only occasionally was involved in outbreaks. Five cases with gastroenteritis related to consumption of cold smoked trout were reported from Finland (Miettinen 1999). Shrimp was implicated as food source in two pregnancy-associated cases with bacteremia in the USA in 1989 (Riedo et al. 1994).

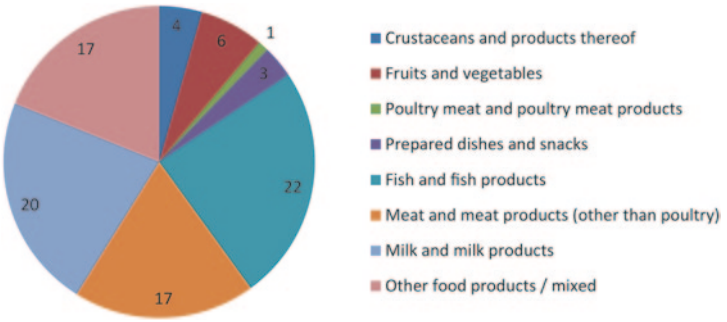

Fig. 10.6 Rapid Alert System for Food and Feed (RASFF) notifications concerning *Listeria monocytogenes*, 2012

In China, an outbreak of gastroenteritis involving 82 cases was traced back to a vacuum-sealed product of cooked, unshelled eggs served to children as a meal during school break (Feng et al. 2013). In Italy, 1566 cases of listeria gastroenteritis were traced to consumption of corn salad in 1997, and 14 cases to rice salad in 1993 (Salamina et al. 1996; Aureli et al. 2000).

The discovery of *L. monocytogenes* mainly in raw and RTE meat, poultry, seafood, and dairy products has prompted numerous product recalls leading to large financial losses for the industry and to numerous health scares. In the European Union, a rapid alert system for food and feed (RASFF) was put in place to provide food and feed control authorities with an effective tool to exchange information about measures taken responding to serious risks detected in relation to food or feed. This exchange of information helps member states to act more rapidly and to respond to a health threat caused by food or feed in a coordinated manner. During the last decade, RASFF messages with information concerning listeria have increased by the factor of three. While in 2012, 90 notifications concerned *L. monocytogenes* (2011: 107, 2010: 108, 2009: 85, 2008: 51, 2007: 37, 2006: 26, 2005: 117, 2004: 122, 2003: 58, 2002: 51, 2001: 36), only 33 alerts were issued in the year 2000. This increase seems to reflect increased awareness about listeria contamination as a potential public health risk. In 2012, fish, meat, and milk products accounted for 66% ($n=59$) of the 90 RASFF notifications (Fig. 10.6).

10.6 Molecular Typing and Molecular-Epidemiological Evidence of Foodborne Transmission

10.6.1 Typing Methods

Subtyping of bacterial isolates is essential for active surveillance and outbreak investigation. If a typing methods shows isolates to be very similar to each other,

transmission is likely and we speak of "making" a cluster ("rule in"). If a typing method shows isolates to be different, transmission is unlikely and we speak of "breaking" a cluster ("rule out"). However, there are various caveats like occurrence of genetic recombinations (falsely ruling out) and occurrence of epidemiologically independent sources (falsely ruling in). Typing results can miss epidemiological relations due to under-discrimination and due to over-discrimination. The resolution can be too crude and too fine, so transmission chains can be overlooked. The challenge is to find a typing method with a resolution that correlates with epidemiological events.

10.6.1.1 Serotyping

Serotyping was the first method available for subtyping *L. monocytogenes* isolates. Serotyping is based on somatic (O) and flagellar (H) antigens. Published references define 13 *L. monocytogenes* serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Allerberger 2003; Chen 2012). Serotypes are determined by reactivity with antisera. Commercially available serotyping sera (available from BD Diagnostics, USA and Denka Seiken Co. Ltd., Japan) include a serotyping scheme for these 13 serotypes. Listeria handbooks do not agree on this issue; for example, the latest edition of "Listeria, Listeriosis, and Food Safety" (Graves et al. 2007) does not list the *L. monocytogenes* serotype 4ab, whereas the "Handbook of *Listeria monocytogenes*" (Chen and Knabel 2008) does. In light of our experience, we question the existence of *L. monocytogenes* serotype 4ab. Taking into consideration the significance of reliable and unambiguous serotyping, especially in epidemiological tracking, an official revision of the *L. monocytogenes* serotyping scheme might be advised. In epidemiological investigations, bacterial serotyping usually is unable to estimate the relatedness of different isolates, as both invasive listeriosis and febrile gastroenteritis are caused mostly by serotypes 1/2a, 1/2b and 4b strains. Serotyping of *L. monocytogenes* therefore has only limited practical value for investigating chains of transmission.

Doumith et al. developed a PCR-based serotyping method. Their multiplex PCR divides *L. monocytogenes* isolates into four groups employing primers annealing to *Listeria* genus specific *prs* and genes specific to serotype-associated phylogenetic lineages of *L. monocytogenes*; this method can differentiate between strains of serotype 1/2a or 3a, 1/2c or 3 c, 1/2b, 3b or 7 and the serotype 4b complex (4b, 4d, 4e) (Doumith et al. 2004a). The *prs* primers are specific for the putative phophoribosyl pyrophosphate synthetase (*prs*) gene of *Listeria* spp.

10.6.1.2 Molecular Typing

Molecular typing refers to any technique and method that is used to characterize microorganisms at the nucleotide level. It supports studies to trace back the source of an outbreak and to identify new risk factors as the strains can be linked more accurately to epidemiological and clinical data. All of this information can be applied

towards improving and better targeting existing infectious disease prevention and control measures and thus presents a clear and immense benefit for the public health and public health policies (Allerberger 2012).

Early studies identified two genomic divisions ("lineages") by means of a variety of genotyping tools (Lee et al. 2012b). An additional lineage, lineage III, was first discovered in 1995 (Rasmussen et al. 1995). More recently, four lineages of *L. monocytogenes* were proposed by dividing lineage III into two separate evolutionary groups (den Bakker et al. 2010). Lineage I includes strains of serotypes 4b, 4d, 4e, 1/2b and 3b. Serotype 4b is implicated in many outbreaks and sporadic cases resulting in lineage I being overrepresented among clinical samples (Lee et al. 2012b). Lineage II encompasses serotypes 1/2a, 1/2c, 3a and 3c. Isolates belonging to this lineage are frequently found in foods and natural environments although serotype 1/2a is also frequently identified among clinical isolates and sometimes implicated in outbreaks. Lineages III originally consisted of serotypes 4a and 4c. It was divided into two subgroups, one subgroup with strains of serotype 4a and the other with strains of serotype 4c (Doumith et al. 2004b). Even though these "new" lineages III and IV have been implicated in occasional sporadic cases, they are markedly less common in human listeriosis than strains of lineage I or II.

Molecular typing of *L. monocytogenes* splendidly complements traditional epidemiological surveillance by providing appropriate discriminatory analyses to foster rapid and early detection of dispersed clusters or outbreaks and to facilitate detection and investigation of transmission chains.

High Resolution Melting Curve-PCR (HRM-PCR)

High resolution melting (HRM) curve-PCR analysis for *L. monocytogenes* was developed by Pietzka et al. (2011). Genomic bacterial DNA (gDNA) is extracted from bacterial cells grown overnight at 37 ° on blood agar plates and a 500-bp fragment located in the virulence gene internalin B (*inlB*) is amplified for subsequent HRM analysis. HRM curve analysis constitutes an inexpensive assay and represents an improvement in typing relative to classical serotyping. This method provides a rapid and powerful screening tool for simultaneous preliminary typing of up to 384 samples in approximately 2 h.

Pulsed-Field Gel Electrophoresis (PFGE)

DNA macrorestriction analysis by pulsed field gel electrophoresis (PFGE) is presently considered the gold standard for subtyping of food borne pathogens like listeria, salmonella, campylobacter and *Bacillus cereus* (Allerberger 2012).

Electrophoresis as an analytical method separates macromolecules such as nucleic acid by their size, charge, conformation and reactivity (Lee et al. 2012a). Classical electrophoresis employs a steady electric field orientated in one direction. This procedure, relying on a single paired electrode, permits the movement of DNA molecules only to a maximal size of 50 kb (Fangman 1978). PFGE is

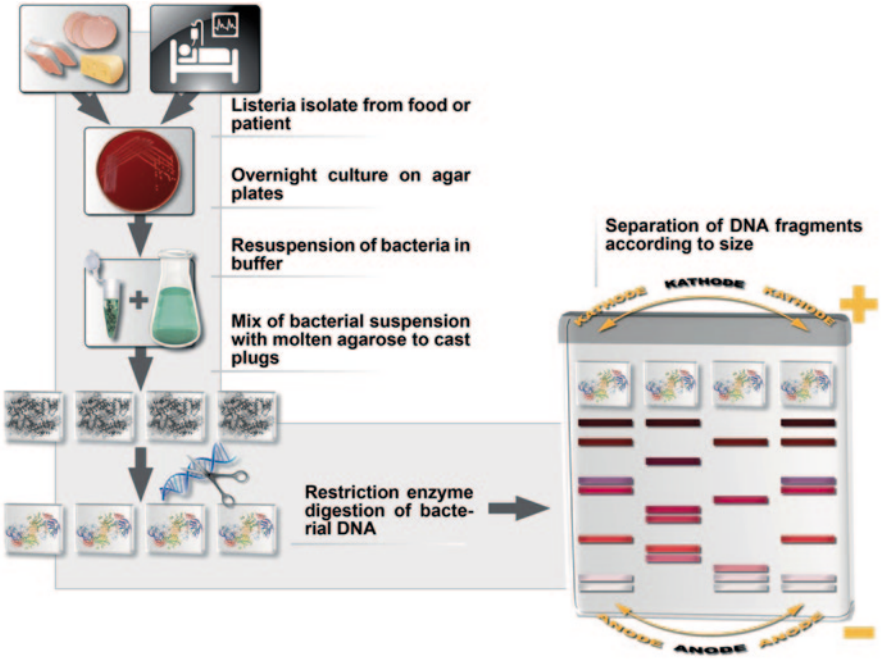

Fig. 10.7 Schematic principles of pulsed field gel electrophoresis (PFGE)

able to separate molecules as large as 12 Mb in size (Orbach et al. 1988). D.C. Schwartz and C.R. Cantor developed this variation of agarose gel electrophoresis, which revolutionized precise separation of DNA fragments greater than 40–50 kb. In PFGE, the orientation of the electric field across the gel is changed periodically ("pulsed") rather than kept constant as it was in conventional agarose gel electrophoresis (Schwartz and Cantor 1984). This technology separates large fragments of unsheared microbial chromosomal DNA obtained by embedding intact bacteria in agarose gel plugs, enzymatically lysing the cell wall and digesting the cellular proteins. The intact DNA is digested with an infrequently cutting restriction enzyme. Subsequent restriction fragment length polymorphism analysis allows differentiation of clonal isolates from unrelated ones. PFGE relies on a direct current electric field that changes orientation and intensity relative to the agarose gel. The rate of the changes is called pulse time, and its duration is an important factor to determine what molecular size range can be separated. The DNA molecules migrate through the agarose gel in a zigzag way, responding to the changes of the electric field. For larger molecules the reorientation takes more time. They become trapped in the matrix if the pulse time is too short (Birren et al. 1988). Figure 10.7 depicts the basic principles of pulsed field gel electrophoresis (PFGE).

Preparation of DNA: *L. monocytogenes* isolates from food samples, environment, animals or patients are grown overnight on blood agar or brain-heart-infusion agar. The cells are harvested from the plates and resuspended in a sterile buffer

solution (TE buffer). Lysozyme is used to break the bacterial cell wall. Molten agarose is added to the bacterial suspension and poured into templates, resulting in the casting of agarose plugs. During the preparation process, shear forces can lead to strand breakage. Thus, bacterial DNA is extracted inside these agarose plugs: The plugs are incubated with a cell lysis buffer (50 mM Tris.HCl pH 8.0, 50 mM EDTA, 1 % sodium lauroyl sarcosine, 0.1 mg/mL Proteinase K) for at least 2 h and then washed several times to clean the plugs from cell and protein debris. At this stage, i.e. after DNA extraction, the plugs can be stored at 4 °C for longer periods of time.

Restriction enzyme digestion: DNA molecules in agarose plugs can be digested with all commonly used restriction enzymes. Many thousand restriction enzymes are known and several hundred are commercially available. Enzymes like *EcoR*I or *Hind*III digest bacterial DNA to fragments of approximately 4 kB in size, which is too small for PFGE (Bhagwat 1992); larger DNA fragments are needed, i.e., enzymes which cleave rarely. For subtyping of *Listeria monocytogenes*, mainly *Asc*I and *Apa*I are used as restriction enzymes, yielding 8–25 DNA fragments with up to 1 MB in size.

Markers: To be able to compare PFGE patterns from different laboratories, entrainment of a size standard is essential. *Salmonella* Braenderup strain H9812 was chosen as a universal standard, which is also used as standard for PFGE-typing of *E. coli*, *Salmonella* spp., and *Shigella* spp. (Hunter et al. 2005).

Electrophoresis: After restriction enzyme digestion, the plugs are loaded onto a comb, which is then transferred into the agarose gel casting stand. The plugs are agarose-sealed with the gel. In the gel, DNA fragments are separated according to their molecular weight: Smaller fragments migrate faster than larger ones. Pulse time (or switch time), voltage and temperature are the main parameters influencing this separation of the DNA. DNA is exposed to an electric field which switches angles in an alternating period of time, called pulse time. The migration rate of DNA fragments in PFGE is proportional to the voltage. An increase in voltage allows separation of larger molecules. Also temperature affects DNA migration rate and the resolution power. At 34 °C, DNA fragments of 50–1000 kb size run twice as fast as at 4 °C (Birren et al. 1988). However, increase of migration speed is often associated with a decrease in resolution power. Usually PFGE is performed at temperatures between 12 and 15 °C.

Analysis: Databases (e.g. Bionumerics from Applied Maths, Brussels, Belgium) are used to store and manage the resulting PFGE-patterns. The simultaneous occurrence of a new PFGE pattern in human cases and in a certain food product is a strong hint for epidemiological coherency.

Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP)-analysis was developed in the early 1990's by Vos et al. and is a registered trademark of KeyGene (Vos et al. 1995). It is based on PCR amplification of restriction fragments of a complete genomic digest. AFLP represents a relatively simple, low cost, rapid and highly discriminatory

method which covers a larger portion of the genome than other typing techniques. In principle the method scans the genome for sequence polymorphisms producing DNA fragments, mainly between 50 basepairs and 700 basepairs in size. The fragments are separated on a denaturing acrylamide gel. The presence and absence of fragments produces a band pattern or AFLP profile comparable to bar codes used for product identification in commerce (Rupptisch 2013). Here it determines a genetic fingerprint. For subsequent data analysis the resulting AFLP profile can be converted into a binary presence—absence (1/0) code, a process known as "scoring" (Kück et al. 2012).

The national *Listeria*-reference laboratory for England and Wales uses fluorescent Amplified Fragment Length Polymorphism (fAFLP) analysis with *Hind*III/*Hha*I as subtyping method for *L. monocytogenes* since 2008. In fAFLP, the *L. monocytogenes* genome is digested with a rare cutting restriction enzyme such as *Hind*III and a more frequently cutting enzyme like *Hha*I. In a second step, adapters containing about 15 bp are ligated to the restriction fragments. These adapters serve as targets for two different primers, one of which has a fluorescent label. Usually, a FAM-labeled *Hind*III and a non-labeled *Hha*I primer are used. Both primers contain the adapter sequence, the specific restriction site sequence and one additional base pair on the 3′ end which extend into the restriction fragment. PCR amplifies only one fourth of the fragments containing the corresponding additional base pair and only hybrid fragments with two different restriction sites. Electrophoretic separation of amplicons on a gel matrix is followed by visualization of the band pattern. While PFGE is known to be a very time-consuming and work-intensive method (starting with a pure culture, first results can be achieved within 3 to 4 days), fAFLP can be completed within 48 h and is easier to perform. Roussel et al. analyzed 109 different *L. monocytogenes* isolates by both methods (Roussel et al. 2013). The isolates were divided by fAFLP and PFGE into three distinguished lineages. Strains known to be epidemiologically associated with one another were found to have unique PFGE and fAFLP types. FAFLP and PFGE divided the strains into 76 and 82 distinct profiles, or types, respectively. The discriminatory index calculated was 0993 and 0996 for fAFLP and PFGE, respectively. The authors concluded that the discriminatory ability of fAFLP was similar to that of PFGE for the subtyping of *L. monocytogenes* isolates and that, "as a less labour intensive technique, fAFLP may be a better method to use than PFGE in investigating outbreaks of human listeriosis and tracking the source of contamination in food processing facilities in real time".

Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA)

The availability of whole-genome sequences had facilitated the discovery of variable number tandem repeats (VNTRs), loci that contain short strings of nucleotides that are repeated, from a few to many times, and which are scattered throughout the bacterial genome. This has led to the birth of Multi-Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) (Lindstedt 2005). Different organisms show variations in the number of these tandem repeated DNA sequences stashed in

different loci. These loci can be multiplexed via PCR and separated by capillary gel electrophoresis. The size of the fragments varies according to the number of repeats in each locus. Results are depicted by numerical codes, giving the number of alleles at each locus tested. These numbers consisting of integers are the MVLA profile and can be compared to those in an existing profile database (Sperry et al. 2008; Jadhav et al. 2012). Murphy et al. called this approach "a valuable tool, which has the capability to provide comparable results when compared with other more established typing methods, including pulsed-field gel electrophoresis" (Murphy et al. 2007). We consider MLVA less discriminatory than PFGE or AFLP.

Whole Genome Sequencing (WGS)

The 'endpoint' of typing techniques is sequencing the whole genome, a method to determine the complete DNA sequence of a single organism. By end of April 2013, GOLD—genomes online database—reported 113 *L. monocytogenes* sequencing projects currently ongoing, with 33 genomes already completed (www.genomesonline.org). To construct the complete nucleotide sequence of a genome, multiple short sequence reads must be assembled based on overlapping regions (de novo assembly), or comparisons with previously sequenced 'reference' genomes (resequencing). The emergence of benchtop sequencers using next generation sequencing technology makes bacterial whole genome sequencing (WGS) feasible even in small research and clinical laboratories. Presently the key challenge is not to produce the sequence data, but to rapidly compute and interpret the relevant information from large data sets (Sabat et al. 2013).

The progress in technology from automated Sanger sequencing (first generation sequencing) to next generation sequencing has revolutionized the field of molecular epidemiology. Sequencing methods still become faster and more affordable from year to year (Ng 2010). Genome sequencing will be the future technique for subtyping pathogens. Comparative whole genome sequencing is capable of discriminating major *L. monocytogenes* clones grouped together by current genotyping methods (Schmid et al. 2014).

The challenge is to fine-tune the resolution to correlate with epidemiological events. Mobile elements ("high clock speed targets") can hamper analysis of whole genomes. The ability to interpret this huge amount of data presently represents the "bioinformatic bottleneck" of whole genome sequencing, preventing the broad usage in routine typing. Innovative bioinformatic projects such as the Cloud Virtual Resource (CloVR) promise to address this bottleneck by automating entire analysis workflows and using online "cloud computing" services for data (Angiuoli et al. 2011a, b).

Sequence-based typing methods are offering new perspectives of enhanced resolution and comparability of typing systems for public health applications. As gene sequencing technology develops further and methods of sequence analysis become even more user-friendly, new typing methods will evolve and promote even more

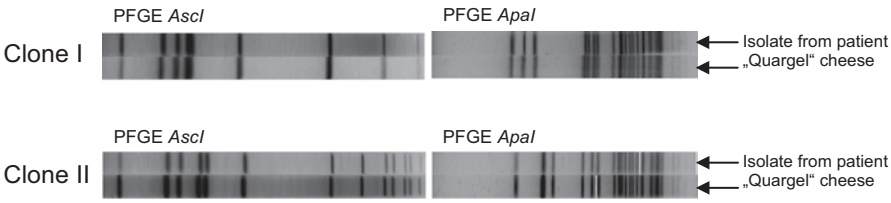

Fig. 10.8 Two unique pulsed field gel electrophoresis (PFGE) patterns (from human cases and the epidemiologically causative food item) in an outbreak affecting 25 persons in Austria, 8 in Germany, and 1 in the Czech Republic in 2009/2010—never seen before. In this outbreak, the shift to a different outbreak clone was probably caused by the change to a new commercial yeast-ripening culture

acceptance of routine molecular typing in public health laboratories (Allerberger 2012; Angiuoli et al. 2011a, b; Riley et al. 2011). Some countries are already systematically using WGS for listeria typing. There is however not yet any agreed EU-wide protocol for analysing and comparing this WGS data across countries.

10.6.2 Interpretation of Typing Results

Adequate molecular typing enables linking pathogen data from human, food, animal, and environmental sources (provided the discrimination correlates epidemiologically). If applied routinely in real time, molecular typing allows for an early detection of national and international clusters and thus facilitates the early identification of potential sources of outbreaks (Fig. 10.8) (ECDC 2013). However, the use of analytical studies to investigate outbreaks of listeriosis has had mixed success (McLauchlin 2011).

If thoroughly investigated, foodborne outbreaks of listeriosis provide an opportunity to identify the food vehicle involved and the factors in the food preparation and handling that contributed to the outbreak. Within the European Union, reporting on foodborne outbreaks is mandatory under the framework of DIRECTIVE 2003/99/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of November 17, 2003 on the monitoring of zoonoses and zoonotic agents. According to this directive, a foodborne outbreak is defined as an incidence, observed under given circumstances, of two or more human cases of the same disease and/or infection, or a situation in which the observed number of cases exceeds the expected number and where the cases are linked, or are probably linked, to the same food source.

While it is clear what constitutes a foodborne outbreak, there is no consensus on criteria for defining a *L. monocytogenes* cluster as a possible foodborne outbreak. Even the term cluster is still under controversial discussion: Within the ECDC's

"Food and Waterborne Diseases (FWD) Molecular Surveillance Pilot Project", the following definition of cluster is considered for *L. monocytogenes* as of April 2013: Three or more strains with identical PFGE pattern name recorded in the last 120 days performing restriction digestion of DNA in agarose plugs with *Asc*I or *Apa*I: "Restriction analysis with a secondary enzyme (*Apa*I) is important in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable. The use of a second enzyme helps us determine relatedness of the isolates being tested by confirming that the PFGE patterns generated with these enzymes are also indistinguishable." (Martinez-Urtaza J, Surveillance & Response Support Unit, European Centre for Disease Prevention and Control (ECDC), Stockholm, personal communication).

In Austria (unlike in Germany or in Switzerland) medical laboratories have a legal obligation to forward human *L. monocytogenes* isolates to the National Reference Centre. From 2009 to 2012, a total of 149 human isolates were PFGE-typed at the Austrian reference centre for listeria: only 67 isolates (45 %) showed unique, individual PFGE patterns. The majority, 82 isolates (55 %), yielded PFGE patterns that fell into clusters (more than one isolate showing a unique PFGE pattern). Lay persons often urge to define a possible foodborne outbreak by the occurrence of just two indistinguishable human isolates, ignoring the fact that many 2-isolate clusters are due to chance and ignoring that any pregnancy-related pair of isolates (mother/child isolates yield identical DNA fingerprinting patterns) would incorrectly be deemed a possible foodborne outbreak. In contrast to the situation in forensic human genetics, where—with the exception of monozygotic twins—indistinguishable DNA fingerprinting patterns prove epidemiological relatedness, in bacteriology epidemiologically unrelated *L. monocytogenes* isolates can yield DNA fingerprinting patterns indistinguishable from each other. Therefore, pure chance yields pairs of epidemiologically unrelated human *L. monocytogenes* isolates with PFGE patterns indistinguishable from each other and pairs of contemporary isolates of human and of food origin, without any causal relation. Figure 10.9 shows PFGE patterns of seven food isolates received by the Austrian reference centre for *L. monocytogenes* in 2012, isolates not linked to one food or food producer. The occurrence of indistinguishable DNA fingerprinting patterns in different food products—epidemiologically unrelated to each other or to human cases—also hampers attribution of human illness to certain food. While much has been learned about food sources for epidemic listeriosis, little is known about food sources of sporadic listeriosis, which, in fact, represents the majority of cases. In Austria, when ≥ 2 *L. monocytogenes* isolates with indistinguishable PFGE pattern combinations are identified within the actual and the previous years, this cluster is evaluated. If at least five isolates were identified within the last 6 months, the cluster is deemed a possible foodborne outbreak.

The United States is a country without legal obligation to forward human *L. monocytogenes* isolates to a reference centre. In the United States, human *L. monocytogenes* isolates sent to state public health laboratories participating in PulseNet, a molecular subtyping network for enteric bacterial disease surveillance, are PFGE typed in a standardized way; the PFGE patterns are then uploaded to a central

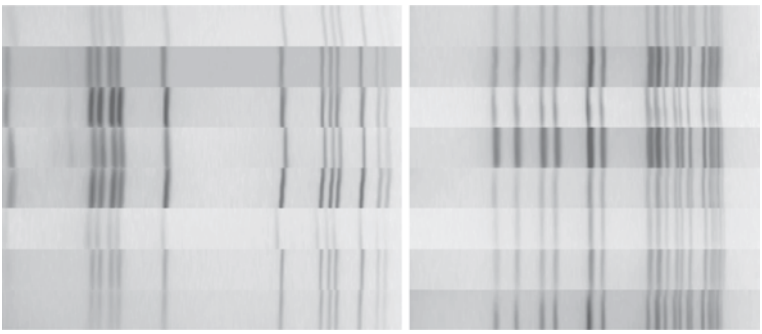

Fig. 10.9 Indistinguishable pulsed field gel electrophoresis (PFGE) patterns from seven different food items from seven individual (geographically and economically unrelated) food producers in comparison with a contemporary isolate from a sporadic human case (source of infection unknown), Austria 2012 (RTE=ready to eat)

database (PulseNet) for national comparisons. When ≥2 *L. monocytogenes* isolates with indistinguishable PFGE pattern combinations are uploaded within a 120-day period, this cluster is evaluated. An investigation is initiated if "epidemiologic indicators suggest a common source" (Cartwright et al. 2013).

In France, there is no legal obligation for private or hospital laboratories to forward human *L. monocytogenes* isolates to the national reference centre (NRC; Pasteur Institute, Paris), but most laboratories forward isolates voluntarily. However, even without legal obligation to forward human isolates to the NRC, physicians and microbiologists have legal obligations to report listeria cases to the public health authorities. At the central level, one dedicated staff member keeps track of the isolates and systematically contacts the labs to ensure that the isolates have been forwarded to the NRC. Each year, the French NRC receives 99–100% isolates of the reported listeria cases. The NRC does perform PFGE typing on every human listeria isolate, using both *Apa*I and *Asc*I enzymes. For endemic listeria strains, the NRC also performs an additional PFGE typing using *Sma*I enzyme to distinguish common *Apa*I/*Asc*I profiles. Until June 2012, the French reference centre was using a threshold of three (>3) indistinguishable human isolates identified over the last 6 weeks for starting an epidemiological investigation. However, the use of that definition was time-consuming and they revised it to set up a new cluster definition based on the strains' characteristics. They now classify *L. monocytogenes* pulsotypes as

endemic (>12 human cases/year), frequent (6–12 human cases/year) or rare (<6 human cases/year). Endemic, frequent and rare pulsotypes respectively accounted for 48, 36 and 15% of the *L. monocytogenes* strains reported during 2009–2011. For endemic pulsotypes, they changed the cluster definition to >6 indistinguishable human isolates identified over the last 6 weeks. For frequent and rare pulsotypes, they kept the old cluster definition (>3 indistinguishable human isolates identified over the last 6 weeks). The French national reference centre for *Listeria monocytogenes* decided to do so by looking back at their cluster investigations results during 2006–2011. During that time period, and using the ">3 indistinguishable human isolates identified over the last 6 weeks" definition, they identified 69 clusters. Among those, 31 were associated with "endemic" pulsotypes and 38 were "non-endemic" clusters (frequent or rare pulsotypes). Among the 31 "endemic" clusters, they suspected the source of contamination in only four out of 31 (13%) and were unable to confirm further sources. By using the ">6 indistinguishable human isolates identified over the last 6 weeks" definition, they would have identified nine "endemic" clusters instead of 31, but still have suspected the source of contamination in 3 out of 31, with a significant reduction of investigative work (Tourdjman M, Institut National de Veille Sanitaire, Saint-Maurice; personal communication).

In Denmark, they do not use one strict rule (Nielsen EM, Statens Serum Institut, Copenhagen; personal communication). Generally, the reference centre starts looking into a cluster when they have three indistinguishable (using PFGE with two enzymes) patient isolates within 3 months. In previous years they encountered a few very common PFGE types among patient isolates (several cases spread over the year for several years), and despite epidemiological investigation, could not find a common source or other epidemiological link. Therefore, they decided to take into account how common a type is before starting any investigation. Food authorities are only contacted, if the reference centre "has some indication for a foodborne outbreak (which is rare)".

In the UK, there is no legal obligation to forward isolates of *L. monocytogenes* from human cases; however there is an obligation for laboratories to report these cases. Isolates sent voluntarily are typed using fluorescent amplified fragment length polymorphism (fAFLP) analysis; PFGE is not used for subtyping listeria (McLauchlin J, Central Public Health Laboratory, London; personal communication).

An EFSA-ECDC collaboration on typing of listeria in RTE products and clinical cases of human listeriosis was initiated in 2010 (Lahuerta 2011). The results provided by this study since 2013 are supposed to contribute to a better understanding of listeriosis epidemiology in the EU and should help to target effective control and preventive measures within both food safety and public health.

Table 10.1 Cases of listeriosis in animals submitted for necropsy and diagnosed at the Institutes for Veterinary Disease Control (Austrian Agency for Health and Food Safety) from 2007 to 2012 (total: 304)

Species	Encephalitic form	Abortion	Septicemic form
Cattle	105	9	
Sheep	103	3	
Goat	71	1	1
Wild-ruminants	6		
Swine		1	2
Brown hare			1
Horse	1		
Total	286	14	4

10.7 Disease Symptoms in Both Animals and Humans

10.7.1 Disease Symptoms in Animals

In general, listeriosis in animals can be classified as six different forms (Selbitz 2011): (a) latent intestinal colonization with shedding via feces, (b) encephalitic listeriosis, (c) septicemic form, (d) metrogenic form, (e) mastitis and (f) ocular manifestation. Table 10.1 summarizes the listeriosis cases in animals, submitted for necropsy and diagnosed at the Institutes for Veterinary Disease Control of the Austrian Agency for Health and Food Safety from 2007 to 2012 (total: 304). The simultaneous incidence of several distinct forms (b–f) in one animal is possible, but constitutes a rare event. Several factors influence the development of these manifestations: site of bacterial entry (oral or nasal mucosa, eye, gastrointestinal mucosa, navel), microbial virulence, host immune competence and way of dissemination (lympho-hematogenous or neurogenic) (Selbitz 2011). Therefore, incubation periods can vary between a few days to 4 weeks.

The most frequent encephalitic form (and other organic manifestations as well) can readily be diagnosed by histological examination with subsequent immunohistochemical detection (i. e. avidin-biotin-peroxidase complex (ABC)-technique) of the pathogen. Suspicion of listerial infection is usually raised by typical morphological features, in a high number of cases upon sole examination of H&E sections. In a retrospective study of 178 encephalitis cases in ruminants, Bagó et al. (2001) found morphological characteristics of listerial encephalitis in 52 cases, which were all confirmed by immunohistochemistry. Moreover, immunohistochemical examination revealed two additional cases of listeriosis, formerly classified as non-purulent encephalitis of unknown etiology. These findings underline the importance of immunohistochemistry in the diagnosis of listeriosis. Figures 10.10 and 10.11 display two histological features of listerial rhombencephalitis in ovine brain with different detection intensity of *L. monocytogenes* (Bagó et al. 2001).

Ruminants are most frequently affected by the encephalitic form (Selbitz 2011) with histological features of a characteristic, predominantly purulent rhombencephalitis and nonpurulent leptomeningitis. Symptoms include elevated temperature

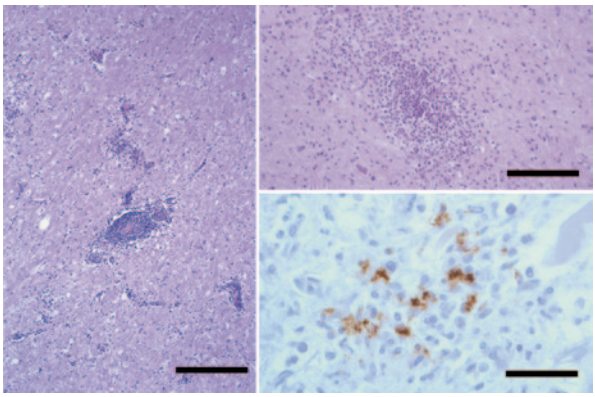

Fig. 10.10 Listeriosis in sheep, "purulent type inflammation". *Left*: monocuclear perivascular cuffing; Hematoxylin and eosin (H&E) staining; bar=250 µm; *Right top*: microabscess-like cluster of neutrophiles; H&E staining; bar=150 µm; *Right below*: immunohistochemical staining of *L. monocytogenes* (brown signals) in an microabscess; bar=25 µm (Reprint by permission of Österreichische Gesellschaft der Tierärzte (ÖGT))

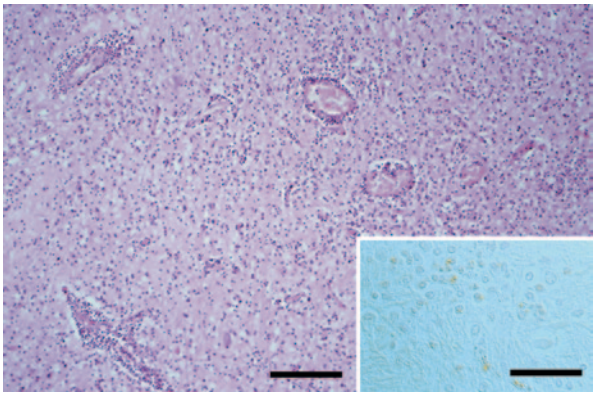

Fig. 10.11 Listeriosis in sheep, "non-purulent type inflammation". Mononuclear perivascular cuffing and diffuse microgliosis; Hematoxylin and eosin (H&E) staining; bar=170 µm; Inset: immunhistochemical detection of sporadic *L. monocytogenes* organisms (brown signals) inside an area of microgliosis; interference contrast microscopy; bar=70 µm. (Reprint by permission of Österreichische Gesellschaft der Tierärzte (ÖGT))

(initially), depression, exaggerated forward or sideward stance, vestibular ataxia, circling, paresis of cranial nervs (nervus facialis, nervus glossopharyngeus, nervus trigeminus), salivation, strabismus, nystagmus, nasal and ocular discharge, reduced pupillary reflex, reduced tongue movement and head tilt (Schweizer et al. 2006; Selbitz 2011; Weiss and Amtsberg 1995; Braun et al. 2002). Young animals during dentition are particularly susceptible for neurogenic dissemination via the

trigeminal and glossopharyngeal nerves due to the increased vulnerability of the oral mucosa. The metrogenic form is characterized by late abortions, premature delivery and frailty of the offspring displaying liver necrosis, abomasal erosions and multifocal accumulations of *Listeria* in parenchymatous organs of the fetus/neonates with or without inflammatory reaction. Metritis or placentitis of the dame is rarely encountered (Dennis 1975; Wagner et al. 2005; Weiss and Amtsberg 1995). The septicemic form primarily affects juveniles (predominantly lambs) and is characterised by fever, general discomfort and diarrhea (Weiss and Amtsberg 1995). The pathomorphological findings are dominated by multifocal necroses in parenchymatous organs and occasionally fibrinopurulent meningitis. Listerial mastitis is a rare form that usually presents with subclinical chronic interstitial mastitis resulting in parenchymal atrophy (Winter et al. 2004). The ocular manifestation of listeriosis is characterised by a granulomatous to purulent keratoconjunctivitis and uveitis/iridocyclitis (Evans et al. 2004; Selbitz 2011). The so-called exposition keratitis is interpreted as a result of cranial nerve paresis and cannot be attributed to the infection itself. Special manifestations like listerial gastroenteritis and granulomatous-suppurative lymphadenitis have also been described (Fairley et al. 2012; Otter et al. 2004; Thompson et al. 2009).

In **swine**, listeriosis usually presents as abortion, septicemia of suckling and fattening pigs as well as encephalitis. Fattening pigs frequently develop hemorrhagic enteritis in the septicemic phase. Animals suffering from cerebral affection display incoordination, torticollis, circling, tremor, and paresis of hind legs (Wendt and Bickhardt 2001).

Listeria infection is occasionally detected in **horses**. Few cases of encephalitic (Rütten et al. 2006), abortive (Welsh 1983), ocular (Evans et al. 2004) and septicemic (Jose-Cunilleras and Hinchcliff 2001; Warner et al. 2012) forms have been reported. The septicemic form is characterised by necrotizing hepatitis and typhlocolitis.

Listeriosis of **dogs** and **cats** is exceptionally rare: septicemic form (Schroder and van Rensburg 1993; Weiss 2005), tonsillitis (Läikkö et al. 2004), cutaneous manifestation (Weiss 2005) and abortion (Sturgess 1989) have been described.

In **poultry**, septicemic listeriosis leading to acute death and encephalitic listeriosis with central nervous system disturbances such as torticollis and drowsiness, has been described. Pathomorphologically, granulomatous and purulent (heterophilic) inflammation of the central nervous system could be demonstrated (Kurazono et al. 2003; Ramos et al. 1988).

Lagomorpha and **rodents** are susceptible to listeriosis as well, usually developing septicemic, metritic and ocular forms (Hoelzer et al. 2012; Peters and Scheele 1996; Selbitz 2011).

In addition, listeriosis has been reported in a multitude of **wild animal species**, whereas the above described courses of disease and lesions can be transmitted to related genera of domesticated species (Hoelzer et al. 2012).

10.7.2 Disease Symptoms in Humans

As *L. monocytogenes* is prevalent in many different foods for human consumption, exposure to this pathogen by the consumption of contaminated food would be considered fairly common. However clinical disease is rare and mainly occurs among the immunocompromised, the pregnant, and the elderly (age ≥60 years). Clinical manifestations range from febrile gastroenteritis to more severe invasive forms including sepsis, meningitis, rhombencephalitis, abortion and perinatal infections.

The median incubation period is estimated to be 3 weeks. Outbreak cases have occurred 3–70 days following a single exposure to an implicated product (Heymann 2008). Non-invasive listeriosis (commonly manifested as febrile self-limited gastroenteritis) usually has a shorter incubation period, which ranges between 6 h and 10 days (American Medical Association et al. 2001). Invasive listeriosis generally needs a much longer incubation period, usually about 8–30 days (Linnan et al. 1988). Goulet et al. (2013) calculated the incubation period of listeriosis by reviewing published literature on patients with a single exposure to a confirmed food source contaminated by *L. monocytogenes*. For gastroenteritis cases, the median incubation period was 24 h with variation from 6 h to 30 days. For invasive listeriosis, the overall median incubation period was 8 days (range: 1–67 days); it differed significantly by clinical form of the disease: pregnancy-related cases showed a median incubation period of 27.5 days (range: 17–67 days), cases with central nervous system (CNS) manifestation a median of 9 days (range 1–14 days) and for bacteremia cases a median of 2 days (range 1–12 days).

Ten percent to 20% of clinical cases are pregnancy-associated (including neonates within the first 3 weeks after birth), and the majority of the rest occurs in non-pregnant immunocompromised individuals or in the elderly. While listeriosis during pregnancy usually presents with flu-like symptoms, which can lead to infection of the fetus causing abortion, premature birth or stillbirth, in non-pregnancy associated cases it mainly manifests as meningoencephalitis or septicemia. The onset of meningoencephalitis (rare in pregnant women) can be sudden, with fever, intense headache, nausea, vomiting, and signs of meningeal irritation, or may be subacute, particularly in an immunocompromised or an elderly host (Heymann 2008; Tunkel et al. 2004).

Rhombencephalitis is an unusual form of listeriosis (Armstrong and Fung 1993). This brain stem encephalitis occurs in previously healthy adults. It is analogous to “circling disease” in sheep. Clinical features are biphasic: fever, headache, nausea and vomiting, lasting several days and then cerebellar signs: cranial nerve deficits and hemiparesis. Cerebrospinal fluid shows increased protein and white blood cell counts; culture is positive in only 50%.

Kasper et al. studied a total of 150 human cases of listeriosis reported in Austria between 1997 and 2007 and found 9.3% to be pregnancy-associated (mother/child illness considered as a single case). (Kasper et al. 2009). Among the 136 non-pregnancy-associated cases, 55.2% were male and 44.9% female. Overall, 131 of 150 human cases (87%) had some type of risk factor or underlying disease associated

with contracting listeriosis. The majority of cases (90.7%) were caused by systemic infections, only 9.3% of cases were local infections. Among non-pregnancy-associated cases the 30 day all cause fatality rate was 28.7% (39/136) and among the pregnancy-associated cases 35.7% (5/14; miscarriage ×3, stillbirth ×1, death in a newborn within 15 days of birth ×1).

The widespread use of immunosuppressive medications for treatment of malignancy and management of organ transplantation has expanded the immunocompromised population at increased risk of listeriosis. The estimated risk for contracting listeriosis is 300–1000 times higher for AIDS patients than for the general population. However, relatively few cases have been reported worldwide among HIV positive or AIDS patients; in Austria listeriosis has been observed in HIV-positive patients only once so far (da Silva et al. 1992; Kasper et al. 2009). The preventive dietary recommendations to avoid the high risk foods in HIV positive persons and the repeated antimicrobial therapy for opportunistic infections may explain the relatively low incidence rate of listeriosis in AIDS patients (Vazquez-Boland et al. 2001).

L. monocytogenes can produce a wide variety of focal infections: conjunctivitis, skin infection, lymphadenitis, hepatic abscess, cholecystitis, endocarditis, peritonitis, splenic abscess, pleuropulmonary infection, joint infection, osteomyelitis, pericarditis, myocarditis, arteritis, and endophthalmitis (Lorber 2010; Guerrero et al. 2004, Allerberger et al. 1989,1992). Cutaneous listeriosis usually presents as papular or pustular lesions on the arms or hands (of veterinarians). Cutaneous listeriosis is observed extremely rarely and is most often acquired as an occupational hazard from infected animals (McLauchlin and Low 1994; Regan et al. 2005; McLauchlin 2011).

In healthy adults, exposure to *L. monocytogenes*-contaminated food usually causes only a short period of fecal shedding without illness. Foodborne transmission of *L. monocytogenes* can also cause a self-limiting acute gastroenteritis (in immunocompetent persons). From the data available in normal hosts in Italy, Illinois (USA) and Austria, it appears that febrile gastroenteritis in normal hosts requires the ingestion of a high dose of several million bacteria (Aureli et al. 2000; Dalton et al. 1977; Pichler et al. 2009). Grif et al. studied the incidence of fecal carriage of *L. monocytogenes* in healthy volunteers (Grif et al. 2003). The PCR results of the subjects indicated an incidence of five to nine exposures to *L. monocytogenes* per person/year. On an average, the incidence of culture-confirmed fecal carriage in healthy adults was two episodes of *L. monocytogenes* carriage per person/year. Fecal shedding was of short duration (maximum four days). The discrepancy between PCR results and the results from conventional culture could be explained by protective host effects. In particular, secretion of gastric acid provides an important protective factor against the passage of potentially pathogenic organisms. Cobb et al. have shown a drastically increased prevalence of *L. monocytogenes* in the feces of patients receiving long-term H_2-antagonists compared to the prevalence in patients with normal gastric secretion (Cobb et al. 1996).

10.8 Discussion of Unresolved Issues

The dose-response relationship of *L. monocytogenes*, which represents an essential component of risk assessment, is still a pivotal question (Hoelzer et al. 2012). Presumably it depends upon the serotype, concentration, virulence and pathogenicity of the involved strain and also on host risk factors (Vazquez-Boland et al. 2001). The infectious dose for systemic listeriosis has not yet been determined.

Although invasive listeriosis occurs primarily in patients with underlying diseases, there are also reports on a rise of listeriosis in previously health persons (Goulet et al. 1998). The mechanism of this appearance has not yet been elucidated.

In recent years, an increasing rate of invasive listeriosis has been reported in several European countries. This increase primarily reflects a higher rate of bacteremic listeriosis in those >65 years of age and is not otherwise correlated with geography, gender, ethnicity, socio-economic factors or infectious serotypes. The causes of this increase remain unknown at present.

Goulet et al. hypothesized that the recently reduced salt content in ready-to-eat food (RTE) products may contribute to the growth of the organism, if present as a contaminant, and increase the likelihood of infection when these products are consumed by susceptible individuals (Goulet et al. 2008). The food industry reduced the salt content of selected products, such as RTE meat products, in response to recommendations in 2002 from food safety agencies, asking for a 20% reduction in average salt intake, spread over five years, in order to prevent disease attributable to hypertension-related conditions. The influence of salt content in food on the general incidence of human listeriosis is unclear.

Morvan et al. have analyzed the evolution of susceptibility to antibiotics in 4186 clinical isolates of *L. monocytogenes* through several decades and found the prevalence of resistant strains in humans at a stable low level of 1.3% (Morvan et al. 2010). Marco et al. tested the in vitro activity of 22 antimicrobial agents against *L. monocytogenes* isolated in Spain (Marco et al. 2000) and found no increase in resistance with sequential analysis over the study period. The question why *L. monocytogenes*, in contrast to all other zoonotic agents, is not showing an increase in antimicrobial resistance to drugs widely used in animal production and in human medicine, remains unresolved.

The emergence of human listeriosis is the result of complex interactions between various factors that may reflect changes in social patterns. Gillespie et al. (2010a, b) studied the association of human listeriosis with neighborhood deprivation and found that listeriosis incidence was highest in the most deprived areas of England when compared with the most affluent, and those affected were more likely to purchase foods from small convenience stores or from local services (bakers, butchers, fishmongers and greengrocers) than were the general population. They hypothesized that small businesses do not have access to the same level of food safety expertise as do larger retail companies, that increased deprivation could be associated with conditions where refrigeration may be inadequate or unavailable and that financial pressures may encourage individuals to store food longer than the food product's safe shelf-life. The exact role of changes in social patterns is still an unresolved issue.

Bacteriophages specific for *L. monocytogenes* have been approved as biocontrol agent against in food (Mahony et al. 2011). Use of phages as biocontrol may inadvertently select for epidemic clones uniformly resistant to all tested phages when grown at temperatures < 30 °C (Kim and Kathariou 2009), should they be present together with other strains of listeria. The role of phage resistance as a contributor to environmental fitness is an unresolved issue.

Changes in the way food is produced and distributed have increased the potential for widespread outbreaks involving many countries as a result of contamination of widely distributed commercial food products. Therefore, more and more states implement PFGE typing of all human isolates and of all food isolates. However, while it is clear what constitutes a foodborne outbreak, there is no consensus on criteria for defining an *L. monocytogenes* cluster as a possible foodborne outbreak. The minimum number of *L. monocytogenes* isolates with indistinguishable PFGE patterns and the respective time period necessary to consider a cluster an indicator for a possible foodborne outbreak, i.e. for starting an epidemiological investigation, remains unresolved.

The establishment of European surveillance and mechanisms for collaborative outbreak investigation is a declared aim of the European Centre for Disease Prevention and Control (ECDC). A centralized database for administration of standardized PFGE patterns of human isolates is supposed to enable public health authorities to link seemingly sporadic cases to transnational outbreaks in the near future. Whether or not PFGE analysis is the right method to be declared as standard in the twenty-first century is another unresolved issue.

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Chapter 11
Brucellosis: It is not only Malta!

Mile Bosilkovski

Abstract Brucellosis is caused by one of the ten species of the genus *Brucella*, of which only several can cause human disease. It is among the most widespread zoonoses in the World. The disease is transmitted to humans by contact with fluids from infected animals or derived food products. The mechanisms by which brucellae manifest their pathogenic features are complex. Lipopolysaccharide (LPS) has a role in evasion of the infected cell and is essential for intracellular survival. Brucellae invade and persist in the host via inhibition of programmed cell death. Despite the high degree of DNA homology within the *Brucella* genus, molecular typing schemes based on the use of multiple locus variable number of tandem repeats analysis (MLVA) have proven to be able to differentiate unrelated *Brucella* isolates which could not be differentiated by classical microbiological methods. Due to its high discriminatory power, MLVA has been successfully used for identification of human outbreaks related with a same source of infection, as well as for confirmation of relapse and laboratory acquired infection. Prevention of human brucellosis may be based on prevention of transmission from animal reservoirs to humans and control or eradication of the infection in the reservoir population. Control and prevention programs in animal brucellosis require effective collaboration between all sections of the community and must be properly planned, coordinated and resourced.

In spite of the huge knowledge on the disease until now, many aspects are not yet clarified, like actual taxonomy and the durability and solidness of the immune response after the disease. Also, there is the need of development of better diagnostic techniques and therapeutical options. The production of an effective and acceptable vaccine for use in humans remains as a special challenge in this field.

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11.1 Introduction

Brucellosis with its acute or chronic form is one of the most widespread zoonoses in the world. In endemic regions brucellosis is recognized to have an important impact on human and animal health, economic development, agricultural trade and even tourism (Bosilkovski et al. 2007). Brucellosis has many synonyms derived from the geographical regions in which disease occurs e.g., Mediterranean fever, Malta fever, Gibraltar fever, Cyprus fever; from the remittent character of the fever e.g., undulant fever; or from its resemblance to malaria and typhoid e.g., typhomalarial fever, intermittent typhoid (Mantur et al. 2007).

11.2 Causative Agents

The genus *Brucella* belongs to the family *Brucellaceae* within the order *Rhizobiales* of the class Alphaproteobacteria. The closest phylogenetic neighbour of the genus *Brucella* is the genus *Ochrobactrum*, a saprophyte that occasionally infects humans (Godfroid et al. 2011).

For long time the genus *Brucella* was comprised by six species, namely *B. melitensis, B. abortus, B. suis, B. canis, B. ovis* and *B. neotomae*. All these species are genetically highly related. In the last few years, several new species were discovered, such as *Brucella ceti, Brucella pinnipedialis, Brucella microti* and *Brucella inopinata*. This classification which is principally based on biochemical capabilities, susceptibilities to dyes and phages as well as varying pathogenicity and animal hosts preference (Al Dahouk and Nockler 2011; Cuttler et al. 2005), is currently accepted by the International Committee on Systematic Bacteriology subcommittee on the taxonomy of *Brucella*, despite the attempts to classify this genus on the basis of genetically high relatedness, according to which it was proposed merging of the *Brucella* species into a single *B. melitensis* species, with the other species to be recognized as biovars of *B. melitensis* (e.g., *B. melitensis biovar abortus 1*) (Verger et al. 1985).

Brucellae are facultative intracellular, small, gram-negative cocci or coccobacilli 0.5–0.7 mm in diameter, and 0.6–1.5 in length. They are non-motile, aerobic, partially acidfast microorganisms, that lack flagellae, capsules, endospores or native plasmids (Lindquist et al. 2007).

Brucella spp. grow on most standard media, for example blood agar, chocolate agar, trypticase soy agar, and serum-dextrose agar. Bovine or equine serum, which is needed for growth by various strains, is routinely added to the basal medium.

Samples for culture are first inoculated into liquid medium such as trypticase soy broth. The organisms grow slowly in culture and blind subcultures should be made every 5 days onto solid medium, e.g. trypticase soy agar. Growth is best at 37 °C. An atmosphere containing 5–10 % CO_2 is needed and is a distinguishing feature for most *B. abortus* isolates (Wright 2000). The colonies are raised and convex, small, smooth and circular, 0.5–1 mm in diameter. They are moist, transparent and glistening (Ganguly 1984).

B. melitensis, B. abortus and *B. suis* have 3, 7, and 5 biovars, respectively. The preferred hosts for *B. melitensis* are small ruminants such as goats, sheep and camels, for *B. abortus* cattle, camels, yaks and buffales, for *B. suis* biovars 1 and 3 pigs, for the other biovars wildlife animals (for biovar 2 wild boar and hare, for biovar 4 caribou and reindeer, for biovar 5 small rodents). For *B. canis* the preferred host is the dog, for *B. ovis* and *B. neotomae* are sheep and desert wood rats, respectively. Finally, the preferred hosts for *B. ceti* are dolphins and whales, for *B. pinnipedialis* seals, and for *B. microti* common voles and red foxes. The host for *B. inopinata* is still unknown (Al Dahouk and Nockler 2011). *B. melitensis, B. abortus* and *B. suis* biovars 1, 3, and 4 are highly pathogenic for humans, *B. canis* shows moderate pathogenicity, and *B. suis* biotypes 2 and 5, *B. ovis* and *B. neotomae* are considered as non pathogenic in humans. The role of the other species is yet to be defined in the future, although there is scarce information for their supposed pathogenicity in humans, although some cases with more serious forms with central nervous system involvement have been reported (Sohn et al. 2003).

Brucellae can survive in the environment for long periods when there is an adequate combination of humidity and temperature. Other factors that affect their survival in the habitat are the nature of the substrate, number of organisms, pH, sunlight and the presence of other microbial contaminants. They can survive in dust from 15 to 40 days, in wet soil for up to 70 days, in liquid manure for up to 2 years, in goat cheese for up to 180 days, and in tap water for up to 60 days. They are quite sensitive to heat and to most commonly used disinfectants, and are killed by pasteurization (Gotuzzo and Carillo 2004). *Brucellae* cannot survive the acidity below pH 4.

11.3 Pathogenic Features and Host Immunity

11.3.1 Pathogenic Features

The principal pathogenic manifestations in brucellosis are mainly based on two characteristic features for this organism: (1) the bacterium exhibits a tendency to invade and persist in the host through inhibition of apoptosis; and (2) it does not bear classic virulence factors, such as toxins, cytolysins, capsules, fimbria, flagella, plasmids, lysogenic phages, resistant forms, antigenic variation, endotoxic LPS, or apoptotic inducers. Moreover, its lipopolysaccharide pathogenicity is not typical.

Urease enzyme is an important determinant of virulence. Urease has the role to protect *Brucellae* in their passage through the stomach when acquired by the oral route (Mantur et al. 2007). After invading and crossing the epithelial surface, *Brucellae* come in contact with humoral mediators and are readily ingested by polymorphonuclear leukocytes and macrophages which then transport them to local lymph nodes. Organisms are retained in this site, but replicate intracellularly and bacteria from lysed cells can infect other cells or disseminate throughout the body. Indeed, from the bulk of ingested *Brucella*, most of them are destroyed within the

phagolysosomes of phagocytic cells of the reticuloendothelial system, while only 15–30 % possess an extremely efficient adaptation to shield themselves from the immune recognition and to manipulate key aspects of host cell physiology (Gorvel 2008). *Brucella* has a type IV secretion system named VirB and periplasmic cyclic beta-1,2-glucans that enable the pathogen to redirect their intracellular trafficking, to avoid fusion and intracellular killing in lysosomes, and finally reaching to their final replicating niche in the endoplasmic reticulum (Gorvel and Moreno 2002; Barquero-Calvo et al. 2007). Once inside the endoplasmic reticulum, *Brucellae* extensively replicate without restricting basic cellular functions or generating programmed cell death (Gorvel and Moreno 2002; Barquero-Calvo et al. 2007). All these activities might promote the chronicity of infection (Skendros et al. 2008).

In order to sustain chronicity, the host environment must provide choline, a necessary precursor for the synthesis of the *Brucella* envelope without which the organisms are not virulent (Comerci et al. 2006). It is also obvious that one of the adaptation keystones is the ability to finely tune the metabolism according to the various nutrients encountered during the infectious cycle (Lamontagne et al. 2010). Susceptibility to intracellular killing differs among species, with *B. abortus* readily killed, and *B. melitensis* rarely affected; this might explain the differences in pathogenicity and clinical manifestations in human cases of brucellosis.

As previously stated, in contrast to other pathogenic bacteria, no classical virulence factors have been described in *Brucella* organisms (Moreno and Moriyon 2002). The smooth lipopolysaccharides and proteins involved in signaling, gene regulation, and trans-membrane transportation, are among the factors suspected to be involved in the virulence of *Brucella*. The smooth lipopolysaccharide helps to block the development of innate and specific immunity during the early stage of infection, prevents the synthesis of immune mediators in inducing host release of inflammatory cytokines, has a role in cell entry and immune evasion of the infected cell, and is essential for intracellular survival. Additionally, the smooth lipopolysaccharide in *Brucella* may be involved in the inhibition of apoptosis of infected cells, thus preventing the programmed cell death.

11.3.2 The Host Response

Antibody response in brucellosis, although extremely useful diagnostically, plays a limited part in the overall host response. It seems that cell-mediated immunity is the principal mechanism of recovery (Skendros et al. 2011). Cell-mediated immunity is also responsible for partial resistance to subsequent reinfection (Gotuzzo 1999).

The serum antibody response to *Brucella* infection in humans is characterized by an initial rise in antibody titers of the IgM class, followed in several weeks by a predominance of IgG antibodies. The titers of agglutinins (IgM, IgA, and IgG), should decline after successful treatment; if they do not, it is necessary to evaluate the patient for the possibility of a relapse or chronic focal disease, for example endocarditis, although they may persist at high levels for months even in cured patients (Al Dahouk et al. 2003).

Control of brucellosis in humans depends on activated macrophages and hence requires development of Th1 type cell-mediated immune response involving IL-2, IL-12, interferon-γ (IFN-γ), and TNF-α. *Brucella* LPS is a weak inducer of the host inflammatory cytokines IL-1β, IL-6, IFN-γ and TNF-α, compared to LPS molecules from many other Gram-negative bacterial pathogens. IFN-γ and TNF-α are considered to be essential for the elimination of the organism (Zhan and Cheers 1995). IF-γ is a key cytokine controlling *Brucella* infection. One of its major functions is the stimulation of *Brucella*-killing effector mechanisms in phagocytic cells (Baldwin and Parent 2002). TNF-α is essential to develop the Th1 type macrophage response generating INF-γ, CD4+, and CD8+ T lymphocytes, and leading to clear the organism (Zhan et al. 1996).

Some brucellar ribosomal proteins have reemerged as immunologically important components. It has been established that the L7/L12 ribosomal proteins are important in stimulating host protective cell-mediated responses (Corbel 1997).

Erythrol is believed to be important for determining tissue tropism for *B. abortus*. Indeed, *Brucella* uses this sugar alcohol in preference over alternatives, and mutants unable to utilize erythrol are severely attenuated in ruminant hosts (Cuttler et al. 2005). It is a well-known fact that the cattle's placental tissue is rich with erythrol, and during gestation, *B. abortus* replicates intensively in placental trophoblasts, which may induce placental integrity disruption and abortion (Ganguly 1984). This also happens in other animals as well, with which the disease is related. The other cause for spontaneous abortion in animals is due to the lack of anti-*Brucella* activity in the amniotic fluid.

The protection in brucellosis is usually short-term and incomplete (Corbel 1997), and lifelong immunity does not occur (Issa and Jamal 1999). As a result, reinfections are possible events (Kasimova and Rentzova 1979). Results from PCR tests show that brucellosis patients who have apparently recovered from the illness still show bacterial load in a considerable proportion, even for several years after treatment, which is indicative of a residual chronic infection (Navarro et al. 2006; Vrioni et al. 2008). However the absence of clinical manifestation of the disease in spite of detectable bacterial load might be explained with the low bacterial replication rate and its control by the host's immune system (Al Dahouk and Nockler 2011).

11.4 Epidemiology

11.4.1 Geographical Distribution of Animal and Human Disease

In developing countries, brucellosis is almost always present in small ruminants, and has no administrative borders. Cattle seroprevalence in the world estimates range between 3 and 15 % (Ghanem et al. 2009; Jergefa et al. 2009). Factors influencing prevalence include production systems, agro-ecological zones, husbandry

practices, contact with wildlife and management factors (Muma et al. 2007). The prevalence of the disease in pigs is generally low, except in parts of South America, Oceania, and South East Asia (Blasco 2010). Within the EU, the epidemiological situation of brucellosis in pigs varies. *B. suis* biovar 2 is the most common agent, but biovars 1 and 3 can also occur. *B. suis* has a much more restricted occurrence than *B. melitensis* and *B. abortus*. *B. canis* is a widespread infection of dogs in many countries.

The incidence of human infections may be a useful indicator of the presence of the disease in animal populations and may be the only source of information for brucellosis surveillance. Unfortunately, the true incidence of human brucellosis is unknown for many countries and regions. The reported incidence of 500,000 cases per year worldwide might be up to 25 times less than the true incidence, having in mind that this disease quite often remains unrecognized mainly due to inaccurate diagnosis and inadequate health network systems, or due to its underreporting mainly associated with poor collaboration from the private health care sector, lack of a standardized case definition for brucellosis, as well as existence of different national policies with regard to diagnosis and treatment of brucellosis cases. The majority of human brucellosis cases are caused by *B. melitensis* (Nicoletti 2010). Infrequently there are cases caused by *B. abortus* and *B. suis*, whereas cases caused by *B. canis* are very rare among humans, even in areas where the infection is common in dogs (Young 1995).

Brucellosis is spread through all continents, but there are regions in the world that are considered to be endemic. Traditionally, endemic regions are Middle East, the Mediterranean region, sub-Saharan Africa, China, India, and parts of South America. In any case, this endemicity of brucellosis tends to be prone to modifications and alternations in the last two decades, as a result of improved diagnostic strategies and implementation of appropriate public health care and veterinary strategies as well as trans-boundary activities for a better surveillance. Also, one cannot neglect the influence of the geopolitical and socioeconomic changes, increased globalization, and transition of the healthcare and veterinary systems (Pappas et al. 2006c). Due to these changes, brucellosis, which up till the nineties in the last century was considered to be endemic in Spain, Portugal, Israel and some year before even in France, nowadays is pronounced diminished, and currently Syria, Mongolia, Turkey, Iran, Iraq, Kazakhstan, Kyrgyzstan, Azerbaijan, Turkmenistan are the countries with the greatest increase in cases. Several countries in Western and Northern Europe (Sweden, Denmark, Norway, Finland, Germany, UK, Netherlands, Belgium, Luxembourg, Austria, Switzerland), Canada, Japan, and New Zealand are believed to possess brucellosis free status; still human brucellosis occurs sporadically in individuals who acquire the infection abroad or by ingestion of illegally imported and unsafe animal products, immigrants from endemic regions, and in occupationally exposed groups (Al Dahouk et al. 2005a; Pappas et al. 2006c). Even today, brucellosis remains as a regionally emerging disease, and cannot be easily contained by border controls.

11.4.2 Sources

The main sources of the disease are almost exclusively infected domestic animals. Wide host range and resistance of *Brucellae* to environment and host immune system facilitate its survival in the population. Various bacteria of the *Brucella* family tend to infect a specific animal species. However, most species of *Brucella* are able to infect other animal species as well. *B. suis* and *B. melitensis* infections can occur if cattle are in contact with infected pigs or goats. This is seen particularly often in some parts of the world (Southern Europe, Israel, Kuwait, and S. Arabia), where *B. melitensis* is found in cattle, and represents a significant source of *B. melitensis* infection. Moreover, abortions in cattle infected with *B. melitensis* have been reported. The same pattern is found (although abortions have not been documented) with *B. suis* biovar 1 in Mexico and parts of Latin America, where cattle are more important than pigs as a source of human infection (Godfroid et al. 2005). The main problem is that there is no applicable vaccine for *B. melitensis* which would successfully prevent disease in cattle, as well as there is no ready vaccine for *B. suis*. It might be too optimistic to state that *B. melitensis* infections in cattle are not sustainable infections and that implementation of control measures for small ruminants without any additional specific measures are likely to solve the problem by exhausting the source of infection in cattle (Godfroid et al. 2005). Probably the same holds true for *B. suis* infection as well. In any case, these assumptions have not been definitively confirmed. One should have in mind that dogs can acquire infection with *B. abortus, B. melitensis* or *B. suis* from aborted ruminants or swine, usually by ingesting fetal or placental material. Then they can excrete these bacteria and may present a serious hazard to humans and domestic livestock. *Brucella canis* is rarely transmitted to humans.

The problem with animal brucellosis is aggravated by wildlife brucellosis which represents a potential zoonotic and human threat (Godfroid et al. 2005). *B. abortus* infection is present in bison and elk in Yellowstone area, *B. suis* biovars 1 and 3 is found in feral swine (domesticated pigs that have returned to the wild) in some regions of the world (USA, Australia). These animals pose a risk to domestic pigs and cattle with which they may commingle (Young 1995). In some regions of USA, feral pigs were infected with *B. abortus* wild type, BA 19, and RB51 vaccine strains besides *B. suis* biovar 1 (Stoffregen et al. 2007). *B. suis* biovar 2 is widely distributed in free-ranging boars and hares within the EU; fortunately, this biovar is not yet an important pathogen for humans. There are observations of *B. melitensis* infected fish in the Nile (El Tras et al. 2010). Therefore, wildlife should be always carefully monitored in order to prevent the re-emergence of brucellosis.

11.4.3 Portal of Entry and Transmission

The portal of entry in either humans or animals are abraded skin or mucosal surfaces (respiratory, gastrointestinal, genital, conjunctiva). Even microtraumas as well as

the smallest disruption of skin integrity and mucosal surfaces are enough to become an entry site for the bacteria.

Infected animals shed brucellae in the environment (pasture and animal barn) through urine, milk and uterine discharges following abortion and subsequent parturition for prolonged periods, making it easily accessible for other animals and humans. Aborted fetuses, placental membranes or fluids, and other vaginal discharges present after an infected animal has aborted or calved may be highly contaminated with infectious *Brucella* organisms, and the number of microorganisms in one gram of placenta is estimated to be 10^{13} (Banai 2010). Animals which are not infected but colonized by the bacteria in the udder can potentially contaminate the milk.

Brucellosis is commonly transmitted to susceptible animals by direct contact with infected animals or via the environment that has been contaminated with the large number of organisms discharged from infected animals. *Brucellae* are spread within the herd primarily by ingestion of contaminated food or water, but *Brucellae* can also be transmitted by inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups, as well as venereally, mainly in *B. suis* infections. Artificial insemination is also described as a way of transmission (World Health Organization 2006). Natural breeding transmits infection in swine and dogs and, to a lesser extent, sheep and goats. *B. canis* transmission may happen by contact with recently aborted animals or with food or environment contaminated by abortions or excreta. Sexual transmission is also an important means of spread, and males can excrete the organisms in large numbers in their semen. Urinary excretion also occurs and is a potential hazard.

After exposure, the likelihood of the animal becoming infected is variable, depending on age, pregnancy status, and other intrinsic factors of the animal, as well as the amount of bacteria to which the animal was exposed. In cattle, sheep, goats and swine, susceptibility to brucellosis is greatest in sexually mature animals. Young animals are often resistant, although it should be noted that latent infections can occur and such animals may present a hazard when mature (World Health Organization 2006).

In countries with effective food hygiene measures preventing foodborne brucellosis, human brucellosis is mainly an occupational disease. Brucellosis is spread to humans through direct contact with infected animals, their carcasses, blood, body tissues, or body fluids, and other animal products or through exposure to a heavily contaminated environment. Direct and environmental contamination may present hazards through inhalation, ingestion, and mucous and skin penetration. Most commonly infected are veterinarians, shepherds, abattoir, dairy and meat industry workers, and personnel in microbiologic laboratories. Besides dealing with aborting animals, hazardous activities also include contacts with infected animals in other circumstances like shearing, dipping, clinical examination, vaccination and treatment, or the disinfection and cleaning of contaminated premises. Risky habits for contracting brucellosis present practices in some parts of the world like skinning stillborn lambs, kids and aborted fetuses, or crushing the umbilical cord of newborn lambs and kids with the teeth (Godfroid et al. 2005).

A possible way of transmission in professionally exposed people also includes inhalation through bacteria rich aerosols which primarily happens in slaughterhouses, barns, and especially in microbiologic laboratories. Aerosol transmission of brucellosis is widely accepted as a potential biohazard in laboratories, and it was estimated that laboratory-associated infections represent 2% of reported cases of brucellosis (Fiori et al. 2000). However, with the new biohazard precautions and with the handling *Brucellae* in biosafety level three cabinets, laboratory infections are no longer frequent.

Other possible way of contracting the disease is through alimentary route, by consumption of products from infected animals which are not adequately thermally processed. This is usually the main source of brucellosis in urban populations. Consumption of unpasteurized dairy products especially raw milk, and soft goat or ewe cheese prepared from unpasteurized milk are the most frequently incriminated food. Consumption of butter and ice cream are also possible means of acquiring brucellosis. Hard cheese is less hazardous because of the activity of propionic and lactic fermentation (Pappas et al. 2005). In certain regions of the world possible way of infection can be ingestion of raw or undercooked liver, spleen, reindeer bone marrow, as well as aborted animals fetuses. Meat products are rarely incriminated food for infection, due to the fact that they are not usually eaten raw, and the number of organisms in meat is low (Pappas et al. 2005).

In endemic areas, due to high family involvement, especially important is the screening of household members of infected persons, in order to determine unrecognized cases (Bosilkovski et al. 2010b).

11.4.4 Various Epidemiological Aspects of Human Brucellosis

A possible occupational way of acquiring the disease also includes numerous incidents of human brucellosis that have been linked to accidental inoculation with vaccine strains, especially in veterinarians during the process of vaccination or during laboratory preparation of the vaccines (World Health Organization 2006). Spraying vaccine into the eyes carries a higher risk for infection than needle stick injuries (Young 1995).

Brucellosis represents a risk for travellers to countries where the disease is endemic, e.g. by consumption of local food prepared mainly from unpasteurized milk products. Travellers may also import infected cheeses or other dairy products into their own countries and infect other people. Imported cases now account for most of the acute brucellosis cases seen in USA and some developed European countries (Melzer et al. 2007).

Human to human transmission is unusual; in rare cases (and mainly theoretically) brucellosis might be transmitted by blood transfusion, sexual transmission, breast feeding, congenitally, and by tissue transplantation.

Brucellosis belongs to the “dirty dozen”, the list of pathogens considered to be most likely used in a bioterroristic scenario (Neubauer 2010). According to the CDC

classification, brucellosis is categorized into group B pathogens due to their low infective dose of only 10 bacteria and the possibility of transmission via aerosols. It is estimated that 50–80 % of exposed persons will develop clinical disease (Neubauer 2010) which is sufficient to cause panic and sustainable human and economic losses (Pappas et al. 2006a).

11.5 Clinical Manifestations

11.5.1 Animal Brucellosis

In animals the infection is long-lasting. The incubation period is quite variable ranging from about 2 weeks to 1 year and even longer. Typically the disease is mild. Characteristic, but not specific signs of brucellosis in most animal hosts are abortion or premature births, infertility, retention of placenta, stillbirth, or birth of weak offspring. There may be also swelling of the testicles in males, and arthritis.

In cows, bacteremia and colonization of the mammary gland and supramammary lymph nodes may lead to suppurative placentitis and abortion. Infected cows usually abort once, but a percentage will abort during additional pregnancies, and calves born from later pregnancies may be weak and unhealthy. Other signs of brucellosis include reduced milk production, mastitis, lowering of fertility with poor conception rates, and arthritis with lameness. In bulls, seminal vesiculitis and orchitis can be seen. In sheep and goats, clinical signs include late term abortion or birth of weak lambs and kids, and mastitis. Localized infections result in orchitis or epididymitis. Arthritis may also be a rare sign in infected sheep and goats. In sows, abortion, infertility, and other reproductive disorders are possible manifestations, as well as orchitis in boars. Less commonly, arthritis, spondylitis, or abscesses in various organs can occur.

In bitches, abortions are typical; in stud dogs, epididymitis, orchitis and scrotal dermatitis, sometimes progressing to complete scrotal necrosis, may occur. Discospondylitis and uveitis are rare events in dogs infected with brucellae. In horses, it causes bursitis called “fistulous withers” and “poll evil”, a swelling of the neck or back. However, pregnant mares may either abort or give birth to weak and vulnerable foals. Clinical signs of brucellosis in camels appear to be very rare (World Health Organization 2006).

11.5.2 Human Brucellosis

In humans, brucellosis is a systemic infection with a very heterogeneous clinical spectrum, which led to the statement: “no disease, not excepting syphilis and tuberculosis, is more protean in its manifestations” (Simpson 1941). The clinical spectrum is ranging from asymptomatic to severe, life threatening forms. Clinical and

laboratory features are often not pathognomonic for human brucellosis and differ widely. *B. melitensis* is the most virulent type and associated with severe acute disease. In general, *B. melitensis* and *B. suis* are more virulent for humans than *B. abortus* and *B. canis,* although serious complications can occur with any *Brucella* species.

In humans the incubation period usually lasts 1–4 weeks, but may be as long as several months, depending on the virulence of the organisms, route of entry, infecting dose and host resistance. The onset may be sudden or more frequently gradual. Symptoms include fever, headache, night sweats, extreme fatigue, malaise, chills, weight loss, loss of appetite, joint pain, back pain, and general aching. The most characteristic among these symptoms is the trias comprising fever, sweating, and arthralgia. Fever may be continuous, intermittent, remittent, or irregular. It can last for months. Therefore, brucellosis is considered as a possible cause of so-called fever of unknown origin. The fever has no distinctive pattern and can range from subfebrile to hyperpyretic values, and in times it can be absent. The sweating is very intensive, mainly at night time, and diaphoresis is present in the absence of temperature. The sweat often is described as exhibiting a characteristic smell of wet hay. Arthralgias are of different intensity and may have a migratory pattern.

Besides the general manifestations of illness, brucellosis can involve any organ or tissue of the body. When clinical symptoms related to a specific organ predominate, the disease is termed "localized". Reported incidence rates range from 6 to 92% (Bosilkovski et al. 2010b). The wide variation in the frequencies of clinical manifestations may reflect the characteristics of the examined population, the nature of the causative agent, geographic variations of the disease, the stage of disease, the diversity of case definition criteria, the diagnostic procedures, and whether the study is retrospective or prospective.

Osteoarticular brucellosis is the most frequent focal form, occurring in about 40% of cases. According to localization, it can present as sacroiliitis, spondylitis, peripheral arthritis, and sometimes also osteomyelitis, bursitis, and tenosynovitis. Brucellar spondylitis is one of the most serious forms not only as localized osteoarticular manifestation, but also in systemic brucellosis. It has been incriminated as the major factor for therapeutic failures, sequel and necessity for surgical treatment mainly due to paravertebral and/or epidural abscesses. It is usually seen in elderly patients. Brucellar sacroiliitis is often present, mainly unilateral. Dominantly affected peripheral joints are hips, knees, and ankles, although any single joint can be involved. The pathogenesis of arthritis in brucellosis is either infective or reactive (Bosilkovski et al. 2004).

Genitourinary localization is second in frequency, with an incidence of 1–20%. Epidydimitis and orchoepidydimitis are the most common genitourinary forms. Prostatitis, dismenorea, salpingitis, cervicitis, pelvic abscesses, are sometimes described, whereas renal involvement is rare. Testicular atrophy is a possible sequela in genitourinary brucellosis.

The reported incidence of neurologic complications is 2–6% of cases. Most common manifestations are meningitis (acute and chronic), encephalitis, myelitis, radiculitis and neuritis (cranial or peripheral), which can present autonomously or

in various combinations. Other CNS manifestations of brucellosis include arachnoiditis, cerebellar syndrome, brain and epidural abscesses, infarction, hemorrhage, cerebral vasculitis, mycotic aneurysms, Guillain-Barré syndrome, chorea, and compressive myelopathy.

Cardiac involvement is rare, occurring in 0–2 % of symptomatic patients. The most common complication is endocarditis, which is also the most common cause of death from brucellosis. Endocarditis can involve both native and prosthetic heart valves. The aortic valve is involved more often than the mitral valve (Gotuzzo and Carillo 2004). Brucellar endocarditis is mainly characterized with fibrosis, hyalinization, and calcifications, rather than appearance of large vegetations. Patients more often die from valve deformity and congestive heart failure before sepsis and embolism, although the latter cannot be excluded as well. Pericarditis, myocarditis, endarteritis, thrombophlebitis, and aortic mycotic aneurisms are also described.

Liver involvement is common, although it has only minor clinical relevancy, e.g. painless hepatomegaly or moderate transaminase elevations. The real complication in brucellosis involving liver include its clinical expression i.e. pain under the right ribcage and/or icter in the presence of more serious changes in the biochemical parameters or abscess formation which could be seen in 3–6 % (Gotuzzo 1999). Rare cases of liver and spleen abscesses, cholecystitis, pancreatitis, ileitis, colitis and spontaneous peritonitis have been reported.

Respiratory form is present in 1–16 % of symptomatic persons. Bronchopneumonia, cavitated pneumonia, interstitial pneumonitis, pulmonary nodules and abscesses, hilar and paratracheal lymphadenopathy, pleural effusions and empyema can be seen.

Hematological complications quite often are a consequence of hemophagocytosis, hypersplenism, and presence of granulomas in bone marrow as part of brucellar infection. Most often present are anemia in up to 75 %, leucopenia in up to 50 %, severe thrombocytopenia in 1–2 %, which may be a cause for death (Gotuzzo and Carillo 2004), and pancytopenia in 5–20 %. In these patients hemorrhage is a possible event, due to liver dysfunction, activation of fibrinolysis and thrombocytopenia. Dissiminated intravasal coagulation (DIC) presents a rare hematologic complication.

A variety of skin lesions have been reported in patients with brucellosis with a frequency of 5–9 %, including rashes, nodules, papules, erythema nodosum, petechiae, purpura, cutaneous granulomatous vasculitis, ulcers, and abscesses. It is considered that they might be a result of local inoculation, dissemination from lymph nodes, or are a consequence of immunological reactions in patients with high antibrucellar antibody titers.

Different ocular lesions have been reported in these patients, including uveitis, nummular keratitis, corneal ulcers, chronic iridocyclitis, retinitis, multifocal choroiditis, or optic neuritis. Uveitis is the most characteristic manifestation, especially in chronic brucellosis. Circulating immune complexes may have a role in the pathophysiology of the uveal damage. Possible sequelae might be cataract, secondary glaucoma, and detachment of the macula (Gotuzzo and Carillo 2004).

Brucellosis during pregnancy is associated with poor fetal prognosis with a high rate of abortion, miscarriage, prematurity and fetal death. Although erythritol is not present in human placental tissue, *Brucella* bacteremia can result in abortion, especially during the early trimesters. In any case, prompt diagnosis and early and proper treatment can be lifesaving for the fetus.

The acute phase of the illness may either submit—spontaneously or more often with proper treatment—or progress to a chronic state. Illness duration for more than 1 year after the original diagnosis is termed chronic brucellosis (Young 1995) and may be present with relapses, development of persistent localized infection, or a non-specific syndrome resembling the chronic fatigue syndrome, or psychiatric symptoms. Relapse is defined as recurrence of characteristic signs and symptoms occurring at some time after the completion of proper antibiotic treatment. Patients with relapse characteristically have objective signs of infection, positive serological tests, and quite often positive blood cultures. In properly treated patients the occurrence of relapses is estimated from 0 and up to 16% (Bosilkovski et al. 2010b). Chronic localized infection is defined as persistence of characteristic signs and symptoms (with or without a positive blood culture) caused by the failure to eliminate a deep focus of infection, such as osteomyelitis, spondylitis, uveitis, or deep tissue abscesses. Finally, in a third group with chronic brucellosis patients suffer from chronic complaints lacking objective signs of infection. In the absence of fever, positive cultures, and serology, patients may suffer from psychiatric symptoms and persistent complaints such as arthralgias, depression, malaise, tremor, anxiety, impotence, insomnia, dizziness, emotional liability, and suicidal ideas. In recent decades with the use of appropriate antimicrobial treatment, the mortality rate in brucellosis has been lower than 1% (Bosilkovski et al. 2010b).

11.6 Diagnosis

Having in mind that brucellosis is a very indolent infection, delay of the diagnosis is more a rule than an exception (Chevrel et al. 2001). The fact that the disease is greatly heterogeneous and nonspecific makes clinical diagnosis difficult. Some hematological and biochemical abnormalities such as leucopenia, lymphocytosis, pancytopenia or elevated liver enzymes are not diagnostic enough to establish the exact diagnosis. As a result, confirmation by laboratory diagnosis is needed when brucellosis is suspected. Laboratory diagnosis of brucellosis includes isolation and identification of *Brucellae* from clinical samples, detection of antigen, molecular methods including PCR, and demonstration of *Brucella* specific antibodies (Mantur et al. 2007).

11.6.1 Bacterial Isolation

The isolation and identification of *Brucella* offers a definitive diagnosis of brucellosis. Blood is the material most frequently used for bacteriological culture. Also,

any fluid or tissue can be cultured (e.g., synovial, pleural, cerebrospinal, prostatic fluid, liver, lymph node), but the yield is usually low. The recommended system of blood culture is the biphasic method of Ruiz Castaneda which uses both solid and liquid medium in the same container (Lindquist et al. 2007). Culture results depend on varying factors including the *Brucella* species, disease stage, isolation method, quantity of circulating bacteria, and previous treatment with antibiotics. Cultural examinations have several disadvantages having in mind that they are time consuming, not sensitive, and hazardous for laboratory infection.

Conventional Castaneda blood cultures are time consuming and seldomly positive before the 4th day of incubation. The majority of blood cultures are positive between the 7th and 21st day. The presumptive *Brucella* isolate should be submitted to a reference laboratory for a precise identification at species and biovar level, which can provide valuable epidemiological information (World Health Organization 2006).

The percentage of cases with positive cultures ranges from 15 to 90 (Pappas et al. 2005; Al Dahouk and Nockler 2011). It also varies according to the clinical form; it is lower in chronic forms. Previous use of antibiotics results in lowering of the positivity of cultural examinations. In the past decades, various technical improvements including BACTEC 9204, Bact/Alert and yield-optimizing methods such as lysis centrifugation have gradually increased the sensitivity of culture methods either in acute or in chronic brucellosis and have significantly shortened the time to detection for *Brucella* spp. in clinical samples from weeks to days, so with these methods the presence of *Brucella* can be detected by the 3rd day of incubation (Araj 1999). Using these automated systems there is no need to incubate bottles more than 14 days (Araj and Kattar 2003; Yagupsky 1999). Recently, a semi-automated metabolic biotyping system based on a selection of 93 different substrates was developed for the identification of *Brucella* and the differentiation of its species and biovars (Al Dahouk et al. 2010). By routine biochemical differentiation methods, *Brucella* spp. may be misidentified as *Moraxella phenylpiruvica* or *Haemophillus species* (Corbel 1997).

As previously stated, aerosol transmission of brucellosis is widely accepted as a potential route of biohazard in laboratories. The WHO has classified brucellosis as a risk group III pathogen for laboratory personnel.

Bone marrow cultures are significantly more sensitive than blood cultures at any stage of disease and even after prior use of antibiotics; moreover, the time to detection can be shortened (Gotuzzo et al. 1986). However, harvesting bone marrow for culture remains an invasive, painful technique, and results are not universally reproducible (Pappas et al. 2005).

The identification of *Brucellae* is achieved by a combination of organism morphology after Gram or Stamp's staining, colonial morphology, CO_2 requirement for growth, H_2S production, selective inhibition of growth on media containing dyes such as thionin or basic fuchsin, as well as urease, oxidase and catalase activity, and the slide agglutination test with undiluted polyvalent anti-*Brucella* serum. Further species and biovar identification requires tests such as agglutination with monospecific sera (A and M), and lysis with the help of Tbilissi and Weybridge brucellaphages (Al Dahouk and Nockler 2011; Lindquist et al. 2007; Pappas et al. 2005).

11.6.2 Serological Diagnosis

Due to the difficulties with culture-based diagnostic methods, the diagnosis of human brucellosis has traditionally been based on the demonstration of high or rising titers of specific antibodies in the serum. The presence of positive serological tests have to be observed in the light of history of exposure and endemicity of the region, history of past brucellosis, as well as the clinical characteristics of the patient (Al Dahouk et al. 2003).

Classical serological tests that are widely used in everyday practice allow the detection of antibodies produced against the lipopolysaccharide of the bacterial cell wall. The most frequently used are Rose Bengal test (RBT) and serum agglutination test (SAT), followed by 2-mercaptoethanol (2-ME), antihuman globulin Coombs, Brucellacapt™, and ELISA. Fluorescence polarization assays (FPA) is a promising alternative to conventional serological tests, it is technically simple and relatively inexpensive, but it is validated only for the detection of brucellosis in animals (Al Dahouk et al. 2003).

The RBT is currently the recommended rapid screening test in emergency departments, but the positive results should always be confirmed by other tests detecting agglutinating and no agglutinating antibodies and by bacteriological culture, particularly in areas with a high incidence of animal brucellosis. The sensitivity of RBT is over 99%, and the specificity is 97% (Gomez et al. 2008). Lateral flow assay (LFA) is a simplified version of ELISA with a great sensitivity and specificity of 97 and 99% respectively (Godfroid et al. 2010). It can be used at all stages of disease, and should be an alternative to RBT for rapid field or bedside testing in poor rural areas without well-equipped laboratories (Al Dahouk and Nockler 2011).

The standard serologic test for detection of brucellosis is the SAT which still remains the most popular diagnostic tool. This is the test against which all others are compared. SAT is a cheap, accurate, easy to perform and reliable test and is still widely used particularly in poorer countries. SAT allows measuring the entire agglutinating capacity, but cannot distinguish individual titers of the different immunoglobulin classes which are important to determine the status of disease (Al Dahouk et al. 2003). As a diagnostic tool SAT has several shortcomings such as false positive reactions due to cross reaction with *Francisella tularensis, Escherichia coli O116* and *O157, Salmonella urbana, Yersinia enterocolitica O:9, Vibrio cholerae, Xanthomonas maltophilia*, and *Afipia clevelandensis* (Pappas et al. 2005). Also, in endemic regions the interpretation of the test could be difficult having in mind that some healthy individuals especially healthy occupational workers may possess serum agglutinins, presumably from previous contact with *Brucellae* leading to past, inapparent, or latent infection. Also, in animals the use of agglutination tests is limited in the differentiation between antibodies resulting from infection or vaccination (Morgan et al. 1969). The next shortcoming are false negative reactions early in the course of infection (Gad El-Rab and Kambal 1998), or because of immunosuppression, the presence of incomplete or blocking antibodies which can mask the agglutination, or so called “prozone” phenomenon (the inhibition of agglutination at

low dilutions due to an excess of antibodies or to nonspecific serum factors), and in chronic cases. Also, the disadvantage for this test is the lack of a defined threshold cut off for agglutination titers considered to be confirmatory for active disease. In non-endemic region a titer of 1/80 is considered positive (Al Dahouk et al. 2003). In endemic regions, a titer of ≥1/160 was accepted as confirmatory. However even lower titers are not unusual in active brucellosis. Early in the course of the disease or in persistent brucellosis, serologically negative patients can be positive in blood culture (Baldi et al. 1996). Obviously the best serological definition of brucellosis is its confirmation by a fourfold or greater rise in *Brucella* agglutination titer between acute- and convalescent-phase serum specimens obtained ≥2 weeks apart and studied at the same laboratory, but quite often it is not practical for the clinician and may delay therapeutic course. STA cannot diagnose *B. canis* infections. Although an infrequent pathogen in humans, *B. canis* is a particular diagnostic challenge because this pathogen does not share cross reacting antigens with the other *Brucella* spp. *B. canis* is a naturally rough species and antibodies against *B. canis* are not detected by tests employing smooth LPS from other *Brucellae* (Araj 1999). This is the same case in *B. ovis* and the rough vaccine strain of *B. abortus RB51*. In these rough strains, diagnosis has been achieved with LPS extracts from rough strains either in ELISA or FPA assays (Cutler 2005).

Some of these shortcomings can be overcome by modifications such as 2-ME, or antihuman globulin Coombs test. 2-ME test is an adaptation of SAT which measures IgG antibodies. This is a useful assay, as it is inexpensive, technologically simple, and proved to be a good test for follow up of brucellosis differentiating between active and inactive infection (Roushan et al. 2010). Antihuman globulin Coombs (AHG) and Brucellacapt™ test are also agglutination tests. They detect non-agglutinating or incomplete antibodies (mainly from IgG and IgA classes), eliminate the prozone phenomenon, and are fairly good for complicated and chronic cases, good for diagnosis of relapses, highly sensitive and specific (Al Dahouk et al. 2003). In addition to SAT, AHG is routinely used in many regions, and the results of this combination may help to overcome the problem of false-negative results and assess the stage of evolution of the disease at the time of diagnosis. Brucellacapt™ (an immunocapture-agglutination technique), is a modification of Coombs test for detection of an incomplete or blocking IgG and IgA antibodies, with similar sensitivity and specificity in diagnosis of brucellosis, but with an advantage that is easier to be carried out.

Complement fixation test (CFT) measures IgM and IgG and is used as a confirmatory test in the diagnosis of brucellosis. High CFT titers provide evidence of active brucellosis. After successful antibiotic therapy CFT titers decline and cannot be detected after 6 months (Buchanan et al. 1974). However, this test is notoriously time and reagent intensive and shows problems encountered in its standardization; so its only value is in detecting antibodies earlier than by agglutination tests. In general, CFT is widely used in animal, but not in human brucellosis.

ELISA measures IgM, IgG, and IgA immunoglobulins, which allows a better interpretation of the clinical situation and overcomes some of the shortcomings of the serum agglutination tests (Pappas et al. 2005). Because of that, ELISA is the test

of choice for complicated and chronic cases when other tests are negative. ELISA is rapid, objective, highly sensitive and specific. Problems are the standardization of the test reagents, variable quality of commercial reagents and interpretation of results, particularly when based on optical density readings alone, which makes difficulties in comparing the results from different laboratories (Young 1995; Al Dahouk et al. 2003). Although ELISA is simple and reliable in detecting different immunoglobulin classes, many laboratories still use classical Coombs test as an extension of SAT for proving non-agglutinating, incomplete or blocking antibodies. This may be due to the higher costs for ELISA, tradition, but also to the fact that some investigators did not find better results using ELISA (Memish et al. 2002). Competitive ELISAs are less prone to cross-reacting antibodies than conventional tests of indirect ELISAs. Its specificity is very high; however, it is slightly less sensitive than the indirect ELISA, and today could serve as an excellent confirmatory assay for the diagnosis of brucellosis in most mammalian species (Nielsen and Yu 2010).

In animals, the SAT and RBT are often the first serological tests and followed by the complement fixation test as a confirmatory test on positive sera with agglutination reactions. In some countries ELISA is used on sera and on pulled milk samples and has replaced the milk ring test because of its easy automation and better outcome in poor quality samples (Nicoletti 2010). Very useful are tests capable of differentiating vaccinal antibodies from antibodies after natural infection like precipitin tests, and competitive ELISA tests (Nielsen and Yu 2010).

11.6.3 Other Diagnostic Possibilities

Antigen detection by ELISA as an acceptable alternative to blood culture for the diagnosis of brucellosis could be an option; however, the number of bacteria present in the blood stream is often low, especially during the acute stages of infection. Antigen ELISA will probably not replace serology in the near future, but might be considered in combination with blood cultures, since results could be available sooner (Al-Shamahy and Wright 1998). Antigen detection methods although potentially useful, have not yet been validated.

Polymerase chain reaction (PCR) provides an additional means of detection and identification of *Brucella* spp. This technique in its conventional and real-time format, allows a rapid and accurate diagnosis of brucellosis and has promising potential. PCR has proven to be more sensitive than blood culture and more specific than serologic tests, both in acute and chronic disease (Al Dahouk and Nockler 2011). Due to the easy availability, whole blood and serum samples are currently preferred for molecular diagnosis of human brucellosis. This technique also appears to be useful in species differentiation and biotyping of isolates.

In clinical settings the majority of genus specific PCR assays target the *bcsp31* gene, which encodes an immunogenic protein. Other targets used for PCR detection of *Brucella* are 16S rRNA gene, 16S-23S internal transcribed spaces region (ITS)

and genes encoding an outer membrane protein (omp2) and the insertion element (IS711). Over the years, numbers of assays for PCR species differentiation have been developed with AMOS PCR being the first and the most widely used species-specific assay (Bricker and Halling 1994). The assay is based on the polymorphism arising from species-specific localization of the insertion sequence IS711 in the *Brucella* chromosome, and can identify *B. abortus* biovars 1, 2 and 4 (without biovar differentiation), *B. melitensis, B. ovis* and *B. suis* biovar 1. The main disadvantage of the AMOS PCR is that it fails to detect all species or all biovars of some species. Recently, a new multiplex PCR assay, suitable for rapid and simple one-step identification and species differentiation was developed (Garcia-Yoldi et al. 2006). This assay, called "Bruce-ladder" successfully identifies and differentiates all *Brucella* species (except *B. inopiata*) as well as vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev 1. Despite the high degree of DNA homology within *Brucella* genus, molecular typing schemes based on the use of multiple locus variable number of tandem repeats analysis (MLVA) have proven to be able to differentiate unrelated *Brucella* isolates which could not be differentiated by classical microbiological methods (Bricker et al. 2003; Le Fleche et al. 2006; Al Dahouk et al. 2007). Due to its high discriminatory power, MLVA technique provides valuable results for epidemiological trace-back analysis, which may identify the source and routes of infection. MLVA has been successfully used for identification of human outbreaks related with a same source of infection, as well as for confirmation of relapse and laboratory acquired infection (Al Dahouk et al. 2005b; Marianelli et al. 2008).

Currently, MLVA is the most suitable molecular method for subtyping *Brucellae* that fulfills all performance criteria recommended for a typing assay that is, typeability, reproducibility, stability, discriminatory power, concordance with other typing techniques and epidemiological concordance (Bricker and Ewalt 2005). Publicly available international database of MLVA-16 profiles based on common set of markers (http://mlva.u-psud.fr/brucella/) is promoting harmonization and allow comparison of profiles globally.

PCR-based laboratory tests are very promising, but they still have some shortages, with a high risk of contamination and high price. Also, infrastructure, equipment and expertise are lacking and a better understanding of the clinical significance of the results is still needed. Although already proven to be a valuable tool when culture fails or serological results are inconclusive, PCR-based laboratory tests cannot be considered as a routine method for diagnosis and follow-up yet.

11.6.4 Assessment of the Disease Activity

Having in mind that quite often definitive assessment of disease activity cannot be based only on clinical parameters, some of the abovementioned methods are also used in the follow-up after treatment. However, until now, no accurate criteria for complete cure exist (Ariza et al. 1992).

For serological follow-up of the disease the determination of *Brucella* specific IgG antibodies as a marker of active infection is most important. Tests allowing determination of IgG (2ME, Coombs, Brucellacpat, ELISA IgG) are considered as suitable tests for follow-up. Rapid fall of the level of *Brucella* specific IgG antibodies is considered to be a convenient measure of therapeutic effectiveness and satisfactory outcome. Persisting high levels of *Brucella* specific IgG antibodies during the follow-up period is almost always present in patients with active disease (Al Dahouk et al. 2003). It is especially difficult to assess the disease activity in two categories of patients.

The first category includes patients who continue to complain of symptoms in the presence of permanently declining or negative serology. Among these may be patients who have an illness with symptoms similar to brucellosis during the follow-up period or patients suffering from a concomitant disease with similar symptoms/signs (i.e. rheumatoid arthritis and brucellosis). However, negative serology does not always exclude persistence of active *Brucella* infection (Payne 1974). In this group of patients it is necessary to make an individual estimate for possible retreatment, careful monitoring (perhaps additional clinical investigations with new blood cultures, ELISA- cytoplasmic protein, PCR) and very often accompanied with psychiatrical consultations as well.

The other group includes patients with no clinical parameters in the presence of permanently high serology. Namely, in a number of treated and cured individuals, *Brucella*-specific IgG can persist for years, so it is not always possible to distinguish serologically between persistent (active) and past infection (Gazapo et al. 1989). Here are also patients who ignore their symptoms and signs or who are not appropriately followed by their physicians, patients who after therapy continue to be professionally exposed to *Brucellae*, patients who had had focal disease but with satisfactory clinical outcome, and patients with high IgG titers at admission. This group of patients necessitates continuous clinical, bacteriological and serological monitoring and usually treatment deferral.

In patients with relapses, at the time of clinical presentation, only 40% show a significant increase in serological titers. In the remaining, seroconversion takes place within 3 months after onset of the symptoms of relapse, at which time these data lose virtually all clinical interest (Ariza et al. 1992). The available serological tests do not allow to distinguish between relapses and reinfections. It is considered that the IgG anti-brucella cytoplasmic protein antibodies, as determined by ELISA, correlate much better with clinical recovery than those to LPS do, and hence, they are a better predictor of cure then anti-lipopolysaccharide antibodies (Baldi et al. 1996). Similar results have been obtained with western blotting. The shortcomings of these methods include their non-quantitative and rather subjective interpretation, the lack of validation data, and the non-availability of reference reagents.

It seems that available molecular techniques are also inappropriate to establish the success of treatment or to predict relapse. Using real-time PCR techniques, *Brucella* DNA can be detected in the majority of treated patients throughout the follow-up period, despite apparent clinical recovery. In a significant number of patients, *Brucella* DNA remains detectable for several months or even years after clinical

cure and in the absence of any symptoms indicative of chronic disease or relapse (Vrioni et al. 2008). On the other hand, relapses were observed in PCR-negative patients (Navarro et al. 2006).

Due to these deliberations, interpretation of serologic tests results in relation to exposure, diagnosis and prognosis of the disease necessitates accurate assessment of clinical history and current status of patients. Interpretation is also dependent of understanding the course of the immune response following infection and treatment of the different types (localized, non-localized) or stages (acute, chronic, relapse/reinfection) of brucellosis (Ariza et al. 1992). In prognostic purposes a useful approach is to have sequential follow-up on both clinical and serological parameters (Bosilkovski et al. 2010a). Considering that available routine serological tests show certain inconsistencies in diagnosis and especially the follow-up of brucellar patients, it is mandatory to develop new laboratory and molecular techniques or to improve the existing ones.

11.7 Treatment

Prompt, timely and adequate administration of drugs is the most important (unfortunately not always the most influential) factor which determines the evolution of the diseases, reduces the complications and prevents emergence of relapses and progression of the disease from acute to chronic (Bertrand 1994). Brucellosis treatment is based on use of antibiotics with intracellular activity, use of combination regimens, and extended treatment duration. At present, monotherapy is not accepted as a treatment strategy for brucellosis, as well as therapy shorter than 45 days (the golden equilibrium of acceptable success, compliance, and lack of significant side effects) (Pappas et al. 2006b).

The currently recommended regimens for the treatment of uncomplicated brucellosis in adults are: (1) oral treatment with doxycycline 100 mg twice daily plus rifampin 600–900 mg daily, both for 6 weeks; or (2) oral doxycycline 100 mg twice daily for 6 weeks plus streptomycin 1 g daily intramuscularly for 2–3 weeks (Ariza et al. 2007). Gentamicin 5 mg/kg daily parenterally in one dose for 7–14 days can be used as a substitute for streptomycin and has shown equal efficacy (Ariza et al. 2007).

In spite that doxycycline-streptomycin regimen is considered the “gold standard” and is proven to be more effective than doxycycline-rifampin (Solera et al. 1995) it has its disadvantages. Streptomycin is not available in certain countries and aminoglycoside administration is only parenteral and not convenient as an outpatient therapy requiring extended healthcare amenities, which is not the case in some brucellosis endemic areas. The doxycycline-rifampin combination for a period of 45 days seems reasonable, inexpensive, and convenient first-line treatment for most endemic areas. One potential problem that could arise with the use of rifampin-containing regimens for the treatment of brucellosis involves the possibility of inducing rifampin resistance to tuberculosis in areas endemic for brucellosis (Al-Hajjaj et al. 2001).

Fluoroquinolones (ciprofloxacin and ofloxacin) in combination with rifampin or doxycycline can be used for the treatment of acute uncomplicated brucellosis as an alternative to the doxycycline-rifampin combination (Falagas and Bliziotis 2006). In children and pregnant women, trimethoprim-sulfamethoxazole in combination with rifampin, is the drug of choice, as well as being a third drug in a complex therapeutic regimens for focal brucellosis (Pappas et al. 2006b).

Relapses are treated successfully with the repeated courses of the same regimens. Traditionally, longer treatment duration (at least 12 weeks) are recommended for *Brucella* endocarditis, spondylitis or neurobrucellosis and more than two drugs combinations in the treatment of endocarditis, neurobrucellosis and localized suppurative lesions. Surgery should be considered in some cases with endocarditis, spondylitis, localized abscesses and some other rare conditions.

The addition of immune response stimulators such as levamisole or IFN $alfa_{2b}$ to the standard regimen in chronic anergic brucellosis seemed to improve the symptoms, however, with no practical application at the present. Corticosteroids should be used only in rare cases with septicemic shock, disseminated intravascular coagulation, iritis and neurobrucellosis complicated by papilloedema, myelopathy, polyneuropathy, and cranial nerve palsies.

The treatment of animal brucellosis is very expensive and often unsuccessful (Kumar 2010).

11.8 Prevention and Control

11.8.1 Prevention in Humans

Humans are not the reservoir of brucellosis; rather human infections are the end result of animal infections, so the elimination of brucellosis from the animal population is the sole mean by which it can be controlled in humans. Therefore, any measure of prevention of brucellosis in men has no influence on the presence of the infection in a defined territory. Prevention of human brucellosis may be based on prevention of transmission from animal reservoirs to humans and control or eradication of the infection in the reservoir population, i.e. infected animals. The transmission of the disease and its incidence is related to the intensity of the prevention activities in the human populations. However, the impact of preventive programs in humans is jeopardized if animal control activities are not implemented or implemented only partially.

The prevention of human infection is based on occupational hygiene and food hygiene in order to reduce the risk of transmission (Morelli 1998). This can be achieved by: (a) direct measures targeted to interrupt the infection cycle by inactivating the pathogenic power of the agent, which is possible in alimentary way of transmission. Prevention of foodborne brucellosis is based on preparation of pasteurized or boiled milk and milk products from treated milk. If local customs and tradition make this difficult to achieve, the cheese should be stored for 6 months

before being eaten. Also, all meat products should be thoroughly cooked before consumption. Drying, salting, smoking and freezing are not reliable methods for killing *Brucellae*; (b) indirect measures targeted to reduce the risk of infection by various activities such as changing behaviors at risk, health education and/or information, e.g. by personal hygiene, adoption of safe working practices, and protective measures for the environment. Professionally exposed persons should wear adequate protective clothing such as coat, rubber or plastic apron, rubber gloves, boots, face shield, goggles or a mask. Public health education should emphasize food hygiene and occupational hygiene. Public educational programs to raise the awareness level of producer groups and general population on relevant aspects of brucellosis should be designed and disseminated through the use of all possible media. Health education programs should pay a particular attention to environmental aspects related to brucellosis transmission. Health education cannot be regarded as effective if specific considerations referring to the community like culture, beliefs, traditions, educational level, social status, occupation, age, etc. are not taken into account (World Health Organization 2006). Also, clinicians practicing in endemic areas must be familiar with the disease and develop a high degree of clinical suspicion (Kumar 2010). Special precautions should be taken by laboratory workers; (c) indirect measures targeted to increase the resistance of humans by vaccination. Up to date, safe and effective vaccines for the prevention of human brucellosis are not available. There have been certain attempts to use vaccines made by peptidoglycan fraction (PI) of lipid extracted cells of *B. melitensis* M15 and subcellular fraction extracted from cell wall preparations of *B. abortus* strain 19 with a protein-polysaccharide complex, which are not in routine use.

11.8.2 Prevention and Control in Animals

Animal brucellosis can often be identified through investigations of human cases. Control and prevention programs in animal brucellosis require effective collaboration between all sections of the community and must be properly planned, coordinated and resourced (World Health Organization 2006). Clearly, there has been much progress in the control and eradication of *B. abortus* in cattle, with many countries free of this infection. However, the control of *B. melitensis* has proved to be much more difficult (Nicoletti 2010).

Prevention in animals includes measures to reduce exposure to infected animals, or to their discharges and tissues. The prevention is composed by activities such as selection of replacement animals, initial isolation and testing of purchased replacements, laboratory control of suspected animals, proper disposal of placentas and non-viable fetuses, control of animal movement, disinfection of contaminated areas, and cooperation with public health authorities (World Health Organization 2006).

Brucellosis control programs that have been proven to be successful in controlling the disease are based on various strategies, including hygienic measures, vaccination, test-and-slaughter of infected animals and combinations. It is not possible

to suggest precise recommendations for control, which may be appropriate for all conditions. The control strategy should therefore be based on a thorough understanding of the epidemiological pattern of the disease, infrastructure, local and regional variations in animal husbandry practices, compliance, cultural practices and animal movements. As a general statement it can be said that once the professional organizations and economic resources are fully adequate, the epidemiological unit of intervention should be defined. Whenever the collective prevalence in the unit is very low (<1% of flocks infected) and uniform, a short term strategy based on test and slaughter programs without vaccination could be applied. In the case where prevalence is moderate or nonhomogeneous, a combination of simultaneous vaccination in young replacements and a test and slaughter in adult animals could be recommended. However, when the disease is highly prevalent (>10% of flocks infected), mass vaccination of all susceptible animals from all animal species involved in the epidemiological cycle is the only reasonable strategy in order to control the disease (Blasco 2010).

Serological tests as well as tests on milk are the usual method of identifying possible infected animals. In most cases, test and slaughter of positive animals is only successful in reducing the incidence if the herd or flock prevalence is very low. As the disease is closer to being eliminated, a test and stamping out program is required for complete elimination. This procedure is expensive and requires cooperation with the animal owners, including compensation for repossessed and destroyed animals. The application of test and slaughter policies is less successful in sheep and goats, because of the less reliable diagnostic tests than in cattle. Test and slaughter is also unlikely to be successful in cattle if the remainder of the herd is unvaccinated, especially in large populations (World Health Organization 2006).

In many countries, vaccination is the most effective and economical means of prevention and control of animal brucellosis. The efforts for brucellosis control with vaccination as the main measure significantly reduce the incidence of the disease in cattle; still they generally met with little success in sheep and goats due, among numerous other factors, to the difficulty encountered by the veterinary services in identifying, vaccinating and monitoring infected flocks, and in controlling their movements. The attenuated (live) strains of *B. melitensis* strain Rev 1 for sheep and goats and *B. abortus* strain 19 and the non-agglutinogenic *B. abortus* strain RB51 for cattle, have proven to be superior to all others. *B. melitensis* strain Rev 1 and *B. abortus* strain 19 in a lesser extent are virulent for humans, and with negligent handling human infections are possible. Any kind of incident like this should be prevented accordingly with appropriate chemoprophylaxis.

11.8.3 Surveillance

The surveillance of human brucellosis is an important topic in the management of brucellosis control/eradication programs. In fact the incidence of human brucellosis is a good index of the presence of infection in animal populations. The epidemiological surveillance of human and animal brucellosis is a key element for

management of prevention and the efficacy of control programs. Actually, one of the crucial factors for the success of an eradication program is the implementation of an effective surveillance system (Morelli 1998). Experience has proven that this system is one of the pillars upon which any monitoring control program, irrespective of the country should be based. The surveillance system must be adapted to the adopted strategy for coping with the disease: prevention of human disease, and prevention, control, or eradication of the infection in the animal population. In addition, other factors that will also determine the type of surveillance system include husbandry systems, marketing methods, and capacities of veterinary services. Brucellosis poses many challenges to designing effective surveillance. This tends to be a chronic disease in both humans and animals, the incubation period is variable and often long, the symptomatology is variable and often minimal, and laboratory confirmations are essential. Also, the human link to the animal reservoir may be ill-defined. This is especially relevant where the animal populations are not well identified, enumerated, or inaccessible (Robinson 1998).

The successful implementation of national brucellosis surveillance, and control programs requires strong intersectoral collaboration between the veterinary and public health sectors. It also requires sustained political commitment in order to assure that the necessary resources, human and financial, are made available for medium and long terms. International technical and scientific collaboration, harmonization of surveillance and control strategies and regulation/legislation activities are also essential for the success of national brucellosis programs (Cosivi 1998).

11.9 Socio-Economic and Public Health Importance

Brucellosis has principal socio-economic and public health importance within countries and is considered significant in the international trade in animals and animal products (Neubauer 2010). Therefore, a worldwide need for the improvement of public health, i.e. medical awareness, surveillance and laboratory diagnostic capabilities, is obvious.

Brucellosis causes appreciable economic losses to the livestock industry and huge economic losses to dairy, sheep, goat and pig farmers in infected areas, resulting from abortions, sterility, birth of weak offspring, decreased milk production, weight loss in animals, lameness, reduced breeding efficiency, veterinary attendance costs, the cost of culling and replacing animals, and vaccination costs (Nicoletti 2010). The disease also constitutes an impediment to free animal movement and adds to the costs of quarantine and testing of export animals. Moreover, it is generally agreed that the impact of the disease in small ruminants is greater in terms of the adverse effects it may have on human health in the rural population due to the high virulence of the agent (*B. melitensis*) to humans and the traditional mode of consumption of sheep and goat animal products.

The principal socio-economic effects of brucellosis in humans are reflected in medical care and reduced productivity (Nicoletti 2010). The disease in humans is

characterized with prolonged illness resulting in loss of vitality, loss of income and manpower, long-term treatment, and medical care costs. The economic losses due to the disease in humans will of course vary, but is by any means very high.

11.10 A Sight in the Future

Human brucellosis still encounters scientists and clinicians with many theoretical and practical challenges. Some of them with mainly theoretical significance are recognition of offered taxonomies, defining the epidemiology and the role of marine mammals, *B. microti*, *B. inopinata* and *B. suis* biovar 2 in human pathology, clearing the uncertainties about the pathogenic mechanisms of *Brucella* spp., such as whether the pathogenic mechanism (arthritis, uveitis, cytopenia) is due to the direct role of the agent or immune reactions. Also, there is the question of the durability and solidness of the immune response after the disease. The detection of a bacterial load years after treatment, in apparently cured patients is very intriguing. Does this mean that *Brucella* infection is lifelong, like toxoplasmosis or herpes viral infections? What will happen in these patients in cases of immunosuppression?

From practical point of view further development of new better diagnostic techniques is necessary to help overcome the above discussed weaknesses of the current tests, the identification of predictors for disease severity, and outcome, discovery of laboratory/molecular criteria capable to differentiate active infection from definite cure (the effect of treatment), and defining what does complete cure mean. Similarly it is necessary to offer improved treatment protocols possibly with less drugs than actually recommended, shorter duration, less relapse and therapeutic failure rates, and better tolerability and price. It is also necessary to define the role of immunomodulation in the treatment of human brucellosis, especially the chronic form. Not to mention that in the future there must be an effort to manufacture perfect brucellosis vaccine that should trigger a solid and life-lasting immunity, protect against infection by *Brucella* species other than those typical of a given host, be innocuous regardless of the physiological state of the animal, not interfere with serological diagnostic tests, not be virulent for humans or carry resistance to antibiotics, not be shed in the environment, be stable and affordable. A special challenge is the production of an effective and acceptable vaccine for use in humans.

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Chapter 12
Q Fever (*Coxiella burnetii*): A Blueprint for Outbreaks

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Abstract About 80 years ago, Q fever research began due to human outbreaks of unknown origin, associated with domestic animals. Since then, some but not all characteristics of this "query" disease, caused by the intracellular bacterium *Coxiella burnetii* were revealed. In this chapter the bacteriology of the bacterium, clinical presentation, epidemiology and transmission of the disease in humans and animals are presented. Domestic small ruminants are the main source of human Q fever. Although Q fever is considered to be an occupational disease, outbreaks have a major public health impact and attract most attention. The Dutch Q fever outbreak, involving 4000 human cases over the years 2007–2010, is an example of how Q fever can re-emerge from an endemic state into an outbreak of unforeseen dimension. In this outbreak the epidemiological link between dairy goats and human cases was confirmed by genotyping for the first time. This was possible due to the previous development of genotyping assays that are applicable on clinical material. Although Q fever seems to be a blue print for outbreaks it is not known yet what factors are essential to cause outbreaks and how they interact. To prevent outbreaks, a better understanding of these factors and their interaction is necessary and research should therefore focus on this.

12.1 Introduction

The first awareness of Q fever was raised by severe outbreaks among abattoir workers in Brisbane, Australia. These outbreaks had been occurring periodically since 1933, but remained undiagnosed until Edward Derrick was assigned to investigate the cause of these febrile illnesses (Derrick 1983). In order to reveal the cause of

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the disease, several experiments were done, but Derrick failed to detect bacteria. This led him to the (wrong) conclusion that the etiologic agent was a virus. Further studies on the virus of this query, "Q", fever were published by Macfarlane Burnet and Mavis Freeman, indicating the rickettsia-like properties of the virus (Burnet and Freeman 1983). At the same time the causative agent of Q fever was also discovered independently in Montana, USA, due to research on Rocky Mountain Spotted Fever (Cox 1938). Later on a laboratory infection revealed the linkage of the two discoveries (Dyer 1938; McDade 1990). So from the early identification of Q fever and the discovery of the etiologic agent it was obvious that the Q fever agent was zoonotic and able to cause severe outbreaks.

The rickettsia-like properties of the agent resulted in the initial designation of the Q fever agent as *Rickettsia diaporica* (diaporica is derived from the Greek word for having the property or ability to pass through [a filter]) by the American group and as *Rickettsia burneti* (after Burnet) by the Australian group) (McDade 1990). In 1948, Philip (1948) proposed a reclassification into *Coxiella burneti*. In this name both Harold Cox and Frank Burnet are honoured for their contribution to the identification of the Q fever agent. At a later time *C. burnetii* has been phylogenetically reclassified from the order of Rickettsiales to Legionellales, based on the sequence of its 16S rRNA (Weisburg et al. 1989).

C. burnetii is an obligate intracellular Gram-negative bacterium. The pleomorphic rods have a diameter of approximately 0.2–0.4 μm and are 0.4–1.0 μm in length (Drancourt and Raoult 2005). In the developmental cycle of *C. burnetii* two distinct variants have been identified: a large cell variant (LCV) and a small cell variant (SCV) (McCaul and Williams 1981). The LCV is the metabolically active and intracellular replicative entity of the bacterium. The LCV transforms into the SCV, which is the spore-like form of *C. burnetii*. In this form the bacterium is highly resistant to environmental stress, such as high temperatures, UV radiation and osmotic pressure. The resistance allows *C. burnetii* to survive in the environment while keeping its infectivity (McCaul and Williams 1981). The SCV can infect host cells, closing the developmental cycle.

Lipopolysaccharide (LPS) is demonstrated in both *C. burnetii* LCV and SCV, although presence of LPS is mainly associated with the SCV (Coleman et al. 2007). Like several other Gram-negative species, *C. burnetii* can display two different LPS phenotypes. The phase 1 phenotype expresses full length LPS which corresponds to the smooth LPS of other Gram-negative bacteria (e.g. *Brucella* spp. and *Enterobacteriaceae*), while the phase 2 phenotype carries LPS that resembles the rough LPS. Phase 2 LPS lacks the O-antigenic region (Toman et al. 2009). Phase 1 bacteria are highly virulent and able to replicate in immunocompetent hosts. This is contrary to phase 2 bacteria, which are avirulent and unable to replicate in immunocompetent animals (Moos and Hackstadt 1987; Andoh et al. 2007). During serial passage in cell culture, phase 1 *C. burnetii* can convert into phase 2 (Hotta et al. 2002). Both LPS phenotypes are inducing phase specific antibodies in infected hosts. Phase 1 antibodies are directed against the full length LPS of phase 1, whereas phase 2 antibodies are assumed to direct against common surface proteins (Marrie and Raoult 1997). The hypothesis is that these surface proteins are also present on the surface of phase 1 *C. burnetii*, but maybe be shielded by the long phase 1 LPS. This may prevent binding of phase 2 antibodies to surface proteins of intact phase 1 bacteria (Hackstadt 1988).

The first complete genome sequence of *C. burnetii* was published in 2003 (Seshadri et al. 2003). Analysis of the original strain isolated from ticks by Davies and Cox in 1938 (called Nine Mile/RSA493) revealed a circular genome of 1,995,275 base pairs. The genetic heterogeneity of different *C. burnetii* strains can be assessed with a number of molecular techniques. Different genotyping techniques have been described, mainly based on the identification of differences between selected loci on the genome (Table 12.1, Massung et al. 2012). Accurate identification of the agent is important to differentiate between strains and to identify epidemiological markers. These markers are at the basis of the molecular epidemiology, which enables the identification of sources of Q fever outbreaks (Arricau-Bouvery et al. 2006). Eventually in future, markers may be discovered in *C. burnetii* genomes that identify strains posing an increased risk for infection. However, it is not known if virulence-encoding genes are located on or associated with the loci used in the current typing methods. So to date, it is not possible to classify the virulence of strains solely based on the available genotyping methods.

An important characteristic for typing systems is the discriminatory power, i.e. the ability to distinguish between unrelated strains. This is determined by the number of types defined by the test method and the relative frequencies of the types. A single numerical index of discrimination is suggested by Hunter and Gaston (Hunter and Gaston 1988). This Hunter-Gaston Diversity Index (HGDI) is based on the probability that two unrelated strains sampled from a test population will be placed into different typing groups. By comparing the HGDI of a typing system the discriminatory power of a method can be identified. It is important to note that the calculated HGDI depends on the panel of strains (i.e. relatedness of the strains), so for an unbiased comparison of typing methods preferably the same panel should be used. Despite its importance for the quality of typing systems the discriminatory power is not assessed for most of the typing systems available for *C. burnetii*. For the ones that were assessed, a HGDI of 0.86 was calculated for RFLP-typing (Jager et al. 1998). For the MLVA typing panels 1 and 2 a HGDI of 0.92 is calculated respectively; for the combination of both a HGDI of 0.99 is calculated (Arricau-Bouvery et al. 2006; Roest et al. 2011a). It is suggested that a HGDI of >0.90 is desirable to interpret typing results with confidence (Hunter and Gaston 1988), indicating the MLVA typing system as a useful typing tool for *C. burnetii*. However, still the heterogeneity of the studied population influences the quality. In comparison with published MST and SNP methods MLVA is probably a more discriminatory typing method for *C. burnetii* (Svraka et al. 2006; Chmielewski et al. 2009; Massung et al. 2012).

12.2 Disease Symptoms in Animals and Humans

12.2.1 Clinical Presentation in Animals

The most important clinical presentations of Q fever in animals that are relevant for the zoonotic properties of Q fever are abortion and stillbirth. Field observations clearly demonstrate *C. burnetii* as a cause of abortion and stillbirth in goats, sheep,

Table12.1 Overview of published genotyping techniques for *C. burnetii* and year of first publication of the technique for *C. burnetii*. (Roest et al. 2013a)

Abbr.	Stands for	Based on	Publ. year	Ref.
RFLP typing	Restriction fragment length polymorphism typing	Analysis of the fragments after digestion with specific restriction enzymes	1990	(Jager et al. 1998; Heinzen et al. 1990)
Com1 typing	Com1 encoding genes sequencing	Sequence analysis of the Com1 encoding genes	1997	(Zhang et al. 1997)
Com1/MucZ typing	Com1 and MucZ encoding genes sequencing	Sequence analysis of the Com1 and MucZ encoding genes	1999	(Sekeyova et al. 1999)
MST	Multispacer sequence typing	DNA sequence variation in short intergenic regions in the genome	2005	(Glazunova et al. 2005)
MLVA	Multiple locus variable number tandem repeats analysis	Variation in the repeat number in tandemly repeated DNA elements on multiple loci in the genome	2006	(Svraka et al. 2006; Arricau-Bouvery et al. 2006)
IS1111 typing	IS1111 repetitive element PCR-based differentiation typing	Identification of different IS1111 insertion elements	2007	(Denison et al. 2007)
RAPD	Randomly amplified polymorphic DNA	Analysis of randomly amplified DNA fragments of the genome	2009	(Sidi-Boumedine et al. 2009)
SNP typing	Single nucleotide polymorphism typing	Differentiating a single nucleotide difference on a locus in the genome by probes	2011	(Huijsmans et al. 2011)

Abbr. abbreviation, *Publ. year* year of first publication of the technique for *C. burnetii*, *Ref.* reference

cattle and cats (van Moll et al. 1993; Guatteo et al. 2011; Berri et al. 2001; Berri et al. 2002; Hatchette et al. 2003; Rousset et al. 2009; Lang 1990; Marrie et al. 1988b). Abortion occurs most frequently at the end of gestation, without preceding clinical symptoms (Arricau-Bouvery and Rodolakis 2005). In goats, abortion, still birth, but also the birth of strong and lively kids can occur after Q fever infection of pregnant animals (Arricau-Bouvery et al. 2005; Roest et al. 2012). When lively kids are born the duration of gestation can be up to 14 days shorter than the normal average gestation duration of 154 days. Experimental infections with *C. burnetii* in pregnant sheep did not result in any abortions (Martinov et al. 1989; Welsh et al. 1958), while in cattle one experimental infection of a pregnant cow resulted in abortion (Lang 1990). The cause of these differences in pregnancy outcome after infection is unknown.

In non-pregnant animals, *C. burnetii* infection is virtually asymptomatic. In cattle, *C. burnetii* infection is associated with metritis and reproduction problems (Lang 1990; To et al. 1998). In dairy goat herds where Q fever abortions occurred, metritis in does can be observed. Weak kids were reported with low body weight and high mortality. Rearing of apparently healthy kids can be complicated by respiratory and digestive tract disorders (Wouda and Dercksen 2007).

12.2.2 Clinical Presentation in Humans

In humans *Coxiella*-infection can manifest in three main clinical presentations: acute Q fever, chronic Q fever and the post-Q fever fatigue syndrome (QFS). Following exposure to *C. burnetii* almost 60 % of the Q fever cases are asymptomatic. Among the 40 % symptomatic acute Q fever patients, the majority will present a non-specific, self-limiting illness. More severe clinical symptoms include fever, headache, chills, atypical pneumonia and hepatitis (Derrick 1983; Maurin and Raoult 1999; Raoult et al. 2005). In the Netherlands, the recent acute Q fever outbreak showed a mortality rate of 1.2 % within approximately 1 month after hospitalization of patients. All lethal cases suffered severe underlying medical conditions (Kampschreur et al. 2010). Acute Q fever is diagnosed in the laboratory following (i) a positive *C. burnetii* specific PCR, (ii) the presence of IgM phase 2 antibodies in serum accompanied by clinical symptoms, or (iii) a fourfold increase of the IgG phase 2 antibody titre. These laboratory findings are also the notification criteria in the Netherlands (Wegdam-Blans et al. 2010).

Chronic Q fever can develop from a primary infection in about 1–5 % of the patients. Chronic Q fever can become manifest years after initial infection. Clinical symptoms include non-specific fatigue, fever, weight loss, night sweats and hepato-splenomegaly, as well as endocarditis (Wegdam-Blans et al. 2012; Raoult et al. 2005). After the recent outbreak in the Netherlands, vascular complications appeared to be more common than endocarditis, but endocarditis may not have become apparent yet (Van der Hoek 2012). Laboratory diagnosis of chronic Q fever includes a high titre of *C. burnetii* phase 1 IgG added by the detection of the Q fever-agents DNA by PCR (Wegdam-Blans et al. 2012).

QFS is another long-term presentation of Q fever. Contrary to chronic Q fever, *C. burnetii* cannot be detected in QSF patients. Furthermore, antibody levels against the bacteria are low or negligible. Symptoms of QFS include prolonged fatigue, arthralgia, myalgia, blurred vision and enlarged painful lymph nodes (Maurin and Raoult 1999). Acute Q fever with hospitalisation was found to be a risk factor for QFS (Morroy et al. 2011). The cause of the development of chronic Q fever or QFS in certain individuals is still unknown.

As in animals, it is suggested that also in pregnant women, *C. burnetii* infection may lead to adverse pregnancy outcome, especially when the acute Q fever remains untreated (Langley et al. 2003; Carcopino et al. 2007). Pregnancy outcomes include spontaneous abortion, intra-uterine fetal death and premature delivery, or low birth weight. In pregnant women, the risk to develop chronic Q fever is assumed to be high (Maurin and Raoult 1999; Carcopino et al. 2009). Besides clinical Q fever also asymptomatic infections may lead to the same risk for adverse pregnancy outcomes (Parker et al. 2006). As above was known during the recent Dutch Q fever outbreak, surveys were set up to investigate the relation between *Coxiella*-infection and pregnancy outcome. In a population-based study, including 1174 women, no relation could be detected between presence of antibodies against *C. burnetii* during early pregnancy and adverse pregnancy outcome (van der Hoek et al. 2011b). An additional study was not supportive to imply a preventive program for seropositive pregnant women as such a program, including serological screening and treatment in case of acute or chronic Q fever, seemed not to be associated with a relevant reduction in obstetric complications in seropositive women (Munster et al. 2013).

12.3 Epidemiology and Burden of Disease in Animals

12.3.1 Host Range

C. burnetii can infect a wide range of animal species. The original isolation in the USA was from the tick *Dermacentor andersoni* (Davis and Cox 1938). Since then *C. burnetii* has been detected in over 40 tick species. Several bird species can also become infected with *C. burnetii*, as experimentally shown (Schmatz et al. 1977a; b; Sethi et al. 1978; Babudieri and Moscovici 1952). Natural infections have been reported in domestic birds and in wild birds (To et al. 1998; Astobiza et al. 2011). In terrestrial as well as in marine wildlife the presence of *C. burnetii* has been confirmed in roe deer, wild boars, rodents, European hare, pacific harbour seal, a Steller sea lion, Northern fur seals and harbour porpoises (Thompson et al. 2012; Astobiza et al. 2011; Lapointe et al. 1999; Kersh et al. 2012; Kersh et al. 2010; Duncan et al. 2012). These data indicate a sylvatic cycle for *C. burnetii*, in which ticks probably play an important role as vector.

In domestic animals, *C. burnetii* has been detected in cats and dogs as well as in domestic ruminants. In cats seroprevalences between 19 and 42% are reported. In dogs seroprevalences up to 22% are detected (Higgins and Marrie 1990; Komiya

et al. 2003a; Marrie et al. 1988a; Boni et al. 1998). In domestic ruminants *C. burnetii* infections are widespread. Seroprevalence levels are estimated up to 82 % in cattle. In sheep and goats average seroprevalences are slightly lower compared to cattle, with values of up to 73 % (Guatteo et al. 2011). Prevalence of *C. burnetii* on cattle herd level as measured from bulk tank milk samples ranges between 32 and 94 % (Angen et al. 2011; Kim et al. 2005; Astobiza et al. 2012). These data indicate that animals that live in close contact to humans have been exposed to *C. burnetii*.

12.3.2 Excretion Routes

Knowledge of the excretion of *C. burnetii* from infected animals is crucial in understanding the transmission routes and risks for human infection. Abortions in *C. burnetii* infected domestic ruminants are accompanied by massive excretion of the bacteria and spread into the environment. This is the most important excretion route of *C. burnetii*, as up to 10^9 organisms per gram placenta tissue are excreted (Arricau Bouvery et al. 2003). However, recent experiments in goats indicate that comparable numbers of *Coxiella* are also excreted during the birth of lively kids (Roest et al. 2012). Also sheep can excrete numerous Q fever-bacteria during normal parturition. This implies that *C. burnetii* can be excreted without clinical signs of Q fever in the herd. This should be taken into account when tracing sources of human Q fever.

C. burnetii has also been detected in faeces, vaginal mucus and milk of infected domestic ruminants (Berri et al. 2001; Arricau Bouvery et al. 2003; Rousset et al. 2009; Guatteo et al. 2006). In goat herds, both in aborting and non-aborting goats, *C. burnetii* DNA has been detected in faeces, vaginal mucus and/or milk (Rousset et al. 2009). In cattle, also variable excretion via faeces, vaginal mucus and milk has been reported, sometimes independent of an abortion history. However, it is suggested that the presence of *C. burnetii* DNA in faeces and vaginal mucus is due to contamination of *C. burnetii* DNA from the environment (Welsh et al. 1958; Roest et al. 2012). Thus, while excretion of high numbers of *C. burnetii* with birth products during parturition is evident, the importance of and the correlation between the excretion routes of *C. burnetii* via faeces, vaginal mucus and milk is much less well established.

12.3.3 Burden of Disease in Animals

It is difficult to assess the burden of disease in farm animals. As an alternative, economic losses can be calculated in farm animal holdings. In the Dutch Q fever outbreak the economic losses have been calculated for goat farms (van Asseldonk et al. 2013). In this paper it is assumed that about 5 % of the animals on Q fever positive farm show clinical signs resulting in production and reproduction losses and that on average 40 % of the pregnant goats on the farms resulted in abortion. However, goats abort in late pregnancy and aborting animals recover rapidly, so milk

production is hardly affected. Furthermore, the economic value of lambs is low. All this results in a relatively limited loss, mainly due to (re)production losses. Costs on farm level increase when mandatory intervention is implemented. In the Dutch Q fever outbreak the intervention costs are estimated at upon 85 Million € (van Asseldonk et al. 2013). These costs consisted of costs for organization (57.96%), culling (21.61%), breeding prohibition (14.19%) and vaccination (6.20%). These costs of intervention were much lower that the total losses in the human domain, which were estimated at 222 Million € (van Asseldonk et al. 2013).

12.4 Epidemiology and Burden of Disease in Humans

Humans usually acquire Q fever by inhalation of *C. burnetii*. Alveolar macrophages and other mononuclear phagocytes are thought to be the primary target cells of the pathogen (Shannon and Heinzen 2009). In these cells *C. burnetii* survives intracellular killing and is able to replicate. A bacteraemia will lead to systemic infection with involvement of the liver, spleen, lungs and bone marrow (Maurin and Raoult 1999). It is assumed that higher doses of *C. burnetii* result in an increasing likelihood of infection and shorter incubation periods (Marrie 1990; Van der Hoek 2012). Human Q fever after oral ingestion has been suggested, but experimental infection of volunteers via contaminated milk did not result in disease although antibody responses were observed (Benson et al. 1963; Fishbein and Raoult 1992). Rare cases of person-to-person transmission, e.g. sexual transmission and blood transfusion, have been reported, but these routes do not seem to play an important role in the epidemiology of Q fever (Marrie 1990; Milazzo et al. 2001). Thus, in humans Q fever is essentially an airborne infection resulting from the inhalation of contaminated aerosols.

12.4.1 Burden of Disease in Humans

Only few studies have been published on the burden of Q fever in humans. In general, following exposure, almost 60% will remain asymptomatic. About 40% will become symptomatic, although the majority will only present mild symptoms of a self-limiting disease. This is reflected in the number of infected persons compared to the number of registered Q fever cases in several outbreaks. In the outbreak that occurred in the Val de Bagnes, Switzerland in 1983, it is suggested that for each patient diagnosed 50 persons remain undiagnosed (Dupuis et al. 1987; Roest et al. 2011b). In the Dutch Q fever outbreaks during the years 2007–2010 it is assumed that for each Q fever patient, 10 additional people were infected (Kampschreur et al. 2012). The hospitalization rate varies and may depend on the clinical experience of the physicians with Q fever. At the start of the Dutch Q fever outbreak hospitalization rates were up to 50%, whereas in the next year the hospitalization rate went down to 21% (Schimmer et al. 2008; van der Hoek et al. 2010).

Long-term effects of infection with *C. burnetii* have been studied as follow-up of the Dutch Q fever outbreak. Twelve to 16 months after the onset of illness severe subjective symptoms, functional impairment and impaired quality of life have been measured (Morroy et al. 2011).

The human disease burden can be quantified via disability-adjusted life years (DALY). For the Dutch Q fever outbreak between 2007 and 2011 the total disease burden is calculated as 2462 DALY, comprising of 22 DALY for acute Q fever, 1481 DALY for QFS, 806 DALY of chronic Q fever and 153 DALY for the 24 people that died (partly) because of Q fever (van Asseldonk et al. 2013). All this indicates that the impact of Q fever can be significant when outbreaks occur.

12.5 Transmission

In humans, Q fever is essentially an airborne infection resulting from the inhalation of contaminated aerosols (Benenson and Tigertt 1956; Tigertt et al. 1961). Aerosols can become contaminated with *C. burnetii* during abortion and parturition of infected pregnant ruminants (Welsh et al. 1958; Benson et al. 1963). Environmental contamination resulting in contaminated aerosols may also follow excretion of *C. burnetii* via feces, vaginal mucus or possibly milk of animals. This contributes to the occupational hazard of Q fever. However, contaminated aerosols are able to travel up to 5 km and infect humans. Windy, warm and dry weather can facilitate this transport (Van Steenbergen et al. 2007; Tissot-Dupont et al. 2004; Schimmer et al. 2010). Also other factors, such as vegetation and soil moisture, seem to be relevant in the dispersion of the bacteria (van der Hoek et al. 2011a). So, direct contact with animals is not a prerequisite for acquiring Q fever, as several outbreaks demonstrate (Salmon et al. 1982; Tissot-Dupont et al. 2004; Tissot-Dupont et al. 1999; Gilsdorf et al. 2008). It is assumed that most animals also become infected via inhalation (Berri et al. 2005), although oral uptake in a heavily infected environment cannot be excluded.

Domestic ruminants appear to be the main source of infection for human Q fever. As indicated earlier, parturition in infected pregnant goats, sheep and cattle result in the massive excretion of *C. burnetii* into the environment. Q fever in cattle seems to result in fewer abortions, probably resulting in a lower risk. Companion animals should also be considered as a source for human Q fever, since several human outbreaks were related to parturient cats and dogs (Marrie et al. 1988b; Pinsky et al. 1991; Komiya et al. 2003b; Buhariwalla et al. 1996). The role of horses and wildlife as a source of Q fever for humans is not clear. In overview, the epidemiology of Q fever can be summarized in a transmission model as presented in Fig. 12.1 (Roest et al. 2009; Roest et al. 2013a).

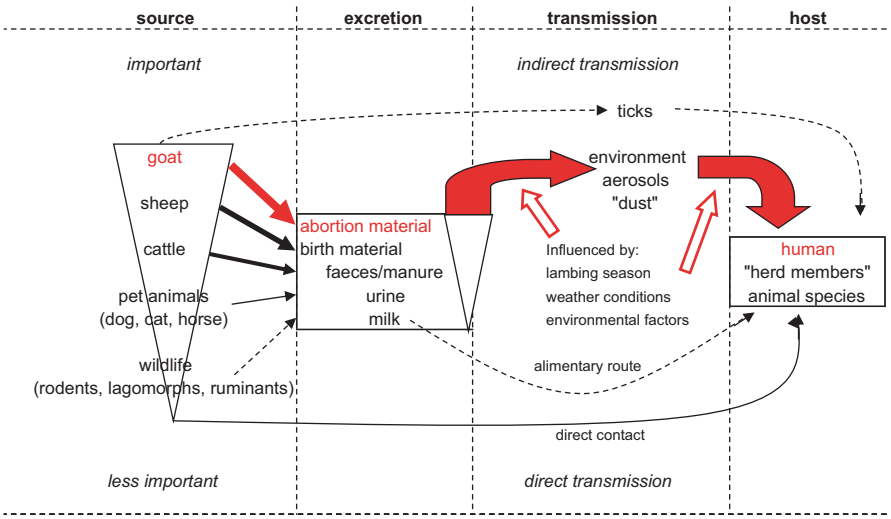

Fig. 12.1 Transmission model for Q fever. Overview of the possible transmission routes of C. burnetii from the animal reservoir to the human (and animal) hosts. The boldness of the arrows indicate the importance of the route, dotted lines indicate possible contributions. The most probable transmission route of C. burnetii in the Dutch Q fever outbreak is indicated in red (Roest et al. 2009, 2013a)

12.5.1 Q Fever Outbreaks

Q fever has a major public health impact when outbreaks occur. Outbreaks are reported frequently and worldwide, involving up to 415 laboratory-confirmed human cases per outbreak. Even higher numbers of human cases are reported, but the attribution to Q fever is unclear as cases are not always laboratory-confirmed (Van der Hoek 2012; Arricau-Bouvery and Rodolakis 2005; EFSA 2010). Outbreaks are usually geographically localised and restricted to one episode. Sheep are identified as the source in the majority of the outbreaks, with goats as 'second best'. Only a few outbreaks of Q fever have been related to infected cattle and cats (Van der Hoek 2012; Arricau-Bouvery and Rodolakis 2005; EFSA 2010). Source identification, however, is mainly based on epidemiological examinations. In most outbreaks, confirmation of the identity of the *Coxiella* strain involved in both host and source, for example by genotyping, is lacking. This is a major drawback in the identification of sources, as sources of Q fever are multiple and *C. burnetii* can be transmitted over larger distances. Thus, identification of the source and confirmation of the relation with human disease is preferably done by genotyping of the involved *C. burnetii* strains.

12.5.2 The Dutch Q Fever Outbreak

Since the 1950s Q fever was present in the Netherlands. However, only a few human cases were reported until the 1970s. In 1975 Q fever became notifiable for

humans (van Vliet 2009) and since that time about 20 cases on average were reported each year. The first human outbreak of Q fever in the Netherlands was reported in 2007: 168 cases were registered in the south of the Netherlands. In 2008 and 2009 the annual number of human cases increased to 1000 and 2355, respectively. Over the years 2007–2010, 4000 human cases were reported making the Dutch Q fever outbreak the largest laboratory confirmed Q fever outbreak ever seen (Roest et al. 2011b).

Until 2005 Q fever was also known to be present in the animal population by serological investigations, although no clinical symptoms were described. This changed in 2005. Slightly ahead of the human outbreak, Q fever problems started in the dairy goat and dairy sheep population with abortion rates up to 80 % per herd (Wouda and Dercksen 2007). Between 2005 and 2009 significant abortions were registered on 28 dairy goat farms and 2 dairy sheep farms. With goat numbers of 600 up to 7000 per herd huge amounts of *Coxiella burnetii* were spread in the environment during abortion and early birth. These bacteria were transported to the neighbouring human population by the prevailing north east winds in pretty dry spring periods. All this took place in the south east part of the Netherlands which is highly populated and has a dense dairy goat industry (Roest et al. 2011b). Eventually, the connection between dairy goats and humans was primary based on epidemiological findings. This connection was confirmed by preliminary genotyping data showing one predominant MLVA type in aborted goats which was also found in infected humans (Roest et al. 2011a; Klaassen et al. 2009). Additional analyses showed also one predominant genotype in humans which was the same as in goats (Tilburg et al. 2012c).

As goats were the suspects of the human Q fever outbreaks, increasingly strong measures were imposed to prevent the spreading of *C. burnetii* in the lambing season. The first measures were implemented in 2008, consisting of the notification of abortions in small ruminant holdings, hygiene measure, especially on manure handling and a voluntary vaccination. In 2009 the measures were extended with more tight notification criteria, including positivity of the bulk tank milk for *C. burnetii* DNA, a transport and breeding ban and a mandatory vaccination. All these measures however did not prevent the increase of human cases in 2009. To ultimately prevent the increase of human cases in 2010 the drastic decision was taken to eliminate all potential high-risk animals. This resulted in the culling of all pregnant goats on Q fever positive farms. All these measures finally resulted in a decline in human cases in 2010 (Roest et al. 2011b). A numberwise summary of the Dutch Q fever outbreak is given in Table 12.2, based on (van Asseldonk et al. 2013; Roest et al. 2011b; Roest 2013).

The question can be raised what the causes of the Dutch Q fever outbreak were. Epidemiological and genotyping investigations confirmed dairy goats and sheep as the cause of the human outbreak. But what caused the Q fever problems in goats? Q fever was present in animal husbandry in the Netherlands for a long period of time, so it was not a new disease for the Netherlands. Several factors are hypothesised to play a role. Firstly the strong increase in the number of dairy goat herds and goat numbers in recent years. In the 10 years before the outbreak the numbers of dairy goats were more than doubled and the average herd size gradually increased to up

Table 12.2 Overview of the Dutch Q fever outbreak year by year. (van Asseldonk et al. 2013, Roest et al. 2011b, Roest 2013)

	2005	2006	2007	2008	2009	2010	2011	2012	Total
Goats									
Number of confirmed Q fever abortion farms (dairy goats and dairy sheep) each year	2	7	7	8	6	0	0	0	30
Number of BTM *Coxiella burnetti DNA* positive farms					62	32	8	3	105
Culled animals	0	0	0	0	7755	50395	0	0	58150
Breeding prohibition	0	0	0	0	0	46130	n.k.	n.k.	46130
Vaccinated animals	0	0	0	0	158019	346463	344424	n.k.	848906
Humans									
Notified	5	10	168	1000	2354	504	81	53	4175
Hospitalised	n.k.	n.k.	83	207	459	n.k.	n.k.	n.k.	749
Deceased	n.k.	n.k.	0	1	7	11	5	1	25

BTM bulk tank milk, *n.k.* not known

to 900 animals. This increase could have influenced the intra-herd dynamics of *C. burnetii* leading to problems. The partly closed status of some herds to prevent infectious diseases like paratuberculosis and caseous lymphadenitis could have made a herd more susceptible for *C. burnetii* or new strains of this bacterium. Secondly the new introduction of a more virulent strain or a genetic shift to a more virulent strain could have contributed to the cause of the outbreak (Roest et al. 2011b).

12.6 Unresolved Issues

The Dutch Q fever outbreak was the first Q fever outbreak in which genotyping of the involved *C. burnetii*-strains was used to confirm the epidemiological link between humans and the cause of the outbreak. Genotyping could be used because of the development of genotyping techniques suitable for *C. burnetii*-DNA positive clinical samples. Because sources of human Q fever can be diverse and because *C. burnetii* can be transported over larger distances genotyping confirmation of epidemiological links is a prerequisite to pinpoint the source of a human outbreak. The necessity of saving clinical material for genotyping purposes should be kept in mind in the diagnostic procedures in both human and veterinary laboratory diagnosis. However, up to now, this is not fully implemented yet.

Q fever is present on cattle farms with seroprevalences up to 94 % (Angen et al. 2011; Kim et al. 2005; Astobiza et al. 2012; Muskens et al. 2011). However, cattle is not associated with human outbreaks (Georgiev et al. 2013), although in a lot of countries cattle industry is bigger than small ruminant husbandry. One possible explanation for this might be the occurrence of cattle related or associated genotypes of *C. burnetii*. Research during the Dutch Q fever outbreak revealed related genotypes in cattle that were much different from the predominant human and goat genotype (Roest et al. 2013b; Roest et al. 2011a; Tilburg et al. 2012a; Tilburg et al. 2012c). This cattle related genotype was not only detected in cattle in the Netherlands, but also in dairy products from Europe and other parts of the world (Tilburg et al. 2012b). It can be assumed that the cattle related genotypes are less virulent for humans. However, it is not known what the molecular differences are between the cattle strains and strains that are responsible for human outbreaks. Knowledge of this can be useful to better understand the infection biology of Q fever in humans and can be beneficial to develop prevention tools such as vaccines.

Finally an important remaining question is 'what are the triggers of an outbreak'. Several factors are known such as *C. burnetii* infected pregnant small ruminants, abortions in goat herds, probably population density, proximity of small ruminant herds to susceptible humans, but also the virulence of the strain (Georgiev et al. 2013). However, which combination of factors triggers an outbreak is unknown. Knowledge on the interaction of these factors and the relative importance of these factors would be very beneficial to come to a (epidemiological) model that can predict the risk for Q fever outbreaks.

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Chapter 13
Cysticercosis: A Preventable, but Embarrassing Neglected Disease Still Prevalent in Non-Developed Countries

We have the Knowledge, We have the Tools … but Cysticercosis Still Affects Humans and Pigs in Underdeveloped Countries, and Spreads to Developed Ones

Agnès Fleury, Edda Sciutto, Aline S de Aluja and Arturo Carpio

Abstract *Taenia solium* cysticercosis affects both humans and pigs. It has been considered an eradicable disease, and yet its prevalence remains stable in most endemic countries, due to the persistence of risk factors usually associated with the marginalization of an important sector of the population. In this chapter we will review key aspects of its epidemiology, clinical features, diagnosis, treatment, and prevention.

13.1 Epidemiology

Taeniosis-cysticercosis complex is, quintessentially, a disease of the poor. Therefore, it is still found in low-income countries where unhygienic conditions prevail. Human and pig cysticercosis are due to the ingestion of eggs produced by

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adult *Taenia solium*, a cestode residing in the human intestine (Sciutto et al. 2000; Larralde & Aluja 2006). Pigs, as natural coprophages, will infect themselves if they are not confined and range about freely searching for food, which may include human feces when open-air defecation exists in that community. If feces were shed by a *T. solium* carrier, the pig will ingest the eggs, which in turn will develop into cysticerci, the parasite larval stages. While human infection may occur by different ways, the most frequent ones seem to be the ingestion of food contaminated with *T. solium* eggs (either coming from a *T. solium* carrier or from fruits and vegetables irrigated with waste water) and/or living together with a teniasic individual. The infection by adult *T. solium* is due to the ingestion of insufficiently cooked pork containing the parasite larval stage, cysticercus cellulosae, commonly known as "pork measles".

13.1.1 Epidemiology in Humans

13.1.1.1 Epidemiology on a Domestic Level

As the parasite life cycle may suggest, infection by the adult parasite will occur essentially in rural areas, where pigs are sacrificed with no sanitary control to be consumed in a party, a marriage or any other local fest. Indeed, in a study conducted in Honduras, where 328 urban and rural inhabitants were examined for intestinal parasites, only one, coming from a rural area, was identified as a *T. solium* carrier (Sánchez et al. 1998). Another study included 606 rural and urban individuals in northern Vietnam, and the only detected *T. solium* carrier was also a rural inhabitant (Somers et al. 2006). The fact that infected pigs are mainly consumed in rural areas had been shown in different studies. Particularly, in a work conducted in rural villages and urban markets of Congo, pigs diagnosed as infected by tongue inspection were found in rural villages only (Praet et al. 2010). Also, it was reported that rural pigs in Mexico are mostly consumed within the locality where they were reared (Morales et al. 2006).

Different reasons could explain the scarcity of data on taeniosis epidemiology. One of these reasons is that the infection is frequently asymptomatic. Symptoms and signs such as pain, weight loss, and fatigue have been described, but they are non-specific, and frequently patients do not attend medical services. Laboratory diagnosis is not easy, requiring proglottid or egg identification in stool, using for example the Kato method (Thienpont et al. 1986), and generally it is not performed in rural communities. Coproantigen ELISA exists (Allan et al. 1992), but it is not extensively used in rural milieus. Moreover, it should be added that in most cultures in endemic countries, speaking of feces-related diseases is still taboo.

The epidemiology of the infection by *T. solium* larvae (cysticercosis) has different characteristics. The continuous and increasing exchanges between rural and urban areas concerning foods and people explain why cysticercosis infection is not confined to rural areas, but can indeed affect all levels of society. A well-known example of this fact was the infection of a New York Jewish family due to the contact with their domestic employee who carried the adult helminth (Schantz et al.

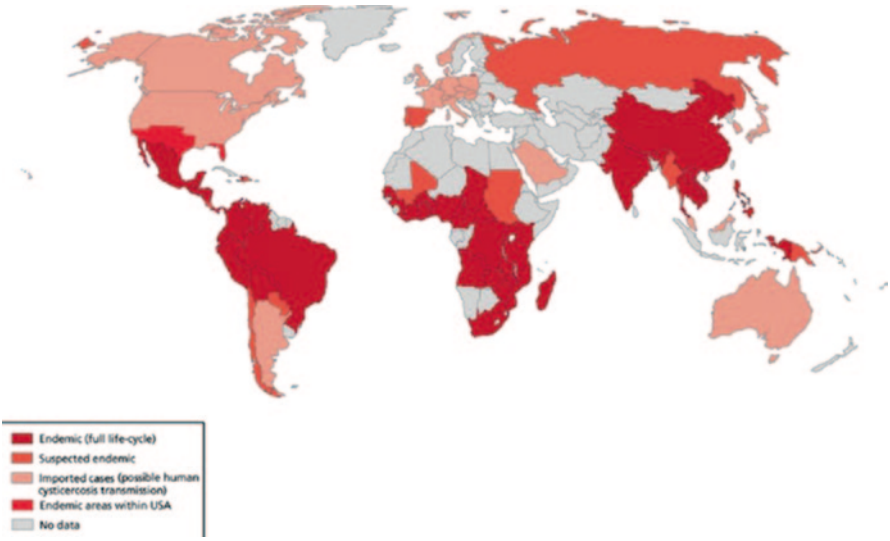

Fig. 13.1 Global spatial distribution of cysticercosis (updated: January 2010). (From: Wertheim et al. 2012)

1992). Reflecting this fact, infected urban inhabitants represent a substantial part of patients in several case series (Fleury et al. 2004; Agapejev 2011).

13.1.1.2 Epidemiology on the International Level

As the parasite life cycle shows, endemic regions will be those where pigs can be in contact with human feces and where sanitary control is not enforced in all areas. For this, almost all non-Muslim underdeveloped countries of Asia, Africa, and Latin America are endemic. The most recent disease distribution map is explicit on this point (Fig. 13.1; Wertheim et al. 2012). The results of the last studies on the subject can be accessed on-line in other work (Fleury et al. 2013b).

Recently, a concern was raised on the increase of diagnosed cases in non-endemic countries (countries where the life cycle of the parasite did not take place, i.e., swine cysticercosis is not present), but where cases of cysticercosis or taeniosis have been detected (Del Brutto and García 2012). Such cases have been reported mainly in the United States of America (Serpa and White 2012; Croker et al. 2012; O'Neal et al. 2011) and in Spain (Del Brutto 2012; Ruiz et al. 2011; Esquivel et al. 2005) due to the increase of human migration from endemic countries. As migration of *Taenia solium* carrier keeps occurring, autochthonous cysticercosis infections have been reported in these countries due to contact with *T. solium* eggs.

Interestingly, key differences in the clinical presentation of cysticercosis between continents have been reported. Extra-neurological presentation seems to be very rare in Latin America (or at least in Mexico) compared with Asia and Africa.

Table 13.1 Prevalence of porcine cysticercosis in different countries

Countries (Ref)	Number of animals	Prevalence (methods used) (%)
Latin America		
Peru (O'Neal et al. 2012)	548	2 (Tongue exam)
		46.7 (EITB LLGP)
Peru (Jayashi et al. 2012)	1153	45.2 (EITB)
Venezuela (Cortez Alcobedes et al. 2010)	52	65.4 (Ab-ELISA)
		42.3 (HP10 Ag-ELISA)
México (Morales et al. 2008b)	562	13.3 (Tongue exam)
Africa		
Kenya (Eshitera et al. 2012)	392	22 (Tongue exam)
		32.8 (HP10 Ag-ELISA)
Cameroon (Ngwing et al. 2012)	499	3.6 (Tongue exam)
		7.6 (Ag-ELISA)
South Africa (Krecek et al. 2012)	261	57 (B158/B60 AgELISA or HP10 AgELISA)
Mozambique (Pondja et al. 2010)	661	12.7 (Tongue exam)
		34.9 (Ag-ELISA)
Burkina Faso (Ganaba et al. 2011)	319	35.4 (Ag-ELISA)
Tanzania (Ngowi et al. 2010)	784	7.3 (Tongue exam)
Uganda (Waiswa et al. 2009)	480	8.6 (B158/B60 Ag-ELISA)
Asia		
India (Prakash et al. 2007)	200 (Brain)	3 (Macro and histopathologicalexam)
India (Sreedevi et al. 2012)	225	11.1 (Pigcarcassesexam)

Likewise, in cases of Central Nervous System (CNS) infection, parasite location in the subarachnoid space and ventricular system seems to be more frequent in Latin America than in India and Africa, where parenchymal location is more frequent (Singh et al. 1997). The reasons for these differences are not known, but genetic factors both of the human host and the parasite could be involved.

13.1.2 Epidemiology in Pigs

Similar to human cysticercosis, swine cysticercosis is present in most rural areas of Africa, Asia, and Latin America. The results of recent studies evaluating the prevalence of swine cysticercosis are presented in Table 13.1.

13.1.3 Burden of Disease

The burden of cysticercosis in humans and pigs and of taeniosis in humans is still difficult to assess, as the disease is frequently asymptomatic both in pigs and humans, and since its diagnosis, at least in humans, depends on the use of modern radiological tools frequently unavailable to the affected population in endemic

Table 13.2 Results of the 2 studies evaluating DALYs (Disability Adjusted Life Years)

	Total YLL[a]		Total YLD[b]		DALYs per thousand persons-year
	Value	95% CR	Value	95% CR	
México (Bhattarai et al. 2012)	7062 (28%)	5509–8818	18,278 (72%)	5891–39,238	0.25 (0.12–0.46)
Cameroon (Praet et al. 2009)	39,017	8195–95,513	6821	14,108–103,469	9 (2.8–20.4)

[a] *YLL* years of life lost due to premature mortality
[b] *YLD* years of lifelost due to time lived in a disability state

Table 13.3 Mortality due to neurocysticercosis

	Mortality (deaths per million population)			
	Age-adjusted annual mortality rates		Crude mortality rates	
	%	95% CI	%	95% CI
United States (Sorvillo et al. 2007)	0.06	0.05–0.07		
Brazil (Santo 2007)	1.68	1.58–1.78		
California (Sorvillo et al. 2004)			0.33	0.27–0.38
Oregon (Townes et al. 2004)			0.29	0.11–0.64

countries. However, several estimations have been made (Bhattarai et al. 2013). Current data on DALYs (Disability Adjusted Life Years), mortality, and economic burden are resumed in Tables 13.2, 13.3 and 13.4.

13.2 Clinical Features

13.2.1 Disease in Humans

Clinical manifestations of neurocysticercosis (NC) vary and strongly depend on the cyst number and location as well as on the host's immune response to the parasite (Carabin et al. 2006, 2011; Carpio 2002). There is a marked clinical heterogeneity across geographical areas. Most cases from the Indian subcontinent present with single lesions, whereas those from Latin America exhibit few viable cysts (Singh 1997; Singh et al. 2010; Vega et al. 2003). These differences are probably due to complex interactions among host, parasite, and environmental factors (Fleury et al. 2010). One of the most intriguing aspects of NC is that presumably a high percentage of individuals with NC remain asymptomatic (Fleury et al. 2010). Some patients develop NC clinical manifestations several years after the parasite lodges in the CNS (Carpio 2002), either by inflammation surrounding the parasite or by a mass effect. The clinical signs of parenchymal cyst location are usually benign; on the contrary, the clinical presentation of extra parenchymal cyst location is life-threatening and may lead to permanent sequels (Estañol et al. 1986).

Table 13.4 Economic burden

		Population in the area	Cost (annual)
California (total hospital charges) 2009 (Croker et al. 2012)		39,434,956	>17 million ($)
West Cameroon (Praet et al. 2009)			
	Global cost		10.3 million € (95% CR 6.9–14.7)
	Human cysticercosis	5,065,382	95.3%
	Porcinecysticercosis	450,000	4.7%
	Individual cost of NCC-associated epilepsy		194 €
Eastern Cape Province, South Africa (2004) (Carabin et al. 2006)			
	Global cost		15–27.5 million €
	Human cysticercosis	7,088,000	73.1–85.4%
	Porcine cysticercosis		14.6-26.9%
	Individual cost of NCC-associated epilepsy		US$ 632–844
Los AngelesCounty (hospital charges) 1991–2008 (Croker et al. 2010)			7.9 million ($)[a]
Peru (Individual total cost of patients during the first 2 years of treatment) (Rajkotia et al. 2007)			996±80 US $[b]

[a] Average annual charge

[b] representing 54% of a minimum wage salary during the first year of treatment and 16% during the second

Most symptomatic parenchymal NC patients show seizures as the only clinical manifestation and their neurological status is usually normal (Carabin et al. 2011; Carpio et al. 1998). Focal neurological deficits, when present, are usually transient over a few days, weeks, or months, with periods of remission and relapse, probably due to different evolutionary stages of the parasite (Carpio 2002). Headache and increased intracranial pressure are frequent in extra parenchymal cyst location patients (Cárdenas et al. 2010; Fleury et al. 2011). This location is found in about one-third of patients. Acute hydrocephalus secondary to intraventricular cysts and chronic hydrocephalus due to arachnoiditis or ependymitis are the most frequent causes of this syndrome (Agapejev et al. 2007). Increased intracranial pressure also occurs in patients with the racemose form of NC and in those with cysticercal encephalitis (Cárdenas et al. 2010; Carpio 2002; Fleury et al. 2011).

Spinal cord cysticercosis is rare (Alsina et al. 2002). Patients experience nonspecific clinical manifestations, such as nerve root pain or spinal cord compression syndromes, according to the level of the lesion. Massive cysticercal infection of striated muscles occasionally produces a clinical picture of generalized weakness associated with muscle pseudohypertrophy.

NC predominantly affects adults in the third and fourth decade of life, being relatively uncommon in children and in the elderly (Kelvin et al. 2009; Sáenz et al. 2006). Most paediatric cases show a single transitional cyst that resolves spontaneously over a few months and do not require any treatment apart from symptomatic

and anti-seizure drugs (ASD) (Kelvin et al. 2011; Singh et al. 2010). However, severe forms of NC may exceptionally occur in younger patients, including cysticercal encephalitis, which results in permanent neurologic sequels, such as amaurosis. Hydrocephalus and intraventricular NC are extremely rare in children (Agapejev et al. 2007; Carpio 2002).

13.2.2 Disease in Pigs

In our experience, pigs rarely show definite signs of the parasitosis. Signs are inconspicuous even in animals harboring several larvae in the brain, although they may show somnolence and remain inactive during longer periods. Convulsions are rare and occur mostly at night, during sleep. No significant hematological change was detected in a sample group of 17 pigs (Royo Martínez 1996). In one study (Prasad et al. 2006) the following symptoms were found to be very specific for cysticercosis: excessive salivation (dribbling of saliva), excessive blinking (5–10 blinks/min) and tearing (trickling tears from the eye) as well as single subconjunctival nodules. Unfortunately, these symptoms have not been confirmed by other authors, and thus pig cysticercosis cannot be clinically diagnosed.

13.3 Diagnosis of Cysticercosis

13.3.1 Diagnosis in Humans

NC diagnosis cannot rely on clinical grounds alone, since no typical clinical picture of NC exists. As previously stated, the most common clinical sign of parenchymal NC is epileptic seizure, which occurs in 60–90 % of cases, followed by headache, motor focal deficits, and psychiatric and cognitive symptoms (Carpio 2002; Rodrigues et al. 2012). Diagnosis of extraparenchymal NC is even more difficult, considering that unspecific symptoms and signs of intracranial hypertension and meningitis may occur, either with or without signs of cerebrospinal fluid inflammation (Cárdenas et al. 2010; Fleury et al. 2011).

NC diagnosis is mainly done by neuroimaging. New imaging techniques, including computed tomography (CT) and magnetic resonance imaging (MRI), have improved the detection of scolex inside cysts, which can be considered pathognomonic of neurocysticercosis (Fig. 13.2) (Lucato et al. 2007; Mont'Alverne Filho et al. 2011). Imaging procedures allow visualizing the vesicular, colloidal, granular-nodular, and calcified phases of the parasite in CNS (Carpio et al. 2013; Escobar 1983) (Fig. 13.2). MRI is more sensitive than CT in diagnosing viable and degenerating cysticerci, as well as cysts located in the ventricles or the subarachnoid space. However, CT is more sensitive to detect calcifications (Carpio et al. 2013).

There is as yet no ideal immunological test for diagnosing NC. The difficulties of developing a sensitive and specific immunological test for NC diagnosis stem from the proper characteristics of the disease. Different immunological tests have been

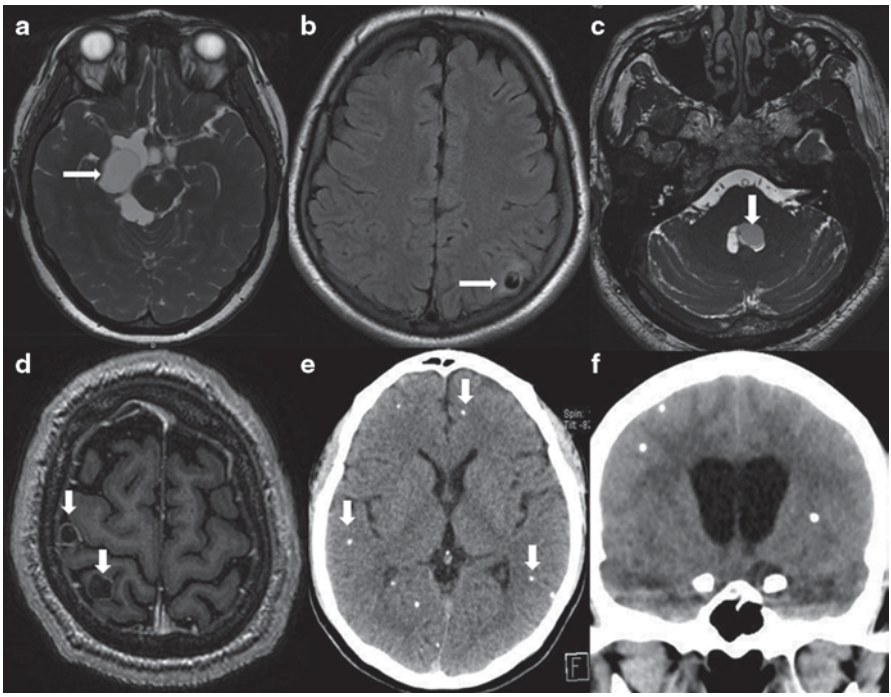

Fig. 13.2 Neurocysticercosis images. **a** Vesicular cysticercus located in subarachnoid cisterns (*MRI*). **b** Vesicular cysticercus located in subarachnoid sulcus (*MRI*). **c** Degenerating cysticerci (*colloidal*) located in the fourth ventricle (*MRI*). **d** Degenerating cysticerci (*colloidal*) located in the subarachnoid space of the convexity (*MRI*). **e** Calcified (*dead*) parasites (*CT scan*). **f** Calcified parasites associated with hydrocephalus (*CT scan*)

developed. The most employed methods aiming to detect specific antibodies are enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) assay (Proaño-Narvaez et al. 2002; Tsang et al. 1989), the two main techniques currently used. These assays are useful to identify individuals who have had systemic contact with the parasite at some time. Seropositivity, however, does not necessarily mean an active systemic infection or central nervous system involvement at any time (Carpio 2002).

Antigen detection by monoclonal or polyclonal antibodies using the ELISA technique has also been developed (Brandt et al. 1992; Fleury et al. 2007, 2013a). Detection of specific antigens in serum or CSF by ELISA in patients with parasites located in the subarachnoid space or the ventricular system is a specific sign of parasite viability and may be used to evaluate treatment response (Fleury et al. 2007, 2013a).

In spite of the current immunological and imaging advances, NC diagnosis is still challenging in many patients. Diagnostic criteria for NC have been proposed (Del Brutto et al. 2001). These diagnostic criteria may be useful to identify patients with parenchymal NC, but not so for patients with extraparenchymal NC (Machado 2010). Thus, a definitive NC diagnosis may require the conjunction of different testing modalities, which can be difficult to obtain in endemic countries.

Table 13.5 Studies comparing sensitivity and specificity of different immunodiagnostic tests

Country (Ref)	Type of swine infection	Method	Antigen	Sensitivity (%)	Specificity (%)
India (Singh et al. 2013)					
	Natural infection	ELISA	Crude lysate	85	98
		ELISA	Cyst Fluid	70	98
		ELISA	Scolex	65	96
		ELISA	Cyst wall	45	98
Peru (Gonzalez et al. 1990)					
	Natural infection	ELISA	Crude lysate	79.2	76.2
		EITB		100	100
México (Sciutto et al. 1998)					
	Experimental infection	Ag-ELISA		83.7	95.9
		ELISA	Cyst fluid	86	95.7
	Natural infection	Ag-ELISA		44.4	45.8
		ELISA	Cyst fluid	55.5	75
		EITB		64.7	59.1
Brasil (Nunes et al. 2000)					
	Experimental + natural infection	ELISA	Crude lysate	85.7	96.4
		ELISA	Cyst fluid	67.8	98.2

13.3.2 Diagnosis in Pigs

Different tools are available to diagnose pig cysticercosis, but the results they yield are frequently divergent.

First, and perhaps the most traditional diagnostic method, is the visual inspection of the inferior surface of the tongue (Leuckart 1879). This is the most frequently used procedure, even though it is a risky operation for the person who performs it and a source of stress to the animal, leading to significant changes in cortisol levels (Pérez-Torres et al. 2012). The method is specific, but it fails to detect all infected animals, as only heavily infected pigs will present parasites in the tongue muscles. Two studies comparing the results of tongue inspection with MRI or necropsy found that tongue-test sensitivity was 70 % and its specificity 100 %, respectively (Gonzalez et al. 1990; Singh et al. 2013).

Another method used is the neck muscle examination: a small, 4–5 cm-long skin incision is cut on the lateral side of the neck, and a trained volunteer inserts two fingers to palpate any nodule (Singh et al. 2013). This diagnostic method is very invasive and may lead to infectious complications, but has shown a sensitivity of 100 % and a specificity of 75 %, respectively (Singh et al. 2013), as neck muscle seems to be one of the most common site of cysticercus infection.

Eyelid examination is also used to some extent: the presence of cyst nodules is tested by direct visualization (Singh et al. 2013). This method is very specific (100 %), but its sensitivity is very low (25 %) (Singh et al. 2013).

A number of serological tests have also been evaluated: detection of specific antibodies using different antigens (whole crude lysate, cyst fluid, scolex, cyst wall) by ELISA and EITB, or specific antigen detection. As shown in Table 13.5, these

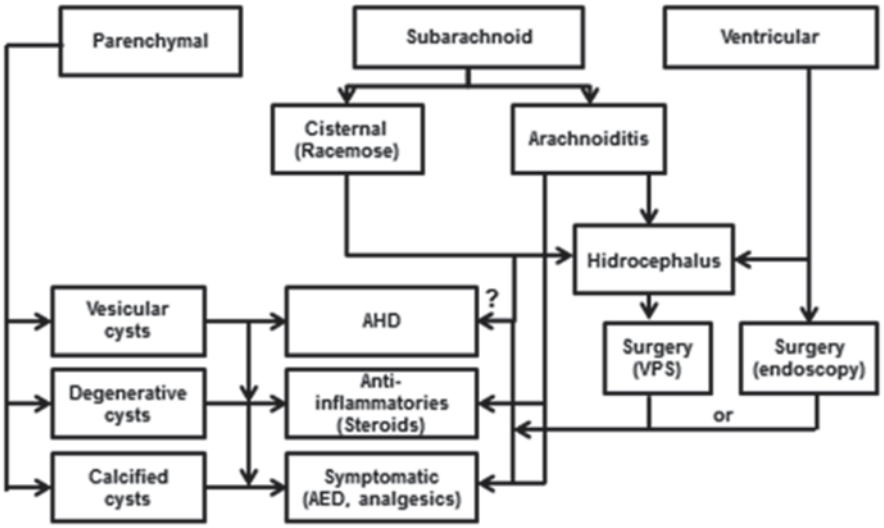

Fig. 13.3 Neurocysticercosis treatment. (From Carpio et al. 2013)

tests have shown varying performance among studies, probably due, in part, to different infection intensity.

Recently, Mexican researchers developed a diagnostic method using portable ultrasonography equipment to examine pig muscles, with very satisfactory results in muscles of pigs (Herrera et al. 2007). In spite of the high cost of the equipment, it is a highly recommended method for the diagnosis of porcine cysticercosis.

13.4 Treatment

13.4.1 Treatment of Humans

NC treatment should be individualized, based on the pathogenesis and natural history of the disease in each patient (Fig. 13.3). Therapy is limited in most cases to symptomatic treatment for patients with seizures. Oral mannitol or glycerol is used in patients with high intracranial pressure, and analgesics should be given for headache. Corticosteroids are often administered for NC, on the premise that they reduce inflammation and edema around dying parenchymal cysts, and are also recommended to treat large subarachnoid cysts and arachnoiditis (Carpio et al. 2013; Fleury et al. 2011). However, the dosage, the treatment duration and form, and most significantly the administration timing, are not clearly defined (Carpio et al. 2008).

Surgery is now almost restricted to ventricular shunt placement for hydrocephalus and to occasional cases of accessible racemose subarachnoid cysts and intraventricular cysts, which are mainly removed by endoscopic approach (Torres-Corzo et al. 2010). Transitional or degenerating cysts, regardless of their size or location, should not be biopsied or removed if differential diagnosis has been discarded, since the parasite is dead and will disappear or be calcified spontaneously (Singh et al. 2010).

NC treatment with anti-helminthic drugs (AHD), praziquantel (PZQ) (Robles and Chavarrıa 1979) and albendazole (ALB) (Xiao et al. 1986) has been available for at least 25 years, but its use has always been controversial. PZQ is an acylated isoquinole-pyrazine with broad anthelminthic activity. Its mechanism of action is not fully understood; however, it is assumed that PZQ changes calcium metabolism and intracellular permeability, with the main effect of inhibiting muscular movements (Garcia-Dominguez et al. 1991). ALB is a benzimidazole with a broad antihelminthic spectrum, which exerts an anticysticercal effect by inhibiting the glucose uptake through parasitic membranes, thus causing energy depletion (Lacey 1990). There is evidence for the efficacy of AHD treatment for viable parenchymal cysts; however, no controlled clinical trials have so far established definitive doses and treatment duration (Carpio et al. 2013). According to placebo-controlled clinical trials (Carpio et al. 2008; Garcia et al. 2004) AHD are effective in about 30 to 40 % of patients, according to the disappearance of viable parenchymal cysts in imaging studies. The most frequent treatment scheme for PZQ is 50 mg/kg/day for 15 days, and for ALB it is 15 mg/kg/day for 8 days (Carpio et al. 2013). A recent Cochrane review (Abba et al. 2010) concluded that the use of ALB in adults with viable cysts is associated with a decrease in the number of cysts, but no difference was detected for ALB for seizure recurrence with untreated patients.

For extraparenchymal cysts the management is even less clear. While AHD have demonstrated efficacy in some cases, it is clear that not all cases respond to the current treatment (Carpio et al. 2008; Fleury et al 2011). This stresses the urge for looking for new treatment alternatives (Diazgranados-Sánchez et al. 2008).

13.4.2 Treatment of Pigs

Several cysticidal drugs have proven to be effective in destroying the parasite (Gonzalez et al. 1997). Indeed, the treatment of infected pigs has been proposed and successfully tested locally (Gonzalez et al. 2012). In spite of their effectiveness, cysticidal drugs have not been extensively used to treat cysticercotic pigs, due to practical reasons: treatment is hardly manageable, and the necessity of waiting several months for cysticerci destruction is costly for rural pig breeders. Possible detrimental effects on the environment through the promotion of drug resistance should be considered as well (Domke et al. 2012).

13.5 Preventive Measures

In spite of the high prevalence of this parasitosis and its impact on human and veterinary health, as well as on economy, its eradication remains a major challenge. Different interventions have been proposed and proved effective for control, albeit with varying practical possibilities.

13.5.1 Focus on Pig-Targeted Actions

Focusing preventive measures on porcine cysticercosis is particularly relevant, since it is the essential step for parasite transmission (Aluja 2008).

In rural communities, pigs become infected because of poverty-related factors, i.e., low hygiene standards and inadequate human feces disposal, which contribute to environment contamination by *T. solium* eggs. In these rural areas, pigs (being natural coprophages) are allowed to freely roam in search of food, favoring them to ingest human feces contaminated with parasite eggs (Copado et al. 2004). This rustic rearing promotes the parasite life cycle, since after ingestion the eggs will evolve to cysticerci and subsequently will be delivered to humans via undercooked infected pork meat (Sciutto et al. 2000).

Compulsory meat inspection aiming to avoid infected pork meat consumption seems a reasonable effort. Unfortunately, while this measure is fully operative in official slaughterhouses, it cannot be enforced in rural, hardly accessible small communities where pigs are clandestinely killed and consumed at the occasion of private fests (Willingham et al. 2010). Educational programs to train the inhabitants of rural communities in good pig sacrificing practices would be a useful intervention to prevent the consumption of undercooked infected meat. Here, too, the challenge will be covering all persons involved in this practice.

Making pig confinement obligatory to avoid contact with human feces would clearly help to interrupt transmission. While this could be a feasible possibility, the poor economic conditions in these rural communities promote the free-roam foraging of pigs to supplement the meager food their owners provide them with.

Porcine cysticercosis is a vaccine-preventable disease. Thus, pig vaccination may provide an additional tool for taeniosis-cysticercosis control and prevention. The first report on successful porcine cysticercosis vaccination was established using total extracts from *T. solium* cysticerci, recovered from naturally infected pigs, as a source of vaccine antigens (Molinari et al. 1997). Since then, various native and subunitary vaccine candidates have been identified, but only a few have been found effective under the complex natural field conditions (Huerta et al. 2001; Morales et al. 2008a; Jayashi et al. 2012). Among them figures the vaccine named S3Pvac and also the HP6/Tsol18 vaccine. S3Pvac vaccine is composed by three small peptides (KETc1, KETc12, and GK1 [KETc7], originally isolated from *Taenia crassiceps* and shared by other cestodes including *T. solium*. Both synthetic S3Pvac (Huerta et al. 2001) and S3Pvac recombinantly expressed in filamentous phages (Morales et al. 2008a, 2011) were successfully tested in the field. HP6 antigen,

originally isolated from *Taenia saginata* (Benitez et al. 1996) and reported to induce high protection levels against cow cysticercosis (Lightowlers et al. 1996), has been found present in *T. solium* (HP6/*Tsol*18) cysticerci and shown a protective effect against porcine cysticercosis (Assana et al. 2010). Finally, infected pigs could be exchanged by vaccinated pigs, better suited to endure the hardship of their lives.

13.5.2 Focus on Human-Targeted Actions

Here, too, different measures have been taken and showed some efficacy. An aggressive hygiene-promotion campaign in rural areas is an obvious start point, since it can eradicate not only *T. solium*, but many other infectious diseases transmitted by human feces (Yap et al. 2012). Among others, this type of measures allowed the control of *T. solium*-related diseases in most European countries in the early twentieth century. However, achieving the same in the currently endemic countries would require an economic and social development that just does not seem achievable in the near future.

Massively administered human cestocidal treatment to reduce the number of tapeworm carriers is another measure which has been applied in different circumstances. Human deworming can be achieved using a single oral dose of niclosamide (2 grs in adults, 1 gr in children) or of praziquantel (5 to 10 mg/kg) (Pearson and Guerrant 1983; Pearson and Hewlett 1985). A priori, niclosamide should be preferred as it is not absorbed at the intestinal mucosa, thus avoiding possible symptoms derived from an occult NC. This strategy has been used in several studies in endemic countries, either administered alone (Cruz et al. 1989; Allan et al. 1997; Sarti et al. 2000; Wu et al. 2012) or associated with other preventive measures (Garcia et al. 2006, 2010).

Health education programs aiming to promote a better understanding of the parasite transmission mechanisms and to improve the hygienic behavior, pig management and sanitary conditions which foster transmission is another measure that has be taken (Keilbach et al. 1989; Sarti et al. 1997; Ngowi et al. 2008; Wohlgemut et al. 2010; Wu et al. 2012).

These interventions, all applied at a small or medium scale, showed generally relevant immediate results, although long-term evaluations are lacking.

Finally, it should be noted that the role of national health authorities in these programs is essential and must be promoted. Researchers alone are not able to implement a (currently inexistent) nationwide program, the only realistic way to eradicate this parasitic disease.

13.6 Unsolved Issues

Cysticercosis is considered a neglected "tools-ready disease" according to WHO (2007), and as a potentially eradicable disease since 1993 (Recommendations of the International Task Force for Disease Eradication). Eradication is feasible, be-

cause—as we have stated earlier—(1) humans and pigs are the only affected species; (2) humans (the definitive host) are the only source of pig *T. solium* infection; and (3) there exist efficient intervention strategies which can interrupt the parasite life cycle.

In spite of this, cysticercosis is still endemic in most countries of Latin America, Asia, and Africa, although its burden is difficult to estimate.

The reasons for this situation are multiple, a major cause being that it is a "forgotten disease of forgotten people" (Hotez 2008), which does not motivate governments to take the necessary measures. As said before, instituting nationwide government control programs is one of the main unresolved issues.

With regard to neurocysticercosis, several items remain unsolved at this point. Focusing on diagnosis, for instance, neuroradiological studies (currently representing the gold standard) are not available to all affected population due to their cost: therefore, improving the immunodiagnostic techniques is mandatory, as all existent tools show low sensitivity, particularly in cases of single-cyst infection. At a therapeutic level, further research is required to understand why some patients do not respond to specific cysticidal treatment, and to develop alternative treatment approaches for these cases. Additionally, managing the inflammatory reaction is a pending problem. Corticosteroids are of great utility to avoid inflammatory complications, but they show severe collateral effects, and their possible role in the lack of response to specific treatment forbids their indiscriminate use. Further research is much needed here, too.

Finally, it should be stressed that taeniosis/cysticercosis is an eradicable disease, and that the firm government commitment in affected countries is key to reach this goal.

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Chapter 14
Toxoplasmosis: A Widespread Zoonosis Diversely Affecting Humans and Animals

Florence Robert-Gangneux, Dominique Aubert and Isabelle Villena

Abstract Infections by the protozoan parasite *Toxoplasma gondii* are widely prevalent in humans and animals worldwide. Since the discovery of parasite's life cycle several studies identified sources of human infection through ingestion of viable tissue cysts in raw or undercooked meat or by ingestion of food and water contaminated with sporulated oocysts after being shed in the feces of infected felids. Prevalence of toxoplasmosis varies between different countries and regions in the same country depending on age, social culture, eating habits and environmental factors. In immunocompetent patients, toxoplasmosis is generally asymptomatic or benign, but severe infections are described in tropical areas due to atypical strains. In immunocompromised patients, *T. gondii* is an opportunistic parasite that may induce life-threatening disease; severe disease could occur in HIV-infected patients or in transplant patients. Finally, in case of congenital toxoplasmosis, infection can lead to abortion, cerebral damage or ocular lesions. New genotyping tools were recently applied to field studies in different continents and revealed a complex population structure for *T. gondii* with a greater genetic diversity than expected. Moreover, a relation between *Toxoplasma* genotype and the severity of infection is described. According to different routes of transmission, hygienic measures can be recommended to avoid *Toxoplasma* infection. These measures can be accompanied by a serological screening of patient at risk for toxoplasmosis as it is recommended

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in France. Although screening is expensive, the important preventive role of health care policies in the decrease of the burden of toxoplasmosis cannot be denied.

14.1 Introduction

The protozoan parasite *Toxoplasma gondii* has a worldwide distribution and is one of the most frequent parasitic infections. This obligate intracellular parasite was first described in the common gundi (*Ctenodactylus gundi*), a rodent from North Africa, by Nicolle and Manceaux in 1908, and was subsequently recognized as the agent of a widespread zoonosis involving humans as well as virtually all warm-blooded animals and birds. However, it took several decades until its entire life cycle was definitively understood in the late 1960s (Hutchison et al. 1969; Frenkel et al. 1970), with the demonstration of the cat as definitive host responsible for oocysts shedding through feces and infection of intermediate hosts. It is now well established that not only the cat, but all felids can reproduce the sexual life cycle of the parasite and participate to the spread of the disease, which explains the wide distribution of toxoplasmosis.

Regarding human infection, the most remarkable events were the first reports on cases of congenital toxoplasmosis in 1939 (Schwartzman et al. 1948), the development of the first serologic test by Sabin and Feldman (1948), and the recognition, in the middle 1970s, that past infection could reactivate in immunocompromised patients (Weiss and Dubey 2009). The high burden of congenital toxoplasmosis led to the progressive implementation of prevention policies in some European countries. During the last decade, the development of new genotyping tools and the multiplication of field studies have increased comprehension of the phylogenetic evolution of *T. gondii* in the world (Mercier et al. 2011), and advances have been achieved in the knowledge on the particular virulence associated with some genotypes (ElHajj et al. 2007).

14.2 A Life Cycle Involving All Warm-Blooded Animals

14.2.1 Definitive Hosts and Contamination of the Environment: Not Only the Cat!

While only felidae can act as definitive hosts and thus shed oocysts in their feces, almost all warm-blooded animals can serve as intermediate hosts. Many host species (birds, rodents, carnivorous or herbivorous animals) from polar to tropical areas have been identified by serology or bioassay. *Toxoplasma gondii* undergoes sexual reproduction in the felid intestine, resulting in the production of millions of environmentally unsporulated oocysts. Oocysts take 1–5 days to sporulate in

the environment and become infective and resistant in environment. Oocysts may survive for months in soil and water, thereby enhancing the probability of transmission to intermediate hosts such as birds, rodents and humans. Cats become infected after consuming intermediate hosts harbouring tissue cysts or directly by ingestion of sporulated oocysts. Animals bred for human consumption and wild game may also become infected with tissue cysts after ingestion of sporulated oocysts in the environment. Humans may acquire *T. gondii* infection via oral uptake of sporulated oocysts from the environment, consumption of raw or undercooked meat containing tissue cysts or via transplacental transmission of the parasite from the non-immune mother to the fetus (Fig. 14.1).

Oocysts are essential in the life cycle of *T. gondii* and cats were everywhere, except the frozen artic. At any given time, approximately 1 % of cats are expected to shed oocysts (even if most cats only shed oocysts for about one week in their life), and this is supported by faecal survey. The number of oocysts shed by naturally infected domestic cats is largely unknown, but probably several millions are disseminated in the environment according to experimental infection studies. Whether naturally infected cats shed oocysts more than once in their life, is unknown. The number of oocysts shed during the secondary infection is usually lower than during the primary infection (Dubey and Frenkel 1974). In addition to domestic cats, wild felids can also shed oocysts. The role of wild felids in parasite transmission to humans may be important especially in areas where the domestic cat, *Felis catus*, is absent (e.g. in the tropical forest). Cats are essential for the maintenance of *T. gondii* in the environment, since infections are virtually absent from areas lacking cats. A single oocyst is able to infect pigs or mice, but pathogenicity depends on the strain, inoculum and infection route (Dubey and Beatty 1988). Oocysts are less infectious and pathogenic for cats than for intermediate hosts (Tenter et al. 2000).

Based on serologic surveys, up to 74 % of the adult cat population may be infected by *T. gondii* (Tenter et al. 2000). In nature, cats are infected by eating small preys harboring tissue cysts, or by ingesting oocysts from soil. Fatal toxoplasmosis is rare and occurs more often in immunocompromised cats and in kittens. Vertical transmission is uncommon and is probably not important for parasite propagation. Cats excrete millions of unsporulated oocysts in their feces after ingesting any of the three infectious stages, the prepatent period is 3–10 days after ingesting bradyzoites and more than or equal to 18 days after ingesting oocysts or tachyzoites. The patent period is only 1–3 weeks, but a re-excretion is possible, at least experimentally, after a second challenge with *T. gondii*, after corticoid treatment or superinfection by *Isospora felis*. Even within a shared habitat, exposure of wild and domestic felids to *T. gondii* may increase or decrease based upon access to prey and dietary preferences (VanWormer et al. 2013). Pet and feral domestic cats showed different prey consumption patterns, with higher predation levels observed in feral cat populations (Fitzgerald and Turner 2000). In regions where humans, livestock, and wildlife live in close contact, wild felid prey frequently included domestic livestock such as sheep, goats, and cattle as well as wildlife (Treves and Karanth 2003).

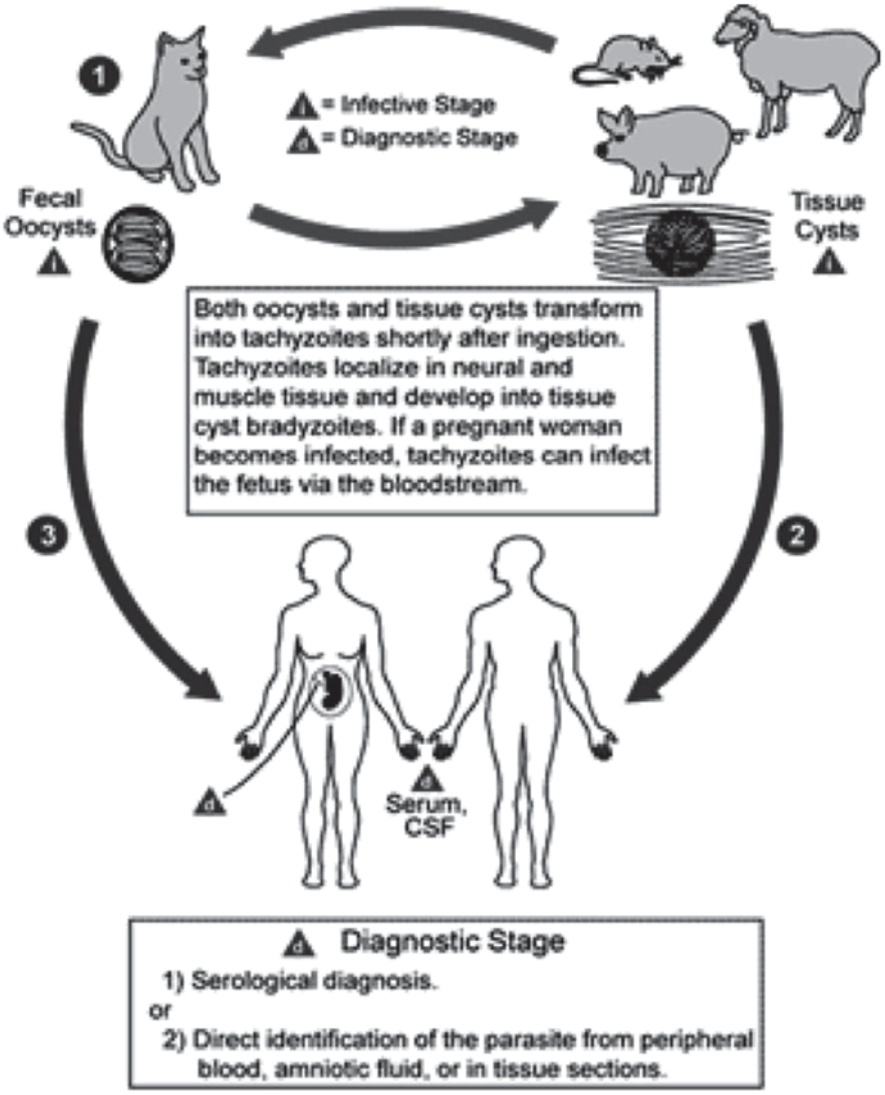

Fig. 14.1 Life cycle of *Toxoplasma gondii*. (Graph obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary)

14.2.2 Life Cycle of T. gondii *in Domestic Animals and Wildlife*

T. gondii is not an obligatory heteroxenous parasite and can propagate clonally (presumably indefinitely) by cycling among intermediate hosts. This can occur vertically through transplacental transmission from mother to offspring (Dubey et al. 1997). Tissue cysts are also orally infectious to carnivorous intermediate hosts, permitting *T. gondii* to bypass the sexual stage in the definitive host (Khan et al. 2007).

Oocysts are responsible for most of the *T. gondii* infections in non-carnivorous mammals and birds, whereas other species are mainly infected by eating animals harboring tissue cysts. *T. gondii* infection in wildlife does not occur with the same probability in any species or place. Wild-living species first have variable levels of susceptibility and exposure. Exposure is largely determined by life history traits, especially feeding behavior. Herbivore intermediate hosts acquire infection via food or water contaminated with sporulated oocysts. Carnivores and omnivores may additionally become infected mainly by ingesting meat containing cysts. Hejlíček et al. (1997) suggested that carnivores have a higher prevalence of antibodies to *T. gondii* due to the cumulative ingestion of infected animals and that seroprevalence would be lower, by decreasing order, in omnivorous, herbivorous, and insectivorous animals.

14.2.3 A Wide Range of Opportunity for Human Infection

Human infection can occur from different transmission routes due to several stages of the parasite (cysts, oocysts and tachyzoites). Even thought the sources of *T. gondii* infection for humans are well known, their relative contribution is still unclear. The major routes of transmission vary between different human populations and depend on social culture, eating habits and environmental factors (Tenter et al. 2000).

The most frequent transmission route appears due to cysts present in meat as a risk-factor analysis indicates that 30–63 % of human infections can be attributed to the consumption of undercooked meat (Cook et al. 2000). All warm-blooded animal species can be infected, but prevalence of infection in meat producing animals are variable among species and countries (Tenter et al. 2000; Dubey 2010). Sheep and pigs seem to be more contaminated while infection seems rarer in cattle. In France and Norway, consumption of undercooked lamb appears to be a stronger risk factor than consumption of pork (Kapperud et al. 1996; Baril et al. 1999), while it is different in Poland where consumption of undercooked pork was identified as the principal risk factor (Paul 1998). Moreover, there are few reported outbreaks due to ingestion of well-identified meat and absence of evidence of source of infection is frequent due to lack of detection of cysts in meat.

Infection through cysts can also occur after organ transplantation when an organ from a *Toxoplasma*-seropositive donor is grafted to a *Toxoplasma*-seronegative recipient. Generally, heart, liver and kidney transplantation are most frequently associated with this route of transmission (Speirs et al. 1988).

Infection through oocysts is another transmission route for humans. Sporulated oocysts are very resistant (especially to most of disinfectants) in the environment. They may survive for several years and disperse through water or soil movements and contaminate vegetables. They may be an important source of infection for humans, and several waterborne outbreaks of toxoplasmosis linked to oocyst contamination of drinking water are reported (Dubey 2010).

The last transmission route of infection is through tachyzoites. Tachyzoites are delicate, they are destroyed by gastric secretions and unable to survive outside its host. So contamination by this route is very rare except in the case of congenital infection due to transplacental transmission from a mother to her fetus after a primary maternal infection firstly acquired during pregnancy. Tachyzoites can be rarely transmitted by transfusion (blood/bone marrow donor) or in case of laboratory accidents. Finally, toxoplasmosis in humans was occasionally reported after transmission by goat milk (Sacks et al. 1982; Skinner et al. 1990).

14.3 Prevalence and Disease Burden in Humans

14.3.1 Pathophysiology of the Disease

The pathophysiology of toxoplasmosis results from the dissemination of tachyzoites. After trans-epithelial passage across the intestinal barrier, tachyzoites invade rapidly monocytes and gain access to the blood flow, and from there virtually to all organs (Robert-Gangneux and Darde 2012). In fact, tachyzoites can invade actively all nucleated cell types, which can explain the variety of clinical manifestations possibly observed, in particular in immunocompromised patients. After the onset of an efficient immune response, tachyzoites are not eradicated, but convert into bradyzoites within cells and persist as cysts lifelong, mostly in muscles, retina and brain.

14.3.2 Toxoplasmosis in Immunocompetent Subjects: Seroprevalence in the World

It is widely admitted that toxoplasmosis is frequently asymptomatic in immunocompetent humans, but may cause mild and non-specific symptoms including fever, asthenia and lymphadenopathy in about 20 % of patients. Therefore, the diagnosis is often retrospective and is based on serology. Estimates indicate that approximately 25–30 % of the world human population is infected by *Toxoplasma* (Montoya and Liesenfeld 2004). Seroprevalence varies among countries, but also among regions within the same country. Low seroprevalence (10–30 %) is observed in North America, in South East Asia, in Northern Europe, and in Sahelian countries of Africa. Moderate prevalence (30–50 %) is found in countries from Central and Southern Europe, and high prevalence in South America and in tropical African countries. Several factors can be put forward to explain this heterogeneity, especially (1) climatic factors affecting survival of oocysts in the environment (Dubey 1998), (2) the prevalence in meat-producing animals, (3) cooking or cultural habits, and (4) socio-economic level and quality of water. Seroprevalence has declined over the last decades in most industrialized countries, probably as a result of combined factors (increased socioeconomic level and improvement of hygienic conditions,

change of farming systems, consumption of frozen meat, and feeding of cats with sterilized food). For example, in France, the seroprevalence in pregnant women was about 80 % in the early sixties, and declined to 54 and 44 % in two national perinatal surveys in 1995 and 2003, respectively, while at the same time the average age of first pregnancy increased (Villena et al. 2010). The seroprevalence observed in the general population is increasing with age and seroprevalence in men and women does not differ for the population aged 45 years and under (Bellali et al. 2013).

14.3.3 Congenital Toxoplasmosis: Determinants of Severity and Various Incidences in the World

Vertical transmission can occur when primary infection is acquired during pregnancy, by transplacental transfer of tachyzoites either during blood flow dissemination or at a later stage. The colonization of the placental tissue by the parasite is probably an important factor in the process, since 50–72 % of placentas from infected fetuses still harbour parasites at birth, which make this biological sample a good tool for diagnosis (Robert-Gangneux et al. 2011). In France, the mean seroconversion rate during pregnancy was estimated to be 6–7 per 1000 seronegative pregnant women (Berger et al. 2008). The frequency of vertical transmission and the severity of fetal damage depend on the stage of pregnancy when maternal infection occurs. Fetal infection occurs in less than 10 % of cases during the first trimester, but increases to 30 % of cases in the second trimester and 60–70 % in third trimester and even more close to delivery (Dunn et al. 1999). The severity of fetal infection is inversely correlated, since neonates are usually asymptomatic in more than 80 % of cases when infected during the third trimester of gestation (Desmonts and Couvreur 1974). Conversely, when transplacental transmission occurs during the first trimester of pregnancy, the consequences on fetal development are severe, often leading to gross abnormalities involving the brain and eye tissues or to abortion. Major sequelae include mental retardation, seizures, microcephalus, hydrocephalus, microphthalmia, cataract, increased intraocular pressure, strabismus, uveitis, retinochoroiditis, and possibly blindness. During the second trimester, fetal infection may have variable consequences, including hepatosplenomegaly, intracranial calcifications, epilepsy, anemia, thrombocytopenia-induced petechiae, pneumonitis or retinochoroiditis. Among the 272 cases collected in 2007 through the French surveillance for congenital toxoplasmosis network (Villena et al. 2010), 11 cases resulted in termination of pregnancy owing to cerebral lesions or fetal death, and 87 % of live-born infants were asymptomatic. The remaining 13 % of cases had intracranial calcifications (14 cases), hydrocephalus (3 cases) and/or retinochoroiditis of variable severity (12 cases). Similar tendencies have been documented in the last four years (data 2012, National Reference Center on Toxoplasmosis, France).

The incidence at birth was about 3 per 10,000 live births in 2007 in France (Villena et al. 2010), which is in the same range as incidence rates reported in other European countries, such as Denmark (2.1/10,000 live births) and Switzerland (4.3/10,000 live births), but is higher than that reported in Sweden (0.73/10,000) or

in a pilot study in Massachussetts (1/10,000) (Guerina et al. 1994) and lower than in Brazil (6/10,000) (Lago et al. 2007).

14.3.4 Toxoplasmosis in Immunocompromised Patients

While toxoplasmosis is usually a mild or asymptomatic infection in immunocompetent subjects, it is a life-threatening infection in immunocompromised patients. Various factors severely impairing the cellular immune response, e.g. HIV infection and immunosuppressive therapies, can lead to severe toxoplasmosis, due to either primary acquired infection or to reactivation of latent infection. Indeed, profound immunosuppression can favour cysts rupture and tachyzoite multiplication and dissemination. Cyst reactivation is mostly localized to the brain and the retina, but can occur in other tissues, as *Toxoplasma* can invade all organs that can be subsequent potential sites for cyst reactivation (Patrat-Delon et al. 2010). This peculiarity puts transplant patients at risk for both reactivation and organ-transmitted infection. The risk for disseminated infection is closely related to the duration and degree of immunosuppression, with hematopoietic stem cell transplant (HSCT) patients being most at risk (Derouin and Pelloux 2008), whereas focal disease, such as cerebral toxoplasmosis or retinochoroiditis, is more commonly observed in HIV-infected patients. In HIV^+ patients, the incidence of toxoplasmosis is closely related to $CD4^+$ T cell counts, with an increasing risk when falling below 100 cells/μL. The incidence of toxoplasma encephalitis has decreased and is now stabilized since the use of highly active antiretroviral therapy; it represents about 200 cases/year in France (Abgrall et al. 2001). In the absence of an implemented recording system, there are no data available regarding the incidence of toxoplasmosis in transplant patient.

14.3.5 New Insights into the Comprehension of Parasite Virulence: The Role of Different Genotypes

Although one single parasite species is responsible for toxoplasmosis in humans and animals, it has been shown since the 1990s that clinical isolates from Europe and USA could be divided into three major genotypes: types I, II and III, which are equivalent to clonal lineages (Darde et al. 1992; Howe and Sibley 1995; Ajzenberg et al. 2002a).

However, more recently new genotyping tools such as multilocus sequence typing were applied to field studies in other continents and revealed a much more complex population structure with a greater genetic diversity, likely reflecting frequent exchanges of strains between hosts as well as recombination of isolates during sexual life cycle within the definitive hosts (Ajzenberg et al. 2004). This led to the generation of recombinant isolates (I/II, I/III or II/III), but also to new clonal

haplogroups, and in some areas, particularly in South America, to atypical genotypes with many unique polymorphisms. Type II strains markedly predominate in humans in Europe (Ajzenberg et al. 2002b; Aubert et al. 2010) and are isolated in more than 90 % of congenital infections in France. Other clonal lineages are occasionally (type III) or exceptionally (type I) described in Europe. The exceptional isolation of atypical strains in France can be related to travels to South America or consumption of imported meat (Pomares et al. 2011). In North America type II strains also predominate (Howe and Sibley 1995), but a higher prevalence of atypical strains is observed, and a clonal haplogroup (haplogroup 12) has been recently identified (Khan et al. 2011). By contrast, atypical genotypes largely predominate in South America, whereas type II isolates are rare (Pena et al. 2008). In Africa, clonal lineages known as *Africa 1-3* haplogroups coexist with type II and III lineages (Mercier et al. 2010). Until now, few data in humans are available from Asia, but some studies reveal a more limited genetic diversity than in South America, the presence of type III strains, and the widespread detection of a clonal lineage in pigs in China (Zhou et al. 2010). Recently, clustering methods were used to organize the marked genetic diversity of 138 unique genotypes into 15 haplogroups that collectively define six major clades (Su et al. 2012).

It is long known that clonal genotypes have variable virulence. Genotypes I and II exhibit a high and low virulence, respectively, whereas genotype III shows intermediate virulence. What emerges from recent epidemiologic studies using new genotyping data is that atypical strains are highly virulent and challenge the concepts of pathophysiology of the disease, at least in some parts of the world. First of all, the fact that infection is usually asymptomatic when it occurs in an immunocompetent subject is not questioned in Europe and North America, but recent experience from French Guiana shows that severe and even lethal toxoplasmosis can be observed in immunocompetent patients infected with atypical strains (Carme et al. 2002). Regarding congenital toxoplasmosis, a comparative prospective cohort study of infected children in Brazil and Europe showed that, independently of treatment, Brazilian children had a five-times higher risk than European children for developing eye lesions, and their lesions were larger, more multiple, more recurrent, and more likely to impair vision (Gilbert et al. 2008). Besides, recent observations have shown that: (i) re-infection with an atypical strain of a previously immune woman could lead to congenital transmission (Elbez-Rubinstein et al. 2009), and (ii) the rate of severe congenital infections was higher when women were infected with an atypical or recombinant strain than with a type II strain, whatever the stage of pregnancy at maternal infection was. Cumulative data (2006–2011) of the French surveillance network for congenital toxoplasmosis show that about 84 % of congenital infections with type II strains are asymptomatic infections, whereas 83 % of infections with atypical or recombinant genotypes are symptomatic. The large predominance of type II strains of low virulence in France (92 % of cases), and more generally in Europe, explains the relatively low burden of disease in these countries.

14.4 Prevalence and Disease Burden in Animals

Contact and interaction between wild fauna, domestic animals, and human beings may lead to an increased risk of transmission of zoonotic pathogens (Artois 1993). Wild mammals and birds are exposed to *T. gondii* through the ingestion of food or water contaminated with sporulated oocysts derived from felid faeces (Dubey and Jones 2008). Additionally, wild carnivores and omnivores may also be infected by feeding on birds and mammals with *T. gondii* tissue cysts.

14.4.1 Wild Life

Serologic studies have assessed *T. gondii* infection in several species of wild animals from Europe (Jakubek et al. 2001; Sobrino et al. 2007; Richomme et al. 2009; Beral et al. 2012) and other continents (Dubey et al. 1999). Mammals like cervids, wild boars, canids, viverrids and mustelids, as well as different kinds of birds, are among those wild animals found with antibodies to *T. gondii*, either at a group or an individual level (Dubey et al. 2004). The modified agglutination test (MAT) has proved to be a very sensitive and specific assay for the serological diagnosis of *T. gondii* infection in many species of wild mammals and birds (Sobrino et al. 2007; Literák et al. 1992; Gauss et al. 2006).

Exposure to *T. gondii* is highest in carnivorous species (Cabezón et al. 2011). High *T. gondii* seroprevalence is also reported in large predator species as lynx and the European wildcat (Sobrino et al. 2007) which is of epidemiological significance because infected felids shed oocysts in the wild environment.

In most species, *T. gondii* infection is generally unapparent, provoking only mild symptoms. However, a limited number of highly susceptible species have been discovered, in which *T. gondii* infection frequently leads to clinical disease and mortality. Marsupials and New World monkeys, which have evolved largely separately from cats, are among the most vulnerable species (Tenter et al. 2000). Fatal toxoplasmosis is also well-documented in hares (Jokelainen et al. 2011). *T. gondii* infection can be present at a high level in many wild birds without any clinical impact, but toxoplasmosis can be clinically severe in pigeons and canaries (Dubey 2002). The ingestion of infected birds is considered an important source of infection for cats (Dubey 2010).

14.4.2 Meat-Producing Animals

Tissue cysts of *T. gondii* contained in meat and meat-derived products may be important sources of infection for humans. However, for public health purposes it is important to note that the tropism of *T. gondii* and the number of tissue cysts produced in a certain organ vary with the intermediate host species. In livestock,

T. gondii tissue cysts are most frequently observed in various tissues of infected pigs, sheep and goats, and less frequently in infected poultry, rabbits, dogs and horses. By contrast, tissue cysts are found only rarely in skeletal muscles of cattle or buffaloes (Tenter et al. 2000). Finally, prevalence in meat-producing animals varies among species and countries and is largely dependent on laboratory measures.

Prevalence of toxoplasmosis is highest in sheep and toxoplasmosis is implicated in 10–20 % of sheep flocks with an abortion problem. The prevalence increases with age, reaching more than 90 % in some studies (Tenter et al. 2000); the prevalence in ewes is more than twice than that in lamb. Viable *T. gondii* have been recovered from as many as 67 % of sheep samples. Among the infected meat, lamb meat is supposed to be a major source of toxoplasmosis worldwide (Tenter et al. 2000). *T. gondii* has been recognized as one of the main causes of infective ovine abortion in New Zealand, Australia, The United Kingdom, Norway and the United States (Dubey and Beatty 1988). Goats appear to be more susceptible to clinical toxoplasmosis and even adult goat may die of acute toxoplasmosis.

Seroprevalence levels are lower in cattle and very variable in pigs (1–60 %) and poultry (0–30 %), depending on their lifestyle (indoor or outdoor). In pigs, clinical symptoms are rare (leading to rare cases of myocarditis and encephalitis (Dubey and Jones 2008)), but the real problem of toxoplasmosis in pigs lies in the fact that tissues of infected animals may contain *T. gondii* tissue cysts. Infections with this parasite are common in pigs worldwide. The prevalence of *T. gondii* infection has decreased significantly with changes in pig production. It was suggested that infected pork products cause 50–75 % of all cases of human toxoplasmosis in the United States, but a large study of Dubey et al. (2005) showed a seroprevalence of 0.57 % and parasite isolation in only eight cases (0.38 %). It is estimated that one pig is consumed by 200–400 individuals, and meat products are often made by combining the meat from different animals, thereby enhancing the transmission risk (Kijlstra and Jongert 2008). Moreover, an upsurge in consumer demand for ‘organically raised’, ‘free range’ pork products has resulted in increasing numbers of hogs being raised in non-confinement systems. By that, the prevalence of *T. gondii* in 33 market pigs raised under certified organic management conditions on 2 farms from Michigan, USA, was 90 % (30/33) leading to the isolation of 17 strains (Dubey et al. 2012). This study indicates that organic pork meat may pose an increased risk of transmitting *T. gondii* to humans. Bayarri et al. (2012) have analysed 50 samples of fresh pork meat and commercial cured ham collected in the city of Zaragoza (Spain) and *T. gondii* was detected in two samples of rib, reflecting a frequency of 8 % positive fresh pork meat.

T. gondii has rarely been isolated from bovine tissue. It is unclear whether this is due to fast elimination of cysts from cattle tissues or to inconsistent cyst formation following infection. In a study analyzing more than 2000 samples of beef, *T. gondii* was not isolated when using a bioassay in cat (Dubey et al. 2005). The prevalence of viable *T. gondii* in chickens produced in intensive farming is usually very low, but may be high in free-range chickens. It is not known how many tissue cysts result in the infection of humans, but ingestion of one cyst is sufficient for a cat to become infected.

During the production of various meat products, meat of many animals is mixed, which also amplifies the risk in cases where only few animals might be infected (Aspinall et al. 2002).

14.4.3 Genotype Distribution

In Europe, the majority of isolates from wildlife are type II strains, with a few type III strains. From 26 *T. gondii* DNA extracts from red fox in Belgium submitted to a genotyping analysis with 15 microsatellite markers (Ajzenberg et al. 2010), 25 were type II and only one type III (De Craeye et al. 2010). Similarly, using six loci microsatellite analysis, only type II strains were observed in 46 French isolates including 21 from wild boar (Richomme et al. 2009), 12 from roe deer, 9 from foxes, one each from mouflon, red deer and mallard (Aubert et al. 2010) and one from tawny owl (De Craeye et al. 2010). Using the same molecular technique, Jokelainen et al. (2011) also identified the clonal type II in 15 DNA extracts from hares in Finland. In a recent study in Central and in Eastern Germany, Hermann et al. (2010) determined the complete genotype for 12 samples tissues from red foxes, using nine PCR-RFLP markers. Interestingly, this study showed evidence of a mixed infection as well as infection with a *T. gondii* genotype that may represent a recombination of *T. gondii* types II and III. Su et al. (2006) developed a standardized restriction fragment length polymorphism (RFLP) typing scheme based on nine mostly unlinked nuclear genomic loci and one apicoplast marker. These markers allow to distinguish the archetypical from atypical types. In addition, these markers can easily detect mixed strains in samples. Mixed infection of *T. gondii* strains in intermediary hosts has previously been reported (Ajzenberg et al. 2002a; Aspinall et al. 2002). In Svalbard, a Norvegian arctic archipelago, 55 artic foxes were found infected with *T. gondii*: 27 harboured clonal type II (17/27 were apico I and 10/27 apico II, respectively) and four had clonal type III (Prestrud et al. 2008). Strains from 22 foxes (40 %) could not be fully genotyped, but two (3.6 %) shared more than one allele at a given locus. Again, the most prevalent genotype in this study was clonal type II with a few types III genotypes. It is noteworthy that type II is also the dominant type in domestic mammals in Europe. For instance, Halos et al. (2010) analysed 433 hearts of sheep by using PCR-restriction fragment length polymorphism and microsatellite markers on parasites isolated after bioassay in mice. All 46 genotypes belonged to type II, except for one strain from the Pyrenees mountains area, which belonged to genotype III, which is the first non-type II genotype found in sheep in Europe (Owen and Trees 1999) and Denmark (Jungersen et al. 2002). This similarity between strains found in wildlife and domestic species in Europe suggests that no clear separation exists between the wildlife and domestic cycles.

In North America, strains of *T. gondii* are more diverse. A recent study analysed 169 *T. gondii* isolates from various wildlife species, and revealed the large predominance of the recently described clonal type 12, followed by the type II and III lineages; these three major lineages accounted for 85 % of strains from wildlife

in North America (Dubey et al. 2011). The strains isolated from wildlife in North America are therefore more diverse, but may also be more different from strains found in the domestic environment than in Europe. Although type 12 has been identified from pigs and sheep in the USA, it may be more specifically found in wildlife (Su et al. 2012). The relative high diversity in *T. gondii* genotypes isolated from wildlife samples compared to those from domestic animals raised the question as to whether distinct gene pools exist for domestic and sylvatic hosts (Wendte et al. 2011).

The high genetic diversity in tropical wildlife in connection with a sylvatic life cycle has been firstly evoked in French Guiana where severe cases of human toxoplasmosis were detected after eating Amazonian undercooked game or drinking untreated river water (Dardé et al. 1998; Carme et al. 2009). These cases were due to highly atypical strains, all with an unique genotype, as determined by microsatellite analysis (Ajzenberg et al. 2004).

14.4.4 Most Frequent Sources for Human Infection: Case-Control Studies and Outbreaks

Different approaches have been used to estimate the relative importance of sources of infection, using risk-factor analyses or estimation of the fraction of an attributable risk, either in the general population (chronic infection) or in cases of seroconversion in pregnant women. These studies clearly identified the ingestion of undercooked meat as a risk factor (Cook et al. 2000; Kapperud et al. 1996; Baril et al. 1999; Berger et al. 2008). However, this result is probably partly due to this risk being easier to characterize than the risk due to ingestion of oocysts. Several reports on toxoplasmosis outbreaks have been published in recent decades, mainly regarding outbreaks associated with the consumption of undercooked meat (Dubey 2010; AFSSA 2005). Moreover, it is difficult to link outbreaks due to *Toxoplasma* contamination because most infections are asymptomatic. In France, only few familial outbreaks were reported due to consumption of raw lamb meat (Ginsbourger et al. 2010).

However, outbreaks can be reported when infections are symptomatic due to virulent strains (e.g. from atypical genotype). In this way, previous studies demonstrated that consumption of game from the Amazonian forest was strongly associated with the risk of developing severe toxoplasmosis 10–20 days later (Carme et al. 2002). An outbreak of toxoplasmosis from December 2003 through mid January 2004 involving 11 cases among the 38 inhabitants of a village in Suriname near the French Guiana border was described (Demar et al. 2007) and more recently a new case of severe toxoplasmosis after consumption of semi-raw game (Brazilian Tapir, locally known as Maipouri) was reported (Groh et al. 2012).

Recently, an emerging risk was observed in France with severe human infection transmitted by imported horse meat; source of infection seems to be linked to consumption of raw horse meat from South America where atypical strains are

circulating; these strains are virulent and responsible for severe congenital toxoplasmosis or death even in immunocompetent persons (Pomares et al. 2011). This risk must be evaluated by survey of imported meat on a large scale and French authorities and EFSA are informed.

Moreover, severe toxoplasmosis was also observed in waterborne outbreaks due to contamination by oocysts. The first large outbreak was reported in British Columbia, Canada where drinking water taken from a surface reservoir was suspected to be contaminated by oocysts from cougar (Bowie et al. 1997). Unfortunately, oocysts were not detected after the outbreak in the reservoir. In contrast, *T. gondii* oocysts were isolated once in Brazil from samples taken from small reservoirs on roof tops. To assess this potential source, the water was filtered through membranes and the filters were fed to pigs and chickens which developed toxoplasmic infection (De Moura et al. 2006). Although detection of oocysts in environmental samples is difficult (Dumètre and Dardé 2007; Jones and Dubey 2010), new methods are currently being developed. One method to detect oocysts in filtered water is based on PCR to detect *T. gondii* DNA (but not viable oocysts) after concentration (Kourenti and Karanis 2004; Villena et al. 2004). Oocysts can also contaminate vegetables and lead to human infection, but cases associated with identified vegetables are very rare. One case-control study of an outbreak of acute toxoplasmosis in Brazil implicated escarole and green vegetables as contaminated sources of infection (Ekman et al. 2012).

While differentiating routes of *T. gondii* acquisition has been historically difficult, a recently recognized oocyst-specific antigen (Munoz-Zanzi et al. 2010) applied in a study on 76 mothers of congenitally infected infants in the United States demonstrated that 78% of these women had oocyst-acquired infections (Boyer et al. 2011).

14.5 Impact on Public Health and Health Care Decision Makers

14.5.1 Implementation of Prevention Measures: Depending on Seroprevalence

14.5.1.1 Hygienic Measures

Hygienic measures can be recommended to seronegative patients (pregnant women or immunocompromised patients) to avoid *Toxoplasma* infection. Current prevention messages address hygienic measures with respect to cats, to the consumption of well-cooked meat and thoroughly washed raw vegetables, as well as to hand-cleaning (Table 14.1). Drinking water has recently emerged as a new risk factor in some countries, depending on the source of the water supply network (surface or ground water) and on the sanitary level or the use of well water. Indeed, several water-borne

Table 14.1 Hygienic measures according to prevention of *Toxoplasma* infection in seronegative persons

Action or situation	Prevention measures
Cat contact	Wash hands carefully after stroking a cat
	Wear gloves when changing cat litter
	Change litter frequently and wash the tray with hot water (>60° C)
	Avoid litter in the kitchen
Meals	Cook the meat well-done or stew
	Avoid microwave cooking for meat
	Avoid raw vegetables at restaurants
	Avoid raw shellfish
	Avoid raw goat milk
Preparation of meals	Wash vegetables, fruits and herbs thoroughly, especially if they grow close to the ground
	Wash hands, knives, any containers and table thoroughly after meat manipulation or cutting
Water	Prefer mineral water to tap water
Gardening or outdoor activities	Wash hands thoroughly and brush nails after any outdoor activities in contact with soil
	Wear gloves for gardening
	Avoid ingestion of water during recreation activities in lakes or rivers

outbreaks have been reported in Brazil, India and Canada (Bowie et al. 1997; Bahia-Oliveira et al. 2003). Besides, ingestion of contaminated water from lakes or rivers during recreational activities has been recently stressed out as a potential source for *Toxoplasma* infection (Jones and Dubey 2010), which could explain the large proportion of unexplained toxoplasmosis in pregnant women as shown in a study in the northern USA (Jones et al. 2009). Environmental contamination by wild felids is more difficult to master, but basic hygienic measures during or after external activities can help to reduce the risk. Finally, the trends of biologic food consumption should not lead to hazardous behaviour. It is worth to take into account that—although rarely—unpasteurized goat milk or raw shellfish may act as vehicles for food-borne toxoplasmosis (Jones et al. 2009).

Recent knowledge on strain virulence should be taken into consideration and these recommendations should now be applied to travellers visiting countries where virulent strains predominate, such as South America or Africa, even if they were previously immune.

14.5.1.2 Serological Screening

Serological screening of pregnant women has been proposed to reduce the burden of congenital toxoplasmosis, and has been implemented in France in 1987, with repeated monthly testing of seronegative women since 1992. The rationale of this

approach relies on the possibility to perform a prenatal diagnosis and to treat the mother in case of seroconversion. Intuitively, this approach is pertinent when the seroprevalence is relatively high, and therefore also the probability of infection during pregnancy. Conversely, in low prevalence countries this approach of a public health policy might not be cost-effective. Therefore, the cost-benefit ratio should be carefully evaluated before the implementation of such screening measures. Other countries have also a screening policy (Austria, Belgium, Italy, Lithuania, Slovenia) (Benard et al. 2008), but the frequency of serologic testing may vary from one to three monthly testings of pregnant women. Some countries do not recommend this screening (United Kingdom, Norway, Finland and more recently Switzerland), arguing either for a too high cost or maternal stress in case of diagnosing a seroconversion or even fetal risk associated with amniocentesis. In France, a trend towards a regular decrease in seroprevalence has been observed since the 1970s, and continues through the three most recent national perinatal surveys in 1995 (55%), 2003 (44%) and 2010 (37%), thus questioning the cost of maintaining a full screening of seronegative women.

Postnatal screening of neonates at risk is another option, which has been implemented in some countries where prenatal screening was not considered to be a health priority, as is the case in Sweden, Denmark, Poland or USA (Massachussets, New England). It allows the treatment of infected neonates in order to reduce the development of eye or neurologic sequelae. The cost of such screening has been also debated in Denmark, where it was estimated that the low burden of disease (1.6 per 10,000 live-born infants) did not justify continuing neonatal screening (Roser et al. 2010).

As for congenital toxoplasmosis, there is no consensus about serologic screening in immunocompromised patients, yet an annual serological testing is usually recommended in HIV-infected patients previously seronegative for *Toxoplasma*. Prevention messages on how to avoid infection should also be the rule, as it is done for pregnant women. Chemoprophylaxis is recommended when the $CD4^+$ T cell count is below 200/μL.

In transplant patients, the decision for *Toxoplasma* antibody screening varies highly among countries and is again mostly explained by the differences in the incidence or prevalence of toxoplasmosis. A recent case-control study (Fernandez-Sabe et al. 2012) showed that primary infection observed in solid organ transplant patients was due to a mismatch D+/R− in about half of the patients, emphasizing the need for primary prevention, through both donor/recipient screening and hygienic measures. In France, serological screening of the organ donor is mandatory and is strongly recommended (in practice, always done) in the recipient, whereas in the USA serologic screening of donors and recipients greatly depends on the transplant center. Serological screening of the donor is also routinely performed in 11 European countries (Derouin and Pelloux 2008). The knowledge of both recipient and donor serological status allows starting primary chemoprophylaxis at the time of transplantation in case of a mismatch, particularly for heart transplant patients. More detailed information on the risk and prevention can be found in a recent review (Robert-Gangneux and Darde 2012).

14.5.2 Impact of Prevention and Screening on the Burden of Disease: Congenital Toxoplasmosis

A recent French retrospective study by Wallon et al. (2013) estimated the incidence of congenital transmission and the proportion of severely impaired infected neonates before and after onset of the French screening policy associated with prevention measures and maternal treatment, i.e. before and after 1992, respectively. They clearly found a significant impact of monthly mandatory retesting of seronegative women on the transmission rate of congenital infection, as well as an impact of the treatment strategy adapted to prenatal diagnosis. The continuous treatment of women with positive prenatal diagnosis with pyrimethamine-sulfadiazine significantly reduced the severity of clinical signs in infected children.

In the United States, several longitudinal clinical studies showed that newborn postnatal screening and treatment was associated with better neurologic and developmental outcome (Guerina et al. 1994; Roizen et al. 1995; McLeod et al. 2006). By contrast, in ancient series, untreated infants, although asymptomatic at birth, developed high rates of ocular lesions or neurologic sequelae or suffered from recurrent episodes of retinochoroiditis (72 %), despite spot treatment at the time of diagnosis and at each recurrent lesion (Phan et al. 2008). More recently, Stillwaggon et al. (2011) evaluated the societal cost of congenital toxoplasmosis and concluded that implementation of a similar prevention program as that currently applied in France (maternal serological screening, prenatal diagnosis and treatment) would be cost-saving at the scale of the USA, even if the seroprevalence is low, with an infection rate of 1 per 10,000 live births. However, the pattern of infection in North America could be more harmful than previously suspected, probably due to the genotypes of circulating strains. This study estimated the cost of mild vision loss and of severe toxoplasmosis at a rate of US$ 500,000 and US$ 2.7 million , respectively.

With the discovery of type non-II strains circulating in North America and being more virulent than those responsible for toxoplasmosis in Europe (McLeod et al. 2012) it has recently been hypothesized that the disease burden in the USA could be due not only to the absence of prenatal management or postnatal treatment. However, the important preventive role of health care policies in the decrease of the burden of toxoplasmosis in France cannot be denied.

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Part II
Zoonoses in food-chain and domestic animals: Focus on antibiotic resistance

Chapter 15
Extended-Spectrum β-Lactamase and AmpC β -Lactamase-Producing Bacteria in Livestock Animals

ESBLs in Livestock—How Big is the Piece in the Complex Puzzle on Antimicrobial Resistance?

Christa Ewers

Abstract In the past two decades we have faced a rapid increase in human infections caused by 3rd-generation cephalosporin resistant bacteria, e.g. *Escherichia coli*, *Klebsiella pneumoniae* and Salmonella, mainly due to the plasmid-encoded production of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases. The primary reservoirs of ESBL/AmpC-producing bacteria are still contentious. However, questions have been raised about the possible role of livestock animal- and food-related reservoirs in the spread of such bacteria, particularly *E. coli* and Salmonella. This is mainly due to the fact that the intense use of antibiotics in this sector, but also in the medical field, undoubtedly contributes to the global rise of antimicrobial resistant bacteria. Entry of multidrug-resistant microorganisms early in the livestock production cycle, their frequent occurrence in healthy animals and food products, together with the finding of common molecular characteristics suggests a livestock animal-to-human transmission. However, links between ESBL/AmpC-producing bacteria from these two sources have been drawn mainly from observational epidemiological data while studies providing unquestionable proofs for transfer directionality and quantifying the risk for human health are limited. In any case, food animal production should be regarded a major player in the expansion of the global bacterial resistome. Therefore, efforts need to be directed towards reducing reliance on antimicrobials in this sector wherever possible. As many field, e.g. medicine, veterinary medicine, animal husbandry, environment and trade, are involved in the complex issue of antimicrobial resistance, isolated, sectorial efforts in the food animal production field will not be that efficient unless concerted efforts from all those involved are applied on a global scale.

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15.1 Resistance Mechanisms in Broad-Spectrum Cephalosporin-Resistant Gram-Negative Bacteria

Beta-lactams are the most widely used antibiotics in clinical practice over the world (Galan et al. 2013) and strong selective pressures upon them have resulted in a continuous increase of antimicrobial resistant isolates (Pitout and Laupland 2008). The β-lactams interfere with the metabolism of the bacterial cell wall by mimicking and thereby inactivating one of the building blocks, i.e. penicillin-binding proteins, used by enzymes to construct peptidoglycan (Prescott 2000). Resistance to β-lactams can be due to mutations in the penicillin-binding proteins, a reduced permeability of the cell wall, and the production of β-lactamase enzymes able to hydrolyze and inactivate the β-lactam-ring, which is by far the most common mechanism in *Enterobacteriaceae*. Penicillin was one of the first β-lactams developed for clinical use in humans, and as early as 1940, β-lactamase activity was described as a penicillin-inactivating mechanism that threatened the use of this critical class of β-lactam antibiotics (Bush and Fisher 2011). With the introduction of new β-lactams (e.g. the penicillin derivatives methicillin and oxacillin; 1st–4th-generation cephalosporins, and the carbapenems such as imipenem and meropenem) with improved activity on specific bacteria and increasing stability to hydrolysis, new β-lactamases emerged and are continuously evolving with the ability to hydrolyze the β-lactam bond in almost all β-lactam-containing molecules (Bush and Fisher 2011). To date >1000 β-lactams, grouped into the four Ambler classes A-D based upon amino acid sequence homology have been described. Class A and C β-lactamases are the most commonly found in *Enterobacteriaceae* and are mainly represented by Extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases (in this chapter referred to as "AmpCs"), respectively (Bush and Fisher 2011; Smet et al. 2010).

Enterobacteriaceae producing ESBLs are resistant to 3rd and 4th-generation cephalosporins and to aztreonam, while they are usually inhibited by β-lactamase-inhibitors, such as clavulanate, sulbactam and tazobactam. They also do not hydrolyze cephamycins and carbapenems. While early ESBLs mainly evolved from natural narrow-spectrum TEM-1/TEM-2- (named after a Greek patient "Temoneira") and SHV-1 ("Sulfhydryl reagent variable")- β-lactamases, CTX-M (Cefotaximase Munich)-type enzymes, all of which reveal an ESBL phenotype, succeeded over the last two decades and currently represent the most common ESBLs (Pitout 2008; Livermore et al. 2007; Canton et al. 2008; Bush and Jacoby 2010). Other groups, such as OXA-, PER- and VEB- β-lactamases have been described more recently and are found only sporadically (Coque et al. 2008). The genes that encode CTX-M-type ESBLs have once been mobilized from the chromosomes of environmental *Klyuvera* spp. and nowadays are predominantly encoded on transferable plasmids in both, clinical relevant and commensal bacteria (Livermore et al. 2007). Currently, members of the *Enterobacteriaceae* family are the most often encountered bacteria possessing ESBL/AmpC enzymes. After their initial observation in humans in the 1980s, ESBL/AmpC-producing *Enterobacteriaceae* emerged in companion animals more than a decade later and shortly after that in food-producing animals as well as in wildlife and various environmental sources (Smet et al. 2010; Guenther

et al. 2011; Ewers et al. 2011). The rapid and extensive dissemination of CTX-M-type ESBLs in medical and veterinary settings, and in the environment is regarded as one of the most successful histories of antimicrobial resistance observed in the antibiotic era, probably driven by one or a combination of the following factors: (i) efficient capture and spread of $bla_{\text{CTX-M}}$ genes by mobile genetic elements, (ii) association of these mobile elements with highly successful bacterial genotypes; (iii) extensive use of extended-spectrum cephalosporins and other antimicrobials which can co-select ESBL-producing strains resulting in high selective pressure (D'Andrea et al. 2013).

AmpC- β-lactamases historically were chromosomal cephalosporinases *in Pseudomonas aeruginosa* and many *Enterobacteriaceae* (Bush and Jacoby 2010; Munier et al. 2010). Since the 1980s, a growing number of genes for AmpC enzymes have escaped from the chromosome of different Gram-negative bacteria onto transmissible plasmids. These "acquired" or "plasmidic" AmpCs (pAmpCs) consequently can now appear in bacteria usually lacking or poorly expressing chromosomal bla_{AmpC} genes, such as *E. coli* or *K. pneumoniae*. Although many Gram-negative bacteria produce a chromosomal β-lactamase (cAmpC), it is the transferable pAmpC that is responsible for most of the multidrug resistance observed in Gram-negative isolates (Bush and Fisher 2011). AmpC β-lactamases mediate resistance to most penicillins, 1st–3rd-generation cephalosporins, cephamycins, and inhibitor- β-lactam combinations (e.g. amoxicillin/clavulanate), but usually not to 4th-generation cephalosporins and carbapenems (Jacoby 2009). According to their original chromosomal producers they once developed from, AmpCs can be divided into five clusters: the *Citrobacter freundii* cluster, represented by CMY-2, the *Enterobacter* cluster with MIR-1 and ACT-1, the *Morganella morganii* group with DHA-1, the *Hafnia alvei* cluster represented by ACC-1, and the *Aeromonas* cluster with MOX-1 (also called CMY-1) and FOX-1 enzymes. Plasmid-encoded *ampC* genes have been known since 1989 when CMY-1 (named after its primary substrate cephamycin) was described in *K. pneumoniae* isolates (Bauernfeind et al. 1989), while currently the most common pAmpC β-lactamase worldwide is CMY-2 (Jacoby 2009). Although to date resistance due to pAmpCs is less common than ESBL production in most parts of the world, it may be (i) more difficult to detect in the clinical laboratory, probably underestimating its occurrence, and (ii) broader in substrate spectrum, probably resulting in significant therapeutic failure (Jacoby 2009).

Broad-spectrum cephalosporins have long been the treatment of choice for serious infections with enterobacterial pathogens. Since ESBL/AmpC-producing bacteria rapidly increased in the past decade, other critically important antimicrobials had to be used alternatively (Pitout and Laupland 2008). However, mobile genetic elements, such as plasmids and transposons, frequently encode not only ESBL/AmpC genes but in addition genes coding for resistance e.g. to aminoglycosides, quinolones, and sulfonamides. Thus, the way was paved for the wide spread of bacteria with broad-spectrum cephalosporin resistance in both hospital settings and the community. As a result, since a couple of years clinicians are increasingly forced to turn to carbapenems (e.g. imipemen and meropenem) to treat infections caused by multidrug resistant bacteria. It may not be surprising that this has already

led to the selection of acquired carbapenemases (e.g. as KPC (*K. pneumoniae* carbapenemase), OXA [Oxacilliniase]-48-like, and NDM-1 [New Delhi metallo-beta-lactamase-1]), one of the most pressing public health threats relating to antibiotic resistance, again first in humans and later and still only incidentally in companion and livestock animals (Nordmann 2013; Fischer et al. 2012, 2013; Stolle et al. 2013; Poirel et al. 2012).

15.2 Livestock Animals and Food as Source of ESBL/AmpC-Producing Bacteria

The primary reservoirs of ESBL/AmpC-producing *Enterobacteriaceae* are still controversial. Several reports described the occurrence of these bacteria in healthy humans (Overdevest et al. 2011; Pitout et al. 2009; Valverde et al. 2004), companion animals (Ewers et al. 2010, 2011; Dierikx et al. 2012; Pomba et al. 2014), wild animals (Literak et al. 2010; Guenther et al. 2012) and the environment (da Costa et al. 2013; Mesa et al. 2006). Since the late 1990s, ESBLs/AmpCs have been detected in poultry (Girlich et al. 2007; Dierikx et al. 2010; Hasman et al. 2005; Kojima et al. 2005; Brinas et al. 2003), pigs (Riano et al. 2006; Tian et al. 2009; Agerso et al. 2012), cattle (Reist et al. 2013; Shiraki et al. 2004; Wieler et al. 2011; Gupta et al. 2003; Frye et al. 2005; Madec et al. 2012) and retail meat (Agerso et al. 2012; Cohen Stuart et al. 2012; Doi et al. 2010; Geser et al. 2012b; Jensen et al. 2006; Kola et al. 2012; Bergenholtz et al. 2009; Jouini et al. 2007) almost globally, and this is why several studies raised questions in particular about the possible role of livestock animal- and food-related reservoirs in the spread of ESBL/AmpC-producing bacteria (Smet et al. 2010; Canton et al. 2008; Ewers et al. 2012; Carattoli 2008; Seiffert et al. 2013; Hunter et al. 2010). Notably, healthy animals might not only carry multidrug-resistant commensal, i.e. non-pathogenic microorganisms, but may particularly be a source for primary or secondary pathogens leading to various diseases (e.g. gastroenteritis, urinary tract infection, wound infection, bacteremia, and septicemia) in healthy individuals or people with weakened immune system (Smet et al. 2010; Canton et al. 2008). One of the earliest descriptions of poultry as carriers of ESBLs and AmpCs was by Brinas et al. (2003), who recovered CTX-M-14-, SHV-12- and CMY-2 β-lactamases in *E. coli* from the feces of healthy chickens in Spain in 2000 (Brinas et al. 2003). After that, studies from numerous countries followed and reported about the frequent presence of different types of ESBLs (e.g. CTX-M-1, -M-2, -M-9, -M-14; SHV-2, and -12; TEM-52 and -106) and AmpCs (e.g. CMY-2 and ACC-1) in *E. coli* and less frequently in Salmonella isolates from poultry (Smet et al. 2008, 2010; Girlich et al. 2007; Ewers et al. 2012; Randall et al. 2006). First indications for the presence of ESBLs/AmpCs in healthy pigs came from Spain, where an SHV-12-producing Salmonella isolate was identified in 1999 (Riano et al. 2006), and from the United States, where researchers detected CMY-2-producing *E. coli* isolates during the years 1998 and 1999 (Winokur et al. 2000). Nearly within the same time-frame, initial reports were published from the first

findings of AmpC-producing Salmonella and CTX-M-2-producing *E. coli* isolates in healthy cattle in the United States (Gupta et al. 2003) and Japan (Shiraki et al. 2004), respectively. Likewise, ESBL/AmpC-producing bacteria were recovered from sick animals, animal manure and the farm environment, indicating additional sources (Smet et al. 2010; Mesa et al. 2006; Ewers et al. 2012; Friese et al. 2013; EFSA 2011; Carattoli 2008).

Total prevalences of ESBL/AmpC-producing bacteria in healthy animals of the major livestock species, i.e. poultry, pigs and cattle, vary largely with study type. Nevertheless, poultry production is currently suspected the major contributor to the selection and spread of 3rd-generation cephalosporin resistant bacteria. Here, percentages of ESBLs (mainly targeted in the cited studies) and/or AmpCs range from 0.1 to 6.9 % for *Salmonella* spp. (Smet et al. 2008, 2010; Ewers et al. 2012; Randall et al. 2006; Allen and Poppe 2002; Taguchi et al. 2006) and from 1.7 to 63.4 % in *E. coli* isolates (Smet et al. 2008, 2010; Girlich et al. 2007; Kojima et al. 2005; Riano et al. 2006; Geser et al. 2012b; Ewers et al. 2012) mainly originating from broilers, but also from turkeys in different European and Asian countries and in North America. Rates of *Salmonella* and *E. coli* isolates producing ESBLs/AmpCs described for pigs are between 0.2 and 4.6 % and between 0.7 and 29.8 % (Smet et al. 2010; Riano et al. 2006; Ewers et al. 2012; Blanc et al. 2006; Wu et al. 2008), those for cattle range from 0.6 to 2.4 % and from 0.7 to 35.4 %, respectively (Smet et al. 2010; Reist et al. 2013; Wieler et al. 2011; Frye et al. 2000; Ewers et al. 2012; Hunter et al. 2010; Gupta et al. 2003; Geser et al. 2011; Snow et al. 2012). The overall heterogeneity of these global data may not simply refer to differences between animal species or national/local variations in pathogen prevalence and susceptibility patterns or to global differences in antimicrobial use. It could be also due to different study designs, i.e. with respect to inclusion criteria (e.g. animal age, production system, previous antimicrobial treatment) and microbiological procedures (e.g. usage of selective or non-selective cultivation media; sample enrichment). However, it was commonly agreed that livestock should be regarded as major player in the maintenance and/or expansion of the global bacterial resistome with a noticeable trend towards a more frequent colonization with ESBL/AmpC-producing bacteria of poultry compared to pigs and cattle (in rank).

Several countries have implemented antimicrobial resistance monitoring systems to provide an estimation of whether the level of resistance has increased or decreased over time and to measure the impact of specific interventions or programs implemented at the national level (MARAN 2013; SVARM 2011; GERMAP 2012; NARMS 2010; DANMAP 2011). Since 2004, Member States of the European Union are required to monitor and report antimicrobial resistance data on Salmonella and Campylobacter on mandatory basis and on indicator bacteria, i.e. commensal *E. coli* and enterococci, on voluntary basis from food producing animals (at farm or slaughterhouse level) and food thereof. Under the framework of Directive 2003/99/EC the European Food Safety Authority (EFSA) collects, analyses and publishes these data in a yearly summary report (EFSA 2013). In 2011, resistance to the 3rd-generation cephalosporin cefotaxime, which is almost indicative of the presence of ESBLs, was observed in Salmonella isolates from *Gallus gallus*, turkeys, pigs,

cattle and meat derived from broilers, at very low or low levels varying between 0 and 3 %, as well as in indicator *E. coli* isolates from *Gallus gallus*, pigs and cattle at levels ranging from < 1 to 6.4 % with variations between countries (EFSA 2013). The median occurrence of resistance to 3rd-generation cephalosporins was very similar to the figures obtained in 2010, indicating a steady trend in the dissemination of ESBLs/AmpCs in the European Union. It appears that *E. coli* is the major source of β-lactamase resistance, which is less frequently found in Salmonella isolates. Overall, lower resistance rates observed in these monitoring studies compared with research studies are likely due to the fact that most reporting countries did not use selective primary isolation media containing cephalosporins, resulting in a lower sensitivity to detect ESBL/AmpC producers. To ensure comparability of official monitoring data across the European Union, the EFSA recently provided technical specifications on the harmonized monitoring (e.g. usage of selective primary isolation media) and reporting (e.g. usage of epidemiological cut offs instead of clinical breakpoints) of antimicrobial resistance (EFSA 2012) . Despite all these obscurities, the percentages of livestock animals colonized with 3rd-generation cephalosporin resistant and/or ESBL/AmpC-producing *E. coli* mostly exceed what has been determined for human healthy carriers so far. While initially, human fecal carriage of predominantly ESBL-producing isolates has been mainly reported in nosocomial outbreaks, these isolates passed into the community almost in the mid-2000s (Canton et al. 2008). Proportions of humans colonized with ESBL-producing bacteria range from 0.6 to 18.2 % in various geographical settings between 2001 and 2011 (Ewers et al. 2012; Seiffert et al. 2013; Geser et al. 2012a; Nicolas-Chanoine et al. 2013), emphasizing the importance of the community reservoir in the evolution and dynamics of ESBL-producing pathogens.

The contribution of food sources to the burden of antimicrobial resistance in humans is another controversial issue in the global crisis of antimicrobial resistance. Contamination of meat products with resistant bacteria may not only contribute to a theoretical spread of these organisms within the human population but also to a rapid transfer of resistance genes from foodborne commensals to human pathogens (da Costa et al. 2013). People could ingest ESBL/AmpC-producing bacteria by consuming the contaminated food directly or through cross-contamination on non-cooked foods. Indeed, the presence of ESBL/AmpC-producing bacteria on retail meat has been documented repeatedly (Hasman et al. 2005; Doi et al. 2010; Geser et al. 2012b; Bergenholtz et al. 2009; Egea et al. 2012). Mainly following selective enrichment procedures, rates of ESBLs/AmpCs detected in beef and pork meat ranged from 0 to 21.7 % (Agerso et al. 2012; Geser et al. 2012b; Jensen et al. 2006; Ewers et al. 2012; Carattoli 2008; Lavilla et al. 2008). Much higher proportions, almost ranging between 30 and 100 % have been reported in several studies performed on raw poultry meat in the recent couple of years worldwide (Overdevest et al. 2011; Hasman et al. 2005; Cohen Stuart et al. 2012; Doi et al. 2010; Kola et al. 2012; Jouini et al. 2007; Ewers et al. 2012; Randall et al. 2011; Egea et al. 2012; Leverstein-van Hall et al. 2011; Kluytmans et al. 2013); lower rates have been documented only exceptionally (Bergenholtz et al. 2009); Warren et al.

2008). Notably, differences in ESBL/AmpC contamination of conventional versus organic (restricted antimicrobial use in animal rearing) poultry meat are not as apparent as probably expected, which was shown in studies from Germany (43.9 vs. 36%) (Kola et al. 2012) and from the Netherlands (100 vs. 84%) (Cohen Stuart et al. 2012). The finding of ESBL-producing *E. coli* (3%) in flocks of chicken egg layers reared in Danish organic systems could indicate that ESBL-colonized 1-day-old chicks were introduced into organic farms or that cross-contamination between conventional and organic flocks during rearing or slaughtering or through an ESBL-contaminated environment accounted for the colonization of animals (Cohen Stuart et al. 2012; Kola et al. 2012). Colonization of 1-day-old chicks and farm environmental contamination with ESBL-producing bacteria has indeed been demonstrated previously (Bortolaia et al. 2010; Laube et al. 2013). The industrial production of broiler meat is the final or bottom level of a 4-step pyramid, below the parent, grandparent, and primary breeder steps and few reports suggest that ESBL/AmpC-producing bacteria are not uncommon in the top of some production pyramids. For instance, in Sweden, transmission of such bacteria from imported breeding chickens was documented by findings of *E. coli* carrying respective resistance genes in environmental samples from hatcheries rearing production animals or breeding stock (parent animals) (SVARM 2011). Obviously, ESBL/AmpC-producing *E. coli* have also been introduced in the Dutch poultry production chain through imported day-old grandparent chickens and the occurrence of these bacteria in the different levels of layers are likely attributed to vertical transmission (MARAN 2013). Another important criteria is age-related and production cycle-dependent colonization of animals. In a longitudinal study from Denmark a significant decrease in the carriage partly of identical ESBL-producing *E. coli* was detected from piglets to weaners and finishers (Hansen et al. 2013). A reverse relationship between the prevalence of antimicrobial resistant bacteria in the intestinal microbiota and animal age has been demonstrated at dairy farms as well, which has been attributed either to a higher fitness of the resistant strains in young calves or selection pressure due to the feeding of waste milk that may contain antimicrobial residues (Geser et al. 2012b; Hordijk et al. 2013). Such findings emphasize that knowledge about the epidemiology of ESBLs/AmpC at the farm level may be of great value for the design of surveillance and intervention studies. With respect to a possible meat contamination during slaughter, recent studies, acknowledging that not the mere presence of ESBL/AmpC-producing bacteria animals, but rather the shedding density may greatly influence the risk of food (and environmental) contamination are of particular interest. In a study from the United Kingdom the proportion of animals, designated high-density shedders ($\geq 1 \times 10^4$ CFU/g) of CTX-M *E. coli* was 8.6, 40.0, and 46.9% from cattle, pigs, and chickens, respectively (Horton et al. 2011). This is in analogy to what has been deduced from investigations on the linkage between super-shedding of enterohaemorrhagic *E. coli* O157 strain and human infection (Chase-Topping et al. 2008). Thus, at the individual animal level, chicken and pigs may pose the major risk of food contamination although the similarity of the median values from each of the animal groups suggested that there is a comparable risk of contamination from these sources (Horton et al. 2011).

Irrespective of the origin, i.e. associated with fecal carriage or with food contamination, CTX-M-1 is by far the most frequent ESBL type identified in all major groups of livestock animals in Europe, followed by CTX-M-14 (most prevalent type in Asia), TEM-52, and SHV-12. Other ESBL types frequently isolated include variants belonging to the CTX-M (-2, -3, -8, -9, -15, -32), SHV (-2, -5) and TEM (-106) families (Ewers et al. 2012; Seiffert et al. 2013). The type of AmpC β-lactamase detected was almost always the CMY-2 variant. ESBL producers have been mostly found in Europe, whereas AmpC producers are particularly common in North America and Asia, mirroring the trends for human isolates in these regions (Liebana et al. 2013). However, CMY-2 is also one of the major β-lactamase isolated from poultry in Europe (Brinas et al. 2003; Doi et al. 2010; Kola et al. 2012; Smet et al. 2008; Blanc et al. 2006; SVARM 2011; MARAN 2009). Different ESBL/AmpC types are linked with distinct plasmid incompatibility groups and these plasmid scaffolds are important factors in understanding the spread of antimicrobial resistant bacteria across the different habitats. For instance, the $bla_{\text{CTX-M-1}}$ gene is frequently carried by IncN, IncFII, IncFIB, and IncI1 plasmids and those plasmids have been observed in human samples as well (EFSA 2013; Carattoli 2013). The most frequent ESBL types in humans worldwide are CTX-M-15 and CTX-M-14 and the $bla_{\text{CTX-M-15}}$ gene has spread in a pandemic fashion, mainly driven by IncF plasmids (Livermore et al. 2007; Nicolas-Chanoine et al. 2008). While CTX-M-14 is also prevalent in poultry and cattle in Asia, suggesting possible transmission scenarios in the respective area, it is rather interesting to note that the most common ESBL type in humans, namely CTX-M-15, has a very low impact in livestock animals. Only few studies detected this enzyme type in bacteria from cattle (Wieler et al. 2011; Madec et al. 2012; Watson et al. 2012), including the CTX-M-15-producing enterohemorrhagic *E. coli* O104:H4 responsible for a recent outbreak of Shiga toxin-producing *E. coli* infection in Germany (Ewers et al. 2011). Incidental findings have been reported from poultry (Randall et al. 2006; Smet et al. 2008) and pigs (Tian et al. 2009; Agerso et al. 2012). On the other hand, the wide dissemination of CTX-M-15 in companion animals (Ewers et al. 2010) clearly shows that other transmission scenarios have to be considered beyond that linked to livestock animals.

The frequent carriage of ESBL/AmpC-producing bacteria by healthy cattle, pigs or poultry and high prevalence of these organisms in food products indicates that food-producing animals may be the origin of at least part of the human infections. Knowledge on the genetic makeup and epidemiology of plasmid and bacterial host is the minimum necessary for further assessing the food-borne risk and may also be valuable for source attribution. It is generally accepted that thorough cooking destroys bacteria in food, while cross-contamination to uncooked food may occur in case of inadequate hygiene measures, and these are important lessons from food-borne outbreaks due to Salmonella, Campylobacter and enterohaemorrhagic *E. coli* (EHEC) bacteria. As we currently cannot determine the magnitude of the mode of transmission of ESBL/AmpC-producing bacteria via the food chain, this theoretical hazard to human health requires further assessment. It may be desirable, that foods should be subject to a monitoring program for antimicrobial resistant bacteria, as

is already done for intestinal pathogenic bacteria. However, potential consequences would very much depend on an estimation of the true risk of food products on the transmission of ESBL/AmpC-producing *Enterobacteriaceae* of zoonotic relevance.

15.3 Antimicrobial Use and Its Impact on the Emergence of ESBL/AmpC-Producing Bacteria in Livestock Animals

Any use of antimicrobial agents, whether in humans, animals and plants, promotes the selection and dissemination of antimicrobial resistant bacteria, regardless if they are pathogens, commensals or environmental strains. This greatly influences the population structure of microbial communities resulting in unpredictable consequences for public health (Pitout and Laupland 2008; EFSA 2013). Even though this is widely accepted, the evidence linking antibiotic use in food-producing animals with resistance in humans is a very controversially discussed issue raised by professional and governmental organizations. Although the dogma is that resistance genes have mainly evolved as a result of the recent use and misuse of antibiotics, we should also consider the previous notion of the ancient origin of antimicrobial resistance, implying that we all basically live in a surrounding filled with antimicrobial resistance genes and their precursors, the so-called resistome (Rolain et al. 2012; D'Costa et al. 2011; Wright 2007). Historically, the assessment of biological consequences due to antibiotic use in food animals was limited to zoonotic intestinal pathogens, such as *Salmonella* spp. and *Campylobacter jejuni*. There was evidence that due to the agricultural use of antibiotics these bacterial pathogens may develop resistance to antimicrobial substances approved for the treatment of humans (Collignon 2005). For instance, the sharp increase in the prevalence of ciprofloxacin resistance among *C. jejuni* isolates from human infections in the United States was associated with the approval of fluoroquinolone-analog enrofloxacin for poultry production in 1990 and led to the ban of all use of this class of drug in chickens at the end of the twentieth century (Collignon 2005; Smith et al. 1999). Remarkably, Australia faces a relative low rate of fluoroquinolone resistance in clinical *C. jejuni* isolates and this is most likely attributed to the fact that this drug was never used in animal production (Unicomb et al. 2006).

The use of antimicrobials can differ in humans and food-producing animals in terms of methods of administration and quantities administered. There are also important variations between and within food-producing animal species, as well as between countries (EFSA 2013; Guardabassi and Kruse 2008). In humans, antibiotics are administered individually to patients for therapeutic purposes, i.e. to treat infection, whereas prophylactic use of drugs to prevent the emergence of infections is the exception. This is quite comparable to what we encounter in small-animal and equine veterinary medicine. In contrast, in industrial farming, antimicrobials are commonly given to a whole group of animals (flocks or pens) to treat infections

at the anticipated onset of disease in a population, as evidenced by the occurrence of clinical signs in individual animals of these groups (metaphylaxis) (Guardabassi and Kruse 2008). Another practice still common in some countries is the use of antimicrobials to spur animal growth and to improve feed efficiency (Gilbert 2012). Likewise depending on national legislations, antimicrobials may also be administered in feed or in drinking water for prolonged periods of time at sub-therapeutic concentrations without the need for veterinary prescription (Aarestrup 2005). Needless to say that this is one of the most intensively discussed issues in terms of antimicrobial resistance. Since 1997, the WHO, together with the Food and Agriculture Organization (FAO) and the World Animal Health Organization (OIE), has consistently recommended restrictions on non-therapeutic uses of antimicrobials in food animals, which resulted in several countries adapting their legislation over time (Silbergeld et al. 2008; Anonymous 2007). The European Union banned most growth promoters (e.g. avoparcin, tylosin phophate, bacitracin zinc, virginiamycin, and spiramycin) at the end of the 1990s because their in-feed application has been associated with the selection of resistance to clinically important antimicrobials in human medicine (Aarestrup et al. 2001). For example, in the Netherlands, 14 % of people living near turkey farms where the growth-promoter avoparcin was used were found to carry Vancomycin-resistant Enterococci (VRE), which are frequent causes of nosocomial infections (Stobberingh et al. 1999). Soon after the remaining growth promoters have been phased out in Europe from January 2006, some types of antimicrobial resistances were indeed substantially reduced and the economic effect on animal health and productivity was found to have been minor (Aarestrup et al. 2001). Obviously, the supposed benefits of growth promoters could alternatively be achieved by improving hygiene, management conditions, and other measures aiming at disease control, such as biosecurity and vaccination (Guardabassi and Kruse 2008). Unfortunately, the United States and other Countries, including Japan and Australia, have not introduced similar restrictions and the practice of feeding growth promoters without veterinary prescription remains widespread on a global scale (Löhren et al. 2008).

Worldwide there are apparent differences in relation to regulation, dispensation and use of veterinary antimicrobials (Valois et al. 2008). Nowadays a wide range of antimicrobial agents are permitted for use in food animal production and these are frequently the same, or belong to the same classes, as those used in the human medicine (EFSA 2013). Due to public health concerns, a much greater scrutiny is now focused on therapeutic use of antimicrobials in food-producing animals, particularly for substances that have analogues in human medicine (Collignon et al. 2009), in particular if these are ranked as “critical important”. To be classified in this category, an antimicrobial agent must serve the following criteria: the antibiotic is used (i) as sole therapy or one of few alternatives to treat human diseases, and (ii) to treat diseases caused by either organisms that may be transmitted via non-human sources or diseases caused by organisms that may acquire resistance genes from non-human sources (WHO 2009). Some antibiotic classes, including 3rd and 4th-generation cephalosporins, quinolones, and aminoglycosides, are defined as “critically important” for both human and animal health, by WHO and OIE respectively.

This raises a particular concern in the prioritized use of these antimicrobials in the veterinary area to assure an appropriate balance between animal health needs and public health considerations (Anonymous 2007). Systematic reviews and guidelines about β-lactams currently licensed for use in food-producing animals have been published previously (Smet et al. 2010; Liebana et al. 2013; Löhren et al. 2008; Burch et al. 2008; Constable et al. 2008). Due to a very broad spectrum, short or zero withdrawal times for milk and the availability of 'long acting' formulations for certain indications ceftiofur and cefquinom are commonly used in veterinary medicine where they are authorized for the treatment of various diseases caused by defined pathogens in cattle and pigs (Liebana et al. 2013; Burch et al. 2008; Constable et al. 2008). The previous authorization of ceftiofur for injection of day-old chickens for prevention of septicemia in some Member States of the EU has been phased out and currently no cephalosporin-containing products are licensed for poultry species in the EU and in many other countries. In Canada, a strong correlation between a reduction in ceftiofur-resistant *Salmonella* Heidelberg and *E. coli* (both producing AmpC) from human infections and retail poultry and withdrawal of ceftiofur use for disease prophylaxis in hatcheries has been reported (Dutil et al. 2010). Usage of ceftiofur and cefquinom and probably also environmental contamination due to the excretion of their metabolites mainly with the urine, may have influenced the emergence of acquired AmpCs and ESBLs in both Gram-negative pathogens and commensals in livestock animals (Aarestrup 2005; Subbiah et al. 2012; Tragesser et al. 2006). In addition, most ESBL/AmpC-producing bacteria carry further resistances to commonly used veterinary drugs, e.g. amoxicillin, sulfonamides, trimethoprim, fluoroquinolones, and aminoglycosides, such that dropping the use of cephalosporins may be only one out of several necessary steps in reducing antimicrobial resistance (EFSA 2013; Silbergeld et al. 2008).

Several experimental and on-farm studies have been published supporting the hypothesis that the veterinary use of β-lactams, but also yet undetermined factors not related to antimicrobial use may select for ESBL/AmpC-producing *Enterobacteriaceae* in animals. There is evidence that parental therapy with ceftiofur significantly increases the likelihood of dairy cows to be colonized with 3rd-generation cephalosporin-resistant *E. coli* (Snow et al. 2012; Tragesser et al. 2006; Volkova et al. 2012). On the other hand, the transfer of a CMY-2-plasmid to *Salmonella* spp. and commensal *E. coli* in cattle was not attributed to ceftiofur treatment (Daniels et al. 2009). Similarly, CTX-M-producing *E. coli* persisted on a dairy farm in the absence of the use of any β-lactam for longer than 6 months (Liebana et al. 2006). Co-selection of 3rd-generation cephalosporin resistance, as evidenced by the isolation of ceftiofur-resistant CMY-2-producing *E. coli* upon administration of florfenicol (used in veterinary medicine only), has been reported recently for French cattle (Meunier et al. 2010). In pigs inoculated intragastrically with an *E. coli* expressing CTX-M-1 an increase in the number of CTX-M-1-producing *E. coli* was highest after administration of cephalosporins and interestingly, this was mainly due to the proliferation of indigenous isolates that probably acquired the ESBL-plasmids via conjugation (Cavaco et al. 2008). The authors pointed out that pigs treated with cephalosporins and sent to slaughterhouses shortly after the end of the withdrawal

time may still shed high numbers of ESBLs (up to 10^6 CFU/g feces), favoring contamination of food products and the environment. An association between the prophylactic ("off-label") use of ceftiofur in 1-day-old piglets for disease prevention and the occurrence of CTX-M-1-producing *E. coli*, which could not be detected on control farms without a recent history of ceftiofur usage, was recently demonstrated (Jorgensen et al. 2007). In a study from Belgium, the administration of amoxicillin in poultry was significantly associated with the emergence of 3rd-generation cephalosporin resistant *E. coli* (Persoons et al. 2011). Likewise, an experimental chicken model clearly illustrated how, in *E. coli*, "old" antimicrobials, e.g. amoxicillin, may co-select antimicrobial resistance to 3rd-generation cephalosporins by favoring resistance plasmid exchange (Dheilly et al. 2012). However, beyond antimicrobials the authors identified poor hygienic condition, lack of acidification of drinking water, repeated feed changes during the production cycle, and hatchery of origin as additional risk factors possibly promoting the spread of resistant bacteria in poultry.

Although clear evidence has been provided for a linkage between antimicrobial use and selection of 3rd-generation cephalosporin resistant organisms in livestock animals, banishing the therapeutic use of antimicrobial agents in these animals is surely not a viable solution. With respect to this, various national veterinary organizations have developed general ethical guidelines to encourage a prudent use of antibiotics in line with a "good veterinary practice" (Guardabassi and Kruse 2008). This would for instance include (i) the use of antimicrobials by veterinary prescription and oversight only (which is a standard prerequisite for the use of veterinary medical products containing antimicrobials for food-producing animals in the European Union already), (ii) decision on therapeutic options guided by susceptibility testing of the identified pathogen, and (iii) prioritization of antimicrobial use according to the critical importance of the respective substance for humans, and several other issues aiming to reduce antimicrobial resistance in livestock animals whilst ensuring animal welfare at the same time. The decision on exceptional off-label use of antimicrobials (i.e. use in a different species, for a different disease, or at a dosage different to that on the label) to avoid causing unacceptable suffering should strictly follow a specific cascade (Directive 2011/82/EC). This is also to ensure a restrictive use of substances of critical importance for human health. In several countries off-label use of cephalosporins in food-producing animals is no longer allowed, after it has long been used in the United States and Europe to prevent early mortality due to septicemia in poultry hatcheries or to treat diarrhea or prevent systemic infection in piglets (Seiffert et al. 2013; EFSA 2013).

Some of the above mentioned national guidelines, which are indeed largely voluntary and thus not as binding as legislation, give specific recommendations for the use of cephalosporins. In the "Formularia", which are the Dutch guidelines for restricted use of antibiotics for all major animal species, drugs of major importance to public health are considered third choice drugs for treatment of infections in food-producing animals, except when no alternative therapy is possible as indicated by susceptibility test of the target bacteria (KNMvD 2007). The German guidelines for prudent use of antimicrobials in veterinary medicine recommend a restrictive

use of antimicrobials ranked as critically important for human health in sick individual animals and only in case microbiological identification and susceptibility testing of the target pathogen have been performed (BTK-AGTAM 2010). Other guidelines are even more detailed, providing specific recommendations for particular indications in different animal species. A general recommendation for the use of broad-spectrum cephalosporins in food-producing animals has been stated during an expert meeting of representatives of the FAO, WHO, and OIE in Rome in 2007. Accordingly, the use of these substances should be limited to cases in which the use of narrower-spectrum antimicrobials is not possible due to resistance problems or a proven lack of clinical efficacy by alternative agents (Anonymous 2007). The US Food and Pig and poultry producers in some countries have already introduced voluntary bans on the use of broad-spectrum cephalosporins, in other countries, e.g. in Germany, the use of 3rd- and 4th-generation cephalosporins in poultry is not allowed at all (DANMAP 2011; MARAN 2012; Cooper 2012). In Denmark, the occurrence of ESBL *E. coli* in pigs at slaughter fell from 11.8% in 2010 to 3.6% in 2011, that of pigs tested on farms from 11% in 2010 to 0% in 2011 after the voluntary ban was introduced in July 2010 (Agerso and Aarestrup 2013). Also in the Netherlands some benefits regarding a drop in antimicrobial resistance are seen after the reduction in the use of antimicrobials (>50%) by the veterinarians over the last couple of years (MARAN 2013). Compared with 2009, a steady decrease in resistance in several animal species, including pigs, to antimicrobials of critical importance for human health has been reported (MARAN 2009, 2013). Although this is very encouraging, long-term implications for animal health cannot be foreseen yet. The overall quantity of antibiotics used in the food-producing animal industry is difficult to assess due to a number of confounding factors in the provided data (e.g. use of different technical units, lack of precise information on specific purpose for antibiotic use, clear separation of antibiotic use for food-producing and companion animals) (Seiffert et al. 2013). Specific national surveillance programs to monitor the consumption of antimicrobials and antimicrobial resistance development have been set up by many countries, for instance Denmark (DANMAP 2011), France (ANSES 2011), the Netherlands (MARAN 2013), Norway (NORM-VET 2012), Sweden (SVARM 2011), Germany (GERMAP 2012), and the United States (FDA 2011). In 2009, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), was launched by the European Medicine Agency (EMA) to develop a harmonized approach for the collection and reporting of data on veterinary antimicrobials based on national sales figures and combined with estimations of consumption in at least the major groups of species (poultry, pigs, veal calves other ruminants, pets and fish) (EMA 2013). To reasonably compare data with the human field, identical technical units are warranted. As there are marked differences in daily dosing between the different antimicrobial agents, pharmaceutical forms and animal species, it has been recommended to implement the DDDA (defined daily dose animals; the assumed average maintenance dose per day per kg body weight for the main indication in a specified species) and the DCDA (defined course dose animal) as technical unit for the analysis of ESVAC data across the European

Union. In some countries, automated data collection systems on herd or animal level are already implemented or under development for the prioritized animal species pigs, poultry and cattle, which are currently also addressed in the EFSA reports on occurrence of antimicrobial resistance (MARAN 2013; EMA 2013; Merle et al. 2012; DANMAP 2012).

15.4 Transmission of ESBL/AmpC-Producing Bacteria or Resistance Genes Between Livestock Animals and Humans and Zoonotic Aspects

A number of ESBL/AmpC-producing bacteria, such as *E. coli*, Salmonella, and other Gram-negative bacteria, are not only of clinical and economic impact in human medicine, but can also cause infections in animals and are potent colonizers of the gut of healthy animals at the same time (Smet et al. 2010; Canton et al. 2008). This makes a transmission of these microorganisms, whether they are antimicrobial resistant or not, and also of related antimicrobial resistance genes, indirectly via the food chain and the environment or directly via contact a plausible scenario (Carattoli 2008).

The transmission and spread of ESBLs/AmpCs among different habitats (i.e. animals, humans, and the environment) is mainly driven by mobile genetic elements, such as transposons, insertion sequences, integrons and plasmids, some of which are homologous in isolates from both food-producing animals and humans. Due to an often occurring physical linkage of multiple genetic determinants on the same plasmid, self-conjugative properties and the capability to acquire additional mobile genetic elements (e.g. insertion sequences and transposons) plasmids have been signified as major drivers in the dissemination of ESBLs/AmpCs in Gram-negative bacteria over the past decade (Bush and Fisher 2011; Carattoli 2013; Naseer and Sundsfjord 2011). On the other hand, clonal expansion of distinct bacterial genotypes, as probably best exemplified by the dissemination of pandemic, multidrug-resistant, highly virulent *E. coli* CTX-M-15-O25b:H4-ST131 clone within health care institutions and the community may largely contribute to the public health burden of antimicrobial resistance (Nicolas-Chanoine et al. 2008). Accordingly, investigating outbreaks due to ESBL/AmpC-producing bacteria, tracing the spread of resistance determinants in epidemiologically linked strains, and comparison of strains on a global scale from different sources substantially relies on a combinational methodological approach, considering both, bacterial host and resistance genes-carrying plasmid. An integral part of plasmid epidemiological surveillance is plasmid replicon typing to determine incompatibility groups. Plasmid multilocus sequence typing (pMLST), by which plasmids are assigned to sequence types (STs) (http://pubmlst.org/plasmid/), allow for an even finer resolution e.g. of same plasmid families revealing identical replicon types. The latter is in analogy to the MLST for bacterial genomes (e.g. for *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli/) which also aims at identifying groups of clonally related strains (Seiffert et al. 2013; Carattoli 2011).

Main drivers of the spread of ESBL/AmpC genes in both humans and animals are IncF, IncI, IncN, IncA/C, IncL/M, and IncK plasmid families (Carattoli 2013). Irrespective of the host, several *bla* genes are frequently, although not exclusively, associated with plasmids of distinct Inc groups, such as $bla_{\text{CTX-M-1}}$ with IncN and IncI1, $bla_{\text{CTX-M-14}}$ with IncK and IncF, $bla_{\text{CTX-M-15}}$ with IncF and IncI1, and $bla_{\text{CMY-2}}$ with IncI1 and IncA/C (Seiffert et al. 2013; Carattoli 2009, 2013). The finding of related β-lactamase types and/or plasmid scaffolds among *Enterobacteriaceae* from humans and livestock in the past suggested an interspecies transfer of ESBL/AmpC plasmids, mainly through the food chain, as possible scenario (Overdevest et al. 2011; Doi et al. 2010; Leverstein-van Hall et al. 2011). For instance, genetically related IncI1 plasmids (i.e. with the same plasmid sequence type) carrying the $bla_{\text{CTX-M-1}}$ gene were determined in *E. coli* and Salmonella isolates from humans, food-producing animals and from meat samples in various European countries (Leverstein-van Hall et al. 2011; Carattoli 2011). Similar reports refer to $bla_{\text{CTX-M-1}}$ gene carrying IncN plasmids that are extensively distributed among different livestock animal species, but were also identified in *Enterobacteriaceae* from humans and retail meat (Randall et al. 2006; Bortolaia et al. 2010; Cavaco et al. 2008; Carattoli 2011; Novais et al. 2007; Moodley and Guardabassi 2009). Lavilla et al. (2008) suggested that foods can be a transmission vector for ESBLs, probably from two reservoirs, namely food animals and food handlers. They found that individuals involved in an outbreak of acute gastroenteritis, in addition to the enteropathogenic organism, shared an ESBL-producing *E. coli* and rated this as indirect demonstration of its transmission from a common food source (Lavilla et al. 2008). However, we should be especially careful in overestimating the conclusions from such observational epidemiological studies, since they do not provide any information about the quantity of risk, i.e. how easy humans become carriers as the result of the consumption of meat and to which frequency a possible transfer either of a bacterial strain or of plasmid-related resistance genes occurs. There is also support for the spread of closely related plasmids and identical ESBL/AmpC types among farmers and their animals (e.g. pigs and broilers) (Moodley and Guardabassi 2009; Dierikx et al. 2010, 2013) indicating that direct contact and/or environmental vectors could play a role in the transmission of antimicrobial resistance, which likewise cannot be quantified to date. In an experimental model mimicking the human gut microbiota an *E. coli* strain of poultry origin established itself very well and easily transferred its plasmid (IncI) and *bla* gene (TEM-52) to commensal human *E. coli* even in the absence of antimicrobial selective pressure (Smet et al. 2011). This may not simply demonstrate a theoretical scenario as supported by previous findings that the prevalence of ESBL-producing *E. coli* among people working in meat-processing companies or among farmers was higher than usually recorded for the general population at the given time and region (Geser et al. 2012a; Lavilla et al. 2008; Dierikx et al. 2013). Moreover, in some studies, patients suffering from gastrointestinal disease due to infections with ESBL-producing Salmonella had more contact with food-producing animals than subjects with susceptible isolates (Gupta et al. 2003; Fey et al. 2000).

We need to be clear that the majority of studies performed around the topic of antimicrobial resistance transfer so far did not particular focus on confirming precise transmission paths, but aimed to explore whether bacteria similar at the genetic level occurred in different animal species and humans, indicating possible epidemiological links (Wu et al. 2013). If such links, mainly created on the basis of observational findings, are indeed consistent with transmission through the food chain, remains inconclusive, unless infections with ESBL/AmpC-producing bacteria cannot be clearly traced back to a food-borne source. This in turn, would implicitly assume that (i) *bla*-gene carrying plasmid and bacteria are transmitted jointly through a single event, and (ii) the strain transmitted is capable of causing disease in humans in a given period of time, which for instance has been the case in one of the first foodborne nosocomial outbreaks due to an ESBL-producing (SHV-1, CTX-M-15) *K. pneumoniae* isolate in Spain (Calbo et al. 2011). Interestingly, and this is best known for *E. coli*, isolates supposed to be non-pathogenic for otherwise healthy individuals seem to be more prone to acquire ESBL/AmpC β-lactamase genes than their pathogenic counterparts are. For instance, among Shiga-toxin-producing *E. coli* (STEC), which are responsible mainly of food-borne infections (i.e. bloody diarrhea and hemolytic uremic syndrome) in humans, only negligible numbers have been determined as ESBL producers so far (Ishii et al. 2005; Torpdahl et al. 2013; Dutta et al. 2013). The Shiga-toxin positive O104:H4-CTX-M-15 *E. coli* outbreak strain from 2011 is one prominent example among the few incidental findings worldwide (Brzuszkiewicz et al. 2011). The rare occurrence of such strains is not merely explainable by the fact that human STEC infections are usually not treated with antimicrobials, unless complications occur. Healthy cattle are the main accepted reservoir of STEC (Wieler et al. 2011) and given that any antimicrobial selective pressure would naturally affect the entire bovine intestinal microbiota, one would expect that the finding of ESBL/AmpC-producers would not be almost limited to non-pathogenic commensal strains. However, so far only few ESBL-producing STEC strains have been published from animal sources, i.e. from cattle in France (O111:H8-CTX-M-15) (Valat et al. 2012) and Germany (O145:NM-CTX-M-1) (Ewers et al. 2014) and from a chicken in the Netherlands (O157-CTX-M-2) (Roest et al. 2007).

Concerning extraintestinal pathogenic *E. coli* (ExPEC), highly virulent strains that are basically decipherable by their virulence gene profile and assignment to phylogenetic group and/or multi locus sequence type, are also not among the majority of strains identified from infected and colonized humans and animals (Ewers et al. 2012). The sudden worldwide increase of *E. coli* clone O25:H4-ST131-CTX-M-15 in hospital- and particularly in community-onset infections is a so far unique exception from that (i.e. with respect to the extent of its spread) (Nicolas-Chanoine et al. 2008; Peirano and Pitout 2010). ST131 is part of the highly virulent phylogenetic group B2 and since its first recognition in 2008 it developed to the most dominant ExPEC genotype worldwide (Nicolas-Chanoine et al. 2008). *E. coli* strains of this clonal group are commonly associated with bacteremia, urinary tract infections and urosepsis, and due to their multidrug resistance discordant antimicrobial therapy and increased morbidity and mortality are increasingly observed (Peirano

and Pitout 2010; Johnson et al. 2010). The number of reported livestock animal and food-associated ST131 isolates, whether they harbor ESBLs or not, is negligible compared to its incredible frequency in humans. While a $bla_{CTX\text{-}M\text{-}9}$ carrying ST131 *E. coli* could be isolated from poultry in Spain (Cortes et al. 2010; Mora et al. 2010), several other researchers failed to detect this genotype among ESBL-producers e.g. from poultry and retail poultry meat (Overdevest et al. 2011; Egea et al. 2012) and from cattle (Madec et al. 2012). In contrast, a much wider distribution of CTX-M-type-producing ST131 *E. coli* strains has been observed in companion animals and horses, only a few years after its first emergence in humans (Ewers et al. 2012, 2014; Dierikx et al. 2012; Pomba et al. 2014). Thus, this clonal group basically circulates among humans, but crossed the species barrier to dogs and cats in particular, suggesting (i) a transmission cycle between companion animals and humans which largely differs from that between livestock and humans or (ii) the inability of the ST131 genotype to stably colonize the gut of livestock animals. In any case it is clear that livestock animals are currently not a threatening source of this highly virulent and multidrug resistant clonal group, while the reasons for low carriage rates with such strains are yet unsolved (Ewers et al. 2012).

Nevertheless, there is evidence for the existence of shared clones of ESBL/AmpC-producing *E. coli* in food-producing animals and humans, indicating that some common genotypes, plasmid and beta-lactamase types could indeed be circulating between them (Lavilla et al. 2008; Lopez-Cerero et al. 2011). Though less prevalent among highly virulent B2 ExPEC strains, ESBL/AmpC-producing *E. coli* are generally dispersed over the entire population of this bacterial species (Ewers et al. 2012). However, there is an apparent accumulation of multidrug-resistant isolates in certain non-B2 genotypes and strains of various clonal groups, including ST10, ST38, ST167, ST405, ST410, ST617, and ST648 are circulating widely across species and sources, i.e. humans, animals and food products (Overdevest et al. 2011; Ewers et al. 2012; Leverstein-van Hall et al. 2011; Cortes et al. 2010; Oteo et al. 2009; Bortolaia et al. 2011). Even so, considerable overlaps in all relevant traits, including bacterial genotype, β-lactamase gene, plasmid Inc group and, where applied, genomic fingerprint, among ESBL-producing *E. coli* isolates from food-producing animals, meat, and humans were infrequently reported (Madec et al. 2012; Randall et al. 2006; Lavilla et al. 2008; Dierikx et al. 2013; Bortolaia et al. 2011; Borjesson et al. 2013). Investigating genomic and plasmid backbone Kluytmans et al. (2013) found just one perfect match between geographically and temporally matched *E. coli* from humans and chicken meat (Kluytmans et al. 2013), implicating chicken meat as a source of human colonization with ESBL-producing *E. coli* isolates. Also other authors found that ESBL-producing *E. coli* from humans were generally different to that from animals, i.e. chickens, cattle, turkey, and pigs in the same region (only 1.2 % [3/258] related strains], as judged from antimicrobial and virulence gene profiles in combination with clonal complexes, suggesting a widespread human-to-human transmission as a strong possibility (Wu et al. 2013). Likewise, in a recent Swedish study there was no indication of the spread of *E. coli* carrying $bla_{CMY\text{-}2}$ from broilers to human clinical settings (Borjesson et al. 2013).

A very good example showing that even with an apparently sufficient molecular data set the direct contribution of livestock animals to the transmission of antimicrobial resistant microorganisms might be overstated, has been published previously. By using comparative whole genome analyses Mather et al. (2013) could demonstrate that, contrary to current belief, the epidemic multidrug-resistant *Salmonella Typhimurium* DT104, responsible for human gastrointestinal infections worldwide, was largely maintained within animal and human populations separately (Mather et al. 2013). They discovered distinguishable bacterial populations existing in human and animal populations living side by side, with limited transmission, in either direction, pointing towards alternative sources for these multidrug-resistant strains. This study emphasizes the critical importance of integrated genotypic data sets, including whole genome analysis, in understanding the ecology of bacterial zoonosis and antimicrobial resistance. In analogy to these data, one could also suggest that ESBL/AmpC plasmids have been acquired separately and time-independent in bacteria residing in animals and humans, followed by an independent micro-evolution of bacterial strains.

To conclude, robust studies providing unquestionable proofs for the livestock animal-to-human transmission and quantifying the true burden for public health are very rare. Due to the lack of sufficient molecular epidemiological data, the initial micro-evolutionary events leading to the introduction and further enrichment of ESBL/AmpC-producing bacteria in food-producing animals remain inconclusive. Overlaps in bacterial strain and plasmid characteristics among humans and food-animals always refer to one of these criteria only. Accordingly, several studies consistently emphasized the key role of plasmid versus clonal dissemination in the spread of ESBL/AmpC genes between animals and humans and *vice versa*. The notion that people who have direct contact with infected animals or contaminated meat, such as farm workers, slaughterers or people working in the food-producing industry, face a particular risk to acquire antimicrobial resistant bacteria requires further consideration in future. Despite these ambiguities, mitigation measures should aim to reduce the prevalence of ESBL/AmpC-producing bacteria in livestock animals and food to lower the overall burden on the bacterial resistome.

15.5 Conclusions

The frequent occurrence of ESBL/AmpC-producing bacteria in livestock animals and food thereof, and its unpredictable impact on food safety and environmental pollution is a major concern in the global debate on antimicrobial resistance. Problems related to antimicrobial resistance are inherently associated with the use of antibiotics in any environment, i.e. related to veterinary and human medicine. Accordingly, its use in the livestock production cycle may be one among other important factors in promoting the development of multidrug-resistant bacteria and genetic resistance determinants of zoonotic relevance. Detailed knowledge on common or separate micro-evolutionary events of bacteria and resistance genes among livestock animals and humans is still lacking while at present the interspecies trans-

fer of related plasmids seems to prevail over the transmission of relevant bacterial genotypes. This would urgently need further consideration in the future, i.e. by performing large scale studies that include human and veterinary settings at the same time and in the same geographic region in combination with highly resolving molecular techniques, such as whole genome and plasmid sequence analysis. One good example for such an approach on national basis is the German collaborative research project RESET—resistances in humans and animals—(http://www.reset-verbund.de/) which is funded by the Federal Ministry of Education and Research. Even if the impact of food-producing animals to antimicrobial resistance in humans cannot be quantified yet, antimicrobial consumption in these animals undisputedly contributes its part in the maintenance or even expansion of the global bacterial resistome. Therefore, antimicrobial use in this sector needs to be kept at levels as low as possible without losing sight of any aspects of animal welfare. Generally, the judicious and rational use of antimicrobials, particularly those that are "critically important" for human health, should be regarded as a naturally ethical issue in the veterinary profession. However, this would be only one step toward preserving the benefits of antimicrobials for people. Concerning the selective effect of antimicrobials and possible transmission routes of multidrug-resistant bacteria and resistance genes, numerous environmental pathways (e.g. water, soil, manure, and mechanical or non-livestock animal vectors) as well as resistance of bacteria to abiotic factors, which may be crucial to predict their ability to recolonize hosts and to colonize new hosts, need to be considered carefully. Beyond antimicrobial consumption animal husbandry, extensive trade, farm hygiene and biosecurity as well as intensive farming are considered the most compelling risk factors contributing to the global emergence of ESBL/AmpC-producing *Enterobacteriaceae* in livestock. In particular, massive movement of animals from reproduction to fattening farms and the global trade of food might facilitate a virtually boundless transfer of multidrug-resistant microorganisms between farms and countries, weakening national mitigation measures considerably. This makes joining transnational forces to contain the risks of spreading antimicrobial resistance one essential part in an overall holistic approach. In this context, risk assessment, i.e. identification of stages within the food production chain (e.g. slaughter of animals, distribution, handling and consumption of foods) that may pose an increased risk of human infection with antimicrobial resistant bacteria, should be generally included as a mature part of this global effort. Based on this, distinct production phases could be specifically targeted while developing and implementing intervention strategies, which on a long term should help to lower the risk of antimicrobial resistance.

Resistance will probably never return to pre-antibiotic use levels; weak market encouragements and increasing difficulty and cost to develop new effective antimicrobial substances have greatly discouraged investment in this area. Not only for these reasons are alternatives to antimicrobials urgently required in veterinary medicine. Implementation and optimization of biosecurity on farms and overall good hygiene practices at all stages of the food chain, i.e. pre- and post-harvest, have already shown excellent results in reducing the impact of multidrug-resistant bacteria in livestock animals. Several other options that have been proposed to lower the burden of antimicrobial resistance, such as pre- and probiotics, competitive exclu-

sion products, vaccines, and bacteriophages have shown variable effects so far, but represent promising approaches that need to be addressed as soon as possible, as there is no more time to lose.

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Chapter 16
Zoonotic Transmission of Antimicrobial Resistant Enterococci: A Threat to Public Health or an Overemphasised Risk?

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Abstract Enterococci are intrinsically resistant to various antimicrobial classes and able to acquire resistance to clinically relevant drugs via horizontal transfer. Consequently, limited therapeutic options are available for treatment of enterococcal infections. Zoonotic transfer of antimicrobial resistance in enterococci has been studied for two decades. The first studies hypothesizing possible animal-to-human transmission of resistant strains and mobile genetic elements are dated 1993. Since then a considerable amount of papers has been published on this subject, providing the groundwork for important decisions limiting antimicrobial use in animal husbandry. In this chapter, the relative contribution by animal enterococci to antimicrobial resistance in human infections was reviewed taking into consideration the potential impact associated with different enterococcal species, animal hosts, epidemiological routes and mechanisms of transfer. The authors conclude that potential zoonotic risks mainly concern horizontal transfer of resistance genes and clonal transmission of multidrug-resistant *Enterococcus faecalis* sequence type ST16. The impact of clonal transmission from food animals to people appears to be negligible for other multidrug-resistant *E. faecalis* and *E. faecium* lineages responsible for hospital infections. Although it has been demonstrated experimentally that antimicrobial resistant enterococci of animal origin can transiently colonise the human digestive tract and transfer their resistance genes to the indigenous microflora, the actual risks associated with foodborne transmission are controversial, mainly limited to poultry meat products and possibly differ between geographical areas. Research is warranted to explore the ecology of enterococcal mobile genetic elements carrying resistance genes of clinical relevance and to develop suitable technologies to perform this type of studies.

Enterococci are commensal bacteria in the intestinal microbiota of humans and animals (Gilmore et al. 2013). *Enterococcus faecalis* and *E. faecium* are the most frequent species in humans and domestic animals, although species distribution has

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a certain degree of host-specificity and is influenced by diet and environmental conditions (Aarestrup et al. 2002; Kühn et al. 2003). Both species are opportunistic pathogens that can cause a variety of infections, especially hospital-acquired infections, including endocarditis, bacteraemia, meningitis, wound and urinary tract infections, and peritonitis (Arias and Murray 2012). *E. faecalis* is the species most frequently isolated from human clinical specimens, followed by *E. faecium* (Hidron et al. 2008; Oteo et al. 2007). Together these two species are ranked as the third cause of bacteraemia in European and in American hospitals accounting for approximately 11–13 % of all bacteraemia cases (Ammerlaan et al. 2013; de Kraker et al. 2013). *E. faecalis* and *E. faecium* are also among the most common pathogens recovered from catheter-associated, skin and soft tissue infections (Arias and Murray 2012).

Enterococci are intrinsically resistant to important antimicrobial classes in clinical practice. Consequently, therapeutic options against enterococci are limited. Treatment of life-threatening infections usually consists of a combination of penicillin (ampicillin or penicillin) and aminoglycoside (gentamicin or streptomycin) (Arias and Murray 2008). Glycopeptides such as vancomycin are the best alternative, if the causative strain is resistant to one or both these drugs or in cases where aminoglycoside use is contraindicated due to nephrotoxicity. Other second tier agents include quinupristin-dalfopristin (for *E. faecium* only) and newer drugs such as linezolid, daptomycin, tigecycline and fifth-generation cephalosporins. Older antibiotics such as chloramphenicol, doxycycline, minocycline and nitrofurantoin may be used for specific indications (Arias and Murray 2008).

Over the last two decades many authors have hypothesized that animal enterococci may serve as a reservoir of resistant strains and resistance genes to humans. Antimicrobial resistant enterococci in food of animal origin gained attention from researchers and public health authorities in the early 1990s. At that time, vancomycin-resistant enterococci (VRE) were emerging as nosocomial pathogens worldwide (Leclercq et al. 1988; Uttley et al. 1988; Sundsfjord et al. 2001). Although initially it was believed that nosocomial use of vancomycin was the only factor selecting for VRE, this assumption was partly revisited after a considerable reservoir of VRE was reported in the community and in production animals in Europe (Bates et al. 1993; Klare et al. 1993; Torres et al. 1994; Goossens 1998; Martone 1998). In 1995, two independent studies established a correlation between usage of the vancomycin-analogue avoparcin as a growth promoter in livestock and occurrence of VRE in chickens and pigs (Klare et al. 1995; Aarestrup et al. 1995). Subsequent studies confirmed that i) there is cross-resistance between avoparcin and vancomycin (van den Bogaard et al. 1997a); ii) avoparcin use was associated with occurrence of VRE in animal faeces and meat products (Bager et al. 1997; Aarestrup et al. 2000a); and iii) occurrence of VRE in food animals and meat products was correlated to occurrence of VRE in faecal samples of community-dwelling humans (Pantosti et al. 1999; Klare et al. 1999; van den Bogaard 2000). The risk that animal VRE could be transmitted through the food chain was regarded as high, since enterococci are particularly resistant to heat, disinfectants and other decontamination procedures used at slaughterhouses (Giraffa 2002). All these evidences provided the basis for establishing and maintaining the ban of avoparcin use enforced in the EU since 1997

in accordance with the precautionary principle (Anonymous 1997). Additional links between occurrence of antimicrobial resistant enterococci in production animals and in healthy humans were hypothesized on the basis of similarities in the patterns of resistance to quinupristin/dalfopristin, erythromycin, tetracyclines, gentamicin and chloramphenicol, as well as in the distribution of genes conferring resistance to these antimicrobials (Welton et al. 1998; Werner et al. 1998; Aarestrup et al. 2000a; Aarestrup et al. 2000b; Del Campo et al. 2003; Klare et al. 2003; Kieke et al. 2006). These findings contributed to support the ban on use of all antimicrobials as growth promoters in the EU, which was ratified through different EU Regulations enforced in 1999 and in 2006 (Anonymous 1998a; Anonymous 1998b; Anonymous 2003). Use of growth promoters is still allowed in the USA, even though avoparcin has never been used in this country.

This chapter is a review of the literature regarding the possible contribution of enterococci of animal origin to antimicrobial resistance problems encountered in human medicine. The topic is reviewed taking into consideration the roles played by different enterococcal species (*E. faecium* vs. *E. faecalis*), animal hosts (food vs. companion animals), epidemiological routes (foodborne transmission vs. transmission by contact with animals) and mechanisms of transfer (clonal transmission vs. horizontal gene transfer). The chapter is organised into five sections addressing genetic basis of antimicrobial resistance in enterococci (16.1), prevalence of resistance in human, animal and food isolates (16.2), evidence of transmission between animals and humans (16.3), genetic links between clinical and animal strains (16.4) and concluding remarks by the authors (16.5).

16.1 Genetic Basis of Antimicrobial Resistance in Enterococci

Enterococci tolerate low concentrations of β-lactams, quinolones, aminoglycosides and lincosamides, and are able to metabolise preformed folic acid, thereby bypassing inhibition of folate synthesis by trimethoprim and sulphonamides (Murray 1990; Hollenbeck and Rice 2012). Moreover, they have a particular ability to acquire exogenous resistance genes via conjugative transposons and plasmids (Werner et al. 2013). This section summarises the genetic basis of antimicrobial resistance in enterococci with focus on acquired resistance to antimicrobials of clinical relevance and potential animal reservoirs.

16.1.1 β-lactam Resistance

Enterococci display intrinsic resistance to cephalosporins and decreased susceptibility to penicillins (Fontana et al. 1990; Zhang et al. 2012). In *E. faecium,* high-level penicillin resistance is mainly associated with mutations or overproduction of *pbp5*, which encodes a penicillin binding protein (PBP5) with low affinity for

penicillins (Arias and Murray 2012). An additional gene (*ddcY*) which mediates ampicillin resistance by altering the pathway of peptidoglycan synthesis was identified in ampicillin-resistant *E. faecium* (AREF) mutants selected *in vitro* (Mainardi et al. 2000). The clinical importance of this resistance mechanism appears to be limited, since this gene is present in a relatively small proportion (17 % out of 29 strains and 25 % out of 8 strains according to different studies) of *E. faecium* strains (Mainardi et al. 2000; Sacco et al. 2010; Zhang et al. 2012). Finally, a further, uncommon mechanism of ampicillin resistance in *E. faecium* is represented by enzymatic drug inactivation. Chromosomal beta-lactamase-encoding genes conferring ampicillin resistance were recently reported in different *E. faecium* strains (Sarti et al. 2012).

Transferability of ampicillin resistance determinants was shown *in vitro*, but the frequency and importance of this event in nature is unknown. Rice et al. demonstrated that *pbp5* is transferable horizontally and, recently, Novais et al. showed that five (28 %) out of 18 *E. faecium* strains isolated from the pig farm environment could transfer ampicillin resistance by conjugation, though the underlying genetic basis of resistance was not investigated (Rice et al. 2005; Novais et al. 2013).

Ampicillin-resistant *E. faecium* (AREF) have been detected in production animals worldwide with considerable differences between countries (see Sect 2). For example, in 2011 occurrence of ampicillin resistance in *E. faecium* isolates from chickens was 3 and 75 % in Denmark and Ireland, respectively (EFSA 2013). In addition, significant temporal variations in occurrence of AREF in production animals could be observed within the same country. In Denmark, prevalence of ampicillin resistance in *E. faecium* was 3 and 23 % among pig isolates in 2010 and 2011, respectively (DANMAP 2011). Notably, AREF are commonly detected in dogs and, to a lesser extent, in cats (Butaye et al. 2001; Simjee et al. 2002; Rodrigues et al. 2002; Moyaert et al. 2006; Damborg et al. 2009; Ghosh et al. 2011, 2012; de Regt et al. 2012).

In *E. faecalis*, penicillin resistance is mainly mediated by mutations in *pbp4* (Ono et al. 2005). Plasmid-mediated beta-lactamases were first described in *E. faecalis* in 1983, but their occurrence remains rare (Murray 1992; Hollenbeck and Rice 2012). Occurrence of acquired penicillin resistance is rare in *E. faecalis* of both human and animal origin.

16.1.2 Aminoglycoside Resistance

Enterococci display intrinsic low-level resistance to aminoglycosides (Galimand et al. 2011; Arias and Murray 2012). High-level aminoglycoside resistance is caused by ribosomal mutations and/or acquisition of genes encoding enzymes that modify the drug (Chow et al. 2000; Arias et al. 2010). The most commonly detected gene is the transposon-associated *aac(6')-Ie-aph(2'')-Ia*, which mediates resistance to gentamicin, but not to streptomycin (Chow et al. 2000; Arias and Murray 2012). High-level streptomycin resistance may result from ribosomal mutations or acquisition of *ant(6')-Ia* (Arias et al. 2010). These mobilisable genes conferring high-level gentamicin and streptomycin resistance have been detected in enterococcal isolates

from production and companion animals worldwide (Torres et al. 2003; Harada et al. 2005; Novais et al. 2005; Jackson et al. 2010; Larsen et al. 2011; Frye and Jackson 2013; Novais et al. 2013). There are no peculiar differences between *E. faecium* and *E. faecalis* in the occurrence of aminoglycoside resistance.

16.1.3 Glycopeptide Resistance

Resistance to glycopeptides such as vancomycin and teicoplanin is mainly mediated by *van* operons which encode modified peptidoglycan precursors terminating in D-Ala-D-Lac or D-Ala-D-Ser and retain lower affinity to glycopeptides compared to the wild-type peptidoglycan precursor terminating in D-Ala-D-Ala (Courvalin 2006). Nine *van* operons have been described to date in *E. faecium* and *E. faecalis*, with *vanA* and *vanB* being the most frequently detected among clinical isolates, with notable country-based differences (Lebreton et al. 2011; de Garnica et al. 2013). Indeed, VanA is the predominant type of glycopeptide resistance reported among clinical isolates in Europe and North America, whereas VanB is more common among isolates in Australia (Cetinkaya et al. 2000; Christiansen et al. 2007; Deshpande et al. 2007). Interestingly, an increased occurrence of *vanB* was recently observed in hospital-associated *E. faecium* isolates in France, Germany and Spain (Bourdon et al. 2011; Klare et al. 2012; Lopez et al. 2012). Vancomycin resistance operons have been described both on chromosome and on plasmids, mainly in association with conjugative transposons Tn*1546* and Tn*1546*-variants (*van A*), and Tn*1547* and Tn*1549* (*vanB*) (Hegstad et al. 2010). Interestingly, glycopeptide resistance operons displaying 79–94 % nucleotide identity to *vanA* operon in enterococci have been described in *Paenibacillus* spp. isolated from soil, indicating that this glycopeptide resistance determinant may have originated in the environment a long time before emerging in clinical settings (Guardabassi et al. 2005). A novel glycopeptide resistance mechanism mediated by mutations in *ddcY* (see paragraph 1.1) was described in *E. faecium* strains obtained *in vitro* through serial exposure to increasing concentrations of glycopeptides (Cremniter et al. 2006). This mechanism of resistance, which leads to cross-resistance to ampicillin and glycopeptides (both vancomycin and teicoplanin) through bypass of PBPs-mediated peptidoglycan cross-linking, has not been described in clinical isolates.

VRE, generally harbouring *vanA,* are still detected in production animals from countries that used avoparcin, although at low prevalence (Lim et al. 2006; EFSA 2013). Noteworthy, avoparcin use was discontinued in Sweden already in 1984 and no VRE were detected in production animals until 2000 (Nilsson 2012). Then, from 2000 to 2005 the proportion of broiler flocks positive for VRE strikingly increased from less than 1>40 %, mainly due to spread of a single *E. faecium* clone for reasons which are still unknown (Nilsson 2012). VRE have not been reported to date in production animals from most countries where avoparcin has never been used (e.g. Canada and Australia), with the exception of the USA where vancomycin-resistant *E. faecium* (VREFm) was reported once in swine (Coque et al. 1996; Diarra et al. 2010; Donabedian et al. 2010; Fard et al. 2011; Tremblay et al. 2011; Tremblay et al. 2012).

VRE have also been isolated from companion animals in various countries (Devriese et al. 1996; Simjee et al. 2002; Manson et al. 2003; Torres et al. 2003; Herrero et al. 2004; Poeta et al. 2005). Independent of host species, occurrence of vancomycin resistance is generally more frequent in *E. faecium* than in *E. faecalis*.

16.1.4 Daptomycin Resistance

Daptomycin resistance is mediated by mutations in two groups of chromosomal genes (Arias et al. 2011; Tran et al. 2013). The first group include genes encoding a three-component regulatory system (LiaFSR) involved in homeostasis of the cell envelope, while the second group of genes encode enzymes (GdpD and Cls) involved in cell membrane phospholipid metabolism (Arias et al. 2011; Humphries et al. 2012; Tran et al. 2013). In addition, mutations in the mannose-specific phosphotransferase system (PTS) were also suggested to be involved in daptomycin resistance in *E. faecium* (Humphries et al. 2012). Resistance to this lipopeptide antibiotic is rare in humans (and it is mainly developed during therapy) and has never been reported in animals (Novais et al. 2004; Hollenbeck and Rice 2012). Nevertheless, a zoonotic potential of daptomycin-resistant *E. faecium* was hypothesized in a recent study showing that proximity of the residence of patients to animal or crop operations was associated with occurrence of daptomycin resistance (Kelesidis and Chow 2013).

16.1.5 Linezolid Resistance

The most frequently reported mechanism of linezolid resistance is associated with mutations in domain V of 23S rRNA described both in *E. faecium* and in *E. faecalis* (Arias and Murray 2012). In addition, transferable *cfr* has been recently reported in *E. faecalis* from cattle, swine and farm sewage in China and *E. faecalis* and *E. faecium* in humans in Spain and Thailand (Cercenado et al 2010; Diaz et al. 2012; Liu et al. 2012; Liu et al. 2013). This gene has been found both on conjugative and on non-conjugative plasmids, often in association with IS*Enfa4*, IS*1216* and IS*256*-like elements, thus showing a high propensity to disseminate by conjugation and recombination events (Diaz et al. 2012; Liu et al. 2012; Liu et al. 2013; Shen et al. 2013).

16.1.6 Streptogramin Resistance

E. faecalis is intrinsically resistant to streptogramins such as quinupristin/dalfopristin, whereas *E. faecium* displays innate low-level resistance to streptogramin B (quinupristin) (Singh and Murray 2005). Several plasmid-mediated streptogramin resistance genes, with *vat*(D) and *vat*(E) being the most frequently detected, have been described in animal and human *E. faecium* isolates worldwide, and yet

unknown resistance mechanisms are likely to exist (Jensen et al. 1998; Soltani et al. 2000; Hammerum et al. 2001; Hershberger et al. 2004; Simjee et al. 2006; De Graef et al. 2007; Jung et al. 2010; Frye and Jackson 2013).

16.1.7 Tigecycline Resistance

The mechanisms of tigecycline resistance are currently unknown. Occurrence of resistance to this tetracycline analogue is sporadic in clinical and animal isolates (Waites et al. 2006; Dowzicky and Chmelarová 2011). Tigecycline-resistant *E. faecalis* isolates were reported in samples from chicken meat and swine in Portugal (Freitas et al. 2011b).

16.2 Prevalence of Antimicrobial Resistance

Prevalence of antimicrobial resistance and distribution of antimicrobial resistance genes vary significantly depending on host species and geographical regions. Local data on prevalence of antimicrobial resistance often reflect the specific patterns of antimicrobial usage within each host species and marked differences between hosts may provide useful epidemiological indications on potential reservoirs of antimicrobial resistance within defined geographical areas. The two following paragraphs summarise available national data on prevalence of antimicrobial resistance in *E. faecium* and *E. faecalis* from human patients and production animals. Most data derive from studies in Europe and the USA, as in these regions occurrence of antimicrobial resistance in animal and human isolates has been monitored for longer time and in a more systematic way compared to other regions of the world.

16.2.1 Patterns of Antimicrobial Resistance in Animal and Human E. faecium

Clinically-relevant antimicrobial resistance phenotypes in *E. faecium* that may be linked to animal reservoirs include resistance to ampicillin, gentamicin, vancomycin and quinupristin-dalfopristin. Resistance to linezolid and daptomycin is rare or even absent in animal populations (see Sect 1.4 and 1.5).

Recent data from Europe and North America show that ampicillin resistance is frequent (≥ 89 %) among human clinical isolates, irrespective of geographical origin (Table 16.1). Ampicillin resistance occurs less frequently among isolates from animal sources, with the exception of turkey meat in the USA in which ampicillin resistance occurred in 75 % of isolates (Table 16.1). Thus it seems that animal sources, mainly poultry, could contribute to occurrence of ampicillin resistance in clinical settings only to a limited extent. A marked difference in the prevalence of

Table 16.1 Prevalence of antimicrobial resistance in *Enterococcus faecium* isolated from humans (clinical isolates) and animals (indicator isolates) within the same country. (Pen, penicillin; Gen, gentamicin; Van, vancomycin; Ery, erythromycin; Qui/Dal, quinupristin/dalfopristin; Tet, tetracycline; Lin, linezolid.)

Country	Isolation year	Origin (no. of isolates)	Prevalence of antimicrobial resistance (%)							Reference
			Pen	Gen	Van	Ery	Qui/Dal	Tet	Lin	
Denmark	2011	Human (619)	93	74	1.3	–	–	–	–	DANMAP2011
	2011	Swine (116)	23	1	1	33	2	62	0	DANMAP2011
	2011	Pork, DK (27)	0	0	0	15	0	7	0	DANMAP2011
	2011	Broilers (107)	3	0	0	15	1	5	0	DANMAP2011
	2011	Broiler meat, DK (83)	2	0	0	19	1	10	0	DANMAP2011
	2011	Broiler meat, imported (64)	28	0	0	61	12	34	0	DANMAP2011
	2011	Cattle	–	–	–	–	–	–	–	DANMAP2011
	2011	Beef (16)	0	0	0	0	0	0	0	DANMAP2011
USA	2009	Humans ([a])	–	19	75	–	–	–	–	The surveillance network USA, 2013
	2004–2005	Humans (280)	89	–	68	–	–	–	0	Waites et al. 2006
	2011	Chicken meat (221)	19	9	0	22	32	43	0	NARMS 2011
	2011	Turkey meat (40)	75	10	0	32	55	82	0	NARMS 2011
	2011	Pork (37)	3	0	0	11	13	49	0	NARMS 2011
	2011	Beef (82)	4	0	0	6	11	22	0	NARMS 2011

[a] 8283 isolates tested for vancomycin resistance and 4772 isolates tested for gentamicin resistance

ampicillin resistance has been observed between isolates from pig (23 %) and pork (3 %) in Denmark, indicating that hygienic measures at slaughter effectively reduce human foodborne exposure to ampicillin-resistant strains of pig origin.

Occurrence of gentamicin resistance greatly varies between countries and host species. In Denmark, gentamicin resistance is high (74 %) among clinical isolates, low (1 %) in pig isolates and absent in meat isolates, indicating that there is no significant animal reservoir of this resistance in this country. Differently, in the USA gentamicin resistance occurs less frequently (19 %) than in Denmark among clinical isolates, but is more frequent in poultry meat isolates (9 and 10 % in broiler and turkey meat, respectively). These data suggest that poultry meat is a potential reservoir of gentamicin resistance for *E. faecium* isolated from human infections in the USA.

Geographical patterns of vancomycin resistance are also useful to infer potential epidemiological links between animals and humans. In Denmark, prevalence of vancomycin resistance is extremely low (ca. 1 %) among clinical and swine isolates, and was not detected among poultry isolates by the conventional monitoring program in 2011 (Table 16.1). On the contrary, vancomycin resistance is widespread in American hospitals, being detected in up to 75 % of clinical isolates (Table 16.1).

In this country, vancomyicin resistance is virtually absent among isolates from production animals, which strongly indicates absence of animal reservoirs. Altogether, it appears that occurrence of vancomycin resistance among human clinical isolates is primarily driven by hospital use of glycopeptides and only marginally influenced by zoonotic transmission from animals.

Different considerations can be made with regard to the occurrence of quinuprisitin/dalfopristin resistance. This phenotype occurs at variable frequency (1–55 %) among isolates from poultry meat both in Europe and in the USA (Table 16.1; EFSA 2013). The frequent recovery of resistant strains from poultry products in Europe is surprising, since the quinuprisitin/dalfopristin analogue virginiamycin, which is likely to select for quinuprisitin/dalfopristin resistance, has not been used since 1999, while it is still used in the USA for growth promotion. Genetic linkage of quinopristin/dalfopristin resistance genes to genes conferring resistance to antimicrobials used in animal production (e.g. macrolides) may explain the persistence of these genes in food animals in Europe (Hammerum et al. 2001). Data on occurrence of quinopristin/dalfopristin resistance in human isolates are not readily available. However, it seems that prevalence of this resistance phenotype is lower in human isolates compared to animal isolates, suggesting that existence of an animal reservoir is possible for quinupristin/dalfopristin resistance (Donabedian et al. 2006; Kieke et al. 2006; Hammerum et al. 2009).

Finally, it is important to note that occurrence of resistance to antimicrobials used in animals like erythromycin and tetracyclines is generally high among animal and meat isolates, with the exception of cattle and beef (Table 16.1). Although veterinary use of these antimicrobials does not constitute a direct risk to public health since they generally are not used for treatment of human enterococcal infections, it may favour co-selection of genes conferring resistance to clinically relevant antimicrobials, as hypothesized for quinupristin/dalfopristin and glycopeptides (Hammerum et al. 2001; Novais et al. 2005).

16.2.2 Patterns of Antimicrobial Resistance in Animal and Human E. faecalis

From a contemporary clinical perspective, the only antimicrobial resistance phenotype in *E. faecalis* that could be significantly linked to animal reservoirs is gentamicin resistance, since resistance to ampicillin, vancomycin and linezolid is rare or even not detected in animal isolates, (Ghosh et al 2012; Liu et al. 2012; 2013). According to recent data, gentamicin resistance is relatively high among clinical isolates in Europe (43 %) and in the USA (29 %) (Table 16.2; Kuch et al. 2012). In Denmark, gentamicin resistance was reported at comparable prevalence in clinical (31 %) and in swine (21 %) isolates (Table 16.1). Similarly, in the USA occurrence of gentamicin resistance in poultry meat isolates was 30–34 %, which is comparable to values observed in clinical isolates (Table 16.2). These data indicate the possible existence of an animal reservoir of gentamicin-resistant *E. faecalis*, though linked to different animal sources in different geographical areas. Interestingly, as observed for *E. faecium*, occurrence of gentamicin-resistant strains is significantly higher in Danish pigs (21 %) than in pork (2 %) (Table 16.2). These data indicate low risk of carcass contamination in pig slaughtering and consequent low human exposure to gentamicin-resistant through consumption of pork.

As observed for *E. faecium*, resistance to erythromycin and tetracycline is widespread among *E. faecalis* isolates from animals and meat (Table 16.2), possibly as a consequence of the massive use of these antibiotics in livestock production.

16.3 Transmission of Antimicrobial Resistance Between Animals and Humans

Transmission of antimicrobial resistance between animal and human enterococci may happen through different epidemiological routes and mechanisms. Humans are exposed to animal enterococci by direct contact with animals and animal-contaminated environments or indirectly, through consumption of contaminated food of animal origin and vegetables from crops treated with animal manure. Once acquired, strains of animal origin may transiently colonise the human digestive tract and transfer mobile genetic elements (MGE) containing antimicrobial resistance genes to the indigenous microflora, including bacteria other than enterococci.

16.3.1 Foodborne Transmission

Foodborne transmission may result from consumption of contaminated animal food products and cross-contamination in the kitchen (Wegener et al. 1997). *E. faecium* and *E. faecalis* generally contaminate raw meat and cheese at concentrations of 10^2–10^4 and 10^5–10^7 colony forming units (CFU) per gram, respectively

Table 16.2 Prevalence of antimicrobial resistance in *Enterococcus faecalis* isolated from humans (clinical isolates) and animals (indicator isolates) within the same country. (Pen, penicillin; Gen, gentamicin; Van, vancomycin; Ery, erythromycin; Tet, tetracycline; Lin, linezolid)

Country	Isolation year	Origin (no. of isolates)	Prevalence of antimicrobial resistance (%)						Reference
			Pen	Gen	Van	Ery	Tet	Lin	
Denmark	2011	Human (513)	-	31	0	–	–	–	DANMAP 2011
	2011	Pigs (117)	0	21	0	54	85	0	DANMAP 2011
	2011	Pork, DK (133)	0	2	0	8	17	0	DANMAP 2011
	2011	Pork, imported (45)	0	4	0	11	36	0	DANMAP 2011
	2011	Broilers (110)	0	0	0	15	17	0	DANMAP 2011
	2011	Broiler meat, DK (34)	0	0	0	18	26	0	DANMAP 2011
	2011	Broiler meat, imported (69)	0	1	0	49	67	0	DANMAP 2011
	2011	Cattle	–	–	–	–	–	–	DANMAP 2011
	2011	Beef, DK (20)	0	0	0	5	20	0	DANMAP 2011
	2011	Beef, imported (30)	0	3	0	7	17	0	DANMAP 2011
USA	2009	Humans[a]	–	29	4	–	–	–	The surveillance network, USA, 2013
	2005	Humans (740)	3	–	5	–	–	0.5	Waites et al., 2006
	2011	Chicken meat (186)	0	30	0	35	63	0	NARMS 2011
	2011	Turkey meat (392)	0	34	0	47	92	0	NARMS 2011
	2011	Pork (334)	0	1	0	4	79	0	NARMS 2011
	2011	Beef (269)	0	0	0	3	18	0	NARMS 2011

[a] 23,826 isolates tested for vancomycin resistance and 14,990 isolates tested for gentamicin resistance

(Giraffa 2002). The hypothesis that antimicrobial resistant enterococci of animal origin could be transferred to the intestine of healthy humans via food is indirectly supported by studies describing clonally related strains in meat products and in the faeces of meat consumers. Donabedian et al. described closely related gentamicin-resistant *E. faecalis* strains in multiple pork samples and one human sample, and indistinguishable strains in a chicken meat sample and a human sample in the USA (Donabedian et al. 2003). Agersø et al. demonstrated clonal relatedness between five vancomycin-resistant *E. faecalis* (VREFs) from turkey meat and from the intestine of healthy humans in Denmark (Agersø et al. 2008). Finally, in an additional study from Denmark, Hammerum et al. described the detection of highly related vancomycin-resistant *E. faecium* (VREFm) isolates in pig samples and in the intestine of a healthy human who reported no contact with pigs but had eaten pork (Hammerum et al. 2004).

Evidence that animal enterococcal strains occurring in food have the ability to colonise the human intestine for a variable time period has been shown by experiments conducted on healthy human volunteers. In an experiment performed on himself, Berchieri established that a minimum concentration of 10^7 CFU of VREFm of poultry and pig origin was necessary to be able to isolate the same strain from faeces for a period of 20 days (Berchieri 1999). Sørensen et al. demonstrated that VREFm from poultry meat and quinupristin/dalfopristin-resistant *E. faecium* from pork ingested at 10^7 CFU could be detected in the faeces of 8 out of 12 volunteers 6 days after ingestions, at different concentrations (Sørensen et al. 2001). One out of 12 volunteers excreted the strain also 14 days after ingestion (Sørensen et al. 2001). In a similar experiment, Lester et al. demonstrated that animal VREFm transiently colonising the human gut could transfer *vanA* to resident commensal *E. faecium* strains in three out of six volunteers, indicating that occurrence of VREFm in food may result in transfer of vancomycin resistance to consumers (Lester et al. 2006). Recently, Al-Ahmad et al. showed that foodborne *E. faecalis* could integrate into dental oral biofilm in 5 out of 6 volunteers for at least 5 days, indicating a potential risk for endodontic infections that may evolve into bacteraemia (Al-Ahmad et al. 2010).

In conclusion, based on the current knowledge, ingestion of antimicrobial resistant enterococci of animal origin can result in colonisation of the human digestive tract for a variable time, likely depending on the numbers of enterococci ingested as well as on host factors, and exchange of MGEs containing antimicrobial resistance genes with the indigenous microflora.

16.3.2 Transmission via Direct Contact with Food Animals

Farm and slaughterhouse workers and veterinarians are the main categories at risk for this transmission route, since they are daily exposed to high numbers of animals. High density of animals and animal excreta implies also a high load of faecal bacteria in farm environments. Various studies showed that genetically related antimicrobial resistant enterococci can be isolated from animal faeces, insects, dust

and air inside and in proximity of farms, which indicates the existence of multiple sources of human exposure to animal enterococci (Graham et al. 2009; Ahmad et al. 2011; Braga et al. 2013; Novais et al. 2013). Evidence of human infections caused by antimicrobial-resistant enterococci transmitted by direct contact with production animals is limited. Das et al. reported a VREFs-infected wound in a worker who was injured while working at a factory packaging chickens (Das et al. 1997). The strain isolated from the wound had the same resistance profile as isolates from the factory and the patient had no risk factors for a VREFs infection, strongly supporting animal origin of the infection (Das et al. 1997).

Different studies reported occurrence of genetically related enterococci strains displaying specific resistance phenotypes in the faeces of animals and healthy farm workers. VREFm clones shared by turkey, turkey farmers and turkey slaughterers and by broiler and broiler farmers were detected in The Netherlands and in Norway (van den Bogaard et al. 1997b; Simonsen et al. 1998; Stobberingh et al. 1999; Jensen et al. 2003). Clonally related quinupristin/dalfopristin-resistant *E. faecium* were isolated from a poultry farmer and his animals in The Netherlands (Jensen et al. 1998). Closely related plasmids and indistinguishable Tn*1546* variants harbouring *vanA* have been reported in genetically unrelated VREFm isolated from poultry and workers within farms (Stobberingh et al. 1999; van den Bogaard et al. 2002; Sletvold et al. 2007), suggesting that *vanA* of animal origin may be horizontally transferred to the intestinal microbiota of farm workers.

16.3.3 Transmission via Direct Contact with Companion Animals

A role of companion animals as reservoirs of antimicrobial-resistant enterococci was first hypothesized in 1996, when van Belkum et al. discovered that 17% of dogs and cats examined harboured VREFm while the incidence among people living in the same area was 2–3%, and that VREFm isolates from a dog, a cat and a human carrier were indistinguishable by pulsed-field gel electrophoresis (PFGE) (van Belkum et al. 1996). The authors concluded their article by raising the question, "which dog poses a greater risk to the postman: the one that barks or the one that wags its tail?" (van Belkum et al. 1996). Companion animals represent potential sources of antimicrobial-resistant bacteria, since they live in close contact with their owners and are often administered antimicrobials belonging to the same classes used for treatment in humans (Guardabassi et al. 2004; Jackson et al. 2009). Antimicrobial resistant enterococci can be isolated from different animal body sites and from faeces, which may represent a source of contamination of domestic and urban environment (Jackson et al. 2009; Ghosh et al. 2011). Ampicillin-resistant *E. faecium* (AREF) were detected in a considerable proportion of dogs and cats in different European countries, being present in 23% (of 183), 30% (of 79) and 76% (of 25) of dogs in the UK, The Netherlands and Denmark, respectively, and in 13% (of 85) of cats in The Netherlands (Damborg et al. 2009; de Regt et al. 2012). In addition, VREFm were detected in 1.4% (out of 71) and 13% (out of 87) of dog faeces samples examined in Portugal and Spain, respectively (Herrero et al. 2004;

Poeta et al. 2005). Occurrence of VRE in dogs was reported also outside Europe. The first VREFm reported in a dog in the USA was shown to harbour a mutated form of Tn*1546* described in human patients (Simjee et al. 2002). A VREFs from a dog with mastitis displayed a PFGE profile prevalent among human isolates in New Zealand (Manson et al. 2003). These studies suggest that VRE and *vanA* may be exchanged between humans and dogs, but the importance and prevalent direction of this transmission route is difficult to assess.

16.4 Genetic Links Between Clinical and Animal Strains

Even if it appears plausible that antimicrobial resistant enterococci of animal origin reach the digestive tract of humans and transfer resistance genes to human-adapted strains, the human health consequences associated to this biological phenomenon are controversial. This section reviews the current knowledge of the genetic similarities between clinical and animal strain populations of *E. faecium* and *E. faecalis*. This information is of paramount importance to assess the risk of zoonotic transmission.

16.4.1 Genetic Links Between Clinical and Animal E. faecium

Based on multilocus sequence typing (MLST), lineages 17 (which include, among others, sequence types ST16 and ST17), 18 (ST18) and 78 (ST78 and ST192) are the most important epidemic lineages associated with nosocomial infections worldwide (Willems et al. 2012). These hospital-associated lineages are generally characterised by ampicillin resistance and are particularly enriched in genes encoding colonisation and adhesion factors, which likely play a role in virulence (Somarajan and Murray 2013). Animal strains rarely overlap with the hospital-associated lineages, with the notable exception of canine *E. faecium* strains. Indeed, AREF belonging to the hospital-associated ST78 and ST192 are frequently detected in dogs, but they generally lack genes encoding putative virulence factors (Damborg et al. 2009). Thus it appears that animal and clinical *E. faecium* strains constitute two distinct subpopulations in relation to ampicillin resistance and occurrence of putative virulence factors. Similar conclusions have been drawn for VREFm. The population structures of *E. faecium* isolated from human patients and animals are generally diverse, and overlap only sporadically (Woodford et al. 1998; Jung et al. 2006; Biavasco et al. 2007; Donabedian et al. 2010; Freitas et al. 2011a; Hammerum 2012; Tzavaras et al. 2012; Getachew et al. 2013). Among lineages of clinical relevance, there are single reports of *vanA*-positive VREFm ST132 (related to ST18) in swine in Portugal, *vanA*-positive VREFm ST78 in rabbit meat, and *vanB*-positive VREFm ST17 in chicken meat and veal in Spain (Lopez et al. 2009). VREFm lineages grouped in clonal complex CC5, which are common among porcine strains of diverse geographical origin, have been sporadically reported as a cause of urinary

tract infections in hospitalised patients (Freitas et al. 2011a). These findings suggest that animal VREFm strains have a limited zoonotic potential, as further substantiated by recent evolutionary studies based on comparative genome analyses, which conclusively showed that animal and clinical strains, although evolutionary linked, constitute different subpopulations or clades that diversified mainly through recombination and acquisition or loss of MGEs and eventually adapted to different ecological niches (van Schaik et al. 2010; de Regt et al. 2012; Galloway-Peña et al. 2012; Willems et al. 2012; de Been et al. 2013; Lebreton et al. 2013).

16.4.2 Genetic Links Between Clinical and Animal E. faecalis

Also in *E. faecalis*, few genetic lineages, namely CC2, CC16 and CC87, are particularly enriched among nosocomial isolates and associated with multidrug resistance. However, differently from *E. faecium*, these hospital-associated clones are phylogenetically closely related to human commensal and animal strains indicating the absence of a clear boundary between clinical and non-clinical strains (McBride et al. 2007; Willems et al. 2011; Palmer et al 2012). CC2 strains including the epidemic VREFs clone ST6 are frequently associated with nosocomial infections and have sporadically been reported in animals such as pigs and natural gilthead seabream (*Sparus aurata*, a saltwater fish species) in Portugal and crows in the USA (McBride et al. 2007; Freitas et al. 2009; Freitas et al. 2011a; Barros et al. 2012; Kuch et al. 2012; Oravcova et al. 2013). Similarly, CC87 strains are associated with nosocomial infections (especially in Poland), but have not been described in animals to date (McBride et al. 2007; Kuch et al. 2012; Getachew et al. 2013). Therefore, the genetic link between animal and human *E. faecalis* seems to be weak for these two lineages. Interestingly, the ecology of CC16 is different and this lineage appears to have a broad host spectrum, since it is well represented both among clinical and among non-clinical isolates (Ruiz-Garbajosa et al. 2006; Willems et al. 2011). In a recent study examining 386 contemporary human *E. faecalis* from hospital and community sources in six European countries, ST16 (the presumed founder of CC16) strains represented 11% and 15% of the total hospital and community-associated strains, respectively (Kuch et al. 2012). Half of the ST16 strains from each source displayed gentamicin resistance, suggesting that gentamicin-resistant strains causing up to 6% of nosocomial infections may be acquired in the community (Kuch et al. 2012). Notably, ST16 was found to be highly predominant among gentamicin-resistant *E. faecalis* isolated from pigs and pork in Denmark in 2001–2002, and represented 9% of 22 *E. faecalis* isolates from endocarditis patients in the same country in 1996–2002 (Larsen et al. 2010). These porcine and human strains were shown to be closely related also by PFGE, strongly suggesting a link between gentamicin-resistant *E. faecalis* ST16 in pigs and human patients in Denmark (Larsen et al. 2010). Gentamicin-resistant ST16 has also been associated with nosocomial infections in Cuba, with urinary tract infections and poultry in Vietnam, and with swine and poultry in Portugal (Freitas et al. 2009; Quinones et al. 2009; Poulsen et al. 2012; Novais et al. 2013). To the best of the authors' knowledge, the

genetic background of gentamicin-resistant strains frequently isolated from poultry meat products in the USA (see Sect 2.2) has not been investigated. Multidrug-resistant ST16 displaying additional resistance to linezolid have been reported in patients in Greece and Thailand (Spiliopoulou et al. 2011; Diaz et al. 2012), and vancomycin-resistant strains have been isolated from American crows (Oravcova et al. 2013). Altogether, it appears that the genetic link between clinical and animal *E. faecalis* mainly concerns this clone.

16.4.3 Genetic Links Between Mobile Genetic Elements in Clinical and Animal Enterococci

Exchange of MGEs carrying antimicrobial resistance genes may represent an additional genetic link between animal and clinical strains. The possible existence of this genetic link is suggested by the fact that most genes conferring resistance to clinically relevant antibiotics are transferrable and indistinguishable MGEs have been detected in animal and human strains (Hegstad et al. 2010; Werner et al. 2013). Transferability of MGEs from animal to human enterococci strains has been demonstrated *in vitro* and/or in animal models, including transferability of gentamicin resistance in *E. faecalis* and of ampicillin, gentamicin and vancomycin resistance in *E. faecium* (Lester and Hammerum 2010; Ghosh et al 2011; Sparo et al. 2012; Novais et al. 2013). A recent study showed that at least half (18/36) of the VREFm strains isolated within the same hospital in the USA in the period 1998–2009 had acquired the *vanB*-containing Tn*1549* via independent insertion events, indicating *de novo* generation of VREFm rather than cross-transmission, presumably mediated by transfer of this resistance determinant from gut anaerobic bacteria (Howden et al. 2013). Analysis of polymorphisms in MGEs harbouring resistance genes can be a useful epidemiological marker, since specific mutations in transposons and resistance genes have been associated with different animal species. For example, a point mutation (G to T) in *vanX* in Tn*1546* has been consistently associated with *E. faecium* isolated from poultry (G) and pigs (T), while both types occur among human clinical isolates (Jensen 1998; Hammerum 2012). Similarly, different *erm*(B) alleles occur at different frequencies among macrolide-resistant *E. faecium* isolates from pigs and poultry, and all variants are present among isolates from healthy and diseased humans (De Leener et al. 2005). These data suggest that animal and clinical *E. faecium* exchange MGEs carrying antimicrobial resistance genes, but the extent of this mechanism of zoonotic transfer remains unknown. In this regard, it is of paramount importance to implement sequence-based methods for typing of enterococcal plasmids allowing reproducibility, transportability, and comparability of data via the Internet, such as those developed for Gram-negative bacteria (Jensen et al. 2010). The risk of horizontal transfer of resistance genes between animal and human strains has not been thoroughly investigated in *E. faecalis* and it would be particularly interesting to compare the MGEs associated with gentamicin resistance genes in animal and clinical isolates.

16.5 Concluding Remarks

Enterococci are among the leading causes of serious human infections like bacteraemia and endocarditis. These infections are generally treated empirically and the consequences of treatment failure may be fatal to the patient, if infection is caused by a strain resistant to first line agents. By this chapter, the authors made an attempt to evaluate to what extent resistance problems in human enterococcal infections are attributable to strains and MGEs of animal origin. The authors' conclusion is that in general clinical *E. faecium* strains are not directly linked to animal sources, whereas the boundary between animal and clinical *E. faecalis* strains is not well defined. A clear overlap is evident for multidrug-resistant *E. faecalis* ST16, which has been associated with both human patients and animals, particularly pigs. Further detailed population genetic analysis of *E. faecalis* is needed to evaluate whether this and other clones shared by humans and animals might favour transfer of antimicrobial resistance from farms to hospitals.

Even though resistance genes of clinical relevance have been widely reported in enterococci isolated from animals, comparison of data on prevalence of antimicrobial resistance in animal, meat and human clinical isolates indicates that zoonotic risks associated with horizontal gene transfer from animal to human enterococci greatly differ depending on geographical region and are restricted to specific types of resistance and animal sources. The major risk seems to be associated to horizontal transfer of gentamicin resistance genes through consumption of poultry meat, especially in the USA, where resistance to this first line agent is relatively frequent in isolates from broiler and turkey meat. Moreover, farm-to-fork transmission of gentamicin resistance genes is plausible, since aminoglycosides are used both in hospitals and in livestock production, but hardly ever used for systemic antimicrobial therapy in the primary health care sector because of parenteral administration and high toxicity. A possible poultry reservoir of quinupristin/dalfopristin-resistant *E. faecium* cannot be excluded on the basis of prevalence data. However, the lack of epidemiological data on strains of human and animal origin hampers quantification of this zoonotic risk. VRE strains causing human infections are not obviously linked to animals as indicated by the fact that they are prevalent in countries where avoparcin has never been used in livestock and VRE are rare or absent in livestock. Indigenous anaerobes in the patient's digestive tract seem to be a more important source of VanB operons than farm animals. As for VanA operons, farm animals have been a reservoir of these vancomycin resistance determinants, as indicated by the recovery of indistinguishable Tn*1546* variants in clinical and animal VREf isolates. However, this zoonotic risk has been significantly reduced by the ban of avoparcin and by the consequent decrease of VRE in livestock.

Little is known about the zoonotic risks associated with direct exposure to animals, including farm and companion animals. The risk of foodborne transmission is significantly higher for poultry meat than for other food products of animal origin, mainly due to lower hygienic standards and higher risk of carcass contamination in poultry slaughtering. Although horizontal transfer of resistance genes from animal

to human enterococci has been demonstrated to occur in human digestive tract under *in vivo* conditions, the magnitude and clinical significance of this phenomenon remain unclear. Further insights into the ecology and epidemiology of MGEs carrying resistance genes of clinical relevance are needed to clarify the public health impact of horizontal gene transfer of antimicrobial resistance from animal to human enterococci.

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Chapter 17
Infections With Multidrug-Resistant Bacteria—Has the Post-Antibiotic Era Arrived in Companion Animals?

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Abstract The increasing prevalence of infectious diseases caused by drug- and multidrug-resistant pathogenic bacteria in companion animals (dogs, cats, horses), particularly in veterinary hospitals, is a worrisome development. Regarding companion animals, currently the four clinically most important groups of multidrug-resistant pathogenic bacteria are methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA), methicillin-resistant *S. pseudintermedius* (MRSP), Extended-ß-lactamase-producing (ESBL) Enterobacteriaceae and multidrug-resistant *Acinetobacter* (*A.*) *baumannii*. Infections caused by these bacteria are often associated with clinical settings and involve mostly wound, skin, ear or urinary tract infections. *S. pseudintermedius* is a typical cause of canine skin infections and until recently regarded as being host-specific. However, the epidemic spread of MRSP together with the changing socio-cultural interaction between companion animals and humans has already resulted in human cases of MRSP infections. Just the opposite development was observed with MRSA. Here, typical hospital-associated (HA) genotypes originating from humans spread into companion animals, now being a substantial cause of disease. In both cases, typical non-zoonotic bacteria turned into zoonotic agents. These findings are just the tip of the iceberg when it comes to the influence of antimicrobial drug usage and multidrug-resistance in speeding up microbial evolution. Concerted action is urgently needed to slow down these processes.

17.1 The Role of Socio-Cultural Developments for the Transmission of Multidrug-Resistant Bacteria Between Man and Companion Animals

The domestication of wild animals is regarded as a pivotal threshold in human evolution, a well-known fact that gives evidence for the prehistoric human relationships with companion animals (Zeder 2006). In this chapter we concentrate on the

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importance of three companion animals, i.e. dogs, cats and horses, as sources or targets of infections with four multidrug-resistant zoonotic bacterial pathogens. We consider the ongoing socio-cultural changes in the relationship between companion animals and humans as a highly important matter in this respect. Here we briefly describe the domestication of dogs as one example of this ongoing development. This is meant to just give a flavor of our vision in terms of the possibility of an exchange of zoonotic pathogens between companion animals and their owners. We envision that the ever closer relationship will increase the risk of exchange of microbiota, and thus also of pathogens.

Although the dog was the first domesticated animal the idea based on molecular clock estimates from mitochondrial DNA that domestication started about 135,000 years (Vilà et al. 1997) is not without controversy. More circumspect estimates of zooarcheologists lead to the conclusion that dog domestication began about 15,000 years ago. Since even a broad analysis of 49,024 autosomal SNPs in 1375 dogs (representing 35 breeds) and 19 wolves failed in unraveling the history of dog domestication, next-generation sequencing of modern and ancient dog genomes is needed to settle this debate (Larson et al. 2012). Nevertheless, DNA analysis revealed that American dogs were more similar to dogs from the Old World than to gray wolves of North America. This outcome supports the idea that dogs accompanied humans crossing the Bering Strait in the late Pleistocene (Leonard et al. 2002), who consequently availed from the dogs abilities in hunting, protection and as pack animals. Moreover, during the process of domestication, dogs have been selected for a set of social-cognitive abilities that enable them to communicate with humans in unique ways (Hare et al. 2002). The archeological finds of canine bones revealed distinctions against contemporary wolves that were pronounced in both skulls and dentition and could be interpreted as results of human intervention in natural selection or may be markers of an early commensal relationship with humans as so called "camp-follower scavengers" (Murugaiyan et al. 2014; Zeder 2006). Skull shape modifications were probably the result of changes in dogs' brain morphology with a decrease of overall size relative to that of wolves and an increase in brain-to-body-size ratio (Schoenebeck and Ostrander 2013; Zeder et al. 2012).

In 2010, the estimated percentage of European households owning at least one dog or one cat was 27 and 24 %, respectively, a demonstration of the tight bond between mankind and domesticated animals, particularly canides, in postindustrial societies. Moreover, if it comes to numbers, 73.643.400 dogs and 84.705.500 cats were counted for European households (fediaf.org 2010). Since people are willing to spend a lot of money for their beloved companions not only for costly extensive medical treatments of seriously ill or elderly patients, but also for a wide range of unusual consumer products and services, pet animals contribute significantly to the economy (Walsh 2009). In 2010 the estimated combined annual turnover of pet food industry and related supply and services throughout Europe reached about € 24 billion (fediaf.org 2010).

It seems to be common knowledge that the human-animal interactions possess the potential to benefit human mental and physical health and wellbeing (O'Haire 2010). However, study results towards the association between pet ownership and

human health were frequently found contradictory (McNicholas et al. 2005). Regarding the psychological part, the most important benefit of pet ownership was identified in terms of companionship (Duvall Antonacopoulos and Pychyl 2010). Nonetheless, a closer look at the complex psychological nature of human and companion animal relationships revealed that the degree of attachment (to the animal) is a stronger predictor of psychological distress than gender, marital status, age, and number of people within a household. However, the causal direction of this association remains unknown at present and needs to be further investigated (Peacock et al. 2012). Pet ownership in elderly people (≥65 years) is frequently discussed as a possibility to avoid loneliness and depression, a rising problem due to population aging in Western societies. Interestingly, only for those people who were both, divorced and living alone, pet ownership demonstrated the potential for being associated with greater satisfaction with life (Himsworth and Rock 2013). Thus, chronically disconnected people tend to substitute social contacts by companion animals (Serpell 2003; Zeder 2006). Moreover, a number of studies confirmed the positive impact of interactions with companion animals on physical health, particularly in patients with heart disease and chronic diseases like cancer (Friedmann and Thomas 1995; Johnson et al. 2003). Anderson et al. (1992) correlated pet ownership with cardiovascular benefits, e. g. lower systolic blood pressure, plasma cholesterol (men only) and triglyceride levels (Anderson et al. 1992).

The socio-cultural background including traditions seems to influence the orientation of companion animal owners towards their animal: Study results by Blouin et al. (2013) suggest that among dog owners, one group values mostly the usefulness of their dog (e.g. protection), another provide the status of surrogate humans, and the third group views the dogs as valuable companions including own (animal) interests (Blouin (2013). The second group uses anthropomorphism, a term which can be defined as the attribution of human mental states (thoughts, feelings, motivations and beliefs) to non-human creatures (Serpell 2003). Moreover, anthropomorphism induces a certain kind of evolutionary selection pressure and a variety of corresponding adaptations, which were not exclusively linked to animal welfare, resulting in a novel ecological niche (Serpell 2003). Beside the socio-cultural effects of this equalization of humans and companion animals, the transmission risk for pathogens rises as a result of offering certain human privileges to companion animals. This should be highlighted by the following study results: Of 102 dog owners, who attended a dog show event in 2009, 88.9% reported to allow at least one dog in the house, 68.5% allow the dog(s) to rest on the sofa, 39.8% allow their dogs to come onto the bed, 93.5% let them lick their hands and 52.8% let them lick their face (Walther et al. 2012b). These exemplarily co-habitation and behavioral pattern give cause to concern with respect to the fact that 60% of all human pathogens were generally regarded as zoonotic (Cleaveland et al. 2001; Woolhouse and Gowtage-Sequeria 2005) and a rising number of opportunistic bacteria exhibit multidrug resistance (Chuang et al. 2010; Stegmann et al. 2010; Wieler et al. 2011). Recent work published by Song et al. proving that family members share microbiota with their dogs, in particular regarding skin microbiota, gives further evidence on the extensive exchange of microbes (Song et al. 2013).

When new zoonotic pathogens emerge, a frequently chosen strategy is to search for the most important infections source, ignoring other species that might strongly influence transmission (Brisson et al. 2011). A considerable problem arises when scientists use the term "reservoir" in this context. Although "reservoir" is defined as the natural permanent infection source of a certain pathogen, some scientists assign rather deliberately any infection source identified as reservoir. This implies that eradication of the respective pathogen in this reservoir will be the key for intervention. This can be illustrated with the emergence of so-called "livestock-associated" (LA-) MRSA. The first report of colonized members of a farmer family and their pigs in the Netherlands in 2004 (Voss et al. 2005) was followed by a rush of scientists from different disciplines in stables, flocks and cages. Early focusing on a limited number of possible host species as infection source can delay biological understanding. This may cause misguiding public health interventions as the important host species of either amplifying or transmissing the pathogen are overlooked (Brisson et al. 2011; Walther et al. 2012a). In case of LA-MRSA, a broad spread took place since 2005, including infections of horses, dogs, cats and many other species. Similarly, the new MRSA variant harboring *mec*C, initially described for humans and ruminants, was subsequently also found in diseased companion animals (Walther et al. 2012a). The finding that particular genetic lineages of Staphylococci are able to infect several host species led to the introduction of the paradigma of extended host spectrum genotypes (EHSG) (Walther et al. 2009).

Knowledge concerning the risks associated with infections of companion animals with multidrug-resistance bacteria is rather limited (Stull et al. 2012). In consequence, interdisciplinary efforts are required to fulfill the demands of zoonotic disease information for both companion animal and non-owning households (Stull et al. 2012). We consider surveillance efforts in terms of a "One health" umbrella as the key starting point. Here we summarize knowledge on four multidrug-resistant bacterial species. We highlight the knowledge gaps of these increasing important in terms of disease burden in companion animals and their zoonotic potential.

17.2 Methicillin-Resistant *Staphylococcus aureus* in Companion Animals

The natural habitat of *Staphylococcus (S.) aureus*, a Gram-positive coccus, is the skin and the anterior nares of humans. Approximately 30% of humans are permanently while up to 50% are transiently colonized. Besides, various animals can be carriers. As an opportunistic pathogen, *S. aureus* plays an important role in both human and veterinary medicine, affecting a broad range of animals.

Methicillin-resistant *S. aureus* (MRSA) are resistant against all ß-lactam antibiotics mediated by the acquisition of the Penicillin-binding protein 2a (PBP2a) encoding *mec*A- or *mec*C-gene. PBP2 is expressed by all *S. aureus* and catalyzes two enzymatic reactions (transpeptidation and transglycosilation). By binding ß-lactam antibiotics, the transpeptidase of PBP2 loses its function which mediates

the bactericidal effect. PBP2a acts as an additional transpeptidase and shows, unlike the native PBP2, a low affinity to ß-lactam antibiotics, thus it compensates the loss of function in PBP2 after therapy with ß-lactams.

Only a short time after the introduction of methicillin, MRSA-infections were reported with an increasing number in hospitals as well as other healthcare settings. This observed restriction led to the denomination of those genotypes as hospital-associated (HA-) MRSA. Along with methicillin-resistance, *S. aureus* frequently acquire resistance against various antimicrobials, leading to reduced therapeutic options. In human medicine, MRSA is one of the most important nosocomial pathogens that provides a significant burden to healthcare settings due to extended therapy, prolonged hospital stay and higher costs (Köck et al. 2010). During the 1990s the MRSA epidemiology changed, as MRSA-infections were no longer restricted to healthcare settings, but were increasingly observed in humans outside the hospital. In comparison to well-characterized HA-MRSA, these strains belonged to different genetic lineages and were termed as community-associated (CA-) MRSA (Chambers 2001). Nowadays, MRSA are important pathogens in both healthcare settings and surroundings without exposure to hospital environments (Köck et al. 2014).

It was not until the late 1990s that the awareness of MRSA as cause of infections in animals increased based on several case reports. In 2004, MRSA of a specific genetic lineage, namely CC398-MRSA, emerged as frequent colonizers of pigs and farmers in close contact. The initial assumed host restriction to swine and other livestock resulted in the denomination as Livestock-associated (LA-) MRSA. Since then, CC398-MRSA have been reported in humans and several other animal species (Köck et al. 2014).

Meanwhile, MRSA have been detected in a wide range of animals. In particular companion animals are regularly identified to be colonized and/or infected with MRSA. The main infection sites in dogs and cats include wounds, urogenital tract, skin and mucosa as well as ears (Walther et al. 2008). Data concerning colonization in dogs and cats are rare and difficult to compare due to heterogeneous study settings. However, these surveys describe colonization in dogs and cats with rates from zero to 4 % in healthy dogs and cats without clinical background and up to 9 % for dogs at a veterinary clinic (Weese 2010). Although there is a lack of knowledge concerning the colonization length, first evidence exists that MRSA-carriage is transient in dogs and cats (Loeffler et al. 2010b). The genotypes of canine and feline MRSA mirror predominant human HA-associated MRSA-lineages within the same geographic region like clonal complexes (CC)8 in France (Haenni et al. 2012), CC5 and CC22 in Germany (Vincze et al. 2013) and CC22 and CC30 in the United Kingdom (Loeffler et al. 2010a), indicating a spill-over from human hospitals to dogs and cats. In addition, CC398-MRSA can be detected in dogs and cats on a regular basis, underlining the potential of this lineage to colonize and infect not only livestock and humans but dogs and cats as well (Köck et al. 2014; Vincze et al. 2014).

In horses, MRSA-infections were described only occasionally after the first report in 1989 and it was not before the mid-1990s that an increasing number of equine MRSA-cases demonstrated the importance of this pathogen. Infections can be associated with both horses in clinical environments and without exposure to

clinics. While nosocomial outbreaks in equine clinics occur on a regular basis resulting in a large number of surgical site infections, MRSA-infections also have a serious impact in sporadic outbreaks without clinical background. Common infections without hospital background include joint, incision, and skin or soft tissue infections (Weese 2010). Similar to colonization studies in dogs and cats, data for horses are difficult to compare and range, depending on the investigated population, from 0 to 5% in horses from farms, up to 11% in horses admitted to equine clinics (van Duijkeren et al. 2010). In contrast to strains from dogs and cats, equine MRSA-lineages do not mirror common regional human lineages. Instead, CC8-MRSA, a prior human epidemic clone with sparse occurrence currently in humans, was reported as most common lineage in horses, leading to speculations on putative advantages of this lineage in equine colonization and infection (Weese 2010). Only recently, CC398-MRSA were reported in several horses from different European countries like Belgium, France and The Netherlands (Van den Eede et al. 2009; van Duijkeren et al. 2010). Recent data point towards the microevolution of a horse-adapted sublineage of ST398 (Abdelbary et al. 2014).

Transmission events between companion animals and humans have been described regularly, showing either humans as infection or colonization source for dogs, cats and horses or vice versa (Faires et al. 2009; Ferreira et al. 2011; Köck et al. 2014; Manian 2003; Weese et al. 2005). Even though, there is evidence for zoonotic transmission between companion animals and humans, longitudinal studies that address the frequency and impact of these events are still missing. Since dogs and cats are regularly infected with MRSA-lineages similar to prevalent regional genotypes causing infections in humans, transmission might be an important factor for colonization and/or infection in both directions. These events need to be unraveled, therefore longitudinal studies as well as case-control studies are urgently needed. The above discussed changes in the socio-cultural relationship between humans and companion animals clearly provide more opportunities for transmission (Walther et al. 2012b). Consequently, owners of MRSA-infected dogs were found more often nasally colonized with MRSA than the average population in Great Britain. Thus, MRSA-infected dogs are already identified as a risk factor for human colonization (Loeffler et al. 2010a).

So far, genetic lineages that were recognized as cause of infections in dogs, cats and horses did not show specific restrictions to one or a limited number of hosts. In contrast, MRSA of these genotypes seem to possess the potential to infect and/or colonize humans as well. Genotypes with the ability to infect a broad host spectrum were termed as extended host spectrum genotypes (EHSG) (Köck et al. 2014; Walther et al. 2009). With the knowledge that (i) MRSA is an important pathogen in companion animal medicine, (ii) MRSA from companion animals are EHSG, (iii) ownership of an MRSA-infected companion animal results in a higher risk of human colonization, and (iv) epidemiology changes over time as proven for MRSA in horses, monitoring and surveillance utilizing molecular typing of MRSA in companion animals is crucial to understand dynamics and spread of MRSA in companion animals.

17.3 Extended-Spectrum Beta-Lactamase (ESBL)-Producing Enterobacteriaceae

Extended-spectrum beta-Lactamases (ESBL) are a diverse group of hydrolyzing enzymes produced by Gram-negative bacteria that inactivate a wide range of beta-lactam antibiotics including broad-spectrum penicillin and cephalosporin up to the 3rd generation. ESBL enzymes in general are encoded on plasmids. They can still be inhibited by clavulanic acid which separates ESBL from AmpC, another type group of beta-lactamases. The *ampC* genes are often chromosomally encoded and occur frequently in Enterobacteriaceae. Their overexpression due to mutations confers broad-spectrum beta-lactam resistance as well.

As carbapenems are not inactivated by ESBL-or AmpC producers, they often present the last treatment opportunity. This is due to the fact that ESBL-producing Enterobacteriaceae often display additional resistances to several other classes of antimicrobial substances, resulting in multidrug-resistant phenotypes. Therefore, ESBLs are a worrying global public health issue as infections caused by these multidrug-resistant organisms are associated with a higher morbidity and mortality and a greater fiscal burden (Dhillon and Clark 2012). Taking into account the increasing worldwide prevalence (central Europe approx. 10% of clinical *E. coli*) and an ever diminishing supply in the antibiotic armamentarium, ESBLs represent a clear and present danger to public health (Dhillon and Clark 2012). Although ESBLs are often commensals, they represent primary or secondary pathogens in healthy individuals in a wide range of diseases such as urinary tract infections, wound infections, pneumonia and septicaemia (Canton and Coque 2006). Cephalosporins are among the most commonly used antimicrobial classes in companion animal medicine, therefore increasing resistance against this class also presents an emerging problem for veterinarians.

Initially ESBL-producing Enterobacteriaceae were only observed in clinical settings where the first nosocomial outbreaks were recorded in clinics in Central Europe in the late 1980s. Since then an explosive worldwide proliferation of ESBLs in human clinical settings has been observed (Ewers et al. 2012). Within the last decade a community-associated onset has taken place. In between ESBL-producing Enterobacteriaceae are isolated from a wide range of companion animals, livestock, environmental samples and wildlife all over the world (Guenther et al. 2011). These ESBL-producing Enterobacteriaceae of animal origin did not only present resistant commensals, but included strains relevant to human health underlining the zoonotic character of ESBL (Carattoli 2008; Ewers et al. 2011a; Hasman et al. 2005; Overdevest et al. 2011; Smet et al. 2010). Considering that horizontally acquired plasmids are encoding these resistances, it is obvious that these developments further enrich the resistome (Gaze et al. 2013).

ESBL-production in companion animals is primarily associated with *Escherichia coli*, but can also occur in other pathogenic Enterobacteriaceae such as *Citrobacter*, *Proteus*, *Salmonella* and *Klebsiella* (Ewers et al. 2011b). About a decade after

the increased numbers of nosocomial infections in human medicine the numbers of ESBL involved in nosocomial infections of companion animals is also rising.

At present over 200 ESBL types are known and have been classified into three major classes: CTX-M, SHV and TEM. These ESBL-encoding genes are frequently located on plasmids, which harbour additional resistance genes and can be transmitted between different Enterobacteriaceae species. Amongst companion animals, ESBLs are mostly described in dogs, cats and horses, whereas their prevalence in other companion animals still remains unknown yet (Ewers et al. 2012). ESBL-producing Enterobacteriaceae are mostly spread by the faecal-oral route and transmission can occur either by direct contact or indirect by contaminated food and water.

As mentioned above different enzymes encode for ESBL-production and the ones present in companion animals are basically the same than the ones, which have been found in human isolates. The first descriptions in companion animals date back to the late 1990's and these early studies reported the presence of mainly TEM and SHV genes in clinical isolates from dogs in Spain, Portugal and Italy (Feria et al. 2002; Teshager et al. 2000).

However, similar to the situation in humans also in companion animals a shift towards CTX-M-genes seems to have taken place over the last decade. These CTX-M-genes have been detected in 2.6–5.6% of all reported clinical and commensal Enterobacteriaceae and overall this gene type accounts for about 25–76% of all Extended-spectrum beta-lactamases originating from companion animals (Ewers et al. 2011b). $Bla_{\text{CTX-M}}$-genes are particularly common in companion animal isolates from Europe, but can also often be found in isolates originating from America, Australia and Asia (Ewers et al. 2012). The most frequent CTX-M-types reported in companion animals are CTX-M-15 and CTX-M-1 (Damborg et al. 2011; Damborg et al. 2012; Dierikx et al. 2012, Dolejska et al. 2011; Ewers et al. 2010; Schink et al. 2011).

Extensive studies on risk factors concerning ESBL-carriage in companion animals are missing yet. However, risk factors identified in human medicine seem to be relevant in the veterinary context as well. Indeed, some studies showed that hospitalization and antimicrobial treatment—in particular with cephalosporins—appear to select for ESBL in horses and dogs (Damborg et al. 2011; Damborg et al. 2012; Dolejska et al. 2011; Maddox et al. 2012). Taking into account these main risk factors, general infection control measures and rational antibiotic use appear to be important steps in the prevention of an intra- and interspecies spread of ESBL-producing Enterobacteriaceae.

Regarding the zoonotic risk of ESBL-bacteria there is currently only limited evidence for a transmission between closely interacting humans and companion animals. One study reported the presence of the same *E. coli* clone harbouring a CTX-M-1 type beta-lactamase in a human and several horses in the same riding centre (Dolejska et al. 2011). Nevertheless, potentially zoonotic ESBL-strains exist in companion animals. One example is the pandemic multidrug-resistant and virulent *E. coli* lineage B2-O25b:H4-ST131-CTX-M15, which represents the dominant ESBL-isolate in most European countries and all over the world (Livermore et al. 2007). Recent studies have reported this lineage to be occurring in companion

animals as well as—also in association with CTX-M-15– among *E. coli* isolates from dogs, cats and horses worldwide (Albrechtova et al. 2012; Ewers et al. 2010; Timofte et al. 2011).

This illustrates that besides the same genes, identical clonal lineages are also shared between humans and companion animals. Thus it seems that transmission of this recently evolved clone has taken place between humans and companion animals or vice versa, either directly or indirectly. Research is needed to assess and prevent the human health risk associated with the companion animals, especially in consideration of the frequent use of cephalosporins in small animal medicine.

Summing up the role of companion animals in human infections caused by ESBL-producing Enterobacteriaceae is currently impossible. There is strong evidence that a decade after these bacteria have established in human medicine we observe an identical epidemiology in companion animals. ESBLs in between have established in commensal and potentially pathogenic Enterobacteriaceae of companion animals. Undoubtedly, this could be a valuable source of human infections and vice versa.

17.4 Multidrug-Resistant *Acinetobacter baumannii* and Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP)

This chapter briefly summarizes trends in infections with two bacterial pathogens, as multidrug-resistant variants of these have only recently been detected in animals. Only initial evidence exists about their zoonotic potential. However, as multidrug-resistance is a potent driver towards bacteria successfully entering new habitats (Gaze et al. 2013; Guenther et al. 2011; Holt et al. 2012), we consider an increasing incidence of infections with multidrug-resistant variants of *Acinetobacter* (*A.*) *baumannii* as well as *Staphylococcus* (*S.*) *pseudintermedius* in the near future. *S. pseudintermedius* until recently was regarded as a non-zoonotic pathogen of dogs and horses. However, case reports in humans encouraged us to include this pathogen (Vincze et al. 2010). This even more as both pathogens display multidrug-resistant types which in the case of infections can hardly be treated by antibiotics. Furthermore, both species are of high tenacity, thus survive in the environment for considerable long time periods. Both form biofilms on abiotic surfaces. This leads to increased survival within human and veterinary clinics. These features are a prerequisite for both microbes to act as nosocomial agents, therefore large-scale desinfections are needed in case of nosocomial outbreaks.

A. baumannii is a facultative pathogen that is isolated both from humans and in animals from different diseases such as pneumonia, catheter-associated genital tract infections, septicemia, skin and soft tissues infections. It is mostly seen in immunocompromised patients (Karah et al. 2012; Kim et al. 2013; O'Shea 2012; Qiu et al. 2012; Renckens et al. 2006). As well-known nosocomial pathogen it is associated

with increased morbidity and mortality in the medical field. Infections in companion animals have only recently been reported (Boerlin et al. 2001; Endimiani et al. 2011; Francey et al. 2000; Müller et al. 2014; Vaneechoutte 2000; Zordan et al. 2011). This is why crucial knowledge on the epidemiology is lacking.

Over the last 20 years multidrug-resistant *Acinetobacter* (*A*.) *baumannii*- strains are increasingly isolated from medical patients, mostly associated with infections in clinical settings. Mortalities of 40–75 % for ventilator-associated pneumonia and septicemia in 28–43 % have been reported for humans (McConnell et al. 2013; Qiu et al. 2012). Some 2–10 % of all nosocomial infections in intensive care units are caused by *A. baumannii*. The rate of multidrug-resistant *A. baumannii* isolates increased during 2002–2006 from 2.1 to 7.9 % (Wadl et al. 2010). In between increased isolation rates of multidrug-resistant *A. baumannii* are also observed in veterinary clinics. In cats and dogs *A. baumannii* has been reported to mainly cause wound and urinary tract infections. The latter ones are often associated with catheterized animals in veterinary hospitals therefore nosocomial origins are most probable (Zordan et al. 2011). An identical development is envisioned as already experienced with MRSA, namely the appearance of this multidrug-resistant pathogen first in human medicine and then with a decades' time lag in veterinary settings (Ewers et al. 2012; Ewers et al. 2011b; Vincze et al. 2014; Wieler et al. 2011).

However, substantial epidemiological data are lacking. Even the reservoir of *A. baumannii* is still unknown, but most probably it is the environment as it can be isolated from soil, water and food. Although pathogenic *A. baumannii* are isolated from skin or surfaces or in stool samples, in general the ubiquitous apathogenic *Acinetobacter spp*. are isolated (Mortensen and Skaar 2012; Peleg et al. 2008). Acinetobacter may be part of the physiological microbiota of animals. However, still this hypothesis needs to be falsified. The question of the transmission direction between humans and animals is therefore also open and needs to be further ascertained (Hamouda et al. 2011; Müller et al. 2014; Peleg et al. 2008). As it is currently not possible to unequivocally identify *A. baumannii* by phenotypical or MALDI-TOF methods it is not always clear whether actually *A. baumannii* was identified or an isolate belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (*Acb* complex) (Dijkshoorn et al. 2007; Peleg et al. 2008). Therefore molecular methods like analysis of the 16S-23S rRNA-gene spacer region, *bla*-OXA-51-like gene, *rpo*B gene or whole genome sequence analysis are increasingly implemented (Chang et al. 2005). As each of the four species belonging to the Acb complex differ in clinical course and thus in different treatment options a correct species identification is of utmost importance (Espinal et al. 2012).

A. baumannii are naturally resistant against trimethoprim. In addition, they have a tendency to develop resistance extremely rapidly by up-regulation of efflux pumps or acquisition of resistant determinants. The intrinsic resistance mechanisms include the small number of porins, the AmpC cephalosporinase (Hamouda et al. 2011) and the multidrug efflux pump AbeABC. Different classes of antibiotics such as aminoglycosides, chloramphenicol, tetracyclines, fluoroquinolones, trimethoprim, and beta-lactams including Carbapenemene can be ejected as substrates (Gootz and Marra 2008; Müller et al. 2014). Resistance to fluoroquinolones is based primarily on mutations in the genes *gyrA* and *parC*, which encode a DNA gyrase

and topoisomerase IV (Peleg et al. 2008). Genomic analysis of multidrug-resistant *A. baumannii* strains demonstrated that the genes that mediate resistance are usually encoded on so-called resistance islands. The 86-kb AbaR1 Island encoding 45 resistance genes is the largest island of resistance described (Fournier et al. 2006). *A. baumannii* has mechanisms that favor horizontal gene transfer and thus the rapid development of multidrug resistant (MDR=multidrug resistant) strains (Dijkshoorn et al. 2007; Giamarellou 2008; Müller et al. 2014; O'Shea 2012). This is why *A. baumannii* genomes harbor a large number of mobile genetic elements such as transposons, integrons class I or insertion sequences (Overdevest et al. 2011; Peleg et al. 2008). Thus in *A. baumannii* isolates ESBLs and Oxacillinases are seen, some even being carbapenemases (Gootz and Marra 2008; Peleg et al. 2008). Carbapenems are currently the most effective antibiotic in the treatment of *A. baumannii* infections. Resistance to these antibiotics significantly reduces therapeutic options. Meanwhile *A. baumannii* strains have been isolated that are resistant to all classes of commercially available antibiotics, which for both medical and veterinary doctors is a tremendous challenge (Dijkshoorn et al. 2007). Some authors argue that regarding *A. baumannii* we are closer to the end of the antibiotic era as with MRSA (Giamarellou et al. 2008; Müller et al. 2014).

Most infections in humans are caused by the so-called EU or international clones I-III. These are characterized by a high level of antibiotic resistance (Dijkshoorn et al. 2007; Giamarellou et al. 2008; Karah et al. 2012; Seifert et al. 2005). *A. baumannii* isolates belonging to these EU clones have also been responsible for outbreaks in German veterinary clinics and veterinary practices (Endimiani et al. 2011; Müller et al. 2014; Vaneechoutte et al. 2000; Zordan et al. 2011). It is clear that *A. baumannii* is a pathogen with zoonotic potential.

A totally different perspective is given by *S. pseudintermedius*. The bacterial species has previously been known as *S. intermedius*, one of the most important causes of pyodermia and otitis, particularly in dogs. In 2005, Devriese et al. described a novel coagulase positive staphylococcal (CPS) species, denominated as *S. pseudintermedius* (Devriese et al. 2005). In the following years, molecular taxonomic investigations revealed that *S. pseudintermedius*, *S. delphini* and *S. intermedius* are closely related CPS species referred to as staphylococci of the intermedius group (SIG). While *S. intermedius* seems to be commonly associated with pigeons, *S. pseudintermedius* is not only a common colonizer of the canine skin, but also an opportunistic pathogen frequently isolated from clinical specimens of dogs, and, to a lesser extent, of cats and various other host species (Bannoehr et al. 2007; Bannoehr and Guardabassi 2012; Kadlec et al. 2010; Ruscher et al. 2008; Sasaki et al. 2007; Solyman et al. 2013). These findings have led to the realization that most canine isolates previously identified as *S. intermedius* should have been classified as *S. pseudintermedius* (Bannoehr et al. 2009; Sasaki et al. 2007). While biochemical features seem to vary among isolates of the same SIG species, sequence based methods, analysis by MALDI-TOF (Matrix Assisted Laser Desorption Ionisation-Time Of Flight Mass Spectrometry) and polymerase chain reaction-restriction length polymorphism (PCR-RFLP) are considered as reliable identification tools for *S. pseudintermedius* (Bannoehr and Guardabassi 2012; Murugaiyan et al. 2014; Savini et al. 2012).

The first methicillin resistant variants of *S. pseudintermedius* were reported sporadically in the late 1990s (Gortel et al. 1999; Piriz et al. 1996). Only a few years later, a sudden rise of MRSP reports followed and meanwhile MRSP are among the most important therapeutic challenges due to their frequent multi-drug resistance phenotype world-wide (Bemis et al. 2009; Perreten et al. 2010; Ruscher et al. 2010). Moreover, MRSP are of particular concern with respect to the increase of nosocomial infections in veterinary medicine and their potential transferability to humans and other animals in the household (Perreten et al. 2010; Perreten et al. 2010; Wieler et al. 2011). Consequently, owners of dogs suffering from *S. pseudintermedius*-infections and veterinarians seem to be at higher risk for nasal colonization and/or contamination than other people. Dog owners who keep more than two dogs also have a significantly higher chance to harbor *S. pseudintermedius* in the nose cavity (Frank et al. 2009; Ishihara et al. 2010; Paul et al. 2011; Vincze et al. 2010; Walther et al. 2012b). A high level of domestic MRSP-contamination, probably mediated by loss of hair and epithelia cells of the infected animal patient, may lead to a higher risk of developing MRSP infections in case of surgical or non-surgical wounds for pet owners (van Duijkeren et al. 2011). A sudden increase of reports concerning cases of severe *S. pseudintermedius*-infection in humans is noticeable and seems to reflect these changes in infection ecology (Riegel et al. 2010; Savini et al. 2013; Stegmann et al. 2010; Van Hoovels et al. 2006). The public health impact of the sudden emergence of a multidrug resistant zoonotic pathogen frequently occurring in companion animals like MRSP needs to be the subject of more detailed molecular and epidemiological studies (Loeffler et al. 2010a).

17.5 Conclusions and Outlook

Human interventional therapeutic use and misuse of antimicrobial drugs both in veterinary and human medicine is a major driver for the enrichment of the bacterial resistome. The increasing isolation of multidrug-resistant bacteria from clinical infections, be they zoonotic or non-zoonotic, is a direct consequence of this recent development. While initially key multidrug-resistant bacteria like MRSA, ESBLs or *A. baumannii* have been isolated from medical clinics, their spread into the community was followed by infections in companion animals. In between, first cases of euthanasia due to failing therapy have been reported. These cases are vivid proof of a post-antibiotic era. Besides the clinical failure of antimicrobial drugs, multidrug-resistant bacteria show a change in habitat specificity in that they enable the bacteria to spread into previously unavailable habitats (Guenther et al. 2011; Holt et al. 2012). One particularly worrisome outcome of this development is the proven spread of MRSA into companion animals, leading not only to nosocomial infections in veterinary hospitals, but also to ongoing transmission between companion animals and their owners (Fig. 17.1). Similar trends are evidenced regarding ESBL-producing Enterobacteriaceae and multidrug-resistant *A. baumannii*. These worrisome developments have fostered the initiation of the EU-wide project CALLISTO to give

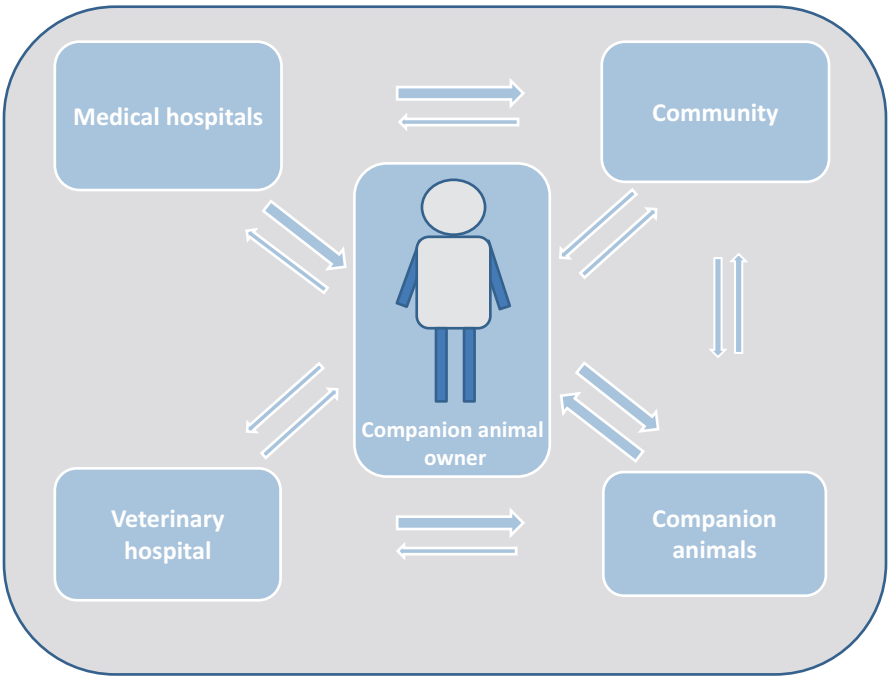

Fig. 17.1 Transmission routes of multi-resistant bacteria between companion animals and humans

ground on further scientific steps in developing strategies against these zoonotic agents (http://www.callistoproject.eu/joomla/). In the case of *S. pseudintermedius* a tendency is observed that this naturally species-specific facultative pathogen is able to infect humans by gaining multidrug resistance. The socio-cultural changes in the co-existence between humans and their companion animals foster these developments. The link between multidrug-resistance and increased zoonotic potential of bacterial pathogens is a key evolutionary challenge we have to tackle. The only way of success is a multi- and interdisciplinary approach, the start of which is a global molecular driven surveillance of multidrug-resistant bacteria. This can only be successful when implemented by a "One health" approach. Such a surveillance will unravel both the microevolution of these bacteria and the directionality of transmission. These data are needed as a base for future intervention studies. However, regardless of these larger research efforts—prudent use of antimicrobial drugs must be a prerequisite for each and everyone in the world of antimicrobial drug usage.

There is a lack of knowledge on their prevalence in veterinary medicine based on the fact that no surveillance is in place. Clearly, effective preventive measures can only be installed based on unequivocal identification and representative epidemiological studies. Here the lack of knowledge is immense. As potential source of zoonotic transmission, data concerning colonization and infection should be determined and monitored continuously. Further, interdisciplinary studies are needed to determine the frequency of colonization and/or infection in owners and their

animals with the same clone. These data can help to identify the impact of companion animals as source of human infection and/or colonization and could raise the owner's awareness that companion animals can be a source of pathogen transmission in general, and in particular for MRSA.

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Part III
Important zoonoses in non-food animals

Chapter 18
Influenza from a One Health Perspective: Infection by a Highly Versatile Virus

Leslie A. Reperant and Albert D.M.E. Osterhaus

Abstract Influenza A viruses (IAVs) are among the most versatile viruses, in terms of host range, pathogenesis, route of transmission, transmissibility efficiency, and evolutionary dynamics. Several IAVs are recognized pathogens of a wide range of avian and mammalian species. On the one hand, they cause mild or asymptomatic infections of the intestinal tract in wild water birds, their natural reservoirs, resulting in annual epidemic cycles fueled by IAV fecal-oral transmission in watershed habitats. On the other hand, they may cause disease manifestations in poultry and mammalian species, including humans, upon sporadic cross-species transmission and during self-limiting outbreaks or large-scale airborne epidemics. In these spill-over host species, clinical signs and symptoms range from inapparent to severe and often fatal respiratory and extra-respiratory conditions. A feature of IAVs in domestic spill-over hosts and humans is their ability to adapt to these new species to eventually be maintained independently of new introductions from their natural host reservoirs. In humans, IAVs of animal origin can be the precursors of pandemic influenza viruses. These pandemic viruses eventually evolve into seasonal influenza viruses that cause recurring epidemics of seasonal influenza. Through their versatile nature, IAVs are a striking example of the flexible nature of zoonotic threats and of the richness of avenues zoonotic pathogens can take to burden public health.

18.1 Introduction

IAVs are Orthomyxoviruses closely related to influenza B and C viruses (Palese and Shaw 2007). They are enveloped single-stranded negative-sense RNA viruses with eight gene segments. These include two genes coding for the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, and six genes coding for several

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other internal proteins, including the polymerase complex, matrix and non-structural proteins. The surface glycoproteins HA and NA interact with cellular receptors and are important antigenic targets of the host's specific immunity mediated by neutralizing and other biologically active antibodies. Most internal proteins are involved in IAV structure or replication.

The replication of IAV RNA genome lacks effective exonuclease proofreading capability, and is known to introduce base mutations at relatively high rates. In addition, the segmented genome allows for reassortment during co-infection with different IAVs, resulting in new viruses containing gene segments of mixed parental origin. The high mutation rate and extensive reassortment have led to an unmatched diversity of IAV lineages (Chen and Holmes 2006). Based on the HA gene, IAVs may have emerged 4000 years ago, from a common ancestor with influenza B viruses, while the split between influenza A/B and influenza C viruses is estimated to have occurred some 8000 years ago (Suzuki and Nei 2002). IAV HA and NA genes are grouped into putatively 18 and 11 different subtypes, respectively. Although most IAV HA and NA subtypes likely split 2000 to 3000 years ago, IAV diversity within each subtype is fairly recent, dating from the past hundred to several hundred years (Suzuki and Nei 2002; Chen and Holmes 2006).

Several IAVs are recognized pathogens of a wide range of avian and mammalian species, and their natural history in their wide host range clearly illustrates the One Health principle, whereby human health, animal health and ecosystem health are closely inter-related. Wild water birds are the natural reservoirs of IAVs (Webster et al. 1992), yet IAV lineages, acquired upon cross-species transmission, have established in domestic animal species. In turn, domestic animals, especially poultry and swine, are the main sources of zoonotic IAV infections. Zoonotic IAVs may spark influenza pandemics in humans, should they acquire efficient human-to-human transmissibility, which they lack upon cross-species transmission. Pandemic viruses eventually evolve into human seasonal influenza viruses, causing annual epidemics that spread globally. While IAV cross-species transmission has occurred probably since IAV emergence several thousands of years ago, their versatile epidemiology and evolutionary dynamics in an expanding host range have been influenced by the human species, especially during the past few hundred years. The relentless growth of domestic swine and poultry populations in the last decades has contributed to increased genetic diversity of IAVs circulating in domestic animals and occasionally infecting humans, expanding the pool of IAVs with pandemic potential (for review see Reperant and Osterhaus 2012).

In this chapter, we will review IAVs population-level epidemiology, evolutionary dynamics and associated host-level pathogenesis of infection in their wide host range. In addition we will describe the adaptive changes associated with IAVs host switch and their sustained establishment in novel host species.

18.2 Population-Level Epidemiology and Evolutionary Dynamics

18.2.1 Natural Reservoirs

The majority of the currently known diversity of IAVs is maintained in avian hosts of the order Anseriformes, Charadriiformes, and Gruiformes, which encompass water birds such as geese, ducks, gulls, waders, and coots (for review see Olsen et al. 2006; Reperant et al. 2013). Wild freshwater birds are considered IAVs natural reservoirs. IAVs harboring most of the 144 combinations of the first 16 and 9 subtypes of HA and NA genes, respectively, have been isolated from wild water birds (Munster et al. 2007). Avian IAVs natural wide host range, counting more than 110 wild bird species from 13 different orders, likely contributes to IAVs remarkable diversity in these species.

The recent discovery of IAVs H17N10 and H18N11 in bats has unexpectedly revealed the maintenance of IAVs by wild mammalian species (Tong et al. 2012, 2013a). Structural and phylogenetic analyses demonstrated substantial divergence and ancient evolutionary relationship between these viruses and other IAVs, predating the split between influenza A and B viruses (Tong et al. 2012, 2013a; Zhu et al. 2012, 2013). Future studies on IAVs in bats are needed to unveil their epidemiology and the role these mammalian hosts have played in IAV evolutionary history.

Other host species to IAVs can be considered spill-over host species, as they initially acquired IAVs upon cross-species transmission from their ancestral reservoirs (see below). However, IAVs have established in domestic animal species, including poultry, pigs, horses and dogs, and in humans. These species now maintain distinct IAVs and have thus become their natural reservoirs, and new players in IAVs cross-species transmission.

18.2.1.1 Epidemiology

IAV epidemiology in wild water birds is characterized by annual epidemic cycles. IAVs typically infect juvenile birds of the year, immunologically naïve to IAVs, or young individuals with limited history of past infections, that congregate during one stage of their life history (Olsen et al. 2006). Morbidity and mortality burdens associated with IAV infection in wild water birds are low, with little evidence of disease or death of infected birds. Some effects of IAV infection on wild birds' health or behavior have been proposed, such as reduced food intake and delayed or shortened migration, but are difficult to evidence (Kuiken 2013).

Avian IAV epidemiology has been particularly studied in three natural host systems: dabbling ducks, waders and gulls (Fig. 18.1). In dabbling ducks, such as mallards (*Anas platyrhynchos*) and common teals (*A. crecca*), annual epidemics generally occur in juvenile birds in fall, as they congregate after annual molt and prepare for migration (Olsen et al. 2006; Munster et al. 2007). The arrival

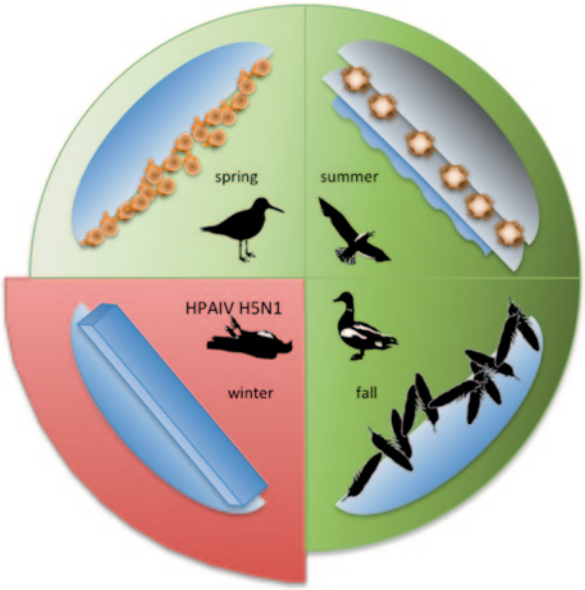

Fig. 18.1 Seasonality of influenza A virus (IAV) outbreaks in wild birds is typically associated with large congregations of birds. IAV epidemics occur in spring in waders, during re-fueling on horseshoe crabs at Delaware Bay; in summer in gulls, when fledging birds leave their nests at breeding colonies; in fall in dabbling ducks, when they congregate before migration. Congregation of water birds over open water bodies along freezing fronts likely fueled highly pathogenic IAV (HPAIV) H5N1 outbreaks in wild birds in Europe during winter of 2005–2006. See text for more details

of migrants from other populations into these congregating groups may play an important role in IAV spread (van Dijk et al. 2013). A seasonal IAV hotspot in waders occurs at the Delaware Bay, in North America, likewise when migrants congregate at this important stopover site during spring (Webster et al. 1992; Hanson et al. 2008). Large populations of waders timely re-fuel during this migratory stopover on spawning horseshoe crabs (*Limulus polyphemus*), creating unique conditions for IAV spread. However, such conditions have not been observed in Europe where IAV prevalence in waders is reported low throughout the year (Olsen et al. 2006; Munster et al. 2007). Lastly, annual IAV epidemics were recently demonstrated in young black-headed gulls (*Chroicocephalus ridibundus*) in northern Europe, when fledging birds from breeding colonies leave their nests in early summer (Verhagen et al. 2014). Targeted sampling of other gull species at the time of fledging may unveil similar IAV dynamics in this family of birds.

The aquatic habitat of wild freshwater birds and their feeding and social behavior likely favor IAV transmission in these species (for review see Olsen et al. 2006; Reperant et al. 2013). IAV infect wild water birds via a fecal-oral and possibly fecal-cloacal route of transmission, which can be enhanced in watershed habitats heavily contaminated by large groups of birds. IAV can persist several months in water, especially at low temperatures and low salinity levels, and such environmental persistence may be important for IAV year-round persistence in wild water birds (Stallknecht and Brown 2009).

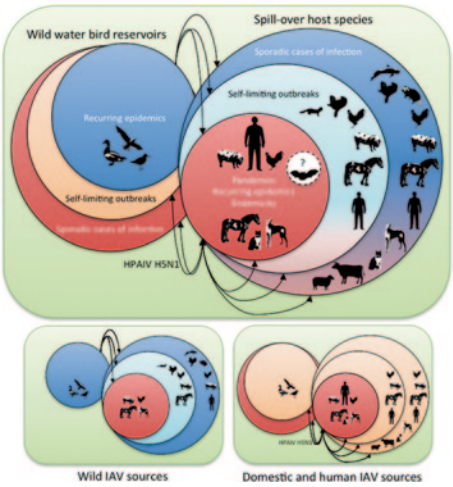

Fig. 18.2 Schematics of influenza A virus (IAV) diversity and evolutionary dynamics in natural reservoir and spill-over host species (based on Dugan et al. 2008**)**. IAV diversity in wild water birds is mainly characterized by co-circulation of a large number of subtypes and lineages and their frequent reassortment; in domestic animal species, by occasional IAV cross-species transmission, reassortment across subtypes and genetic and antigenic drift; and in humans by rare IAV cross-species transmission, reassortment within subtype and genetic and antigenic drift

18.2.1.2 Evolutionary Dynamics

The remarkable diversity of wild bird IAV lineages is puzzling. High mutation rates and frequent reassortment characterize IAV evolutionary dynamics in wild bird reservoirs (Chen and Holmes 2006; Dugan et al. 2008) (Fig. 18.2). However, these dynamics largely differ between genes coding for internal and surface proteins.

IAV internal genes show high levels of genetic identity resulting in highly conserved proteins. It suggests that these genes are under strong purifying selection, have reached fitness peaks, and may not elicit strong immune responses in wild birds. The conservation of these proteins allows widespread reassortment with exchange of functionally equivalent internal gene segments (Dugan et al. 2008). The NS gene may be under some form of balancing selection that has resulted in the co-circulation of two alleles that can be found in combination with any HA and NA subtype. In contrast, while the HA and NA genes within each subtype show high levels of genetic identity, the HA and NA genes of different subtypes are remarkably divergent. The absence of intermediate subtypes indicates strong natural selection likely resulting from cross-immunity. Frequent mixed infections are reported in wild birds, favoring reassortment between HA and NA subtypes, leading to a large number of possible combinations. However, the current diversity of IAVs within each HA and NA subtypes in wild birds is recent and suggestive of population bottlenecks, during which IAV diversity may be periodically purged by the sweep of genes with high fitness, eliminating other lineages (Dugan et al. 2008).

IAVs circulating in gulls are generally distinct from those circulating in ducks and waders, and mostly of the H13 and H16 subtypes. This demonstrates that for these subtypes, lineage divergence likely resulted from physical separation upon host switch. A limited number of IAV subtypes circulating in ducks have evolved in allopatry and geographical isolation, such as H15 present apparently exclusively in Australia. Most IAVs of wild water birds, however, can be grouped in large phylogenetic groups associated with broad geographical regions, corresponding to continents, hemispheres, or major water bird migration flyways, indicating limited geographical separation (Dugan et al. 2008). Reassortment is frequent between IAVs of different gene constellations within these groups, but only occasional between IAVs of distinct geographical phylogenetic groups.

18.2.2 Spill-Over Host Species

Several IAVs infect a wide range of host species other than wild water birds, including poultry, marine mammals, ungulates, carnivores and humans, which can be considered spill-over host species (Table 18.1) (for review see Webster et al. 1992; Reperant et al. 2009; Taubenberger and Kash 2010; Reperant and Osterhaus 2012). In these species, IAVs may cause sporadic cases of infection, self-limiting outbreaks, pandemics and recurring epidemics, or reach endemicity. IAVs infecting spill-over host species, including those currently circulating independently of introductions from wild birds, have all originated from avian IAVs acquired upon cross-species transmission.

In contrast to what is observed in wild water birds, morbidity or mortality burdens can range from inapparent to high. Interestingly, most IAVs isolated from wild spill-over host species cause sporadic cases of infection without further on-going host-to-host transmission, or cause self-limiting outbreaks that eventually die-out in these species. In contrast, most IAV lineages that established and currently circulate in spill-over host species, independently of cross-species transmission, emerged in domestic animals, including poultry, swine, horses and dogs, and in humans (Fig. 18.3). An exception warranting further research is bats, which may maintain distinct IAVs (Tong et al. 2013b). Domestic species and humans, which maintain distinct IAVs, have become their natural reservoirs and new players in their cross-species transmission.

18.2.2.1 Epidemiology

Non-Established IAVs

Sporadic IAV infections or incidental serological evidence thereof have been reported in a wide range of avian and mammalian species, including humans, as well as in amphibians and reptiles (Table 18.1). Animal species that are in direct or indirect

Table 18.1 List of influenza A virus subtypes and lineages detected in spill-over host species. (Based on following reviews: Brown 2000; Swayne and Halvorson 2001; Swayne 2007; Peiris 2009; Reperant et al. 2009; Taubenberger and Kash 2010; Reperant and Osterhaus 2012)

Pathotype	Influenza subtype	Infected species	Species of origin	Detection	Sustained
LP	H1N1	Human (1918–1957/1977–2009)	Unknown	Isolation	Yes
LP	H1N1	Human (1976)	Swine	Isolation	Yes
LP	H1N1	Human (2009–present)	Swine	Isolation	Yes
LP	H1N1	Swine	Avian	Isolation	Yes
LP	H1N1	Swine	Human	Isolation	Yes
LP	H1N1	Turkey	Human	Isolation	Yes
LP	H1N1	Turkey	Human	Isolation	Yes
LP	H1N1	Turkey	Swine	Isolation	Yes
LP	H1N2	Swine	Reassortant avian/swine/human	Isolation	Yes
LP	H1N7	Swine	Reassortant equine/human	Isolation	Yes
LP	H2N2	Human (1957–1968)	Reassortant avian/human	Isolation	Yes
LP	H3N1	Swine	Reassortant avian/swine/human	Isolation	Yes
LP	H3N2	Human (1968–present)	Reassortant avian/human	Isolation	Yes
LP	H3N2	Dog	Avian	Isolation	Yes
LP	H3N2	Swine	Avian	Isolation	Yes
LP	H3N2	Cat	Canine	Isolation	Yes
LP	H3N2	Swine	Human	Isolation	Yes
LP	H3N2	Swine	Reassortant avian/swine/human	Isolation	Yes
LP	H3N3	Harbour seal	Avian	Isolation	Yes
LP	H3N3	Swine	Avian	Isolation	Yes
LP	H3N8	Horse	Avian	Isolation	Yes
LP	H3N8	Dog	Equine	Isolation	Yes
LP	H4N5	Harbour seal	Avian	Isolation	Yes
LP	H4N6	Harbour seal	Avian	Isolation	Yes
LP	H4N6	Swine	Avian	Isolation	Yes
LP	H7N7	Harbour seal	Avian	Isolation	Yes
LP	H7N7	Horse	Avian	Isolation	Yes

Table 18.1 (continued)

Pathotype	Influenza subtype	Infected species	Species of origin	Detection	Sustained
LP	H9N2	Swine	Avian	Isolation	Yes
LP	H10N7	American mink	Avian	Isolation	Yes
LP	H1N1	Human	Swine	Isolation	No
LP	H1N2	Human	Swine	Isolation	No
LP	H3N2	Human	Swine	Isolation	No
LP	H7N2	Human	Avian	Isolation	No
LP	H7N3	Human	Avian	Isolation	No
LP	H7N7	Human	Harbour seal	Isolation	No
LP	H7N9	Human	Avian	Isolation	No
LP	H9N2	Human	Avian	Isolation	No
LP	H10N7	Human	Avian	Isolation	No
LP	H10N8	Human	Avian	Isolation	No
LP	H5N1	Cat	Avian	Isolation	No
HP	H5N1	Dog	Avian	Isolation	No
HP	H5N1	Human	Avian	Isolation	No
HP	H5N1	Leopard	Avian	Isolation	No
HP	H5N1	Owston's palm civet	Avian	Isolation	No
HP	H5N1	Stone marten	Avian	Isolation	No
HP	H5N1	Tiger	Avian	Isolation	No
HP	H7N3	Human	Avian	Isolation	No
HP	H7N7	Human	Avian	Isolation	No
LP	H1	Raccoon	Avian	Serology	Unknown
LP	H1/H3/H7	Bothrops snake	Unknown	Serology	Unknown
LP	H1/H3/H7	Crotalus snake	Unknown	Serology	Unknown
LP	H1/H3/H7	Frog	Unknown	Serology	Unknown
LP	H1/H3/H7	Toad	Unknown	Serology	Unknown
LP	H12	Seals	Unknown	Serology	Unknown
LP	H13N2	Pilot whale	Avian	Isolation	Unknown

Table 18.1 (continued)

Pathotype	Influenza subtype	Infected species	Species of origin	Detection	Sustained
LP	H13N9	Pilot whale	Avian	Isolation	Unknown
LP	H1N1	Skunk	Human	Isolation	Unknown
LP	H1N1	Cattle	Swine	Isolation	Unknown
LP	H1N1	Cattle	Unknown	Serology	Unknown
LP	H1N1	Deer	Unknown	Serology	Unknown
LP	H1N1	Goat	Unknown	Serology	Unknown
LP	H1N1	Sheep	Unknown	Serology	Unknown
LP	H1N3	Balaenopterid whale	Avian	Isolation	Unknown
LP	H2	Sheep	Unknown	Serology	Unknown
LP	H2N2	Cattle	Unknown	Serology	Unknown
LP	H3	Raccoon	Avian	Serology	Unknown
LP	H3	Ringed seal	Unknown	Serology	Unknown
LP	H3	Seals	Unknown	Serology	Unknown
LP	H3N2	Cattle	Human	Isolation	Unknown
LP	H3N2	Cattle	Unknown	Serology	Unknown
LP	H3N2	Deer	Unknown	Serology	Unknown
LP	H3N2	Goat	Unknown	Serology	Unknown
LP	H3N2	Sheep	Unknown	Serology	Unknown
LP	H3N2	Water buffalo	Unknown	Serology	Unknown
LP	H3N2	Yak	Unknown	Serology	Unknown
LP	H4	Seals	Unknown	Serology	Unknown
LP	H4N2	Raccoon	Avian	Serology	Unknown
LP	H4N6	Raccoon	Avian	Serology	Unknown
LP	H7	Ringed seal	Unknown	Serology	Unknown
LP	H7	Seals	Unknown	Serology	Unknown
LP	H7N7	Cattle	Unknown	Serology	Unknown
LP	H7N7	Ringed seal	Unknown	Serology	Unknown
LP	H7N7	Sheep	Unknown	Serology	Unknown
LP	N1/N4/N6	Ringed seal	Unknown	Serology	Unknown

Table 18.1 (continued)

Pathotype	Influenza subtype	Infected species	Species of origin	Detection	Sustained
–	Unknown	Broad-snouted caiman	Avian	PCR	Unknown
–	Unknown	Chinese allogator	Avian	PCR	Unknown
–	Unknown	Nile crocodile	Avian	PCR	Unknown
–	Unknown	Schneider's dwarf caiman	Avian	PCR	Unknown
–	Unknown	Baikal seal	Unknown	Serology	Unknown
–	Unknown	Belugas	Unknown	Serology	Unknown
–	Unknown	Caspian seal	Unknown	Serology	Unknown
–	Unknown	Chinese allogator	Unknown	Serology	Unknown
–	Unknown	Common mink whale	Unknown	Serology	Unknown
–	Unknown	Dall's porpoise	Unknown	Serology	Unknown
–	Unknown	Harp seal	Unknown	Serology	Unknown
–	Unknown	Hooded seal	Unknown	Serology	Unknown
–	Unknown	Reindeer	Unknown	Serology	Unknown
–	Unknown	Ringed seal	Unknown	Serology	Unknown
–	Unknown	Schneider's dwarf caiman	Unknown	Serology	Unknown
–	Unknown	Sea lion	Unknown	Serology	Unknown

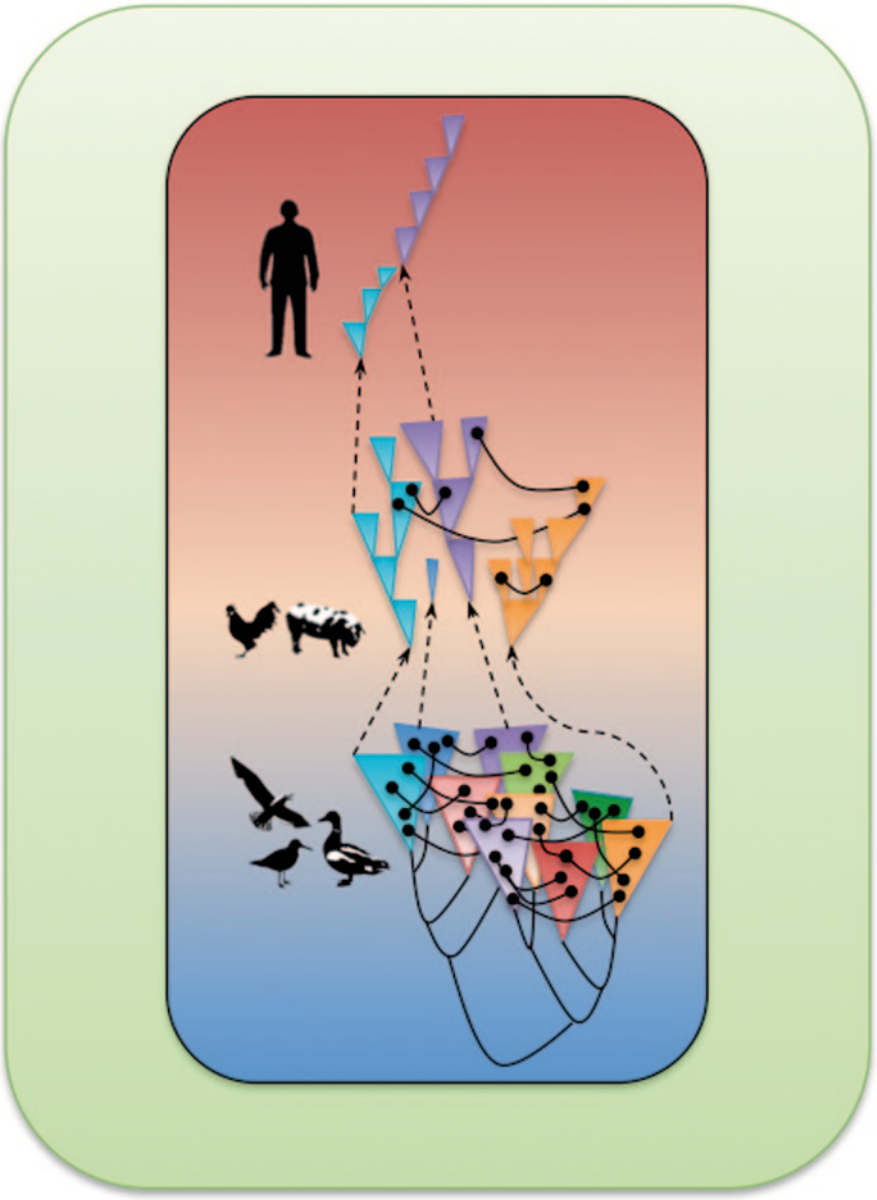

Fig. 18.3 Influenza A virus (IAV) epidemiological dynamics in natural reservoir and spill-over host species. Wild water bird natural reservoirs sustain recurring IAV epidemics and may transmit these viruses to spill-over host species, where they can cause sporadic cases of infection, self-limiting outbreaks, pandemics and recurring epidemics, or reach endemicity. Domestic animals and humans maintain adapted IAVs, and bats may maintain evolutionary distinct IAVs Spill-over species maintaining adapted IAV may also transmit these viruses to other host species, resulting in sporadic cases of infection, self-limiting and sustained outbreaks. These include spill-back infections and self-limiting outbreaks of highly pathogenic IAV (HPAIV) H5N1 in wild water birds

contact with IAV host reservoirs or spill-over host species, e.g. due to predation, shared food and water resources, or overlapping habitat, may become infected with IAVs upon cross-species transmission (for review see Reperant et al. 2009). IAV cross-species transmission between wild and domestic animal species and humans is not infrequent, including IAV transmission from humans to animals (Table 18.1). In humans, serological surveys demonstrated limited zoonotic exposure to IAVs in wild waterfowl hunters and bird banders (Gill et al. 2006; Gray et al. 2011). Contact with domestic poultry and swine is associated with an increased likelihood of zoonotic IAV exposure (Gray et al. 2007). Outbreaks of zoonotic IAV infection in humans have increased in the past decades, in parallel with the relentless growth of domestic swine and poultry populations worldwide (for review see Reperant and Osterhaus 2012). Such infections may be at the basis of the emergence of novel pandemic IAVs (see below).

IAV cross-species transmission may lead to sustained spread of the virus, with on-going host-to-host transmission in the new host species. This may result in self-limiting outbreaks that eventually die-out, or may spark large-scale epidemics that result in IAV establishment and continued circulation in the species concerned. Self-limiting outbreaks of IAV infection, following cross-species transmission, have been reported in wild and domestic avian and mammalian species, and in humans. The reasons behind the self-limiting nature of these outbreaks are little understood. IAVs may not acquire full adaptation to efficiently spread in a new host species upon cross-species transmission, and on-going host-to-host transmission may remain limited below levels allowing sustained spread. In particular, adaptation of avian IAVs of wild bird origin to terrestrial poultry is thought necessary for their establishment in these species (Swayne 2007).

Alternatively, the number of immunologically naïve hosts in the population may be insufficient to sustain IAV transmission. This may be due to rapid depletion of the pool of susceptible hosts during outbreaks. For example, large self-limiting IAV outbreaks in wild harbor seals (*Phoca vitulina*) have resulted in high mortality and morbidity burdens, resulting in the death of up to 25 % of local populations (Lang et al. 1981; Webster et al. 1981; Geraci et al. 1982; Hinshaw et al. 1984; Callan et al. 1995). The depletion of the pool of susceptible hosts, associated with the patchy nature of seal populations and short annual breeding seasons—that replenish the pool of susceptible hosts only once a year—may explain the self-limiting nature of the outbreaks. There is currently no evidence of continued circulation of seal-adapted IAVs. Similarly, self-limiting outbreaks of avian IAVs in farmed American mink (*Vison vison*) (Klingeborn et al. 1985) and domestic swine (Brown 2000), resulting in high mortality or morbidity burdens, likely depleted the pool of susceptible hosts in the affected farms, and containment prevented further spread.

The presence of pre-existing immunity in spill-over host populations may also contribute to the self-limiting nature of IAV outbreaks in domestic species and humans upon cross-species transmission. For example, IAV H3N8 of wild bird origin caused epidemics in horses in China during three consecutive years. Yet, the virus did not establish itself in the equine population (Guo et al. 1992). Circulation of a previously established equine IAV H3N8 and vaccination against this virus may have played a role in the eventual extinction of avian IAV H3N8 in horses. In humans, an outbreak of respiratory disease in a military camp in North America in

1976 caused by IAV H1N1 of swine origin turned out to be self-limiting (Goldfield et al. 1977; Top and Russell 1977). The simultaneous circulation of another IAV subtype—seasonal IAV H3N2—at the time of the outbreak, and/or the presence of pre-existing immunity against AIV H1N1 may have contributed to limiting the spread of this virus in the human population (Gaydos et al. 2006).

Established IAV Lineages in Animal Species

Occasionally, IAVs with efficient on-going host-to-host transmission in a new host species may sustainably spread, reaching endemicity or causing recurring epidemics in the species concerned. A limited number of IAV lineages have adapted to domestic animals, including poultry, swine, horses and dogs, and to humans. They are maintained independently of cross-species transmission from wild water birds or other spill-over host species. The conditions allowing IAV establishment and continued circulation in spill-over host species remain little understood.

Domestic swine and poultry harbor the highest diversity of established IAVs in spill-over host species, with several co-circulating subtypes and lineages (Brown 2000; Alexander 2007). A wide range of poultry species, including ducks, geese, chickens and quails, are susceptible to IAV infection. Species diversity, high density and population turnover, trade and mixing during co-raising or at animal markets, are believed important determinants for the evolution, emergence, establishment and diversification of swine and poultry IAV lineages (for review see Reperant and Osterhaus 2012). IAVs in swine and poultry typically are endemic over large geographical areas, if not globally, without displaying clear seasonal patterns of infection (Brown 2000; Alexander 2007). Endemicity in these species is likely maintained by the continued replenishment of susceptible hosts through trade or breeding.

Endemic IAV infection may go unnoticed in swine and poultry, because of low morbidity and mortality burdens. However, IAVs may cause detectable disease, especially in young animals immunologically naïve to IAV. In domestic poultry, IAVs may also develop increased pathogenicity, defining IAV pathotype in these species. When IAV-associated mortality is low in poultry, IAVs are qualified as low pathogenic avian influenza viruses (LPAIVs). In terrestrial poultry, mainly chickens and turkeys, LPAIVs of the H5 and H7 subtypes can evolve into highly pathogenic avian influenza viruses (HPAIVs), causing ravaging outbreaks with up to 100 % mortality (Swayne 2007). High poultry density in industrial farms is thought to favor HPAIV evolution and emergence. Because of their high mortality burdens—also due to the rapid implementation of stamping out and other control measures—HPAIVs may rapidly run out of susceptible hosts to infect, and outbreaks typically are self-limiting in adequately managed poultry populations. HPAIVs of the H5N1 subtype are a notorious exception. These viruses became endemic in poultry in South-East Asia and Africa, and rapidly diversified following their emergence more than 15 years ago (Chen et al. 2006; Li et al. 2010). While these viruses may silently circulate in aquatic poultry, they cause usually winter epidemics in terrestrial poultry. Subclinically infected aquatic poultry may be crucial for HPAIV H5N1 maintenance in domestic birds (Hulse-Post et al. 2005).

Table 18.2 List of pandemic influenza A viruses in humans. (For review see Taubenberger and Kash 2010)

Influenza subtype	Lineage	Year of introduction	Years of circulation
H1N1	Avian	1918	1918–1957
H2N2	Reassortant avian/human	1957	1957–1968
H3N2	Reassortant avian/human	1968	1968–present
H1N1	Human	1977	1977–2009
H1N1	Swine	2009	2009–present

A limited diversity characterizes IAVs circulating in horses and in domestic carnivores. Reports of equine epidemics of respiratory disease in past centuries suggest that equine IAVs have circulated in horses for several hundred years (Taubenberger and Morens 2009). However, only two IAV subtypes have been isolated from horses to date (for review see Reperant et al. 2009). Their emergence likely resulted from IAV cross-species transmission from avian hosts. IAVs of the H7N7 subtype emerged in horses in the mid 1950s and have not been isolated since 1980. IAVs of the H3N8 subtype emerged in the early 1960s and currently cause recurring seasonal epidemics in equine populations worldwide, reminiscent of seasonal human influenza.

Equine IAVs H3N8 infected domestic dogs in the early 2000s and have subsequently become endemic in canine populations in North America and parts of Europe (Crawford et al. 2005; Daly et al. 2008). In South-East Asia, IAV H3N2 of avian origin have become endemic in canine populations in the late 2000s and have subsequently spilled-over from dogs to domestic cats (Song et al. 2008; Song et al. 2011). These viruses may cause severe epidemics in both species. Future studies are awaited to unveil IAV evolutionary and epidemiological dynamics in domestic carnivores.

Established IAV Lineages in Humans

IAVs of animal origin with efficient human-to-human transmissibility can be at the origin of rare influenza pandemics in humans (Table 18.2). In particular, the high incidence of zoonotic IAV infections of any particular subtype in humans, are feared to provide these viruses with opportunities to acquire efficient transmissibility, sparking an influenza pandemic. Outbreaks of sporadic IAV infections result from frequent cross-species transmission while on-going host-to-host transmission remains limited. Recently, cross-species transmission of a number of swine and poultry IAVs to humans resulted in large outbreaks often associated with relatively high morbidity and mortality burdens. These include outbreaks of HPAIV H5N1 and H7N7, swine IAV H3N2, and most recently LPAIV H7N9, which have caused several hundred cases of human infection (Table 18.3). Evolution of these viruses by mutation and/or reassortment into a transmissible form may produce a novel pandemic IAV.

Table 18.3 List of recent zoonotic influenza A viruses causing large outbreaks in humans

Pathotype	Influenza subtype	Host of origin	Year	Number of cases	Ref.
HPAIV	H7N7	Avian	2003	89	(Koopmans et al. 2004)
HPAIV	H5N1	Avian	1997–present	666	(WHO 2013b)
LPAIV	H3N2	Swine	2011–present	309	(WHO 2013a)
LPAIV	H7N9	Avian	2013–present	137	(CDC 2013)

Pandemic IAVs cause severe epidemic waves in humans, typically with high attack rates, morbidity and mortality burdens. They infect a large proportion of the human population worldwide, which typically has little pre-existing immunity against them (Taubenberger and Kash 2010). Introduction of a pandemic IAV in the human population results in so-called antigenic shift, replacing one of the existing seasonal IAV subtypes. Pandemic waves in humans currently spread around the world within a matter of months. The viruses continue to circulate and evolve into seasonal influenza viruses after the pandemic that they caused, replacing one of the hitherto circulating seasonal IAVs. Such strain replacement suggests the existence of some levels of cross-immunity between IAVs of different subtypes. As a result, only a limited number of AIV subtypes have been established in the human population. The severity of the seasonal epidemics that follow pandemics typically declines with time, at least in part due to the building-up of specific immunity and the increasing inability of the IAVs to escape from the specific antibody landscape building up over time. As has been documented for IAVs H3N2 seasonal waves of influenza tend to emerge in South-East Asia, where multiple peaks of seasonal AIV infections are observed year-round. These viruses are seeded into countries of the northern hemisphere during the winter season, followed by their spread to countries of the southern hemisphere in the subsequent winter season there (Russell et al. 2008).

At least in part due to naïve, immature or impaired immune responses, infants, the elderly and individuals with comorbidities are particularly at risk of severe IAV infection that may be further complicated by bacterial infection. (Taubenberger and Morens 2008). Because of abundant social contacts, school-age children are considered major spreaders of IAVs in the human population. Serological surveys have established that most individuals of seven years of age or older have been infected at least once by seasonal IAVs and are partially protected against IAV re-infection due to immune memory (Bodewes et al. 2011). Nevertheless, healthy young adults may be severely affected during IAV pandemics, due to limited cross-protection from previous infections against a novel pandemic IAV.

Spilled-over IAVs Spilling Back to Wild Bird Populations

IAVs circulating in spill-over host species rarely spill back to wild bird populations. Adaptation of IAVs to spill-over host species is thought to hinder their ability

to infect wild water bird species. This has been demonstrated experimentally for a number of poultry IAVs, which inefficiently replicated in water birds (Swayne 2007). Yet, little is known about the extent of cross-species transmission of IAVs circulating in aquatic domestic poultry to related wild water bird species, and their subsequent spread.

Likewise, HPAIVs usually do not evolve in wild water birds, and if these birds become infected following cross-species transmission from poultry, the infection may not always be as severe as in domestic birds. However, since 2002, HPAIVs of the H5N1 subtype have spilled back from infected poultry to a wide range of wild bird species (Sturm-Ramirez et al. 2004), which can be considered spill-back hosts in this regard; (for review see Reperant et al. 2013) (Fig. 18.3).

HPAIV H5N1 infections have caused inapparent to fatal disease in wild birds, following sporadic infection or during self-limiting outbreaks in South-East Asia, the Middle East, Europe and Africa. Wild water birds, as well as birds from other orders, including corvids and birds of prey, appeared to be severely affected. The routes of transmission of HPAIV H5N1 likely included the oral and respiratory routes, contrasting with the fecal-oral transmission of LPAIV. Predation or the scavenging on infected carcasses may be a unique route of transmission of HPAIV H5N1 in corvids and birds of prey.

Wild bird species little affected clinically by HPAIV H5N1 may contribute to their geographical spread (Gilbert et al. 2006; Keawcharoen et al. 2008; Reperant et al. 2010). In Europe, HPAIV H5N1 outbreaks in wild birds occurred during winter periods and were associated with the 0 °C isotherm, suggesting that congregation of water birds on open water bodies along freezing fronts fueled the epidemics (Reperant et al. 2010) (Fig. 18.1). To date, there is little evidence that HPAIVs of the H5N1 subtype are maintained in wild bird populations, in the absence of repeated introductions from poultry.

18.2.2.2 Evolutionary Dynamics

A limited number of IAV subtypes and lineages circulate in spill-over host species, and most extant lineages emerged within the past hundred years. However, in domestic animals, like in humans, co-circulation of multiple IAV subtypes and lineages is reported. Although IAVs that are endemic in poultry and, to a lesser extent, in swine have diversified similarly to the diversification in wild birds, the diversity in spill-over host species is usually more restricted than that in wild water bird reservoirs (Fig. 18.2). In humans, and to a lesser extent, in domestic animals, IAV lineages show evolutionary patterns associated with genetic and antigenic drift, whereby mutations in the antigenic sites of the surface glycoproteins allow IAVs to escape antibody mediated immunity that builds up in the population. IAV diversity is greatest in poultry and swine and results from repeated introductions of new IAVs, relatively frequent reassortment as well as genetic and antigenic drift in these species (Brown 2000; Olsen 2002; de Jong et al. 2007; Alexander 2007). In horses, two main lineages of IAV H3N8 co-circulate (Lai et al. 2001). Distinct phylogenetic

groups associated with broad geographical areas indicate some geographical isolation of IAVs of domestic species, despite international trade.

IAV antigenic drift is most pronounced in humans, probably due to a strong population herd immunity landscape associated with human population size, age structure, lifespan and geographical structure (Taubenberger and Kash 2010). A limited number of combinations of amino-acid changes in HA antigenic sites of seasonal IAVs result in significant antigenic variations, leading to immune escape (Smith et al. 2004). Human seasonal IAVs antigenic drift is clustered, with such combinations of amino-acid changes occurring typically every 3 to 5 years. Single lineages usually predominate during human influenza seasons, with serial replacement of strains (Fig. 18.2). However, reassortment between IAV lineages within the same subtype appears more frequent than previously thought (Rambaut et al. 2008).

18.3 Host-Level Pathogenesis of Infection

18.3.1 Natural Reservoirs

IAVs maintained in wild water bird reservoirs are all of low pathogenic pathotype. These LPAIVs typically cause inapparent intestinal tract infection in wild water birds (Fig. 18.4). Cloacal shedding of high LPAIV loads lasts several days to a week, although prolonged shedding for weeks has been reported under experimental conditions. Wild water birds become infected following ingestion of water-borne IAVs that reach the intestine, or may contract the virus via the cloaca (Webster et al. 1992).

LPAIVs generally do not cause detectable damage in the intestinal tract of naturally or experimentally infected water birds, despite large numbers of infected cells detected by immunohistochemistry (for review see Kuiken 2013). Most infected intestinal epithelial cells are those of the intestinal crypts at the base of the villi of the large intestine and epithelial cells of the Bursa of Fabricius. It has been suggested that the rapid turnover of intestinal epithelial cells coincides with the duration of LPAIV replication cycle (Daoust et al. 2011). Intestinal epithelial cells in the crypts continuously divide, pushing infected cells towards the tip of the intestinal villi. Lytically infected cells may be released into the intestinal lumen, as they reach the tip of the villi and naturally shed into the lumen. Although visible intestinal damage may not occur, LPAIV infection of the gut may have clinical impact on wild water birds, such as impaired digestive functions. This may affect food intake, growth, migratory abilities, and reproductive success. Carefully designed studies are needed to address this unresolved question (Kuiken 2013).

LPAIV infection can cause gross and histological lesions in other organs than the intestinal tract in experimentally infected water birds (for review see Kuiken 2013). In particular, tracheitis, pneumonia and airsacculitis, associated with viral replication in respiratory epithelial cells in the airways and air sacs have been described in several studies. Intra-nasal inoculation of mallards resulted in mild pharyngitis and

tracheitis associated with LPAIV replication in respiratory epithelial cells and this route of inoculation may reproduce respiratory infection of dabbling ducks during feeding. However, aerosol inoculation of domestic ducks with LPAIV resulted only in intestinal infection. Furthermore, neither lesions nor antigen-positive cells have been detected in the respiratory tract of naturally infected water birds, despite massive LPAIV infection of the intestinal tract. In accordance with these pathological findings, LPAIV pharyngeal shedding is typically limited in water birds.

Little is known on wild bird immune response to LPAI. The absence of lesions is associated with the absence of recruitment of inflammatory cells to the intestinal site of infection, suggesting a limited innate immune response (Daoust et al. 2011). However, immunoglobulins, including secretory IgX (equivalent of mammalian IgA) are produced and detectable within a few days following experimental infection in domestic ducks, and may be present along the intestinal mucosa (Higgins et al. 1987; Magor et al. 1998). Avian adaptive immune responses against LPAIVs may protect at least in part against re-infection with IAVs of the same, and perhaps to a lesser extent, of different but related subtypes (Latorre-Margalef et al. 2013). The extent and duration of immunity against LPAIV re-infection in wild birds remain unknown, yet may be essential in determining LPAIV epidemiological and evolutionary dynamics in their natural host reservoirs.

18.3.2 Spill-Over Host Species

18.3.2.1 Avian Spill-Over Host Species

LPAIVs of wild bird origin probably behave similarly in aquatic poultry as in wild water birds. In terrestrial poultry, LPAIVs of wild bird origin can cause clinical infection, principally of the respiratory tract and occasionally of the intestinal and uro-genital tracts, with little mortality (Mo et al. 1997; Swayne and Halvorson 2001) (Fig. 18.4). Infection with LPAIV results mainly in drop in egg production and respiratory signs, and occasionally, in intestinal and uro-genital signs. Pharyngeal and cloacal shedding typically lasts several days to a week. Experimental inoculation of chickens demonstrated LPAIV replication in epithelial cells in the airways and lungs in association with inflammatory conditions like rhinitis, tracheitis and pneumonia. LPAIV pathogenicity varies greatly between poultry species. Pneumonia may be complicated by secondary bacterial infection. Rarely nephrosis and nephritis are observed. Intra-venous inoculation of AIVs of wild bird origin has been shown to result in infection of renal tubular epithelial cells and intestinal epithelial cells in chickens (Swayne and Slemons 1990).

In terrestrial poultry, LPAIV of the H5 and H7 subtypes can evolve into HPAIV. These viruses cause severe systemic infection resulting in more than 75 % mortality, mainly in chickens and turkeys (Swayne and Halvorson 2001; Swayne 2007) (Fig. 18.4). They are abundantly shed from both the respiratory and intestinal tracts. HPAIVs can infect endothelial cells and parenchymal cells in a wide range of organs, including respiratory tract, heart, pancreas, liver, intestine, kidneys, adrenal

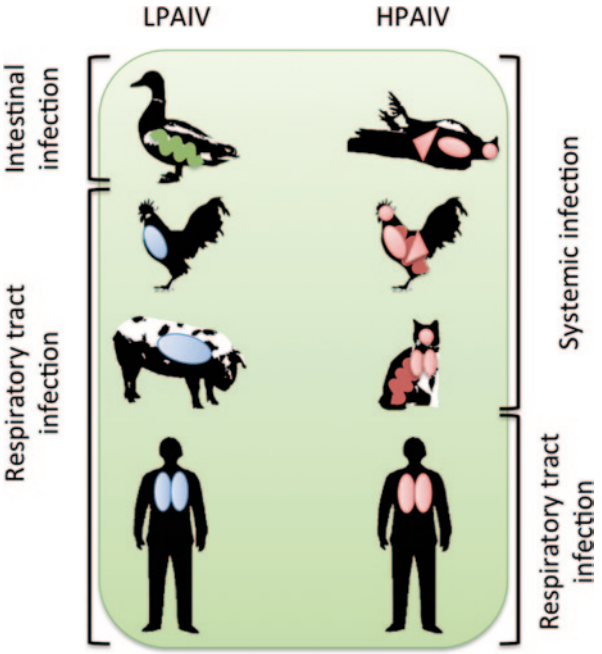

Fig. 18.4 Pathogenesis of influenza A virus (IAV) in natural reservoir and spill-over host species. LPAIVs cause typically inapparent intestinal tract infection in wild water birds, respiratory and occasionally intestinal and uro-genital infection in poultry, and respiratory infection in mammals. HPAIVs H5N1 cause systemic infection in wild birds, poultry and mammals, with frequent infection of the respiratory tract, brain, liver and intestines. Note that surprisingly, HPAIV H5N1 is rarely detected in the intestines of wild birds

glands, and brain in poultry. HPAIV infection results in hemorrhages, coagulation failure and organ failure, and occasionally neurological signs, when birds survive the acute phase of the disease. However, gross and microscopic lesions of inflammation, necrosis and hemorrhages may be rare, because sudden death can occur as early as 24 h after infection.

HPAIVs usually do not infect water birds or cause inapparent infection in these species. However, HPAIV H5N1 have the ability to infect a wide range of avian hosts, including aquatic poultry and wild birds (for review see Swayne 2007; Reperant et al. 2013). HPAIV H5N1 pathogenicity in aquatic poultry and wild birds varies greatly and ranges from asymptomatic infection to fatal systemic disease, depending on the virus lineage and bird species involved. In contrast to LPAIVs HPAIVs of the H5N1 subtype do not infect the intestinal tract of water birds, and cloacal shedding is rare. HPAIVs H5N1 are mainly shed from the pharynx of wild birds, and clinical signs include respiratory and neurological manifestations. Organs most often found infected are the respiratory tract, pancreas, liver, kidneys, adrenal glands and brain in these species (Fig. 18.4). Infected cells are parenchymal cells of most organs, and IAV antigen-expression in these cells is associated with lesions of inflammation and necrosis. In contrast to poultry, endothelial cells are not infected in most species of wild birds. Exceptions include whooper swans (*Cygnus cygnus*) and tufted ducks (*Aythya fuligula*), in which endothelial infection by HPAIV H5N1 and severe hemorrhages are reported (for review see Reperant et al. 2013). The timeframe of development of clinical signs varies among wild bird species. Clinical disease may start a few days to more than a week after infection.

Our current understanding of the immune response of poultry against LPAIVs or HPAIVs is incomplete (Suarez and Schultz-Cherry 2000; Swayne 2007). Neutralizing antibodies against the HA- and to a lesser extent NA-proteins provide protection against disease. The role of cellular immunity is less clear and may reduce viral shedding and help accelerate recovery upon infection. Overall, the immune response of terrestrial poultry against IAVs appears stronger than that of aquatic poultry. Vaccination of poultry against IAV elicits principally neutralizing antibodies against the HA protein, but does not fully abrogate infection, and hence limited shedding can occur in vaccinated birds. Vaccination of poultry against HPAIV H5N1 may be an effective tool to reduce the virus prevalence in endemic countries but stamping-out measures, combined with testing and reactive culling of poultry, are often necessary for the eventual eradication of HPAIVs.

18.3.2.2 Mammalian Spill-Over Host Species

IAVs that sporadically cross from avian to mammalian hosts, including zoonotic IAVs infecting humans, may cause from inapparent to severe respiratory and systemic infections. Clinical disease upon sporadic IAV infection is best documented in humans. In general, patients infected with zoonotic IAVs present with conjunctivitis or mild influenza-like illness (Peiris et al. 2007). However, in a number of cases, they may develop a severe or fatal lower respiratory tract infection characterized by necrotizing bronchiolitis, interstitial pneumonia, and diffuse alveolar damage.

Infections with HPAIVs of the H5N1 subtype display a unique pathogenesis. In addition to a respiratory route of infection, HPAIV H5N1 may infect mammalian species, including humans, via an intestinal route of infection. In most mammals, HPAIV H5N1 infections severely affect the lower respiratory tract as well as of other organs, including liver, kidney, heart, pancreas, intestine and brain (for review see Reperant et al. 2009) (Fig. 18.4). Lesions of inflammation and necrosis are associated with antigen-expression in parenchymal cells in these organs. Lungs, liver and brain are the most frequently infected organs in most mammals, resulting in respiratory and neurological clinical signs. In humans, the lungs are the primary sites of infection, although evidence of HPAIV H5N1 replication in other organs has been reported, including in the brain and intestinal tract (Peiris et al. 2007). Lung lesions are typically severe, with diffuse epithelial necrosis, the presence of pulmonary oedema and hemorrhage, and massive infiltration of inflammatory cells. Elevated levels of cytokines may contribute to the severity of the inflammatory response in fatal cases of human infection. These lesions can result in respiratory distress and death within 10 days.

Most IAVs that efficiently transmit in mammalian species, including pandemic and seasonal IAVs in humans, typically cause both upper and lower respiratory tract infection, resulting in rhinitis, tracheo-bronchitis, bronchiolitis and interstitial pneumonia (Kuiken and Taubenberger 2008) (Fig. 18.4). Clinical signs range from mild nasal secretions and coughing to severe pneumonia, which may be complicated by

bacterial infection, acute respiratory distress and death. In general, infection is more severe and located along the entire respiratory tract in individuals with immature or impaired immune responses, with little history of past infections or with history of past infections by IAVs of a different subtype. These include infants and the elderly or individuals with comorbidity; individuals of a species occasionally infected by IAVs, like harbor seals and farmed mink; and individuals infected by a novel IAV subtype, like pandemic IAV in humans or a novel IAV strain in domestic species. In contrast, the infection is usually less severe and more localized to the upper region of the respiratory tract in individuals with optimal immune functions or a history of past infections with the same or related virus strains. These include healthy individuals infected with seasonal or endemic IAVs.

The pathogenicity of IAV infection in mammals appears also largely virus strain-dependent. In general, IAVs that recently crossed the species barrier and some drift variants that escape population immunity tend to cause more severe disease, with the infection being located in both upper and lower respiratory tract in immunologically naïve individuals (Kuiken and Taubenberger 2008). In contrast, IAVs that recurrently circulate in mammalian populations tend to cause less severe disease, with the infection being located in the upper regions of the respiratory tract. However, since IAV pathogenicity depends on both host and viral factors, the disease outcome upon IAV infection is highly variable. In humans, mild (e.g., 1968 and 2009), moderately severe (e.g., 1957) and severe (e.g., 1918) pandemics have occurred, with the severity of certain seasonal IAV epidemics matching that of mild pandemics (Taubenberger and Morens 2009).

In mammals, neutralizing immunoglobulins and secondary cellular immune responses, which tend to be stronger and longer-lived in the deeper regions of the respiratory tract, provide protection against re-infection with the same or closely related IAV (Doherty et al. 2006). While neutralizing immunoglobulins are largely strain specific, secondary cellular immune responses likely provide some cross-protection against heterosubtypic strains (Rimmelzwaan and Osterhaus 1995; Rimmelzwaan et al. 2007). Pre-existing immunological memory in mammalian populations may not only protect against severe lower respiratory disease upon re-infection, but also exert selective pressures on IAVs, possibly resulting in changes in tropism patterns along the host respiratory tract (Reperant et al. 2012a).

18.4 Crossing Species, Crossing Scales: Adaptive Changes and Gain in Efficient Transmissibility

18.4.1 Host Switch

IAV crossing species barriers from natural host reservoirs to other avian or mammalian spill-over hosts can be accompanied by differences in epidemiological and evolutionary signatures, and different pathogenesis patterns. This may include

changing tissue tropism patterns and variable severity of resulting disease. IAV tissue tropism and disease patterns are determined by the ability of the IAV to infect and replicate in target cells and stimulate host immune responses. These IAV replication processes involve virus-host interactions that occur at molecular and cellular levels, and govern the extent of IAV adaptation to avian and mammalian hosts.

18.4.1.1 IAV Adaptation to Avian Hosts

IAV tissue tropism and patterns of infection in avian hosts are largely determined by the distribution of IAV receptors in the avian intestinal and respiratory tracts, and by IAV pathotype. The HA proteins of IAVs mediate their attachment to sialylated glycans of a variety of structural conformations, expressed on the surface of host cells. The HA proteins of IAVs circulating in wild water birds and in most poultry species have a preferred receptor binding affinity for sialic acids with α2,3 linkage to galactose (SAa2,3Gal) (for review see Taubenberger and Kash 2010; Reperant et al. 2012b), and recognize both *N*-acetylneuraminic acids (Neu5Ac) and *N*-glycolylneuraminic acids (Neu5Gc) (Suzuki et al. 2000). In ducks and terrestrial poultry, these receptors predominate and are abundantly expressed on the surface of intestinal and respiratory epithelial cells (Kuchipudi et al. 2009; Pillai and Lee 2010).

Likewise, the NA proteins of IAVs circulating in avian hosts typically have syalidase specificity for SAa2,3Gal. The frequent reassortment of IAV HA and NA genes in wild water birds indicates that these genes are generally compatible and balanced across subtypes and do not point to specific HA/NA combination with higher fitness (Dugan et al. 2008). In terrestrial poultry, an in-frame deletion of approximately 20 amino-acids from the stalk region of the NA protein is associated with reduced enzymatic activity and may represent a common adaptive change in IAVs circulating in these species (Taubenberger and Kash 2010).

In some terrestrial poultry species, such as quails and turkeys, the HA proteins of circulating IAVs can have a dual receptor binding affinity for both SAa2,3Gal and sialic acids with α2,6 linkage to galactose (SAa2,6Gal). The latter are expressed on intestinal and respiratory epithelial cells of terrestrial poultry, but are absent or rare on those of ducks (Wan and Perez 2006; Kimble et al. 2010; Pillai and Lee 2010). Because SAa2, 6Gal receptors are preferentially recognized by IAVs that spread efficiently in the human population, independently of cross species transmission (see below), terrestrial poultry may contribute to the generation of IAVs with pandemic potential.

The localized and systemic nature of LPAIV and HPAIV infection, respectively, in terrestrial poultry (and in other avian hosts for HPAIV H5N1) is determined by the HA protein cleavage site, mediating HA cleavage and fusion of the viral and cellular envelopes (for review see Swayne 2007; Taubenberger and Kash 2010; Reperant et al. 2012b). The HA protein cleavage site of most avian IAV and poultry LPAIV is composed of a conserved Q/E-X-R motif that requires the presence of extra-cellular trypsin-like proteases to be cleaved. Tryspin-like proteases are secreted

in the intestine and are present in the respiratory tract of birds. In contrast, the HA cleavage site of HPAIV is characterized by the insertion of multiple basic amino-acids, resulting in a R-X-R/K-R motif. It is recognized by ubiquitous intra-cellular furin-like proteases present in cells of many organs. HPAIV multi-basic cleavage site thus contributes to the ability of these viruses to cause systemic infection beyond the intestinal and respiratory tracts in birds.

Little is known about the determinants of efficient replication of avian IAV in birds. However, avian IAV tend to be more sensitive to low temperatures than mammalian IAV. Avian IAV replicate efficiently at bird body temperature of 41 °C, while their replication at 33 °C is reduced *in vitro*. A glutamic acid residue at position 627 in the PB2 protein in part governs IAV cold-temperature sensitivity (Massin et al. 2001). The PB2 627E residue is highly conserved in avian IAVs, although a lineage of HPAIV H5N1 with substitution E627K in the PB2 protein has evolved and circulates in bird populations.

18.4.1.2 IAV Adaptation to Mammalian Hosts

The HA proteins of IAVs circulating in mammals have variable receptor binding affinity for SAa2,3Gal, SAa2,6Gal, or both types of receptors (for review see Taubenberger and Kash 2010; Reperant et al. 2012b). In most mammalian species, the HA proteins of circulating IAVs recognize both Neu5Ac and Neu5Gc, except for human IAVs, which only recognize Neu5Ac (Suzuki et al. 2000).

In general, in mammals, SAa2,3Gal receptors are abundantly expressed on respiratory epithelial cells located in the deeper regions of the respiratory tract, including bronchioles and alveoli (Shinya et al. 2006; van Riel et al. 2007, 2010). The affinity of avian IAVs for these receptors coincides with the location of avian IAV infection in the lower respiratory tract of mammals, including humans, and in part determines the severity of the resulting disease.

Similar to avian IAVs, IAVs circulating in seals, horses and dogs have a preferred receptor binding affinity for SAa2,3Gal (Connor et al. 1994; Matrosovich et al. 2000). In these species, SAa2,3Gal receptors are abundantly expressed on respiratory epithelial cells in the trachea and bronchi, as well as in the bronchioles and alveoli (Suzuki et al. 2000; Maas et al. 2007; Ning et al. 2012). In contrast, SAa2,6Gal are rarely expressed. The abundance of IAV receptors in both upper and lower respiratory tract may partly determine the relative severity of IAV infection and frequent development of bronchiolitis and pneumonia in these species.

The HA proteins of IAVs circulating in humans typically have a preferred receptor binding affinity for SAa2,6Gal and those circulating in swine typically have a dual receptor binding affinity for both SAa2,3Gal and SAa2,6Gal (for review see Taubenberger and Kash 2010; Reperant et al. 2012b). Some pandemic IAVs also have been shown to have dual receptor binding affinity; however, most seasonal IAVs, which evolved from pandemic IAVs, have predominant receptor binding affinity for SAa2,6Gal. Likewise, an increase in NA enzymatic specificity for SAa2,6Gal occurred from the emergence of pandemic IAV H2N2 in humans in 1957 to recent seasonal IAV H3N2 (Kobasa et al. 1999).

In both swine and humans, SAa2,3Gal and SAa2,6Gal are expressed on the surface of different respiratory epithelial cells (van Riel et al. 2007, 2010; Van Poucke et al. 2010; Trebbien et al. 2011). SAa2,3Gal predominate in the deeper regions of the respiratory tract. They are expressed mainly on non-ciliated respiratory epithelial cells and type II pneumocytes, which are the most abundant cell types in the bronchioles and alveoli, respectively. In contrast, SAa2,6Gal predominate in the upper regions of the respiratory tract. They are mainly expressed on ciliated respiratory epithelial cells and type I pneumocytes. Ciliated respiratory epithelial cells are the most abundant cell type in the nasal cavity, trachea and bronchi. A similar distribution of SAa2,3Gal and SAa2,6Gal receptors is described in the respiratory tract of the ferret, which provides a most relevant animal model of human IAV pathogenesis.

While the abundance of avian IAV receptors in mammalian lower respiratory tract correlates with the severity of avian IAV infection in mammals, the predominance of SAa2,6Gal in the upper respiratory tract of humans, swine and ferrets correlates with milder disease, as described for seasonal and/or endemic IAVs in humans, ferrets and swine. Remarkably, the presence of IAV receptors—whether SAa2,3Gal or SAa2,6Gal—in the upper regions of the respiratory tract, i.e. nasal cavity, trachea and bronchi, is common to those mammalian species that sustain epidemic spread of IAVs. Infection of the upper respiratory tract is considered essential for on-going host-to-host transmission of IAVs in mammals (for review see Taubenberger and Kash 2010; Sorrell et al. 2011; Reperant et al. 2012b) (see below).

The HA proteins of IAVs that efficiently spread in mammalian species all have a cleavage site with a single arginine motif, corresponding to LPAIV in poultry, and are cleaved by extra-cellular trypsin-like proteases. Such proteases are present in the respiratory tract of mammals (for review see Reperant et al. 2012b). In contrast, the multi-basic cleavage site of HPAIVs H5N1 contibutes to the systemic nature of the infection in mammals.

The replication in mammalian cells of IAVs circulating in mammals is typically more efficient than that of avian IAVs. Mammalian IAVs typically replicate efficiently at low temperatures corresponding to those recorded in the mammalian upper respiratory tract. Substitution E627K in the PB2 protein is conserved among many mammalian IAVs and may confer resistance to cold temperatures (Massin et al. 2001). This PB2 substitution introduced in a range of IAV genetic backgrounds, including of avian and mammalian origin, increases IAV replication and pathogenicity in mammals (for review see de Wit et al. 2008; Taubenberger and Kash 2010; Reperant et al. 2012b). It improves binding of PB2 and NP proteins to assemble into viral ribonucleoproteins (vRNP) in mammalian cells, increasing viral transcription, replication and production. Interestingly, however, this substitution is not present in equine IAVs of the H7N7 subtype and in equine and canine IAVs of the H3N8 subtype, suggesting that host factors likely affect the evolution of this mutation. More surprisingly, this substitution is also absent in the pandemic IAV H1N1 of 2009, and introducing the mutation in this virus genome did not result in enhanced replication or increased pathogenicity of the virus (Herfst et al. 2010).

The nuclear transport of some mammalian IAV vRNPs was recently shown to be mediated by different importins-α than those mediating transport of avian IAV vRNPs (Gabriel et al. 2011). Six isoforms of importin-α, which recognize vRNP nuclear localization signals as part of the classical nuclear import pathway, have been described in humans and chickens. While avian IAV vRNP nuclear import was shown to be dependent upon importin-α1 and importin-α3, increased use of importin-α1 and a switch from importin-α3 to importin-α7 correlated with efficient IAV replication in mammalian cells, and impaired replication in avian cells. These changes were associated with substitution D701N in the PB2 protein and N319K in the NP protein in a mouse-adapted strain of avian IAV H7N7. Substitution D701N in the PB2 protein is found in many mammalian IAVs and in avian IAVs that caused severe disease in humans, including HPAIV H5N1. It is considered one of a number of genetic markers differentiating avian from human IAV isolates. However, and again quite surprisingly, this substitution, like E627K, is absent in pandemic IAV H1N1 of 2009, and its introduction into the genome of this virus did not enhance replication nor increase pathogenicity (Herfst et al. 2010).

Elevated levels of pro-inflammatory cytokines have been reported in fatal human cases of 1918 pandemic IAV H1N1 infection, and in fatal and severe cases of HPAIV H5N1 infection (for review see de Wit et al. 2008; Taubenberger and Kash 2010; Reperant et al. 2012b). The NS1 proteins of these viruses are potent inhibitors of the antiviral effect of the innate immune response. Escape from host antiviral responses may lead to the development of a so-called cytokine storm, further contributing to the severe respiratory symptoms of these infections. However, NS1 mutations associated with such escape from host antiviral responses are not present in most IAVs circulating in humans, and thus do not appear to be markers of IAV host adaptation.

18.4.2 Transmissibility

Although several markers of IAV adaptation to avian or mammalian hosts, associated with sustained IAV circulation in these species, have been identified, the determinants of the effective transmission of progeny viruses produced during infection to a new individual host are incompletely understood (Sorrell et al. 2011).

Receptor binding affinity for SAa2,6Gal and high replication levels in the upper respiratory tract appear essential for transmission of IAVs among humans. Only two amino-acids, changing IAV H1N1 receptor binding affinity from SAa2,6Gal to SAa2,3Gal, abrogated contact transmission of 1918 pandemic IAV H1N1 in the ferret model (Tumpey et al. 2007). Conversely, the introduction of one mutation in the HA gene of avian IAV H9N2 conferred receptor binding affinity for SAa2,6Gal and improved contact transmission of the virus in ferrets (Wan et al. 2008). To date, specific residues of IAV HA proteins of the H1, H2, H3, H4, H5, H7 and H9 subtypes affecting their receptor binding affinity for SAa2,3Gal or SAa2,6Gal have been described (for review see Reperant et al. 2012b). However, receptor binding affinity

for SAa2,6Gal is not sufficient to confer IAV transmissibility. A number of mutations have been found to decrease binding affinity of HPAIV H5N1 for SAa2,3Gal and increase their binding affinity for SAa2,6Gal; however, none of the mutated viruses efficiently spread by contact or aerosols in the ferret model (Chutinimitkul et al. 2010; Herfst et al. 2012).

Additional mutations, notably in the vRNP and matrix genes, affect IAV transmissibility in animal models. In particular, identified mutations improving IAV replication in mammalian cells were shown to confer or improve IAV transmissibility. In combination with the HA and NA proteins of the 1918 pandemic IAV H1N1 (with binding affinity for SAa2,6Gal), PB2 protein with E627K substitution allowed aerosol transmission of an avian IAV in ferrets (Van Hoeven et al. 2009). Conversely, substitution K627E in the PB2 protein of human seasonal IAV H3N2 impaired its aerosol transmission in guinea-pigs (Steel et al. 2009). Substitution D701N in the PB2 protein also improved contact transmission of HPAIV H5N1 and aerosol transmission of human seasonal IAV H3N2 in guinea-pigs (Steel et al. 2009). Lastly, the M gene was recently shown critical for the high transmissibility of 2009 pandemic IAV H1N1 in guinea-pigs (Chou et al. 2011).

A set of mutations in the HA and PB2 proteins were recently found to allow aerosol transmission of HPAIV H5N1 in the ferret model (Herfst et al. 2012). These included two mutations in the HA gene known to change receptor binding affinity of HPAIV H5N1 from SAa2,3Gal to SAa2,6Gal; two additional mutations in the HA gene, of which one affected glycosylation of the protein; and PB2 E627K substitution. Reassortment of HPAIV H5N1 with 2009 pandemic IAV H1N1 also resulted in viruses that efficiently transmitted via respiratory droplets in ferrets and guinea-pigs (Imai et al. 2012; Zhang et al. 2013). In most of these reassortant viruses, mutations in the HPAIV H5N1 gene conferring SAa2,6Gal were present, while several genes of pandemic H1N1 origin were shown to improve transmissibility, including PA, NP, NA, M and NS genes.

18.5 Conclusions

The highly diverse populations of IAVs circulating in wild bird species exist as a large pool of functionally equivalent and so often interchangeable gene segments that form transient gene constellations (Dugan et al. 2008). Occasionally, some of these gene constellations may lead to IAV infection of other avian or mammalian species, including humans. The ability of these viruses to productively replicate and sustainably spread among these new host species still poses numerous questions, likely to be addressed by studying more different IAVs in an increasing number of host species. IAV host switch and adaptation to novel host species, transmissibility, and pathogenicity are each dependent on complex multifactorial, interrelated and mutual interactions between virus and host and are likely under largely different selective pressures (Taubenberger and Kash 2010). Striking similarities though, in pathogenesis and transmissibility of IAV infections in newly invaded avian and

mammalian species, including humans, may point to common pathways that lead to adaptation and sustained presence in these newly 'colonized' species.

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Chapter 19
Important Zoonoses in Animals: Parapoxviruses: Extraordinary "Ball of Wool" Particle Shape—Masters of Local Infection and Immune Escape

Mathias Büttner

Abstract Parapoxviruses (PPV) represent a separate genus within the subfamiliy *Chordopoxvirinae* of the family *Poxviridae*. Species members of the genus *Parapoxvirus* (PPV) within the family *Poxviridae* cause contagious pustular dermatitis in small ruminants (prototype Orf virus, ORFV) and mostly mild localized inflammation in cattle (bovine papular stomatitis virus, BPSV and pseudocowpox virus, PCPV) and red deer in New Zealand (Parapoxvirus of red deer, PVNZ). All PPVs show a widespread and worldwide occurrence and are known to be zoonotic, leading to circumscribed skin lesions in humans, historically known as milker's nodules. Human PPV isolates are often ill defined concerning their allocation to an animal origin. PPV cause local infections and are specialized to counteract skin and mucous membrane defense mechanisms after virus entry into damaged skin by micro-lesions or wounds. Sophisticated escape mechanisms mediated by virulence genes of PPV have been elucidated in recent times. Whole genome sequencing (WGS) of various PPV species genomes allows more detailed and comparative determination of virus peculiarities. After PPV transmission to man, molecular conservation of the animal's virus genome can indicate the origin of the virus. Due to their high stability in the environment and local persistence in the host PPV have established as a worldwide problem in livestock especially in small ruminants. With regard to diagnostic differentiation after manifestation in human patients as well as for reliable differentiation from notifiable diseases in livestock PPV still pose a challenge for clinicians and microbiologists.

19.1 Introduction

Parapoxviruses (PPV) represent a separate genus within the subfamiliy *Chordopoxvirinae* of the family *Poxviridae* (ICTV 2012). In the genus Parapoxvirus the Orf virus (ORFV) is the type species and there are three additional species defined

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so far: *Bovine Papular Stomatitis virus* (BPSV), *Pseudocowpoxvirus* (PCPV) and *Parapoxvirus of red deer* in New Zealand (PVNZ). There are other tentative species attributable to the genus such as camel and chamois contagious ecthyma virus and sealpox virus. A poxviral disease of red squirrels in Great Britain was supposed to be caused by a parapoxvirus, but molecular analysis revealed a so far unrecognized poxvirus (SQPV) of the subfamily *Chordopoxvirinae* (McInnes 2006). The virus species belonging to the PPV genus have been defined according to the host species they got adapted to during evolution. The prototype member of the PPV genus, Parapoxvirus ovis or Orf virus (ORFV), is endemic in most sheep and goat raising countries in the world causing contagious ecthyma (CE) or "Orf", a term probably derived from the old Icelandic word "hurf" meaning a wound scab.

19.2 Virus Properties

The PPV consist of a single, linear, double-stranded DNA molecule of 130–140 kbp with typical hairpin loops at the termini containing repeats of about 3.5 kbp. In contrast to other member genera of the *Poxviridae* they contain an unusual high G+C content (~64%). Only recently complete sequence information of the defined PPV species ORFV, bovine papular stomatitis virus (BPSV) as well as pseudocowpox virus (PCPV) is available (Delhon et al. 2003; Hautaniemi et al. 2010; McGuire et al. 2012; Mercer et al. 2006; Friederichs et al. 2014). The genomes known so far encode an estimated 133 genes in BPSV or 132 in ORFV from which 88 are conserved in all *Chordopoxvirinae*. Central parts of the genome are highly conserved and contain genes essential for virus replication, particle packaging and export (Mercer et al. 2006; Haig and Mercer 2008). Gassmann et al. (1985) showed for the first time that the virus species ORFV, BPSV and PCPV can be differentiated by end to end hybridization with species-specific probes. Pathogenesis-related genes are found in the terminal regions of the genomes. Comparative sequence analysis has been performed for the virus interferon resistant (VIR) gene, the interleukin 10 (IL-10) encoding gene, the GM-CSF inhibitory gene and the gene coding for the vascular endothelial growth factor (VEGF-E) that has been found to be unique for PPV among the *Chordopoxvirinae* (Haig and Mercer 2008; Meyer et al. 1999). Although the 132 putative genes in ORFV are present in different isolates, substantial sequence variations may occur (Mercer et al. 2006). Highest sequence differences among PPV species are found in the near terminal-ends of the right and left hand of the genomes.

PPV genomes show an unusual plasticity when viruses undergo passages in cell culture (already after six passages) that can result in deletions (Hautaniemi et al. 2011). Duplications and rearrangements of genes as well as major deletions of non-essential genomic regions can occur after serial cell culture passages and adaption to cell lines. Stable deletions can lead to attenuation of the virus (Cottone et al. 1998; Büttner and Rziha 2002). PPV differ from orthopoxviruses within the subfamily *Chordopoxvirinae* not only by genome particularities, but also structurally

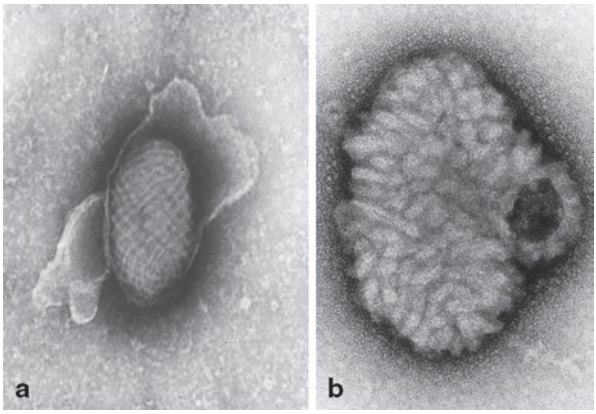

Fig. 19.1 **a** Typical regular crisscross arrangement of tubular core filaments in PPV virion "ball of wool morphology" **b** Disruption of regular tubule formation in virus particle with deleted 10kDa fusion protein encoding gene, loss of virion morphological integrity

in terms of (i) the size, shape and virion axial ratio, and (ii) the arrangement of the viral core protein filaments. The PPV virion is smaller than for example the vaccinia virus (VACV) and its shape is an elongated oval. The tubular viral core subunits are unlike those of other poxviruses regularly arranged (like a coiled-up rope) giving the particle a "ball of wool" morphology. The 10 kDa fusion protein, a homologue to the VACV protein A27L plays a role for PPV morphological integrity since deletion of this gene leads to particle deformation (Büttner and Rziha 2002) (Fig. 19.1). Infectious virus is found in different forms of maturation similar to VACV as intracellular non-enveloped immature virions, extracellular single enveloped mature virions and extracellular double enveloped mature virions (Tan et al. 2009). The outer envelope of the mature particles is thought to contain also proteins of the host cell. Among the 30–40 virion polypeptides (10–220 kDa) 10–13 surface polypeptides have been identified. A major envelope protein (39–45 kDa) is a potent immunogenic agent, but does not induce protective immunity.

19.3 Epidemiology, Host Range, Immune Response

Parapoxviruses (PPV) cause infections and disease worldwide predominantly in domestic and wild life ruminants. PPV infections were also reported from pinnipeds (sealpox), Japanese serow, reindeer, musk oxen, red deer, chamois and cats (Haig and Mercer 2008; Fairley et al. 2008). All PPV species known so far can infect man and lead to localized erythema, papules or pustules commonly called milker's nodule (MN), paravaccina or pseudocowpox (PCP). In general a precondition for successful infection and manifestation of PPV lesion in man as well as in animals is broken skin or micro-wounds. PPV infections are neglected zoonoses, often underestimated due to the frequent benign resolution of the lesions as well as the lack of specific aetiology formulated by physicians. However, cases of severe disease can occur in severely immune-compromised persons. Milker's nodules virus isolates or

viral DNA sequences from infected persons usually are traced back to the source of infection mainly sheep or cattle, but there are no clear-cut molecular correlates to the originally transmitted animal virus, since a matching pair of isolates usually is not available. This may be the reason why human PPV isolates or more common PPV–DNA fragments amplified from human lesion material or biopsies is often attributed to PCPV. However, recently more detailed molecular analysis and whole genome sequencing revealed the close relationship between the PPV animal host origin identity and the zoonotic transmission to man (Friederichs et al. 2014). As in animals human infections with PPV occur worldwide, especially in countries with intense sheep and goat breeding. The rate of subclinical infections in domestic small and large ruminants is unknown, but it is well known that Orf infections in sheep flocks do not induce long lasting immunity and can re-emerge in half to one year intervals. Nowadays ORFV transmission from sheep and goat to humans dominates the zoonotic infections in contrast to former contact infections from cow's teats when milking by hand was practiced (milker's nodule). In sheep thorough investigation of post infection immune reactions revealed a fairly normal adoptive T- and B-cell response. However, it can be concluded that local immune responses at the site of virus entry are successfully evaded by PPV to allow productive replication and establishment of a lesion (Haig 2006; Haig and McInnes 2002). Like in other poxviral genomes there are a variety of PPV virulence genes coding for immune evasive proteins that primarily counteract innate immune responses (Büttner and Rziha 2002; Haig 2006). One exceptional PPV unique virulence gene encodes the vascular endothelial growth factor, VEGF-E, that is responsible for angiogenesis and keratinocyte migration/proliferation (Meyer et al. 1999; Wise et al. 2012).

Little is known about immune reactions in PPV infected persons. Cutaneous infiltrates in affected humans have been characterized by immunohistochemistry showing an influx of CD3-positive T-lymphocytes of which the majority was CD4-positive. On infiltrated lymphocytes CD30 was detected as a marker for stimulated cells indicating a Th2 rather than a Th1 immune response (Rose et al. 1999). In humans there are no reports about PPV inter-individual transmission, e.g. by close contact from a diseased individual to another. PPV infections lead to antibody development with virus binding capability (Western Blot and ELISA reactive), but virus neutralizing antibodies are only found after repeated infections or several booster injections. The ewe's colostrum obviously does not protect her lambs from infection. Vaccines for sheep have been developed long time ago beginning with scarification trials using scab material and nowadays attenuated cell culture adapted ORFV strains are used as live vaccines, but with limited success. At least in sheep vaccination can prevent the severity of pustule development in lambs and protect them for a certain time from re-infection in a flock. There are no special studies that evaluate the prevention of virus spread by consequent and repeated vaccination. The resistance of PPV, especially of the mature enveloped particles, against environmental influences has to be taken in account with regard to contaminated pastures, fodder and wool of the animals (Robinson and Petersen 1983).

19.4 Diagnosis

Electron microscopy is a method of choice when enough virus particles can be expected (10^5 or more particles) in a sample (biopsy material, skin specimen). In samples from affected persons it is extremely difficult to find PPV virions in electron microscopy because of a lack of sufficient tissue and frequent pre-diagnostic treatment of lesions. This is why currently electron microscopy has been replaced or at least is accompanied by molecular diagnosis, predominantly polymerase chain reaction (PCR). A highly sensitive real time PCR protocol became established for rapid and reliable detection of PPV genome presence in tissue samples (Nitsche et al. 2005). Conventional PCR followed by electrophoresis of amplified DNA fragments still is of importance for further molecular analysis, e.g. sequencing. The latter is used to differentiate PPV species and to perform molecular epidemiology. The most popular target region for this purpose is the open reading frame (ORF) 011 containing the B2L gene, an orthologue of the vaccinia virus Copenhagen F13L gene, that encodes the major envelope protein p37K (Sullivan et al. 1994).

Whole genome sequencing techniques, e.g. using Miseq/Illumina instruments with paired end sequencing and a read length of 150 bp in each direction, become increasingly popular and affordable. For intense further studies PPV laboratory strains are essential, although cell culture isolation of PPV out of field samples is tedious and time consuming. Primary ruminant or human fibroblasts are the most permissive cells followed by Vero cells that have been successfully used for virus propagation (Büttner and Rziha 2002). Long incubation (up to seven days) of inoculated cells and blind passages can become necessary for successful PPV isolation. All experiences so far demonstrate that PPV, in contrast to many other poxviruses, does not replicate in the chorioallantois membrane of embryonated eggs.

Serology is of little value in PPV diagnosis. Antibody development is readily induced, mainly directed against the major envelope protein (B2L gene), but antibody screening in ruminants is of poor significance due to the high and widespread subclinical infection prevalence in the population. There are no commercial antibody assays available. Therefore only experimental ELISA protocols have been developed that can also be used to test human sera (Hartmann et al. 1985). Serum neutralizing assays are limited to plaque reduction in permissive cells for testing of hyper-immune sera or monoclonal antibodies (Czerny et al. 1997).

19.5 Clinical Features

Orf and bovine papular stomatis are widespread infections of sheep and cattle. Normally a mild and localized inflammatory disease is seen in cattle (Fig. 19.2). In sheep and small ruminants a benign form of Orf is differentiated from malignant forms mostly complicated by bacterial super-infections (Fig. 19.3). In cows and ewes painful inflammatory manifestation with ulceration can occur at the udder which is attributed to PCPV and Orf infection, respectively. After zoonotic transmission a localized skin lesion can develop in humans (Fig. 19.4). The so-called

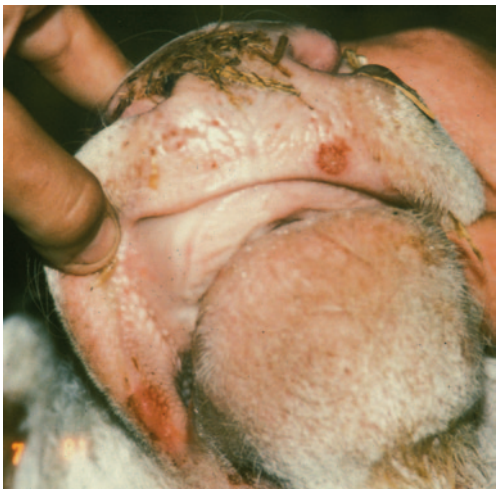

Fig. 19.2 Muzzle of cow with lesions of bovine popular stomatitis (BPS): circumscribed herds of inflammation in the skin and mucosa

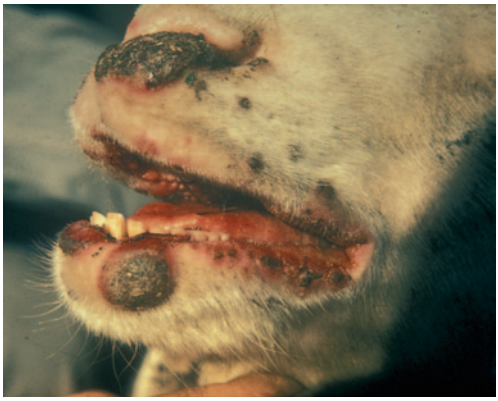

Fig. 19.3 Contagiuos pustular dermatitis (CPD) "Orf" affected sheep with proliferative lesions around the mouth, inner lips and nose. Secondary bacterial infection complicates lesion healing and can exacerbate severe forms of Orf

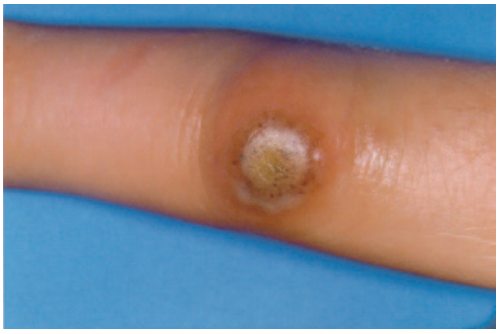

Fig. 19.4 Manifestation of zoonotic Parapoxvirus infection of man (milker's nodule). Local (mostly self-healing) lesion showing a more proliferative nodule-like character. The typical central impression in the lesion (pox navel) as known from *Orthopoxvirus* infections is missing

"milker's node" is characterized by papulae and pustulae irrespective of the PPV that has been transmitted from animals. It is caused by close contact with infected animals mostly domestic ruminants. Human PPV infections are considered to be rare and rather fortuitous events, infection rates of individuals at risk (e.g. slaughterhouse workers, veterinarians, farmers and animal care takers) are way higher. Also people not occupationally handling animals can get infected, particularly by visiting a petting zoo or by household exposure when handling contaminated furs, skin, or meat (Bayindir et al. 2011; Nougairede et al. 2013). Mass infection of people has been described linked to ritual sheep slaughter in celebration of Islamic holidays (Nougairede et al. 2013; Uzel et al. 2005). The epitheliotropic PPVs enter via scarified or otherwise broken skin and subsequently can replicate locally in the human epidermis. Occasionally large and painful nodules develop, which are mostly localized on the hands, especially the index finger and less frequently the face (most likely by smear infection). Disease manifestation via localised lesions is benign and generally resolves within max. 1–2 months even without any medical treatment (Leavell et al. 1968). However, there are individual cases described in the literature of severe complications linked to immune-suppression (Larcher et al. 2009; Lederman et al. 2007). In human PPV infections laboratory diagnosis is important to differentiate VACV, monkey pox, varizella zoster, other herpes virus infections or allergic skin reactions. PPV induced lesions normally do not show a central impression, but rather an inflammatory focal pustule or a more proliferative papule. A common feature of PPV is the stimulation of vascular endothelial cells to proliferation leading to a tumour like clinical outcome described as "giant orf" in man (Ballanger et al. 2006; Geerinck et al. 2001). In sheep severe lesion development is known as bloody lesion or exhibits a cauliflower like proliferation, mostly complicated by bacterial super-infection.

Human cases are commonly designated as PCPV infections, but human PPV isolates are poorly characterized by molecular tools. So far there are no prototype laboratory strains for human-derived PPV. As mentioned more sequence information is available to get a more detailed impression about similarities and differences among the established PPV species including the complete genome sequence of three Orf strains, one BPSV stain and two PCPV strains from humans and reindeer (Delhon et al. 2003; Hautaniemi et al. 2010; Mercer et al. 2006; Friederichs et al. 2014). Different PCR protocols have been established for amplification of coding regions in the ORFV genome. Sequence information of PCR fragments, mainly from the B2L coding region, have been used for comparative investigations of various PPV isolates from domestic and wildlife ruminants.

Clinical diagnosis of BPS and Orf in sheep is complicated by different similar inflammatory disease manifestations in the skin and mucous membranes caused by other viruses such as Foot and Mouth Disease (FMD), Bluetongue (BT), Bovine Viral Diarrhea—Border Disease (BVD, BD) and bovine herpesvirus (BHV-1) rhinotracheitis. Rapid and reliable differential diagnosis for such communicable and economically important diseases is essential.

19.6 Therapy

There is no specific therapy for PPV infections in man and animals. For local and systemic virocidal treatment in humans and lambs cidovir has been described to be effective (Nettleton et al. 2000; Gallina and Scagliarini 2010). Prophylactic vaccination against Orf for sheep and goats is practised since long time, but to-date has not resulted in a potent vaccine providing long term sterile immunity. At least safe cell culture attenuated vaccines have been developed that protect sheep from severe forms of Orf (Mayr et al. 1981; Nettleton et al. 1996).

19.7 Prospects

In close orientation on the model VACV the search for non-essential genes or genomic regions to be replaced by a gene of interest for vector expression was successful using cell culture propagated attenuated ORFV. Meanwhile a variety of ORFV vaccine vectors have been described to provide solid protection in laboratory animal infection models and even against lethal swine diseases such as pseudorabies and classical swine fever. Although PPV possess efficient immune evasive mechanisms, on the other hand they are potent immune-modulators providing adjuvant properties. The latter seems to be of special advantage under non-permissive conditions in hosts where the immune evasive properties of PPV are not functional. Experiences about the beneficial use of an immune stimulating product containing inactivated ORFV are well known in veterinary medicine (Haig and Mercer 2008; Büttner and Rziha 2002). The potent PPV mediated immune stimulation is effective even in tumour models as recently reviewed by Rintoul et al. (2012).

The recent emerge of a new poxvirus in squirrels and the occurrence of PPV in unusual hosts such as cats raises concern about a so far unknown pool of PPV like poxviruses in nature.

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Chapter 20
Orthopoxviruses—Plagues of Mankind, Strategists in Immune Evasion, Teachers in Vaccination

Claus-Peter Czerny

Abstract Smallpox, the mankind's greatest plagues caused by *Variola virus* (VARV), is officially eradicated since 1979. The reason for this success was the clear picture of the disease, the compulsory immunization worldwide with the *Vaccinia virus* (VACV), but essentially the fact that VARV is not zoonotic and has no reservoir in the environment. VARV belongs to the subfamily *Chordopoxvirinae* of the family *Poxviridae* and is classified in the genus *Orthopoxvirus* (OPXV). OPXVs are closely related and cross-protective to each other after infection of their hosts. Zoonotic OPXVs pathogenic for humans are *Vaccinia virus* (VACV), *Buffalopox* virus (BPXV), *Cowpox virus* (CPXV), *Monkeypox virus* (MPXV), and *Camelpox virus* (CMLV).

20.1 Introduction

Smallpox, the mankind's greatest plagues caused by *Variola virus* (VARV), is officially eradicated since 1979. The reason for this success was the clear picture of the disease, the compulsory immunization worldwide with the *Vaccinia virus* (VACV), but essentially the fact that VARV is not zoonotic and has no reservoir in the environment. VARV belongs to the subfamily *Chordopoxvirinae* of the family *Poxviridae* and is classified in the genus *Orthopoxvirus* (OPXV) (http://ictvonline.org/virusTaxonomy.asp?version =2012; King et al. 2012). OPXVs are closely related and cross-protective to each other after infection of their hosts. Zoonotic OPXVs pathogenic for humans are *Vaccinia virus* (VACV), *Buffalopox* virus (BPXV), *Cowpox virus* (CPXV), *Monkeypox virus* (MPXV), and *Camelpox virus* (CMLV).

Vaccinia virus is the type species of the genus and the poxvirus studied most intensively. It was used in the vaccine that successfully eradicated smallpox under the direction of the World Health Organization (WHO) (Fenner et al. 1988). It was a striking accomplishment in the history of infectious diseases and resulted in the last human smallpox case being reported in 1977 in Somalia. Up to now, the nomenclature of the different poxvirus species is normally derived from the host

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from which it was usually isolated. VACV received its name from the Latin word "vacca" (=cow), because it was believed for a long time that it was isolated from cowpox diseases and used for human vaccinations. When Edward Jenner applied a suspension from the infected, pustulous skin of a cow to a human, and proved that this treatment protected against a smallpox challenge in 1796, he demonstrated that pox disease in cows is related to a similar disease in humans and provided the first scientific description of a human vaccination by detailing the efficacy of "scarification". However, today VACV and CPXV can be distinguished clearly with modern diagnostic tools. The origin and natural history of VACV are unknown and remain a riddle of virology (Baxby 1981). The animal poxvirus used by Jenner almost 200 years ago for the first human vaccinations was probably a genuine CPXV, whose original strain no longer exists. Later, naturally occurring VACV stains may have found their way into vaccine production and have since then been cultured without interruption in laboratories to produce the smallpox vaccine (Smith 2007). There are also some hints that VACV came from horses. Today, research on VACV continues, since it is a virus model for basic research. However, VACVs are also important expression vectors that have been used as a recombinant live vaccine against many infectious diseases since its establishment (Mackett et al. 1982; Panicalli et al. 1982). In recent years, recombinant VACV strains, highly attenuated and defective in replication such as the modified VACV Ankara (MVA) have attracted much attention (Sutter and Moss 1992; Volz and Sutter 2013). Those strains are safer for vaccinees due to several deletions in their genomes. *Buffalopox virus* is a close variant of the VACV. The disease was first described in India (Ramkrishnan and Anathapadmanabham 1957) and discovered around the time of smallpox epidemics and the launching of VACV vaccination programs. It is still associated with sporadic outbreaks in Asian buffaloes (*Bubalus bubalis*) in Bangladesh, India, Indonesia, Pakistan, Egypt, Russia, and Italy (Mayr and Czerny 1990b; Essbauer et al. 2010). The FAO/WHO Joint Expert Committee on Zoonosis declared buffalopox an important zoonotic disease (FAO/WHO 1967). Today, buffalopox is recognized as an emerging contagious viral zoonosis of domestic buffaloes (Singh et al. 2007) and cows (Yadav et al. 2010; Goyal et al. 2013). The genuine *Cowpox virus* is one of the earliest described members of this genus. The cowpox disease is as old as smallpox, but it was recognized much later. The origin and first outbreaks of cowpox are unknown (Mayr and Czerny 1990c). Historically, the common term "cowpox" was applied indiscriminately to all diseases in cows with local or generalized pustular lesions and pox exanthema on the skin and mucous membranes of milking cows' udders and teats (udder pox). This included zoonotic transmission of these diseases to humans (milkers) through close contact with infected cows. Etiologically speaking, cowpox diseases are not uniform. Besides the genuine CPXV, VACV and members of the genus *Parapoxvirus* (Stomatitis papulosa, Milker's node, Ecthyma) can also infect cows and produce a similar clinical picture. In recent years, VACV infection in cattle, as well as the transmission to humans, are being reported an emerging disease, especially in Brazil (Damaso et al. 2000; Leite et al. 2005; Trindade et al. 2007; Quixabeira-Santos et al. 2011). However, only the disease caused by CPXV should be designated as "cowpox". *Monkeypox virus* was discovered in 1958 during

an outbreak in a facility for captive monkeys in the zoological garden of Copenhagen, Denmark (Von Magnus et al. 1959). Since then, several outbreaks have been reported in European and American zoos, and MPXV has revealed a propensity to induce disease in a large number of animals within the mammalia class from pan-geographical locations (Essbauer et al. 2010; Parker and Buller 2013). Human monkeypox, which causes a vesiculo-pustular rash illness clinically indistinguishable from smallpox, is a viral zoonotic disease that occurs mostly in the rainforests of Central and Western Africa. It was initially discovered to cause human infection in 1970 in Western Africa and the Congo Basin (Lourie et al. 1972; Damon 2011). Monkeypox has presumably been active in sub-Saharan Africa for thousands of years, ever since humans acquired the virus through direct contact with infected animals (Nalca et al. 2005). After the cessation of VACV immunization against smallpox, MPXV infections are now emerging as a public health concern. Human monkeypox is almost clinically identical to ordinary smallpox, and therefore, since the global eradication of smallpox in 1979, much attention has been paid to monkeypox as a smallpox-like disease and possible agent of bioterrorism. Additional attention was brought to bear on this virus when, in the spring of 2003, it emerged for the first time outside Africa in the Western Hemisphere, and caused a cluster of cases in the Midwest of the USA. The disease was traced back to MPXV infected rodents imported from Ghana, West Africa. *Camelpox virus* has been known since the Middle Ages and is one of the most important infectious and contagious diseases in camels. CMLV causes a smallpox-like illness in the natural host. It is widespread worldwide in camelids and is enzootic throughout camel-rearing areas in Asia and Africa, but not in Australia (Baxby 1972; Mayr and Czerny 1990d; Duraffour et al. 2011). CMLV is responsible for severe economic losses due to high morbidity, relatively high mortality in younger animals, loss of condition and reduced milk production in lactating animals. The disease occurs in the Bactrian camel (*Camelus bactrianus*) and in the Dromedary camel (*Camelus dromedarius*). It is unknown to South American tylopods (llama and related species). CMLV has been long suspected to cause the illness in a unique host, the camel. Since 2011, however, it has been confirmed that under certain circumstances, CMLV can be mildly pathogenic in humans (Bera et al. 2011).

20.2 Virus Properties

Orthopoxviruses are large and complex virions that measure 320 × 270 nm in diameter. Their morphology is pleomorphic and brick-shaped. A lipoprotein surface membrane displays tubular units of about 10 nm. Negative contrast electron microscopy (EM) images show that the surface membrane encloses a biconcave or cylindrical core, which contains the genome. It consists of a single, linear, double-stranded and covalently closed DNA molecule, which is organized in a nucleoprotein complex. One or two lateral bodies appear to be present in the concave region between the core wall and the inner membrane (King et al. 2012). The optimum pH

is around 7.6. The virus is labile to fat solvents (chloroform, ether), formaldehyde (1–3 %), oxidizing agents, acids, and all virucidal disinfectants at pH values below 7 and above 9. The surface membrane is removed by non-ionic detergents and sulfhydryl reducing reagents. Under natural conditions, dry scabs can remain infectious for several weeks. A cell-free virus is inactivated in 1–2 h at +56 °C. When lyophilized, it remains stable for unlimited periods at temperatures of +4 °C and under.

Virus morphogenesis is best studied for VACV. It begins following DNA replication and expression of early, intermediate and late genes. During its replication cycle, VACV and obviously most of the poxviruses, produce two different forms of virions. Particle assembly is initiated with the formation of crescent-shaped membrane structures in the intermediate compartment between the endoplasmic reticulum and the trans-Golgi network. Replicated DNA is packaged, forming fully infectious, mature MV particles. The mature virus, also known as the intracellular mature virus (IMV), represents ca. 95 % of fully mature progeny virus particles and remains in the cytoplasm until cytolysis. Depending on the virus strain, a proportion of ca. 5 % of MV becomes enwrapped by an additional lipid double layer of intracellular membranes derived from the trans-Golgi network or endosomes. These "wrapped" virus particles (WV) or intracellular enveloped viruses (IEV) can leave the cell before a cytopathic effect (cpe) is detectable in cell cultures. Externalization of WV occurs via association and transport by the cellular microtubule network to the cell periphery, as well as by fusion of the outermost membrane layer with the cell membrane to form double enveloped extracellular virus (EV) particles. They can be bound to the cell surface as a cell-associated enveloped virus (CEV). However, in an infected body, EV is responsible for rapid distribution. Both envelopes of MV and EV contain different proteins and they are therefore antigenically distinct from each other. Antigenic sites, which are responsible for the induction of protective immunity, are localized exclusively in the virus envelope, especially in proteins responsible for virus attachment or associated with the fusion event. In the MV envelope, the six proteins A17, A27, A28, D8, H3, and L1 are predominantly responsible for the induction of neutralizing antibodies. The proteins A14.5 and A17 anchor A27 within the envelope, whereas a conserved sequence within the H2 subunit of the VACV entry/fusion complex is important for interaction with the A28 subunit and specifically enhances the neutralizing antibody response to the A28 protein (Nelson et al. 2008). In the EV envelope, there are six major proteins: A33, A34, A36, A56, B5, and F13. B5 and A33 induce neutralizing antibodies. F13 is involved in wrapping and is target of the potent antiviral ST-246. The species-specific A56 glycoprotein is the hemagglutinin. Besides the induction of protective immunity, MV and EV envelope proteins play significant roles in virus entry, wrapping, and release. Entry and egress of poxviruses to/from cells are complex events and are far from general comprehension. Monoclonal antibodies (Mabs) are able to influence both events, however, VACV deletion mutants, which miss targets of several neutralizing Mabs remain infective in vitro. The entry of poxviruses into mammalian cells is divided into two main phases. Attachment of the virions to the cell surface, followed by a fusion entry event, delivers the viral core into the cytoplasm. It is not clear whether poxviruses use a cellular receptor for entry. The attachment phase is

mediated by electrostatic interactions between virions and glycosaminoglycans or laminin on the cell surface. During recent years, it has become evident that a multi-subunit entry/fusion complex (EFC), consisting of at least twelve highly conserved viral proteins in MV particles, is necessary for fusion with the cell membrane and entry of the core into the cytoplasm (Moss 2012). Virus egress is mainly achieved by EV proteins.

The first complete DNA sequence of a VACV (strain Copenhagen) was published in 1990 (Goebel et al. 1990). Publication of several other VACV complete genome sequences followed (http://www.poxvirus.org). With the impressive developments in next generation sequencing techniques a lot of further complete genome sequences can be expected in near future. VACV genomes are ranging from 178 kbp (VACV MVA) to 192 kbp (VACV Copenhagen), with typical hairpin loops at the termini containing repeats of about 12 kbp (Goebel et al. 1990; Antoine et al. 1998). In accordance with most of the other genera of *Poxviridae,* VACV contains an A/T-rich genome (~67%). From the proteins encoded by the genomes known so far about 100 are present in virions and 89 are conserved in all *Chordopoxvirinae* (Smith 2007). Approximately half of the VACV genome is non-essential for virus replication in cell culture. Central parts of the genome are highly conserved (ca. 100 kbp) and contain genes essential for virus replication, transcription, and particle packaging (polymerases and other enzymes, structural proteins). Genes related to pathogenesis, host range, and tropism are found in the terminal regions of the genomes (e.g. immunomodulators, anti-apoptotic proteins). Genome and phylogenetic analyses based on host-range and structural protein genes confirmed that *BPXV* clustered closely with VACV rather than with other OPXVs (Dumbell and Richardson 1993; Singh et al. 2006; Bera et al. 2012). Among the genus *Orthopoxvirus,* traditionally defined *CPXVs* possess the largest genomes with over 220 kbp; roughly 30 kbp larger than the average size of VACV genomes. The hairpin loops at the termini contain repeats of about >50 kbp in some strains (Smith 2007). Like other OPXVs, 30–40% of the CPXV genome encodes proteins that play important roles in virus pathogenesis and host range (Upton et al. 2003). "White cowpox" variants also occur as a result of a deficit in terminal sequences of the red CPXV genome, affecting approximately 11.4% of the genome (Archard and Mackett 1979). The genome of *MPXV* is 196.86 kbp (MPXV Zaire-96-I-16) in size and encodes 191 different proteins. The central region of the MPXV genome is almost identical to that of other OPXVs, including VARV. However, the terminal regions of the MPXV genome, which encode virulence and host range factors, differ substantially from the other OPXV species (Shchelkunov et al. 2001). MPXV is a distinct species, which evolved from an OPXV ancestor independently of VARV. CMLV is taxonomically distinct from members of the genus *Parapoxvirus,* which cause contagious dermatitis in camels also (Mayr and Czerny 1990d; King et al. 2012). More than 60 different CMLV strains have been isolated worldwide since 1972 (Duraffour et al. 2011). The entire genome sequences of two CMLV strains from Kazakhstan (CMLV-M-96) (Afonso et al. 2002) and Iran (CMLV-CMS) (Gubser and Smith 2002) have been published. Genomes of 205.770 bp (CLMV M-96) and 202.205 bp (CLMV CMS), respectively, contain more than 211 putative genes. The hairpin

loops at the termini contain repeats of about 6 kbp. The analysis of CMLV genome sequences has revealed that it is most closely related to VARV, the causative agent of smallpox, sharing all genes involved in basic replicative functions and the majority of genes involved in other host-related functions (Afonso et al. 2002; Gubser and Smith 2002; Duraffour et al. 2011). Particularly the arrangement of ORFs close to and within the ITRs of CMLV and VARV showed a higher degree of similarity in comparison with other OPXVs.

20.3 Epidemiology, Host Range, Immune Response

Vaccinia virus is a zoonotic agent with a broad host range. It can be transmitted to virtually all mammals and from affected animals to humans, where it causes manifest infections with both local affections and generalized disease. Virulent VACV strains are particularly dangerous for immunosuppressed persons, where generalized vaccinia may be fatal. During the era of smallpox vaccination in humans, vaccinia infections in ruminants, pigs and other animal species often originated from farm children after primary vaccination. The local pustules became dry and fell off as scabs. VACV survived for a long time in those materials. It finds its way into dust and spreads in the form of airborne particles. However, it is primarily transmitted from humans to animals by direct contact and to a certain extent also by biting flies and other arthropods (Mayr and Czerny 1990a). Small lesions on the skin and mucous membranes promote transmission. In the population, an infected animal with manifest disease may spread the virus, which thereby adapts to the species through animal-to-animal passages. Virus spread amongst animals results in an increase in virulence. However, foci of VACV in animals remain mostly locally restricted. The virus does not spread in an epidemic manner. Starting in 1999, several VACV strains were shown to be responsible for zoonotic disease affecting more than 1100 dairy cattle and up to 80 % of their handlers in rural tropical rainforest and woodland savanna areas in South-east Brazil (Trindade et al. 2007). Several VACV-like viruses have been isolated and named according to their geographic origin, i.e. isolates Aracatuba, Cantalago (CTGV), Guarani, Passatempo (Leite et al. 2005). In addition, a virus has been isolated from sentinel mice (isolate Belo Horizonte). In Brazil, the emerging disease seems to be endemic. The reasons for the occurrence of VACV in these areas of South America may be long-term survival of vaccine strains in nature. However, recent phylogenetic studies provided evidence that it is unlikely that Brazilian isolates have derived from a single vaccine strain used over the past century (Trindade et al. 2007). The epidemiology of BPXV should be reconsidered more than three decades after termination of the smallpox eradication campaign in humans. It has been speculated that BPXV emerged from the VACV Lister vaccine strain, which was used to produce smallpox vaccine in buffalo calves in India. Gradually, the vaccine strain adapted in buffaloes and became pathogenic, causing outbreaks in buffaloes. Thereafter, BPXV outbreaks have been occurring frequently in different parts of the country, affecting both buffaloes and humans (Baxby and

Hill 1971; Bhanuprakash et al. 2010b; Gurav et al. 2011). Zoonotic cases of BPXV have also been reported in unnatural hosts such as cows (Yadav et al. 2010; Bera et al. 2012). Improved dairy hygiene practices and wearing gloves while milking may help to prevent the spread of infection to other animals and humans. Until 40 years ago, the genuine CPXV was found in milking cows with occasional zoonotic transmission on the hands of milkers via contact with pocks on the teats. The most likely means of transmission of CPXV is direct contact. The virus primarily enters the body through broken skin, but can also be contracted from dust containing the virus via the mucous membranes of the respiratory and digestive tracts (Mayr and Czerny 1990c; Baxby and Bennett 1997). Occasionally, a large number of animals may contract the infection while grazing. Cowpox can also be transmitted indirectly, e.g. during milking via the milk pail (Ghosh et al. 1977). In addition, different invertebrates can spread CPXV in a purely mechanical way. Intrauterine transmission can occur during the course of generalized disease. The virus passes into the environment by way of pustules from the skin and mucous membranes, which have dried up and peeled off. Ocular secretion, as well as such of the upper respiratory and the digestive tracts, may also be responsible for transmission. Cowpox epidemics in intervals of 10 years are known to have occurred in Germany, England, France, Poland and Russia. Cowpox had a purely economic significance during the period in which smallpox vaccination took place. It occurred sporadically, only occasionally reaching epidemic proportions. In the 1950s, epizootic cowpox in cattle broke out in Holland, Great Britain, France, and Brazil. After 1970, and especially since the termination of worldwide compulsory vaccination against smallpox, however, cowpox has attracted more attention, in view of the risk of its transmission to humans as a zoonosis (Mayr and Czerny 1990c). Today, CPXV is believed to be endemic to Eurasia with a very broad range of host species, although cowpox-like illness in cows is a rare event. The last three decades in Europe have witnessed an increasing number of reports of the infection among a broad range of domestic animals and animals kept in zoological gardens including cats, primates and pet rodents, as well as human CPXV infection. A new epidemiological aspect emerged when cowpox infections were observed in various carnivorous species (Felidae), in some cases in a generalized form. Cowpox in cats was first detected in 1977–1978 (Marennikova et al. 1977; Thomsett et al. 1978). Later, CPXV was also found in leopards, lions, ocelots, cheetahs, and jaguars (Webster and Jefferies 1984). The first zoonotic transmission of CPXV from a cat to a human was documented in 1985 in the Netherlands (Martland et al. 1985). Since then, more than 400 cases of CPXV infections in domestic cats have been recorded, although it is supposed that many infections go unnoticed by veterinarians and/or owners. More than 30 outbreaks have been discovered in zoo animals between 1960 and 2014. Cat-to-cat transmission is apparently rare. The outcome of the infection seems to depend on the CPXV strain, route and site of administration, and most likely the dose of infection. The real incidence of CPXV in cats may be reflected by serological data. Here, a variation from 0 up to 16% of cats with antibodies against OPXV is reported from England, Norway, Austria, Germany and Finland (Czerny et al. 1996; Tryland et al. 1998; Juncker-Voss et al. 2004). There is seasonal variation, with an accumulation

of CPXV infections in late summer and autumn. Cats as predators are exposed to CPXV, while hunting rodents serve as a reservoir for CPXV. Human CPXV cases are mainly caused by direct contact with cats, in rare cases, by direct contact with zoo animals (Baxby and Bennet 1997). In recent years, transmission from pet rats to other host species including humans has "emerged", but the infections usually caused mild and self-limiting lesions (Wolfs et al. 2002; Martina et al. 2006; Hümer et al. 2007; Campe et al. 2009). In the search for a virus reservoir, infections in laboratory rats have already been discovered (Krikun 1974). The virus, which was first isolated from the lungs of diseased rats, was given the name "pneumotropic virus of white rats", but was later identified as a CPXV, which also occurs in wild rats (*Rattus norvegicus*). The virus took an unapparent course and led to systemic diseases (pneumonia, exanthema). Long-term ecological studies on CPXV infections have provided evidence that wild rodents harbour CPXV, although the virus has been rarely isolated and infection rates vary seasonally (Marennikova et al. 1984; Crouch et al. 1995). Serological assays and PCR data from Great Britain, Norway, Sweden, Finland, Russia and Germany have confirmed that different vole species, mice, and gerbils serve as a natural reservoir for CPXV (Mayr and Czerny 1990c; Essbauer et al. 2010). CPXV infections are rooted predominantly in voles (e. g. *Myodes glareolus*). The virus is excreted with urine and feces. Hunting cats tend to be accidentally infected by their prey, and they themselves represent a potential threat to their owners. CPXV can also infect monkeys. An epizootic infection was observed in a colony of 80 New World monkeys in Germany, consisting of various species including a group of marmosets and those of the *Saguinus* species. During the summer and autumn seasons of 2002, 30 animals died of unknown diseases. A complete patho-histological and virological investigation with complete genome sequencing confirmed that the OPXV isolate was a CPXV variant (Mätz-Rensing et al. 2006; Carroll et al. 2011). The origin of this poxvirus outbreak remains unexplained. The virus was designated as "Calpox virus". The CPXV isolates investigated so far display a high and more complex heterogeneity in comparison to other OPXV species. Higher genotypic variation was found in German CPXV isolates than in those from Scandinavia and the UK, which suggests the potential for the geographically independent evolution of these viruses and perhaps differences in their rodent reservoir species (Meyer et al. 1999). The data also showed that several CPXV strains seem to co-circulate regionally and timely in Germany, while in England one CPXV strain seems to dominate. Both previous and current literature describe "cowpox" as a disease with a monophyletic agent. Genotypic data presented in a recent study indicates that CPXV is not a single species, but a composite of up to five species, which can infect cows, humans, and other animals (Carroll et al. 2011). *Monkeypox* outbreaks could be observed in several chronological circles. The infection affects monkeys and humans. MPXV was discovered in *monkeys* in 1958, when two outbreaks of a non-fatal pox-like disease were observed in two shipments of cynomolgus monkeys (*Macaca cynomolgus*) arriving in Copenhagen (Von Magnus et al. 1959). Skin eruptions developed between 51 and 62 days after arrival, and approximately 25 % of the animals developed a clinical disease (Essbauer and Meyer 2007). The virus isolate was named

Monkeypox virus and given recognition as an own species of the genus *Orthopoxvirus* (Essbauer et al. 2010). This MPXV Copenhagen strain is regarded as the reference strain within the species. In the following 10 years, a total of nine monkeypox outbreaks were observed in captive monkey colonies in the US, the Netherlands, and France, and several isolates were recovered. The natural route of transmission is unknown (Arita et al. 1972; Parker and Buller 2013). Although MPXV was discovered in 1958, the virus was first identified in humans in 1970. At that time, it was found that a smallpox-like disease in humans, living in tropical rainforest areas in several countries in Western and Central Africa, was caused by MPXV (Jezek et al. 1988). Smallpox had just been eliminated from these countries. The discovery of MPXV led to a WHO-initiated intensive investigation of the human disease to address public health importance and to determine whether MPXV might represent a threat to the global smallpox eradication campaign. Initial epidemiologic studies conducted during 1970–1979 detected a total of 47 cases of human monkeypox near the rainforests of sub-Saharan Africa, of which 38 occurred in the Democratic Republic of Congo (DRC) and the remainder in Cameroon, the Central African Republic, Gabon, Cote d'Ivoire, Liberia, Nigeria, and Sierra Leone (Breman et al. 1980; Nalca et al. 2005). Seven of the 47 reported infections were fatal (case fatality rate 15%). Secondary transmission was determined to be the most likely cause of infection in 4 cases (8.5%), with secondary attack rates of 7.5% among close family members living in the same household and 3.3% among all susceptible contacts. This was much lower than the 37–88% observed with smallpox (Nalca et al. 2005). Monkeypox was not considered to be a serious health risk at that time because there was no evidence of sustained transmissibility in humans. After the end of the active WHO surveillance program in 1986, only 13 cases of human monkeypox were reported until 1996. In 1996/97, however, the largest outbreak ever recorded occurred in the Kasai Oriental region of the DRC (Hutin et al. 2001; Rimoin et al. 2007; Essbauer et al. 2010). The remarkable rise in secondary cases between 1970–1986 surveillance data and the 1996/97 outbreak data (28 *versus* 78%) was due to a less specific case definition used in the 1996/97 outbreak. A substantial proportion of cases were possibly chickenpox (Di Giulio and Eckburg 2004; Rimoin et al. 2007). In 2005, the emergence of occasional and sporadic human MPXV infections was reported for the first time from a rural farming area in Southern Sudan, which differs ecologically tremendously from the tropical rainforest. A follow-up investigation by the WHO supported the hypothesis of recurrent carry-over from local, supposed animal reservoirs (Damon et al. 2006). MPXV was once restricted to specific regions of Africa, but its environment has expanded, even intercontinentally (Parker and Buller 2013). In 2003, human monkeypox was first recognized outside Africa in the USA (Reed et al. 2004). As of July 8, 2003, 71 cases of human monkeypox have been reported to the CDC and 49% of the cases were laboratory confirmed. Trace-back investigations have determined that all confirmed human cases of monkeypox were associated with captive prairie dogs obtained from an animal distributor. These animals appear to have been infected through contact with African rodents. Before that 2003 US outbreak, human monkeypox had never been reported in the Western hemisphere (Nalca et al. 2005). The reservoir for MPXV is still

unknown. However, there is data available to suggest that monkeys are, similar to humans, incidental hosts, and that the reservoir is likely to be one or numerous species of rodents or squirrels which inhabit the secondary forest of West and Central Africa (Khodakevich et al. 1988). To understand how human monkeypox is derived from an animal source, initial efforts focused on monkeys. Serological surveys of Asian monkeys were negative, but specific antibodies were detected in eight species of monkeys living in Western and Central Africa. However, since MPXV does not cause persistent infections, attention was then directed to terrestrial and arboreal rodents (Essbauer and Meyer 2007). Several epidemiological studies were conducted in the DRC, and in 1985, attention focused on animals found near villages in which cases of human monkeypox had recently occurred. MPXV-specific antibodies were found in two squirrel species (*Funisciurus aneryhrus* and *Heliosciurus rufobrachium*) and the virus was recovered from a diseased squirrel (Arita et al. 1985). A subsequent seroprevalence study, carried out as part of the 1996/97 outbreak investigation in the DRC, showed that 39–50% of *Funisciurus* spp. and 50% of *Heliosciurus* spp. squirrels were seropositive (Hutin et al. 2001). In conclusion, conditions that facilitate outbreaks of human monkeypox in Africa include an enzootic circulation of MPXV in animals living in agricultural areas and forests surrounding human settlements, consuming of poorly cooked meat of infected wild or forest animals, and close contact with animals such as hunting, skinning, and playing with carcasses (Meyer et al. 2002). Monkeypox cases have not been reported in East Africa, where human contact with wildlife is more limited. Whether this lack of human disease is due to the absence of MPXV in rodents is unknown (Salzer et al. 2013). Sporadic outbreaks of CMLV infections have been reported among camels from a wide range of countries in Asia and Africa, especially in the Middle East (Bahrain, Iran, Iraq, Oman, Saudi Arabia, United Arab Emirates and Yemen), in Africa (Algeria, Egypt, Ethiopia, Kenya, Mauritania, Morocco, Niger, Somalia and Sudan), and in Asia (Afghanistan, India, Pakistan, the Asian part of Russia, Turkmenistan, and United Arab Emirates) (Baxby 1972; Wernery and Kaaden 2002; Balamurugan et al. 2009; Bhanuprakash et al. 2010a; Touil et al. 2012; Ayelet et al. 2013). Although the occurrence of camelpox is reported frequently by field veterinarians and camel herders, and despite the fact that CMLV is genetically the closest known virus to VARV, the epidemiology of the disease remains poorly studied. A reservoir other than camels is unlikely to exist (Duraffour et al. 2011). The CMLV infection is usually restricted to camels and causes localized skin lesions. CMLV spreads rapidly in camel populations, affecting young animals in particular. Occasionally the infection leads to a generalized form of the disease (Bera et al. 2011). Once CMLV has established itself in a herd, it will further be transmitted to other camelids both directly by contact between infected and susceptible animals through skin abrasions or via aerosols, and indirectly via a contaminated environment (Khalafalla and Mohamed 1996). Virus in dry scabs or crusts remains infectious for 4.5 months or longer. CMLV is also frequently shed with saliva and secretions from the eyes, the upper respiratory tract, and the digestive tract to the environment, such as water, which then becomes the source of infection (Khalafalla 2007). Feed, grazing and contaminated objects play an important

role in the spread of the infection. Epidemics occur every 3–5 years, mostly in the warm, humid seasons. Various studies have indicated that the incidence of camelpox outbreaks increases during rainy seasons, with the appearance of more severe forms of the disease. This may be due to the fact that moisture may enhance virus stability in the environment and increase subsequent transmission to susceptible animals. Virus transmission could be also associated with the involvement of arthropods, such as *Hyalomma dromedarii* ticks, which are abundant during rainy seasons and may serve as a mechanical vector of the virus (Wernery et al. 2000; Ayelet et al. 2013). CMLV was regarded to be strictly host-specific and thought not to infect humans or animal species, such as cattle, sheep and goats. However, it has already in the 1980s been speculated that humans handling camels were infected with CMLV (Kriz 1982). The first conclusive piece of evidence for a zoonotic CLMV infection in humans was reported in 2011 (Bera et al. 2011). The outbreak in Dromedarian camels (*Camelus dromedaries*) in the Northwest region of India between December 2008 and May 2009 involved camel handlers and attendants with clinical manifestations over fingers and hands.

Poxviruses possess a repertoire of genes encoding multiple proteins that efficiently counteract the host immune responses, especially innate reactions of the immune system (Essbauer et al. 2010). CPXV contains the largest genome and possibly infects the widest range of host species. Consequently, CPXV encodes the largest operative group of genes, representing the whole spectrum of immune evasive mechanisms poxviruses have acquired during coevolution with their hosts. CPXV is believed to be the most ancient poxvirus and the virus most closely related to a common ancestor virus. This is in contrast to VARV, which lacks many of the putative ancestral genes, and therefore most likely explains its restriction to a single host—the human being. Once humans and animals have recovered from OPXV infections, they possess a protective immunity persisting for several years.

The eradication of smallpox was accomplished through the compulsory vaccination of mankind with live VACV, a benign relative to VARV, usually administered percutaneously by scarification of the skin with a bifurcated needle. Live poxvirus vaccines is the preferred method of prophylaxis. However, depending on the strains, a relatively high number of adverse clinical side effects had to be accepted at that time, where smallpox was widely distributed. Today, smallpox does not exist anymore in the environment. However, due to fears that VARV from an unregistered stock or MPXV may be used in bioterrorism, VACV remains in the public eye as a conventional poxvirus vaccine. Many countries have acquired stocks of first and second-generation VACV vaccines (Essbauer et al. 2010). But due to the well-known complications, only highly attenuated VACV strains may be used as vaccines in future. Attenuated VACV strains have been obtained by repeated passage in cell culture. They are considerably less virulent for humans and animals, and their ability to induce protective immunity against smallpox remains unchanged. The LC16m8 (Hashizume 1975) derived from a from single plaque of VACV Lister, and the modified VACV strain Ankara, which was developed in the 1970s by 571–576 passages on primary chicken embryo fibroblast cells (Mayr et al. 1978), are regarded as promising so-called third generation smallpox vaccine candidates—

attenuation through sequential passage (Moss 2011). Replication of VACV MVA is usually accompanied by a cytopathogenic effect (cpe) in primary chicken embryo fibroblast cells (CEF), leading to cytolysis after 3 days. Virus harvests contain up to $10^{7.0}$–$10^{8.5}$ $TCID_{50}$/ml (Mayr et al. 1978). The VACV strain MVA has proven to be harmless and has good immunogenic properties. A dose should contain at least $10^{7.0}$–$10^{8.0}$ $TCID_{50}$/ml of the virus. The strain may also be used for vaccinations in veterinary medicine against VACV, BPXV, CPXV, and CMLV (Mayr and Czerny 1990a, b, c, d; Hafez et al. 1992). The preferred method of prophylaxis employs live virus vaccines for healthy animals or after an outbreak has started in a herd or a zoo (emergency vaccination). For primary vaccination, two parenteral injections should be given intracutaneously or subcutaneously at an interval of 3–4 weeks. In the event of an emergency, one injection is sufficient. Re-vaccination should occur after 2–3 years. The MVA vaccines should contain at least $10^{7.0}$–$10^{8.0}$ $TCID_{50}$ per dose to ensure a high degree of efficacy. Vaccination of zoo animals has been used over many years to protect elephants and rhinoceroses kept in zoos and circuses (Mahnel and Mayr 1994). Protection is based on cellular immunity, which begins 3–5 days p.i. and on humoral immunity, which develops after 8 days p.i. It is based on neutralizing antibodies against various antigenic sites on the envelopes of MV and EV. Neutralizing antibodies persist for 2–3 years and are considered to prevent generalization of the virus. It has been shown that camels infected intradermally or subcutaneously with high doses of the vaccine strain VACV Elstree did not develop any signs of illness and were protected from subsequent challenge with pathogenic CMLV strains (Baxby et al. 1975; Hafez et al. 1992; Duraffour et al. 2011). However, the virulent VACV strain Elstree could induce generalized infections among young susceptible camels. Additionally, three live camelpox vaccines, of which two are commercialized, have been evaluated. They contain the CMLV strains Jouf-78 (Hafez et al. 1992), $VD_{47/25}$ (Nguyen et al. 1996), and Ducapox 298/89 (Wernery and Zachariah 1999). In Saudi Arabia, the CMLV strain Jouf-78, an attenuated CMLV strain passaged 80 times in cell culture, has been shown to offer full protection from CMLV challenge. In camels, this strain is not pathogenic when given intradermally or subcutaneously at a dose of $10^{6.8}$ $TCID_{50}$ (Hafez et al. 1992). From field studies, a single dose of the vaccine ranging from 10^3 to 10^4 $TCID_{50}$ was advised for full protection. The attenuated CMLV strains $VD_{47/25}$ and CaPV298-2 were innocuous in camels during experiments in Mauritania and in the United Arab Emirates (Nguyen et al. 1996). However, in both cases protection from severe to lethal CMLV infection could not properly be evaluated, because it was difficult to guarantee the naïve status of animals enrolled due to the endemicity of the disease in the countries at the time of the study (Nguyen et al. 1996; Duraffour et al. 2011). In Morocco, an inactivated camelpox vaccine derived from the CMLV strain T8 with an adjuvant has proven to induce CMLV-neutralizing antibodies (Khalafalla and El Dirdiri 2003). Once MPXV is identified as the disease agent, quarantine and immediate ring vaccination are important public health protective procedures. The perceived risk of deliberate usage of smallpox and the possible severe side effects of the currently available smallpox vaccine recommend use of safer next generation vaccines (Artenstein 2008). Although the new vaccines are likely to improve safety,

it is not a foregone conclusion that they will in fact provide adequate immune protection, particularly in immunodeficient populations.

The advances in biotechnology that allow insertion, deletion and interruption of genes in specific genomic sites of VACV, such as immunomodulating and host-range genes, enable a targeted attenuation of viruses while maintaining immunogenicity (for review see Jacobs et al. 2009). Today, VACV is used in many laboratories worldwide as a vector for the generation of recombinant vaccine candidates, capable of safely expressing foreign proteins. Due to their safety, replication defective strains such as VACV MVA are favored for this instance. They have already proved their potency as an immunization vector over years (Sutter and Moss 1992; Volz and Sutter 2013).

20.4 Clinical Features

Vaccinia virus infections in mammalians can develop as most common local affections and a generalized disease. Latent infections are unlikely. The incubation period is 2–3 days. Lesions develop on restricted areas of the mucous membranes, skin or on other organs. A generalized disease develops in immunosuppressed humans and animals. It arises as a result of a massive propagation of the virus in the primary target organs and progresses through viremia. In the local form of the disease efflorescences (*stadium maculosum*), which arise on the skin and the mucous membranes, develop subsequently into pustules, when necrosis of the blisters' central region (pox navel) occurs. It takes about 5 days for the pustules to fully develop. If there is no secondary bacterial infection, the pustules dry up within a few days and fall off as scabs after 1 week. In the inner organs, a localized proliferation initially occurs at the point, where the virus is multiplying. The generalized form of the infection begins with general signs of disease, e.g. anorexia, restlessness, and salivation. The incubation period is between 3 and 7 days. When the fever has subsided, pox pustules gradually appear over the whole body. Complications are acute ulcerations. In the absence of complications, the generalized form progresses rapidly and the symptoms vanish. In domestic and farm animals VACV has an affinity to fetal organs (Mayr and Czerny 1990a). Therefore, abortion and fetal malformation must be expected in susceptible animals. Newborns are most at risk, as the vaccinia infection easily becomes generalized. The prognosis is good in most cases. For decades, VACV was used for the immunization of humans during the smallpox eradication campaign. Besides the outstanding achievement of VACV as a vaccine, several serious complications have been reported, with the frequency depending on the vaccine strain (Smith 2007; Essbauer et al. 2010). The complications included, e.g. progressive vaccinia, generalized vaccinia, vaccinia eczematum, sometimes neurological disorders (encephalitis), myocarditis or ocular complications. Numerous strains exist which differ in their biological properties and virulence in humans and in animal models. Clinically, buffalopox resembles VACV infections. In buffaloes, the disease is characterized by a local pox exanthema and localized pock-lesions on the muzzle,

Fig. 20.1 Diverse severity of cowpox virus infections in domestic cats

udder, teats, inside of thighs, scrotum, base of the ears, inner surface of earflap and eyes in mild form (Mayr and Czerny 1990b). In individual cases, buffalopox can also develop as a severe systemic disease of a cyclical pattern with generalized lesions. Severe local pocks affecting the udder and teats can lead to mastitis, thereby undermining the productivity of milk animals (40–70 % reduction) and thus having an impact on the respective dairy industry (Essbauer et al. 2010; Prabhu et al. 2012). The disease may be transmitted to humans who are in close contact with affected buffaloes. BPXV infection in humans has been reported with lesions confined to the hand, wrist and thumbs, with or without swollen regional glands and fever (Ghosh et al. 1977; Singh et al. 2007). Cowpox in ruminants resembles local affections with pustule formation, and a generalized systemic disease (Mayr and Czerny 1990c). There is no evidence of latent CPXV infections. The incubation period generally lasts for 3–7 days. The disease begins with a moderate fever above 40.5 °C as early as 3 days after inoculation. Shortly after or at the same time as the fever intensifies, pocks appear on the muzzle, udders, teats and testes, and only very rarely on other parts of the body. The pustules reach maturity after 8–11 days and then dry up into a scab. Typical for an infection with CPXV is that it is frequently accompanied by hemorrhagic symptoms, resulting in scabs with a darker color than those occurring after VACV infection. Localized pox lesions usually take a benign course unless the tops of broken pustules become infected with bacteria or abscesses or ulcers form. The generalized infection, however, often leads to a more severe course of disease and show a cyclic pattern affecting the skin and mucous membranes, internal organs and the central nervous system. They are the result of virus replication in organs of primary affinity such as bone marrow and the liver, following viremia. High fever and heart or circulatory complications are the dominant symptoms. In the advanced stage of the disease, central nervous disorders become apparent. The prognosis is favourable for the localized form of the disease. Abortions occur in pregnant animals. Younger animals are more seriously affected than older ones. In domestic and also large cats, slight or severe multiple skin lesions (seen primarily on the head, oral cavity, neck, forelimb or paws), conjunctivitis or purulent ocular discharge develop upon infection (Fig. 20.1). Occasionally, systemic infections occur which may end fatally if inner organs such as the lungs (e.g. necrotizing pneumonia), co-infections or immunodeficiency are involved (Bennett et al. 1989; Czerny et al. 1996).

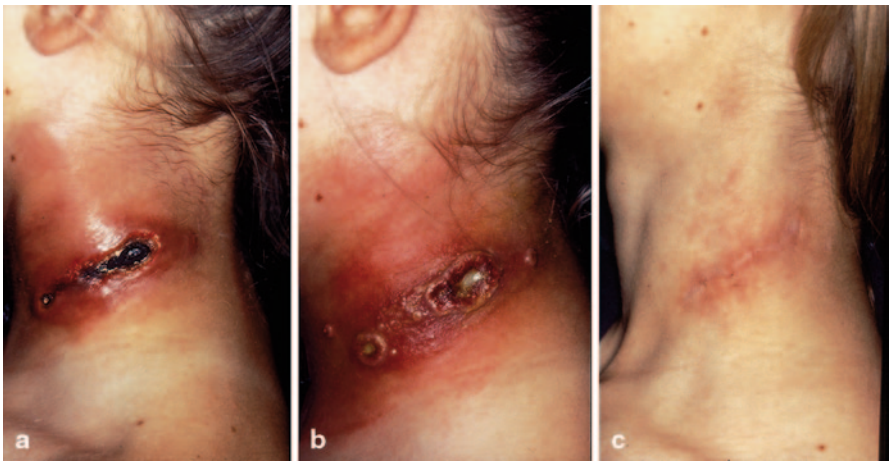

Fig. 20.2 Cowpox virus infection of a 13-years-old girl transmitted by a domestic cat. Typical necrosis and healing of the lesions within 8 weeks

Human *CPXV* infections after direct contact with pustule material, e.g. during milking, or from house pets or zoo animals, are generally mild and self-limiting, but can take a severe course. They are characterized by localized, pustular skin lesions, mostly on the hands. Scratches or abrasions of the skin after contact with cats may determine localization of the lesions elsewhere, e.g. on the face, neck, arms or feet (Fig. 20.2). Pocks heal after 3–8 weeks (Czerny et al. 1996; Schulze et al. 2007). Systemic involvement with fever, lymphangitis, lymphadenitis, conjunctivitis and disseminated pox exanthema may also develop. Fatal outcomes of human CPXV infections have only been reported for immunocomprised individuals thus far (Eis-Hübinger et al. 1990; Czerny et al. 1991; Pelkonen et al. 2003).

In case of MPXV infection in monkeys, usually no clinical signs are detected until the rash appears. They develop from generalized skin eruptions into papules on the trunk, face, palms, and soles. Papules subsequently evolve into vesicles, pustules and scabs, which usually fall off 7–10 days after onset of the rash. The severity of the disease varies depending on the host species. During an outbreak in the zoological garden of Rotterdam, the disease was mild in Cynomolgus monkeys (*Macaca cynomolgus*), but more severe in orangutans (*Pongo* sp.) (Arita et al. 1972). Human MPXV infection can cause severe illness and even death (Frey and Belshe 2004; Johnson et al. 2011). Monkeypox is clinically very similar to the ordinary forms of smallpox, including flulike symptoms, fever, malaise, back pain, headaches, and characteristic rash (Nalca et al. 2005). In the 1980s, studies on human monkeypox showed an incubation period of 10–14 days and an infectious period during the 1st week of the rash (Essbauer and Meyer 2007). A characteristic 2-day prodrome with fever and malaise occurs in most patients before the rash develops. The only feature differentiating human monkeypox from smallpox is lymph node enlargement. Lesions develop virtually simultaneously and evolve together at the same time, forming papules, vesicles, pustules and crusts before resolving and leaving scars. This

process takes 2–3 weeks. Severe eruptions can cover the entire body, including the palms and soles. Chickenpox, a disease caused by the unrelated varicella zoster herpesvirus, became the primary differential diagnostic challenge (Jezek et al. 1988). Only the disease, which emerged in the United States in 2003, was mainly mild in nature. Among 69 of 71 patients for whom data was available, 18 (26%) were hospitalized. Two children had a serious clinical illness. The median incubation period was 12 days (range: 1–31 days). The aggregate clinical signs and symptoms were similar to those described in outbreaks of monkeypox in Africa. Unlike the African outbreaks, the US outbreak did not result in any fatalities and there was no documented human-to-human or secondary transmission. Clinical manifestations of *Camelpox* range from unapparent infection to mild, moderate, and less common, severe systemic infection and death (Czerny and Mayr 1990d; Ayelet et al. 2013). Young calves and pregnant females are more susceptible (Wernery and Kaaden 2002; Al-Zi'abi et al. 2007). The incubation period for the adult camel is usually 6–7 days (range 3–15 days). Newborn foals of non-immune camels fall ill between 3 and 5 days after being exposed to the virus at birth. The disease is characterized by fever, enlarged lymph nodes, and skin lesions. The latter appear 1–3 days after the onset of prodromal symptoms (fever and salivation), starting as erythematous macules, developing into papules and vesicles, and later turning into pustules. The first symptom is usually an edema of the entire face. Then pustules form on the eyelids, lips, nostrils, and margins of the ears. In severe cases, secondary bacterial infections of the eyes and ears may cause blindness. Skin lesions may spread to the neck, throat, mammary glands, extremities, inguinal region, and perineum (Mayr and Czerny 1990d). In the generalized form, a typical pox exanthema may cover the entire body and pox lesions are found to be associated with the mucous membranes of the mouth, respiratory, digestive and genital tracts (Wernery and Kaaden 2002). Pregnant female camels tend to suffer abortions. Secondary infections result in suppuration beneath the pock scabs, abscesses in the subcutis, muscles, lymph nodes and lungs, and general pyemic symptoms. In such cases, the animals experience exhaustion, loss of appetite, diarrhea, and rapid loss of weight. They die quickly or after a few months due to total exhaustion. In general, young animals (2–3 years old) are most severely affected. The mortality rate in infected herds ranges from 25 to 100% for young animals, and from 5 to 25% for older animals. Mortality is higher among male (8.8%) than female animals (4.4%). Skin lesions may take up to 4–6 weeks to heal.

20.5 Diagnosis

Clinical lesions caused by OPXV are mostly typical for poxvirus infections, but not sufficient for an etiological diagnosis of the disease in humans and animals. However, the typical pock morphology caused by CPXV is very specific. Single lesions consist of three significant features: a central dark-red to black colored necrosis surrounded by an elevated margin, which is girdled by a sharp haemorrhagic

edge. This picture, especially the necrosis, enables the differentiation of CPXV from VACV and parapox virus infections. Differential clinical diagnosis of MPXV infections reflects mainly on smallpox and other causes of vesiculopustular rashes such as chickenpox, whereas clinical symptoms of camelpox are very similar to viral diseases, such as contagious ecthyma (parapoxvirus) and papillomatosis (papillomavirus) (Wernery and Kaaden 2002; Ayelet et al. 2013). Due to their large size and typical particle morphology, the preferred method for a rapid OPXV diagnosis in fresh samples of skin and mucous membrane pustules, swabs or bioptates is electron microscopy (EM). The brick shape of the virions with lateral bodies and a central core are typical morphological characteristics. However, it is not possible to differentiate within the genus *Orthopoxvirus* by EM at the species level. Approved classical methods for further characterization is virus inoculation in animals, especially laboratory animals. Of all the common laboratory animals, rabbits, guinea pigs, rats, and mice are susceptible to OPXVs. After intracutaneous injection of CPXV into the rabbit skin, indurated lesions with a dominant hemorrhagic character and central necrosis can be observed. Virus isolation and classical differentiation of OPXV is possible based on the character of primary foci on the chorioallantoic membrane (CAM) of embryonated chicken eggs, which have been pre-incubated for 10–12 days. Flattened, fairly rounded pocks (1.5–4 mm in diameter) and a red central hemorrhagic area arise at 72 h post inoculation. Afterwards the infection becomes generalized throughout the CAM along the blood vessels. Generalization includes the embryo and its internal organs. During this stage the embryo dies. After generalization, titers between $10^{7.5}$ and $10^{8.5}$ EID_{50}/ml can be found in the CAM. All OPXVs replicate in a broad cell spectrum of virtually all *cell cultures* from mammals and birds. Particularly African Green Monkey cell lines (Vero, MA-104) are recommended (Czerny et al. 1994). Virus growth is accompanied with a cythopathic effect (cpe). It begins with rounding of the cells and results in lysis, cell fusion, and the formation of syncytia. Optimum virus harvests contain $10^{7.0}$–$10^{8.5}$ $TCID_{50}$/ml. Histologically, intracytoplasmic A and B-type inclusion bodies (Guarnieri's bodies) can be detected. The acidophilic A-type inclusion bodies are viroplasm zones at an advanced stage of virus development, in which mature particles dominate. The basophilic B-type inclusion bodies are zones in which early virus development takes place. The lesions include ballooning degeneration of keratinocytes, prominent spongiosis, dermal edema, and acute inflammation. Eosinophilic A-type inclusion (ATI) bodies in the cytoplasm of infected cells are phenotypic features, which distinguish CPXV from other OPXV species (Essbauer et al. 2010). In contrast to VACV inclusion bodies, those of CPXV are large, compact, less granular, and occur only in small numbers. A-type inclusion bodies consist of a 160-kDa protein—one of the most abundantly synthesized late proteins—and involve other factors (e.g. structural protein P4c), which drive the inclusion of mature virions (Patel et al. 1986). There is no cross-reactivity between surface antigens of OPXV and the other genera of the family *Poxviridae*. Only the nucleoprotein antigen, extracted from virus suspensions with 0.04 M NaOH and 56 °C treatment, is regarded to be highly cross-reactive among members of *Chordopoxvirinae* (King et al. 2012). But all members within the genus OPXV are serologically closely related. Today,

ELISA, immunofluorescence assays (IF) and Western blotting (WB), together with neutralization and plaque reduction tests (NT/PlRT), are common. A species-specific differentiation of OPXVs by serological assays is only possible using monoclonal antibodies (Mabs) (Czerny et al. 1994, 1997). Especially, in case of CPXV isolates species-specific Mabs proved to differentiate between different CPXV groups. This can be important when isolates from rodents are to be distinguished from the ectromelia virus (ECTV; mousepox virus), which is the only rodent poxvirus known. Molecular biological methods differentia-ting between OPXV are well established. Besides hybridization techniques and restriction enzyme analysis of the DNA, several conventional PCR assays have been used to distinguish OPXVs from the species or strain level. Since 2001, real-time PCRs have been developed (e.g. Czerny et al. 2001; Kulesh et al. 2004; Balamurugan et al. 2009; Gavrilova et al. 2010; Li et al. 2010; Shchelkunov et al. 2011; Venkatesan et al. 2012a). A DNA oligonucleotide microarray with the TNF receptor gene crmB (Lapa et al. 2002) and loop-mediated isothermal amplification (LAMP) assays are alternative rapid methods for OPXV detection or differentiation (Iizuka et al. 2009; Venkatesan et al. 2012b). Today, due to rapid next generation sequencing techniques, reliable phylogenetic analysis is becoming increasingly related to complete genome sequencing. Genotypic data presented in a recent study indicates that CPXV is not a single species, but it has different genotypes and may be subdivided into at least five genetically distinct monophyletic clusters (Carroll et al. 2011). A genomic analyses based on the whole genome from nine CPXV strains circulating in Europe, as well as three genomes of CPXV from Great Britain, Moscow and Germany, sequenced previously (Brighton Red, GRI-90, GER 1991–1993), clearly suggests that CPXV, as currently recognized, is a polyphyletic assemblage. In other words, a composite of up to five CPXV species can infect cows, humans, and other animals. Therefore, the current CPXV taxonomic rank must be divided into multiple species which each represent a monophyletic lineage. Indirect diagnosis via the detection of OPXV-specific *antibodies* in human and animal sera is possible in individual cases by examining paired sera in hemagglutination inhibition (HI) assay, neutralization (NT) and plaque reduction test (PlRT), Western blotting (WB) or different types of ELISAs (Czerny et al. 1996; Tryland et al. 1998). Besides IgG ELISAs, an OPXV-specific IgM assay was recently described and applied to determine acute-phase humoral immunity to MPXV in the 2003 US outbreak (Karem et al. 2005).

20.6 Therapy

There are currently no specific, licensed antiviral drugs for the treatment of zoonotic OPXV infections in humans and animals on the market. This is significant due to the number of recent cowpox and moneypox cases reported in humans. *Symptomatic treatment* is intended to prevent secondary bacterial infections of the lesions. Localized skin and mucous membrane inflammations after CPXV infection were treated symptomatically with antibiotics or iodine and halogen-based ointments, acidic

solutions, sprays or embrocations. In some cases, due to delayed diagnostics, lesions were surgically excised (Campe et al. 2009; Ninove et al. 2009). Humans, as well as animals, can be protected against the infection with zoonotic OPXVs by passive immunization administering immune serum against VACV. The experience with the use of VACV vaccine to prevent smallpox suggests that antibody preparations could be generated that would be active against VARV and VACV (Casevall 2002). Neutralizing and protective antibodies to VACV and OPXVs have been described that target viral envelope antigens. However, often and especially in case of immunosuppressed individuals, it is too late for serum therapy, which does not prevent the local affections from developing into a generalized systemic disease (Eis-Hübinger et al. 1990; Czerny et al. 1991). Additionally, reservations against polyclonal human immune sera arise due to safety reasons. The origin of those biological compounds is not characterized very well. The advances in antibody engineering may enable the construction of immunoglobulin libraries in near future, allowing the establishment of highly affine species-specific and neutralizing antibodies, designed against well-defined antigenic sites, with key roles in virus replication. Antivirals against poxviruses exist since the 1950s. Although not approved and licenced by the health authorities, over the last years, potent antiviral molecules active *in vitro* and *in vivo* against poxviruses, including OPXVs, have been developed. The acyclic nucleoside analogue and viral DNA polymerase inhibitor *cidofovir* [(S)-1-(3-hydroxy-2-phosphonylmethoxy-propyl)cytosine, HPMPC] is a broad-spectrum antiviral drug that acts against many DNA viruses, including herpes, adeno, polyoma, papilloma, and poxviruses. Among poxviruses, cidofovir has demonstrated in vitro activity against OPXVs (VACV, VARV, CPXV, MPXV, CMLV, ECTV), molluscipox (molluscum contagiosum) and parapox (orf) virus (De Clercq 2002; Quenelle et al. 2004; Nalca et al. 2008; Andrei et al. 2010; Hostetler 2010; Duraffour et al. 2011) and proved to inhibit growth of *in vitro* and *in vivo*. Since 1996, cidofovir has been licensed under the name VistideTM (Gilead) for clinical treatment of cytomegalovirus (CMV) retinitis in patients with AIDS. Cidofovir has not been used to treat OPXV infection in humans, but has been tested extensively in laboratory animals (Smee 2008). The anti-poxvirus activity of cidofovir *in vivo* has been shown in different models of infection when the compound was administered either intraperitoneally, intranasally (aerosolized) or topically. In immunocompromised human patients, cidofovir has been used successfully for the treatment of recalcitrant *molluscum contagiosum* virus and *orf* virus. Cidofovir must be administered intravenously and accompanied by probenecid and hydration to avoid renal toxicity. Modified forms of cidofovir such as the lipid derivative CMX001 (HDP-cidofovir) with improved oral bioavailability have already shown some promise in a mouse model of OPXV infection (Quenelle et al. 2004; Hostetler 2010; Andrei et al. 2010). Cidofovir remains a reference compound against poxviruses and holds potential for therapy and short-term prophylaxis. CMLV resistant to cidofovir and its analog HPMPDAP could be obtained *in vitro* after passaging the virus 20–40 times in increasing concentrations of the drug (Smee et al. 2002; Duraffour et al. 2009). Sequencing of the viral DNA polymerase gene led to the mapping of mutations conferring resistance (Gammon et al. 2008; Duraffour et al. 2009).

A relatively new drug, *ST-246* (Tecovirimat, Arestvyr™), is currently considered to be effective for treatment of OPXVs. It is a small-molecule identified through a high-throughput screen of a library of compounds for its ability to inhibit the cytopathic effect of poxviruses in vitro (Yang et al. 2005). ST-246 has proven to be a potent inhibitor of OPXV egress from cells. The drug targets the F13 protein (p37) in VACV and its homologs in other OPXV species (Grosenbach et al. 2010). This protein is highly conserved among OPXVs, particularly in the region targeted by ST-246, explaining the genus-wide susceptibility to the drug. ST-246 treatment of infected cells inhibits plaque formation and reduces the formation of enveloped virus without affecting the production of intracellularly retained mature virus particles or EV-associated proteins. Thus viral dissemination is dramatically reduced. ST-246 is effective in vitro against a number of OPXV strains, including VARV, VACV, CPXV, ECTV, MPXV, and CMLV (Yang et al. 2005; Quenelle et al. 2007; Nalca et al. 2008; Smith et al. 2011; Duraffour et al. 2013). Further testing demonstrated that ST-246 is orally bioavailable, well tolerated, nontoxic, and thus reduces virus dissemination also in vivo. No observable adverse effect level in mice measured at 2000 mg/kg and the no observable effect level in non-human primates measured at 300 mg/kg, which is 20 to 30 times the effective antiviral dosing for these species, respectively. ST-246 has been demonstrated to be very effective in numerous animal models for lethal poxviral disease including VACV, ectromelia (ECTV), CPXV, rabbitpox, MPXV, and VARV in mice, ground squirrels, prairie dogs, rabbits, and non-human primates, essentially providing full protection as a postexposure prophylactic and therapeutic. An excellent safety profile of ST-246 for humans was demonstrated in Phase I and II clinical trials, and blood exposures exceeding levels demonstrated to be efficacious in animals are readily achievable in humans (Jordan et al. 2008). Many evaluations of ST-246 efficacy have been performed in immunocompetent animals. However, the risk of severe poxviral disease is greater in immunodeficient hosts. The efficacy of ST-246 in preventing or treating lethal poxviral disease in immunodeficient mice has also been confirmed (Grosenbach et al. 2010). Delaying treatment until 72 h after infection reduced ST-246 efficacy in some models but provided full protection from lethal challenge in most. These findings suggest that ST-246 may be effective in controlling smallpox or other pathogenic OPXVs in some immunodeficient human populations for which the vaccine is contraindicated. ST-246 showed potent antiviral activities against VACV infections in humans under emergency use. For the Cantagalo virus (CTGV) and other VACV viruses being the etiologic agents of a pustular disease in dairy cows and dairy workers in Brazil, the effect of ST-246 on virus replication was confirmed (Santos-Fernandes et al. 2013).

Recently, the synthesis and characterization of new series of *thiazolo[3,2-a]pyrimidine-6-carboxylate* derivatives 3a-f and 4a-f was reported for therapy of BPXV and CMLV (Umesha et al. 2013). The probable mode of action was determined through in silico study by docking the receptor methionyl-tRNA synthetase and human inosine-5′-monophosphate dehydrogenase (IMPDH) for antibacterial and antiviral activities, respectively.

Much work still needs to be performed (especially in nonhuman primates), before a licensed drug will be available to treat human OPXV infections. However, the threat of smallpox as a bioweapon and the emerging threat of human monkeypox, among other poxviral diseases, highlight the need for further effective poxvirus countermeasures (Henderson et al. 1999). Cidovovir and its lipid derivative CMX001 (Chimerix Inc, NC, USA), as well as ST246 have gained "Investigational New Drug" (IND) status, permitting their emergency use for the treatment of life-threatening VACV infections.

20.7 Prospects

Due to the ability of VACV to take up a large amount of foreign genes, today the development of recombinant VACV vector vaccines is of great interest for medical applications. However, the former vaccine strains used during the smallpox eradication campaign are not at the forefront, but highly attenuated, and therefore safer, VACV strains derived over hundreds of passages in cell culture are of interest. The modified VACV Ankara is an exceptionally safe vaccine virus and is one of the most promising candidates for this purpose. Preclinical studies are under way to select promising MVA vaccines for clinical testing and the evaluation of immune correlates of protection. Over a period of time, BPXV has established itself in buffaloes and has evolved to be increasingly pathogenic to the new host, besides evolving further to infect both cattle and humans. The occurrence of any pathogenic poxvirus, which spreads efficiently from human-to-human, could be considered an immediate public health concern. The human outbreaks of VACV-like viruses in Brazil and BPXV in India illustrate how vulnerable the human population is to the emergence and re-emergence of viral pathogens from unsuspected sources (Bera et al. 2012). Infections of ruminants (especially cows) with the genuine CPXV is a rare event worldwide. Instead, other mammalian species are targets of the virus. A salient infectious chain is the transmission of CPXV from felidae to humans. Worldwide one lethal case has been occurred in an immunocompromised individual (Eis-Hübinger et al. 1990; Czerny et al. 1991). Recent investigations on full genome sequencing data have clearly proven that CPVX isolates are polyphyletic (Carroll et al. 2011). The most recent publications on taxonomy refer to this aspect, but they have not yet corrected the monophyletic view, as this is an ongoing process and more important findings are expected in the near future (King et al. 2012). Next generation sequencing techniques will facilitate the evaluation of much larger data pools than those currently available. Therefore, CPXV differentiation and the identification of natural reservoirs will continue to be fascinating research areas in the next few years. *MPXV* was once restricted to the jungles of Central and Western Africa. The disease, unlike smallpox, is a typical zoonosis in that most cases occur as a result of direct contact with an infected animal. The 2003 US outbreak demonstrated that the environment of MPXV has expanded. Hence, MPXV is another example that demonstrates how vulnerable we are to so-called exotic pathogens (Essbauer et al.

2010). Following cessation of the broad VACV immunization in the 1980s, MPXV infections are now emerging as a public health concern (Song et al. 2013). The similarity of African monkeypox cases to smallpox cases, has led to fears that the virus could be used as a bioterrorism agent (Nalca et al. 2005; Parker et al. 2007). This is inasmuch important as 50 % of the population around the globe < 30 years are unvaccinated and are most vulnerable in the event of smallpox re-emergence (Singh et al. 2012). Such concerns have resulted in increasing efforts to elucidate poxvirus pathogenesis and to develop anti-OPXV vaccines and drugs. As a zoonotic agent, MPXV is far less sensitive to typical eradication measures since it is maintained in wild-animal populations. MPXV infections could be risky for immunocompromised individuals in particular. Thus, a change in the natural transmission patterns of MPXV infections in Sub-Saharan Africa may strongly be influenced by the increase of an HIV-infected population and *vice versa*. CMLV is of considerable economic importance due to its high morbidity, relatively high mortality in younger animals, loss of condition and reduced milk production in lactating animals. Although transmission to humans may be rare, camelpox must be considered as a zoonosis, which can cause illness, particularly in immunosuppressed persons. Similar to VACV-like viruses circulating in wild animals in Brazil, BPXV in buffaloes, humans and cows India, CPXV in Europe, and MPXVs in the Democratic Republic of Congo, CMLV may also be a potential public health concern due to evolutionary changes occurring naturally or due to laboratory manipulations. New information pertaining to OPXV persistence in nature would significantly improve surveillance and control programs. Recently it was suggested to eradicate CMLV from the globe such as smallpox and rinderpest or poliomyelitis (Bray and Babiuk 2011). The idea of eradication has traditionally been associated with activities on a global scale, but such a massive effort would not be required for a disease like camelpox, which is confined to a specific region.

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Chapter 21
Elimination of Rabies—A Missed Opportunity

Thomas Müller, Conrad M. Freuling, Charles E. Rupprecht, Leonard Both, Anthony R. Fooks, Tiziana Lembo, Lea Knopf, Deborah J. Briggs and Louise H. Taylor

Abstract Rabies is one of the oldest known zoonoses. Recognized etiological agents consist of at least 15 proposed species of lyssaviruses with primary reservoirs residing in the Orders Carnivora and Chiroptera. A plethora of viral variants, maintained by a diverse set of abundant hosts, presents a formidable challenge to a strict concept of true disease eradication. Despite the availability of affordable and efficacious animal and human vaccines, today however dog rabies continues to escalate unabated across much of Asia and Africa, causing millions of suspect human exposures and tens of thousands of human rabies deaths annually. By identifying what hampers global human rabies elimination this chapter emphasizes that, given the global epidemiology of rabies, the "One Health" concept is key to solving the problem. Next to state of the art human rabies prevention, immunization and experimental therapy, it is obvious that human rabies can only be eliminated through rabies control at the animal source. This 'paradigm shift', however, needs new grassroot initiatives as well as political will and the closing of ranks of all stakeholders in the near future.

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21.1 Global Epidemiology of Rabies—A Retold Story

Ancient world writings from as early as Mesopotamian and Greek times suggest that rabies (lyssa, hydrophobia) is the oldest recognized zoonosis, perhaps as old as mankind. The first written record of rabies is in the Mesopotamian Codex of Eshnunna of Babylon (circa 1930 BC), which mentioned preventive measures to be taken by owners of dogs suspected of having contracted rabies and further detailed heavy punishments for dog owners in case another person was bitten by their rabid dog and later died (Dunlop and Williams 1996). Rabies is defined as an acute, progressive, incurable viral encephalitis that is transmitted following bites by infected mammals. Whilst the name rabies is derived from the Latin name for 'madness', the ancient Greek term 'lyssa' presumably means 'violent'; with the root "lud" used in the name of the genus lyssavirus (Jackson 2013a).

For millennia, the disease has been considered a scourge for its prevalence and a dual public horror and biomedical travesty (Rupprecht et al. 2008). There is probably no other zoonosis known today which has caused such anxiety to people, has been so intensively studied and about which so many common and scientific reports have been published. In the following sections we will attempt to elucidate the complex epidemiology of rabies, including the diversity of its causative agents and its reservoir hosts. Furthermore, we will explain (i) why rabies is still considered a neglected zoonotic (or tropical) disease, (ii) what progress has been made in the elimination of canine and wildlife mediated rabies, (iii) what hampers global rabies elimination and (iv) what new initiatives are being taken to break the current stagnation of the elimination process and find ways out of the dead end.

21.1.1 Lyssavirus Diversity

For a long period of time it was believed that rabies was caused by a single lyssavirus. It was not until the discovery of bat-associated lyssaviruses in the second half of the last century that this perception changed. Today it is commonly accepted that rabies disease is caused by a plethora of different lyssaviruses, negative-strand RNA virus species (previously genotypes) of the Lyssavirus genus, family Rhabdoviridae of the Mononegavirales order (Dietzgen et al. 2012). Intriguingly, almost all lyssaviruses are associated with Chiroptera and hence rabies is the most significant viral zoonosis associated with bats (World Health Organisation 2005). The genus has been subdivided into virus species which share certain morphological and structural characteristics with the rabies virus (RABV), the causative agent of classic rabies in animals and humans: the prototype RABV, African Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus-1 (EBLV-1), EBLV-2, Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), Shimoni bat lyssavirus (SHIBV), and West-Caucasian bat virus (WCBV). Recent detections of novel lyssaviruses named Bokeloh lyssavirus (BBLV) (Freuling et al. 2011), Ikoma lyssavirus (IKOV)

(Marston et al. 2012), and Lleida bat lyssavirus (LLEBV) (Ceballos et al. 2013) further support the delineation of lyssavirus species. There is reason to believe that, with reinforced and enhanced surveillance, the diversity of lyssaviruses will further expand.

Lyssaviruses can be further segregated into phylogroups on the basis of their genetic and antigenic relatedness as well as their biological properties, e.g. pathogenicity, induction of apoptosis, cell receptor recognition, and immunogenicity. So far the great majority of lyssavirus species, including RABV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, KHUV, and IRKV belong to phylogroup I. BBLV also clusters with this group (Freuling et al. 2011). The African lyssaviruses LBV, MOKV and SHIBV were assigned to phylogroup II (Badrane et al. 2001; Kuzmin et al. 2010). Studies revealed that WCBV does not show any cross-neutralisation with any member of the two phylogroups and may form an independent phylogroup (Hanlon et al. 2005; Kuzmin et al. 2005). Interestingly, despite the close phylogenetical relationship of IKOV and WCBV, a lack of cross-neutralization was demonstrated in a recent study (Horton et al. 2014).

There is evidence for broad spectrum cross-neutralisation and cross-protection within phylogroup I (Brookes et al. 2005; Hanlon et al. 2005). This phylogroup includes all currently available vaccines for both human and veterinary use. However, no or only limited cross-protection of classical rabies vaccines with phylogroup II lyssaviruses or other more divergent viruses was measured (Fekadu et al. 1988; Hanlon et al. 2005). Given that almost all bat lyssaviruses have caused fatal spillovers into humans and terrestrial mammals (Johnson et al. 2010a), broader reacting vaccines may be useful (Evans et al. 2012). Recombinant vaccine viruses or reverse genetic constructs encoding the glycoprotein genes of different lyssaviruses may have the potential to offer protection against those lyssaviruses that are incorporated into a given vaccine (Weyer et al. 2007).

Within each lyssavirus species genetic diversity may vary more or less within sublineages corresponding to distinct variants circulating in specific geographical regions and/or particular reservoir hosts in complex ecological communities. It is assumed that limited diversity of a specific variant corresponds to a dynamic equilibrium (or genetic stasis) resulting from a relatively conserved and long term virus-host co-evolution and co-adaptation. Such stability is observed frequently among bat lyssavirus variants (Rupprecht et al. 2008). However, viral variants are also transmitted regularly outside a single host species to a variety of other species, based in part on normal ecological interactions.

21.1.2 Reservoir Hosts

21.1.2.1 RABV

While all mammals are susceptible to the disease, the primary reservoirs for sustained rabies cycles are species of the orders *Chiroptera* (bats) and *Carnivora*. The lyssavirus reservoir in its entire complexity is cryptical yet. The prototype

RABV is the only lyssavirus species known to circulate in bats and also independently in terrestrial reservoirs. Amongst the latter, particularly canine species (family Canidae) are reservoir hosts of RABV in most parts of the world. Rabies maintained and transmitted by domestic dogs (*Canis familiaris*) is by far the most important independent cycle with relevance for public health (Tang et al. 2005). Canine rabies causes most of the annual tens of thousands of human rabies cases or leads to contacts that require costly medical intervention. Developing countries in Asia and Africa suffer most from the burden of the disease (Knobel et al. 2005).

21.1.2.2 Americas

The Americas are the only continent where just one single lyssavirus species is present—the classical RABV; the reason for this remains elusive. In North America, independent infectious cycles exist in raccoons (*Procyon lotor*), skunks (*Memphitis ssp*), red foxes (*Vulpes vulpes*), grey foxes (*Urocyon cinereoargenteus*), coyotes (*Canis latrans*) and arctic foxes (*Alopex lagopus*) (Blanton et al. 2012). The arctic fox seems to transmit the arctic variant of RABV circumpolarly in Europe, Asia and North America (Mansfield et al. 2006a; Mork and Prestrud 2004). Historically, canine rabies (introduced by European Colonization) was predominant in the Americas especially in Central and South America causing many human casualties. After mass immunizations of dogs and strict control measures, canine rabies has been successfully controlled in many countries in the Americas (Rupprecht et al. 2008; Vigilato et al. 2013). With the control of canine rabies and with more sophisticated surveillance methods, other species have been identified as reservoirs for RABV, e.g. the crab-eating fox (*Cerdocyon thous*) (Favoretto et al. 2006) and white-tufted-ear marmoset (*Callithrix jacchus jacchus*) (Favoretto et al. 2001). The latter is the first report of the order Primates as a primary source of rabies infection for humans in a restricted geographic area. The small Indian mongoose (*Herpestes auropunctatus*) was introduced to many Caribbean islands during the latter part of the nineteenth century for the purpose of rodent population control. This species is now implicated as the principal wildlife reservoir for rabies on the islands of Cuba, Grenada, Puerto Rico and Hispaniola (reporting from the Dominican Republic only) (Nadin-Davis et al. 2008).

The Americas is also unique in its Chiroptera reservoirs for RABV variants. Almost all bat species have been identified as hosts harbouring distinctive bat-adapted RABV variants (Banyard et al. 2011, 2013a). While RABV in insectivorous bats only causes sporadic spillovers, infected hematophagous bats in central and south America cause severe losses by transmitting the disease to livestock animals and occasionally causing human deaths (Kuzmin and Rupprecht 2007). It has not yet been elucidated whether rabies was present in the New World prior to the arrival of the first European settlers in the middle of the previous millennium (Vos et al. 2011). Phylogenetic analysis of RABV strains, however, suggests that historical spillover events have occurred from the Chiroptera to the Carnivora order (Badrane and Tordo 2001). In fact, the RABV raccoon lineage in the South-eastern USA and

the RABV skunk lineage in South-Central USA are related ancestrally to bat rabies virus variants (Velasco-Villa et al. 2008). Natural bat-to-carnivore host-switching is documented regularly, confirming the possibility of such historical events. While most of these spill-over events lead to dead-end infections, sustained infection cycles of bat RABV lineages have been reported from foxes (Daoust et al. 1996) and skunks (Leslie et al. 2006).

21.1.2.3 Africa

The cosmopolitan lineage of canine RABV is believed to have spread across the African continent during European colonization. The complexity of the current rabies epidemiology affecting many host species seems a result of historical introductions, translocations, emergence in free roaming and feral dogs and adaptations to wildlife species (Rupprecht et al. 2008). Rabies cycles are sustained by carnivore hosts, particularly by canine species (family Canidae). Besides the domestic dog as the major host reservoir of classical RABV in Africa, other wildlife canids such as jackals (*Canis adustus and Canis mesomelas*) and bat eared foxes (*Otocyon megalotis*) are assumed reservoirs for RABV (Bingham et al. 1999; Sabeta et al. 2007). For instance, spotted hyenas (Crocuta crocuta) were shown not to maintain an independent RABV transmission cycle (Lembo et al. 2007). However, the ecology of canine rabies in Africa is complex (Bingham 2005).

RABV also occurs in other small carnivores in Southern Africa. Whether members of the family Herpestidae appear to be responsible for a transmission cycle of a distinctive variant of RABV remains the subject of further scientific discussion (Nel et al. 2005). Uniquely, there has also been sustained transmission of the cosmopolitan RABV lineage among kudu antelopes (*Tragelaphus strepsicetus*) in Namibia as result of their social behaviour causing up to 50,000 deaths, corresponding to around 20 % of the kudu population (Mansfield et al. 2006b; Scott et al. 2012).

21.1.2.4 Europe

The earliest references to rabies come from ancient sources in Europe (Neville 2004). From then until the nineteenth century, most reports on rabies were on dog mediated urban rabies. However, rabies epidemics were also described in wildlife, e.g. in wolves and foxes (Blancou 2004). In most European countries canine mediated rabies was successfully controlled by traditional veterinary measures or compulsory vaccination of dogs (Müller et al. 2012). Nowadays, Turkey remains the only South-Eastern European country where dog mediated rabies persists (Johnson et al. 2010b). In the middle of the twentieth century, rabies in red foxes (*Vulpes vulpes*) emerged and spread across Europe. By the mid-1980s, except for Scandinavia, the British Isles and Southern Europe, large parts of mainland Europe were affected. Rabies spread came to a standstill in particular when oral rabies vaccination (ORV) field trials were implemented (Wandeler 2004). At present, fox rabies is still

endemic in the Balkan and Eastern Europe, whilst large parts of Western and Central Europe have been freed from fox rabies, thanks to EU supported implementation of ORV programmes (Müller et al. 2012). Besides the red fox, the second most affected wildlife species is the raccoon dog (*Nyctereutes procyonoides*), an alien animal introduced to Europe from East Asia. Recently, rabies cases were reported from the golden jackal (*Canis aureus*) in Bulgaria, another member of the Canidae family (Johnson et al. 2006a). However, it is not yet clear, whether the raccoon dog or the golden jackal represent independent rabies reservoir hosts.

Other alien species in Europe, the North American raccoon (*Procyon lotor*) and the small Indian mongoose (*Herpestes auropunctatus*) are now firmly established in Germany and Western Balkan, respectively, reaching fairly high population densities as in their ancestral habitats (Cirovic 2006; Vos et al. 2012). A sustained introduction of a RABV variant into those species would leave veterinary authorities facing a significant challenge in terms of rabies control (Vos et al. 2012).

21.1.2.5 Asia

Domestic dogs represent the major reservoir and vector for the disease in Asia. Particularly in countries like India and China, dog transmitted rabies causes thousands of deaths per year (Suraweera et al. 2012; Wu et al. 2009). Besides dog rabies, the disease has also been reported from wildlife species in many Asian countries. Recently in 2013, a rabies epidemic occurred in Taiwan that had been thought to be rabies-free for decades, with the majority of rabid animals being ferret badgers (*Melogale moschata*) (Chiou et al. 2014). In fact, it is suggested that rabies in ferret badgers is an independent transmission cycle in China (Liu et al. 2010; Zhang et al. 2013).

A rabies epidemic in red foxes in the Aegean region of Turkey has been reported (Johnson et al. 2006b), and in other countries in the Middle East fox rabies has emerged in recent years (Seimenis 2008). In Russia and other countries of the former Soviet Union, rabies is also maintained by wild canids, particularly foxes, while local dog epizootics occur only sporadically in some territories (Kuzmin et al. 2004). The red fox serves as the main reservoir, but the steppe fox (*Vulpes corsac*) and the golden jackal participate in rabies virus circulation in the steppe and desert territories (Kuzmin et al. 2004). It is not clear whether the Eurasian wolf (*Canis lupus lupus*) is involved in RABV circulation, but there are some reports especially from Mongolia (Botvinkin et al. 2008). Phylogenetic data indicate that, except for arctic foxes in the polar region and raccoon dogs in far Eastern Russia, an association of RABV variants with host species is less obvious (Kuzmin et al. 2004). In Central Asia fragmentary surveillance data indicate that the majority of cases are reported from dogs. Although wildlife rabies has been reported only scarcely, it seems to play a role in the epidemiology of the disease (Gruzdev 2008). For instance, in the Kirgiz Republic the red fox is considered a major wildlife reservoir (Taichiev and Botvinkin 2006).

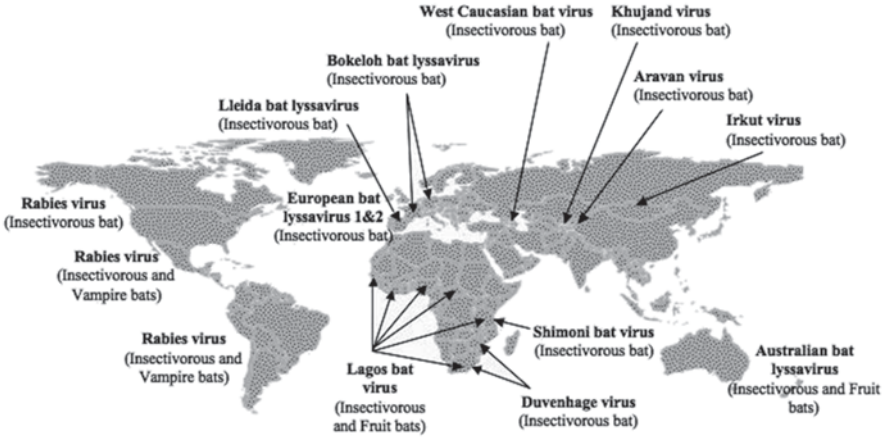

Fig. 21.1 Lyssaviruses isolated from bats. Not included are Mokola virus (MOKV) and Ikoma lyssavirus (IKOV) as they were isolated from terrestrial animals.

21.1.2.6 Other Lyssaviruses

Intriguingly, all recognized and proposed lyssavirus species within the genus have reservoirs in Chiroptera (Fig. 21.1), except for MOKV and IKOV (Banyard et al. 2013a). Bats have particular traits that may promote the maintenance and transmission of lyssaviruses. It is hypothesised that all lyssaviruses originated from a precursor bat virus and therefore bats, with their more than 1100 recognized species to date (more than 16% of mammalian species), are the ultimate historical source of carnivore rabies infections, based in part upon epidemiological, ecological, and phylogenetic inferences (Badrane and Tordo 2001; Calisher et al. 2006). In Europe, bat rabies is caused by various lyssaviruses. EBLV-1 accounts for more than 95% of all bat rabies cases in Europe and is mainly found in Serotine (*Eptesicus serotinus*) and Isabelline bats (*E. isabellinus*) (Schatz et al. 2013). In contrast, EBLV-2 has only been detected sporadically, always associated with *Myotis* bats (*M. daubentonii and M. dascyneme*) in the Netherlands, United Kingdom, Switzerland, Germany and Finland (Banyard et al. 2013a). Bat rabies cases in Europe are seemingly less frequent than in the Americas; however, in many European countries bat rabies surveillance is still inadequate, despite international recommendations (Schatz et al. 2013). Recent discoveries of novel bat lyssaviruses WCBV (2001), BBLV (2010) and LLEBV (2012) from *Miniopterus schreibersii* and *M. nattererii*, respectively, (Ceballos et al. 2013; Freuling et al. 2011; Kuzmin et al. 2005) indicate that the diversity may be even higher.

In Africa, LBV and DUVV have been associated with several bat taxa, including *Eidolon*, *Epomophorus*, *Miniopterus*, and *Nycteris* species (Hayman et al. 2012). Recently, SHIBV was isolated from an insectivorous *Hipposideros* bat in Kenya (Kuzmin et al. 2010). In Australia, variants of ABLV exist among several species of *Pteropus*, as well as in insectivorous *Saccolaimus flaviventris* (Foord et al. 2006).

At present, three lyssavirus species are known to circulate in Asian bat populations. Single representatives of ARAV and KHUV were isolated from Eurasian Microchiroptera, i.e., *Myotis blythi* and *M. mystacinus* (Arai et al. 2003; Botvinkin et al. 2003). IRKV was isolated from infected *Murina leucogaster* in Irkut (Kuzmin et al. 2005) and most recently in China (Liu et al. 2013). One human rabies case after IRKV infection was reported in the Russian Far East (Leonova et al. 2009).

Despite MOKV being isolated from a variety of mammal species, including shrews (*Crocidura* spp.), rodents (*Lophuromys* spp.), and unvaccinated and vaccinated domestic cats and dogs (Sabeta and Phahladira 2013) and IKOV being isolated from an African civet (*Civettictis civetta*) from Tanzania (Marston et al. 2012), these species are the only lyssaviruses never to have been isolated from bats. Given the fact that Chiroptera are considered key reservoir species for lyssaviruses, there is reason to believe that the reservoir hosts of those lyssaviruses are bats, too. However, such reservoirs for MOKV and IKOV still remain to be identified.

Now that it is understood that each lyssavirus has evolved to fit a particular ecological niche and that continuing evolutionary progression yields new variants and new threats to human and animal health, concerted global actions to combat rabies in terrestrial animals become increasingly important.

21.1.2.7 Self-Made Problems

The underlying diversity of the neurotropic, negative-stranded RNA viruses responsible for infection, combined with adaptation to the central nervous system in a broad spectrum of abundant, widely distributed mammalian hosts, may seem to hinder serious contentions for disease abatement (Rupprecht et al. 2008). For a long time, unfortunately, the global rabies situation has not changed dramatically, despite local successes in rabies control in particular reservoir hosts such as dogs or foxes in Europe (Müller et al. 2012), Latin America (Vigilato et al. 2013) and North America (Blanton et al. 2012). Hence, disease distribution still encompasses all continents, with the exception of Antarctica. There is ample evidence that for centuries human socio-cultural evolution as well as population growth and human related activities (e.g. the introduction of alien wildlife species, livestock, and the translocation of domestic and wild animals) has affected rabies epidemiology and control. It is likely that in the future these factors will also affect the (re-) emergence of rabies (for a review, see Freuling et al. 2013a). Furthermore, ineffective rabies control measures at the animal source may well create new rabies related problems in wildlife. Mongoose rabies on the Caribbean islands and the recent re-emergence of fox rabies in Turkey, for example, is considered a result of independent spillover infections from rabid dogs (Johnson et al. 2003; Nadin-Davis et al. 2006a; Vos et al. 2009). The latter phenomenon is observed in many other countries in Asia, Africa and the Americas as well. It is speculated that even growing urbanisation, man-made environmental changes (e.g. deforestation) or climate change will put human populations at risk of exposure to rabies in the future if supraregional rabies control programmes are not implemented as soon as possible (Freuling et al. 2013b).

21.2 State of the Art of Human Rabies Prevention, Immunization and Experimental Therapy

Rabies is a highly neglected, but also a vaccine-preventable, zoonotic disease (Briggs 2012). For practical purposes, rabies should be considered as universally fatal, once clinical signs manifest. However, progress is being made in the basic understanding of the design and application of biologics to prevent, and in the future treat, rabies (Rupprecht et al. 2006). Vaccines may be applied to at-risk populations prior to exposure (pre-exposure immunization), or biologics are provided through post-exposure prophylaxis (PEP), administered to individuals after viral exposure, but before the onset of illness (Tables 21.1 and 21.2).

The administration of PEP occurs after a thorough risk assessment, predicated in part by viral pathogenesis, the local epidemiology of rabies, the mammalian species involved, and the circumstances of each specific exposure (Table 21.2). Exposure is defined as occurring either via bite (any penetration of the skin by the teeth of a rabid animal) or non-bite (transdermal or mucosal contact with virus-infected material, such as brain tissue) routes. Almost all human cases are caused after the bite from a rabid mammal. After such an event, PEP begins by thorough washing of the wound with soap and water. In previously unvaccinated persons, rabies immune globulin (RIG) is infiltrated into and around the bite, as an initiation of passive immunization. The RIG may be human in origin (HRIG) or heterologous, obtained from other species. The first of several doses of rabies vaccine is also administered at the same time as RIG. The use of RIG on day 0 bridges the time after viral exposure, but before the active induction of rabies virus neutralizing antibodies (VNA) from vaccine administration (Both et al. 2012). Well documented failure of PEP is very uncommon (Shantavasinkul et al. 2010a). Survivorship is virtually assured if prophylaxis is begun in a timely and appropriate manner after exposure. However, there is no proven treatment after the manifestation of rabies as an acute progressive encephalomyelitis.

21.2.1 Preventive Vaccination

Vaccination of certain occupational groups, such as first responders in rabies management, is recommended, because the actual risk of viral exposure is higher than in the general population-at-large (Table 21.1). Prior pre-exposure immunization simplifies PEP, because the immune response is primed and is thought to provide a degree of protection against unrecognized exposures, which by definition should be negligible, if proper personal protection equipment is used, safe recognized techniques are employed, and exposure protocols are followed. If pre-immunized persons are exposed knowingly to rabies virus, booster doses of vaccine are used to induce an anamnestic response. Also, RIG is unnecessary under such circumstances, because VNA are likely present already, and the RIG may form immune complexes and interfere with development of a normal anamnestic response. Pre-immunized

Table 21.1 Rabies immunization recommendations

Criteria for pre-exposure immunization[a]			
Exposure category	Nature of risk	Typical populations	Pre-exposure regimen[b]
'Continuous'	Virus present continuously, usually in high concentrations. Specific exposures may be unrecognized. Bite, non-bite, or aerosol exposures	Rabies research workers.[c] Rabies biologics production workers	Primary course. Serologic testing every ~ 6 months. Booster immunization if antibody titer falls below 'acceptable' level[c, d]
Frequent	Exposure usually episodic, with source recognized, but exposure also may be unrecognized. Bite, nonbite, or aerosol exposures	Rabies diagnostic workers, [c] cavers, veterinarians and staff, and animal control and wildlife workers in areas where rabies is enzootic. All persons who handle bats	Primary course. Serologic testing every ~ 2 years. Booster vaccination if antibody titer is below 'acceptable' level
Infrequent (greater then population-at-large)	Exposure nearly always episodic with source recognized. Bite or nonbite exposures	Veterinarians and animal control staff working with terrestrial animals in areas where rabies is uncommon to rare. Veterinary students. Travelers visiting areas where rabies is enzootic and immediate access to appropriate medical care including biologics is limited	Primary course. No serologic testing or booster vaccination
Rare (population-at-large)	Exposure always episodic. Bite or nonbite exposure	Population-at-large, including individuals in rabies-epizootic areas	No vaccination necessary, unless exposed

Post-exposure immunization. All PEP should begin with immediate thorough cleansing of all wounds with soap and water. Persons not previously immunized: RIG, 20 IU/kg body weight, as much as possible infiltrated at the bite site (if feasible), with the remainder administered IM; cell culture vaccine, ID or IM (i.e., deltoid area), one each on days 0, 3, 7, and 14. Persons previously immunized: Two doses of modern cell culture vaccine, ID or IM (i.e., deltoid area), one each on days 0 and 3. No RIG should be administered. Pre-exposure immunization with modern cell culture vaccine; prior PEP with modern cell culture vaccine; or persons previously immunized with any other type of rabies biologic and a documented history of an 'acceptable' rabies virus neutralizing antibody response to the prior vaccination

[a] Adapted from recent WHO and U.S. Advisory Committee on Immunization Practices (*ACIP*) guidelines

[b] Pre-exposure immunization. Pre-exposure immunization consists of three doses of cell culture vaccine, ID or IM (i.e., deltoid area), one each on days 0, 7, and 21 or 28. Administration of routine booster doses of vaccine depends on exposure risk category as noted above

[c] Assessment of relative risk and any extra monitoring of immunization status of laboratory workers is the responsibility of the laboratory supervisor (as an example, see guidelines in the current edition of the United States Department of Health and Human Services' Biosafety in Microbiological and Biomedical Laboratories)

[d] Routine Pre-exposure booster immunization consists of one dose of modern cell culture vaccine, ID or IM (i.e., deltoid area). An acceptable antibody level is a 1:5 titer (complete inhibition in the RFFIT at a 1:5 dilution, approximately equivalent to 0.1 IU/ml) or ~ 0.5 IU/ml. Boostering is recommended if the virus neutralizing antibody titer falls below this level, as long as the person remains at risk of viral exposure

Table 21.2 Rabies risk assessment and prophylaxis considerations

Animal type	Evaluation and disposition of animal	Post-exposure prophylaxis recommendations
Dogs and cats	Healthy and available for 10 days observation	Should not begin PEP unless animal develops signs of rabies[a]
	Rabid or suspected rabid	Initiate PEP immediately[b]
	Unknown (escaped)	Consult public health officials
Bats, foxes, mongoose, raccoons, skunks, other carnivores	Regard as rabid unless geographic area is known to be free of rabies or until animal is proven negative by diagnostic tests	Initiate PEP[c]. Consider factors such as provocation, suggestive clinical signs, severity of wounds, type of exposure, and timeliness of diagnostic results (24–48 h) for decisions regarding immediate initiation, or to delay pending test results
Livestock, insectivores, most rodents, and lagomorphs (rabbits and hares)	Consider individually	Consult public health officials; bites of rats, mice, hamsters, guinea pigs, gerbils, chipmunks, squirrels, shrews, and other small mammals almost never require PEP

Adapted from recent WHO and U.S. Advisory Committee on Immunization Practices (*ACIP*) guidelines

[a] If clinical signs compatible with rabies develop during a ~10-day confinement and observation period, the animal should be euthanized and tested immediately. Depending on circumstances, initiation of PEP may be delayed pending a laboratory report, if results may be obtained promptly in ~24–48 h

[b] If the bite was unprovoked or resulted in severe wounds, prophylaxis of the bitten person should begin immediately with rabies immune globulin and modern cell culture vaccine. Rabies PEP may be discontinued if the test is negative

[c] If available, the animal should be euthanized and tested as soon as possible. Holding for an observation period is not recommended since the potential viral shedding period prior to clinical signs has only been determined for dogs, cats, and ferrets

individuals should remain vigilant in recognizing potential viral exposures and seek appropriate PEP ad hoc.

If exposures occur, but are unrecognized, the pre-immunized individual may still succumb to rabies after exposure, albeit very rarely, as did one documented case in a previously vaccinated but non-boosted Peace Corps worker, who was bitten by her own puppy, that died of a disease compatible with rabies. Travelers should be advised to consider pre-exposure vaccination based upon their planned activities and destination, as certain critical biologics may not be always readily available (Jentes et al. 2013).

21.2.2 Preventive Vaccination of Children?

An estimated 10–16 million people undergo rabies PEP worldwide each year following exposure to proven or suspected rabid animals (Both et al. 2012). The pediatric population is of special importance related to animal bites and rabies exposures. Of note, the first ever patient to receive successfully Pasteur's rabies vaccine was a child presenting with multiple deep bite wounds in July 1885. This case

exemplifies that children in particular are at a high risk of exposure to rabid dogs. It is estimated that, on average, 50 % of rabies deaths concern children under 15-years of age. Children 5–10 years of age are particularly exposed, because they are often unable to discern abnormal animal behavior, they are watched less by their parents than younger children, they like playing with dogs and, due to their size, they are frequently bitten on the head and neck which carries a higher risk of contracting rabies (Ichhpujani et al. 2008; Knobel et al. 2005). Because of the large numbers of affected children, rabies is the seventh most relevant global infectious disease with regards to the years of life lost (Jackson 2008).

Several surveys have confirmed the disproportionate toll of rabies among children, resulting from an insufficient supply of rabies biologics, including RIG and modern cell-culture vaccines (Pancharoen et al. 2001a, b; Sriaroon et al. 2006; Wilde et al. 1996). Studies in Tanzania have shown that up to 55 % of victims are children (Hampson et al. 2008; Mazigo et al. 2010). In a South African survey, 48.8 % of rabies exposures were children < 10 years and 21.8 % were 11–20 years (Weyer et al. 2011). A study across eight Asian countries revealed that 43 % of all patients were children and teenagers (Dodet et al. 2008a). A study focusing on rabies in China demonstrated that children under 15 constitute 25 % of the nation's rabies deaths (Song et al. 2009). Another study in Cambodia showed that, among 44 rabies-positive cases, 37 % were 15 years old or younger, and RIG was administered free of charge only for children with wounds on their upper arms and faces (Ly et al. 2009).

It is important to mention that the need for RIG can be avoided by the use of pre-exposure-prophylaxis (PrEP), as previously vaccinated bite victims only receive two booster doses with vaccine and no RIG is required. PrEP may be useful for certain populations at high risk of rabies infections, e.g. children in endemic areas containing many unvaccinated stray dogs, or those in very remote areas such as the Amazon who suffer continuous exposure to vampire bat bites. PrEP can also be applied to international travellers, e.g. in the UK an annual number of 3700–5700 rabies vaccine prescriptions were dispensed during 2009–2011 (ePact database).

The field of rabies immunization benefits from over a century of classical scientific insights, but also lingers in the legacy of Pasteur towards certain conservative tendencies, particularly related to the basic use, types, doses, routes, and schedules of biologics (Wu et al. 2011). As background, several recent publications have reviewed in depth the history, basic approach, available products, and biological basis for rabies immunization (Briggs 2011; Rupprecht and Plotkin 2013; World Health Organisation 2013). Other updates, specific details and current recommendations on rabies immunization may be found at the websites (http://www.who.int/rabies/en/) of the World Health Organization (WHO) and the Advisory Committee on Immunization Practices (ACIP), United States (http://www.cdc.gov/vaccines/acip/recs/index.html).

The etiological agents of rabies consist of diverse RNA viruses in the Family Rhabdoviridae, Genus Lyssavirus (Kuzmin et al. 2009). While all lyssaviruses cause rabies, historical and current rabies biologics are produced only using rabies virus strains. Modern rabies biologics will protect against all known rabies virus

variants, as well as several related lyssaviruses. However, as cross-reactivity against distantly related lyssaviruses of phylogroup II and III may be much less than ideal, the development of a pan-lyssavirus vaccine would be highly desirable, especially for laboratory workers handling these viruses (Evans et al. 2012).

The original anti-rabies biologics of the late ninetieth and early twentieth century consisted of rabies viruses propagated in mammalian nervous tissues, such as rabbits, small ruminants, or suckling mice. These nerve tissue origin (NTO) vaccines are no longer recommended for human immunization, although such products are still in use in the developing world. Due in part to poor potency, over the course of a month, 14–21 administrations of NTO vaccines occurred by the intra-cutaneous route applied over the abdominal region. Such NTO biologics were gradually replaced by safer and more potent cell culture vaccines during the late twentieth century. Modern rabies cell culture vaccines include the human diploid cell vaccine (HDCV), purified chick embryo cell vaccine (PCEC), purified duck embryo vaccine (PDEV), and purified Vero cell rabies vaccine (PVRV). Besides an improvement in overall quality, the use of HDCV, PCEC, PDEV, and PVRV allowed a decrease in the total number of vaccine doses applied to ~ 4–5, and the application via the intramuscular (IM) route at a volume of 0.5–1.0 mL.

Rabies vaccines and RIG are listed among the WHO Essential Medicines for both adults and children, but access across the developing world is often insufficient. In fact, shortages of rabies vaccine and especially RIG are common in developing countries and bite victims may need to travel long distances to obtain any PEP and may present to medical personal with substantial delays. Also costs are a restriction, since the price for modern tissue-culture vaccine vials ranges from $ 7 to 20 in many low-income countries and multiple vials are required per patient depending upon the PEP regimen used (Hampson et al. 2011; Quiambao et al. 2005). To save costs compared to IM vaccination, ID vaccination makes use of less vaccine, with small amounts of vaccine being injected into the skin at multiple sites on the first day of the course to elicit a potent immune response (0.1 ml for each ID injection versus one 0.5 or 1 ml vial for each IM injection) without limitations in safety, immunogenicity, nor efficacy for both pre-exposure immunization as well as in use for PEP (Khawplod et al. 2012; Shantavasinkul et al. 2010b, b; Shantavasinkul and Wilde 2011; Warrell 2012).

The disadvantage of ID regimens is that vaccine leftovers in partially used vials must be discarded after several hours to avoid the risk of contamination. Additionally, ID vaccination is technically more demanding, is subject to pharmaceutical regulations, and may result in more frequent local adverse events. However, where feasible, due to a high turnover of animal bite victims, local health clinics, and well trained staff, switching from IM to ID immunization will make rabies PEP more accessible and affordable (Hampson et al. 2011). Besides alternative routes, shorter vaccine schedules are envisioned, based in part upon the evidence provided in animal models, the basic immunological response to rabies vaccines, epidemiological investigations, and human clinical trial data (Khawplod et al. 2012; Robertson et al. 2010; Rupprecht et al. 2009, 2010).

Table 21.3 Ten generalized observations on human rabies immunization in the twenty first century.(Adapted from Rupprecht and Plotkin 2013)

Antibodies to the viral glycoprotein appear to be the most important in rabies immunization
Anti-IgG antibodies begin to appear within ~7–14 days of rabies vaccination in healthy subjects
A basic prime-boost strategy seems most effective in rabies pre-or post-exposure vaccination
Modern cell culture vaccines are superior to nerve tissue origin products in safety and immunogenicity
Intradermal vaccine regimens provide comparative effectiveness compared to intramuscular use
Routine serologic monitoring is unnecessary except in the immune-compromised or after major deviations from standard recommendations
Host factors, such as age, genetic background, etc., will affect an ideal immune response to vaccine
The role of cell-mediated immunity is not well documented in rabies prophylaxis
Timely and appropriate wound care, infiltration of rabies immune globulin, and administration of potent cell culture vaccines virtually assure human survivorship, even after severe bite exposures
Most people die of rabies because they do not receive appropriate access to modern rabies biologics

After vaccination, antibody titers serve as surrogates of response, but do not directly correlate with absolute protection against a fatal productive infection, because other immunological factors also play a role in prevention of disease (Briggs 2011). Hence, there is no known absolute "protective antibody" level for all humans. Minimum arbitrary standards are based empirically on presumed activity of rabies virus-specific antibodies, e.g., VNA, for a given exposure scenario and on repeatable values for paired sera. For example, a VNA titer of 0.5 IU/mL by WHO standards, or of ≥1:5 (approximately 0.10 international units/mL) or higher, is evidence of adequate immunization in persons at either constant or frequent risk of exposure, at 6-month or 2-year intervals, respectively, as a measure of baseline immunity, as recommended by the United States Advisory Committee on Immunization Practices. A single booster vaccine dose is administered if the VNA level is lower than recommended, based on a determination of risk (Table 21.1). After a century of use, several generalizations are apparent in the application and use of human rabies biologics today (Table 21.3). Future alterations in the methods used to measure basic rabies vaccine potency are anticipated to reduce the dependency on the use of animals and help better resolve a cumulative understanding of human response to precise doses of vaccine by more comparative techniques (Stokes et al. 2012).

21.2.3 Improvements to PEP

While current biologics to prevent rabies are extremely effective, progressive development of other products is necessary. Modern inactivated cell culture vaccines and HRIG are vastly improved over historical NTO vaccines, but such products are expensive (especially in the developing world where they are most needed), are often in scarce supply, and may carry a perceived theoretical risk of adventitious

agents. Hence a major focus in rabies prevention has concentrated on the need for potent, inexpensive PEP, especially different routes, fewer vaccine doses, shorter schedules, and replacement of costly HRIG, while retaining activity against a wide variety of diverse lyssaviruses (Both et al. 2012).

Besides HRIG, there are other products to provide passive immunity. In contrast to the relatively poor quality equine anti-rabies serum used in the past that resulted in high adverse reactions, such as serum sickness in up to 40 % of human recipients, modern purified equine rabies immune globulin (ERIG) products are safer, more potent, and more affordable than older cruder products, and are less expensive than HRIG. Such ERIG products have been used effectively in conjunction with vaccine in human rabies PEP, particularly in developing countries. Due to its potency and lack of apparent significant local or systemic effects, purified ERIG products seem the only immediate alternative, should the supply of available HRIG be threatened by shortages, contamination, or other limitations (Quiambao et al. 2009). Such use of heterologous products might be considered a temporary antecedent until the availability of more novel replacements, such as monoclonal antibodies (mAbs) (Both et al. 2013a, b).

Particularly countries with chronic shortages of HRIG and ERIG would benefit greatly from replacement of these scarce and expensive polyclonal preparations, especially in Africa which suffers from an estimated 24,000 human rabies deaths per year, with less than 2 % of exposed patients receiving RIG (Knobel et al. 2005)

In the past, hybridomas that secrete rabies virus antigen-specific mAbs have been generated, and mAbs were selected on the basis of isotype, antigen and epitope specificity, virus strain specificity, affinity, and neutralizing activity. In a WHO consultation in 2002, a number of mAbs were proposed for inclusion into an antibody cocktail (Müller et al. 2009) and several mAb combinations were designed based on stringent criteria (Table 21.4). In addition to the mAbs developed by the WHO Rabies Collaborating Centres, several other rabies-specific mAbs are being investigated and some are currently in advanced stages of clinical development, namely mAb cocktail CL184, developed by the Dutch company Crucell, and mAb 17C7, developed by Massachusetts Biologic Laboratories (Bakker et al. 2008; Sloan et al. 2007).

Preclinical studies on Crucell's mAb cocktail CL184 demonstrated that the two-component mAbs recognize non-overlapping and non-competing epitopes on different epitopes of the rabies virus glycoprotein, and that the two mAbs show reciprocal neutralization of escape mutants. Broad *in vitro* neutralization against a panel of lyssaviruses was observed, and the mAbs were capable of *in vivo* protection in the hamster model (Bakker et al. 2008). Two phase 1 studies were undertaken by Crucell to investigate the safety and tolerability of the mAb cocktail in healthy humans (Bakker et al. 2008). The tolerability was demonstrated to be acceptable and no relevant negative effects on the immune response after vaccination were observed. Following these observations in phase I clinical trials, three phase II trials have been undertaken in the USA, Indonesia and India (Bakker et al. 2008). In another approach, neutralizing mAb 17C7 was generated by using transgenic mice which express human antibody genes (Sloan et al. 2007). Preclinical studies have

Table 21.4 Critera for antibody selection and testing by the WHO Rabies Collaborating Centres. (Source: WHO consultation 2002)

a) Criteria for mAb selection
The history of the hybridomas used for production including the relative risk of their contamination with certain agents, e.g. Foot and Mouth Disease Virus (FMDV), Transmissible Spongiform Encephalitis (TSE) agents) and use of FCS (foetal calf serum) should be available
A production of a minimum of 100 IU per ml of crude hybridoma supernatant should be obtained
Stability expressed as loss of antibody secretion production should not exceed 10% upon passaging
In-vitro cross-reactivity should be measured by the Rapid Fluorescent Focus Inhibition Test (RFFIT) or Fluorescent Antibody Virus Neutralisation (FAVN) test on a selection of rabies and rabies-related viruses isolated from the following host species and geographical areas, including dogs from Asia, Africa and the New World, mongoose(s) from South Africa, and ABLV from Australia
***In vitro testing of candidate* mAbs**
History of hybridomas should be established in writing
Use of FCS should be avoided. Low serum or no serum media are preferred
Each laboratory should establish a mini master cell bank with a minimum of ten vials
Tests for mycoplasma, bacteria, etc. in T25 cm^2
30 passages must be performed (with freezing aliquots after every ten passages)
Culture batch (500 ml) on roller bottles
Purify IgG on protein A column and determine IU/mg
Compare supernatant of passages 0, 10, 20, 30 (a 30% variation ± for naturalisation from test to test is acceptable) and determine isotype at passages 0 and 30
Purify IgG at a concentration of at least 1000 IU/ml

shown that mAb 17C7 can confer protection equivalent to HRIG in the hamster challenge model, tested either alone or in combination with rabies vaccine (Sloan et al. 2007). Clinical trials for this mAb, in collaboration with the Serum Institute of India, have been undertaken to assess the safety in healthy adults.

The administration of such mAbs has theoretical advantages over RIG, including that comparatively small volumes of mAbs would have to be inoculated for equivalent active protein content, because specific neutralizing activity per mass of protein is higher, so mAbs may be optimal for lessening the trauma and pain of local wound infiltration with a source of passive antibodies. Secondly, safety issues arising from the possibility of adventitious agents associated with human or animal blood products would be alleviated by bulk production under modern GMP conditions in cell culture. Despite progress shown under research conditions, the acceptability of murine mAbs for rabies PEP have been questioned, in that a human anti-mouse response may present a significant drawback by the consequent effects on kinetics and antigenic targeting (Müller et al. 2009). If murine or other heterologous mAbs are used, human anti-species responses might be expected. However, these may not be necessarily deleterious, because mAbs would only be used once in rabies PEP. As is the case for HRIG, mAbs would not be re-administered, should a person be re-exposed in the future. In a previously vaccinated subject, rabies PEP in

these situations consists of vaccine only on days 0 and 3. Moreover, if such mAbs are recognized as foreign antigens, with an expected shortened serum half-life, this potentiated clearance may be advantageous by minimizing the opportunity for interference with an active immune response on behalf of the vaccinated host, while effectively neutralizing virus, prior to induction of host VNA. This is one of the primary reasons cited for homologous or human mAbs, as opposed to heterologous mAbs.

To be most effective, conventional PEP should be applied prior to viral invasion of the nervous system. However, PEP may still be effective even after virions have accessed the nervous system. For example, in experimental animal infections, analysis by RT-PCR revealed the presence of rabies virus-specific RNA in the brain of animals within hours of direct viral inoculation. Yet, when animals were administered a mAb 24 h after inoculation, a majority survived an infection in which all controls succumbed (Dietzschold 1993). Neither viral antigens nor virus-specific RNA were detected when these survivors were euthanized a month later. These data and others demonstrate that virus "neutralization" and clearance are less than fully understood (Dietzschold et al. 2008; Hamir et al. 2011). Thus, if produced in a cost-effective manner, anti-rabies mAbs may be useful in future human PEP. Such biologics that abrogate infection even after virus enters the CNS present exciting new possibilities for intervention with significant advantages over historical polyclonal RIG.

21.2.4 Treatment of Clinical Rabies?

Prevention of viral exposures is still the optimal approach in rabies prevention, or when this fails, application of modern PEP before onset of illness. However, in some circumstances, alternatives regarding experimental treatment of clinical rabies may be warranted (Willoughby 2007). Unfortunately, the use of many different products, such as RIG and Ig fragments, cytosine or adenine arabinoside, interferon, acyclovir, antithymocyte globulin, steroids, vidarabine, tribavirin, inosine pranobex, ribavirin, etc., has not demonstrated substantive utility (Jackson et al. 2003). Historical case histories of recovery after clinical signs in other mammals suggest that host defenses may be exploited, if the patho-physiological mechanisms of disease can be better understood. Moreover, reviews of human case series and findings of apparent natural acquired immunity suggest that human rabies may not be uniformly fatal, but rather behave as a continuum (Feder et al. 2012; Gilbert et al. 2012). One recent classical example can be used as an illustration. During 2004, a 15 year-old female Wisconsin resident was bitten by a bat on her hand. The small wound on her finger was cleaned, however, PEP was not administered. Approximately 1 month later, she developed generalized fatigue and paresthesia of her left hand, diplopia, ataxia, nausea, and vomiting. On the fourth day of illness, blurred vision, left leg weakness, and ataxia were noted, and later fever, slurred speech, nystagmus, and tremors of her left arm; she was admitted to a pediatric facility. On the

second day of care, the presence of rabies virus-specific antibodies in the patient's CSF and serum were documented. However, attempts to isolate virus, detect viral antigens, or amplify viral nucleic acids from skin biopsies and saliva samples were unsuccessful. An experimental treatment, termed the "Milwaukee Protocol", was initiated, which combined anti-excitatory and antiviral drugs, including ketamine, ribavirin and amantadine, in conjunction with supportive intensive care (Willoughby et al. 2005). Neither rabies vaccine nor RIG was administered because of the patient's VNA response and the theoretical potential for harm from an altered immune response. After more than 70 days of hospitalization, the patient recovered, with only minor neurologic sequelae (Hu et al. 2007). She became the first person to survive clinical rabies without a history of prior vaccination. While promising, this experimental protocol has been attempted in several other human rabies cases without success, and remains controversial (Aramburo et al. 2011; Hemachudha et al. 2006; Rubin et al. 2009). While there is no established therapy that is effective for patients who develop rabies, efforts should continue on basic viral pathogenesis research, design of anti-viral compounds, development of relevant surrogate animal models and protocols that mimic supportive intensive care, and experimental applications in human cases where ethical/legal approvals and modern teams and facilities exist for thorough application and evaluation (Franka and Rupprech 2011; Jackson 2013b; Lingappa et al. 2013; O'Sullivan et al. 2013; Smith et al. 2011; Willoughby et al. 2008, 2009).

21.3 Human Rabies can be Eliminated Through Rabies Control at the Animal Source!

21.3.1 Canine Rabies Control

In areas of the world where rabies maintenance and transmission to humans are driven by domestic dogs, interventions targeted at this host species can result in reductions in dog, other species and human cases. Indeed multiple historical and current examples demonstrating the feasibility of this concept both in developed and developing countries exist (see Lembo et al. 2013 for a review), providing optimism that well-implemented dog rabies management can deliver tangible benefits for public health. The epidemiological theory and practicalities of dog rabies control have been extensively covered elsewhere (Knobel et al. 2013; Lembo et al. 2013). Here we will therefore focus on current issues and trends with the aim of providing up-to-date evidence-based recommendations.

In the pre-vaccination era dog movement tracing and restriction, muzzling, case notification, and culling constituted "classical" measures to control dog rabies (Blancou 2003; Théodoridès 1986). In many developing country settings, local and national authorities still promote the reduction of host population densities through removal or sterilization as a means to decrease rabies incidence on the assumption

of density-dependent transmission. The limits of this approach are emphasised by general failure in controlling the disease (Lee et al. 2001; Windiyaningsih et al. 2004). Recent research points to a lack of evidence for the role of density in the transmission of rabies in domestic dogs (Hampson et al. 2009), calling for further research into other factors (for example animal and human behaviour, sanitation of habitats), which may influence contact rates and reproduction (Morters et al. 2013). In addition, drastic dog removal through culling is often unacceptable to local communities and can be counter-productive in that it reduces the overall herd immunity as a consequence of the acquisition of new unvaccinated dogs or of dogs being hidden by their owners during vaccination campaigns. Given the considerable animal welfare concerns associated with these approaches, removal should only be carried out in the case of suspect rabid animals using methods that are considered acceptable on ethical and scientific grounds (Close et al. 1996, 1997; WSPA 2008).

Regarding the impacts of dog sterilization, the limited evidence available does not allow the determination of its role as a sole means to reduce rabies incidence in the absence of dog vaccination (Morters et al. 2013). An additional issue is that of costs associated with surgical sterilization, hence current research looks into the development of potentially more affordable immunocontraceptive rabies vaccines (Rupprecht and Wu 2009). Yet measures to promote responsible dog ownership as part of sustained large-scale dog vaccination programmes, as well as legislative measures, such as, for example, mandatory dog registration and identification, tie-up orders and abandonment legislation, are likely to represent the key approaches for cost-effective dog rabies control.

Since the advent of effective animal vaccines, regular mass dog vaccination reaching a continuum of 60–70 % of the dog population (Coleman and Dye 1996; Hampson et al. 2009) has become the mainstay of successful dog rabies control and eventual elimination. The most cost-effective and logistically feasible strategy to reach the vast majority of domestic dogs is parenteral central-point vaccination (Kaare et al. 2009; Kayali et al. 2003), although in remote locations this approach may need to be combined with more intensive and costly house-to-house delivery (Kaare et al. 2009). Despite evidence for improved coverage in certain settings (Estrada et al. 2001), the use of oral delivery strategies, which was advocated in the late 1990s as an alternative vaccination approach in circumstances where dogs are “stray” or not easy to handle (WHO 1998), has not become widespread. There are a number of reasons for the limited uptake of oral vaccination of dogs: (1) although often unrestrained, most domestic dogs in rabies-endemic areas are owned and are therefore accessible for parenteral vaccination (Kaare et al. 2009; Kayali et al. 2003); (2) techniques are available for catching dogs that are not used to being restrained (e.g. using nets handled by experienced dog catchers); (3) oral vaccines are more costly than injectable vaccines; and (4) there are concerns over safety of the oral rabies or vector virus strains for humans.

Once large-scale vaccination interventions are underway an essential aspect is to determine whether the campaigns are reaching an adequate number of dogs, i.e. to estimate the vaccination coverage. This generally requires an initial determination

of the dog population size, which can also be of value in the preparatory phase (e.g. to estimate the amount of supplies needed). Techniques to estimate dog population sizes exist, but are based on surveys of a sub-set of the population and therefore require multiple extrapolations, which may provide unrealistic estimates (Knobel et al. 2013). Complete dog counts should therefore be integrated within nationwide censuses of the human population.

Building capacity for and allocating resources to surveillance is an essential part of dog rabies management activities, although high standards of surveillance are met only in a few countries, with official records often underestimating the true number of rabies cases. Efforts should therefore focus on developing inexpensive and user-friendly approaches to improve reporting and communication, and on increasing local awareness to ensure that all bite and rabies cases reach the responsible central-level authorities (see also Sect. 21.4).

It is clear that there are no technical barriers to the implementation of large-scale mass vaccination programmes (Lembo and Partners for Rabies Prevention 2012). However, the elimination of rabies requires additional essential components, which are more challenging to address (see Sect. 21.4): effective engagement and cooperation amongst key players in rabies prevention and control, appropriate legal frameworks, high levels of awareness amongst affected communities, correct and cost-effective provision and utilisation of human biologics, and sustained commitment towards mobilizing and maintaining an adequate level of resources.

21.3.2 Wildlife Rabies Control

Wildlife rabies control was historically disregarded given the greater priority given to dog rabies control and a lack of appreciation of wildlife reservoirs until the 2nd half of the twentieth century. In Europe, at a time when the elimination of dog rabies was accomplished by strict implementation of hygienic measures, registration and mass vaccination of dogs in the 1970s, fox rabies had emerged and had already spread to vast areas requiring fundamental changes in rabies control policies (Müller et al. 2012). Similarly, in North America canine rabies was successfully controlled during the late 1970s, but since that time, rabies has been maintained in multiple mesocarnivore and bat species (Blanton et al. 2012). Particularly, a raccoon rabies epizootic occurred in the early 1950s in Florida. Infected raccoons from enzootic areas were later translocated to the mid-Atlantic region in the late 1970s and caused a large raccoon rabies epizootic that is still active (Jenkins et al. 1998).

Theoretically, wildlife rabies in any species can be controlled either by drastic decimation or mass vaccination of the primary reservoirs, and thus reducing the number of susceptible animals below an endemic threshold ($R0 < 1$) (Aubert 1992). In Europe, early attempts aiming exclusively at a drastic decimation of the fox population (e.g., by hunting, trapping, poisoning, and gassing) failed (Aubert 1999). In fact, elimination of a reservoir species is impractical, expensive, and ethically and ecologically unacceptable unless it is an invasive introduced species (Rupprecht

et al. 2001). It appeared that disadvantages, e.g. removing mature individuals from the population, outweigh any advantages (Winkler and Jenkins 1991). Likewise, trapping of foxes, coyotes (*Canis latrans*), and/or poisoning of skunks to control rabies in North America also resulted in no significant reduction of rabies cases (Rosatte 2013). Although parenteral vaccination of trapped wild animals principally worked (Aubert 1994; Rosatte et al. 1990), this was no option for large scale control of rabies.

21.3.3 Pioneering Work

In the late 1960s research was initiated to develop oral rabies vaccine for red foxes (*Vulpes vulpes*) and resulted in the discovery of a modified-live rabies virus vaccine strain that effectively immunized foxes when given orally (Baer et al. 1971). In parallel, the concept of mass baiting of a wild species was proven feasible, which led to the first release of an oral rabies vaccine (ORV) targeting red foxes in the wild in Switzerland in 1977 (Steck et al. 1982). This was a breakthrough in wildlife rabies control and also triggered further field trials in other European countries in subsequent years (Wandeler 2000, 2004). Whilst at the very beginning appropriate tools had still to be developed from scratch, pioneering developments, including efficacious and safe oral rabies virus vaccines, adequate fox-adapted ORV strategies, machine-made baits, and automated computer supported aerial bait distribution, were technical milestones on the road to success in subsequent decades (Müller et al. 2012).

21.3.4 Success Stories and Setbacks

Since 1989, in Europe the European Union (EU) has been co-financing costs for disease elimination in member states and neighbouring none-EU countries. This co-financing policy has been a strong incentive advancing the EU to becoming the driving force for fox rabies control in Europe (Demetriou and Moynagh 2011). As a consequence, during the past three decades, ORV programmes have been implemented in 24 European countries. The maximum total area ever covered at least once with vaccine baits in Europe between 1978 and 2012 encompassed almost 2.6 million km². A total of ten different commercial modified live rabies virus vaccines, attenuated and recombinant, were used in ORV campaigns (Freuling et al. 2013b). As a result, the number of animal rabies cases in Europe decreased from 18,588 cases in 1980 to 6130 cases in 2012 with fox-mediated rabies having virtually disappeared from vast areas of Western and Central Europe. Despite this tremendous success European countries had to face several setbacks resulting in delay in rabies elimination at the regional level. Reasons were multifaceted, including: over-optimistic interpretation of initial success, which may have led to a premature declaration of areas as being “rabies-free,” often followed by a reappearance of the

disease; missed opportunities for shared cross-border activities; increasing fox densities; lack of long-term funding; violation of elementary principles in rabies control; absence of complementary measures; inadequate bait distribution; insufficient epidemiological analysis; other disease priorities; deficient surveillance; inadequate intra- or inter-area information exchange; decreasing public awareness; and inferior adaptation of vaccination strategies to changing epidemiological or environmental conditions (Müller et al. 2012; Stöhr and Meslin 1996). A comparative analysis of implemented ORV programmes between 1978 and 2010 revealed that rabies control took between 5 to 26 years depending upon the country (Freuling et al. 2013b). The proportion of land area ever affected by rabies and an index capturing the size and overlap of successive ORV campaigns were identified as factors having statistically significant effects on the number of campaigns required to both control and eliminate rabies. Repeat comprehensive campaigns that are wholly overlapping eliminate infection much more rapidly and are less costly in the long-term (Freuling et al. 2013b; Selhorst et al. 2005). While it needs comparatively less money to reduce the number of rabies cases by 50 % or even 90 % using ORV, disproportionally greater effort is required in the final phase of an ORV programme, with a median of 11 additional campaigns required to eliminate disease once incidence has been reduced by 90 % (Freuling et al. 2013b).

Besides Europe, ORV has been applied with progress towards eliminating rabies in red foxes in Ontario, Canada (MacInnes et al. 2001) and also to successfully control a canine rabies virus variant that had spilled over into coyotes in Texas (Sidwa 2005). Additional programmes in this area are focused on containing and eliminating a variant of gray fox (*Urocyon cinereoargenteus*) rabies (Rosatte 2013; Slate et al. 2009). Elsewhere in the world wildlife rabies control efforts using ORV where conducted in Turkey (Ün et al. 2012) and Israel (Linhart et al. 1997).

In an effort to contain the raccoon rabies and its spread in Ontario, a Point Infection Control programme near the point of discovery has been established. This can involve trapping and euthanizing raccoons and skunks within a 5 km radius of each case in a new area. It always includes trapping, (parenterally) vaccinating and releasing (TVR) raccoons, skunks, foxes, and feral cats within a further 5–10 km radius. Additionally, vaccine baits are distributed outside of, but surrounding the TVR zone as a final buffer against the spread of the disease (Rosatte et al. 2001).

21.3.5 Before Starting

Before considering the use of ORV for mass vaccination of free-ranging wildlife scientific assessments of the rabies situation, knowledge of the main target species. are required. In the absence of functioning dog rabies control programmes, wildlife rabies is often blamed for sustaining the disease. While this may partly be true, the main focus should remain on targeting dogs as these cause the vast majority of human cases and rabies PEP (World Health Organisation 2005). Because a multitude of stakeholders and interests are involved guidance should be sought.

Next to official documents (European Commission 2002; World Health Organisation 2005) and current literature (Rosatte 2013) another novel tool is the Blueprint for Rabies Prevention and Control. Besides canine rabies a second module has been developed and incorporated into the website, comprising the control of rabies in wildlife populations, specifically in red foxes (http://foxrabiesblueprint.org/) using ORV. The Blueprint for Fox Rabies Prevention and Control has been developed by global rabies experts from the "Partners for Rabies Prevention". It is not meant to replace existing material or national guidelines but rather to serve as an easy-to-use guide to assist countries in understanding how to prevent and control fox rabies using ORV. The Blueprint brings together relevant information on rabies prevention from specific international health organizations (WHO, OIE), published data from the field, and expert knowledge as well as including case reports on effective fox rabies control and elimination.

21.3.6 Challenges

On a global scale, considering the large areas that need to be covered to eliminate wildlife rabies in Europe, Asia, North America, and other parts of the world, cost-benefit analyses are required before implementing wildlife control measures.

Economic costs are an important issue, especially for less developed countries, requiring novel cost-effective vaccination strategies to be applied under different ecological conditions. Furthermore, whilst ORV of foxes and raccoon dogs does not present problems, ORV of multiple species of wildlife reservoir hosts, e.g. raccoons, skunks and mongoose in North America still remains a major challenge (Rupprecht et al. 2008). Although rabies was successfully controlled in red foxes, the Arctic variant of rabies virus is still maintained in striped skunks in small foci in southwestern Ontario, Canada (Nadin-Davis et al. 2006b). In fact, both raccoons and skunks are currently the primary terrestrial reservoirs of rabies in eastern North America, and commercially available vaccines do not produce sufficient levels of immunity, particularly in skunks (Slate et al. 2009). In addition oral vaccination of raccoons with vaccine baits requires 4–8 times as many baits per unit area as those required for the control of fox rabies (Rosatte 2013). Although the expansion of raccoon rabies has been halted using ORV, the disease is far from being eliminated. The fact that raccoons and mongoose have also been introduced as alien species to Europe highlights the need for combined research efforts to find solutions, such as the development of oral vaccines for multiple species as well as optimal baits and baiting strategies in the presence of multiple reservoirs.

21.3.7 Rabies Control in Bats?

Although bats are the ancestral reservoir for lyssaviruses, control efforts are limited per se by the accessibility and the status of protection of bats in many parts of

the world. Therefore, any specific population control measures aimed at reducing disease prevalence in bats are both prohibited or unwarranted a scientific point of view (Brass 1994). In keeping with ongoing conservation measures the main focus of activities should be rather on (i) the establishment of adequate surveillance, (ii) increasing public awareness about bat rabies and (iii) targeted pre- exposure immunization and PEP.

21.4 What Hampers Global Human Rabies Elimination?

The cosmopolitan and generalist nature of rabies virus, characterized by multiple hosts maintaining a range of variants, is of concern in the context of disease eradication (Rupprecht et al. 2008). However, when we consider epidemiological and operational criteria, the elimination of canine rabies, which is responsible for the greatest number of human deaths, is a feasible objective (Lembo et al. 2010). Theoretically, all human rabies deaths could be prevented using timely PEP. However, limitations in biologics availability and prohibitive costs make this unlikely. A more practical solution would be to prevent the vast majority (approx. 95 %) of human exposures by elimination of canine rabies and then use timely PEP to treat exposures from other animal sources.

So, what hampers the elimination of human rabies on a global scale? While identifying key constraints preventing the achievement of this goal, this section aims to (a) demonstrate that most of these challenges can be addressed and (b) identify areas towards which efforts should be directed.

21.4.1 Low Political Awareness

Limited awareness accounts for the unsatisfactory level of political commitment and investment towards dog rabies elimination. A reason for this is that, while much scientific knowledge has been generated demonstrating the need for and feasibility of eliminating canine rabies (Lembo et al. 2010), there have been insufficient efforts to communicate research outputs to key stakeholders, resulting in weak translation of evidence into policy. For instance, despite the well-recognised impacts of rabies on local communities and national economies (Knobel et al. 2005), there is still a very limited appreciation of the true scale of the human disease problem within high-level policy making bodies. One explanation is that rabies typically affects the poorest segments of the population with most deaths occurring “at home”, because victims are too ill or too poor to travel to hospitals (Hampson et al. 2008). The high fatality rate of rabies resulting in almost certain death means that there are no “walking wounded” and therefore most cases fail to reach the attention of health authorities generating little or no incentive for action. This attitude is exacerbated by a widespread perception that the elimination of human rabies through dog rabies vaccination is unfeasible because of the presence of many “stray” dogs that cannot

be handled and due to a complex epidemiology involving wildlife reservoirs, despite evidence suggesting that in fact stray dogs comprise a negligible part of the dog population (Gsell et al. 2012; Kayali et al. 2003; WHO 1988) and that canine rabies epidemiology is dog-driven in most endemic areas (Lembo et al. 2008).

Despite these constraints, progress has been made towards greater prioritisation of rabies in international agendas through high-level consensus amongst the scientific community, political advocacy and core partnerships (Lembo et al. 2011). Such achievements include the recognition by major international organizations, notably the World Health Organization (WHO), the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO), of the feasibility of global canine rabies elimination and the declaration of this as a joint goal with a tentative timeline by region (OIE 2011; WHO 2011), as well as the inclusion of rabies on global health agendas (e.g. the World Health Assembly 2011, and the OIE Global Conference on Rabies Control 2011) (http://www.who.int/neglected_diseases/en/).

While the currently limited engagement of central-level national authorities is a harder challenge to overcome, bridging the gap between knowledge generation and policy-level application represents a key area to address to move the rabies elimination agenda forward. Steps that should be taken include a better understanding of the policy making landscape and its mechanisms, as well as the development of communication strategies tailored for policy making bodies.

21.4.2 Low Awareness at the Grass-Roots Level

Many avoidable human rabies deaths occur because of insufficient awareness on effective PEP measures at the community level, especially in very remote areas (Hampson et al. 2008). Increasing awareness can therefore save lives. This has been successfully achieved by involving communities in the prevention and control process. Initiatives such as the “Adopt a village” programme in India, where local women involved in maternal-child health care activities were trained in rabies prevention, or the global World Rabies Day campaign have generated considerable societal support, while also demonstrating that affected communities have a critical need to express their concerns about rabies (Sudarshan et al. 2013).

However, questions still remain regarding the most effective avenues to reach communities at risk. For instance, while it is recognised that children are an important risk group (Kilic et al. 2006), uncertainties remain about the type of interventions that may be most effective for this audience. The emphasis so far has been on school-based interventions (Lapiz et al. 2012), despite the fact that in remote rabies-endemic areas non-schooled children still represent a large proportion of the community. Furthermore, interventions at the school level rarely involve parents, who are likely to play an important role in shaping children’s health behaviour, or key community members (e.g. community leaders), despite their potential role in encouraging community participation in rabies management initiatives. There is little reason why educational programmes that address children within the wider

community context could not enable positive behaviour change in rabies prevention and control; exploratory research in these areas is likely to provide valuable insights.

21.4.3 Rabies Biologicals are Still Inaccessible or Unaffordable for Those Most in Need

In situations where exposed individuals are aware of the need to obtain prophylaxis after a suspect exposure, a common constraint to overcome is that of inaccessibility or unaffordability of rabies biologicals. There are a number of reasons for this: (1) despite the international push for provision of rabies biologics free-of-charge, bite victims in low-income countries often have to incur costs that are beyond their financial means (Knobel et al. 2005); (2) no systems are in place to reduce or harmonize the currently very variable prices of PEP; (3) regional disparities exist in PEP regimens; and (4) mechanisms for supply and distribution of PEP are unsatisfactory, often failing to reach remote areas that need these products the most.

More economical, but equally efficacious administration regimens, such as the intradermal route (Brown et al. 2008; Brown et al. 2011; Hampson et al. 2011; Warrell et al. 2008), and reduced (four-dose) schedules (Rupprecht et al. 2009), are therefore being promoted, with a number of rabies-endemic countries now in the process of incorporating these changes into their healthcare systems.

Public Private Partnerships (PPPs) have also played an important role both at the regional and global levels. In some regions, for example, PPPs have improved accessibility to PEP in remote regions (Lapiz et al. 2012). Globally, joint efforts by international health organizations, donors and private industry are progressively resulting in the establishment of vaccine banks (http://www.oie.int/support-to-oie-members/vaccine-bank/). Such initiatives aim to provide high quality vaccines (supplied by vaccine producers selected through an international call for tender) to beneficiary countries upon official request from their Veterinary Services, with priority given to developing countries with the lowest GDP for emergency vaccination. Broad PPPs, such as for example the Partners for Rabies Prevention, have made advances in the development of global strategies for rabies prevention (Lembo et al. 2011) and could go a long way in generating options for vaccine pricing strategies appropriate to low-income countries.

21.4.4 Limited Surveillance and Diagnostic Capacities

Limited surveillance and diagnostic capacities are enduring issues in regions of the world where the highest number of rabies deaths occur. Because of the resulting under-reporting and under-diagnosis, rabies is falsely perceived as an insignificant health issue leading to low priority and hence further neglect (Fooks 2005). A lack of integrated surveillance systems, involving both the central- and local-level human and animal health sectors, is a key gap in surveillance capacity in developing

countries (Banyard et al. 2013a, b; Molyneux et al. 2011). Another limiting factor is that international standards for rabies surveillance and evaluation of interventions are entirely reliant on laboratory confirmation of cases post-mortem, which requires the establishment of rigorous systems for the collection and submission of samples, and adequate laboratory infrastructure and capacity to perform rabies diagnostics (Banyard et al. 2013a, b; Fooks et al. 2009; Meslin and Kaplan 1999). To this end, simplified techniques have been developed for sample collection, preservation, and diagnosis in poorly-resourced settings (Durr et al. 2008; Lembo et al. 2006). The reality, however, is that very few countries in rabies-endemic areas are likely to meet global surveillance and diagnostic standards in the foreseeable future and this should not further delay the implementation of large-scale rabies elimination programmes. Approaches based on clinical case finding by local communities were the primary surveillance tool in the final stages of rinderpest eradication (Mariner et al. 2012; Mariner and Roeder 2003). Given the distinctive nature of rabies, recognition among local communities in affected areas is high (Hampson et al. 2009) and therefore participatory surveillance has the potential to play a key role in assessing the impacts of rabies interventions. Efforts should therefore be made to provide affected communities with user-friendly tools for rapid case reporting using, for example, mobile phone technologies (Vital Wave Consulting 2009), and to shift the focus from laboratory-based surveillance as the sole means to evaluate intervention efforts.

Available Databases and Reporting Systems on Animal Rabies

On a global scale there are two main international organisations which traditionally host databases dedicated to or including detailed data on rabies: The World Animal Health Organisation (OIE) and the World Health Organisation (WHO). Since its creation in 1924 the OIE collects and disseminates animal health information from its member countries. The World Animal Health Information Database contains detailed information on rabies occurrence, categorized both by domestic animal or wildlife as per first administrative division and as officially reported by the veterinary services of the 180 OIE member countries. Over several decades, WHO has collected human and animal rabies data annually through designated national rabies focal points of its 194 member states. These data were fed into the rabies dedicated 'Rabnet' database with similar data as specified above, including human rabies cases. Regrettably, in the absence of regular reporting from most WHO Member States, the 'Rabnet' site has been closed until further notice.

Additional global databases that collect, among other diseases, epidemiological information on rabies include EMPRES-i managed by the Food and Agriculture Organisation (FAO) (FAO 2013), and ProMedMail (ISID 2013). Both of these sources emphasize newly reported outbreaks or unusual rabies events, rather than providing a comprehensive picture on the rabies burden and its evolution over time and space.

On a regional scale, a few rabies-dedicated databases have been established as a result of large scale regional rabies control initiatives. The Pan American Health Organisation (PAHO) hosts the 'Sistema de Información Epidemiológica' (SIEPI) and the WHO Collaboration Centre for Rabies Surveillance and Research in Germany maintains the Rabies Information System (Rabies Bulletin Europe) for Europe. Both databases provide fairly realistic data on the burden and species affected in countries of Latin American and Europe, respectively. Other regional organisations like the African Union/Interafrican Bureau for Animal Resources have attempted to centralise the reporting of animal health data, including rabies, but data are less accurate and often incomplete.

21.4.5 Companion Animal Health Issues

An additional issue in rabies-endemic countries arises from the limited capacity within veterinary services to address animal health problems related to companion animals given the greater focus on livestock health/agricultural problems. This problem can be overcome by providing training and support for veterinary services to handle domestic dogs and dog diseases. However, a more challenging issue to address is that for most veterinary and medical professionals in Africa and Asia interventions involving domestic dogs are viewed as low status, because they target an animal species of no economical value and an underrepresented segment of the human population, the rural poor. Yet, lessons can be learnt from areas of the world, such as Latin America for example, were national elimination programmes led by an influential government sector (public health) have resulted in dramatic declines in human and canine rabies (Schneider et al. 2007).

21.4.6 Administrative and Financial Issues in the Operationalisation of One Health Initiatives

Given the nature of rabies, the responsibility for its control should be borne by a broad range of sectors, hence the importance of inter-sectoral and multi-disciplinary collaboration in rabies management initiatives (Lembo et al. 2011). However, the range of "players" that ought to be involved makes operationalisation problematic because of difficulties in harmonising administrative and management structures across sectors, and planning and implementing joint financing mechanisms involving different ministries.

The creation of inter-sectoral zoonotic units or task forces provides a practical solution towards simplifying administrative processes. However, an important issue is the need for large initial investments to achieve high-level dog vaccination coverage. Building the body of evidence by demonstrating the cost-beneficial impacts

of dog rabies control due to reduced expenditure on costly human post-exposure vaccines is an important step towards the integration of budgets across ministries in order to ensure sustained financial support, and some progress has been made in this respect (Zinsstag et al. 2009). The effectiveness of inter-sectoral strategies for canine rabies elimination has already been proven in the Americas (Schneider et al. 2007) and there is no reason why the Latin American model could not be applied elsewhere. Raising awareness towards prioritisation of rabies in national agendas and budgets is also going to be important to ensure the sustained financial commitment necessary for long-term impacts of dog rabies control operations. PPPs have a role to play in this respect, as well as in persuading major international donors that investing in canine rabies elimination is worthwhile (Lembo et al. 2011).

21.4.7 Sustainability Issues

Programme sustainability is crucial if canine-mediated human rabies is to be eliminated. There are some aspects of building sustainable programmes that will necessarily be common across various geographical locations including: implementation and enforcement of national laws and regulations focusing on rabies prevention activities; establishment of adequate surveillance systems; continuing education and awareness for public health officials, national veterinary services and citizens; access to effective rabies biologics, including high-quality vaccines for dogs; rabies vaccine banks for dog vaccination; and sufficient funding to ensure programme continuation. Approaches to ensure programme sustainability will in all likelihood vary depending upon the country. For example, in the province of Bohol, Philippines, a small fee charged for registration of dogs has supplemented financial support provided by the provincial government to maintain programme sustainability (Lapiz et al. 2012). However, this approach may not work in other settings and may be responsible for insufficient turnout, hence low vaccination coverage (Zinsstag et al. 2009). Whatever the approach to building sustainable rabies prevention and control programmes the involvement and continued support of local communities is vital. Governments should also understand that rabies prevention and control requires a long term commitment. However, the planning and implementation of sustainable rabies control programmes will result in multiple public health benefits for local communities that are currently living at daily risk of exposure as well as public health officials that are responsible for the welfare of their communities.

21.5 New (grassroots) Initiatives

All human rabies deaths are preventable with existing tools, yet rabies still kills tens of thousands of people a year. Too often, interventions have a local impact, but because they are not maintained, rabies resurges (Rupprecht et al. 2008). The situation is well understood, and there is a growing consensus that low education

at all levels, and a lack of political will, inter-sectoral collaboration and funding are the main obstacles to realizing a 'rabies free' future (Gongal and Wright 2011; Lembo et al. 2011).

Victims of dog bites still die due to a lack of knowledge about rabies, or insufficient access to affordable PEP. Government indifference, driven partly by limited data to assess rabies' true impact, and poor inter-sectoral collaboration means that rabies in dogs remains uncontrolled. Clearly, new initiatives are needed to break the cycle of neglect, with a focus on not only identifying the hurdles to better rabies control, but also ways over or around those hurdles (Dodet and Africa Rabies Expert Bureau 2009; Dodet et al. 2008b; Lembo et al. 2011).

21.5.1 GARC—Uniting a Community Using Technology

Aiming to address the neglect that has hampered rabies control for so long, the Global Alliance for Rabies Control (GARC) was established in 2007. GARC's vision was to build a true One Health model of control, involving all relevant sectors and everyone who had an interest in, and a desire to improve, rabies control worldwide (GARC 2012). Everyone has a role to play in rabies control, whether they are running a country-wide control programme educating others about the need to vaccinate animals, advocating at international levels for increased investments in rabies control, or understanding how to protect themselves against rabies in the case of an animal bite.

In a few short years, GARC and its partners have united their efforts, built a worldwide community and linked them to information and tools to prevent rabies using websites, emails, an electronic newsletter, social media tools and a webinar. As internet technology continues to increase its global reach, GARC enables communication and information sharing in all directions, across continents. Resources developed by GARC and other large organizations are shared with the community and vice versa, to the benefit of all.

21.5.2 Partners for Rabies Prevention—The First PPP for Rabies

Public private partnerships are increasingly recognized as an effective and necessary means of harnessing all of the relevant skills needed to tackle problems such as neglected diseases (Croft 2005; WHO 2002; Widdus 2005).

The first meeting of the Partners for Rabies Prevention (PRP), a truly inter-sectoral PPP initiated by GARC, was held in 2008. For the first time, key representatives for rabies within WHO, FAO of the United Nations OIE, rabies vaccine manufacturing companies, academics, implementers, and Non-Governmental Organizations sat together informally, with each having a voice in discussions on how to tackle the neglect of rabies with practical ways forward. Each participant

contributed their varied skills and experience to the collaboration (Lembo et al. 2011; Taylor and Partners for Rabies Prevention 2013). A roadmap that identified the key barriers to better rabies control implementation and the knowledge gaps that need to be filled, was developed by consensus and has guided all of the group's work since then (Lembo et al. 2011).

The PRP's work involves three main approaches: increasing awareness of the scale of the problem of rabies; providing access to educational tools for both the public and professionals to use in their communities; and building the evidence to advocate at international levels for improved commitments to rabies control. These approaches start to address all of the factors hampering global human rabies elimination. The ultimate aim is to change the political indifference that surrounds rabies control in so many countries by building the evidence that canine rabies elimination is feasible, practical and the only long term cost-effective strategy to stop needless deaths due to rabies (Taylor and Partners for Rabies Prevention 2013).

21.5.3 World Rabies Day—Empowering the Community to Increase Awareness and Education

One of the first initiatives of the PRP was to launch the World Rabies Day campaign (GARC 2012), an initiative run by GARC. By making rabies and rabies control efforts newsworthy, the campaign brings attention to neglected communities affected by rabies and recognition to those addressing the problem (Briggs and Hanlon 2007; Cleaveland et al. 2010). The World Rabies Day campaign is based on a simple model of asking everyone interested in rabies control to use the resources provided and do whatever they can to increase rabies awareness, education or control in their community around 28th September. Over the first 5 years, participants in more than 150 countries have held events educating 182 million people and vaccinating 7.7 million animals (GARC 2012). Whilst GARC facilitates World Rabies Day events, hosts the resources and coordinates reporting, this is a community-based initiative with educational materials and experience contributed and adapted by the communities themselves. This grassroots movement demonstrates not only the demand for more attention to be paid to rabies control, but also a huge desire to do something about it. It is recognized and endorsed by international organizations such as WHO and OIE (OIE 2011; WHO 2013a)

21.5.4 Community Based Rabies Control—Demonstrating Practical Success

Community engagement is important not only for educational projects, but also for compliance with disease vaccination efforts; otherwise large scale disease control efforts imposed from above sometimes fail to produce the expected results (Atkinson et al. 2011). The PRP saw the need to support a pilot rabies control project

that demonstrated that rabies control was feasible, and that community engagement is a critical factor in its implementation and sustainability. In the Bohol Rabies Prevention and Elimination Project, carried out in the Philippines, there was a huge emphasis on community engagement well before any animal was vaccinated, and a large dependency on volunteerism from the community to enable the project to run cost-effectively (Lapiz et al. 2012). The project, which improved quality of life of the island of Bohol's 1.13 million inhabitants, reduced cases of human rabies to zero within 2 years and established a dog registration programme that ensures sustainable funding for rabies vaccination (Lapiz et al. 2012).

21.5.5 Tools for Professionals—Rabies Blueprints and Stepwise Approach towards Rabies Elimination

Through online resources and an electronic newsletter, the many members of GARC's community who are actively engaged in rabies control worldwide are able to stay updated. However, poor access to current international guidelines and advice on how to implement them for professionals was identified by the PRP as a problem that needed to be addressed. The online Blueprint for Rabies Control was developed to provide a comprehensive practical guide, and the first One Health resource for rabies information, to those in positions to design and implement rabies control programmes (Lembo and Partners for Rabies Prevention 2012). Two sections have been developed, rabies control in dogs for canine rabies endemic countries, and rabies control in wildlife, with both including human rabies prevention information (Partners for Rabies Prevention 2011).

To complement the Canine Rabies Blueprint, a Stepwise Approach towards Rabies Elimination (SARE) was developed. This acts as a planning and evaluation tool for progress made towards elimination of human and dog deaths due to rabies (Partners for Rabies Prevention 2014). SARE describes a series of epidemiological or other stages that a country would progress through (from uncontrolled disease with an unknown burden, to the elaboration of a national rabies control strategy, to implementing it, to ultimately achieving elimination of human and canine rabies cases). The ability of countries to assess their rabies control efforts and progress towards elimination will be an important step in the refinement of both country and regional control efforts in rabies.

21.5.6 Community-Lead Innovations

Although the principles of rabies control are well established, a 'one size fits all' implementation process is unlikely to work. Practices need to be culturally appropriate to ensure the compliance essential to success (Atkinson et al. 2011). Volunteerism may work well in some places, but not in others; funding mechanisms for mass

vaccination programmes will vary across countries. By making the appropriate tools accessible to all, the chances of local innovation solving some of the age old problems that have hindered rabies control are maximized. From devising vial sharing schemes for intradermal PEP (Bharti et al. 2012), to making existing legislation work to increase canine vaccination rates in rural communities (Partners for Rabies Prevention 2012), to utilizing health promoters, to improving communication channels (Partners for Rabies Prevention 2010), individuals and communities are showing tremendous innovation. Connecting a community allows such innovative ideas to be shared, replicated or adapted to the benefit of all.

21.5.7 Advocating for Political Change

Community-led initiatives are grounded in reality and can be highly effective, but may have only a very localized impact. The combination of leadership from a coordinated group of international organizations and experts in rabies with a grassroots base of advocates and practical implementers is a powerful recipe for change that the PRP is aiming to foster. International organizations bring their expertise and strategy at a large scale, those on the ground bring local adaptation, compliance and advocacy, and both sides put pressure on governments who ultimately must run sustainable rabies control programmes.

Recently established and reinvigorated regional networks of rabies experts in canine rabies endemic areas of Africa, Asia and the Middle East have become a powerful force in driving advocacy for cross border collaborations and moving towards effective regional rabies control and elimination strategies (Dodet 2007; Dodet and Africa Rabies Expert Bureau 2009; Dodet et al. 2013). Already progress is apparent, such as World Rabies Day catalyzing improvements in rabies control from governments and investments from foundations (Cleaveland et al. 2010) and the new strategic Framework for the Elimination of Human Rabies in South-East Asia (WHO Regional Office for South East Asia 2012). With the recent increase in international support for the control of neglected diseases (WHO 2013a, b), rabies is starting to move up the international health agenda. The PRP group and others are seeking to build on this momentum, and put rabies specifically on the agenda of international organizations' governing assemblies of member countries.

21.5.8 Conclusions

The pioneering work of Louis Pasteur's first vaccination trials in humans paved the way for today's efficient pre-exposure immunization and PEP and preventive vaccination in animals. It is a tragedy that at the beginning of the twenty-first century rabies is still an imminent danger for humans and animals alike. Because interventions focusing solely on disease prevention in humans have no effect on the reduction of infection in the reservoir hosts, the most effective way to combat human

rabies infections is to control the disease by mass vaccination of the animal source, e.g. dogs and wildlife. Unfortunately, many people and public health officials still feel that the wildlife problem makes rabies uncontrollable. However, it is a matter of fact that eliminating canine rabies reduces the human death toll profoundly. Moreover, spillovers from wildlife into dogs are rarely seen where dog rabies had been eliminated.

The availability of effective rabies control strategies, existing experience (especially in Latin America) and the support of the scientific community are evidence that the elimination of dog-transmitted rabies can be achieved in the short term. As a result, WHO is developing a global strategy for the elimination of dog-mediated rabies with the collaboration of international strategic partners such as FAO and OIE, and with strong participation of stakeholders and civil society groups. WHO strongly promotes global human rabies prevention mainly through the elimination of rabies in dogs as well as a wider use of the intradermal route of rabies vaccination which reduces the volume and the cost of cell-culture vaccine needed for PEP by ~60%. The use of these measures will be integral to reaching the goals for human and dog rabies elimination, set for all Latin American countries by 2015 and South-East Asia by 2020 (World Health Organisation 2013). In South-East Asia, a 5-year plan (2012–2016) currently aims to halve the estimated number of human rabies deaths across rabies-endemic countries.

The targets set by the WHO are supported by various partners, including the Bill & Melinda Gates Foundation. In cooperation with WHO, the Bill & Melinda Gates Foundation initiated a pilot programme in 2009 to eliminate canine rabies in three rabies-endemic territories, namely the KwaZulu Natal province in South Africa, the South-Eastern part of the United Republic of Tanzania and the Visayas archipelago in the Philippines. This pilot programme aimed to verify that human rabies can be prevented through the control of rabies in the canine vector population. The programme has also helped to refine strategies for canine rabies elimination which could be implemented in other countries in the region. In the Philippines, the successful concept of the Bohol rabies elimination project initiated by the provincial government and supported by GARC has been so convincing that it is being replicated in similar projects on other islands in the region. The elimination of canine rabies in these three areas would ensure that >50 million people do not live in fear of rabies.

Amongst OIE Member Countries, rabies was identified as a priority disease for developing or implementing new technologies, and the recent adoption of resolution WHA66.12 on neglected diseases brought more attention to rabies at the World Health Assembly. The time is ripe to make human rabies history. Therefore, a specific resolution, e.g. through the World Health Assembly would further emphasize the claim for human rabies elimination in the near future. Scientific and practical evidence supporting the feasibility of elimination is building, and there is momentum towards policy change coming from international organizations. This increasing momentum together with advocacy efforts such as the World Rabies Day campaign will increasingly persuade national governments to make political commitments and to allocate sufficient financial and human resources for rabies control.

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Part IV
Zoonoses in Domestic Animals

Chapter 22
Dogs and Transmission of Infection to Man, "Respected Member of the Family?"

Frans van Knapen and Paul Overgaauw

Abstract Numerous reviews on dog zonoses address long-lasting lists of zoonotic infections, observed worldwide or very specifically in certain regions only. Here we describe the average family dog in the Western hemisphere owned by an average family without sufficient knowledge about potential hazards their pet might transmit to family members.

This chapter is based on semi quantitative risk analysis in order to rank potential health risk transmitted from family dogs to human. Surprisingly every day risk is different from the generally expected potential risk according to traditional ranking of hazards (zoonoses) in dogs in general. Attention is given to human behavior regarding the family dog and responsible dog ownership. Modern trends include pet travel or pet import from endemic to non-endemic areas, without sufficient knowledge amongst pet owners or public health institutes. Of great value is information provided by ESCCAP (www.esccap.org) with information for European countries (veterinarians and pet-owners) on prevalences and prevention of parasitic infections in dogs and cats in the major languages of Europe.

Eventually attention is paid to new trends in dog feed such as feeding bones and raw meat. This may have serious consequences for the spread of ordinary zoonoses like Salmonella and parasitic infections not only between dogs, but also to family members.

A last point of attention is the prevention of attracting wild life zoonoses via dogs to family members (eg. *Echinococcus multilocularis* and *Baylisascaris* spp.).

Authorities responsible for public health should be encouraged to pay more attention, not only in providing more regulations, but primary in enforcement of existing rules and stimulating responsible pet-ownership. Companion animal veterinarians and (local) public health authorities, including physicians, should contribute equally in zoonoses prevention programs ('One health' approach).

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22.1 Introduction

Until about the turn into the twenty-first century zoonoses were regarded by the medical profession as 'just a number' of the well-known infectious diseases in the modern ('western') part of the world. As infectious diseases in that part of the world were overcome by increased public hygiene, food safety, vaccinations and proper antibiotic treatment if necessary, there seemed no need for further attention. However, a number of events has attracted public and political awareness for new and re-emerging zoonoses such as the rapid spread of SARS in 2002 (Peiris et al. 2003), West Nile virus throughout the United States (Shephard et al. 2006), the outbreaks of avian influenza in South East Asia, but also in Europe (Shortridge et al. 2003) with varying types (H5N1, H7N7) leading to human casualties which alarmed public health authorities in many countries.

In 2004 a joint WHO/FAO/OIE consultation on emerging zoonoses was held in Geneva (WHO/FHO/OIE 2004). Other incidents fostered this change such as antibiotic resistance supposed to be related with animal treatments (MRSA, ESBL) (Weese 2010; Friese et al. 2013), and the tremendous spread of ordinary grey ticks (*Ixodus ricinus*) and consequently Lyme disease in humans (Van der Giessen et al. 2010). A central question to be answered is what role do veterinarians play in public health in the twenty-first century (WHO 2002). In this chapter we will concentrate on some relevant dog zoonoses and the role of the dog owner in infection and prevention.

Dog zoonoses and public health has been addressed recently in full detail (Macpherson et al. 2013). A traditional approach to deal with zoonoses is to use a template including the biology of the germ, epidemiology, disease in humans and animals, diagnosis, and prevention or control of the disease. Often it means a mere enumeration of zoonoses found in dogs without further going into relative health risks for dog owners or the population at large through environment pollution (Tan 1997). Depending on the endemicity of particular zoonoses in certain areas of the world more attention may be paid to these (e.g. echinococcosis and toxocarosis, Carmena and Cardona 2013; Desplazes et al. 2011).

In this chapter we will concentrate on pet-ownership in an average (north) west European country without endemicity for diseases like leishmaniosis, rabies or heartworm disease. We pay attention to the average family dog in an average family with more or less awareness about potential health risks, however, enjoying having a pet.

22.2 Risk Analysis

The relative risks for human health consequences or the attribution because of having dogs for the huge variety of dog zoonoses is largely unknown or studies carried out remain to be inconclusive. This is due to failure in examining both pet owners and their pets simultaneously and comparing isolates by genotyping, serotyping or

other identification methods to suggest a one way cross infection or having a common source for infection elsewhere. There are, however, also indications that some infections may be transferred from humans to their dogs, such as MRSA (Levebvre et al. 2009) and *Giardia* spp. The zoonotic potential of *G. duodenalis* is considered as evident, based on findings of assemblages A and B in man as well as in dogs. Authors automatically concluded then a one-way transmission route to the human (Marangi et al. 2010; Dado et al. 2012). Knowing the infectious potential of the *Giardia* parasite, this does not exclude at all an interaction between owner and his dog. Evidence on the contribution and frequency of the zoonotic potential is (still) lacking (Sprong et al. 2009).

Each risk analysis starts with summing up potential biological hazards (H), in this case zoonoses, in an area and is followed by hazard characterization including prevalence figures in the reservoir (dogs), virulence for humans, transmission routes, and survival of the agent in the environment. These criteria are then weighed, mostly based on experts opinions.

The second step is exposure assessment (E). Who is exposed to the potential hazard and for how long or how often. How much of the potential pathogen is needed to become a health risk? This inevitably is directly related to human behaviour in relation to their dog.

The third step is to calculate the impact of getting infected, e.g. how serious is the disease, what is the chance for complications Disability-adjusted life years (DALY's), and what economic consequences may be expected (labour hours lost). Each of the parameters can be ranked in classes 1–5, where 1 stands for negligible and 5 for the most serious possibility. Ranking is based on literature data, own observations (measuring) or expert opinions, thus arbitrarily.

The final risk assessment can be achieved by multiplying the outcome of hazard characterization, exposure assessment and impact (H × E × I=number). The outcome is nothing more than a ranking order of the potential health hazards and has no absolute meaning. Since these figures have been estimated for a large Dutch small animal practice (Berends 2006) we shortly refer to the results. Table 22.1 provides the top five of zoonoses in dogs according to the hazard characterization ranking order. In Table 22.2 the ranking order of exposure to dog zoonoses is pro-

Table 22.1 Ranking order of biological hazard characterization in dogs

1.	*Rabies*
2.	*Capnocytophaga canimorsus*
3.	*Leptospira spp.*
4.	*Salmonella spp.*
5.	*Campylobacter spp.*

Table 22.2 Ranking order of exposure assessment

1.	*Dermatophytes*
2.	*Pasteurella mutocida, P. canis*
3.	*Staphylococcus aureus/intermedius/ pseudointermedius*
4.	*Campylobacter*
5.	*Salmonella*

Table 22.3 Ranking order of potential human health risks due to dog zoonoses

1.	*Campylobacter*
2.	*Pasteurella spp.*
3.	*Salmonella spp.*
4.	*Staphylococcus spp.*
5.	*Giardia duodenalis*

vided. Eventually in Table 22.3 the top five important dog zoonoses in an average small animal clinic are mentioned. We will use this point of departure because the intensive contact clinicians have with dogs will certainly be comparable to that of the owner with only one important exception: the duration of exposure. Since this is also subject to the owner's behaviour which will be discussed in the next paragraph.

22.2.1 Having Dog as Pet

Companion animals enhance the psychological and physiological well-being of the human because of psychological support, friendship, and even good health practices (exercising or reducing stress). Dogs play an important role in the development and in the treatment of behavioral problems of children, the well-being of the elderly, and decrease work leave through illness and visits to the doctor (Beck and Meyers 1996). Pet ownership certainly will lead to health benefits, although not all (social) studies have been based on correct methodologies (Koivulsilta and Ojantlava 2006).

Here we deal with potential biological hazards (zoonoses) that may have negative health consequences for the owner. The starting point is Table 22.3, which ranks the zoonoses with potential health risks for individuals with short but intensive contact, such as veterinarians or breeders. It may be assumed that exposure to the potential hazards mentioned will be much higher and long lasting when the owner (family) is involved. The actual top five zoonoses, however, will remain similar and concerns fecal-oral route of transmission (*Campylobacter, Salmonella, Giardia*), direct contact (*Staphylococus* spp.) and injuries as result of bites, licking, and scratching (*Pasteurella multocida, P. canis*) .

22.2.2 Human Behavior

As pets are increasingly considered as member of the family, physical contact is very common. Cuddling, stroking, and playing with the animals are all normal behaviour of dog owners and even more their kids. It is part of enjoying pet animals, but pets have become more often substitutes for childbearing and child care, sometimes leading to excessive pet care and intensive contacts (Chomel and Sun 2011).

It is remarkable that during dining of the owner, dogs are often allowed to approach, to beg for snacks, and being stroked or even worse are allowed to join the dinner. Therefore, it would be evident to at least wash hands after the fun and before a meal. The number of potential pathogens such as enterobacteriaceae (Westgarth

et al. 2008) or parasite eggs (Keegan and Holland 2010) from the fur of most animals, including dogs, is easily detectable and can be washed off by flushing water and ordinary household soap. It is self-evident behaviour to do so before sitting down for dinner or after visiting a washroom, which is part of upbringing and education.

Licking in the face, or sharing an ice cream is part of showing mutual affection and is allowed by as much as 50% households (Overgaauw et al. 2009). The idea that the tongue of a dog is clean and may even be used to clean up wounds by licking is widespread among the public and sometimes even among first aid health professionals (Verrier 1970). In literature, increasingly indications can be found that licking by a dog may lead to infections (Booij-Vrieling 2010; Haesebrouck et al. 2009); or serious health consequences in individual cases (Shewring and Rushforth 1990; Wade et al.1999; Overgaauw and Van Knapen 2012).

Allowing dogs to sleep in the bedroom (33–56%) or even to sleep in the bed of the owner (18–50%) is certainly contributing to the transmission of zoonoses including parasites (Overgaauw et al. 2009; Chomel and Sun 2011). Intensive contact with skin and nose, *Staphylococcus* spp., even when the dog is healthy and without skin lesions, may lead to contamination with antimicrobial resistant strains (MRSA) (Kempker et al. 2003; Cain 2013).

Having one or more dogs in the household means that soil from outdoors is regularly spread throughout the house. Dogs were regularly reported to have soil transmitted parasitic infections in their fur (Keegan and Holland 2010) with as yet unknown consequences for the owner however it should be kept in mind that even *Toxocara* eggs may be easily found in household dust from dog owner houses (Overgaauw and Boersema 1998).

Amongst soil transmitted diseases serious consideration must be given to toxoplasmosis, because dogs might act as active distributors of oocysts from the environment. Dogs regularly and actively roll in faeces of other animals or are eating these (Frenkel et al. 1996; Nijsse 2013).

An important role of the veterinarian is to make owners aware of the potential risks and that personal hygiene and proper cleaning of the house/kitchen is necessary, without frightening them. Awareness and responsible pet ownership are the most important issues to achieve a healthy relationship between owner and dog.

22.2.3 Responsible Pet Ownership

The benefits of pet ownership come with obligations. This is called responsible pet ownership and includes among others that owners provide preventive (e.g., vaccinations, parasite control) and therapeutic health care for the life of pet(s) (AVMA 2012) to prevent transmission of pathogens to the human. Responsible pet ownership also means that dog owners should realize that the other half of the population have no dogs or may even not like dogs. Annoyance about dog faeces in the streets or noisiness is common. Dog owners can contribute to public discussions in communities to show their attitude to regular deworming or parasite control, health certificates and to clean up the faeces of their dog while walking. It is surprising to see the differences between dog owner's attitudes in various countries in Europe. While

British dog owners are used to clean dog faeces, Dutch citizens are not sufficiently stimulated to do so. Only 39% of dog owners report to regularly clean up faeces of their dog (Overgaauw et al. 2009).

22.3 New Trends

22.3.1 Emerging Zoonoses

'Emerging zoonoses' are zoonoses that are newly recognized or newly evolved or that have occurred previously, but show an increase in incidence or expansion in the geographic, host, or vector range' (WHO/FAO/OIE 2004). A major issue of spreading zoonoses with dogs is transport from endemic areas to non-endemic areas. In the EU without borders such transports only require mandatory health certificates, signed by an official veterinarian, and proper vaccinations (e.g. rabies). Rabies, *Echinoccocus granulosus* and *Leishmania* are in many countries therefore 'imported' infections. Import of canine babesiosis (not a zoonosis) and probable settlement in Western Europe including its vector (*Dermacentor reticulatus*) are a clear sign of dragging off zoonoses (Daugschier 2001). Harmonization of parasite control in Europe has taken place by the independent organization ESCCAP (www.esccap.org) in order to enable veterinarians to inform clients about the differences of endemic diseases in the various European countries and how to prevent contracting or importing infectious diseases when traveling with dogs (holidays, animal assisted-dogs) or purchasing dogs from abroad. Obviously, this is not only of value regarding parasitic infections, but also for bacterial or viral zoonoses (e.g. brucellosis, rabies), however, comparable informative websites for veterinarians do not exist yet. Transports from dogs originating from Eastern Europe (Romania, Bulgaria) and Mediterranean countries (Spain, Italy) for commercial enterprise to the Northwest of Europe increase the spreading of unwanted and unexpected zoonoses to non-endemic areas such as cystic echinococcosis (Carnena and Cardona 2013). The shifting frontier lines from the South (e.g. *Leismania*) and Middle Europe towards Western and Northern Europe (e.g. *E. multilocularis*) also suggest changes of habitats whether or not supported by climate change. On the ESCCAP website these changes are regularly updated.

22.3.2 Feeding of Raw Meat to Dogs

Although many dog owners in the Western world feed their pets industrially processed food, an upward trend is emerging towards feeding home-made food instead of pre-prepared food, which may consist of leftovers, home-made and prepared meals, bones, raw meat, and organ meat. Outbreaks of Salmonella have been described, which could be linked to contaminated animal food. Care takers and family

members can become infected with *Salmonella*, not only when preparing food that contains raw meat, but also after being in contact with infected animals that excrete the bacteria (Finley et al. 2006; Lefebvre et al. 2008; Schlesinger 2002), and after being in contact with infected food bowls, for example when cleaning them (Weese et al. 2006). Dogs regularly lick their anus and it has been found that over half of all dog and cat owners allow their pets to lick their hands or faces. By this way, bacteria may be spread. Especially individuals with a impaired resistance are at a greater risk of becoming ill. Therefore, it is recommended that dogs living with children, elderly and those being used therapeutically in caring for people, are not allowed to eat fresh meat. As long as dogs are fed with commercially available (complete) diets, which are free from pathogens (canned, dry food), there exists no danger in getting infected through the feed chain.

22.3.3 Contact with Wildlife Zoonoses

Hunting dogs that are allowed to feed on waste of wild animals, or free ranging dogs hunting or scavenging for their own feed, are at more risk of picking up zoonoses that may have serious consequences for the owners health. In *Echinococcus multilocularis* (fox tapeworm) endemic areas (see www.esccap.org), there is a real risk that (hunting) dogs may become infected with this tapeworm. Because *E. multilocularis* easily grows in dogs the threat of spreading it to owners is also realistic. Monthly deworming in endemic areas is strongly recommended (Hegglin and Deplazes 2013). Moreover in areas with raccoons and raccoon dogs in wildlife in large parts of Europe a new threat is the appearance of *Baylisascaris* spp. in dogs, too. This emerging zoonosis, potentially leading to severe larva migrans infection in animals, humans, and particularly in children needs further attention (Okulewicz and Buńkowska 2009; Lee et al. 2010).

22.3.4 Dogs and Transmission of Human Viruses

The increasing role of influenza A virus and human disease and the threat of new pandemics of new types (hemagglutinin (H) and neuraminidase (N)) also reflects dogs and cats. So far canine influenza (H3N8) does not cause harm to humans, but human pathogenic influenza isolates are able to infect dogs and cats and these animals may play a role in interspecies transmission and spread of influenza virus (Song et al. 2008). Small animal practitioners may play an important role in early warning systems for influenza in humans and dogs (Beeler 2009).

Recently, it was shown that human noroviruses can survive in dog's gastrointestinal tract. This major source for human diarrheal disease worldwide is suggested to be transmitted from human to dog and consequently may be transferred to others (Summa et al. 2012).

Table 22.4 Additional potential health risks for dog owners depending on behaviour, travelling or import of dogs form areas endemic for zoonoses not covered by Table 22.3

1.	*Echinococcus granulosus*
2.	*Echinococcus multilocularis*
3.	*Toxocara spp.*
4.	*Bayliscascaris spp.*
5.	*(Influenza/norovirus)*
6.	*(Toxoplasma)*

Additional potential health risks for dog owners depending on behaviour, travelling or import of dogs form areas endemic for zoonoses that are not covered by Table 22.3, are presented in Table 22.4.

22.4 Authorities Involvement

With respect to emerging zoonoses, early warning and surveillance are important issues (Van der Giessen et al. 2010). Although active surveillance systems exist particularly for livestock and wildlife, no such system exists for pet animals.

Notifiable diseases from pet animals in the Netherlands include brucellosis, campylobacteriosis, echinococcosis, leptospirosis, rabies, salmonellosis, toxoplasmosis, tuberculosis and yersiniosis. Data are however scarce and undoubtedly underreporting occurs. This may be due to improper diagnosis or ignorance. Enforcement of the existing legislation should be the first goal of authorities. Community administration however is regularly confronted with complaints of citizens about dogs and dog behaviour and indirectly about dog owner behaviour. This was mentioned before and requires more attention of local authorities. Responsible pet ownership should get more attention and may be stimulated locally. Dogs enduring health certification by a veterinarian at regular intervals including proper parasite control, vaccinations, and general health checks, may be rewarded with a recognizable medal to their collar. In such way the dog owner may show (social control) his or her public responsibility. Moreover, the obligation to clean up one's own dog's faeces should be stimulated by the national government in those countries where this is not yet commonly practiced.

22.5 Conclusions

The role of the companion animal veterinarian is not only to care for animal diseases, but is increasingly becoming important in the field of veterinary public health.

Livestock veterinarians and official veterinarians have taken up this responsibility already for long. Disease detection, reporting and prevention are important issues. Companion animals, including dogs, may act as important sentinels for public

health. Veterinarians should advice more often to pet (dog) owners about health education regarding husbandry, dog behaviour and responsible pet ownership. Co-operation with community health services and the local administration should be part of the contribution of small animal practitioners in the twenty-first century 'One health' approach (Trevejo 2009)

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Chapter 23
Cat-Related Zoonoses: Killing You Softly with Feces and Fleas

Andreas Sing

Abstract In many countries worldwide, cats have become "man's really best friend". In the following chapter the public health relevance of cats will be highlighted by introducing the most relevant zoonotic pathogens including *Toxoplasma gondii, Bartonella henselae, Toxocara cati*, *Rickettsia felis*, enteropathogenic bacteria and the emerging cat-related pathogen *Corynebacterium ulcerans*.

23.1 Introduction

The family *Felidiae* of the Order *Carnivora* is of comparatively recent evolutionary origin with a proposed common ancestor living <11 million years ago (Agnarsson et al. 2010; Johnson et al. 2006). Based on morphological and molecular data 38 extant and two extinct species can be divided into eight major lineages: the two "big cat" clades of the pantherine lineage with four roaring and one non-roaring species and the Asian leopard cat group, the ocelot lineage, the caracal group, the puma group, the baycat group, the *lynx* genus and the domestic cat lineage (Agnarsson et al. 2010; Serpell 2000; Johnson and O'Brien SJ 1997; Johnson et al. 2006).

The latter lineage originated in the Mediterranean region about 6–7 million years ago (Johnson et al. 2006) and consists of six small cat species from which four, i.e. the domestic cat (*Felis catus*), the European wildcat (*F. silvestris*), the African wildcat (*F. libyca*) and the sand cat (*F. margarita*), diverged later on. Based mainly on morphological, archeological and behavioural findings *F. libyca* is hypothesized to be the most likely ancestor of the domestic cat. Even etymological findings support this idea, since the word for cat in many languages (e.g. English *cat*, fourth century Latin *cattus*, the ancient Greek *κατος*, the medieval Byzantine Greek *κατης* [Nicholas 1999], Spanish *gato*, French *chat*, German *Katze*, Lithuanian *katė,* the modern Arabic *quttah*) is thought to be derived from the Nubian word for cat, *kadiz*.

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Domestication of cats started presumably 10,000–25,000 years later than the domestication of dogs (Germonpré et al. 2009) and probably began in Egypt about 4000 years ago, where cats even enjoyed a sacred status and were associated with the cult of the cat-shaped deity Bastet. One strain of etymological speculation links the English diminutive *pussy* and the Romanian word for cat *pissica* to this Egyptian goddess (Serpell 2000). Several reasons for cat domestication have been discussed in the literature based on its hunting properties: (i) protection of grain storages by killing small rodents (indirect benefit from hunting); (ii) use as a companion animal for bird hunting (direct benefit from hunting); (iii) use for religious reasons or as status symbol due to its resemblance of a lion, the king of the carnivorous hunters (Serpell 2000; Jores 2004).

Interestingly and in a pronounced contrast to dogs, the domestication process of cats did not induce major changes either in the physical shape or in the behaviour of domestic cats when compared to wildcats.

From its sacred origins in Ancient Egypt, the domestic cat spread—supported by its remarkable "sea sickness resistance"- often by ships across the Mediterranean—first throughout Europe and finally world-wide (Engels 1999, Jores 2004). While during the first millennia of the human-cat relationship the animal enjoyed valuable respect nearly in all human cultures, the esteem of cats dramatically changed in the Middle Ages at least in Christian Europe and Japan when demonic features were attributed to them; the respective iconic pictures commonly propagated in fairy tails are those of a cat sitting on the hunchback of a witch or even being a companion of the devil. Probably the previously as benevolent cat attributes described features such as (female) fertility and sexuality were now disregarded as demonic for cultural or religious reasons and consequently the cat was transformed into a symbol of these now as vicious connotated features. These associations were also based on facets of the natural behaviour of cats, e.g. their obvious independence, affectionate nature, search for physical caresses, sexual behaviour and promiscuity which all were now interpreted in the light of a sexualized, often misogynic perspective (Serpell 2000). After this emotional set back in the history of the human-cat relationship things clearly improved in the last few centuries making the cat now in many countries "man's really best friend" with the numbers of household cats outnumbering those of domestic dogs.

23.1.1 Cat Demography

The worldwide cat population is estimated to exceed 500 million, of which approximately 220 million are domestic cats according to the International Federation for Animal Health Europe (IFAH 2012). The largest pet cat population lives in the United States with about 74–95.6 million cats present in 30–39 % of US households (data from 2007 to 2012: The Humane Society of the United States 2014; Batson 2008), followed by Russia (17.9 million), Indonesia (15 million), Brazil (14.8 million), Panama (12 million) and China (10.7 million) (Batson 2008). In

Europe (excluding Russia) about 70 million cats are living in about 24 % of European households; France (11.4 million), UK (8.5 million), Germany (8.2 million) and Italy (7.5 million) ranked as the four countries with the highest pet cat populations in Europe in 2012 (FEDIAF 2012; PFMA 2013). Interestingly, the highest rates of households with cat ownership in Europe are found in the East, e.g. in Romania (45 % of households), Latvia (40 %) and Hungary (36 %). The Australian Companion Animal Council reported 2.4 million cats in 23 % of Australian households (ACAC 2010). Cat ownership is relatively low in African countries besides South Africa and Tanzania (each 2 million) and Spanish-speaking Southern America besides Argentina (3.7 million) and Chile (1.7 million).

In a random sample of 2,980 UK households in 2007 pet cat ownership was significantly correlated with female sex of the owner (OR 1.63), having a garden (OR 3.66), living in a semi-urban or rural environment (OR 1.30) and having a higher education degree (OR 1.36) (Murray et al. 2010). In the US more than 75 % of the persons taking care of a cat within a household are women; this rate is highest among 19–29 year-old women (AVMA 2012). Indirect evidence for a higher proportion of women caring for cats might be drawn from a US multi-centre study on 110 patients with pet-afflicted bite wounds showing that 72 % of people bitten by cats were women, while only 38 % of dog bites documented in the study population affected women (Talan et al. 1999).

23.1.2 Public Health Impact of Cats

Cats may kill humans directly by attacking, violating or—seldomly and then reaching literally fame (Anderson 1955)—eating them. About 14,009 deadly attacks by large carnivorous cats on humans have been recorded world-wide in the twentieth century, the vast majority caused by tigers—probably a gross underestimation (Löe and Röskaft 2004). Also pet cats may attack humans; about 400,000 cat bites occur every year in the US, the incidence of cat-bite related injuries is estimated to be 18/100,000 (ca. 108,000 bites per year) (WHO 2013a).

While these literally crude data show quite obviously a direct impact of cats on human health, the zoonotic health risks of cats to humans are much less visible and indirect.

Starting from a comprehensive risk assessment review on companion animal-associated zoonoses by the Robert Koch-Institut, Germany (Weber and Schwarzkopf 2003), and based on an intensive literature review as well as expert interviews of 14 scientists from the fields of veterinary medicine, human medicine and microbiology a list of eight, three and six pathogens with a public human health risk scored as “high”, “low” and “unsure”, respectively, for the German situation was compiled at the Veterinary University Hannover (Möbius 2013). *Bartonella henselae*, *Capnocytophaga* spp., *Pasteurella multocida*, Orthopoxvirus, *Cryptosporidium parvum*, *Toxoplasma gondii*, *Microsporum canis* and *Trichophyton* spp. were ranked

among the "high risk" zoonotic pathogens, *Coxiella burnetii*, *Dipylidium caninum* and *Echinococcus* spp.) among the "low risk" group and *Campylobacter jejuni*, *Escherichia coli*, *Leptospira* spp., *Salmonella* spp., *Toxocara* spp. and *Giardia* spp. among the ambiguous zoonotic agents. Pathogens for which only a theoretical transmission risk exists (e.g. Lyssa viruses in a rabies-free country) or for which only anecdotal case reports could be found in the literature were excluded. A similar, but not risk-oriented list on zoonotic infectious diseases of companion animals including cats was recently published by Day et al. as an electronic document for the *Emerging Infectious Diseases* journal of the CDC, Atlanta, USA (Day et al. 2012). Table 23.1 comprises the most important cat-associated zoonotic pathogens.

In the following we will mainly concentrate on pathogens with significant public health importance or a quite unique association with cats. Mainly bite-transmitted pathogens (e.g. *Capnocytophaga* spp. and *Pasteurella* spp.) and dermatophytes (e.g. *Microsporum* spp. and *Trichophyton* spp.) will not be dealt due to their quite special transmission pathways. Antibiotic resistant bacteria with the potential of zoonotic transmission, especially Methicillin-resistant *Staphylococcus aureus* will also not be addressed in this chapter.

23.2 The Usual Suspects

23.2.1 Toxoplasma gondii

23.2.1.1 The Pathogen—Life Cycle and Transmission

The only known definitive hosts for *T. gondii* are members of the family *Felidae* in which this obligate intracellular coccidian protozoan of the Phylum *Apicomplexa* lives in the intestines to complete its sexual life cycle by producing unsporulated oocysts. These oocyts are shed in the cat's feces and need to sporulate for 1–5 days in the environment to become infective. Intermediate hosts, e.g. birds and rodents, but principally any warm-blooded species including humans, get infected by ingesting soil, water or plant material contaminated with oocysts. Shortly after ingestion, oocysts transform within the intermediate host into tachyzoites which travel mainly within macrophages primarily into neural or muscular tissue (but principally in any tissue) where they develop into bradyzoites. Several thousands of bradyzoites form tissue cysts which are ingested by cats when feeding on their prey, e.g. the "regular" intermediate hosts. Moreover, cats may also become infected directly by ingestion of sporulated oocysts. Humans may become infected (i) by eating undercooked meat of animals harboring tissue cysts, (ii) by consuming food or water contaminated with oocyst harbouring cat feces, (iii) by ingesting oocysts via otherwise contaminated environmental samples—either directly by a cat (e.g. via fecally contaminated soil or pet cat litter boxes) or indirectly by another animal (e.g. by insect vectors such as cockroaches or flies) (Torrey and Yolken 2013) or by dogs carrying oocysts in their fur after having rolled in cat feces due to their behavorial trait of

Table 23.1 Cat-transmitted zoonotic diseases. (modified from Möbius 2013 and Day et al. 2012)

Pathogen	Disease in cats	Disease in humans	Transmission
Anaplamsa phagocytophilum	Fever, polyarthritis	Fever, polysystemic disease	Tick bite
Ancylostoma spp.	Mostly asymptomatic	Cutaneous larva migrans	Soil
Bartonella henselae	Mostly asymptomatic, subclinical fever, endo-/myocarditis	Cat scratch disease, bacillary angiomatosis, fever, endocarditis, lymphadenopathy	Bite, scratch, minor trauma
Bordetella bronchiseptica	Bronchopneumonia	Bronchopneumonia, sinusitis, meningitis, septicemia (mainly in immunosuppressed people)	Aerogenous
Capnocytophaga spp.	Asymptomatic oral carriage	Bite wound, bacteremia, endocarditis, sepsis	Cat bite
Campylobacter spp.	Diarrhea	Diarrhea, Guillan-Barre syndrome	Fecal-oral
Chlamydophila felis	Pharynigitis, conjunctivitis, rhinitis	Pneumonia, conjunctivitis	Aerogenous, cat tear fluid
Corynebacterium ulcerans, toxigenic	Asymptomatic or bilateral rhinitis	Diphtheria	Aerogenous or direct (skin) contact
Coxiella burnetii	Subclinical, Q fever, abortion, stillbirth	Q fever	Aerogenous, direct contact
Cryptosporidium spp.	Diarrhea	Diarrhea	Fecal-oral, water-borne
Dipylidium canis	Mostly asymptomatic	Rarely diarrhea, pruritus ani	Oral ingestion of flies
Echinicoccous spp.	Mostly asymptomatic	Cystic or alveolar echinococcosis	Fecal-oral
Escherichia coli, e.g. EHEC	Asymptomatic or diarrhea	Diarrhea, haemolytic uremic syndrome	Fecal-oral
Francisella tularensis	Septicemia, pneumonia	Tuleremia (different organ systems)	Bite, contact to body fluids
Giardia spp.	Asymptomatic or diarrhea	Asymptomatic or diarrhea	Fecal-oral, water-borne
Leishmania spp.	Cutaneous or visceral leishmaniasis	Visceral leishmaniasis (polysystemic disease)	Sand fly bite
Leptospira spp.	Often asymptomatic	M. Weil (leptopsirosis with renal or hepatic involvement)	Water-borne
Lyssa virus	Rabies	Rabies	Cat bite
Microsporum canis	Skin disease	Skin disease	Direct skin contact
Mycobacterium tuberculosis, M. bovis	Tuberculosis	Tuberculosis	Aerogenous, very rare (only case reports)

Table 23.1 (continued)

Pathogen	Disease in cats	Disease in humans	Transmission
Orthopox viruses	Cat pox	Cat pox	Direct skin contact
Pasteurella spp.	Asymptomatic or upper respiratory tract infection	Wound infection, sepsis	Cat bite
Rickettsia felis	Asymptomatic	Flea-borne spotted fever	Cat flea bite, cat flea feces
Salmonella spp.	Asymptomatic or diarrhea	Diarrhea	Fecal-oral
Sporothrix schenckii	Cutaneous or disseminated mycosis	Cutaneous or disseminated mycosis	Contact to cat fluids via skin wounds
Staphylococcus aureus incl. MRSA	Asymptomatic or skin infection	Skin infection	Direct contact
Streptococcus spp.	Asymptomatic or skin infection	Skin infection	Direct contact
Strongyloides stercoralis	Bloody diarrhea, anemia	Polysystemic	Soil
Toxocara spp.	Toxocariasis	Toxocariasis	Fecal-oral
Toxoplasma gondii	Asymptomatic	Toxoplasmosis	Fecal-oral
Trichophyton spp.	Skin mycosis	Skin mycosis	Direct contact
Yersinia enterocolitica	Subclinical or yersiniosis, e.g. diarrhea, abdominal pain, arthritis, septicemia	Yersiniosis, e.g. diarrhea, abdominal pain, arthritis, septicemia	Fecal-oral

so-called xenosmophilia (Frenkel and Parker 1996)-, (iv) via blood transfusion or organ transplantation, or (v) transplacentally (Hill and Dubey 2002).

23.2.1.2 Epidemiology in Cats and Humans

Globally, seroprevalence in cats ranges from around 5% to more than 90% and is higher in feral and stray cats than in domestic pets (Dabritz and Conrad 2010; Dubey and Jones 2008; Nutter et al. 2004). Seroprevalence rises with cat age (Vollaire et al. 2005); e.g. in a Belgian study on 567 health domestic cats seroprevalence increased from 2% in kittens below 12 months of age to 44% in 7-year old cats (De Craeye et al. 2008). Probably less than 1% of mainly young cats—often being infected for the first time during their first outdoor hunting experiences—are shedding oocysts once in their life-time for an average period of one week and up to three weeks, although reported rates of shedding domestic cats range from 0 to 34% (Dabritz and Conrad 2010; Barutzki and Schaper 2011; Epe et al. 2004). A single cat may excrete more than 50 million oocysts per day (Dabritz and Conrad 2010: data are based on 73 experimentally or naturally infected cats from 5 different studies) with 3–810 million oocyts per cat infection. With an estimated 40 g of daily feces production per cat, the amount of annual fecal production by feral cats was calculated to reach up to 2.4×10^6 t in the US matching up to 2.4×10^{15} oocyts deposited in the US environment (Dabritz and Conrad 2010; Torrey and Yolken 2013). It should be noted, however, that laboratory methods for oocyst detection especially in environmental samples are not very sensitive; direct measurements and source detection in outbreak situations are therefore often not possible.

Seroprevalence in humans shows a similar range as in cats, i.e. from about 3% to around 90% in different human populations and geographical regions (Dabritz and Conrad 2010). In most European, Central/South American and North American countries, about one fourth to two thirds of the—from a public health point of view—most relevant human population, i.e. pregnant women or women of childbearing age, show anti-*Toxoplasma* IgG antibodies (Dabritz and Conrad 2010; Nogareda et al. 2013; Galvan-Ramirez et al. 2012). In some countries including France the prevalence has been fallen in the last two decades, probably due to hygienic and awareness building measures (Petersen et al. 2010; Nogareda et al. 2013).

The global annual incidence of the most severe form of toxoplasmosis, i.e. congenital toxoplasmosis, was estimated in a recent WHO review to be 190,100 cases matching an incidence of 1.5 cases of congenital toxoplasmosis per 1,000 live births (Torgerson and Mastroiacovo 2013).

23.2.1.3 Disease in Cats

Infection in cats is usually asymptomatic or subclinical with only minor symptoms such as short-term diarrhea, lymphadenopathy or fever. Overt disease is more likely in cats with immunosuppression, including young kittens and cats with e.g. feline

leukemia virus (FELV) or feline immunodeficiency virus (FIV) infection. During the parasite's extraintestinal tachyzoite phase and depending on the affected organ system, pneumonia with cough and breathing problems, longer episodes of diarrhea, uveitis, iritis, chorioretinitis, myocarditis or encephalitis may develop. Very rarely, sudden death may occur especially in very young kittens.

23.2.1.4 Disease in Humans

Most infections in humans are asymptomatic. If symptomatic, mainly immunosuppressed people are concerned. Lymphadenopathy, sometimes associated with fever, fatigue, muscle pain, sore throat and headache, is the most frequently observed acute clinical form of postnatally acquired toxoplasmosis in humans. Most important clinical findings in immunosuppressed patients include encephalitis, chorioretinitis or pneumonitis due to reactivation of bradyzoites which are otherwise immunologically well controlled in tissue cysts and might develop into tachyzoites during phases of immunosuppression. Recently, evidence is rising that latent *Toxoplasma* infection might be linked to behavourial or mental changes and psychiatric disease, e.g. an association with self-directed violence (Pedersen et al. 2012), suicidal behaviour (Groër et al. 2011) or automotive accidents (Flegr et al. 2002); however, if and which *Toxplasma*-specific underlying causes might be involved is not yet understood (Flegr 2013).

Congenital toxoplasmosis is caused by transplacental infection when a non-immune mother infected for the first time during pregnancy transmits tachyzoites to her fetus. The clinical spectrum of congenital toxoplasmosis ranges from asymptomatic infection to severe syndromes including hydrocephalus, microcephaly or intracranial calcifications—leading to mental and/or psychomotoric retardation—and chorioretinitis causing vision impairment or even blindness. Stillbirth or death in the neonatal period may also occur.

23.2.1.5 Public Health Importance

A recent WHO review estimates the global burden of congenital toxoplasmosis to be as high as 1.20 million DALYs (Torgerson and Mastroiacovo 2013). The highest burdens were seen in South America and in some Middle Eastern—especially in low-income—countries. For their calculations, the WHO review authors took into account different disease patterns, manifestations and severities possibily due to different pathogenic strains and their respective geographical distribution.

In the 1990s, about 750 deaths per year were attributed to toxoplasmosis in the US (Mead et al. 1999), while more recent estimates report 327 deaths per year making *Toxoplasma* the food-borne pathogen with the second most annual deaths after *Salmonella* spp. (Batz et al. 2011). These estimates of lethal outcomes might mainly refer to infections acquired by immunosuppressed people via ingestion of tissue cysts. However, due the larger amount of people affected oocyst-transmitted

infections may be both clinically and from a public health point of view more severe than tissue cyst-induced infections as mainly concluded from outbreak situations (Beneson et al. 1982; Burnett et al.1998; Teutsch et al. 1979; Bowie et al. 1997).

On a global scale, the relative contribution of human infection via oocysts from food or drinking water contaminated with cat feces on the one hand and from tissue cysts by eating undercooked meat is very difficult to establish and probably differs for different geographical locations and human populations due to different environmental conditions, socio-cultural customs and also the pathogenicity of the prevalent *Toxoplasma* strain. Interestingly, case-control studies failed to explain up to 40 % of human infections due to any known risk factor (Petersen et al. 2010). In one European multicentre study from France, Italy, Belgium, Denmark and Sweden between one to two thirds of infections could be attributed to consumption of undercooked meat products and 6–17 % to soil contact (Cook et al. 2000). However, considerable seroprevalence data in vegetarian human populations from India (Rawal 1959: 21 %), among Seventh Day Adventists in the US (Roghmann et al. 1999: 18 %) and in Amerindian aborigines from Venezuela (Chacin-Bonilla et al. 2001: 43.5–62.4 %) as well as symptomatic outbreaks of human toxoplasmosis mainly linked to probably contaminated drinking water sources (Balasundaram et al. 2010; Petersen et al. 2010; Dabritz and Conrad 2010), e.g. the most prominent and best analysed 1995 Vancouver outbreak presumably caused by cougars shedding oocyts (Aramini et al. 1999; Aramini et al. 1998; Bowie et al. 1997), or to contaminated vegetables (Ekman et al. 2012) suggest a significant proportion of oocyst-transmitted toxoplasmosis at least in some circumstances (Peterson et al. 2009). Moreover, when finding with a novel sporozoite-specific serological test that 78 % of 76 mothers of congenitally infected infants in a US-wide survey had a primary infection with oocysts Boyer et al. speculated that a major number of congenital toxoplasmosis cases and at least four North American epidemics are due to infection via oocysts (Boyer et al. 2011).

Data from 11 epidemiological studies performed from 1990 to 2006 in different European, African and American countries analysing soil contact associated with seropositivity found odds ratios (OR) from 1.4 to 10.3 suggesting a possible risk of oocyst-transmitted infections via cat feces (Dabritz and Conrad 2010). However, direct daily contact with cats or cat-ownership was not associated with an increased risk of *T. gondii* infection in one US and two European case-control studies (Cook et al. 2000; Kapperud et al. 1996; Jones et al. 2009). In contrast, the OR for owing a cat was 1.25 in a study from the Czech Republic (Kolbekova et al. 2007). Interestingly, owing three or more kittens was associated with an adjusted OR (aOR) of nearly 28 in the very same US study which showed a “protective” effect of owing one or two cats (aOR 0.6) (Jones et al. 2009).

In conclusion, there is considerable indirect evidence for a direct role of oocyst-shedding cats in *Toxoplasma* transmission to humans. Unfortunately, although oocyts have been detected both in environmental and in water samples by different methods (Dabritz and Conrad 2010), often the implicated sources suggested by epidemiological considerations cannot be corroborated or certified by direct detection of oocysts due to the lack of laboratory facilities or methodologically based

sensitivity problems. Therefore, the public health impact of directly cat-associated, i.e. oocyst-transmitted, toxoplasmosis cannot be clearly calculated.

23.2.1.6 Public Health Measures

When assessing the risk of directly cat-associated transmission, it should be noted that probably one oocyst might be enough to cause infection as data from experimentally infected swine suggest (Dubey et al. 1996). Considering the mean of 50 million oocysts daily excreted by a single freshly infected cat methods preventing oocyst contact, ingestion or inhalation are obviously reasonable. Care should especially be taken when dealing with sandboxes where cats which do usually not defecate randomly, but rather select places for defecation, often deposit and subsequently cover their feces resulting in the accumulation of more than 1 million oocyts per square foot in certain areas of sandboxes (Torrey and Yolken 2013).

Interestingly, in a 19 months-lasting German study on more than 18,000 feline fecal samples the proportion of *T. gondii*-positive samples collected between January and June was significantly lower than between July and December (Herrmann et al. 2010).

Cat-related public health measures for reducing the risk of toxoplasmosis might therefore include (i) hygienic measures such as wearing gloves when and washing hands after gardening, working with soil (especially in places where domestic and above all feral or stray cats might defecate) or having contact with cat feces; (ii) thoroughly washing of vegetables and fruits; (iii) restricting pet cats', especially kittens' access to wild rodents (although estimated bradyzoite rates for rodents are quite low, e.g. 0.1–0.4 % in Germany); (iv) avoiding disposal of cat feces into drinking water sources (e.g. the respective Californian legislation (Dabritz and Conrad 2010)); (v) adopting a stray cat policy reducing roaming of unowned and not sufficiently supervised cats; (vi) preventing contact of cats and their feces to highly susceptible intermediate host animals such as swine, lambs or chicken (Dubey 2010); (vii) development of a feasible and robust vaccine for cats (Dabritz and Conrad 2010; Petersen et al. 2010).

23.2.2 Bartonellosis

23.2.2.1 The Pathogen

The genus *Bartonella* comprises a group of Gram-negative facultative intracellular bacteria with a unique life cycle involving one or few closely related mammals as reservoir hosts and different bloodsucking arthropods as vectors. Currently, 29 *Bartonella* species and three subspecies are listed (www.bacterio.net) which have been identified in a wide range of domestic and wild animals, including at least 12 causing disease in humans and 10 zoonotic species from cats and/or dogs (Cicuttin

et al. 2014; Chomel et al. 2012; Harms and Dehio 2012; Zangwill 2013). Following transmission by an arthropod vector, bartonellae colonize a not yet definitely identified primary niche which probably involves migratory cells as well as additional cell types and are transported to the vascular endothelium where they persist intracellularly (see Harms and Dehio 2012 for an intense review of the molecular pathogenesis of *Bartonella* spp.). From the primary niche the bartonellae invade erythrocytes where they finally persist to be again taken up by bloodsucking arthropods for transmission to another host. Both vector (including its ecology) and host (including the primary niche as first step in the intrahost replication cycle) factors are responsible for the reservoir host specificity of the respective *Bartonella* species (Harms and Dehio 2012). Their life style both in the primary and the intraerythrocytic niches allow the bartonellae to escape the immune system, to replicate within their host, to relapse from time to time and to evade antibiotic treatment.

The majority of human infections is caused by three *Bartonella* species of which one is zoonotic and has cats as reservoir hosts, i.e. *Bartonella henselae*. The natural and most important arthropod vector both for direct intra- (i.e. cat-to-cat) and probably also for indirect interspecies (i.e. cat-to-human) transmission is the cat flea *Ctenocephalides felis* (Breitschwerdt and Kordick 2000; Mosbacher et al. 2011; Harms and Dehio 2012). Transmission to humans is thought to occur mainly via *B. henselae*-contaminated cat flea feces after inoculation by a cat scratch or a cat bite; interestingly, *B. henselae* DNA could be isolated from gingiva and claw beds of domestic cats in the US (Lappin and Hawley 2009).

Besides in cat fleas, *B. henselae* has also been identified in hard ticks from Europe and North America (i.e. *Ixodes ricinus* and *Ixodes pacificus*) suggesting a possible role of *Ixodes* spp. as vectors as it is proven for another *Bartonella* species (Mosbacher et al. 2011; Reis et al. 2011).

While cats are the natural reservoir host for *B. henselae*, infection has also been shown serologically or by DNA detection in a variety of accidental hosts including dogs, coyotes, cattle, horses and deers (Mosbacher et al. 2011).

23.2.2.2 Epidemiology in Cats and Humans

Bartonellosis has a worldwide distribution. In cats, seropravalence usually ranging from 40 to 70 % is much higher in warm, humid regions in which high flea infestation is expected (Breitschwerdt and Kordick 2000; Mosbacher et al. 2011). Seroprevalence for pet cats was determined to be for instance 21 and 24 % in South Africa and Zimbabwe, respectively (Kelly et al. 1996), 23 % in Austria (Skerget et al. 2003) and Turkey (Guzel et al. 2011), 26.7 % in Taiwan (Chang et al. 2006), 29.6 % in Catalonia, Spain (Pons et al. 2005), 41.1 % in France (Gurfield et al. 2001), 35.4 % in Sicily (Mansueto et al. 2012), 32 % in Jordan (Al-Majali 2004), 3.7–75 % in different parts of the US (Jameson et al. 1995; Case et al. 2006; Nutter et al. 2004) and 71.4 % in northeastern Spain (Solano-Gallego et al. 2006). An absolute exception is the seroprevalence of 0 % in 100 domestic and feral cats from Norway (Bergh et al. 2002). Hunting for prey, having access to outdoor environments, living

previously as a stray cat or living with other pet cats in the same household were found to be associated with a higher risk of seropositivity (Al-Majali 2004; Gurfield et al. 2001). When compared with pet cats from the same geographical region, feral cats show usually significantly higher seroprevalence rates, e.g. in Sicily (35.4% vs. 68.3%; Mansueto et al. 2012), in North Carolina, USA (75% vs. 93%; Nutter et al. 2004), in Taiwan (26.7% vs. 40%; Chang et al. 2006) or in Turkey (22.9% vs. 31.7%; Guzel et al. 2011). In contrast, seroprevalence in shelter cats seems to be bimodal, i.e. either most cats or only very few show a positive serology (Breitschwerdt and Kordick 2000) with flea infestation as the most important risk factor. Prevalence of bacteremia in both domestic (DC) and stray cats (SC) is usually lower than the respective seroprevalence with 0% in DC in Madrid, Spain (Gil et al. 2013), 4% in Catalonia and northeastern Spain (Pons et al. 2005; Solano-Gallego et al. 2006), 3% in Brazil (DC; Braga et al. 2012), 5.8% (DC) and 18.6% (SC), respectively, in China (Yuan et al. 2011), 16% (DC) and 40% (SC), respectively, in Australia (Branley et al. 1996), 14.9% in Argentina (Cicuttin et al. 2014), 15.7% (DC) and 35.5% (SC) in La Rioja, Spain (Gil et al. 2013), 16.5% (DC; Gurfield et al. 2001) and 37.2% (SC; Heller et al. 1997) in different parts of France, 16.7% (DC) and 31.3% (SC) in Taiwan (Chang et al. 2006), 17% in Algeria (SC; Azzag et al. 2012) and New Zealand (DC; Joseph et al. 1997), 25% (DC) and 26% (SC) in the Netherlands (Bergmans et al. 1997) or 39.5% in California, USA (Chomel et al. 1995). Risk factors for *B. henselae* bacteremia are grossly those associated with seroprevalence.

In free-ranging or captured big cats such as lions, panthers and cougars *B. henselae* infection could also be documented serologically or directly by PCR and/or bacterial culture. Seroprevalence rates ranged from 0% in 44 Amur tigers (Goodrich et al. 2012), 18% for panthers in Florida, USA and 28% for cougars from Texas, USA (Rotstein et al. 2000), 17% for lions and 31% for cheetahs in Africa collected between 1982 and 2002 (Molia et al. 2004), 29% in African lions (Pretorius et al. 2004), 37% for Californian mountain lions (Girard et al. 2012), 30% in captive wild felids, 35% for mountain lions, to 53% in bobcats from California, USA, respectively (Yamamoto et al. 1998). Bacteremia with *B. henselae* detected either by culture and/or PCR was found in 1.5% in 65 African lions (Pretorius et al. 2004), in 3.4% of 58 lions (Molia et al. 2004), in 6% of in Iriomote wild cats (*Prionailurus iriomotensis*) from Japan (Tateno et al. 2013), in 15% of neotropical felids mostly of the genus *Leopardus* from a Brazilian shelter (Guimaraes et al. 2010) and in 35% in feral cats (*Felis catus*) from St. Simons Island, Georgia, US (Hwang and Gottdenker 2013).

In humans, the annual number of infections has been estimated to range between 22,000 and 24,000 in the US, with about 2,000 cases requiring hospitalization (Jackson et al. 1993). Thousands of cases may occur yearly in Europe (Chomel et al. 2006). In the US, there seems to be a seasonal distribution of human cat-scratch disease (CSD) incidence, with the majority of cases occurring between July and January (Carithers 1985; Reynolds et al. 2005; Jackson et al. 1993). This pattern might be due to the seasonal breeding patterns of domestic cats, the acquisition of kittens as family pets, and the peak temporal presence of the cat flea among cats (Anderson and Neuman 1997).

Screening of healthy human blood donors in different industrialised countries, e.g. Sweden, France, Austria and the US, found a seroprevalence with anti-*B. henselae* antibodies of 2–6% (Mosbacher et al. 2011; Breitschwerdt and Kordick 2000). However, studies in certain human populations with a presumably higher risk of attracting infection, e.g. veterinarians, cat owners or farm workers, detected higher seroprevalence rates. Seropositivity was 9.6% in Chinese farm workers (Zhang et al. 2008), 15% in Japanese veterinarians (Kumasaka et al. 2001), 45% and 53.3% in Polish veterinarians and cat-owners, respectively (Chmielewski et al. 2007), and 51.1% in veterinarians from Austria (Nowotny et al. 1997). In contrast, a Taiwanese study among veterinary clinic staff found only a seropositivity of 1.7% (Chang et al. 2006). Also, a seroprevalence rate of 7.1% which is in the range of that found in the normal population was detected in attendees of a veterinary conference in Ohio, USA (Noah et al. 1997). Moreover, no association of anti-*B. henselae* antibody positivity with cat ownership was seen in two studies from Austria (Skerget et al. 2003) and Germany (Rath et al. 1997), respectively. Taken together, these data suggest that frequent or even close contact with cats per se does not necessarily lead to *B. henselae* infection and other factors, e.g. flea infestation of cats, have also to be considered.

Only very few studies analysing anti-*B. henselae* antibody seroprevalence in HIV-positive CSD- or BA-asymptomatic patients have been published; most of them do not differentiate between *B. henselae* and other *Bartonella* spp., have only very small study population sizes and/or lack control groups. Despite these biases and shortcomings, most of the study authors, however, postulate a higher seroprevalence in HIV-patients than in the HIV-negative population (Blanco et al. 1999; Frean et al. 2002; Pons et al. 2008; Trataris et al. 2012), while another study found no significant differences between both groups (Tsukahara et al. 1999).

23.2.2.3 Disease in Cats

Naturally infected cats are usually asymptomatic, although *B. henselae* bacteremia may last for months to years. In experimentally infected cats, in cats infected with non-reservoir-adapted *Bartonella* species (i.e. non-*B.henselae* species) or in immunosuppressed cats, e.g. with a co-infection with Feline Immonodeficiency Virus (FIV), symptoms such as lymphadenopathy, fever, mild transient anemia or cardiac and renal lesions might be present (Breitschwerdt and Kordick 2000).

23.2.2.4 Disease in Humans

Cat scratch disease (CSD) in immunocompetent people and bacillary angiomatosis (BA) in mainly immunosuppressed patients are the prominent clinical syndromes caused by *B. henselae* (Zangwill 2013). Typically, CSD is a benign and self-limiting disease in humans, presenting with lymphadenopathy (>90% of patients) mainly of an upper extremity or the neck and often showing suppuration (15–30%), low-grade

fever (26–60 %), a primary cutaneous lesion at the inoculation site (25–90 %), malaise and weight loss (10–45 %) (Zangwill 2013). Rare symptoms include erythema nodosum, figurate erythemas, thrombocytopenic purpura, Perinaud's oculoglandular syndrome, encephalopathy, hepatic granulomas, osteomyelitis, pulmonary disease, optic neuritis and endocarditis. In most cases, the clinical findings resolve spontaneously after 6–12 weeks, while lymphopathy may persist for weeks to months. BA is an uncommon, but severe and potentially fatal disease especially seen in AIDS patients and caused by the pathogen's direct and indirect proangiogenic features (Harms and Dehio 2012). Hallmarks of cutaneous manifestations are often multiple (up to hundreds) erythematous, highly vasculated exophytic lesions or subcutaneous nodules. Basically any organ might be involved, but besides the skin mainly bones, liver (i.e. peliosis hepatitis) and spleen are affected. Due to their intracellular life style as "intruders below the radar" bartonellae in their niches are difficult to reach by antibiotics (Harms and Dehio 2012). So far, no randomized clinical trials showing an effective antibiotic treatment for CSD exist (Prutsky et al. 2013), although guidelines based on expert consensus have been published (Rolain et al. 2004). BA is usually treated with macrolides or tetracyclines (Zangwill 2013; Rolain et al. 2004).

23.2.2.5 Public Health Importance

Data obtained by multi locus sequence typing (MLST) of feline and human *B. henselae* isolates indicate that certain sequence types (ST), e.g. ST1, ST2, ST5 and ST8 might be associated with zoonotic transmission and human disease, while others, e.g. ST6 and ST7, are more restricted or even exclusively found in cats (Arvand et al. 2007; Iredell et al. 2003). However, due to different geographic distributions, e.g. that in Asia which differs significantly from that in other parts of the world, a bias regarding an overemphasized association of certain STs with human disease cannot be excluded (Arvand et al. 2007; Bouchouicha et al. 2009; Li et al. 2006; Gil et al. 2013). An overrepresentation of ST1 among human isolates was reported by Arvand analysing 182 strains from Europe, North America and Australia, by Iredell on 37 strains from France, Germany, the US and Australia and—together with ST8– by Gil et al. from northeastern Spain (Arvand et al. 2007; Iredell et al. 2003; Gil et al. 2013). Moreover, among 26 *B. henselae* strains isolated from stray cats in China comprising 18 different STs nearly two thirds belonged to ST1 which is associated with human disease (Yuan et al. 2011). ST1 was also predominant in 9 *B.henselae* strains obtained from cats in Buenos Aires, Argentina, followed by ST8, ST5 and ST6 (Cicuttin et al. 2014). In contrast, ST1 does probably not present the greatest public health risk in the UK: in an England-based MLST study on 118 *B. henselae* strains isolated from humans and cats the vast majority (85 %) of zoonosis-associated strains belonged to ST2, ST5 and ST8, respectively, while 74 % of the feline isolates belonged to ST4, ST6 and ST7 also indicating that a few, uncommon STs were responsible for the majority of symptomatic human infections in the UK (Chaloner et al. 2011). Interestingly, ST1 and ST5 were found to be significantly

more common in countries outside Europe than in England, ST5 and ST7 more common in continental Europe than in England and ST4 and ST6 more common in England than in the rest of the world (Chaloner et al 2011). ST1 was also found to be only rare or even absent in North West European countries, but dominating in the Mediterranean region (Arvand et al. 2007). In a Spanish study analysing both 35 feline strains and the to date largest number of human isolates ($n=46$) ST5 was by far the most frequent ST among both feline and human isolates comprising more than half of all STs in the respective group (humans: 54.3 %; cats: 61.5 %) (Gil et al. 2013). Moreover, ST5– besides ST7 -was also the most often identified ST in 42 German pet cat isolates (Mietze et al. 2011). Both in England (Chaloner et al. 2011) and Spain (Gil et al. 2013), ST5 and ST8 were among the three STs most frequently associated with symptomatic human infection.

However, in contrast to most studies using MLST or MLVA as molecular epidemiological tools, two complimentary French studies (Li et al. 2006; Li et al. 2007) and one Japanese study (Yanagihara et al. 2010) performing the more discriminatory multispacer typing (MST) did not find statistically significant differences in genotypic diversity between human and feline isolates. The reason for this discrepancy is not yet clear, but may be explained by the predominance of different zoonosis-associated strains in different geographic regions.

Not only an association between certain *B. henselae* strains and zoonotic transmission, but also with clinical presentation in humans, either with CSD or endocarditis, has been shown in different studies suggesting the existence of strains with a higher pathogenic potential for humans (Bergmans et al. 1996; Gil et al. 2013)

23.2.2.6 Public Health Measures

Except for recommendations to avoid cat contact, cat owners should be advised to screen their cats for fleas and to remove these vectors thus prohibiting exposure in- and outside of human habitats. Immunosuppressed people should be advised not to adopt stray or flea-infested cats (Stützer and Hartmann 2012). Antibiotic treatment of individual cats has not been proven to be effective in eliminating the carrier status (Mosbacher et al. 2011) and is useless in cats older than 2 years of age due to the self-limiting character of the disease (Breitschwerdt 2008; Stützer and Hartmann 2012). However, it has been proposed to consider doxycyline treatment of both symptomatic and asymptomatic cats younger than 2 years of age when living in the same household with an immunosuppressed human to reduce the bacterial load (Stützer and Hartmann 2012). On a public health scale implementing ectoparasite eradication and prevention programs including the use of acaracides might be an option to decrease *B. henselae* transmission both within the cat population and from cats to humans (Mosbacher et al. 2011; Breitschwerdt 2008).

23.2.3 Toxocara cati

23.2.3.1 The Pathogen—Life Cycle and Transmission

Toxocara cati, the cat roundworm, is the most common endoparasite in cats. As its close relative, *Toxocara canis*, it belongs to the ascarid nematodes in the order *Ascaridida,* superfamily *Ascaridiodea,* family *Toxocaridae*. Its definitive host are cats, in which they live as adults within the lumen of the small intestine. Cats might get infected by ingesting viable, embryonated eggs from contaminated sources (e.g. soil or paratenic hosts such as mice, other rodents, earthworms, ants or soil-dwelling invertebrates). After ingestion within 2–4 h, the eggs hatch in the duodenum to release juvenile larvae which penetrate the small intestine, enter the circulation, migrate via the bloodstream or the lymph vessels throughout the body and may invade any organ, especially the liver (after 24 h), the heart or the lungs (after another 12–24 h). Similarly to the human roundworm *Ascaris lumbricoides*, the larvae migrate especially in young kittens through the lungs, penetrate the trachea, enter the esophagus, get swallowed and finally reach the lumen of the small intestine where they mature and mate. A single female worm produces about 200,000 non-embryonated eggs/day. After a prepatent period of around 8 weeks the eggs are excreted *per vias naturales* together with the cat feces into the environment. Embryonation occurs in the soil within 2–6 weeks after deposition depending on the temperature and environmental conditions. In adult animals with some degree of acquired immunity the larvae may also remain as dormant larvae in any tissue without reaching the intestine. Besides infection by ingestion of eggs, transmammary or lactogenic transmission is also possible and probably the major route of infection in kittens; larvae migrated to the mammary glands of lactating queens may infect nursing kittens throughout the entire lactation period. Vertical transmission and infection by the ingestion of encapsulated larvae harbouring paratenic hosts (preferably mice, but also earthworms, cockroaches or other invertebrates) may also happen. In contrast to *T. canis* in dogs, where transplacental transmission is of major importance for the completion of the parasite's life cycle, this route of transmission has not been described for *T. cati* in cats (Macpherson 2013; Strube and Heuer 2013; Rubinsky-Elefant et al. 2010).

Humans may become infected by accidental ingestion of embryonated eggs via cat-feces contaminated soil (therefore its classification as a primarily telluric zoonosis or saprozoonosis by the WHO) or—less frequently—food (Fillaux and Magnaval 2013). The infectious dose of 100 eggs has been determined for *T. canis* in a single human volunteer (Chaudhuri and Saha 1959). Probably more rarely transmission can be achieved through consumption of encysted larvae in raw or undercooked paratenic hosts (e.g. chickens, sheep, cattle). Mainly children may also get infected by eating invertebrate paratenic hosts, e.g. earthworms (Macpherson 2013). A transmission by direct animal contact, e.g. via eggs in fur, has so far been debated for *T. canis* only; however, most of the few detectable eggs sticking to dog fur are non-embryonated and therefore non-infective (Overgaauw and van Knapen 2013; Macpherson 2013).

In contrast to cats, humans are aberrant or dead-end hosts with respect to the completion of the *Toxocara* life cycle. Infective larvae may hatch after ingestion of eggs, but—in contrast to the human-adapted *A. lumbricoides* larvae—the juvenile stages fail to develop to mature adult worms. Instead, they may migrate throughout the human body and finally get encysted for months or years in basically any organ, causing inflammatory or immunologically driven damage to the respective tissue they happen to reach (Macpherson 2013; Overgaauw 1997; Despommier 2003).

23.2.3.2 Epidemiology in Cats and Humans

T. canis and *T. cati* eggs are morphologically very similar and basically only distinguishable by slight size differences (74.8 × 86.0 µm vs. 62.3 × 72.7 µm, respectively [Fahrion et al. 2011]) making microscopical species differentiation probably impossible in a routine laboratory setting (Macpherson 2013). While molecular diagnostic tools for differentiating *T. canis* from *T. cati* eggs or larvae have recently been developed, serological techniques to differentiate reliably between *T. cati*- and *T. canis*-specific antibodies in either definite host species or humans do not yet exist (Macpherson 2013, Fillaux and Magnaval 2013).

Therefore, basically all available epidemiological data on *Toxocara* spp. or toxocariasis are not *T. cati*-specific, but will reflect more likely the situation for both *Toxocara* species. It is likely that for convenience reasons most studies deduct the respective *Toxocara* species from the name-giving animal in which they were detected. In Europe, infection rates for *T. cati* in cats are reported to range from 8 to 76 %, for *T. canis* in dogs from 3.5 to 34 % (Strube and Heuer 2013; Epe et al. 2004; Barutzki and Schaper 2003). Interestingly, in a study applying species-specific ITS-2-based PCR only about two third of *Toxocara* eggs were *T. canis* and about one third *T. cati* in dog feces, while all eggs from cats were *T. cati* (Fahrion et al. 2011); the finding was explained by the canine behaviour of coprophagy causing *T. cati* to be present also in dog feces.

Toxocariasis is one of the most prevalent parasitic zoonoses worldwide occurring from the sub-arctic to the tropics (Rubinsky-Elefant et al. 2010; Macpherson 2013). The global prevalence in humans is influenced by a variety of environmental, geographic, cultural and socioeconomic factors as well as individual components, e.g. susceptibility to *Toxocara* infection due to immunity, co-infection, genetics, age, gender, nutrition and the behavior of both the human and the definitive hosts (Congdon and Lloyd 2011; Macpherson 2013). Poverty, lack of education, a high percentage of untreated or uncontrolled definitive hosts, poor hygienic conditions both for humans and the definitive hosts, and warm climate allowing longer survival of embryonated eggs in the environment are associated with higher prevalences (Macpherson 2013). Human seroprevalence in industrialised countries ranges from 0.7 to 44 % with the majority of studies indicating prevalences far below 20 % (Macpherson 2013); in children who are most at risk prevalences between 2.5 % in Germany and 7.3 % in the US are reported (Overgaauw and van Knapen 2013). In tropical and/or less industrialised countries seroprevalences are much higher

ranging from 30 to 92.8% (Strube and Heuer 2013; Overgaauw and van Knapen 2013; Macpherson 2013; for a intensive review of human seroprevalence surveys published since 1990, see Rubinsky-Elefant et al. 2010).

Mainly for theoretical deliberations the majority of human toxocariasis infections has been considered to be caused by *T. canis*. These arguments are mainly based on data from Iceland (Overgaauw 1997; Rubinsky-Elefant et al. 2010), where the importation of foreign dogs was banned for *Echinococcus* control reasons from 1909 to 1991, dog ownership was prohibited in the capital Reykjavík since 1924 and a serological study failed to detect anti-*Toxocara* antibodies in 307 adult Icelanders (Woodruff et al. 1982). This study, however, did not give any epidemiological information on the study population, e.g. on any exposure risks such as cat contact, on *T. cati* prevalence in cats, cat population density, environmental load of *T. cati* eggs or their survival in soil under Iceland's climate conditions. Moreover, neither was *Toxocara* infection completely absent in dogs from Iceland during this time-period or shortly thereafter (Richter 1981) nor was the percentage of *Toxocara* eggs shedding cats (12.5%) very high when compared to the situation in cats from other countries (Ágústsson and Richter 1993; Sandholt 2004) making it an oversimplification to claim that a lack of *Toxocara* seropositivity in the study population proves a much lesser transmission risk of *T. cati* to humans when compared to *T. canis*. Besides that, this belief might also be fostered by experimental data showing that *T. canis* larvae migrate faster than *T. cati* through the body of infected mice (Overgaauw and van Knapen 2013; Strube and Heuer 2013).

23.2.3.3 Disease in Cats

Usually, *Toxocara* spp. do not cause pathological changes in the definitive host species. In cases of high infection intensities after transplacental transmission *T. canis*-infected canine puppies might show a pot-bellied appearance, failure to thrive and, in rare cases, death (Macpherson 2013).

23.2.3.4 Disease in Humans

T. cati larvae may reach basically any organ where they finally get encysted. Experimental data with mice suggest that *T. cati* migrate more slowly and/or less often into the CNS or the eye when compared to *T. canis* (Overgaauw and van Knapen 2013; Strube and Heuer 2013). Syndromes of toxocariasis include visceral larva migrans (VLM), which might be characterized by fever, hepatosplenomegaly, abdominal pain, pulmonary disease, asthma-like symptoms, anorexia, weight loss, fatigue, occasionally urticaria, and eosinophilia; ocular larva migrans (OLM), characterized by a usually unilateral granulomatous retinitis causing visual impairment and possibly blindness; neurotoxocariasis (NT), characterized by progressive neurologic disease; and covert toxocariasis (CT), in which chronic abdominal pain or other nonspecific symptoms can develop (Despommier 2003; Macpherson 2013;

Rubinsky-Elefant et al. 2010). Both VLM and OLM most frequently affect children, VLM more often those aged less than 5 years of age, OLM usually those 5–10 years old. OLM is considered to be much rarer than VLM and probably caused by a lower level of infection. Human toxocariasis is usually diagnosed serologically and can be treated with albendazole.

23.2.3.5 Public Health Importance

Since *Toxocara* eggs need 2–6 weeks outside the definitive host to finally contain infective larvae, the most important transmission pathway for humans is via ingestion of soil. Consequently, children are more at risk than adults due to their less strict hygienic behaviour and their tendency to put possibly egg-contaminated objects and soil or larvae-containing paratenic hosts such as earthworms into their mouth.

Direct cat contact is obviously of minor importance, since studies have shown conflicting results on a possible association between seropositivity and cat ownership (Paludo et al. 2007: OR 1.957 in Brazil; Jarosz et al. 2010: OR 2.0; Won et al. 2008: OR 1.2 in the US; Rubinsky-Elefant et al. 2008: OR 0.57 in Brazil; Woodruff et al. 1982). A possible infection route by *Toxocara* eggs sticking on the cat's fur seems unlikely, since eggs have to be embryonated to cause infection which would need time. Moreover, cats—when compared to dogs—show less often xenosmophilic behaviour which might lead to get contaminated with soil- or feces-derived embryonated *Toxocara* eggs.

23.2.3.6 Public Health Measures

To date no specific national control programs against *Toxocara* spp. have ever been attempted.

Of the four main reservoirs of parasite infection (intestinal infections in the definitive host, eggs in the environment, larvae in paratenic hosts, larvae in the definitive host), the easiest to control are the definitive hosts, e.g. cats and dogs (Macpherson 2013). Therefore, prevention of the initial contamination of the environment is the most obvious approach, for instance by regularly deworming cats (and dogs) [see recommendations from CAPC 2014 or ESCCAP 2010], preventing defecation of pet animals in public areas (especially in playgrounds), reduction of free-roaming cats and dogs, fostering pet hygiene and educating the public (Overgaauw and van Knapen 2013). However, the paradoxical finding known for *T. canis* eggs that low levels of egg exposure are more successful in establishing a patent infection in dogs than larger egg amounts might hamper public health measures, if this finding is also valid for *T. cati* and cats (Macpherson 2013).

When addressing hygienic measures for animals, the differences in canine and feline defecation patterns is important: in contrast to dogs, cats tend to defecate in more thoroughly selected and less open places, e.g. in corners, often covering and hiding their feces thereafter. Therefore, playgrounds and especially sandpits

are probably much more important for cat-transmitted *Toxocara* eggs than for those excreted by dogs.

For exposure prophylaxis with respect to *Toxocara* eggs, especially children should be educated about hand hygiene and taught to avoid geophagy.

23.3 Epononymous, but Probably Seldom (or Not Relevant): *Rickettsia felis, Chlamydophila felis, Cryptosporidium felis* and *Opisthorchis felineus*

23.3.1 Rickettsia felis

23.3.1.1 The Pathogen

Rickettsia felis is an obligate intracellular Gram-negative bacterium which is commonly attributed to the spotted fever group (SFG)—more recently to the transitional group (TG)—of *Rickettsia* (Parola 2011; Reif and Macaluso 2009; Pérez-Osorio et al. 2008; Abdad et al. 2011). It has been definitely described in 2002 (La Scola et al. 2002). The cat flea (*Ctenocephalides felis*) is the primary and so far only known natural vector and the reservoir of *R. felis*, although *R. felis* has been found in many other arthropods including ticks, fleas and chigger mites (Reif and Macaluso 2009). *R. felis* was shown to be prevalent in cat fleas from more than 20 countries on five continents with positivity rates ranging from 2 to 70 % (Parola 2011). It has been detected in several peri-domestic species including cats, dogs and opossums, but the definitive mammalian host(s) has not yet been identified. Humans might possibly get infected via flea saliva or—as it is the case with *R. typhi*—via flea feces through dermal micro-traumas.

23.3.1.2 Epidemiology in Cats and Humans

Due to antibodies cross-reacting against antigens from different *Rickettsia* spp., species-specific seroprevalence data are often difficult to obtain. In seroprevalence studies for different mammalian species cats have shown the highest antibody positivity rate so far with up to 70 % in a report from Chile (Labruna et al. 2007), but also lower rates of 4–15 % have been described in several parts of the US (Bayliss et al. 2009; Case et al. 2006).

In humans, serological studies have shown an antibody prevalence of 4.4 % in HIV-positive patients from Spain (Nogueras et al. 2014), of 3.2 and 6.5 % in healthy individuals from Catalonia (Nogueras et al. 2006) and from southern Spain (Bernabeu-Wittel et al. 2006), respectively, and of 17.8 % in volunteers from the Caldas province, Colombia (Hidalgo et al. 2013).

So far, about 100 human clinical infections have been described either in case reports or in diagnostic studies from more than 20 countries of five continents including Brazil, Mexico (Zavala-Castro et al. 2009), the US, Sweden (Lindblom et al. 2010), France, Germany, Spain, Thailand, Sri Lanka (Angelakis et al. 2012), Taiwan, South Korea, Laos, Israel, Tunesia (Kaabia and Letaief 2009), Algeria (Taleb et al. 2013; Mokrani et al. 2012), Egypt, Kenya (Maina et al. 2012; Richards et al. 2010), Senegal (Socolovschi et al. 2010), St. Kitts from the West Indies (Kelly et al. 2010), Australia (Williams et al. 2011), New Zealand (Lim et al. 2012) (countries not specified by an own reference were cited in Renvoisé et al. 2009, Parola 2011; Pérez-Osorio et al. 2008).

23.3.1.3 Disease in Cats

The majority of cats are probably asymptomatic (McElroy et al. 2010).

23.3.1.4 Disease in Humans

Clinical manifestations of human *R. felis* infection may include fever, fatigue, headache, maculopapular rash, an eschar, neurological signs including meningitis-like symptoms (Lindblom et al. 2010), hepatosplenomegaly (Zavala-Castro et al. 2009), digestive disease or pneumonia (Parola 2011; Renvoisé et al. 2009). It is suspected to be an important cause of uneruptive "fever of unknown origin" in Africa (Socolovschi et al. 2010; Mediannikov et al. 2013b; Maina et al. 2012) and a vesicular fever disease known among the local population in Senegal as "yaaf" (Mediannikov et al. 2013a). The disease is usually self-limiting and is most often treated with doxycycline. So far, there have been no reports of flea-borne spotted fever causing death (Abdad et al. 2011).

23.3.1.5 Public Health Importance

The geographic distribution of *R. felis* in the cosmopolitan cat flea reinforces the hypothesis that *R. felis* might be found in most, if not all, human populations where cats and other domestic animals are kept as pets (Abdad et al. 2011). Unfortunately, most case reports do not comment on possible animal contact. One report from Australia indicates that direct contact with cat fleas harbouring cats might be a risk for transmission, since three siblings, their grandmother and a neighbour showed serological and clinical signs of *R. felis* infection, all of them having had contact to a cat family, while the children's parents without cat contact remained asymptomatic (Williams et al. 2011).

23.3.1.6 Public Health Measures

The individual and public health measures recommended for prevention of bartonellosis might also be useful for avoiding *R. felis* infection due to the supposed similar transmission via *Ctenocephalides felis*.

23.3.2 Chlamydophila felis

23.3.2.1 The Pathogen

Chlamydophila felis (previously named as Chlamydia psittaci var. felis) is an obligate intracellular bacterium, which grows in the cytoplasm of epithelial cells where it produces inclusion bodies. Other species in the genus include the epidemiologically by far more important *Chlamydophila pneumoniae* and *Chlamydophila psittaci* as well as *Chlamydophila pecorum, Chlamydophila abortus* and *Chlamydophila caviae*. Transmission occurs via aerosols or secretions from the eyes or noses of infected cats (Sykes 2005).

23.3.2.2 Epidemiology in Cats and Humans

Feline chlamydiosis is the most common cause of pneumonia and acute or chronic conjunctivitis, particularly in kittens, but occasionally also in adult cats (TerWee et al. 1998; Yan et al. 2000; Sykes 2005). Seroprevalence data range from 6 to 58 % in studies from China (Wu et al. 2013), Slovenia (Dovč et al. 2008), Spain (Millán and Rodríguez 2009), Sweden (Holst et al. 2006), Japan (Yan et al. 2000), Slovakia (Halánová et al. 2011), Italy (Di Francesco et al. 2004) and the US (Nasisse et al. 1993). Stray cats, cats from catteries or animal shelters and cats younger than 1 year of age seem to be more affected by *Cp. felis* infection in different studies. PCR studies showed only a low *Cp. felis* prevalence in asymptomatic cats of less than 5 % (Sykes 2005). Not many studies were done in human populations; seroprevalence in the normal population and in veterinarians was 1.7 and 8.8 % in a study from Japan (Yan et al. 2000). In all serological studies, however, cross-reacting antibodies might affect the positivity rate.

23.3.2.3 Disease in Cats

Clinical signs of *Cp. felis* infection are sneezing, transient fever, inappetence, weight lost, nasal discharge, rarely lameness or lethargy and possibly, but not yet proven reproductive tract disease including stillbirth or abortion (Sykes 2005). Chlamydial disease may be complicated by coinfection with other microorganisms. Cats are usually treated with doxycycline for several weeks (Sykes 2005).

23.3.2.4 Disease in Humans

Cp. felis infection may be associated occasionally with conjunctivitis and/or very rarely respiratory tract disease (Browning 2004). In a few serological studies using antibodies reported to be relatively specific for *Cp. felis* antibodies, *Cp. felis* was found to be at best an uncommon cause of community-acquired pneumonia (CAP) in less than 0.5 % of CAP patients from Canada (2/539; Marrie et al. 2003) or Japan (1/506; Miyashita et al. 2005), respectively.

23.3.2.5 Public Health Importance

Exposure to *Cp. felis* is possible when handling infected cats via contact with their aerosols or contaminated body fluids, e.g. tear fluid. The zoonotic potential of *Cp. felis*, however, appears to be very low. Only sporadic cases with possible cat-to-human transmission have been reported (Browning 2004). So far, only one PCR-proven case has been published showing identity of an human and a feline isolate in a HIV-positive patient with chronic conjunctivitis and his recently acquired cat (Hartley et al. 2001). Moreover, in one Japanese patient presenting with CAP and serological evidence of possible *Cp. felis* infection cat contact was documented (Miyashita et al. 2005).

23.3.2.6 Public Health Measures

Care should be taken especially by immunosuppressed people when handling cats with conjuncitivits and/or upper respiratory tract infection. A vaccine for cats is available.

23.3.3 Cyrptosporidium felis

23.3.3.1 The Pathogen

Cryptosporidium spp. are coccidian obligate intracellular parasites mainly infecting epithelial cells of the gastrointestinal tract. They have been reported from a large variety of different hosts including humans. The most important species are *C. parvum* and *C. hominis*, while *C. felis* is one of several species identified in different mammals. Infection occurs via ingestion of viable oocysts, often by drinking contaminated water, eating contaminated food and/or fecal-orally.

23.3.3.2 Epidemiology in Cats and Humans

Fecal oocyst shedding by cats ranges from 0 % to around 30 % according to data from Spain (Gracenea et al. 2009), the Netherlands (Overgaauw et al. 2009),

Australia (Palmer et al. 2008), Canada (Shukla et al. 2006), Colombia (Santin et al. 2006), Brazil (Coelho et al. 2009), the UK (Gow et al. 2009; Tzannes et al. 2008), Italy (Rambozzi et al. 2007) and the US (Ballweber et al. 2009; Mekaru et al. 2007; Nutter et al. 2004; Lucio-Forster et al. 2010).

Worldwide, around 100 isolations of *C. felis* from humans have been reported to date (for a review see Lucio-Forster et al. 2010 and Raccurt 2007; Das et al. 2006; Cieloszyk et al. 2012; Llorente et al. 2006; Cama et al. 2008; Elwin et al. 2012; Matos et al. 2004). In the majority of cases immunosuppressed people were affected, mainly HIV-positive adults (about two thirds of all *C. felis*-positive individuals for whom clinical information regarding their immunostatus is known), but also obviously immunocompetent children from mainly developing countries (Lucio-Forster et al. 2010). For instance, *C. felis* was the underlying *Cryptosporidium* species in 0.4 % of 14,469 human stool samples obtained in England and Wales between 2000 and 2008 (Elwin et al. 2012), in 1 % of 108, 98 and 175 samples from Spain (Llorente et al. 2007), from the Netherlands (Wielinga et al. 2008) and from children in Kenya (Gatei et al. 2006), respectively, in 2.5 % from 40 samples from India (Das et al. 2006) and in 4.3 % from 109 *Cryptosporidium*-infected children from Peru (Cama et al. 2008).

23.3.3.3 Disease in Cats

Oocyst shedding cats are most often asymptomatic. Occasionally, infection may be associated with persistent diarrhea, especially in younger and/or immunocompromised cats (Lucio-Forster et al. 2010).

23.3.3.4 Disease in Humans

In humans, *Cryptosporidia* may cause an enteric disease with watery diarrhoea, abdominal pain and nausea, occasionally accompanied by low grade fever or headache. Immunocompromised individuals and children are especially at risk to develop prolonged or profuse diarrhea. Deaths due to exsiccation may occur mainly in severely immunosuppressed patients or malnourished children in developing countries. In rare cases, mainly in immunocompromised patients, also other organ systems might be infected, e.g. the respiratory tract, the pancreas or the bile ducts. Asymptomatic infections are also reported.

23.3.3.5 Public Health Importance

Case-control studies from several countries were not able to show that contact with companion animals is associated with an increased risk of acquiring cryptosporidiosis (for a reviw see Xiao and Feng 2008). While in some of the few case reports on human *C. felis* infections the patients had previous cat contact (Matos et al. 2004),

no anamnestic cat contact was reported in others (Cacciò et al. 2002; Llorente et al. 2006). Similarly, in a Swiss-US American study by Morgan et al. three HIV-positive *C. felis*-infected patients were reported to have had contact to a cat, while two others denied the presence of cats in their household (Morgan et al. 2000). Therefore, it may be concluded that pet cats impose only a minimal risk for zoonotic transmission of cryptosporidiosis in general and for *C. felis* in particular (Lucio-Forster et al. 2010; Raccurt 2007).

23.3.3.6 Public Health Measures

Despite an only minimal risk of zoonotic transmission, especially immmuncompromised persons should be advised to minimize contact to cat feces and to observe basic hand hygienic measures.

23.3.4 Opisthorchis felineus

Opisthorchis felineus and its close relatives *O. viverrini* and *Clonorchis sinensis* are the three most common liver flukes involved in human health. *O. felineus* was named after one of the definite host species in which it was first described, e.g. cats from Pisa, Italy (Rivolta 1884). Although the species name suggests a zoonotic risk due to cat contact, humans—as other fish-eating mammals including cats—acquire an infection by eating raw fish harbouring the trematode's metacercariae (Pozio et al. 2013; Petney et al. 2013). Therefore, cats do not pose a direct zoonotic risk for human infections, although in some regions they might be required for maintaining the parasite's life cycle.

23.4 The (Un)usual Suspects from the Gut

Cats may excrete a plethora of bacteria, viruses and parasites via their feces (Barutzki and Schaper 2003, 2011; Gow et al. 2009; Waap et al. 2013; Weber et al. 1995; Weese 2011; Spain et al. 2001; Philbey et al. 2009; Paris et al. 2014; Hill et al. 2000; Morato et al. 2009; Dantas-Torres and Otranto 2014; Ballweber et al. 2010; Hoelzer et al. 2011; Esch and Petersen 2013). However, for most of the respective pathogens the presumed zoonotic potential is mainly based on isolation of identical species, subtypes, serotypes or—in better circumstances—of similar genotypic strains from within the pet and human population. However, clearly defined outbreak or case reports with molecular proof of cat-to-human transmission are basically lacking for most enteric pathogens. Moreover, the relative contribution of cat-associated transmission to the overall zoonotic disease load for most of these pathogens is not clear and probably very low for most of them when compared to other zoonotic sources, as it is the case with campylobacteriosis or salmonellosis in which chicken predominate the picture when compared to pet animals.

Recent reviews on the zoonotic potential of *Giardia* spp., of which different genotypic assemblages are relatively host-specific (Ballweber et al. 2010), and non-typhoidal salmonellosis (Hoelzer et al. 2011) suggest only a minimal risk of cats as source for human infection with the respective pathogens. However, indirect epidemiological data suggest at least a potential zoonotic risk, since a multivariable analysis matched on age group which was conducted in Michigan, USA revealed that children with *Salmonella* infections had reported more commonly than controls contact with cats (matched OR = 2.53) (Younus et al. 2010).

Importantly, also antibiotic-resistant *Salmonella* strains have been isolated from cats (Low et al. 1996; Van Immerseel et al. 2004).

An analysis of *Campylobacter* strains isolated from humans and animals in Switzerland using different genotyping methods showed that only very few genotypes among human and cat isolates were similar (Keller et al. 2007). In an extensive study from the Netherlands comparing *Campylobacter* genotypes of strains obtained from pet cats, pet dogs, pet-owners and non-pet owners by MLST an increased risk for human *C. jejuni* or *C. coli* infection was associated with dog-ownership (OR 2.5 in an overall model, 9.2 in a model for campylobacteriosis of probable pet origin), but not with cat-ownership (Gras et al. 2013). In contrast, another Dutch study found an OR of 1.7 and 2.0 of cat-ownership for human infection with *C. jejuni* and *C. coli*, respectively (Doorduyn et al. 2010), but interestingly no association with visiting other cat-owning households. Although pet ownership was estimated in both studies to contribute in 10–25 % to human *Campylobacter* infections, the route of transmission—e.g. pet-to-human or human-to-pet or a common infection source such as food items—cannot be inferred from these modelled attributions (Gras et al. 2013; Doorduyn et al. 2010).

With respect to pathogenic *Escherichia coli*, only a few genetic association studies and/or case reports exist indicating a minor zoonotic potential for cat-related human infections.

A Brazilian MLST analysis of *Enteropathogenic Escherichia coli* (EPEC) strains has shown close genetic similarity between O111:H25 and O125:H6 strains of human and cat origin (Morato et al. 2009). Two case reports on human and cat infections caused by a molecularly identical strain of *Enterohemorrhagic E. coli* (EHEC) have been published from Germany (Busch et al. 2007) and Argentina (Rumi et al. 2012), respectively. However, infection of pet cats with the classical *E. coli* O157:H7 seems to be extremely rare (Kataoka et al. 2010).

Since most of the cat infections due to the above mentioned enteric bacteria are asymptomatic and remain unnoticed, it might be reasonable to advise immunosuppressed people, especially people with HIV-infection, not only to get their pets tested for *Salmonella, Campylobacter* or *Cryptosporidium* when these develop diarrhea, but also to educate about proper hygienic measures when handling animals in general or to avoid exposure to them even if they are asymptomatic (Spain et al. 2001).

23.5 An emerging Cat-Related Pathogen: Toxigenic *Corynebacterium ulcerans*

23.5.1 The Pathogen

C. ulcerans are facultative anaerobic, non-motile, non-sporulating, unencapsulated, pleomorphic, partially acid-fast Gram-positive rods. Similar to *Corynebacterium diphtheriae*, the classical diphtheria agent of humans, and *Corynebacterium pseudotuberculosis*, the agent of caseous lymphadenitis in sheep and goats and a very rare zoonotic pathogen, *C. ulcerans* may harbour a lysogenic beta-corynephage bearing the *tox* gene. Toxigenic strains may produce the *tox*-encoded diphtheria toxin (DT) which is responsible for the systemic symptoms of diphtheria. Since the final taxonomic description of *C. ulcerans* is only from 1995 (Riegel et al. 1995) and the differentiation between *C. ulcerans* and *C. pseudotuberculosis* by classical biochemical tools might be difficult (Torres et al. 2013; Berger et al. 2014), prevalence data on or case reports of *C. ulcerans* infections which were published before 1995 or which do not explain their differentiation methods used have to be handled with care.

23.5.2 Epidemiology in Cats and Humans

Epidemiological data on the prevalence of diphtheria-causing *Corynebacterium* spp. in cats do not exist. Since the first description of *C. ulcerans* in two cats from Scotland in 2002 (PHLS 2002; Taylor et al. 2002) only sporadic and often asymptomatic infections have been identified in cats mostly as part of public health measures during the search for an infectious source in case of a human index patient.

Human diphtheria is a WHO-notifiable disease. In 2012, there were 4,489 cases reported globally with 2,500 estimated deaths (WHO 2013b). On a global scale, no WHO data exist on the relative contribution of the three potentially toxigenic *Corynebacterium* species. In 24 member countries of the European Diphtheria Surveillance Network (DIPNET) except the high diphtheria endemic country Latvia *C. diphtheriae* and *C. ulcerans* accounted for 53 reported human infections each between the period from 2000 to 2009 (Wagner et al. 2012). In many industrialized countries, cases of diphtheria-like infection caused by toxigenic *C. ulcerans* have recently outnumbered those caused by toxigenic *C. diphtheriae* (Wagner et al. 2010; Bonmarin et al. 2009; Zakikhany and Efstratiou 2012; Torres et al. 2013).

23.5.3 Disease in Cats

In most cases in which human *C. ulcerans* infections were associated with pet cat contact the respective cats were either asymptomatic or presented with bilateral

nasal discharge probably due to an underlying Feline Calicivirus (FCV) infection (Hatanaka et al. 2003; De Zoysa et al. 2005). In analogy to dogs (Lartigue et al. 2005), also skin ulcers might be expected.

23.5.4 Disease in Humans

Toxigenic *C. ulcerans* may cause classical respiratory diphtheria or diphtheria-like syndromes as well as cutaneous diphtheria. Classical diphtheria is an upper respiratory tract illness characterized by sore throat, low fever, and an adherent eponymous pseudomembrane (Greek: διφθέρα "pair of leather scrolls") on the tonsils, pharynx, and/or nasal cavity (Bonnet and Begg 1999). Systemic sequelae may appear after several days including myocarditis and peripheral neuropathy. Fatal cases of zoonotic *C. ulcerans* infections have also been reported (Hatanaka et al. 2003; Wellinghausen et al. 2002; Hogg et al. 2009; Mattos-Guaraldi et al. 2008; Tiwari et al. 2008). Treatment of respiratory diphtheria is by immediate antitoxin application and subsequent antibiosis with usally penicillin or erythromycin (Bonnet and Begg 1999).

Cutaneous diphtheria usually presents as skin ulcer and is often caused by a minor skin trauma; frequently, toxigenic *C. ulcerans* are isolated from an infected wound together with *Staphylococcus aureus* and *Streptococcus pyogenes* as additional pathogens.

Non-toxigenic strains may very rarely be associated with human disease, e.g. as cause of bacteraemia or skin ulceration (Corti et al. 2012).

23.5.5 Public Health Importance

C. ulcerans infection was originally associated with consumption of raw milk and dairy products or contact with cattle, but since the first isolation of toxigenic *C. ulcerans* from two domestic cats from the same household in Scotland (PHLS 2002; Taylor et al. 2002) *C. ulcerans* has increasingly been isolated from domestic animals such as pet dogs (Lartigue et al. 2005; Hogg et al. 2009) and cats (De Zoysa et al. 2005). Although most human cases reported in the last decade in the United Kingdom (Wagner et al. 2010), France (Bonmarin et al. 2009) and Germany (Sing and Heesemann 2008) were associated with pet animal contact—this sums up to 94% of case-patients for which the respective information was available within Europe from 2000 to 2009 (Wagner et al. 2012)-, epidemiological links of human infections by toxigenic *C. ulcerans* exclusively restricted to cat contact have only been reported in two patients from Japan with six and nearly 20 pet cats, respectively (Urakawa et al. 2013; Hatanaka et al. 2003), one patient with four cats from Belgium (Detemmerman et al. 2013) and in two patients from France (Bonmarin et al. 2009).

Moreover, isolation of an identical toxigenic *C. ulcerans* strain from a cat and its owner has been documented so far twice in the case of an asymptomatic pet cat and a person with pharyngeal diphtheria-like illness (Berger et al. 2011a) and in a HIV-patient with an axillary lymph node abscess (Yoshimura et al. 2013). Indirect evidence for zoonotic transmission comes from a case of cat bite-transmitted cutaneous diphtheria in which toxigenic *C. ulcerans* were isolated from the infected wound; however, no *C. ulcerans* could be detected in the biting cat (Berger et al. 2011b). Additionally, toxigenic *C. ulcerans* was isolated from a pharyngeal pseudomembrane of a Japanese woman with refractory pharyngitis and discharge material from her cat's eyes suggesting zoonotic transmission (Kamada et al. 2012). Interestingly, when ribotyping 50 human and seven feline *C. ulcerans* isolates from the UK all ribotypes generated by the cat isolates were found among the human isolates; moreover, six out of seven of the cat strains belonged to one of the predominant ribotypes seen among the clinical strains, i.e. U1, U2 and U4 (De Zoysa et al. 2005).

Besides toxigenic *C. ulcerans*, also non-toxigenic *tox*-bearing (NTTB) *C. diphtheriae* strains have been isolated during contact tracing due to a human diphtheria index patient from the ears of two pet cats with otitis in the US (Hall et al. 2010) and from an asymptomatic cat in Belgium (Detemmerman et al. 2013). Interestingly, all feline isolates from both continents showed a 1-bp deletion at nucleotide 55 in the *tox*-gene explaining the non-production of a functional DT; moreover, the *rpoB* sequence of the US strains showed less than 98 % identity when compared to other *C. diphtheriae* suggesting a novel subspecies. The authors of both studies speculate that cats might also serve as reservoirs for—a possibly cat-specific subspecies of—*C. diphtheriae*.

23.5.6 Public Health Measures

In contrast to *C. diphtheriae*, *C. ulcerans* was for a long time thought to be exclusively transmitted from animal to human. However, in one instance from 1996 person-to-person transmission has been suspected due to isolation of *C. ulcerans* from two siblings (Bonnet and Begg 1999 citing a personal communication). Although this case has never been published and probably only a very minor risk of human-to-human spread might exist, the English public health authorities at that time have recommended that the public health response to human *C. ulcerans* infection should be the same as that for *C. diphtheriae*, e.g. isolation and treatment of the index case, tracing and taking nose and throat swabs from close contacts as well as providing prophylactic antibiotics and booster vaccination for close contacts (Bonnet and Begg 1999). However, in a more recent joint publication by authors from the WHO Regional Office for Europe, Public Health Ontario, Canada and DIPNET it was stated that currently no direct evidence of person-to-person spread of *C. ulcerans* or *C. pseudotuberculosis* has been found (Wagner et al. 2012).

Currently, no recommendations regarding an antibiotic treatment of a toxigenic *C. ulcerans* carrying animal exist. In some instances dogs or cats as zoonotic sources of a human index case have successfully been treated using antibiotics, some-

times over a prolonged period (Berger et al. 2011a, b; Hogg et al. 2009). There are also no proven vaccines specifically directed against *C. ulcerans*. Scanty data from clinical case reports showing attenuated clinical symptoms in some patients as well as from cytotoxicity assays using a very limited number of clinical isolates indicate that despite of differences in the aminoacid sequence of DT from *C. diphtheriae* and *C. ulcerans* the currently used diphtheria toxoid vaccine might also protect from diphtheria due to infection with toxigenic *C. ulcerans* (Schuhegger et al. 2008).

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Chapter 24
Public Health and Rodents: A Game of Cat and Mouse

Bastiaan G. Meerburg

Abstract Rodents are the most abundant order of living mammals, distributed on every continent except Antarctic and represent 43 % of all mammalian species. Beside causing food losses and infrastructural damage, rodents can harbour pathogens that may cause serious problems to human and animal health. Unfortunately, rodent-associated problems are not an issue of the past as some may have thought, even not in the developed world. This chapter describes four factors that determine the risk and severity of human infection by zoonotic pathogens of rodents: human behaviour, human health condition, rodent ecology & behaviour and pathogen ecology & persistence. It provides an overview of these factors, their interrelation and also some directions for further research. Main conclusion of this chapter is that although science has come a long way already and we have won some small victories over the rodents, the game of cat (i.e. humans) and mouse is far from being settled.

The order of *Rodentia* is the most abundant and diversified order of living mammals and represents in total about 43 % of all mammalian species (Wilson and Reeder 1993; Huchon et al. 2002). Rodents are distributed on every continent except Antarctica and include many of the most abundant mammals. For many centuries, opportunistic rodent species have been considered as serious pests because of the damage they cause to crops, stored produce or infrastructure and the role they play in the transmission of pathogens to humans and livestock. Improved public sanitation conditions like safe drinking water, the introduction of sewers and the development of efficient anticoagulant rodenticides in the 1950s resulted in an improved public health situation and created the illusion that rodent-associated problems in the developed world had become an issue of the past.

More recently, however, the concern about rodents in both the developing and developed world has grown again because of various reasons. These reasons are the following:

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- The distribution and abundance of various rodent species may be significantly affected by changes in land use (e.g. reforestation projects, urbanization);
- Climatic change may improve living conditions for certain rodent populations;
- Growing outdoor leisure activities increase the exposure of humans to rodents and their excrements and hence the transmission risk of rodent-borne pathogens;
- In some countries the government has receded from rodent control and put it out to contract to private companies. This has led to a serious lack of insight in the spreading and abundance of rodent populations, which is important to monitor the potential introduction and spread of rodent-borne pathogens;
- The human world population is growing rapidly and thus more food is needed. Rodents are responsible for huge pre- and postharvest losses (Meerburg et al. 2009b; Htwe et al. 2012);
- Environmental concerns, toxicological safety regulations and budget reductions have diminished rodent surveillance and rodenticide-based control in many countries;
- The increasing extent of resistance of rodents against second-generation rodenticides has reduced the efficacy and flexibility of rodent control (Pelz 2007; Buckle et al. 2013; Endepols et al. 2012);
- Rodents still play an important role in spreading (re-)emerging zoonotic diseases (Meerburg et al. 2009a).

Rodent presence can have serious implications for public health and be potentially hazardous as they amplify pathogens from their environment by forming reservoirs of zoonotic disease (Webster and Macdonald 1995; Gratz 1994). With reservoirs it is meant that rodents can harbour disease-causing organisms and thus serve as potential sources of disease outbreaks, but always via a vector (tick, sand-fly etc.). Besides as reservoirs, rodents can also act as carriers, which means that rodents that show no or limited disease symptoms but harbour the disease-causing agent, are capable of passing it directly onto humans (Meerburg et al. 2009a).

Two main transmission routes of pathogens can be distinguished (Meerburg et al. 2009a): the direct route (when rodents are carriers) or the indirect route (when rodents function as reservoir and transmit a pathogen through means of a vector), see Fig. 24.1. In the latter, this vector is often an arthropod, but can also occasionally be other animals, such as livestock. Rodents that are (either by accident or on purpose) ingested by livestock can transfer pathogens. When food originating from this livestock is not thoroughly cooked, this may lead to human morbidity (Meerburg et al. 2004).

If we now look at the risks and severity that are imposed by rodents to human health, there are several factors that are of importance (Fig. 24.2).

The first one is human behaviour. People with frequent outdoor leisure activities or which fulfil specific occupations (e.g. in the military, animal trapping or forestry) or those that live in degraded environments will be more exposed to rodent-borne zoonoses than others (Clement et al. 1997; Muliæ and Ropac 2002; Hukic et al. 2010, Sauvage et al. 2007; Bonnefoy et al. 2008). Exposure is the key word here, thus, for example also people that keep rodents as pets may experience higher risks

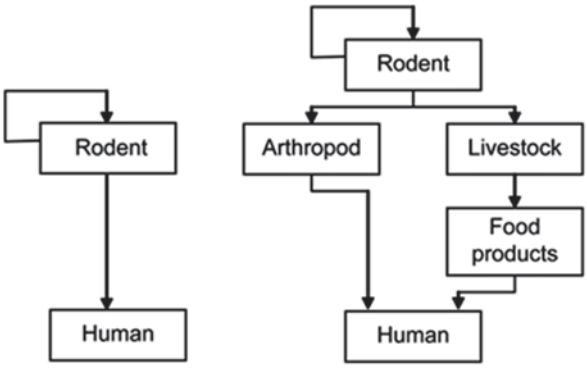

Fig. 24.1 The two main transmission routes, the direct route (*left*) and the indirect route (*right*). (Reproduced from Meerburg et al. 2009a)

Fig. 24.2 Four factors determine the risk and severity of human infections by zoonotic pathogens of rodents: human behaviour, human health condition, rodent ecology & behaviour and pathogen ecology & persistence

of zoonotic infection. The risk of keeping pet rodents will be discussed more in detail later in this chapter.

The second factor is the human health condition. Generally, zoonoses pose unique transmission and disease risks if people are not in good health, such as immunocompromised persons, neonates, the elderly or pregnant women (Mani and Maguire 2009; Hemsworth and Pizer 2006) or may effect persons in specific age groups. As an example Streptobacillus moniliformis may be mentioned, the primary cause of rat bite fever in North America. Children under 12 years of age are mainly infected, and demonstrate an acute syndrome of fever, rash, and polyarthritis. Some years ago, a fatal case-report was reported, a 14-month-old-boy, who was exposed

to filthy living conditions and whose family had pet ferrets. Presumably, the boy was bitten by rodents as autopsy revealed a possible bite mark (Banerjee et al. 2011).

Also aspects such as vaccination coverage may influence the factor human health condition. From a number of studies, it is known that wild rodents can be reservoirs for orthopoxviruses (Tryland et al. 1998, Kinnunen et al. 2011). More recently, pet rats were discovered as a new potential source of local outbreaks with cowpox. However, smallpox-vaccinated patients tend to develop less severe reactions and heal more quickly (Vogel et al. 2012). Thus, there is a direct link between actions of public health services (vaccination) and the recovery rate (severity of infection) of infected persons.

A third factor of importance is rodent ecology and behaviour. As mentioned in the introduction, rodent ecology may differ over the years, depending on climatic factors, feed abundance and predation (Witmer and Proulx 2010). During a study in Namibia, mice entered buildings during the post-harvest stage, which may represent a period of food shortage for these mice in the field (Monadjem et al. 2011). If these species are coincidently contracted with zoonotic pathogens, this may increase the risk of human infection. In a study from Cambodia, it was demonstrated that the rainy season is favourable for transmission of leptospires in rodents, particularly in rain-fed fields (Ivanova et al. 2012). Here, the human risk of contracting *Leptospira* spp. is determined strongly by the ecology of the rodents: in rice-fields, forest, secondary forests and their interface with agricultural fields the potential of humans for contracting leptospirosis infection is the highest (Ivanova et al. 2012).

But the link between rodent ecology and human health risks is not depending solely on one rodent species. Also the presence of other non-reservoir rodent species is important. In a recent study from Panama it was demonstrated that hantavirus prevalence in wild reservoir (rodent) populations and reservoir population density increased when small-mammal species diversity was reduced (Suzán et al. 2009). These authors thus claim that high biodiversity is important to reduce transmission of zoonotic pathogens among wildlife hosts (Suzán et al. 2009). Also host relationships form part of the rodent ecology and there can be significant differences in such relations within the same rodent species. In a study where host-tick relationships of the yellow-necked mouse (*Apodemus flavicollis*), a critical host in the maintenance of the zoonotic disease tick-borne encephalitis, were investigated it was demonstrated that the transmission potential was not evenly distributed among the yellow-necked mice population. The authors found that 20 % of hosts most infested with ticks were accountable for 80 % of the transmission potential, and that these hosts were identified as the sexually mature males of high body mass (Perkins et al. 2003). This leads to the impression that control efforts targeted at this host group would reduce the transmission potential significantly.

In the past, seasons of exceptionally high rainfall were thought to increase rodent populations (because plant growth would lead to abundant seeds and insects) and thus outbreaks of some rodent-borne diseases (Engelthaler et al. 1997; Brown and Ernest 2002). However, we now start to discover that such relationships between rodent population dynamics and precipitation are complex and non-linear. This was also the main conclusion after some scientists studied the El Niño phenomenon in

deserts of southwestern North-America (Brown and Ernest 2002). In agricultural contexts it is also difficult to predict exactly the breeding ecology of species. A recent study from the Philippines compared two rodent species *R. argentiventer* and *R. tanezumi* during four cropping seasons (two dry and two wet). The expectation was that *R. tanezumi* breeding would occur throughout the season, whereas the breeding of *R. argentiventer* would be strongly cued to the generative stage of rice crops (Htwe et al. 2012). However, it was found that their breeding ecology was exactly similar, with the onset of the breeding season at the tillering stage of the rice crops. The conception of adult females was highest during booting and ripening of the rice and the highest litter size occurred at booting and ripening of the rice (Htwe et al. 2012). Such information is essential in order to optimize the management of rodents in order to reduce harvest losses and pathogen transmission risks.

The fourth factor is pathogen ecology and persistence. Many of the mechanisms that mediate pathogen ecology and persistence only start being uncovered. Concerning hantaviruses in rodents, several host factors, including sex steroids, glucocorticoids, and genetic factors, are reported to alter host susceptibility and may contribute to the persistence of hantaviruses in rodents (Easterbrook and Klein 2008). Moreover, because of the recent discovery of structural and non-structural proteins in humans that suppress type I interferon responses, it is now thought that immune responses of rodent hosts could be mediated directly by this virus (Easterbrook and Klein 2008). In laboratory rats it was observed that *Leptospira interrogans* serovar Copenhageni initially disseminates extensively throughout the host, prior to clearance from all tissues except the kidneys, suggesting that the kidneys are immune privileged sites and that this is not caused by tissue tropism (Athanazio et al. 2008). In a study in black rats (*Rattus rattus*) in Madagascar, an important difference in plague resistance between rat populations from the plague focus (central highlands) and those from the plague-free zone (low altitude area) was confirmed to be widespread (Tollenaere et al. 2010). Moreover, these authors discovered that sex influenced plague susceptibility, with males slightly more resistant than females (Tollenaere et al. 2010). It is difficult whether this phenomenon is caused by rodent ecology, pathogen ecology or a combination of both. This is also the case with other findings. In Belgium, a close association between the distribution of hantavirus-infected bank voles and wet habitat types was found (Verhagen et al. 1986). In another, more recent, study from this country, a direct relation between climate and the incidence of human cases of nephropathia epidemica (NE) due to Puumala virus (PUUV) infection was found. High summer and autumn temperatures, 2 years and 1 year respectively before NE occurrence, relate to high NE incidence (Tersago et al. 2009). In the United States, human cases of Hantavirus Pulmonary Synodrome (HPS) were clustered seasonally and temporally by biome type and geographic location. In this study, exposure sites were most frequently found in pinyon-juniper woodlands, grasslands, and Great Basin desert scrub lands, at elevations of 1,800 m to 2,500 m (Engelthaler et al. 1997). This might be caused by presence of rodent reservoir hosts in these areas, but perhaps also because of favourable environmental conditions for pathogen survival. Pathogens do not only persist in the host itself, but may also survive for longer periods of time throughout the environment. For

example, *Yersinia pestis* biotype Orientalis remains viable and fully virulent after 40 weeks in the soil and is then able to continue its role in plague epidemiology (Ayyadurai et al. 2008). Moreover, if factors such as pH, viscosity and salt concentration are optimal, *Leptospira* spp. are thought to be able to survive in fresh water under low-nutrient conditions for over 100 days (Trueba et al. 2004).

It is clear that for eradication strategies more work has to be done on the pathogenesis of the various zoonotic pathogens which can be transferred by rodents. Especially the further development of genetic tools could lead to a better understanding of the virulence and survival mechanisms that are used by pathogens to ensure their persistence in different ecological niches and host reservoirs.

Often, there is a relation between the different factors and complex relationships between pathogen prevalence and rodent density appear likely. In North-Western Europe the main disease-causing hantavirus species is the Puumala virus (PUUV). The reservoir species for PUUV is the bank vole (*Myodes glareolus*), which exists in specific habitats. The risk for PUUV transfer from the bank vole to the human population via excretion of the virus in the environment is dependent on a myriad of biotic and abiotic risk factors, either rodent-, virus- or human-related, that vary in time and space. In a study from Finland, the effect of PUUV infection on the winter survival of bank voles was investigated (Kallio et al. 2007). These authors demonstrate that PUUV infected bank voles had a significantly lower overwinter survival probability than antibody negative bank voles. Thus, the pathogen is able to influence the host population dynamics. During a study on the ecology and demographics of hantavirus infections in rodent populations in the Walker River Basin of Nevada and California, it was found that antibody prevalence could vary within repeatedly sampled sites from 0 to 50% over the course of several months (Boone et al. 1998). In Tanzania, an African rodent (*Mastomys natalensis*) is thought to be the principal source of human infections with *Leptospira* spp. In a study where the dynamics of infection were modelled and in which the climatic conditions in central Tanzania were included, a strong seasonality was visualised in the force of infection on humans with a peak in the abundance of infectious mice between January and April in agricultural environments (Holt et al. 2006). In urban environments, however, dynamics were predicted to be more stable and the period of high numbers of infectious animals runs from February to July (Holt et al. 2006). In countries in Northern-Europe (Germany, Denmark) there are also regional differences visible in the level of encountered *Leptospira* spp. infected-rats (Runge et al. 2013, Krøjgaard et al. 2009). Why these differences occur, is not yet fully understood.

As mentioned before, the risk of transmitting zoonotic pathogens to humans is largest if the exposure risk is maximal. Handlers and owners of pet rodents are often in direct contact with them and may experience significant risks. Some years ago, there was an outbreak of 28 cases of multidrug-resistant *S. enterica* Serotype Typhimurium in the United States. After the outbreak, 22 patients were interviewed. Of them, 13 (59%) had had contact with rodents purchased from retail pet stores (Swanson et al. 2007), while 2 patients (9%) acquired salmonellosis through secondary transmission from a primary patient who had been exposed to rodents. Moreover, 7 patients (32%) had no identified rodent exposure. Matching isolates

were obtained from one submitted urine specimen and 27 stool specimens from patients (Swanson et al. 2007). These authors warn that consumers and animal workers should be aware that rodents can shed salmonellae and should expect rodent excrements to be potentially infectious. Thus, handling of pet rodents may result in health risks, especially for children. When handling pet rodents, their cages or bedding, the hands should be thoroughly rinsed with water and soap. Animal vendors should be aware if substantial diarrhoea-associated complications or death occurs among rodents intended for sale (Swanson et al. 2007).

Some years earlier, an human infection with Lymphocytic Choriomeningitis Virus (LCMV) in the United States was found by the CDC to be associated with pet rodents (hamsters and guinea pigs). Here, the risks extended also beyond the owners of these pets. In this particular case, LCMV was responsible for the death of 3 immunocompromised persons (organ transplant recipients) who received these organs from pet rodent owners (Anonymous 2005). More recently, workers at a rodent breeding facility in the United States were confronted with a LCMV infection. In total, 52 current and former employees of the facility were tested, and 13 of them (25 %) demonstrated a recent LCMV infection (Anonymous 2012).

Exotic rodents may introduce pathogens that were previously unknown to continents. For example, in 2003 a monkeypox outbreak in pet distribution facilities in the US occurred after import of infected African prairie dogs (Anonymous 2003). In total, monkeypox was confirmed in 35 persons, of which none died, but the outbreak required vaccination of 30 persons with smallpox vaccine.

Commercially-traded wild prairie dogs were also responsible for an outbreak caused by *Francisella tularensis* type B in Texas. Antibodies to this pathogen were found in one person that was exposed, thus leading to the first evidence of tularemia transmission from prairie dog to human (Avashia et al. 2002). Problematic was that in the period June-July 2002, more than 1000 prairie dogs were distributed from the facility where the pathogen emerged, to locations in 10 other U.S. states and 7 other countries (Avashia et al. 2002). These had to be traced back and were all euthanized. However, this case underlines the health risks to humans who handle wild-caught animals and underscores the speed of transportation of exotic species and their pathogens over the globe (Avashia et al. 2002).

A human cowpox virus infection is an uncommon and potentially fatal skin disease, which is confined to major parts of Europe. Patients may sporadically contract the pathogen by contact with infected cows, cats or small rodents. However, recently there is also a report from Germany (Munich), where 8 patients were infected by pet rats they had purchased at a local supplier (Vogel et al. 2012). Thus, pet rats can be considered as a novel potential source of local outbreaks of human cowpox virus infections.

Also, dermatophytes can be transferred to humans by rodents. In Switzerland for example, 9 isolates of the fast-growing dermatophyte species *Arthroderma benhamiae* were isolated from 8 children and 1 adult. Eight of the 9 patients had had previous contact with rodents, mostly with guinea pigs (Fumeaux et al. 2004). In another study, where the frequency and types of dermatophytes among both Guinea pigs and rabbits were determined (Kraemer et al. 2012), *Trichophyton mentagrophytes*

was determined to be the most common dermatophyte in pet Guinea pigs and rabbits, but asymptomatic carriers were regularly observed only in Guinea pigs. Consequently, pet guinea pigs carrying dermatophytes can be considered as a serious zoonotic risk for their owners, especially children (Kraemer et al. 2013).

An Australian patient who experienced an infection with *Streptobacillus moniliformis*, the causative agent of rat-bite fever, obtained this pathogen not because she was bitten by rats, but because she had had contact with her pets, including cuddling and kissing them (Papanicolas et al. 2012). This is a risk as *S. moniliformis* forms part of the commensal flora of the rat's oropharynx (Elliott 2007).

But not only handling or keeping pet rodents can impose a risk. Also commensal rodent species (species that live in or around a house or a farm) may lead to health risks. The risk of bites by rats inflicted in urban environments (often in substandard dwellings) and the spread of infection to humans is substantial. In the United States, there a hundreds of rat bite reports each year, while the number may even be underreported by a factor of at least ten (Bonnefoy et al. 2008; Hirschhorn and Hodge 1999). Next to rat bites, ectoparasites that are associated with these rodents can spread additional infectious organisms. The rodents are sometimes also carrying endoparasites or other pathogens which may contaminate the local environment. A literature review on helminths in rodents in South East Asia showed that the highest helminth species richness was found in *Rattus tanezumi*, *Rattus norvegicus* and *Rattus argentiventer*, which are found in more human-dominated habitats such as agricultural areas or human settlements (Chaisiri et al. 2010). In a study in Tokyo, Japan, 17% of the brown rats (*Rattus norvegicus*) from urban areas carried leptospires in their kidneys and cases in human patients could directly be linked to these rats via DNA-analysis (Koizumi et al. 2009). Moreover, rodents in agro-ecological surroundings can be infected with *Salmonella* spp. and *Campylobacter* spp. and transfer these bacteria to livestock or amplify their number in the farm environment (Meerburg and Kijlstra 2007). In this way, a resident infected rodent population could lead to continuously returning infections in the farm environment, with all the negative consequences for both livestock and farmers. The exact risk dimension of livestock-pathogen-human-wildlife interactions is not yet known for many pathogens. Two pathogens may serve as an example here: *Coxiella burnetii*, the causative agent of Q-fever, and Hepatitis E virus (HEV). Concerning *Coxiella burnetii,* it has been implicated in many studies that rodents function as reservoirs for Q-fever, but their exact role in pathogen maintenance, geographic spread and transmission still remains to be clarified (Meerburg and Reusken 2011; Webster et al. 1995). Problematic in determining the exact contribution of rodents is that basic wildlife and domestic cycles of *C. burnetii* infection can operate independently, but will overlap in many instances as many areas in the world are occupied by both domestic and wild animals (Meerburg and Reusken 2011), which makes it hard to unravel their exact contribution. In a recent study from Japan (Kanai et al. 2012) in which wild *Rattus norvegicus* were caught near a pig farm where HEV was present, it was demonstrated that in these rodents there was a relatively high prevalence (17.9%). Consequently, these authors conclude that *R. norvegicus* may be a carrier of swine

HEV in endemic regions, but that the HEV contamination risk due to rats in human habitats remains unclear (Kanai et al. 2012).

Consequently, there remains much work for scientists to be done. Concerning the factor human behaviour, the use of Geographical Information System (GIS) technology could prove to be a useful tool for the identification of endemic foci and high-risk areas for numerous pathogens that are transmitted by rodents. Such technology was recently tested in a study in Cyprus (Psaroulaki et al. 2010), where rats were used as disease sentinels and tested for seropositivity on six microbial agents. By optimizing this technology, more information could be acquired about possible outbreak areas, which facilitates informing the general public by public health officials.

When considering the factor human health condition, one should remember that the world population will increase the coming decades and also that the average age of the world population will increase. Thus, the number of people that may experience significant health effects when infected by zoonoses is growing. We do not yet know the exact dimension of the problem, but it is something to keep in mind.

High resolution remote-sensing could also prove useful to monitor the factor rodent ecology and behaviour. This was recently done in Kazakhstan, where great gerbil burrow systems were observed by means of satellite images (Addink et al. 2010). The occupancy rate of these burrows is a strong indicator for the probability of a plague outbreak. By monitoring the density of great gerbil burrow systems, or locating new or expanding foci, a direct contribution could be made to surveillance and control efforts (Addink et al. 2010). Of course, with such techniques it is not possible to monitor the ecology and behaviour of all rodent species. To gain more insight into the population dynamics and habitat preferences of rodents, field studies will remain necessary. By collection of small mammals in several habitat types, an action which was recently undertaken in Albania (Rogozi et al. 2012), one can gain more knowledge of the reservoir ecology in a country, and thus acquire more possibilities for reliable risk assessments for rodent-borne diseases. Moreover, also rodent identification via molecular methods, e.g. molecular barcoding using short genetic markers (Galan et al. 2012) may be useful as this will lead to a quicker and more accurate species identification. The previous will also prove its worth, if rodent dynamics and ecology will change in the future because of climatic change.

Concerning the factors pathogen ecology and persistence, there are also new opportunities. Fecal samples of wild rodents that were collected in California and Virginia were surveyed in order to obtain an initial unbiased measure of the viral diversity in the enteric tract (Phan et al. 2011). Viral RNA and DNA were randomly amplified. Phylogenetic analyses of full and partial viral genomes revealed many previously uncharacterized viral species, genera, and families, and close genetic similarities between some rodent and human viruses even reflected past zoonoses (Phan et al. 2011). In another recent study, a comparative approach was used to study microparasite species richness across rodent species according to the latitude where they occur (Bordes et al. 2011). The results demonstrated that virus species richness increased towards tropical latitudes, and that rodent litter size seemed to decrease when microparasite species richness increased independently from the

latitude. The authors thus claim that rodent species in the tropics harbour higher parasite species loads effectively, at least in terms of species richness for viruses, and that parasite species richness influences rodent life-history traits (Bordes et al. 2011). This information is also important for reliable risk assessments.

Finally, we may conclude that humankind has come a long way. We increased our knowledge and understanding and have gained some small victories over the rodents. However, there is no definitive victory over them yet, and although they are not able to defeat the cats (in this case the humans), they do still pretty well in avoiding capture. Let's hope that further scientific progress will lead to a better understanding about rodents and their risk for public health and that the contest between cat and mouse may end in a favourable way for humankind.

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Chapter 25
Equine Zoonoses: Consequences of Horse-Human Interactions

Roberta M. Dwyer

Abstract Fortunately, horses have a limited number of diseases that can be transmitted to humans under natural circumstances. However, due to the close contact of horses with many people as work, exhibition or companion animals, human exposures to horse diseases can be more numerous than for other large animals. Some equine zoonoses are significant and emerging, such as Hendra virus, while others are well known: anthrax, rabies and salmonellosis. International and national transportation of horses for competition and sales also require steadfast testing and surveillance of equine infectious diseases to reduce the risk of spread. This chapter will review the zoonotic diseases of horses that can spread to humans via natural exposure: anthrax, brucellosis, cryptosporidiosis, dermatophilosis, Hendra virus, glanders, leptospirosis, MRSA, rabies, ringworm, salmonellosis, trichinosis, VEE and vesicular stomatitis. These diseases have been divided into categories of significant risk to public health and moderate or low risk based on North America. This categorization may vary from country to country on other continents.

25.1 Introduction

Fortunately, zoonotic diseases of horses are limited in number in comparison to other species. For some diseases it is easy to categorize their high public health significance, such as rabies and Hendra virus, because of their high human mortality. For others it is challenging since in some areas of the world disease in animals can cause significant human illness and death due to the limited availability of health care and appropriate antibiotics, vaccines and prophylactic measures. Prompt disease diagnosis in horses and traceback to exposed people are also challenges for existing resources. Other diseases have low public health significance but may be more severe with immunocompromised individuals. For other equine zoonoses, human disease due to horses is unknown (e.g., leptospirosis), but is certainly possible and may have been documented in journals to which the author does not have access. This chapter will discuss only those diseases for which the disease in the horse

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can cause or potentially cause disease in people, which is why pathogens like West Nile Virus (the horse is a dead end host) are not included.

25.2 Anthrax

The Gram positive bacteria *Bacillus anthracis* causes anthrax in horses and many other species of animals. Bacterial spores are very resistant to temperature extremes and can be harbored in contaminated soil, infected animals and infected carcasses.

25.2.1 Clinical Signs in Horses

In horses, ingestion, inhalation and skin penetration (possibly by biting flies) are the primary modes of transmission. After an incubation time of 3–7 days, horses can exhibit fever, colic, dyspnea, edema of the ventral neck, thorax and abdomen, leading to death. Anthrax is usually rapidly fatal. A dead animal that has unclotted blood exuding from body orifices is often strongly suggestive for anthrax, and bodies should not be moved, nor opened for necropsy. When consulting on an anthrax-suspected animal, veterinarians should wear full personal protective (PPE) gear such as disposable coveralls, face shield (or eye goggles and an N-95 mask), disposable boots and gloves. The animal's hide and hair can contain anthrax spores, and individuals in contact with anthrax cases should seek medical advice about prophylactic antibiotics. Regulatory veterinarians and public health officials should be consulted for proper carcass disposal.

25.2.2 Transmission to Humans

Exposure of broken skin, inhalation, or ingestion from inadequately cooked contaminated meat are all potential means of transmission. Tissues, hair, hide, products made from contaminated animal tissues and contaminated soil can all contain infective spores. Soil can be contaminated from exposure to infected animals and their carcasses.

25.2.3 Public Health Significance

Anthrax has been used as a bioterrorism weapon and is endemic in several countries, therefore constituting a significant public health issue. Human exposure comes from compromised skin contact or inhaling spores from infected animals

including hair, hide, and products made from these tissues. Also, ingesting contaminated horse meat that is undercooked can cause human disease.

25.3 Brucellosis

Brucellosis is a disease worldwide caused by several types of *Bacillus* bacteria. Brucellosis in horses is caused by the bacteria *Bacillus abortus*.

25.3.1 Clinical Signs in Horses

Two types of lesions predominate in equine brucellosis: fistulous withers (septic supraspinous of the 2nd and 3rd thoracic vertebrae) and poll evil (atlantal bursitis of the 1st and 2nd cervical vertebrae). Other infections of bursae, bones and joints can also occur, as can abortions (Denny 1973; Ocholi et al. 2004; Carrigan et al. 1987; Collins et al. 1971; Denny 1972). Fistulous withers starts with localized inflammation and swelling along the withers and develops into an abscess containing abundant purulent material. Fistulous tracts can also develop into deeper tissue. Poll evil develops in a similar fashion with inflammation, swelling and a draining abscess.

25.3.2 Transmission to Humans

Broken skin coming in contact with contaminated animals, animal tissues and (in abortion cases) vaginal fluids, fetus, and placenta are modes of transmission. Veterinarians, laboratory and abattoir workers and barn personnel are also at risk via the airborne route. Ingestion of unpasteurized milk and dairy products is another means of transmission common with cattle and goats. Mare milk is drunk by people outside of the U.S., although documented cases of human transmission via horse milk were not found in the literature.

25.3.3 Public Health Significance

While equine brucellosis is rare in the U.S., its incidence could be higher in other geographic areas. Human brucellosis needs to be treated with antibiotics and some patients can become severely ill. *B. abortus* has been identified as an agent that could be used in a deliberate bioterrorism attack (http://www.bt.cdc.gov/agent/agentlist.asp).

25.4 Cryptosporidiosis

Cryptosporidium spp. are coccidian parasites found in multiple domestic and wild animals, with a worldwide distribution. The organism causes diarrhea and other gastrointestinal symptoms; asymptomatic carriers have also been documented. In horses, *C. parvum* primarily causes disease in foals less than six months of age. One study found no oocyst fecal shedding among 301 horses in the USA (Atwill et al. 2000). A new *Cryptosporidium* horse genotype has been recently identified in foals in New York (Burton et al. 2010).

25.4.1 Clinical Signs in Horses

Foals can develop a severe watery diarrhea leading to dehydration, lethargy and even death.

25.4.2 Transmission to Human

People contract cryptosporidiosis via the fecal oral route, either by direct or indirect means.

25.4.3 Public Health Significance

No documented case of human cryptosporidiosis specifically contracted from horses could be found by the author. However, *C. parvum* is zoonotic; it can be shed from multiple animal species; persists in the environment; and is a cause of water contamination. Healthy individuals usually have a self-limiting disease with proper medical care. More severe disease is found in immunocompromised people, especially those with AIDS.

25.5 Dermatophilosis

Dermatophilus congolensis is an actinomyces that causes skin infections in horses, commonly known as “rain rot” or “rain scald.” This disease is common in North America and can range from localized skin lesions to diseased areas on significant portions of the skin. The disease seems to be more common in rainy seasons when skin integrity may be compromised.

25.5.1 Clinical Signs in Horses

Lesions are commonly seen over the dorsum of the horse and start as papules, with hairs crusting together. When crusts are removed, the underlying skin is eroded. Chronic lesions are alopecic and scaly. The diagnosis is usually made based on exposure to wet weather conditions and the typical lesions over the back and rump of the horse.

25.5.2 Transmission to Humans

Skin contact with infected horse skin, or to scabs, especially in moist, humid conditions, is the primary means of transmission (Burd et al. 2007). As with horses, the bacteria gains entry to skin which has abrasions or is compromised versus healthy, intact epidermis.

25.5.3 Public Health Significance

Reports of a limited number of cases indicate that the disease is self-limiting in people with proper hygienic care.

25.6 Hendra Virus

Hendra virus was initially named morbillivirus; further research, however, indicated it is of the family Paramyxoviridae and genus Henipavirus. This is an emerging disease with high fatality in horses and humans in Australia. The initial documented case of disease occurred in 1994 in Queensland, Australia, when a horse trainer, stablehand and multiple horses developed acute illness. The trainer and 14 horses died. Subsequent outbreaks occurred in 1995, 2008, 2009, 2011, 2012 and 2013. The disease has high mortality in horses and people (Tulsiani et al. 2011). The natural host is fruit bats, which do not get sick from the virus.

25.6.1 Clinical Signs in Horses

Horses become acutely ill with fever, tachycardia, difficulty breathing, neurological signs and deterioration. Death can occur in 1–2 days; the case fatality rate is 75 %.

25.6.2 Transmission to Humans

People are thought to contract the disease by close contact with body fluids of infected horses during physical examination, treatment or necropsy. Full PPE needs to be used when examining a Hendra virus-suspected horse, and personnel exposure to the horse should be minimized until a diagnosis is made. No evidence exists that implicates a bat-to-human transmission, or a human-to-human transmission.

25.6.3 Public Health Significance

Because of the high mortality rates in horses and people, this is an emerging disease of high significance. A human vaccine is not available and antivirals do not appear to be effective (Playford et al. 2010). An equine Hendra virus vaccine was released in 2013 (Mahalingam et al. 2012).

25.7 Glanders

Glanders is a bacterial disease of horses, donkeys and mules caused by *Burkholderia mallei* (Dvorak and Spickler 2008). While it has been eradicated from most countries, equine cases have been documented in areas of Asia, Africa and South America. It is considered endemic in some Middle Eastern countries with recent equine cases diagnosed in the United Arab Emirates (2004), Kuwait (2008), Bahrain (2010) and Lebanon (2011). The increase in equine trade across the region in the past decades, along with multiple other factors, are considerations for recent increased detection (Wernery and Wernery 2012).

25.7.1 Clinical Signs in Horses

Glanders can cause acute, chronic or latent disease. Horses develop nasal ulcers, and nodules in nasal airways. A thick, yellow nasal discharge (sometimes bloody) is evident, along with fever, dyspnea, cough, debilitation and intermittent fevers. Cutaneous lesions include skin nodules which break and ulcerate, producing a purulent yellow discharge. Associated lymph nodes may also get enlarged and possibly rupture.

25.7.2 Transmission to Humans

People can become infected via inhalation, ingestion, through abraded or cut skin, and via mucous membranes.

25.7.3 Public Health Significance

Increased risk exists with horse caretakers, veterinarians, laboratory and abattoir workers and butchers. Human infection can be treated with antibiotics, although without appropriate treatment, glanders can have a 95% case fatality rate. *Burkholderia mallei* is considered a potential bioterrorism agent. It was used as a weapon against horses and humans in World Wars I and II.

25.8 Leptospirosis

Leptospirosis is a disease of worldwide distribution caused by a bacterial spirochete. *Leptospira interrogans* is the pathogenic species in horses with several serovars commonly associated with horses: Bratislava, Grippotyphosa, Icterohaemorrhagiae and Pomona among others (Hines 2007). Numerous domestic and wildlife species can shed leptospires in urine.

25.8.1 Clinical Signs in Horses

Leptospirosis has been associated with abortion, stillbirth and neonatal disease, recurrent uveitis (moon blindness) and systemic disease in horses. Systemic disease can cause fever, lethargy, anorexia, icterus and occasionally renal failure.

25.8.2 Transmission to Humans

Humans are exposed by urine-contaminated water and materials being ingested, inhaled or coming in contact with abraded skin or mucous membranes. Contact with aborted fetuses, placenta and fluids is another potential route of exposure, emphasizing the need for farm workers and veterinarians to always wear gloves and protective clothing when handling these tissues. The author could find no documented case of a naturally occurring human case of leptospirosis being directly linked to a horse.

25.8.3 Public Health Significance

Leptospirosis is a disease of worldwide distribution. The role of equines in human leptospirosis is unknown since the serovars common to horses are also shared with other domestic and wildlife species.

25.9 Methicillin resistant Staphylococcus aureus (MRSA)

MRSA is a type of *Staphylococcus aureus* bacteria that is resistant to beta-lactam antibiotics and has gained much publicity with human hospital patients contracting infections that can become life-threatening. In the community, less than 2 % of people are colonized in the nose with MRSA and show no clinical signs. In horses, MRSA first became noted in the 1990's associated with outbreaks in veterinary hospitals and has since been reported in the U.S., Canada, Ireland, Austria, the Netherlands, Switzerland and Israel (Hartmann et al. 1997; Schwaber et al. 2013). Equine MRSA colonization in the nasal cavity appears to be transient; prevalence of colonization has been reported at rates of 1–10.9 %, and it is now considered that MRSA can be transmitted between horses and people (Weese and vanDuijkeren 2010).

25.9.1 Clinical Signs in Horses

Equine MRSA infections have been associated with soft tissue wounds, post-operative and intravenous catheter sites, and bone, joint, uterine and skin infections. Some infections are fatal. MRSA are cultured in equine hospitals commonly from equipment that is close to the horse's nose (feed and watering equipment, twitches, etc.) (Weese and vanDuijkeren 2010; Weese 2004).

25.9.2 Transmission to Humans

MRSA colonization in equine veterinary personnel shows higher rates than the general population. As such, it is now considered that MRSA can pass from horses to humans, and humans to horses through contact with infected or colonized patients and contaminated equipment (Weese et al. 2005).

25.9.3 Public Health Significance

Few reports exist demonstrating human MRSA clinical infections associated with horse contact (Weese et al. 2005, 2006; van Duijkeren et al. 2011). However, this may be due to the lack of a complete history taken from human patients, inability to follow up with animal cultures, underreporting as well as a multitude of other reasons. The disease is an emerging one documented in several countries. Additionally, the reporting of a new MRSA strain (Schwaber et al. 2013) in horses and humans emphasizes the need for continued diligent surveillance, education of horse owners and veterinary staff, rigorous biosecurity in hospitals, and hygiene practices on farms. Much more needs to be learned about MRSA in horses and its true significance to public health.

25.10 Rabies

Rabies is a viral disease of significant public health importance. The virus can infect mammalian species, and is fatal once clinical signs begin. While treatment for exposed humans is available, no proven post-exposure prophylaxis exists for animals (Brown et al. 2011).

25.10.1 Clinical Signs in Horses

Rabies in horses is known as the "great imitator" since horses can present with a variety of clinical signs such as colic, lameness, lethargy and neurologic signs. Nervous system signs include progressive lethargy (the "dumb" form of rabies) to aggression (the "furious" form of rabies). Behavior changes, self-mutilation, ataxia, and loss of function of defecation and urination have also been seen. Once clinical signs are seen, the disease progresses until death, usually within 5–10 days (Green 1993).

No rabies treatment for infected horses is currently available. The rabies vaccine is considered a core recommended vaccine by the American Association of Equine Practitioners.

25.10.2 Transmission to Humans

Exposure to saliva from infected horses is a source of transmission to people via bites, saliva in open wounds or mucous membranes. When working with a rabies-suspect animal, individuals should wear eye goggles, an N-95 mask, disposable gloves and coveralls. Laboratory personnel performing a necropsy or testing brain tissue for rabies should take additional precautions as they are at higher risk.

25.10.3 Public Health Significance

While the author could find no recorded cases of a horse transmitting rabies to a human, the possibility exists. Many people have received post-exposure prophylaxis (PEP) for rabies after handling confirmed cases of the disease in equids. The PEP is known to be very effective especially when promptly started. The disease is of significant public health importance due to high mortality in untreated people. Horses should be properly vaccinated. Animals with neurologic disease or rapid onset of death should be tested for rabies so that prompt PEP can be administered to exposed people when necessary.

25.11 Ringworm

Ringworm caused by *Trichophyton* spp., is common in many mammalian species, including horses. It can also be caused by *Microsporum* spp. Both causes of ringworm are zoonotic (Pascoe and Knottenbelt 1999).

25.11.1 Clinical Signs in Horses

Ringworm (*Trichophyton* spp.) can cause initial circular areas of hair loss with a silvery, scaly appearance to the underlying skin. These areas can be localized, in a region of the body, or generalized across the body. Lesions can expand and nearby areas can coalesce. Common sites include the neck, shoulder and girth areas. *Microsporum* spp. can cause hair loss on the face and legs, but since this organism can be spread by insects, lesions can appear on the body whereever insects land.

25.11.2 Transmission to Humans

Ringworm is spread between animals and people by direct and indirect transmission via touching contaminated skin, equipment, the horse's environment and other materials. Organisms can be found in the soil and are hardy in environmental conditions. People should wear protective gloves when handling horses with ringworm and infected horses and equipment should be kept away from healthy horses. Disinfection of the premises and horse items is also important to controlling an outbreak.

25.11.3 Public Health Significance

Ringworm is treatable in animals and people. The disease does have public health significance since the disease can spread through people who are in close contact, such as school children. However, the disease has a very low mortality in healthy individuals.

25.12 Salmonellosis

Salmonellosis is a common disease of animals and humans with worldwide distribution. *Salmonella enterica* subsp. *enterica* causes equine disease. While multiple serovars have been identified in horses, S. Typhimurium, S. Javiana and S. Newport have been commonly found in the U.S. Any serovar isolated from animals is a potential zoonotic pathogen.

25.12.1 Clinical Signs in Horses

Horses can develop a multitude of disease conditions from ingesting this bacteria: severe acute diarrhea, septicemia, abortions, and localized infections. Horses can also be inapparent carriers.

25.12.2 Transmission to Humans

Direct and indirect contact with infected animals, their equipment and environment are the most common exposures to people. Any animal that has diarrhea should be handled with disposable gloves and protective clothing with close attention to hand hygiene. Personnel who are caring for sick animals should have strict biosecurity protocols in place to prevent human and additional animal exposure. Salmonellosis is also a food-borne illness when contaminated, undercooked horse meat is consumed by people (Anderson and Lee 1976; Espie et al. 2005).

25.12.3 Public Health Significance

Salmonellosis is a significant global health concern. In the U.S., transmission of *Salmonella* spp. from horses to humans can occur, but few cases are actually adequately documented (Bender et al. 2001; Morse et al. 1978). Individuals caring for sick animals, veterinarians, pathologists and laboratory personnel are at higher risk and need to wear appropriate PPE. In other countries, the incidence of salmonellosis directly linked to exposure to horses may be higher, especially in countries where consuming undercooked horsemeat is customary.

25.13 Trichinellosis

The intestinal nematode *Trichinella* is a common concern in humans eating undercooked contaminated pork and wild boar meat. However, horsemeat can also be the cause of trichinellosis in people. Contaminated horsemeat has been traced back to Eastern Europe, Poland, Serbia, Mexico, Canada and the USA. *Trichinella spiralis, T. britovi* and *T. murelli* have been detected in horsemeat (Ancelle 1998; Ancelle et al. 1993; Liciardi et al. 2009; Murrell et al. 2004).

Horses ingest the parasite from being fed *Trichinella* contaminated meat products, and it has been shown that some horses will eat meat voluntarily (Murrell et al. 2004), especially those in poor nutritional condition.

25.13.1 Clinical Signs in Horses

Unknown

25.13.2 Transmission to Humans

Ingesting raw or undercooked infested horsemeat is the mode of transmission.

25.13.3 Public Health Significance

Since multiple human outbreaks of trichinosis have occurred due to horsemeat, the disease has public health importance. Educational efforts need to be directed to the public to cook horsemeat to 160 °F (71 °C) unless the meat has been cured or irradiated to kill the parasite.

25.14 Venezuelan Equine Encephalomyelitis (VEE)

VEE is caused by a virus of the family Togavirus and was first associated with equine disease in the 1930s in Venezuela. The virus has been found in Mexico, Central and South America, and in the U.S.

Antigenic variants I-AB and I-C have caused epizootics of disease in horses; I-D and I-E and other subtypes are enzootic in geographic areas where they cycle between mosquitoes and rodents. However, in 1993 and 1996 two VEE outbreaks in southern Mexico were caused by the variant I-E in equids (Oberste et al. 1998). The last outbreak in the U.S. was in 1971.

25.14.1 Clinical Signs in Horses

After horses are bitten by mosquitoes or other biting flies carrying the virus, severe neurological signs develop in 12–16 h. Central nervous system signs of blindness, circling, head tilt, head pressing, ataxia and lethargy are shown by affected horses. Horses become recumbent and death is usually 5–14 days after clinical signs begin. The case fatality rate is 38–80 % (Scherer et al. 1972; Tigertt and Downs 1962). Equine vaccines for VEE are commercially available in the U.S.

25.14.2 Transmission to Humans

Horses are the source of the virus during disease outbreaks via biting insects. Mosquitoes ingest blood and virus from an infected animal, and the virus is amplified in the salivary glands and gut. This virus-laden fluid is injected into the next victim the mosquito bites. VEE virus is found in the body fluids of infected horses; direct contact with other horses and humans, however, does not appear to be a routine route of transmission.

25.14.3 Public Health Significance

While VEE infection in people results in influenza-like symptoms with low mortality, the sporadic nature of epizootics and the mutation potential for this virus make it unpredictable. VEE virus is also a potential bioterrorism agent.

25.15 Vesicular Stomatitis

Vesicular stomatitis is caused by the vesicular stomatitis virus (VSV) of the Rhabdoviridae family. In the U.S., serotypes New Jersey and Indiana are found, and other serotypes cause disease in South America. The disease is endemic in the U.S., Mexico and Central and South America (McCluskey 2007).

25.15.1 Clinical Signs in Horses

The disease is transmitted primarily by insect vectors, although other means of transmission are possible. The reservoir for VSV is yet unknown. After an incubation time of 3–7 days, horses can develop a fever, vesicles on the lips, tongue, oral mucosa, hard palate and coronary band. These vesicles eventually burst and leave ulcers and erosions. The vesicle fluid and skin lesions are sources of virus. Ventral edema and scabbing of the udder and prepuce are also possible. The disease is self-limiting and resolves with good nursing care.

25.15.2 Transmission to Humans

Human infections are rare and occur primarily in laboratories. However, these individuals are highly trained in biosafety and know to use appropriate PPE. Individuals who handle VSV cases without gloves are at higher risk.

25.15.3 Public Health Significance

VSV has a low public health impact due to the mild disease it causes in people, and its uncommon occurrence.

25.16 Summary

While not all equine zoonoses have significant or known public health significance, several are classified as emerging diseases such as Hendra virus and MRSA. Currently six equine diseases are listed as potential bioterrorism agents by the Centers for Disease Control and Prevention: anthrax, brucellosis, cryptosporidiosis, glanders, *Salmonella* spp. (food safety), and VEE (http://www.bt.cdc.gov/agent/agentlist.asp). More horse disease information can be found in the textbooks such as Equine Infectious Diseases by D.C. Sellon and M.T. Long (Elsevier, 2007; second edition due in 2013); Equine Internal Medicine, 3rd edition by S.M. Reed, W.M. Bayly and D.C. Sellon (Elsevier, 2010); and Veterinary Clinics of North America: Equine Practice, Vol.16 No.1, December 2000, Emerging Infectious Diseases by P.J. Timoney (ed), (Saunders, 2000). A compact, concise public health manual can be found in Control of Communicable Diseases Manual, 19th edition, D.L. Heymann (ed) (American Public Health Association, 2008).

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Chapter 26
Animal Bites and Zoonoses: From A to Z: Alligators to Zebras

Ellie J. C. Goldstein and Fredrick M. Abrahamian

Abstract Worldwide, animal bite-related injuries to humans are a common daily occurrence. Injuries can range from minor puncture wounds to extensive crush injuries and even amputations and death. Increasing population, continued spread of habitation, and the popularity of owning various types of both traditional and non-traditional pets has made it easier for humans to have contact with various types of animals. In general, the oral flora of the biting animal will be concordant with the bacteria isolated from the wound. These oral flora organisms often originate from the normal oral residents and environmental flora as well as the skin and intestinal bacteria of the animals food sources and prey.

26.1 Summary

Worldwide, animal bite-related injuries to humans are a common daily occurrence. Injuries can range from minor puncture wounds to extensive crush injuries and even amputations and death. Increasing population, continued spread of habitation, and the popularity of owning various types of both traditional and non-traditional pets has made it easier for humans to have contact with various types of animals. In general, the oral flora of the biting animal will be concordant with the bacteria isolated from the wound. These oral flora organisms often originate from the normal oral residents and environmental flora as well as the skin and intestinal bacteria of the animals food sources and prey.

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26.2 Introduction

A wide variety of domestic and wild animals are reported to bite people worldwide each year causing injuries that range from minor infections to debilitating and even lethal injuries. The increasing population, continued spread of habitation, and popularity of owning various types of both traditional and non-traditional pets has made it easier for humans to have contact with various types of animals. It is therefore not surprising that this increased exposure can consequently result in more bite injuries.

Most bite wounds are minor injuries that go unreported. Patients often self-administer first aide and usually do not seek or require medical attention. In rural areas and non-industrialized settings, medical care is often hard to obtain and not readily available. In contrast, residents of industrialized countries, especially those in urban and suburban areas, often seek care when incurring moderate to severe bite injuries in an emergency department or in a physician's office. When these injuries are reported, it is usually from small, limited studies that focus on a specific aspect or complication of injury, or concentrate on unusual or resistant organisms. These small often retrospective studies form the only basis for the medical decision-making and treatment of bite wounds that is currently employed worldwide.

Bite wounds can consist of lacerations, evulsions, punctures, scratches, and crush injuries. Although the majority of patients never seek, or do not require extensive medical care, awareness of the magnitude of the infectious complications from bites is necessary. The bacteria associated with bite infections may come from the environment, the victim's skin flora, or most frequently, the oral flora of the biter which can also be influenced by the microbiome of their ingested prey and other food.

The public health implications for animal bite wounds includes not only the cost of therapy, but that of resultant physical disability, both acutely and in the long term, days lost from work or school, costs of hospitalization, and insurance claims. In 2003, the Insurance Information Institute noted that the average dog bite claim cost insurers $ 16,600 totaling greater than $ 320 million annually, in addition to direct care costs of approximately $ 165 million (Quinlan and Sacks 2003).

26.3 Animal Ownership/Contact

In 2013, the Humane Society of the United States estimated that 83.3 million dogs (47% of United States households) and 95.6 million cats were kept as pets, and 56% of households having some variety of pet (http://www.humanesociety.org/issues/pet_overpopulation/facts/pettownership_statistics.html). In addition, Americans keep 8.3 million pet birds, 4.6 million horses, 57.6 million fish, 750,000 ferrets, 3.2 million rabbits, 3.5 million rodents, 1.5 million snakes as well as a variety of other animals (https://www.avma.org/KB/Resources/Statistics/Pages/Market-research-statistics-US-pet-ownership.aspx).

In the United Kingdom, it is estimated that 45 % of households have pets including 8.5 million dogs and 8.5 million cats (http://www.pfma.org.uk/pet-population/). Across Europe there are 70 million pet-owning households (excluding Russia), who own at least one pet animal. This estimation includes 25 % cats and 26 % dogs (http://www.fediaf.org/facts-figures). There is an estimated 17.8 million pet cats and 12.2 million pet dogs in Russia, ranking second only to the United States on the number of pets per capita (http://www.gov.mb.ca/agriculture/statistics/agri-food/russia_pet_food_en.pdf). The Australian Companion Animal Council reported 3.4 million dogs in 36 % of households and 2.4 million cats in 23 % of Australian households (http://www.acac.org.au/pet_care.html). Additionally, there are over 1 million other pets including horses, rabbits, guinea pigs and other small animals (http://www.acac.org.au/pet_care.html). Estimates for other geographic areas are incomplete.

As more people begin to backpack, enjoy ecotourism and push habitations into more rural areas, there is a potential for increased contact with wild animals. Although no estimates exist for the numbers of these contacts, this growing trend and exposure could certainly result in a higher incidence of bite injuries.

26.4 Dog Bites

The Health Care Utilization Project reported on emergency department visits and hospital stays related to dog bites for 2008 noted 316,200 emergency department visits (103.9 per 100,000 population) and 9,500 hospital admissions (2.5 % of patients with bite injuries) related to dog bites which had increased 86.3 % since 1993 (www.hcup-us.ahrq/gov/toolssoftware/ccs/ccs.jsp). Children less than 10 years old had a higher rate of emergency department visits (199.3/100,000 population) than other age groups. Patients residing in rural areas were more likely to visit emergency departments (119.3/100,000 population) and be hospitalized than those from urban settings (29.4/100,000 population). Emergency department visits for dog bites were more common in the Midwest and Northeast United States and lowest in the Western United States. Skin and soft-tissue infections were the cause of 43 % of hospitalizations followed by open wounds to the extremities (22.1 %).

The average cost of a dog bite hospitalization was $ 18,200 which was about 50 % more than for other injury-related hospital admissions with an average stay of 3.3 days. Approximately 58 % of dog bite admissions required a surgical procedure, most commonly debridement of an infected wound followed by suturing of wounds, muscle and tendon repair-related procedures and incision and drainage of abscesses. In the United Kingdom, Hospital Episode Statistics recorded 6,450 admissions for dog bites or “strikes” in a period of 12 months in 2012, a 5.2 % rise compared the prior 12 months period (http://www.pfma.org.uk/pet-population/, http://www.gov.mb.ca/agriculture/statistics/agri-food/russia_pet_food_en.pdf). The incidence of dog bites worldwide has been estimated by extrapolating similar above information and using population statistics to estimate their occurrence (http://www.rightdiagnosis.com/d/dog_bite/stats-country.htm).

It has been estimated that one of every 2 Americans will be bitten in their lifetime, usually by a dog. Dog bites occur in 4.7 million Americans yearly (Sacks et al. 1996) and account for 800,000 medical visits, including approximately 1 % of all emergency department visits (Weiss et al. 1998). Most dog bites (85 %) are provoked attacks by either the victim's own pet or a dog known to the victim and occur during the warm weather months (Goldstein et al. 1980). Bite wounds that require attention are often those to the extremities, especially the dominant hand. Facial bites are more frequent in children younger than 10 years and lead to 5–10 deaths per year, often because of exsanguination (Lockwood 1997).

Larger dogs can exert more than 450 lbs./in.^{2} of pressure with their jaws, which can lead to extensive crush injuries. Home insurance companies may decline to insure homeowners with certain breeds of dogs that are considered to be more aggressive than others. Even insured owners may not be able to find insurance again, once a dog has been reported as a biter. These breeds often include: chows, Rottweilers, Dobermans, Akitas, wolf hybrids, bull terriers, pit bulls, and shepherds.

Patients who present early after an incident often are concerned about crush injuries, care of disfiguring wounds, or the need for rabies or tetanus immunization (Goldstein et al. 1980). Between 2 and 30 % of wounds will become infected and may require hospitalization (Goldstein 1992; Brook 1987; Talan et al. 1999). Patients presenting later than 8 hours after injury usually have established infection. Infections can range from localized cellulitis or abscess to septic arthritis, osteomyelitis, tenosynovitis and rarely, severe sepsis and septic shock. The distribution of bite wounds is shown in Fig. 26.1 (Talan et al. 1999). Fatal infections may occur in certain compromised hosts, as in patients with asplenia, cirrhosis or on steroids which may be due to *Capnocytophaga canimorsus* (Brenner et al. 1989). Women who have undergone radical or modified radical mastectomy or patients with preexisting edema of an extremity due to any cause are at increased risk of infection.

Dog bite wound infections are predominantly related to the microbiology of their oral flora (Goldstein et al. 1980; Brook 1987; Talan et al. 1999). Table 26.1 lists common pathogens found in dog wound infections (Talan et al. 1999). *Pasteurella multocida* is a major and important pathogen but one should note that *Pasteurella canis* is more frequently isolated than *Pasteurella multocida* subspecies *multocida*. Compared to cat bites, this may, in part, account for the greater potential for infection from cat bite wounds. The spectrum of organisms associated with dog bite wound infections is much greater and includes streptococci, staphylococci as *Staphylococcus intermedius* and *Staphylococcus aureus* and anaerobic organisms (Talan et al. 1999). In one large study, the median number of strains isolated from infected dog bite wounds was 2–7.5. Non-purulent, but infected wounds had two strains per specimen compared to 7.5 strains for abscesses (Talan et al. 1999). Forty-eight percent of dog bites grew both aerobes and anaerobes, including 67 % of abscesses, 62 % of purulent wounds, and 13 % of non-purulent wounds (Talan et al. 1999). No growth occurred less than 10 % of the time which may suggest that other fastidious organisms may be involved in infected bite wounds.

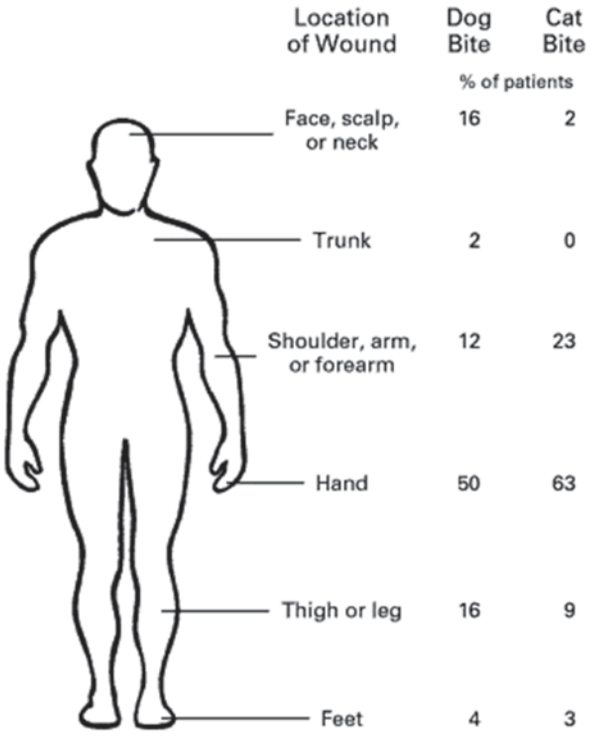

Fig. 26.1 Anatomic distribution of 50 dog and 57 cat bite wound infections. (Source: Talan et al. 1999 with permission)

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been cultured from a variety of companion animals including dogs, although not yet reported as isolated from infected dog bite wounds in humans. MRSA should be considered as a potential causative secondary invader, especially in patients who are not responding to initially administered antibiotics that often do not exhibit activity against MRSA and those known to be colonized or have had prior infections with MRSA.

The antimicrobial treatment of non-infected dog bite wounds that present less than 24 h after injury remains a controversial issue. The Cochrane reports do not recommend prophylactic antimicrobials for these wounds but the data is flawed as it is based on only a few biased studies with small numbers of patients and incomplete bacteriology (Medeiros and Saconato 2001). Recently, a report (Quinn et al. 2010) on the outcomes of 94 patients and in a cost-model using sensitivity analysis across rates of infections from 0 to 16%, determined that "if the risk of wound infection was greater than 5%" then antibiotics could decrease that rate and be "cost effective." It is the authors' opinion that all moderate to severe bite wounds, especially those to the hands, injuries associated with moderate to severe swelling (pre-existing or as the result of trauma), bites in immunocompromised hosts and in proximity to a bone or joint, except those not clinically infected and more than a few days old, should be considered contaminated with potential pathogens and treated.

26.5 Cat Bites

Worldwide, cat bites account for 2–50 % of injuries related to animal-bites. They are second only to dog bites in terms of incidence. In Italy, the incidence of cat-related injuries is 18 per 100,000 population, while in the United States, there are an estimated 400,000 cat bites and 66,000 related visits to emergency departments every year (http://www.who.int/mediacentre/factsheets/fs373/en/).

Wounds inflicted by cats are frequently scratches or tiny but somewhat deep punctures located on the extremities, which are at higher risk of becoming infected (Lucas and Bartlett 1981). Figure 26.1 shows the comparative distribution of cat bite wounds compared to dog bite wounds (Talan et al. 1999). Deep puncture wounds over or near a joint, especially on the hands may result in osteomyelitis and septic arthritis. *Pasteurella multocida* has been isolated from 50 to 70 % of healthy cats and is a frequent pathogen in cat-associated wounds (Goldstein 1992; Talan et al. 1999).

Patients who present with an infected cat bite often have more severe infections than those with infected dog bites, which may be attributed to the higher incidence of *Pasteurella multocida* and *Pasteurella septica* in cat bites compared to dog bites (Table 26.1). Cat scratches are likely to get infected from the cats grooming themselves and inoculating *Pasteurella multocida* onto their claws. *Erysipelothrix rhusiopathiae* has been isolated from 4 % of cat bite wounds (Talan et al. 1999).

Most cat bite wounds are mixed aerobic infections (64 %) with a median number of isolates per wound of five to seven per specimen. There are 5 isolates per specimen (range, 0–12) for non-purulent but infected wounds compared to 6.5 per specimen (range, 0–13) for purulent wounds and seven per wound (range, 3–13) for abscesses (Talan et al. 1999). Five percent of infected wounds do not grow a pathogen, suggesting that there are other fastidious organisms present that require special media for isolation. Cougar, tiger, and other feline bites also yield *Pasteurella multocida* (Burdge et al. 1985; Kizer 1989). Tularemia has likewise been transmitted by cat bites (Capellan and Fong 1993). Cat bites may be treated differently in a court of law as cats are more independent and therefore are not subject to the same scrutiny as dog bites.

26.6 Venomous Snake Bites

Approximately 600 species of venomous snakes exist worldwide with an estimated 5 million people bitten annually. In the United States, only 50–70 % of venomous snake bite victims are envenomated as the remainder are “dry” bites. Still, considerable morbidity and mortality occurs from these injuries. There are an estimated 2.4 million envenomations and 94,000–125,000 deaths annually, resulting in 400,000 amputations and other complications, such as infection, tetanus, scarring, contractures, and psychological consequences. Access to health care and the availability of antivenom decreases the severity of the injuries and improves patient outcomes (http://www.who.int/mediacentre/factsheets/fs373/en/).

The majority of snake bites occur in Africa and Southeast Asia and are most common among people living in rural, resource-poor settings, especially agricultural

Table 26.1 Common aerobic and anaerobic bacteria isolated from infected dog and cat bite wounds. (Source: Talan et al. 1999 with permission and updated)

Organism	Frequency (%)	
	Dog ($n=50$)	Cat ($n=57$)
Aerobes		
***Pasteurella* species**	*50*	*75*
P. multocida subspecies *multocida*	12	54
P. multocida subspecies *septica*	10	28
P. canis	26	2
P. dagmatis	4	7
P. stomsatis	12	4
P. multocida subspecies *gallicida*	2	0
Pasteruella species, other	2	0
***Staphylococcus* species**	*46*	*35*
S. aureus	20	4
S. epidermidis	18	18
S. warneri	6	11
***Streptococcus* species**	*46*	*46*
S. mitis	22	23
S. mutans	12	11
S. pyogenes	12	0
S. sanguis II	8	12
S. intermedius	6	4
***Neisseria* species**	*32*	*35*
N. weaverii	14	14
***Corynebacterium* species**	*12*	*28*
Group G	6	5
C. minutissimum	4	7
C. aquaticum	2	14
***Moraxella* species**	*10*	*35*
***Enterococcus* species**	*10*	*12*
***Bacillus* species**	*8*	*11*
***Pseudomonas* species**	*6*	*5*
P. aeruginosa	2	0
***Weeksella* species**	*4*	*7*
***Capnocytophaga* species**	*2*	*7*
Anaerobes		
***Fusobacterium* species**	*32*	*33*
F. nucleatum	16	25
F. russii	2	14
***Porphyromonas* species**	*28*	*30*
P. gulae	4	11
P. canoris	4	9
P. macacae	6	7
***Prevotella* species**	*28*	*19*
P. heparinolytica	14	9
P. intermedia	8	0
***Propionibacterium* species**	*20*	*18*
P. acnes	14	16

Table 26.1 (continued)

Organism	Frequency (%)	
	Dog ($n=50$)	Cat ($n=57$)
***Bacteroides* species**	*18*	*28*
B. tectus	14	28
B. fragilis	2	2
B. ovatus	2	0
***Peptostreptococcus* species**	*16*	*5*
P. anaerobius	8	5

workers, women and children. The socioeconomic impact and burden of snake bites on these families and communities is increased in these settings (http://www.who.int/mediacentre/factsheets/fs373/en/). While snake bites are uncommon in Europe, a review of the literature from 1970 to 2010 found 7,992 snake bite reports with an even distribution between Northern, Southern and Central Europe, including Russia and Turkey (Chippaix 2012). Most bites occurred between May and September, and 15% were considered severe.

Venomous snakes, usually vipers (rattlesnakes, copperheads, cottonmouths, and water moccasins), bite approximately 8000 people in the United States yearly, of which five or six result in death, usually in children or the elderly, who receive either no or delayed antivenom therapy (Russell 1969). The majority of bites occur in young men in the Southwestern United States between April and September (Gold et al. 2002).

Envenomation can cause extensive tissue destruction and devitalization that predisposes to infection from the snake's normal oral flora. Sparse data exists on the incidence and bacteriology of snakebite infections. In rattlesnakes, the oral flora appears to be fecal in nature because the live prey usually defecates in the snake's mouth coincident with ingestion. Common oral isolates include *Pseudomonas aeruginosa*, coagulase-negative staphylococci, *Proteus* and *Clostridium* species, *Bacteroides fragilis* and *Salmonella arizonae* (*Salmonella* groups IIIa and IIIb) (Russell 1969; Goldstein et al. 1979).

Several treatment guidelines have been published (Lavonas et al. 2011; Walk 2012). In Nigeria, the Health Ministry has attempted to make antivenom more readily available to rural endemic areas and have created a "hub-and-spoke" strategy as a component of "antivenomics" to improve the selection and purchasing of locally appropriate antivenoms (Habib 2013).

26.7 Monkey/Simian

Bites from monkeys typically occur in people who keep them as pets, use them for medical research and those who travel to cities and countries that have a high prevalence of free roaming monkeys (e.g., Gibraltar, Bali, certain parts of India). Monkey bites account for 2–21% of animal bite injuries (http://www.who.int/mediacentre/

factsheets/fs373/en/). In India for example, monkeys are second to dogs for animal bite injuries. Approximately, 11% of bite wounds in United States military personnel stationed in Afghanistan are due to *Macaca mulatta* monkeys, often kept by locals as pets (Mease and Baker 2012).

An emergency department in Los Angeles (Kizer 1979) reported that 1.7% (5/332) of patients who presented during 1975 for bite wounds had monkey bites. Rates of the percentage of monkey bite wounds have been reported from a number of countries and range from 3.2% in India to 0.7% in Israel (Eslamifar et al. 2008; Gross and Torok 1984; Ichhpujani et al. 2008). In the United Kingdom, an animal facility reported 85 (67 incidents in men and 18 in women) monkey bites in handlers over a 6 year period (Tribe and Noren 1983). In addition, persons who visit or work in wilderness areas and national parks worldwide where monkeys reside are also at higher risk. These animals are often considered "mischievous" and will scour for food if hungry. Simple wound management of monkey bites may not prevent potential infectious complications and a protocol for post-bite exposure treatment has been published (Tregle et al. 2011; Newton and United States Armed Forces 2010).

The spectrum of isolates from humans bitten by monkeys is similar to those isolated from human bite wounds. There is a predominance of α-hemolytic streptococci, enterococci, *Staphylococcus epidermidis*, *Neisseria* and *Haemophilus* species, *Eikenella corrodens* and anaerobes including *Bacteroides* and *Fusobacterium* species (Goldstein et al. 1995). Monkeys can naturally acquire *Bartonella quintana* (trench fever) and they may potentially act as vectors (O'Rourke et al. 2005). Transmission of viral diseases is a major health and economic concern with monkey contact and bites (Estrep et al. 2010). Non-human primates are susceptible to a variety of pathogens that bear significant homology to human pathogens. These same viruses pose a potential health issue to humans and can include herpes B virus, Simian varicella virus, Rhesus cytomegalovirus, gamma-herpesviruses, lymphocryptoviruses, herpes saimiri, Rhesus Macaque rhadinoviruses and others (Estrep et al. 2010).

In monkeys from Nepal the prevalence of selected enzootic primate-borne viruses through positive antibody response among 39 rhesus monkeys in Katmandu has been reported (Jones-Engel et al. 2006). The various viruses found included simian foamy virus (97.4%), *Cercopithecine herpesvirus* 8 (94.9%), simian virus 40 (89.7%), and *Cercopithecine herpesvirus* 1 (64.1%; also known as B virus). Documented cases of B virus infection in humans have mostly been attributed to monkey bites. However, other less commonly reported modes of transmission have been due to scratches and percutaneous inoculation with infected materials (Centers for Disease Control and Prevention 1998). A fatal case of B virus infection has been reported following a mucocutaneous exposure (Centers for Disease Control and Prevention 1998).

Other reports of bite-related primate-borne viral infections in humans have included simian foamy virus (especially from ape bites) and monkeypox infections (Calattini et al. 2007; Mutombo 1983; Schweizer 1997), yellow fever virus with the arbovirus carried from one host to another, primarily between monkeys, from monkeys to humans, and from person to person. Sylvatic (or jungle) yellow fever

occurs in tropical rainforests where monkeys are infected by wild mosquitoes and then pass the virus to other mosquitoes that feed on them. The infected mosquitoes bite humans entering the forest, resulting in occasional cases of yellow fever. The majority of infections occur in young men working in the forest (e.g., for logging) (http://www.who.int/mediacentre/factsheets/fs100/en/).

Hepatitis A virus can infect various monkey species such as chimpanzees, owl monkeys, cynomolgus monkeys, rhesus monkeys, stump-tailed monkeys, African green monkeys, tamarins, marmosets and squirrel monkeys. The transmission of human Hepatitis A virus from experimentally infected animals to humans has occurred and been documented (http://www.who.int/mediacentre/factsheets/fs100/en/). Still unknown is the susceptibility of humans to true simian Hepatitis A virus strains (http://www.who.int/mediacentre/factsheets/fs100/en/).

While man is the natural host for Hepatitis E virus, chimpanzees, cynomolgus monkeys, rhesus monkeys, pigtail monkeys, owl monkeys, tamarins and African green monkeys are reported to be susceptible to natural infection with human strains of hepatitis E virus making transmission possible.

The monkeypox virus can cause a fatal disease in humans. It is similar to human smallpox, although typically much less serious. It occurs primarily in remote villages in Central and West Africa, near tropical rainforests. The monkeypox virus is transmitted to people from a variety of wild animals and it spreads in the human population through human-to-human transmission (http://www.who.int/mediacentre/factsheets/fs100/en/).

Malaria due to *Plasmodium knowlesi* also known as "monkey malaria" can occur in humans while staying in rainforests or their fringe areas in Southeast Asia, within the range of the natural monkey hosts and mosquito vector of this infection. These areas include parts of Cambodia, China, Indonesia, Laos, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Vietnam. Travelers to forested areas of Southeast Asia where human *Plasmodium knowlesi* infections have been reported should protect themselves against mosquito bites between dusk and dawn to prevent infection and take the usual chemoprophylaxis where indicated (http://www.who.int/mediacentre/factsheets/fs100/en/).

26.8 Bears

Bear attacks occur worldwide and are, in part, an ecological conflict, often where humans visit or decide to work or live in areas where these large carnivores inhabit (Cardall and Rosen 2003; De Giorgio et al. 2007; Frosch et al. 2011; Herrero 1970; Herrero and Fleck 1990; Mihailoviv et al. 2011; Nabi et al. 2009; Risholt et al. 1998; Tough et al. 1993; Vougiouklakis 2006). In Norway, from 1971 to 1995 there were 80 incidents involving human-bear interactions of which there were 4 fatalities and 6 injuries many of which were due to bites (Risholt et al. 1998). A review of wild animal bites in Kashmir from 2005 to 2007, noted that 51.2 % (104/203) were caused by black bears (Nabi et al. 2009). In North America, human injuries from

grizzly bears (*Ursus arctos horribilis*) in the national parks have been reported at a rate of one person per 2 million visitors (Herrero 1970).

From 1900 to 1985, 162 bear-inflicted injuries (approximately 2 attacks per year) were reported in the United States and Canadian national parks (Herrero and Fleck 1990). Although bear-inflicted human injuries and death are uncommon (Floyd 1999), as the remote bear habitat decreases and humans enter wilderness areas for living and recreation, there are more chances of encounters between bears and humans. These have occurred in all hemispheres where bears are resident and some have even occurred in zoos (Mihailoviv et al. 2011).

The bacteriology of bear oral flora and bear bite wounds to humans is limited to a few studies and case reports (Floyd et al. 1990; Kunimoto et al. 2004; Lehtinen et al. 2005; Parry et al. 1983; Rose 1982). The bacteria isolated from bear bite wounds include *Serratia fonticola*, *Serratia marcescens*, *Aeromonas hydrophila*, *Bacillus cereus*, and *Enterococcus durans*. Lehtinen et al. reported a case of a 56-year-old male who sustained several bite wounds from a brown bear (*Ursus arctos*) that grew *Streptococcus sanguis*, *Neisseria sicca*, *Bacillus* species, and *Mycobacterium fortuitum* (Lehtinen et al. 2005). Those patients that survived a bear attack required air-lifting to nearby medical facilities.

Rabies virus infection in bears has also been reported (Centers for Disease Control and Prevention 1999). However, to our knowledge there have not been any reports of rabies transmission from bears to humans.

26.9 Pigs

Pigs are aggressive animals and their bite injuries are a common occupational hazard, and may also occur to those who own pigs as pets. Nogalski et al. reported 5.13% (96/1,872) of animal-related injuries seen between 2001 and 2004 were related to pigs, either bites or battering or both (Nogalski et al. 2007). These injuries were more often from rural (88.5%; 85/96) areas than urban (11.5%; 11/96). Injuries, often on extremities, commonly occur during capture, transport, or immobilization of the pig (Barnham 1988; Nishioka 1994; Van Demark and Van Demark 1991). Unusual pig bites have included a de-gloving injury to the penis and the prolapsed rectum of a child (Georgiou et al. 2001; Gangopadhyay et al. 2002). In Southeastern Brazil, a case series from a teaching hospital reported 23 pig bites from 1987 to 1990 and estimated the annual incidence to be 1.5/100,000 population (Nishioka et al. 1994). They reported a male to female ratio of 6.7:1 and a median age of 36 years for victims.

Only a few human wound infections after a pig bite have been reported (Barnham 1988, Ejlertsen et al. 1996; Escande et al. 1996; Goldstein et al. 1990). Organisms isolated from these patients included *Streptococcus agalactiae, Streptococcus equisimilis, Streptococcus suis, Pasteurella aerogenes, Proteus species, Escherichia coli, Bacteroides* species including *Bacteroides fragilis*, *Pasteurella multocida*, coagulase-negative *Staphylococcus*, *Streptococcus milleri* and *Myroides*

odoratimimus (Ejlertsen et al. 1996; Escande et al. 1996; Goldstein et al. 1990; Maraki et al. 2012). Identification of bacteria isolated from pig bites in humans is problematic because they cannot be identified or are misidentified by commercial kits and conventional methods (Lindberg et al. 1998).

Recent studies have shown a high prevalence of nasal MRSA colonization in people commonly in contact with live pigs (Khanna et al. 2008; Köck et al. 2009; Smith et al. 2009; Van Cleef et al. 2010). Molecular characterization of MRSA found in pigs and humans in contact with pigs has revealed a *Staphylococcus aureus* protein A (spa) type t108 and sequence type (ST) 398 (van Belkum et al. 2008; van Loo et al. 2007; Huijsdens et al. 2006). Clonal spread of MRSA and transmission through family members of a pig farmer, his co-workers, and his pigs have been reported (Huijsdens et al. 2006).

Although rare, rabies infection has been reported worldwide in pigs (DuVernoy et al. 2008; Morehouse et al. 1968; Yates et al. 1983; DuVernoy et al. 2008; Luo et al. 2013). However, to our knowledge there have not been any reports of rabies transmission from pigs to humans, although there is a report of a rabid pig biting humans playing golf in India (articles.timesofindia.indiatimes.com/2012-03-01/chennai/3111278). Although there are no reports of bite transmission, Hepatitis E virus infections in humans has been associated with pig contact and ingestion of raw pig products (Ruggeri et al. 2013; Dalton et al. 2013).

Pigs may also carry *Clostridium difficile* (Fry et al. 2012), and toxigenic PCR ribotypes found in pigs correspond to PCR ribotypes associated with human disease in hospitalized patients in the Netherlands (Koene et al. 2012). Although not a bite related infection, *Clostridium difficile* disease may potentially be transmitted to humans.

26.10 Horses

Humans and horses have shared a close relationship with one another for over thousands of years. Throughout the world, millions of people have had contact with horses through recreation, sporting, or for occupational reasons. In the United States, it is estimated that there are 4,856,000 pet horses kept by 1.5% of households (1,780,000) with an average of 2.7 horses per household (https://www.avma.org/KB/Resources/Statistics/Pages/Market-research-statistics-US-pet-ownership.aspx). In the United Kingdom, it is estimated by the British Horse Industry Confederation (http://www.bhic.co.uk/downloads/sizescope.pdf) that there are 1 million horses kept by 550,000 owners which extrapolates to 17 horses per 1000 population or 4.3 per km^2. Horse riding is estimated to be a more popular sport than rugby, cricket or fishing, with greater than 7% of the population riding at least one time annually.

It is estimated that between 3 to 4.5% of all animal bites are due to horses (Langley and Morris 2009). Carithers reported that 5 out of 157 (3%) animal bites seen in children in Jacksonville, Florida over a 20-month period were due to horse bites

(Carithers 1958). In England, over a 2-year period, a local hospital reported 622 patients with horse-related injuries of which 24 (3.8%) were bite wounds (Edixhoven et al. 1981). In that series, few had extensive muscle damage and most injuries healed uneventfully. In Lublin, Poland, (Nogalski et al. 2007) it was noted that 2.4% of animal-related injuries were due to horses and that they occurred in equal frequency in urban and rural areas. In contrast, horse bites accounted for 17% of animal bite injuries and were second only to dog bites (69%) in eastern Turkey, during a period of 2 years (Emet et al. 2009). However, in Pune, India only 0.4% of bite cases were due to horses (Shetty et al. 2005).

Horses and zebras share the same genus, so we suspect that their oral flora will likely be similar. Most reports of the bacteriology of horse bite wounds in humans have revealed infections to be polymicrobial with a mixture of aerobic and anaerobic organisms (Dibb et al. 1981; Peel et al. 1991; Benaoudia et al. 1994). *Actinobacillus lignieresii* has often been reported in infected wounds of humans bitten by horses (Dibb et al. 1981; Peel et al. 1991; Benaoudia et al. 1994). *Actinobacillus* species, specifically *Actinobacillus suis* has been found to be a part of normal horse oral and upper respiratory tract floras (Bisgaard et al. 1984; Kim et al. 1976) and has been isolated from a horse-bite (Peel et al. 1991). Horses are known to carry MRSA (Hartmann et al. 1997; Van den Eede et al. 2013; Schwaber et al. 2013), including the ST398 and ST568 strains (Gómez-Sanz et al. 2013), but no reports of horse bite infection due to MRSA have yet been reported.

26.11 Komodo Dragon

Komodo dragons migrated from Australia to the Indonesian Islands of Rinca, Flores and Gili Motang. These largest living lizards can reach a length of approximately 10 feet and weigh up to 150 pounds. They are held in captivity in many zoos around the world. They are carnivores and eat mostly carrion but will prey on birds and mammals. Komodo dragons can bite people and some attacks may lead to death.

The myth of the Komodo bite's lethal ability is related to its oral bacterial flora has recently been disproven by the finding of venom glans in their oral cavity (Fry et al. 2009). It is postulated that the venom is able to kill smaller, more appropriate sized prey but that the larger prey die from delayed wound sepsis due to open tear wounds and secondary infection obtained at watering holes. Goldstein et al. 2013 studied captive Komodo oral flora and found a variety of aerobic gram-negative rods (1–8 per specimen), especially *Enterobacteriaceae*, aerobic gram-positive bacteria (2–9 per specimen), especially *Staphylococcus sciuri* and *Enterococcus faecalis*, and anaerobes (1–6 per specimen), especially *Clostridia*. As with other carnivores, captive Komodo oral flora is simply reflective of the gut and skin flora of their recent meals and environment, and is unlikely to cause rapid fatal infection. To our knowledge, there have not been any reports on the bacteriology of infected human wounds from Komodo bites.

26.12 Alligator/Crocodile

Both the alligator population and human encounters with alligators have increased in the United States (Langley 2005). The same situation exists for crocodiles in Australia and Asia (Caldicott et al. 2005; Gruen 2009; http://factsanddetails.com/Asian.php?itemid=2432&subcatid =434). From 1948 to 2004, there were 376 injuries and 15 deaths reported from alligators in the United States (Langley 2005). In 2009, 11 provoked and 8 unprovoked alligator attacks were reported in Florida (Florida Fish and Wildlife Conservation Commission Historic Alligator Bites on Humans in Florida) (Langley 2005). Unprovoked bites were defined as bites on human beings by wild alligators, which were not provoked by handing or intentional harassment. A review of crocodile attacks in Asia notes that the Nile and salt water crocodiles are most likely to prey on humans, the former most commonly in sub-Saharan Africa and the latter in New Guinea, Borneo and the Solomon Islands. They also note 18 of 31 fatal attacks by salt water crocodiles occurred in Australia between 1970 and 1996 (http://factsanddetails.com/Asian.php?itemid=2432&subcatid =434).

The microbiology of human wounds inflicted by alligators or crocodiles is limited to a few case reports where wound cultures grew mainly aquatic environmental organisms as well as bacterial flora from the skin and intestines of prey such as *Aeromonas hydrophila*, *Enterobacter agglomerans*, *Citrobacter diversus*, *Enterococcus* species, and *Clostridium* species (Flandry et al. 1989; Wamisho et al. 2009). In Malawi (Wamisho et al. 2009) a review of 5 patients bitten by crocodiles grew *Citrobacter* species and other investigators have isolated *Vibrio vulnificus*, *Citrobacter* species, *Burkholderia pseudomallei*, *Pantoea agglomerans*, *Bacteroides melaninogenicus*, *Aeromonas hydrophila*, *Serratia fanticola*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. Caimans are very aggressive and bites from Brazil have been reported. It was speculated that the oral flora of the caiman might be similar to that of the alligators in the United States (Hertner 2006). The oral and cloacal flora of wild crocodiles in the Mexican Caribbean included *Aeromonas hydrophila*, and also *Salmonella arizonae* and *Salmonella typhi* which could pose a threat to humans from both bites and from the aquatic environment (Charruau et al. 2012).

26.13 Rodents/Rats

It is estimated that annually there are 20,000 rat bites in the United States with children the most common victims and most wounds to the face or hands (Hirschhorn and Hodge 1999; Ordog et al. 1985; Elliott 2007; Glaser et al. 2000). Most cities in the United States are populated by the Norway rat, black rat or the house mouse. Rats and other rodents have also gained popularity as pets. The Centers for Disease Control and Prevention (CDC) in the United States initiated a limited national surveillance program for animal bites in 1971 and reported that 4.3 % of the 196,684

cases were due to rat bites (Moore et al. 1977). In Philadelphia, 622 rat bite cases were confirmed from 1974 to 1996 (Hirschhorn and Hodge 1999). It is estimated that approximately 100 persons are bitten by rats in New York City annually and the Department of Health has a proactive, active surveillance "rat-indexing" program that inspects properties and tries to abate substandard living environments to reduce this number (Bragdon et al. 2012; Childs et al. 1998).

Infections from rat bites occur in less than 2% of bitten individuals. Ordog et al. (Ordog et al. 1985) conducted a prospective study of 50 patients with uninfected rat bite wounds and found that only one patient developed an infection. The bacterial isolates they cultured from wounds were mostly skin flora organisms such as *Staphylococcus epidermidis, Bacillus subtilis*, alpha-hemolytic *Streptococcus*, and diphtheroids. In Tanzania, 34 Type-II male diabetic patients with peripheral neuropathy were bitten by rats during sleep which resulted in 4 deaths and 17 minor or major amputations (Abbas et al. 2005). *Actinobacillus equuli* has been isolated from the nasopharynx of laboratory mice and rats and *Actinobacillus lignieresii* has been isolated from the nasopharynx of laboratory rats (Lentsch and Wagner 1980). Cases of rat bite-associated infections in humans with *Corynebacterium kutscheri* (Holmes and Korman 2007) and *Leptospira* (Gollop et al. 1993; Luzzi et al. 1987) have also been reported.

Most attention has been focused on rat bite fever, an ancient disease caused by *Streptobacillus moniliformis*, a fastidious highly pleomorphic, filamentous, gram-negative rod, and *Spirillum minus*, a short, tightly coiled gram-negative rod (Elliott 2007). *Streptobacillus moniliformis* infection is more common in North America while *Spirillum minus* is more common in Asia. Rat bite fever is rare in the United States and its incidence is unknown since it is not a nationally reportable disease.

From 1996 through 1998, Norway rats (*Rattus norvegicus*) from downtown Los Angeles were examined and seroprevalence rates in rats were 25.9% for *Rickettsia typhi*, 6.7% for Seoul virus and 73.1% for hepatitis E virus (Smith et al. 2002). Fifty-two percent of blood specimens collected from rats grew *Bartonella elizabethae*-like isolates when cultured. However, in local skid row residents, the prevalence of antibodies to hepatitis E virus was 13.6%, *Bartonella elizabethae* 12.5%, *Bartonella quintana* 9.5%, Seoul virus 0.5%, and *Rickettsia typhi* 0%.

26.14 Sharks

The International Shark Attack File reported 118 alleged shark attacks in 2012 of which 80 were confirmed as unprovoked and 16 were provoked. The number of unprovoked attacks has steadily increased since the early 1900s and may reflect increased opportunities of interaction due to water sports and time spent in the seas by humans. Conversely, the shark population has declined worldwide. From 1999–2009 there were 455 shark attacks in the United States, and the United States (including Florida, Hawaii and Puerto Rico) is the most common country where they occur (http://www.flmnh.ufl.edu/fish/sharks/isaf/isaf.htm). The majority of attacks

occurred in Florida (294 attacks), Hawaii (42 attacks), South Carolina (32 attacks), and California (30 attacks). Worldwide, during the same period, 700 shark attacks were reported with 51 (7.3%) being fatal attacks.

The highest numbers of attacks worldwide, in order of decreasing frequency, were reported from Florida, Australia, Hawaii, South Africa, and California. There were seven fatalities due to unprovoked shark attacks reported in 2012 resulting in a fatality rate of 8.8% (http://www.flmnh.ufl.edu/fish/sharks/isaf/isaf.htm). The United States fatality rate was 1.9% compared to 22.2% in the rest of the world. Approximately 60% of incidents occur in surfers and boarders compared to 22% in swimmers and 8% in divers. Shark attacks obviously have an economic on the specific vacation spots and beaches involved.

The oral aerobic flora of a male great white shark from Connecticut waters was obtained and various isolates of *Vibrio* species such as *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio fluvialis* were recovered. Other isolates included *Pseudomonas putrefaciens*, "gold-pigmented" *Staphylococcus* species, *Citrobacter* species, and *Micrococcus* species (Buck et al. 1984).

Vibrion carchariae, has been isolated from an infected wound in a shark bite victim swimming off the South Carolina coast (Pavia et al. 1989) and in two cases of infections following shark bites in Australia, the wound cultures grew *Vibrio parahaemolyticus* and *Aeromonas caviae*, and the other *Vibrio alginolyticus*, *Aeromonas hydrophila*, *Proteus* species, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Clostridium freundii*, and an *Enterococcus* species (Royle et al. 1997). Most of these isolates are aquatic organisms and should be considered when selecting antimicrobial prophylaxis (Rtshiladze et al. 2011).

Interaminense et al. (2010) cultured the oral flora of sharks involved in human attacks in Recife, Brazil and found the majority were enterobacteria such as *Enterobacter*, *Citrobacter, Proteus* species, *Providencia alcalifaciens, Escherichia coli, Moellerella wisconcensis* and *Leclercia adecarboxylata*. Other Gram-negative organisms isolated included *Vibrio* species, *Burkholderia cepacia, Acinetobacter* species and *Pseudomonas* species. Gram-positive strains were also isolated including coagulase-positive and coagulase-negative *Staphylococcus* species, *Enterococcus* species, *Micrococcus* species and viridans streptococci.

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Part V
Zoonoses of Wildlife Species

Chapter 27
Vector-Borne Zoonoses

Filipe Dantas-Torres and Domenico Otranto

Abstract Vector-borne zoonoses (VBZ) constitute a group of illnesses caused by a plethora of pathogenic agents affecting animals and humans worldwide. A range of vectors, such as mosquitoes, ticks, fleas, phlebotomine sand flies, lice, and kissing bugs, may transmit numerous bacteria (e.g., *Rickettsia conorii*, *Rickettsia rickettsii*, and *Borrelia burgdorferi*), protozoa (e.g., *Babesia divergens*, *Babesia microti*, *Plasmodium knowlesi*, and *Trypanosoma cruzi*), helminths (e.g., *Dirofilaria immitis*, *Dirofilaria repens*, *Onchocerca lupi*, and *Thelazia callipaeda*), and viruses (e.g., Crimean-Congo haemorrhagic fever virus, West Nile virus, and tick-borne encephalitis virus) to animals and humans. The burden of several VBZ is still considerable in poor rural areas in tropical and subtropical regions; some of these diseases represent a current public health concern in developed countries as well. A number of factors, such as increases in travel and trade, climate and land-use changes, and socioeconomic and political upheavals, may drive or alter the dynamics of VBZ in animals and humans. In this chapter, we provide an overview about selected aspects of VBZ affecting animal and human populations around the world. Unresolved issues regarding the epidemiology and control of this group of zoonoses are also addressed.

27.1 Vector-Borne Zoonoses

Vector-borne zoonoses (VBZ) constitute a group of diseases caused by a wide range of pathogenic organisms, including bacteria (e.g., *Rickettsia conorii*, *Rickettsia rickettsii*, and *Borrelia burgdorferi*), protozoa (e.g., *Babesia divergens*, *Babesia microti*, *Plasmodium knowlesi*, and *Trypanosoma cruzi*), helminths (e.g., *Dirofilaria immitis*, *Dirofilaria repens*, *Onchocerca lupi*, and *Thelazia callipaeda*), and viruses (e.g., Crimean-Congo haemorrhagic fever virus, West Nile virus, and tick-borne encephalitis virus) (Colwell et al. 2011; Dantas-Torres et al. 2012b; Kilpatrick and

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Randolph 2012; Otranto et al. 2013). These pathogenic agents may be transmitted to animals and humans through the bite (during blood feeding) of a variety of arthropods, such as mosquitoes (family Culicidae), fleas (order Siphonaptera), lice (order Phthiraptera), phlebotomine sand flies (subfamily *Phlebotominae*), black flies (family Simulidae), biting midges (family Ceratopogonidae), kissing bugs (subfamily Triatominae), fruit flies (subfamily Steganinae), and ticks (order Ixodida). Some diseases such as plague, a flea-borne disease that claimed the lives of thousands of people since the Byzantine Empire (Gage and Kosoy 2005; Raoult et al. 2013), have been part of humankind for long time and have changed the course of our history. In recent years, a number of new VBZ have also been described (Dantas-Torres et al. 2012b), with direct implications for the diagnosis of this group of zoonoses, whose clinical features may overlap (Paddock et al. 2008).

The epidemiology and distribution of VBZ are influenced by a set of factors, but the presence of animal hosts and arthropod vectors in a given area is a *sine qua non* condition for the enzootic and zoonotic cycles to occur. For instance, small mammals may act as hosts for a number of pathogens (e.g., *Babesia microti* and *Borrelia burgdorferi*) potentially causing disease in humans and may also serve as hosts for arthropod vectors, such as ticks (Dantas-Torres et al. 2012a). Importantly, the complex interactions between animal hosts, arthropod vectors and people occurring in the enzootic and zoonotic transmission cycles of vector-borne pathogens partly explain the difficulties faced by public health authorities trying to control this group of illnesses.

The burden of VBZ is still heavier in poor rural areas in tropical and subtropical regions, where arthropod vectors find a perfect environment for their perpetuation and where access to health care services is often deficient. For example, leishmaniasis and Chagas disease are still one of the leading causes of disability worldwide and responsible for thousands of deaths, mainly in rural and peri-urban areas of several developing countries (Alvar et al. 2006; Bern et al. 2008; Christou 2011; Lozano et al. 2012). Furthermore, climatic, socioeconomic and political changes have caused a profound impact on the epidemiology and distribution of VBZ, some of which currently represent a public health concern in industrialized countries as well (Vorou et al. 2007; Otranto et al. 2013). In the present chapter, we provide an overview on selected aspects of VBZ affecting animal and human populations around the world. Unresolved issues regarding the epidemiology and control of this group of zoonoses are also addressed.

27.2 Morbidity and Mortality

From a global perspective, VBZ such as leishmaniasis, Chagas disease, and African trypanosomiasis are still causing a considerable burden in terms of morbidity and mortality in a number of countries around the world. Particularly, the burden of such VBZ is still evidently heavier in low-income countries as compared with high-income countries. On the other hand, several VBZ such as tick-borne encephalitis

and Lyme disease are also increasing in many industrialized countries (Bhate and Schwartz 2011). Nonetheless, it is difficult to estimate the actual burden of VBZ, mainly in developing countries due to the absence of surveillance and/or deficiencies in the national case notification system. Furthermore, many cases of VBZ remain without a definitive diagnosis, particularly in remote rural areas where the access to basic health care services is still incipient.

Leishmaniasis is a group of phlebotomine sand fly-borne diseases caused by species of *Leishmania*, which are prevalent in at least 98 countries and three territories in all continents, except Oceania (Alvar et al. 2012); the *Leishmania* species circulating presently in Australia is apparently restricted to kangaroos. The global burden of leishmaniasis in terms of morbidity and mortality has recently been reassessed. It has been estimated that approximately 0.2–0.4 million visceral leishmaniasis cases and 0.7–1.2 million cutaneous leishmaniasis cases occur each year in endemic regions (Alvar et al. 2012). Remarkably, more than 90% of all visceral leishmaniasis cases reported worldwide occur in six countries: India, Bangladesh, Sudan, South Sudan, Brazil, and Ethiopia. Similarly, Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru are responsible for 70–75% of estimated cutaneous leishmaniasis incidence. It was also estimated that 20,000–40,000 leishmaniasis deaths occur each year. Nevertheless, the number of cases and deaths that remain underreported in remote rural areas is probably higher than currently appreciated.

Trypanosomiases are neglected tropical diseases caused by *Trypanosoma* species, which might be transmitted by different arthropod vectors. American trypanosomiasis (Chagas disease) is caused by *Trypanosoma cruzi*, which is primarily transmitted by kissing bugs. In spite of the efforts towards the elimination of the vectorial transmission of *T. cruzi* in endemic areas, Chagas disease is still a leading cause of morbidity and mortality worldwide. Indeed, it is estimated that 10–20 million people are infected with *T. cruzi*, mostly in Latin America, causing between 20,000 and 50,000 deaths per year (Tarleton and Curran, 2012). Differently, African trypanosomiasis (sleeping sickness) is caused by species of *Trypanosoma* (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*), which are transmitted by tsetse flies (*Glossina* genus). The disease is restricted to sub-Saharan Africa, where 70 million people are estimated to be at risk of infection. Importantly, the number of cases of sleeping sickness in Africa decreased about 82% in recent years (e.g., 37,991 cases in 1998 and 6,743 cases in 2011), which is the result of the control efforts towards the elimination of the disease from this continent (Simarro et al. 2012).

Tick-borne bacterial diseases such as Lyme disease, Rocky Mountain spotted fever, Mediterranean spotted fever, granulocytic anaplasmosis, monocytic ehrlichiosis, and Q fever constitute emerging public health concerns worldwide (Christou 2011; Dantas-Torres et al. 2012b). For instance, Lyme borreliosis is on the rise in Europe, where more than 50,000 cases are reported each year. Similarly, over 250,000 cases of Lyme borreliosis were reported between 2000 and 2010 in the United States (Dantas-Torres et al. 2012b). Furthermore, human granulocytic anaplasmosis has been increasing in incidence and expanding its distribution in the United States in recent years (Christou 2011).

Arboviruses (i.e., arthropod-borne viruses) such as yellow fever virus, dengue fever viruses, Japanese encephalitis virus, West Nile virus, tick-borne encephalitis, Chikungunya virus, Rift Valley fever virus, and Crimean-Congo haemorrhagic fever virus are responsible for considerable morbidity and mortality worldwide (LaBeaud et al. 2011). For instance, the World Health Organization official estimates indicate that 50–100 million dengue infections occur annually around the globe (WHO 2009), but a more recent estimate increased this figure to 390 million dengue infections per year (Bhatt et al. 2013). Moreover, around 200,000 cases and 30,000 deaths by yellow fever are estimated to occur each year in tropical areas of Africa and Latin America, respectively (WHO 2013). Similarly, more than 67,000 cases of Japanese encephalitis are estimated to occur each year throughout most Asia and parts of western Pacific, with a case-fatality rate ranging from 10–30% (Hu et al. 2012). Furthermore, more than 5300 West Nile virus disease cases occurred in 2012 in the United States, representing the highest number of cases reported to the Centres for Disease Control and Prevention since 2003 (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm).

The recent introduction and subsequent emergence of several exotic VBZ (e.g., West Nile virus, Chikungunya virus, and thelaziasis) worldwide appears to be a function of several biotic and abiotic factors, including increased travel and trade, which ultimately favoured the introduction and/or the expansion of arthropod vectors. Nonetheless, the emergence or re-emergence of endemic VBZ (e.g., Lyme disease, tick-borne encephalitis, leishmaniasis, and malaria) has been attributed to climate changes, land-use and social changes, which have greatly impacted on the ecology of vector species (Cowell et al. 2011; Kilpatrick and Randolph 2012).

27.3 Drivers and Dynamics of VBZ

At the local level, the probability (or the risk) of being exposed to and, thus, becoming infected by a vector-borne pathogen depends on the contact with competent vectors and reservoirs, which in turn may be influenced by several factors (e.g., living conditions, labour and leisure activities, knowledge of the disease transmission and control). From a broader perspective, the dynamics of VBZ in a given area are driven by several biotic (e.g., animal and vector population densities) and abiotic factors (e.g., land-use and social changes, increased travel and trade), which may vary in time and space.

In the past three decades, a number of exotic VBZ were introduced and established into new areas. For instance, canine thelaziasis is a VBZ caused by the spirurid nematode *Thelazia callipaeda*, which is transmitted by the fruit fly *Phortica variegata* (Fig. 27.1) (Otranto et al. 2013). The disease was initially thought to be restricted to the former Soviet Union and to countries in the Far East (the People's Republic of China, South Korea, Japan, Indonesia, Thailand, Taiwan, and India), but nowadays it is widespread in Europe (Otranto et al. 2013). Cases have been reported in several European countries, including Italy, Belgium, France, Portugal, Spain, and Switzerland (Caron et al. 2013; Otranto et al. 2013). The emergence of thelaziosis in

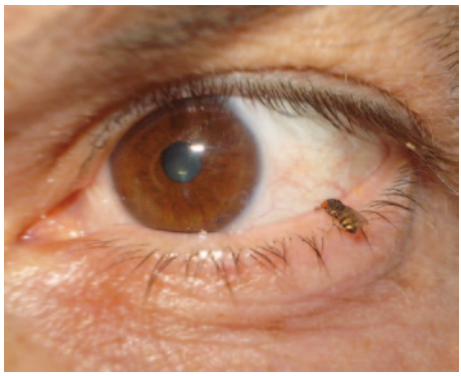

Fig. 27.1 *Phortica variegata*, the vector of *Thelazia callipaeda,* feeding on a human eye

several European countries has been attributed to several factors, such as movement of infected animals, local changes in vector ecology and distribution, as well as to increased awareness of medical physicians, veterinary practitioners and parasitologists. Indeed, the accelerating increase in trade and travel is considered as the main driver of exotic vector-borne pathogen introductions into non-endemic areas in the past five decades (Kilpatrick and Randolph 2012). For instance, infected livestock and people as well as migratory or dispersing birds are potential carriers for the introduction of exotic pathogens (e.g., Crimean-Congo haemorrhagic fever, Chikungunya virus, and West Nile virus) into new regions (Kilpatrick and Randolph 2012).

Upon arrival in a new area, an exotic pathogen needs to find suitable reservoir hosts and vectors to establish locally. For example, the immigration waves of Europeans from leishmaniasis endemic countries (e.g., Portugal, Spain, and Italy) into the New World, since the arrival of the Conquistadores, resulted in multiple introductions of *Leishmania infantum*, the causative agent of zoonotic visceral leishmaniasis, into this region (Kuhls et al. 2011; Dantas-Torres et al. 2012c). The establishment of *L. infantum* in the New World was likely facilitated by the presence of highly susceptible human populations (due to malnutrition and co-infections with pathogens endemic in those areas) and of competent animal hosts, such as foxes, domestic dogs and other wild animals co-habiting the same environment (Desjeux 2004; Dantas-Torres et al. 2012c). Afterwards, the existence of a ubiquitous competent vector (*Lutzomyia longipalpis*), migration waves from rural to urban areas and increased trade have probably favoured the rapid spread of zoonotic visceral leishmaniasis in some Latin American countries such as Brazil (Desjeux 2004; Kuhls et al. 2011; Dantas-Torres et al. 2012c). In addition, the presence of an established vector population and of susceptible hosts that are immunologically naïve to the introduced pathogen might result in explosive epidemics of VBZ (Kilpatrick and Randolph 2012).

The emergence of endemic VBZ might be driven by several factors, which may bring large contingents of non-immune people into contact with vectors. Road building, oil prospecting, mining, farming, irrigation, forestry development, tourism as well as political upheavals, military conflicts, and natural disasters (e.g. the hurricane Katherine) have been associated with the emergence of several VBZ such as dirofilarioses, leishmaniasis, malaria, yellow fever, and tick-borne encephalitis

(Desjeux 2004; Cowell et al. 2011; Kilpatrick and Randolph 2012; Antinori et al. 2013). For example, the largest outbreak of cutaneous leishmaniasis recorded in Colombia occurred during 2005–2009 in soldiers of the Colombian Army, when roughly 40,000 cases were detected (Vélez et al. 2012). In this outbreak, military incursions into the jungle, with the mission to combat illicit crops and the guerrilla, resulted in exposure of soldiers and military dogs to infected phlebotomine sand flies. Similarly, the emergence of malaria by *Plasmodium knowlesi* in south-eastern Asia has been associated with males who had a history of visiting or staying for some days in jungle areas, where wild macaques and anopheline mosquitoes maintain the natural cycle of the parasite (Singh et al. 2004; Lee et al. 2011; Antinori et al. 2013).

While deforestation (for road building, establishment of grazing areas, crop plantations, etc.) may be associated with the emergence of VBZ, reforestation has also been linked with changes in local abundance of animal hosts and arthropod vectors of certain pathogens. For instance, the emergence of Lyme disease in north-eastern United States in the mid-twentieth century has been partly attributed to the rise in deer (*Odocoileus virginianus*) and tick (*Ixodes scapularis*) populations as a result of reforestation occurring during the twentieth century (Barbour and Fish 1993). On the other hand, forest fragmentation in eastern regions of Canada and the United States and changes in predators' communities have been associated with increases in the relative abundance of wildlife hosts of *Borrelia burgdorferi* and in the infection prevalence in nymphal ticks (Logiudice et al. 2008; Levi et al. 2012). Certainly, forest fragmentation and/or reforestation may exert profound changes in wildlife host communities, with direct effects on arthropod vectors and associated pathogens. The effects of these changes in the dynamics of several VBZ may be unpredictable.

Socio-economic and political changes may also increase the risk of VBZ. For example, the upsurge of tick-borne encephalitis in central and eastern European states has been correlated with poverty and household expenditure on food, after the collapse of the Soviet Union (Sumilo et al. 2008). Indeed, changes in land-use, reduced use of pesticides, increased unemployment and poverty might have resulted in increased interactions between people and infected ticks. The linkage between poverty and VBZ is well documented for some neglected diseases, such as leishmaniasis, African trypanosomiasis and Chagas disease. For example, the cracked walls and damp earth floors, together with an absence of sanitation and inadequate garbage collection in impoverished urban and peri-urban settings create phlebotomine sand fly breeding sites and increase the risk of leishmaniasis (Alvar et al. 2006). In the same way, people living in poor rural communities in Latin America, in proximity to forest areas and under precarious housing conditions and underlying poverty are at risk of VBZ, such as Chagas disease (Briceno-Leon 1987).

27.4 Clinical and Diagnostic Considerations

Patients (whether animal or human) suffering from VBZ may present with a suit of clinical signs and symptoms, ranging from a single, localized skin ulcer to severe, life-threatening systemic disease. For instance, canine and human patients affected

by American cutaneous leishmaniasis caused by *Leishmania braziliensis* may present localized skin ulcers or mucocutaneous lesions, whereas those suffering from zoonotic visceral leishmaniasis by *L. infantum* (syn. *L. chagasi*) may exhibit systemic clinical signs (e.g., weight loss, fever, lymph node enlargement, and hepatosplenomegaly) with potential fatal *exitus* (Lainson and Shaw 2005; Dantas-Torres 2009). Other species such as *Leishmania amazonensis* may induce a range of clinical signs in dogs and humans and eventually produce a visceral form of leishmaniasis that may be confounded with *L. infantum* infections (Lainson and Shaw 2005; Dantas-Torres 2009),

Human patients affected by spotted fever rickettsiosis may present with unspecific clinical signs and symptoms (e.g., flu-like illness) in the early stages of the disease (Dantas-Torres 2007), potentially leading to misdiagnosis, delays in treatment initiation, and death. Certainly, the variety of clinical signs and symptoms animals and humans may present when suffering from VBZ make the clinical diagnosis a challenging task for physicians and veterinary practitioners working in both endemic and non-endemic areas. In endemic areas, they may be more used to typical clinical presentations of endemic VBZ, but atypical cases in immunosuppressed individuals and co-infections may further complicate the clinical diagnosis.

Vector-borne nematodes may cause ocular infestations in animals and humans (Otranto and Eberhard 2011). For instance, thelaziosis is an emerging VBZ of animals (e.g., dogs and cats) and humans caused by *T. callipaeda*, whose adults live primarily under the nictitating membrane of the eye and may induce lacrimation, epiphora, conjunctivitis, keratitis and even corneal ulcers (Otranto and Eberhard 2011). However, several other vector-borne nematode species may invade the eyes of animals and humans and must be included in the differential diagnosis. Indeed, we have recently received in our laboratory several nematode specimens from human patients, which were initially suspected to be *T. callipaeda*. As examples, a rare nematode (*Pelicitus* genus) was retrieved from the eye of a human patient from the Amazon region in Brazil (Bain et al. 2011), an enigmatic *Dirofilaria* sp. molecularly close to *Dirofilaria immitis* was characterized from a human patient from northern Brazil (Otranto et al. 2011a), and cases of *Onchocerca lupi* ocular infestations were diagnosed in patients from Turkey and Tunisia (Otranto et al. 2011b, 2012a). Remarkably, *O. lupi* is a little known, but emerging parasite of dogs in parts of the United States and Europe (Labelle et al. 2013; Otranto et al. 2012b), whose zoonotic potential was underestimated until recently. Finally, human infestations by *Dirofilaria* spp. often result in pulmonary nodule formations, which may be erroneously diagnosed as malignant neoplasm, hence representing a further challenge to physicians (Genchi et al. 2011).

A number of rickettsial organisms have been described in recent years and some of them have been implicated in human disease (Dantas-Torres et al. 2012b; Oteo and Portillo 2012). For example, *Rickettsia massiliae* was originally described from ticks in 1994 from France and recently found to be a ubiquitous emerging pathogen of humans (Vitale et al. 2006; Parola et al. 2008; García-García et al. 2010). All published cases of *R. massiliae* infections were clinically similar to Mediterranean spotted fever suggesting that many cases of *R. massiliae* infections are likely to be

misdiagnosed as by *Rickettsia conorii* infections (Parola et al. 2008). The same is true for *Rickettsia parkeri*, which has been described as a human pathogen in the United States and South America, where the disease is likely to be misdiagnosed as other infectious illnesses, including Rocky Mountain spotted fever, dengue fever, and leptospirosis (Romer et al. 2011).

The above mentioned examples illustrate how difficult the clinical diagnosis of VBZ may be and underline that medical physicians and veterinarians, as well as public health authorities should be prepared to react promptly in face of atypical cases in endemic and non endemic areas. It is vital to avoid delays in the treatment initiation, which may eventually result in the death of the patient (Dantas-Torres et al. 2012b).

27.5 Advancements in VBZ Diagnosis

There have been several advancements in the field of diagnosis of VBZ in the past few decades. The refinement of serological tests and the development of molecular biology tools, along with the decipherment of the genomes for several infective agents, have improved considerably our capacity to diagnose VBZ in animals and humans, allowing the detection and characterization of new, emerging pathogens. Moreover, the adoption of a holistic diagnostic approach has culminated in the discovery of new human pathogens, including several bacterial species. For instance, until recently *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, was the only rickettsial agent to be definitely associated with human diseases in the United States. In 2004, the use of a comprehensive diagnostic approach (including serological testing, immunohistochemical staining, cell culture isolation, and molecular methods) led to the description of the first cases of rickettsiosis by *Rickettsia parkeri*, a spotted fever group first identified 65 years ago in Gulf Coast ticks (*Amblyomma maculatum*) collected from the southern United States (Paddock et al. 2004). Remarkably, *R. parkeri* has been detected in *Amblyomma maculatum* and *A. triste* in several South America countries, which suggests that the distribution of this *Rickettsia* in the western hemisphere is probably wider than currently known. Furthermore, a case report from Argentina suggested that *R. parkeri* infections in this region are likely to be misdiagnosed as other infectious diseases, including Rocky Mountain spotted fever and dengue fever (Romer et al. 2011). The use of molecular tools has been proven essential for the correct aetiological diagnosis of tick-borne spotted fever rickettsioses in the United States and other countries of the western hemisphere.

Human malaria has traditionally been associated to four human-adapted *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*. Recently, a fifth species (namely, *Plasmodium knowlesi*) was implicated in several cases of human malaria across south-eastern Asia (Antinori et al. 2013). These cases were initially misidentified as *P. malariae* infections by microscopy, but refined molecular study revealed a large focus of naturally acquired

human infections by *P. knowlesi* in Malaysian Borneo (Singh et al. 2004). Importantly, the initial suspicion that another *Plasmodium* species was involved in human malaria in Malaysian Borneo was based on clinical (e.g., fever with chills and rigor, headache, cough, and vomiting), epidemiological (e.g., mostly adults), and laboratory data (e.g., high parasitaemia, over 5000 parasites per μl) (Singh et al. 2004). Once again, the '*P. knowlesi* example' underlines the importance of using a comprehensive approach, coupling clinical and epidemiological data with modern diagnostic test results towards a correct diagnosis of VBZ.

The use of molecular biology in epidemiological studies has also advanced our understanding of the transmission dynamics and origin of several pathogens causing VBZ. For long time, *Leishmania chagasi* was considered to be the agent of zoonotic visceral leishmaniasis in the New World and referred to as a distinct species from *L. infantum*. However, recent molecular analyses confirmed the Old World origin of the so-called '*L. chagasi*', which is now widely accepted as a synonym with *L. infantum* (Kuhls et al. 2011). Another example of the utility of molecular tools in the diagnosis of VBZ is represented by the first ever described case of zoonotic ocular infestation by *O. lupi* in a patient from Turkey (Otranto et al. 2011b). The nematode detected in a subconjunctival mass was detected on the superonasal quadrant of bulbar conjunctiva, but cut during the surgical removal. The nematode was initially identified as belonging to the genus *Onchocerca* and later on molecularly characterized as *O. lupi*. After this first occurrence, additional cases were detected in human patients from Turkey and Tunisia (Otranto et al. 2012a) as well as in dogs in Greece (Otranto et al. 2012b).

The development of rapid tests including point-of-care assays is also an important advancement in terms of diagnosis of VBZ such as leishmaniasis and Chagas disease. For instance, a new rapid test (PATH-Lemos rapid test) for the point-of-care diagnosis of Chagas disease was compared with a commercially available rapid test (Chagas STAT-PAK, Chembio). As compared to the reference test (the Ortho T. cruzi ELISA, Johnson & Johnson), the PATH-Lemos rapid test demonstrated an optimal sensitivity of 99.5 % and specificity of 96.8 %, respectively, while the Chagas STAT-PAK showed a sensitivity of 95.3 % and specificity of 99.5 % (Barfield et al. 2011). These results show that both rapid tests present high levels of sensitivity and specificity, representing reliable tools for screening and diagnosis of Chagas disease. As another example, a new rapid test (DPP® CVL rapid test) was recently made available for the diagnosis of canine visceral leishmaniasis in Brazil. A study reported high specificity (96 %) and sensitivity (98 %) in diseased dogs, but low sensitivity (47 %) in identifying parasite-positive dogs without clinical signs (Grimaldi et al. 2012). Nonetheless, this test is currently being used as a screening test by public health authorities in Brazil as part of the zoonotic visceral leishmaniasis control program in this country (Dantas-Torres et al. 2012c).

Undoubtedly, these advancements in the field of diagnosis of VBZ will influence the clinical practice of medical physicians and veterinary practitioners. Hopefully, these tools will also be made available for point-of care use in poor rural settings in VBZ-endemic countries in order to improve the diagnostic standard for VBZ in these areas.

27.6 Unresolved Issues

The epidemiology and distribution of VBZ is changing due to several factors, such as unplanned urbanization, illegal deforestation, changing demographics, economic crisis, increased global movement of animals and people, changes in human behaviour, land use and practices (Cowell et al. 2011; Kilpatrick and Randolph 2012). As a consequence, our understanding regarding several aspects (from aetiology to control) of VBZ has also changed in recent years. For example, the refinement and widespread use of genetic tools has greatly improved our capacity of detecting and identifying microorganisms in animals, humans and arthropods. Indeed, a number of microorganisms have recently been detected in arthropods (e.g., ticks) and some of them have been implicated in human disease (Dantas-Torres et al. 2012b). On the other hand, the pathogenic potential of several recently described organisms remain largely unknown and further research is fundamental to predict the emergence of novel VBZ in animals and humans.

The diagnosis of VBZ in animals and humans has advanced considerably in the past decades. For example, rapid immunochromatographic diagnostic tests and PCR-based tools have been developed (Dantas-Torres et al. 2012c), even if most of these tools are still largely restricted to research institutes and reference diagnostic centres. Indeed, the effective implementation of these new diagnostic tools in the field is far from reality in some VBZ-endemic areas. Considering the diversity of pathogenic agents that may potentially infect animals and humans in tropical and subtropical regions, the use of accurate diagnostic methods is fundamental to improve clinical practice. Similarly, a precise and rapid aetiological diagnosis is pivotal to expedite treatment decisions.

It is acknowledged that the control of VBZ in animals and humans is a difficult task due to the inherent complexities involved in the transmission cycles of these diseases. When a VBZ is established in a given area, it is very difficult to eradicate the pathogen and/or the vector or even to reduce the burden of disease. An appropriate example is the case of zoonotic visceral leishmaniasis, which is still causing considerable morbidity and mortality in endemic areas. In Brazil, for example, a control program against zoonotic visceral leishmaniasis has been in place since more than 50 years ago with no impact on the incidence of the disease in dogs and humans (Romero and Boelaert 2010). For any VBZ to be controlled, it is fundamental to reduce the contact between susceptible hosts (animals and people) and vectors. A number of tools for reducing the contact of animals with arthropods (e.g., phlebotomine sand flies, mosquitoes, ticks) have been developed in recent years, such as collars and spot-on pipettes containing active compounds with insecticide and repellent activity (Dantas-Torres et al. 2012c). Insecticide impregnated bed nets may reduce the exposure of humans to arthropod vectors and thus contribute to VBZ control (Kroeger et al. 2003). Unfortunately, most people living in poor rural communities, tropical and subtropical regions of the world cannot handle the costs of preventive tools. In such cases, public health authorities should elaborate and implement governmental programmes for the control of VBZ in order to reduce the burden of these diseases in animals and humans living in poor rural communities.

More research and continuous education for medical physicians and veterinarians is pivotal for controlling VBZ. Certainly, medical physicians and veterinarians are in the front line and should be up-to-date regarding recent advances in VBZ and base their clinical practice on the highest quality scientific evidence available. Improving case detection and reporting in developing countries is central to have more precise estimates of the burden of VBZ in these countries, which in turn is important to define priority in terms of control and management. In this context, the adoption of a One Health approach towards the management of VBZ has also been emphasized in recent years (Day 2011; Dantas-Torres et al. 2012b) and will be a critical step towards the control of these diseases. Indeed, it is imperative to reduce the gap of communication between medical physicians and veterinarians dealing with VBZ worldwide, particularly (but not exclusively) in developing nations. Certainly, this is a simple but important step towards reducing the burden of VBZ in animals and humans around the world.

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Chapter 28
Bat-Related Zoonoses

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Abstract The many emerging infectious diseases associated with Chiropteran species can have major impacts on both ecosystem and public health. As such, the scope of this chapter is to provide an overview of those potential bat-related zoonoses and their clinical relevance to people. With increased disease surveillance and a trend toward more human contact with bat populations, it is likely that additional zoonotic diseases will continue to be identified. Bat infection dynamics are driven by a complex interplay of ecological, immunological, behavioral, and anthropogenic factors. Interdisciplinary work will be needed in the future to better understand the drivers of disease emergence in bat populations and ultimately mitigate the threats that face both people and bats themselves.

Bats are increasingly implicated as hosts of zoonotic and potentially zoonotic pathogens. As a whole, chiropterans now represent the largest known reservoir of emerging viruses (Calisher et al. 2006; Wong et al. 2007). Amongst the 60 viral species currently associated with bats, 59 are RNA viruses of importance in the current generation of emerging and re-emerging human infections (Wong et al. 2007). Lyssaviruses, paramyxoviruses, filoviruses, and coronaviruses are amongst those pathogens impacting the health and well-being of both people and non-human animals around the globe. In comparison to the studies conducted on viral infections, much less attention has been paid to the non-viral pathogens of zoonotic importance within bat populations (Frick et al. 2010; Reichard et al. 2009). This is changing, however, as more research is now being conducted to detect and describe bacteria

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ranging from vector-borne to enteric pathogens, as well as protozoan parasites, and fungal agents in a variety of bat hosts.

The many emerging infectious diseases associated with chiropteran species can have major impacts on both ecosystem and public health (Calisher et al. 2006; Mühldorfer 2013; Wibbelt et al. 2010; Wood et al. 2012). As such, the scope of this chapter is to provide an overview of those potential bat-related zoonoses and their clinical relevance to people. With increased disease surveillance and a trend toward more human contact with bat populations, it is likely that additional zoonotic diseases will continue to be identified. Bat infection dynamics are driven by a complex interplay of ecological, immunological, behavioral, and anthropogenic factors (Hayman et al. 2012). Interdisciplinary work will be needed in the future to better understand the drivers of disease emergence in bat populations and ultimately mitigate the threats that face both people and bats themselves.

28.1 Viral Zoonoses

28.1.1 Rhabdoviridae

28.1.1.1 Rabies and Rabies-Related Viruses

Among bat-associated viral zoonoses, rabies (RABV) is certainly one of the most widespread in a broad range of bat species and around the world, with several new lyssaviruses identified in recent years. In several countries considered to be free of terrestrial rabies, rabid bats and human cases of bat-associated rabies have been identified in the last decades, such as in Australia where three human cases have occurred and in the United Kingdom, with one human case in Scotland (Banayrd et al. 2011; Fooks et al. 2003). In Latin America, more human rabies cases are now related to bat exposure (especially vampire bats) than to dog bites (Condori-Condori et al. 2013).

In Latin America, a review of the literature through 1990 reported 330 cases of bat-transmitted human rabies (Schneider et al. 2009). These cases, along with PAHO data to the end of 2006, revealed 637 reported cases of bat-transmitted human rabies in Latin America. Of 199 human cases transmitted by bats during the period 1996–2006, 146 (73%) were transmitted by vampire bats, 16 (8%) by non-vampire bats, and 37 (19%) with no species reported (Schneider et al. 2009). For instance, in Peru, during 2002–2007, 293 (77%) of the rabies cases diagnosed were associated with vampire bats, whereas 87 (23%) were related to dog rabies virus variants (Condori-Condori et al. 2013). It was also shown that vampire bat rabies variants spread gradually and involve different vampire bat subpopulations with different transmission cycles. Bovine paralysis caused by rabid vampire bat bites also has a major economical impact on cattle production in Mexico and several South American countries (Streicker et al. 2012). Emergence of rabies in insectivo-

rous bats in several countries in Latin America (such as Argentina, Brazil, Chile, Peru, and Uruguay) has also been reported.

In North America, rabies remains an important public health concern in the United States, with most human cases associated with bat rabies virus variants. Cases of rabies virus infection in bats are widely distributed across the continental United States (Patyk et al. 2012). Between 2001 and 2009, more than 205,439 bats were submitted for rabies virus diagnosis, and 6.7% of these bats were rabid. Increased odds of a submitted bat being rabid were associated with species that exhibit inconspicuous roosting habits, bats originating in the Southwest, and bats submitted for diagnosis during the fall (Patyk et al. 2012).

In Europe, bat rabies cases are principally attributed to two lyssaviruses, namely European bat lyssavirus-1 (EBLV-1) and European bat lyssavirus-2 (EBLV-2). Between 1977 and 2011, 961 cases of bat rabies were reported, with the vast majority (>97%) being attributed to EBLV-1, frequently isolated in The Netherlands, Northern Germany, Denmark, Poland and also in parts of France and Spain (Schatz et al. 2013). Most EBLV-2 isolates originated from the United Kingdom (UK) and The Netherlands, and EBLV-2 was also detected in Germany, Finland and Switzerland. There have been 25 suspected cases of EBLV-2, of which 22 have been confirmed. In addition, limited isolations of unique lyssaviruses from European insectivorous bats were reported in south-west Russia in 2002 (West Caucasian bat virus), in Germany in 2010 and France in 2012 (Bokeloh bat lyssavirus) (McElhinney et al. 2013; Picard-Meyer et al. 2013), and Lleida bat lyssavirus was recently identified in a bent-winged bat (*Miniopterus schreibersii*) in Spain (Aréchiga-Ceballos et al. 2013). A few human cases related to bat exposure have also been reported from Europe.

In Asia, limited reports on identification of lyssaviruses or antibodies to lyssaviruses have been published (Liu et al. 2013b). It is certainly a part of the world where new variants are likely to be identified in the near future, when better wildlife rabies surveillance will be set in this part of the world. Many aspects of the ecology of lyssaviruses in bats need still to be investigated, such as low prevalence of infection, potential survival to infection and effective shedding of the virus.

The incubation period of rabies in humans is typically 2 to 8 weeks, but can be as short as 10 days and as long as 6 years. Initial signs include headache, slight fever, malaise and pain at the bite wound. The disease which lasts from 2 to 6 days without medical support, progresses to paralysis of the muscles of deglutition, hyperesthesia and generalized convulsions. Death ensues shortly thereafter (Hoar et al. 1998). In bats, infection rate and mortality is usually low, although this has been studied in few species. Experimental studies in vampire bats indicate that a high viral load is necessary to induce mortality, with either no observable clinical signs or squeaking, tremor, paralysis and loss of appetite (Aguilar-Sétien et al. 2005). In cattle infected by vampire bats, rabies is mainly expressed by paralysis with a rather long incubation period (25–150 days or more) and lasts for 2–5 days before causing death (Hoar et al. 1998).

28.1.2 Paramyxoviridae: Henipaviruses

28.1.2.1 Hendra, Nipah, and Menangle Viruses

Several important zoonotic paramyxoviruses have been associated with animal and human deaths in Australasia since the end of the twentieth century. The henipaviruses are naturally harbored by Pteropid fruit bats (flying foxes) and some microbat species.

Hendra virus: In Australia, Hendra virus was first recognized in 1994 when 21 horses and two humans were infected, leading to the death of 13 horses and one human. As of December 2012, a total of forty-five outbreaks of Hendra virus have occurred in north-eastern Australia, all involving infection of horses (Aljofan 2013). As a result of these events, 90 animals (88 horses and two dogs) have died or been euthanized. These cases have all occurred in Queensland and in north–east New South Wales. Case fatality rate in humans is 60 % (4 of 7 recorded cases) and in horses 75 %. Human infections with Hendra virus range from mild influenza-like illness to fatal respiratory or neurological disease. Infected people initially develop fever, headaches, myalgia (muscle pain), sore throat and a dry cough. They could also have enlarged lymph nodes, lethargy and vertigo. The incubation period ranges from five to 14 days. Hendra virus is transmitted to people through close contact with infected horses or their body fluids. To date, no human-to-human transmission of Hendra virus has been documented. No specific treatment is available, but a vaccine has been developed for immunization of horses and is available since the end of 2012. The following signs have all been associated with Hendra virus cases in horses, but not all these signs will be found in any one infected horse: rapid onset of illness, increased body temperature/fever and heart rate, discomfort/weight shifting between legs, depression and rapid deterioration with either respiratory and/or nervous signs. Respiratory signs include respiratory distress, increased respiratory rates, nasal discharge at death that can be initially clear until progressing to stable white froth and/or stable blood-stained froth. Nervous signs include wobbly gait, apparent loss of vision in one or both eyes, aimless walking in a dazed state, head tilting and circling, muscle twitching, urinary incontinence and inability to rise. Horses get infected when very high concentrations of virus material are deposited directly under trees in what is call the ‘drip zone’ and almost no virus is deposited once the horses leave the perimeter of the trees. This area of the trees where the spats and the urine of feeding flying foxes will be dropped potentially poses an extremely high risk for horses (Australian Veterinary Association: http://www.ava.com.au/hendra-virus).

Nipah virus (NiV): In Malaysia and Singapore, in late 1998 and early 1999 an outbreak of human disease characterized by febrile encephalitis among pig farmers, which appeared to be linked to cases of respiratory and neurological disease in commercially farmed pigs, was described as well as in 11 employees at a slaughter plant in Singapore (Aljofan 2013; Clayton et al. 2013). There were 265 patients, of whom 105 died, reported as having NiV induced viral encephalitis, mostly among adult

males who were involved in pig farming or pork production activities. However, the reported number of patients who survived the acute NiV encephalitis was 160 with 7.5% prevalence of relapsed encephalitis (12/160 patients) more than 24 months after the outbreak. Of the 89 patients previously known to have non-encephalitic or asymptomatic Nipah virus infection, three (3.4%) developed late-onset encephalitis. Most patients presented with a severe acute encephalitic syndrome, but some also had significant pulmonary manifestations. The Malaysian outbreak was controlled by the culling of over one million pigs and strict quarantine measures on pig movements.

Nipah virus re-emerged in 2001 in outbreaks of human disease in India and Bangladesh. Since 2001, outbreaks of NiV infection have occurred almost annually in Bangladesh, with many outbreaks featuring smaller clusters of cases (Clayton et al. 2013). A second outbreak in India, close to the Bangladesh border, was reported in 2007. Sequencing and genetic characterization of these isolates revealed that they were closely related to, but distinguishable from the causative agent of disease in Malaysia. Since the emergence of NiV in Bangladesh and India, over 200 human cases have been identified, with an overall case fatality exceeding 70%. Most cases are related to consumption of unwashed fruits or palm juice contaminated by fruit bats secretions (saliva, urine, fecal materials). Outbreaks in Bangladesh and India were characterized by bat-to-human and human-to-human transmissions. *Pteropus* spp. serve as the wildlife reservoir for NiV across a wide area of South-east Asia, including countries from which no known outbreaks have emerged such as Cambodia, Thailand, Indonesia and Papua New Guinea. Seropositive bats for henipaviruses were also detected in Madagascar, Ghana and a henipavirus, or henipa-like virus, also appears to circulate in both fruit bats and microbats in China (Clayton et al. 2013).

Menangle virus: The Menangle virus, another paramyxovirus, was first identified in 1997 after a piggery in Menangle (New South Wales) experienced a high number of stillbirths (Aljofan 2013; Hoar et al. 1998). Two workers at the piggery became ill with unexplained, flu-like symptoms, but subsequently recovered. Investigations later found that the virus was transmitted from a nearby population of flying foxes, through pigs which act as a carrier of the virus. Bats appear to be an asymptomatic host, with infection caused through contact with body fluids for infected animals.

28.1.3 Filoviridae

28.1.3.1 Marburg and Ebola Viruses

Marburg virus: Marburg virus causes sporadic outbreaks of severe hemorrhagic disease in sub-Saharan Africa. Bats have been implicated as likely natural reservoir hosts based most recently on an investigation of cases among miners infected in 2007 at the Kitaka mine, Uganda, which contained a large population of Marburg virus-infected *Rousettus aegyptiacus* fruit bats (Amman et al. 2012).

In July and September 2007, miners working in Kitaka Cave, Uganda, were diagnosed with Marburg hemorrhagic fever. The likely source of infection in the cave was Egyptian fruit bats (*Rousettus aegyptiacus*) based on detection of Marburg virus RNA in 31/611 (5.1%) bats, virus-specific antibody in bat sera, and isolation of genetically diverse virus from bat tissues (Towner et al. 2009). The virus isolates were collected 9 months apart, demonstrating long-term virus circulation. The bat colony was estimated to be over 100,000 animals using mark and re-capture methods, predicting the presence of over 5000 virus-infected bats. The genetically diverse virus genome sequences from bats and miners closely matched. These data indicate common Egyptian fruit bats can represent a major natural reservoir and source of Marburg virus with potential for spillover into humans.

A study conducted at Python Cave in Uganda, where an American and a Dutch tourist acquired Marburg virus infection in December 2007 and July 2008, found that about 2.5% of more than 1600 bats captured between August 2008 and November 2009 were actively infected with the virus, seven of which yielded Marburg virus isolates (Amman et al. 2012). Moreover, Q-RT-PCR-positive lung, kidney, colon and reproductive tissues were found, consistent with potential for oral, urine, fecal or sexual transmission. The combined data for *R. aegyptiacus* tested from Python Cave and Kitaka mine indicate low level horizontal transmission throughout the year. However, Q-RT-PCR data showed distinct pulses of virus infection in older juvenile bats (~6 months of age) that temporarily coincide with the peak twice-yearly birthing seasons. Retrospective analysis of historical human infections suspected to have been the result of discrete spillover events directly from nature found 83% (54/65) events occurred during these seasonal pulses in virus circulation, perhaps demonstrating periods of increased risk of human infection.

Ebola virus: Evidence of Ebola virus antibodies was reported in various bat species in Africa (Pourrut et al. 2009) and of Ebola-Reston virus in *Rousettus amplexicaudatus* bats from the Philippines (Taniguchi et al. 2011), respectively. In Africa, 1030 animals were captured in Gabon and the Republic of Congo, including 679 bats, 222 birds and 129 small terrestrial vertebrates, and were tested for evidence of infection by Ebola virus (Leroy et al. 2005). Of the infected animals identified during these field collections, immunoglobulin G (IgG) specific for Ebola virus was detected in serum from three different bat species (4/17 *Hypsignathus monstrosus*, 8/117 *Epomops franqueti* and 4/58 *Myonycteris torquata*). Viral nucleotide sequences were detected in livers and spleens in other bats from the same populations (4/21, 5/117 and 4/141, respectively). No viral RNA was detected in kidney, heart or lung in these animals after amplification by polymerase chain reaction (PCR) and no viral nucleotide sequences were revealed in any of the other animal species tested.

Twelve years after the Kikwit Ebola outbreak in 1995, Ebola virus reemerged in the Occidental Kasaï province of the Democratic Republic of Congo (DRC) between May and November 2007, affecting more than 260 humans and causing 186 deaths (Leroy et al. 2009). During the latter outbreak several epidemiological investigations were conducted to identify the underlying ecological conditions and

animal sources. Qualitative social and environmental data were collected through interviews with villagers and by direct observation (Leroy et al. 2009). The local populations reported no unusual morbidity or mortality among wild or domestic animals, but they described a massive annual fruit bat migration toward the southeast, up the Lulua River. Migrating bats settled in the outbreak area for several weeks, between April and May, nestling in the numerous fruit trees in Ndongo and Koumelele islands as well as in palm trees of a largely abandoned plantation. They were massively hunted by villagers, for whom they represented a major source of protein. By tracing back the initial human-to-human transmission events, it was shown that in May the putative first human victim bought freshly killed bats from hunters to eat. This study provided the most likely sequence of events linking a human Ebola outbreak to exposure to fruit bats, a putative virus reservoir. Such findings support the suspected role of bats in the natural cycle of Ebola virus and indicate that the massive seasonal fruit bat migrations should be taken into account in operational Ebola risk maps and seasonal alerts in the DRC (Leroy et al. 2009).

28.1.4 Coronaviridae

28.1.4.1 SARS- and MERS-Coronaviruses

Severe acute respiratory syndrome (SARS) was first reported in February 2003 in China. When the World Health Organization declared the outbreak over on 5 July 2003, more than 8000 cases (and almost 800 fatal) had been reported in 32 countries worldwide (Field 2009; Wang et al. 2006). Initial symptoms are flu-like and may include fever, myalgia, lethargy symptoms, cough, sore throat, and other nonspecific symptoms, leading to sever pneumonia. The only symptom common to all patients appears to be a fever above 38 °C (100 °F). Shortness of breath may occur later.

A succession of phylogenetic and epidemiological findings suggested that SARS had a wildlife origin, and that ‘wet markets’ in southern China were the origin of the outbreak. Subsequently, two groups independently identified SARS-like coronaviruses (SARS-CoV) in species of bats in China. Li et al. (2005) reported serological and molecular evidence of a cluster of SARS-like coronaviruses in several species of free-living horseshoe bats (*Rhinolophus* spp.) in southern China. They contend that the virus responsible for the SARS outbreak in humans in 2003 emerged from this cluster of viruses, and that bats are the origin of the SARS coronavirus. *Rhinolophus* species are more likely to foster host shifts of coronaviruses than other bat species; this propensity, when combined with the potential for close contact between bats, civets and humans in the wildlife trade in southern China, supports SARS-like coronaviruses as being the source of the SARS coronavirus (Field 2009). The majority of the coronaviruses originated from African, Asian and European bats (Corman et al. 2013). In addition to SARS-CoV, four human coronaviruses (HCoVs), termed HCoV-OC43, -229E, -NL63 and -HKU1 are known.

Recently, a sixth HCoV was described, the MERS-CoV, which can cause coughing, fever, and pneumonia. This virus emerged in Saudi Arabia in 2012 and has been reported in some other Gulf States, France, Germany, Italy, Tunisia, and Britain [all cases to date can be epidemiologically linked to Saudi Arabia, Qatar, United Arab Emirates and Jordan]. The MERS virus so far (September 2013) has killed 51 people out of 135 confirmed cases of infections worldwide (ProMed, MERS-COV (71) 20130919). Close relatives of this betacoronavirus termed MERS-CoV and of HCoV-229E exist in Old World bats and HCoV-NL63 could be grown in immortalized bat cells, demonstrating the zoonotic potential of previously reservoir-bound bat CoVs. The recent description of a bat CoV related to MERS-CoV in Mexican bats (Anthony et al. 2013) and in bats from Saudi Arabia (Memish et al. 2013) emphasized the relevance of investigating neo-tropical bats for CoVs.

Identification of sequences of a group C betacoronavirus (β)-CoV in bat guano was recently reported (Wacharapluesadee et al. 2013). The detection of nucleic acid of this group C (β)-CoV and the previous isolation of viruses from bat feces and urine warrant some concerns that guano miners might be exposed to bat pathogens in fresh excreta as well as in soil substances. Therefore, bat guano miners should use preventive measures of personal hygiene and improved barrier protection to reduce the possibility of exposure to zoonotic pathogens.

28.1.5 Other Viral Pathogens

Many other viruses have been isolated or detected by molecular methods or by the presence of specific antibodies in bats, such as Hantaan virus in various bat species in Asia and Africa (Hance et al 2006; Hoar et al. 1998; Wong et al. 2007); Japanese encephalitis virus in China (Liu et al. 2013a), Venezuelan equine encephalitis virus in vampire bats and antibodies in bats from Guatemala (Hoar et al. 1998). In their review, Hoar et al. (1998) reported also detection of Chikungunya virus in African bats (*Scotophilus* sp.), Rio Bravo virus in Mexican free-tailed bats and Rift valley fever virus in bats from the Republic of Guinea.

In Uganda, four human cases of Kasokero virus isolated from *Rousettus aegyptiacus* bats living in the Kasokero cave occurred in laboratory workers (Kalunda et al. 1986). Infected laboratory workers had fever, headache, abdominal pain and diarrhea, sever myalgia and arthralgia. Signs lasted 7–10 days and were followed by complete recovery. It was demonstrated that 67 % of 74 bats from that cave were seropositive for Kasokero virus. Kyasanur virus has been isolated from bats in India and the Vesicular Stomatitis Virus (New Jersey type), which causes flu-like symptoms in infected humans, has been isolated from bats in Panama and Guatemala (Hoar et al. 1998).

28.2 Bacterial Zoonoses

28.2.1 Enteropathogenic Bacteria

28.2.1.1 Salmonella, Shigella, Yersinia, and Campylobacter

Enteric pathogens such as *Salmonella*, *Shigella*, *Yersinia* and *Campylobacter* species have occasionally been found in bats (Mühldorfer 2013). A variety of different *Salmonella* serotypes have been isolated from apparently healthy and diseased bats. Almost all of them are serotypes with a broad host-range. *Salmonella* Enteritidis and *Salmonella* Typhimurium have been frequently identified, which belong to a small group of *Salmonella* serotypes mainly associated with disease in humans and animals. Both serotypes have been isolated from organ tissues of three individual bats of the family Vespertilionidae that were found dead or severely injured near human habitations (Mühldorfer 2013). It was also reported in vampire bats (Hoar et al., 1998). In Trinidad, of 377 tested bats, representing 12 species, four bats (1.1 %) were positive for *Salmonella* spp., 49 (13.0 %) were positive for *E. coli*, and no bats were positive for *E. coli* O157 or *Campylobacter* spp. (Adesiyun et al. 2009). Isolated serotypes of *Salmonella* included Rubislaw and Molade, both from *Noctilio leporinus*, a fish-eating bat, Caracas recovered from *Molossus major*, and *Salmonella* Group I from *Molossus ater*, both insect-eating bats. Of the 49 isolates of *E. coli* tested, 40 (82 %) exhibited resistance to one or more antimicrobial agents.

Shigella, causing a dysenteric infection in humans, was isolated from a *Molossus bondae* bat in Colombia (Arata et al. 1968). *Shigella* strains of serogroups B to D have been isolated from mega- and microbats of diverse feeding habitats (Mühldorfer 2013). *Shigella flexneri* in particular was detected in more than 3 % of bats investigated.

A high prevalence of different Yersinia species (~35 %) was detected in the feces of 70 insectivorous *Myotis myotis* collected from natural populations in Poland (Mühldorfer 2013). Most of the *Yersinia* species isolated from bats are widely distributed in the environment and rarely associated with disease in mammals and birds. Cases of systemic *Y. pseudotuberculosis* infection have been described once in an adult insectivorous bat *(M. myotis)* found dead in Germany after hibernation (Mühldorfer 2013) and a bat in England (Hoar et al. 1998), respectively.

28.2.2 Vector-Borne Bacteria

28.2.2.1 Borrelia, Bartonella and Neorickettsia

Several *Borrelia* and *Bartonella* species and the causative agent of Potomac horse fever disease *Neorickettsia risticii* have been detected in blood and organ tissues of bats (Mühldorfer 2013). The majority of infected animals appear to be healthy, only

two vespertilionid bats (*Pipistrellus* sp. and *Natalus tumidirostris*) revealed severe borrelial spirochetemia.

In recent years, many new *Bartonella* species have been isolated or detected from bats around the world, including the United Kingdom, Kenya, Guatemala, Peru (Bai et al. 2012) Taiwan (Lin et al. 2012), France and Mexico (Stuckey et al. unpublished data). Phylogenetic analyses of *Bartonella* strains derived from bats identified several distinct phylogroups indicating the presence of a variety of novel *Bartonella* species in bats. It is notable that bats of the same species as well as bats of the same geographic origin and ecological niche (i.e. *Desmodus rotundus*, members of the family Vespertilionidae) shared closely related strains of *Bartonella*. It is not known if these *Bartonella* species are zoonotic. Furthermore, soft ticks (family Argasidae) and other ectoparasites commonly found on bats or in bat habitats are infected with *Bartonella*, *Borrelia* and *Rickettsia* species, posing a potential risk of intra- and interspecies transmission cycles between bats, humans and domestic animals (Mühldorfer 2013).

28.2.3 Other Bacterial Pathogens

A variety of pathogenic *Leptospira* species have been identified in bats in Asia, Europe, Australia and the Americas (Hoar et al. 1998; Mühldorfer 2013). The prevalence of leptospiral infections in bats varied from almost 2–35% depending on the sample size of the respective study. The family Phyllostomidae comprised the majority of microbats infected with *Leptospira*, whereas in obligate insectivorous species (i.e. families Vespertilionidae and Molossidae) leptospiral infection with pathogenic strains has occasionally been found. In Australia, native flying fox populations (genus *Pteropus*) were suggested as possible carriers of pathogenic *Leptospira* responsible for infections in humans and other animals because of high bacterial detection rates in kidney (11%) and urine samples (39%) and high seroprevalences (18, 28%) (Mühldorfer 2013). Similarly, bats from Madagascar and Comoros islands harbor a notable diversity of *Leptospira* spp.; a finding similar to the diversity found in a comparable investigation of bats in the Amazon region (Lagadec et al. 2012; Matthias et al. 2005). Leptospirosis incubation is 1–32 days (median 9 days) and median duration is 14 days. Most symptomatic patients develop a mild illness consisting of fever, chills, headache and myalgia. Severe forms of the disease may manifest in acute renal failure, hepatitis, jaundice, myocarditis and meningoencephalitis and outbreaks of severe pulmonary hemorrhagic leptospirosis have occurred resulting in high morbidity and mortality (Leshem et al. 2011).

A few other zoonotic agents, such as *Coxiella burnetii*, the agent of Q fever or *Mycobacterium bovis* were isolated from bats in Morocco and southern USSR and from captive Indian fruit bats in England, respectively (Hoar et al. 1998). Agglutinin antibodies against *Brucella* were detected in 5 of 53 vampire bats captured in areas of Brazil where incidence of brucellosis in cattle was high (Ricciardi et al. 1976). Several *Pasteurella* species (i.e. *P. multocida*, *P. pneumotropica* and *Pasteurella*

species B) have been identified as primary pathogens in bats responsible for a variety of localized and systemic infections in European bat species; most *Pasteurella* strains isolated from organ tissues of 29 vespertilionid bats represented *P. multocida* ssp. *septica* (85 %) and capsular type A (75 %) (Mühldorfer 2013).

28.3 Protozoan Parasites

28.3.1 Trypanosoma, Toxoplasma, Coccidia, and Leishmania

Few parasites of bats are known to be pathogenic to humans and are usually transmitted mechanically via an intermediate vector (Hoar et al 1998). Many species of trypanosomes can infect bats, but one of main concern is *Trypanosoma cruzi*, the agent of Chagas disease. Recently a new genotype of *T. cruzi*, associated with bats from anthropic areas and which could be a potential source of infection to humans was described (Marcili et al. 2009). Chagas disease is commonly transmitted by reduviid bed bugs. In humans, the disease is characterized by high fever, adenitis, anemia and facial edema in the acute form and myocarditis in the chronic form. Pathogenicity of bat trypanosomes for humans is not clearly established. *T. cruzi* has been detected in vampire bats, *Desmodus rotundus*, which can be of concern in term of zoonotic transmission, as these bats feed on mammals, including humans (Ramirez et al. 2013).

Infection of bats with *Toxoplasma gondii* has been reported based on serological studies and more recently on its isolation from bats in Brazil (Cabral et al. 2013; Sun et al. 2013). Therefore, consumption of undercooked bats could be a source of human infection. In bats, systemic toxoplasmosis caused by *T. gondii* was diagnosed in two juvenile, captive flying-foxes (*Pteropus conspicillatus* and *P. scapulatus*), which died following respiratory distress. One animal displayed clinical signs suggestive of neurological disease (Sangster et al. 2012).

Coccidia of the genus *Eimeria* have been isolated from several species of bats in many parts of the world (Hoar et al. 1998). Many new *Eimeria* species have been reported (McAllisher et al. 2012). Prevalence in bats is usually low (< 1–5 %) and it is not known if they are pathogens for humans (Hoar et al. 1998).

Leishmaniasis is a zoonotic disease caused by parasites of the genus *Leishmania*. It has expanded beyond its natural range and is becoming increasingly urban (Shapiro et al. 2013). Using PCR and PCR-RFLP, *Leishmania* (Viannia) *braziliensis* was detected in two bats (Chiroptera) in Mato Grosso do Sul, Brazil, an endemic area. The animals testing positive were found in both a rural site and an urban site. These results indicate the need for further research into the viability of *Leishmania* in bats. It could have implications for public health in that part of Brazil, given the large populations of urban bats, their mobility, and their ability to roost at close proximity to humans within residences and other buildings (Shapiro et la. 2013).

28.4 Fungal Pathogens

28.4.1 Histoplasma, Coccidioides and Other Fungal Infections

Despite the emergence of white nose syndrome caused by *Pseudogymnoascus destructans,* which destroyed an estimated 6–7 million bats in North America in recent years (first reported in 2007 in some New York state caves), the main zoonotic fungal diseases related to bats are histoplasmosis and to a lesser extend coccidioidomycosis and a few other fungal infections also identified in bats (Hoar et al. 1998).

Histoplasmosis: Histoplasmosis is caused by *Histoplasma capsulatum*, a dimorphic fungus that is endemic in the Americas and parts of Asia and Africa (Hoar et al. 1998). There are two varieties that are pathogenic to humans, var. *duboisii* and var. *capsulatum*. The former exists only in Africa, while var. capsulatum is most prevalent in regions of North, Central, and South America, but has also been reported from parts of Africa, Southern and Eastern Europe, Eastern Asia, and Australia (Cottle et al. 2013). It grows as a mold in soil enriched with bird or bat guano; human infection occurs after inhalation of the dust generated when such soil is disturbed. Visiting caves, collecting or being exposed to bat guano are the main sources of human contamination from bats (CDC 2012; Cottle et al. 2013; Hoar et al. 1998; Jülg et al. 2008; Kajfasz and Basiak 2012; Schwarz and Kauffman 1977). The threat of *Histoplasma capsulatum* infection in bat-inhabited caves should be emphasized to travelers and also to physicians (Kajfasz and Basiak 2012). Bats usually are healthy carriers and shed the fungus in their feces. In humans, clinical manifestations in humans vary according to host immunity and exposure intensity, ranging from asymptomatic infection (in most healthy persons exposed to a low inoculum; about 80 % of the time) to life-threatening pneumonia with respiratory failure (Cottle et al. 2013; Hoar et al 1998). Between these extremes, clinical presentations include acute or subacute pulmonary disease, pericarditis, rheumatological syndromes with erythema nodosum, progressive disseminated disease, and mediastinal complications. Acute pulmonary histoplasmosis in returning travelers typically presents as a flu-like illness with high-grade fever, chills, headache, nonproductive cough, pleuritic chest pain, and fatigue. Chest radiographs often show diffuse reticulonodular infiltrates and mediastinal lymphadenopathy. Symptom onset is usually 1–3 weeks following exposure and most individuals recover spontaneously within 3 weeks. Disseminated disease is a rare complication, more likely to occur in persons with severely impaired cellular immunity (Cottle et al. 2013). The African species, *H. capsulatum* var *duboisii,* is associated with cutaneous lesions and occasionally infection of long bones (Hoar et al. 1998).

Coccidioidomycosis: *Coccidioides immitis*, causing coccidioidomycosis, also known as valley fever in California, has been isolated from bat guano (Krutzsch and Watson 1978). Coccidioidomycosis is a systemic disease caused by *Coccidioides immitis* and *C. posadasii* spp., which are predominant in arid zones of the American continent, mainly in the Southwestern United States and the northern states of Mexico, as well as other regions with different environmental conditions

(Welsh et al. 2012). Some countries of Central and South America are also endemic zones. Most infected patients are asymptomatic. Disseminated disease develops in less than 5 % of clinically affected individuals. Culture, biopsy, and DNA probes are used for fungus identification. Prognosis is related to low antibody detection and a positive intradermic skin reaction to coccidioidin. Immunosuppressed patients and pregnant women require special attention in diagnosis, therapy, and prognosis. Amphotericin B in its different forms, itraconazole, and fluconazole, are the most frequently used treatments. Both fungi have been detected in bats and bat guano (Krutzsch and Watson 1978; Cordeiro et al. 2012). In Brazil, *Coccidioides posadasii* was recovered from *Carollia perspicillata* bat lungs (Cordeiro et al. 2012). Immunologic studies detected coccidioidal antibodies and antigens in *Glossophaga soricina* and *Desmodus rotundus* bats.

Candidiasis: Candida albicans, which causes mucocutaneous candidiasis ("thrush" or oropharyngeal candidiasis) in the mouth or throat of humans, was isolated from liver, kidney, spleen and intestinal content of several bats captured in Nigeria (Oyeka 1994). The most common symptom of oral thrush in humans are white patches or plaques on the tongue and other oral mucous membranes. It was indicated that bat consumption is common in that country and people could get infected by improper handling of bats or consumption of raw or undercooked bat meat (Oyeka 1994). In a recent study conducted in Brazil, 7 (12.3 %) of 57 bats showed yeasts in their feces. Five species of the genus *Candida* were isolated: *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. pelliculos* (Botelho et al. 2012).

Other fungal infections: Other fungal infections have been described in bats, some of which could potentially be transmitted to humans. Bats are susceptible hosts and reservoirs for *Histoplasma capsulatum, Paracoccidioides brasiliensis*, and *Sporotrichum schenckii* (Raymond et al. 1997). *Sporothrix schenckii, Scopulariopsis* sp. and *Cryptococcus neoformans* have been isolated from bats or bat guano in the Americas (Hoar et al. 1998; Kajihiro, 1965). Blastomycosis is a granulomatous disease of mucous membranes. *Blastomyces dermatitidis* has been isolated from the lungs of an asymptomatic insectivorous bat (*Rhinopoma hardwickei hardwickei*) from India, and insectivorous bats orally inoculated with *B. dermatitidis* transiently shed viable organisms in their feces. Mexican free-tailed bats (*Tadarida brasiliensis*) intraperitoneally injected with *B. dermatitidis* developed systemic blastomycosis and excreted viable fungi in their feces. Apparently, bats can serve as both hosts and vectors for *B. dermatitidis* and may be potential sources for human infection (Raymond et al. 1997).

28.5 Summary

Bats themselves have an undeniable impact on our planet; with over 1200 chiropteran species identified to date, bats comprise one-fifth of all mammalian species globally and provide critical ecosystem services ranging from pollination to insect control (Wibbelt et al. 2010). Their vast numbers, capability of flight, and a variety

of ecological, immunological, and socioeconomic factors also enable bats to transmit an increasingly recognized spectrum of pathogens (Calisher et al. 2006; Mühldorfer 2013; Wibbelt et al. 2010; Wood et al. 2012). The potential for the emergence of zoonoses in particular will continue to increase as human development encroaches on bat populations. As such, future research will be needed to monitor infection and better understand those underlying drivers of disease.

Addendum Since this chapter was written, the largest outbreak of Ebola virus ever reported occurred in West Africa (Guinea, Liberia and Sierra Leone) with more than 5,300 human cases and more than 2600 deaths by mid September 2014 and another unrelated outbreak occurred since July 2014, with 66 cases including 37 deaths in the area of Djera, Equateur Province in the Democratic Republic of Congo. In both outbreaks, the index case was a person who had been exposed to bushmeat (WHO, 2014).

As far as Bartonella and bats, a recent publication linked Bartonella mayotimonensis, which had been described as a cause of human endocarditis in North America (Lin et al., 2010) to a bat reservoir, as this Bartonella species was isolated from several bats from Finland (Veikkolainen et al. 2014). It clearly indicate that bats can be the reservoirs of zoonotic Bartonella species.

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Chapter 29
Cystic and Alveolar Echinococcosis: Fraternal Twins both in Search of Optimal Treatment

Dominique A. Vuitton and Enrico Brunetti

Abstract 'Echinococcosis' applies to 2 rather different diseases, due respectively to E. *granulosus*, 'cystic echinococcosis' (CE), and to *E. multilocularis*, alveolar echinococcosis (AE). Both species differ by their usual animal reservoir, i.e. mostly domestic animals, for E. granulosus, and mostly wild animals, for E. *multilocularis*. Other species of *Echinococcus* may also cause disease in humans, E. oligarthrus and E. *vogeli* have a clinical presentation similar to that of CE and AE, respectively. No specific disease has been attributed until now to the recently identified species, E. shiquicus. Based on their genetic characteristics, E. *granulosus* has been split into several species: E. *granulosus* sensu stricto (ex-sheep strain), *E. felidis,* E. equinus (ex-horse strain, never recognized to infect humans), *E. ortleppi* (ex-cattle strain), and E. canadensis (ex-camel, pig and cervid strains).

The larval stage of *Echinococcus* spp., also called 'metacestode' is characterized by the germinal layer surrounded by the laminated layer. The germinal layer forms 'buds', then 'vesicles' (cysts), which are filled with a water-like liquid ('hydatid/cyst/vesicle fluid'). These cysts may be single (typically for CE) or multiple and aggregated (typically for AE). Fertility is characterized by the budding of 'protoscoleces' from the germinal layer, and their release into the cyst fluid. The most striking differences between E. *granulosus* and *E. multilocularis* lie in the histological aspect of the metacestodes, respectively big cysts with little inflammatory reaction and numerous protoscoleces in CE, and multiple aggregated small cysts with an impressive granulomatous periparasitic infiltration, associated with dense fibrosis, and few or no protoscoleces in AE, hence the different clinical presentations and complications. Pseudo-cysts, due to massive necrosis of the central part of lesion, may occur in advanced AE.

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CE is usually maintained by the synanthropic domestic cycle (dog/domestic ungulate) and represents a persistent zoonosis in rural livestock-raising areas where humans cohabit with dogs fed on raw livestock offal where the cysts are present. A cycle in wild animals allows E. *multilocularis* to subsist in nature. It includes voles of a number of different species depending on the area and the lagomorph *Ochotona curzionae* (on the Tibetan plateau of China), as intermediate hosts, and foxes, wolves and dogs, and less commonly other carnivores as definitive hosts. Larval AE infection with symptoms close to those observed in humans has been recognized in a number of domestic animals and in zoo animals or in exotic pets. Environmental factors play a critical role in E. *multilocularis* infection in wild animals, resulting in a heterogeneous geographical distribution of the parasite; in many countries, fox urbanization has considerably increased the human population at risk. The burden of both diseases in terms of DALY lost or economic cost has long been underestimated both in humans and animals.

Host-parasite interactions in larval echinococcosis depend on a subtle balance between the various immunological mechanisms involved in protection of the host versus tolerance to the parasite. Very rare in AE, IgE-dependent allergic reactions and eosinophilia occur in CE when there are fissures or ruptures in the cyst. AE lesion progression is faster in patients with immune suppression; immunosuppressive treatments in patients with cancer or inflammatory diseases, more frequently administered since the beginning of the XXIst century, have modified the epidemiology and the presentation of AE in Europe during the last 2 decades. XXIst

The diagnosis of both diseases in humans relies on imaging. Both CE and AE may remain asymptomatic for a long period of time; and spontaneous death of the metacestode does occur in many infected patients. Ultrasound (US) examination is the basis for diagnosis in patients with liver-related signs and symptoms and for mass screening. Fortuitous diagnosis of CE or AE on abdominal US images taken for another indication is not rare. Complementary imaging exams at diagnosis and for follow-up include CT-scan, Magnetic Resonance Imaging, and for AE only, Positron Emission Tomography 1 and 3 hours after IV injection of 18F-FluoroDesoxyGlucose (FDG-PET). Diagnosis is confirmed by specific serology; however, serological tests are far less sensitive in CE than in AE. The WHO-Informal Working Group on Echinococcosis has proposed a US-based classification of CE cysts (CE-1 to CE-5), and a staging system for AE (PNM classification) which should be used by clinicians to better assess the evolution potential and severity of the disease as well as to facilitate comparison between case series.

Until 1980, the treatment of both CE and AE relied only on surgery. Depending on the class, stage, and size of the lesions, a variety of options are now available. For CE it includes short-term (3 months in average) albendazole treatment for small cysts or, at the opposite of the spectrum, long-term albendazole (> 6 months to several years) treatment for disseminated non-operable CE, the PAIR (puncture, aspiration, injection, reaspiration) percutaneous technique for middle-sized liver cysts without daughter vesicles. The current trend for surgery is to remove the whole cyst (‘total cystectomy’) whenever possible to avoid recurrence; ‘partial cystectomy’ with sterilisation of the cyst content by protoscolecide agents is easier to perform in

all settings, but more prone to be followed by recurrence and dissemination. In AE, albendazole treatment is the basis for care management: given for a minimum of 2 years after radical surgical resection of the liver lesions, it must be administered for life in all other cases. The current trend for surgery is to avoid palliative surgery and to prefer percutaneous or perendoscopic drainage and stenting of the obstructed bile ducts and/or percutaneous drainage of the central necrotic cavity in advanced cases. In CE as well as in AE, appropriate monitoring of albendazole and of its possible adverse effects is mandatory, and long-term follow-up of each patient is crucial. For both diseases too, however, there is an urgent need to test new chemical or biological compounds for the treatment of patients who cannot be treated by albendazole, and to evaluate therapeutic strategies, including the various types of surgical operations, by prospective studies.

29.1 The *'Echinococcus'* Genus: A Single Genus for an Increasing Number of Species, and Two Rather Different Diseases in Humans

Echinococcosis and *Echinococcus* Echinococcosis is the disease caused by cestode helminths of the genus *Echinococcus (E.)*. Actually, this generic term applies to two rather different diseases, due respectively to *E. granulosus*, 'cystic echinococcosis' (CE), also previously designated as 'hydatid disease' or 'hydatid cyst', a condition already recognized at Hippocrates' times, and to *E. multilocularis*, alveolar echinococcosis (AE), a tumour-like disease only identified 160 years ago (Virchow 1855). In addition, both species differ by their usual animal reservoir, i.e. mostly domestic animals, for *E. granulosus*, and mostly wild animals, for *E. multilocularis*. However, for more than a century, since the characterization of the '*Taenia echinococcus*' by the German scientist Carl Theodor Ernst von Siebold and the diagnosis of the first human cases of AE in the 1850s, there was no distinction between the two species (Vuitton et al. 2011). Other species of *Echinococcus* may also cause disease in humans, albeit far more rarely and only in South America; they are *E. oligarthrus* and *E. vogeli*, both responsible for 'neotropical' echinococcosis (D'Alessandro and Rausch 2008). The clinical symptoms of 'polycystic echinococcosis', due to *E. vogeli*, are very close to those of AE and species diagnosis relies on the south-American residence of the patient and on molecular identification (Grenouillet et al. 2013), and the very rare cases of *E. oligarthrus* infection, which present as single cysts (D'Alessandro and Rausch 2008); their clinical presentation is similar to that of AE and CE, respectively, and these two species will not be described in detail in this chapter. Finally, a new species of *Echinococcus* was disclosed recently in wild animals and in dogs on the Tibetan plateau, China, *E. shiquicus* (Xiao et al. 2005); however, no disease in humans has been attributed to this species until now.

A New Taxonomy A taxonomic revision of the members of the genus *Echinococcus* (Cestoda: Taeniidae) has been proposed, which follows molecular phylogeny. The full description of the genomes of *E. multilocularis* and *E. granulosus* is now available (Tsai et al. 2013) as well as the complete mitochondrial genome for all species (Nakao et al. 2007). *E. multilocularis* still appears as a single species, although variations in its genome, best detected by using the *EmsB* microsatellite marker, may identify strains and has allowed epidemiologists to track its geographical spreading in the northern hemisphere (Knapp et al. 2010). Conversely, *E. granulosus* has been split into several species, which were previously considered as strains, numbered from G1 to G10 and characterized by their most usual intermediate animal host. The phylogenetic relationships of the various 'strains' of *E. granulosus* with *E. oligarthrus*, *E. vogeli*, *E. multilocularis*, and *E. shiquicus*, have also been studied (Nakao et al. 2007). It is now acceptable to differentiate, within the previously named '*E. granulosus*', *E. granulosus* sensu stricto (ex-sheep strain), *E. equinus* (ex-horse strain), *E. ortleppi* (ex-cattle strain), and E. *canadensis* (ex-camel, pig and cervid strains, also named '*E. granulosus* sensu lato') (McManus 2013). *E. felidis* was also described as a distinct species although it is phylogenetically closely related with *E. granulosus* sensu stricto (Huttner et al. 2008). Sister species relationships were confirmed between *E. ortleppi* and *E. canadensis*, and between *E. multilocularis* and *E. shiquicus*. The basal positions of the phylogenetic tree are occupied by the so-called 'neotropical' endemic species, *E. oligarthrus* and *E. vogeli*, whose definitive hosts are derived from carnivores that emigrated from North America after the formation of the Panamanian land bridge (Nakao et al. 2007).

As no restriction to a single animal host has been identified for the various 'new' species described from the previously named '*E. granulosus*', and no marked differences have been identified between the clinical presentation of CE depending on the species, for the following description, '*E. granulosus*' will be used as a general name for all three new 'zoonotic' species, *E. granulosus* sensu stricto, *E. ortleppi*, and *E. canadensis*. *E. equinus* has never been recognized to infect humans; it thus cannot be considered as 'zoonotic'; and the pathogenicity of *E. felidis* to humans is unknown.

Cystic Versus Alveolar: Similar Structure of the Metacestode, but Different Lesions, Depending on the Immune Response of the Intermediate Host The larval stage of *Echinococcus* spp., also called 'metacestode' is characterized by the *germinal layer*, a syncitial mono-layer of parasite cells surrounded by the *laminated layer,* an acellular layer of polysaccharides which is the interface between the parasite and the host's cellular immune response while allowing exchanges between them (Vuitton and Gottstein 2010). The germinal layer forms 'buds', then 'vesicles' (cysts), which are filled with a water-like liquid ('hydatid fluid' or 'cyst/vesicle fluid'). These cysts may be single (typically for CE) or multiple and aggregated (typically for AE, hence the adjective 'alveolar', i.e. composed of alveoli), and small (from 1 mm to 1 cm, typically for AE) or large (from 1 to 20 cm, typically for CE) (Eckert and Deplazes 2004). Fertility is characterized by the growth and budding of 'protoscoleces' from the germinal layer, and their release into the cyst

fluid. In all species of *Echinococcus*, the 'protoscolex' is that multicellular organism that ensures the transformation of the larva into an adult worm in the intestine of the definitive hosts after a process of evagination when the cysts are ingested, with the offal of domestic intermediate hosts (for CE) or with the rodent intermediate hosts as prey (for AE) by the appropriate carnivores. Hundreds to thousands of worms then settle in the small intestine of the definitive host where they attach through their hooks and suckers, reach maturity, characterized by egg-containing last segments within 2–3 weeks, and release them into the environment with the feces for an average of one month (Eckert et al. 2001a; Torgerson et al. 2011). Each species of *Echinococcu*s was in the past carefully described based on the shape and length of its segments, hooklets, and suckers, since the morphology of the adult stage (worm) was thought to be species-specific: the molecular taxonomy has now shown that different species can have similar morphology and subtle differences are not important for species differentiation (Eckert et al. 2001a; Torgerson et al. 2011; Thompson and Eckert 1983; Thompson et al. 2003). The most striking differences lie in the histological aspect of the respective metacestodes. In CE, cells of the host's immune response have nearly totally disappeared when the cyst is disclosed in an intermediate host, either animal at slaughtering or human at diagnosis, and the cyst with its germinal and laminated layer is surrounded by a peri-cystic fibrous area. The cyst may contain hundreds of protoscoleces. After damage to the germinal layer (rupture or fissure) occurs, the cyst may either degenerate or produce 'daughter vesicles', usually inside and more rarely outside the germinal layer, which gives a 'multivesiculated' appearance to the lesions, quite different from the 'multi-cystic' aspect of neotropical echinococcosis or from the 'alveolar' aspect of AE (da Silva 2011; Rogan et al. 2006) (Fig. 29.1). In AE, the laminated layer is surrounded by a layer of epithelium-like macrophages ('epithelioid cells'), then concentric layers of cells of the immune response, including macrophages, lymphocytes, eosinophils and giant cells, of cells involved in fibrosis development, such as fibroblasts and myofibroblasts, and of collagen bundles and various components of the extracellular matrix (Bresson-Hadni et al. 2007). This 'granulomatous' periparasitic infiltrate is usually bordered by T-lymphocytes, at the immediate proximity of the liver or lung parenchyma. In AE, the extent of the periparasitic infiltrate and the presence or absence of protoscoleces in the parasite vesicles depend on the susceptibility of the host to the development of the metacestode, and may thus be different in the various species of intermediate hosts (Vuitton and Gottstein 2010; Bresson-Hadni et al. 2007; Vuitton 2003). Necrosis may also be observed in the periparasitic infiltrate; in degenerating lesions, especially after years of evolution in humans, massive necrosis may occur in the center of lesions, with the constitution of necrotic cavities, which give a 'pseudo-cystic' appearance to AE lesions; however, such pseudo-cysts have irregular walls and are filled with solid and liquid necrotic debris, and are thus in no way similar to the real 'cysts' of CE (Bresson-Hadni et al. 2006) (Figs. 29.1 and 29.2).

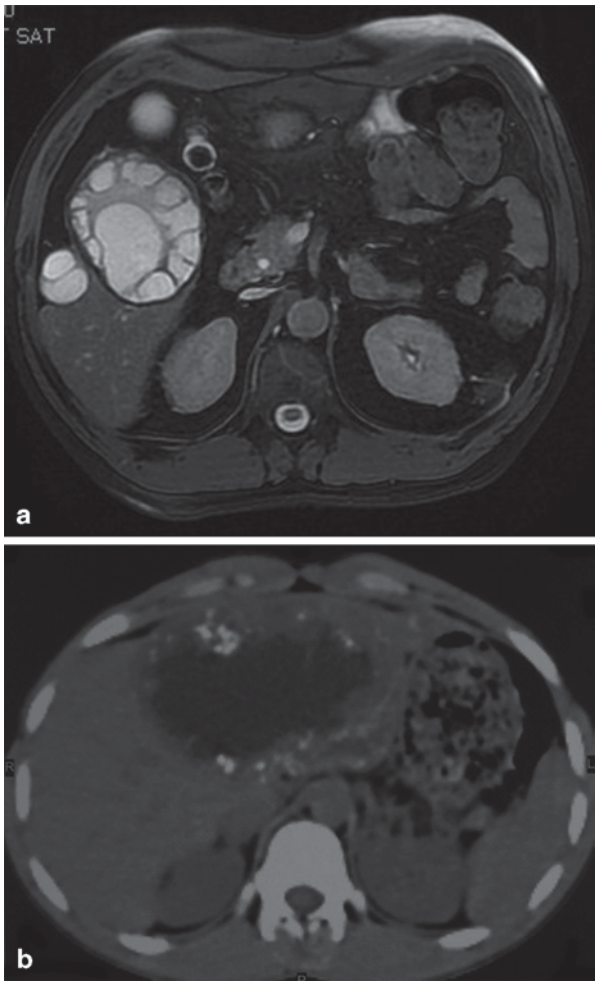

Fig. 29.1 Cystic (CE) and alveolar (AE) echinococcosis typical images. **a** CE: daughter vesicles in a liver cyst, as shown at Magnetic Resonance Imaging; **b** AE: necrotic cavity in the centre of a huge lesion invading the right and left liver, as well as the liver hilum; at the periphery of the hypodense necrotic cavity, hyperdense calcifications are well shown in the pseudo-tumoural lesion by computer tomography (CT)

29.2 The Parasite Life Cycle and the Burden of *Echinococcus* spp. in Animals

E. granulosus **(i.e.** ***E. granulosus sensu stricto, E. ortleppi,*** **and** ***E. canadensis*****)** The adult cestode develops in the small intestine of the definitive host, a carnivore (Eckert et al. 2001a; Torgerson et al. 2011) (Fig. 29.3). The last segments (proglottids) of this worm (3–5 mm long) contain oncospheres (eggs); they are released into the intestine, and the oncospheres are dispersed on the grass with the faeces of the host. When the oncospheres are eaten by the intermediate host (usually a mammal), the hexacanth embryo, released into the duodenum, passes through the intestinal wall and most usually settles in the liver or in the lung as 'cysts' (also called 'hydatids'). Domestic animals such as sheep, goats, cattle, horses, camels, swines,

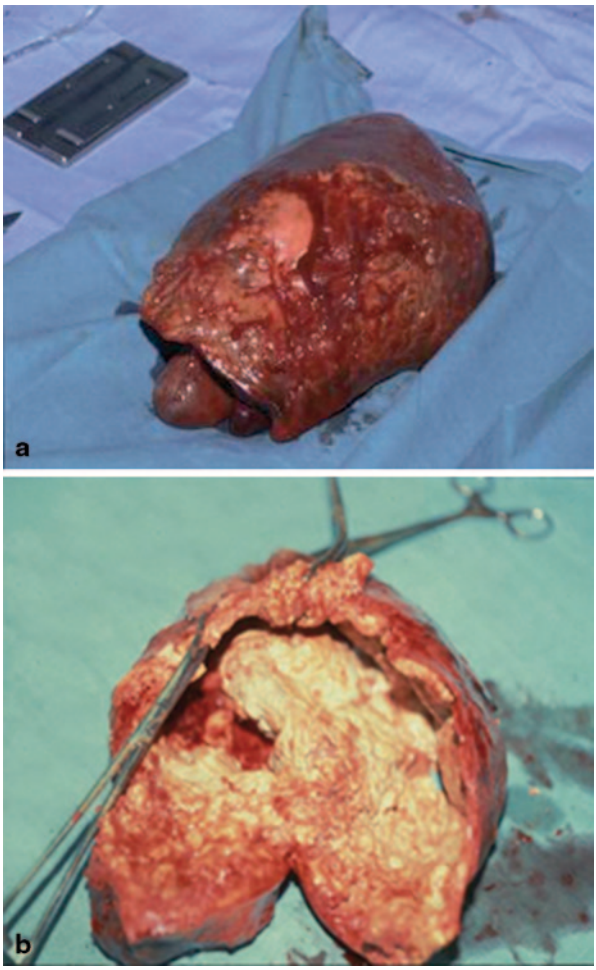

Fig. 29.2 Alveolar echinococcosis; macroscopical view after surgical hepatectomy. **a** Fibrous aspect of the lesions at the surface of the liver. **b** Necrotic cavity in the centre of a very advanced lesion

caribous or reindeers serve as intermediate hosts. Commonly, dogs are infected by eating raw infective offal containing parasite cysts. Humans become accidentally infected either by touching dogs with contaminated hair, or by ingesting vegetables, water or soil infected by dog faeces (Fig. 29.3). Less commonly, the cycle involves a wild carnivore such as the wolf, jackal, or coyote and a wild herbivore such as wild elk, caribou, reindeer or other cervids. As mentioned above, the animal species-specificity of the various parasite species is not strict. Cattle, for instance, may be infected by the so-called 'sheep strain' (currently, *E. granulosus* sensu stricto) or the 'camel strain' (currently *E. canadensis*) as well as by the so-called 'cattle strain' (currently *E. ortleppi*).

CE is usually maintained by the synanthropic domestic cycle (dog/domestic ungulate) and represents a persistent zoonosis in rural livestock-raising areas where humans cohabit with dogs fed on raw livestock offal. Infection of dogs by the

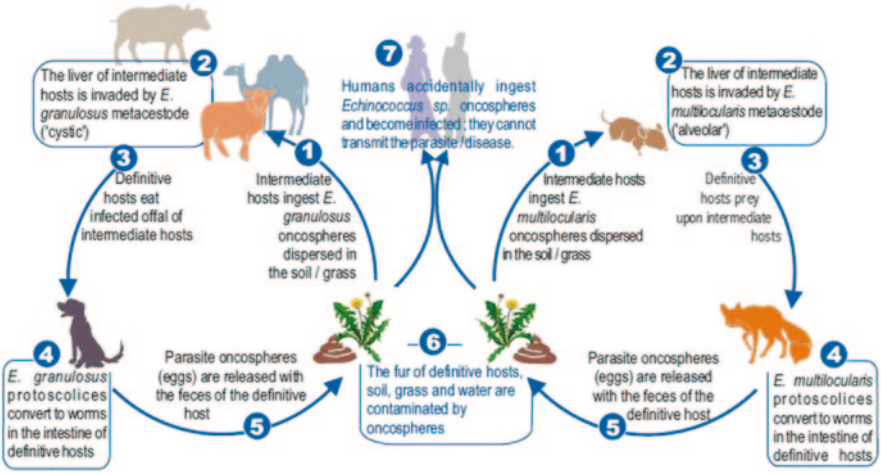

Fig. 29.3 Parasite cycles of *E. granulosus* (*left cycle*) and *E. multilocularis* (*right cycle*)

worms of *E. granulosus* does not cause specifically identified disease or symptoms. Infection of domestic ungulates by the metacestode (larval form) of *E. granulosus* is characterized by single or multiple cysts, usually in the liver or the lung of the animals. The burden of *E. granulosus* infection in domestic animals has long been neglected and was considered to be minimal, since generally the infected animals do not present any overt disease. Moreover, in rural family-type farming the fact that cysts might affect offal was not considered to be of great economical importance. However, more recent evaluation estimates the annual livestock production loss of at least US $ 141,605,195 and possibly up to US $ 2,190,132,464 (Budke 2006).

Risk factors for domestic animal infection by *E. granulosus* metacestodes have been recently reviewed in an exhaustive analysis of the current literature (Otero-Abad and Torgerson 2013). Prevalence of CE differs between study locations or different livestock origin; there are seasonal variations in CE prevalence, as found by abattoir meat inspection; high altitudes and increasing annual rainfall are significant risk factors, as well as the age of the host for many farm species: higher CE prevalence is observed in old animals compared to young ones, and the number of cysts in a farm animal also increases with age. The gender of the intermediate host has also been identified as a possible determinant of CE, with females more at risk in most studies. Among host species, on a farm, small ruminants have frequently been observed with the highest rates of infection; but cattle have also been identified in many studies as bearing the highest prevalence of CE of those observed in farm species. Farm location, but especially management factors are important determinants for infection: e.g. pigs reared in intensive conditions had significantly lower CE prevalence compared to pigs reared in free-range conditions or on family-owned farms, while sheep and goats from mixed farming systems showed higher rates of hydatid infection compared to small ruminants from pastoral systems (Otero-Abad and Torgerson 2013).

Livestock represents the parasite reservoir, while the infection of definitive hosts, mainly dogs, is responsible for the maintenance of the parasite cycle in animals and for the transmission to humans. The most important risk factor is the potential access of dogs to raw (uncooked) and infected offal, and thus depends on their food sources, access to the location where animals are slaughtered, access to livestock carcasses, rural location of dogs, whether dogs are free to roam (with a higher risk for stray dogs, in rural as well as in peri-urban areas, close to dumps or open-air abattoirs), the type of dog, its age (younger dogs being more at risk), and also the knowledge of the owners about echinococcosis and their socioeconomic background. Conversely, indoor or chained dogs are less at risk, and activities that prevent the consumption of livestock offal by dogs, such as the proper disposal of animal carcasses by incineration/burial or avoiding home slaughtering, protect dogs from infection. Dog's gender does not seem to be a significant risk factor (Otero-Abad and Torgerson 2013).

E. multilocularis A cycle in wild animals allows *E. multilocularis* to subsist in nature (Fig. 29.3). The adult cestode usually develops as a worm (2–4 mm long) in the small intestine of the fox (Deplazes and Eckert 2001). The last segments are released into the intestine; the oncospheres (eggs) they contain are dispersed on the ground with the faeces where they contaminate the grass. Oncospheres are surrounded by an envelope which allows them to resist very low temperatures (to −40 °C) but they die at +60 °C. When eggs are eaten by the intermediate host, usually a wild rodent, i.e. voles of a number of different species, depending on the endemic area, and in the lagomorph *Ochotona curzionae*, on the Tibetan plateau of China (Vuitton et al. 2003), the hexacanth embryo is released into the duodenum, passes through the intestinal wall, and enters the liver where it usually settles. AE lesions consist of a mass of small vesicles produced by a single or several embryos, which may occupy a considerable part of the liver. The metacestode may then directly invade all organs and tissues close to the liver, or disseminate in microthrombi to any possible organs through the hepatic veins, heart and pulmonary and systemic arteries. The life cycle is completed when the intermediate host, containing infected larvae with protoscoleces, is eaten by foxes. Dogs are the most common definitive hosts in some areas, such as China (Vuitton et al. 2003). Less commonly, other carnivores including raccoon dogs, coyotes and cats can also serve as definitive hosts (Torgerson et al. 2011; Vuitton et al. 2003).

As for *E. granulosus* infection, gut infection by worms of *E. multilocularis* species does not result in clinical symptoms or disease, in foxes as well as in dogs or other definitive hosts. As most of the intermediate hosts are wild rodents or lagomorphs, there is no special veterinary or economical impact from the infection of the intermediate hosts by the metacestode. However, in all endemic areas, including Europe and Asia, larval infection with symptoms close to those observed in humans have more and more often been recognized in a number of domestic animals, such as horses, pigs, boars, or even dogs which appear to also serve occasionally as intermediate hosts (Vuitton et al. 2003; Scharf et al. 2004; Bottcher et al. 2013; Ueno et al. 2012). In addition, AE cases observed in zoo animals, such as monkeys or lemurs, have received much attention in the recent years in Europe and Japan

(Rehmann et al. 2005), and AE was also observed in exotic pets, such as chinchillas (Staebler et al. 2007).

Environmental factors play a critical role in *E. multilocularis* infection in foxes, resulting in a heterogeneous geographical distribution of the parasite (Vuitton et al. 2003; Giraudoux et al. 2013). From continental to regional scales, AE forms discrete patches of endemicity within which transmission hotspots of much larger prevalence may occur. Each transmission ecosystem has its own characteristics, and lies on a subtle interplay between altitude, climate, landscape characteristics, land use, and predator/prey relationship (Vuitton et al. 2003; Giraudoux et al. 2013). Regional meteorological conditions, such as low temperatures or high annual precipitations, have been reported as being associated with the infection rates. The importance of the availability and predation level on potential intermediate hosts for the successful transmission of *E. multilocularis* is well demonstrated. Changes in land use, such as promotion of permanent pastures, deforestation, or privatization of the land, are prone to influence *E. multilocularis* infection in small mammals and foxes, by favouring periods of high densities of small mammals with exclusive preying of foxes on these potentially infected mammals (Giraudoux et al. 2003; Wang et al. 2004; Pleydell et al. 2004). Involvement of dogs in the life cycle of *E. multilocularis*, as it occurs in rural western China, has been shown to be responsible for higher prevalence of AE in humans than fox infection alone (Torgerson et al. 2011; Vuitton et al. 2003). On the other hand, despite a higher prevalence in foxes from rural areas when compared with urban areas of Europe, there is a high infection pressure frequently reported in the periphery of the cities, and fox urbanization has become a new threat, also by considerably increasing the human population at risk (Robardet et al. 2011; Deplazes et al. 2004).

29.3 Epidemiology of Echinococcosis in the World

CE in the World The disease is prominent in rural areas where humans, dogs, and sheep/goat and cattle coexist closely, with poor housing conditions and low level of sanitation and is most common in the regions of the world where raising livestock is a major industry. The estimated minimum global human burden of human CE averages 285,000 disability-adjusted life years (DALYs) or an annual loss of US $ 194,000,000 (Budke 2006). CE is found in the entire Mediterranean littoral including North Africa; former USSR; East Africa, with highest prevalence found in Turkana region, in Kenya, and in Sudan; and South America, particularly Uruguay, Argentina, and Chile, but also parts of Peru and Brazil (Eckert et al. 2001b). Highest prevalence is observed in Western China, where an average of 1–5% of the population may harbour a cyst when screened systematically using ultrasound examination of the liver (Torgerson et al. 2011). The disease is present in Nepal, India, Pakistan and Bangladesh; it is still frequent in North Africa, Turkey and all Middle-East countries, where it had been identified since Greek/Roman Antiquity. It is re-emerging in several countries where it had been partially controlled after the

Second World War, and especially in all endemic ex-USSR territories, e.g. Bulgaria, Romania, in Europe, and all central Asia republics (Eckert et al. 2001b; Torgerson et al. 2011).

Because of active control campaigns, New Zealand and Australia, which were highly endemic in the past, are now far less infected, and most CE human cases are in immigrants from endemic areas (Eckert et al. 2001b; Torgerson et al. 2011). This is also true for the USA as well as for most of countries of the European Union, including Italy, Spain and Portugal, which were still highly endemic in a recent past. Strict regulations on slaughtering and control campaigns have contributed to decrease very significantly the transmission to humans despite persistent low level infection in domestic animals which can still be tracked by veterinary inspection, especially in Spain, Greece, Southern Italy, and France (especially in Sicily, Sardinia and Corsica) (Eckert et al. 2001b; Torgerson et al. 2011). In the USA and most European countries, CE is mostly—or only—diagnosed in immigrants from the still endemic countries. The diagnosis should thus be evoked for any suspect cystic lesion of the liver when it occurs in immigrants or travellers. A special situation occurs in the United Kingdom, where low level of infection by *E. granulosus* sensu stricto in sheep and in humans is still present in Wales, but most of animal infection is due to *E. equinus*, with horses as intermediate hosts and apparently no transmission to humans (Romig et al. 2006). Non-published results from abattoir surveillance including species genotyping seem to indicate a potential resurgence of *E. ortleppi* infection including human cases in Europe, e.g. in France, which should raise attention of public health authorities (F. Boué and F. Grenouillet and the FrancEchino Network, personal communication).

AE in the World Although always limited to the Northern hemisphere and restricted to specific geographical areas characterized by their ability to sustain the proper functioning of *E. multilocularis* lifecycle among its definitive and intermediate hosts, AE endemic regions have never ceased to extend, from the discovery of the first human case in 1852 in southern Germany. Initially only diagnosed in the mountainous areas of Jura and Alps in central Europe (from Eastern France to Western Austria), and in Russia, from Moscow area and Siberia to the far Eastern region of Kamchatka, AE cases were subsequently recognized during the first half of the twentieth century in Alaska, in Turkey, and in northern Japan, where the parasite was 'artificially' introduced by foxes imported from the Kuril islands to Rebun island, then to Hokkaido, to fight agriculture pests [see references on the history of AE epidemiology in (Vuitton et al. 2011)]. Since the 1940s, the epidemiological situation seemed quite stable, with AE considered to be a rare disease, even in the endemic area. The rare occurrence and low prevalence of AE in so-called 'endemic areas', at usually less than 10/100,000 in regions with the highest contamination pressure (infection of 70% of foxes) was explained both by the rare encounter of humans with infected fox faeces, and by the natural resistance of humans as intermediate host for the parasite (Vuitton and Gottstein 2010; Vuitton 2003). This might also explain why, despite mild-level infection in all Central/Northern states of the USA and Southern provinces of Canada, so few AE cases had been observed in North America.

However, the situation has changed radically since the 1980s. Western China is now likely the world region with the highest number of human AE cases (Vuitton et al. 2003; Craig 2006). Nine provinces and autonomous regions are concerned, including Qinghai, Xinjiang, Gansu, Sichuan, Tibet, and Ningxia in Western China where human cases and animal epidemiology are very well documented, and where both types of echinococcosis are found. In Inner Mongolia in North-Western China, as well as Heilongjiang and Jilin in North-East China, *E. multilocularis* infection of animals and human AE cases have been observed, but the epidemiological situation is less known. Prevalence of the disease may reach 10% of the population in some 'high risk' districts of Gansu and Ningxia, 100 times higher than in the previously known endemic areas of Europe. China is, with Turkey and central Asia, the only region in the world where CE and AE are known to coexist in some communities, and sometimes in the same patient (Wen et al. 1992), or in the same definitive host (Vuitton et al. 2003). Estimates show that in China there are about 380,000 cases of echinococcosis (including CE and AE) and 50 million people are at risk of *Echinococcus* spp. infection (Wang et al. 2012) From the analysis combining human capital method with DALYs to analyse the indirect CE economic burden on a total of 2018 CE patients attending the hospitals in Xinjiang, China, between 2004 and 2008, the per-person direct medical cost for the treatment of echinococcosis was estimated of US $ 1493.12 and the per-person direct non-medical cost of US $ 19.67. The indirect economic cost was US $ 1435.96 per person, and the disability-adjusted life-years (DALY) lost was approximately 1.03 DALY/person (Wang et al. 2012)

Since the 1990s, the recognized extent of the endemic area has progressed a lot in Europe, with fox *E. multilocularis* infection disclosed in all countries, except the United Kingdom, Spain and Portugal, and also now present in Western France, Northern Germany, and Eastern Austria, regions that were not previously known to host *E. multilocularis*-infected foxes. Emergence (or recent recognition?) of the disease in the Baltic States is the most striking epidemiological finding of the beginning of the twenty-first century. Lithuania, in particular, now definitely appears to be a major endemic area, with 80 patients diagnosed between 1997 and 2006 for a total population of 3,535,547 inhabitants, 57% of foxes, several farmer dogs, and various intermediate hosts found infected by *E. multilocularis* (Bruzinskaite et al. 2007). Meanwhile, in Japan and in Europe, fox *E. multilocularis* infection has reached the city, thanks to the increased number and the 'urbanization' of foxes, which poses new and unresolved questions for the prevention of AE (Deplazes et al. 2004).

29.4 Diagnosis and Follow-up of Echinococcosis in Humans

Clinical Presentation of CE CE presents usually as a cyst ('hydatid cyst'), in the liver or in the lung, which may remain clinically silent for a long time and is often discovered incidentally during routine abdominal ultrasound (US) examination or

chest X-ray. All other anatomical locations are possible but rarer, as shown in a large collection of cases in Australia where the most frequent locations of 1802 cysts were liver (63 %), lung (25 %), muscles, (5 %), bones (3 %), kidney (2 %); spleen, brain (1 %), and heart, breast, prostate, parotid and pancreas (< 1 %) (Torgerson et al. 2011). Similar figures are given from more recent series of cases. For the diagnosis of liver cysts, the following signs and symptoms may be observed: right upper quadrant discomfort; urticaria; episodes of itching; right upper quadrant palpable mass. Clinical symptoms are usually absent until the cyst has reached 10 cm in diameter; a cyst is rarely palpable until it has reached 15–20 cm. Physical examination of the liver may be normal or may disclose an enlarged and regular liver. If the cyst is located in the anterior liver, a round, painless tumour can be palpated.

A complication is most often at the origin of hepatic CE diagnosis (Fica et al. 2012). The observed signs and symptoms are mainly jaundice by compression of or rupture into the bile ducts, or anaphylactic shock, eosinophilia, urticaria, and/or acute abdominal pain in case of cyst rupture in the peritoneal cavity. Cyst rupture may be favoured by any abdominal trauma, often related to sport practice in children of endemic areas. Compression of the common bile duct, portal or hepatic veins, or inferior vena cava is uncommon. Rupture of the cyst (usually into the bile ducts) is more common: depending on the case series, at diagnosis, 30–40 % of hepatic cysts diagnosed in hospital settings have ruptured or become infected (Fica et al. 2012). Among lung cysts referred to surgeons, complicated cysts are also frequent, including lung abscess, pleural involvement, pneumonitis and fibrosis in 10.38, 13.21, 7.55 and 11.32 % cases, respectively (Ghoshal et al. 2012). However, when a cyst is found at mass screening in asymptomatic subjects, in more than 75 % of cases its latent asymptomatic evolution without complications may last more than 10 years (Frider et al. 1999; Wang et al. 2006). In a recent study in India, 26 % of liver cysts were either ruptured or infected when referred to surgeons (Malik et al. 2010). Cysts located near the diaphragm can erode it and extend into the pleural and pericardial cavities, the lung, or the bronchi through perforation. Cysts close to the peritoneal cavity may rupture into the peritoneum or into the duodenum, stomach, colon, or right renal pelvis. These ruptures may lead to extra-hepatic CE by the dissemination of *E. granulosus* germinal layer fragments and of protoscoleces, and favour the development of ‘daughter vesicles’ [a more appropriate denomination than ‘daughter cysts’, if their mode of development is considered (da Silva 2011)] in or out the initial cyst. More commonly, the rupture of cyst occurs into bile ducts, and is revealed by cholestatic jaundice, cholangitis, or biliary pain. Some ruptures into bile ducts may be clinically silent, and are thus only disclosed during an operation.

Cough, hiccups, and chest pain are the main symptoms of lung cysts when there are not diagnosed by chance on a chest X-ray (Ghoshal et al. 2012). Cyst rupture is also a frequent opportunity for diagnosing lung cysts: rupture in the bronchi may be followed by elimination of cyst fluid and materials (membranes, protoscoleces) by cough.

In endemic areas, mass screening programmes have been implemented for the past 20 years, using US and serology, and have become a common circumstance of diagnosis. Such mass screening campaigns have shown that liver cysts had a very

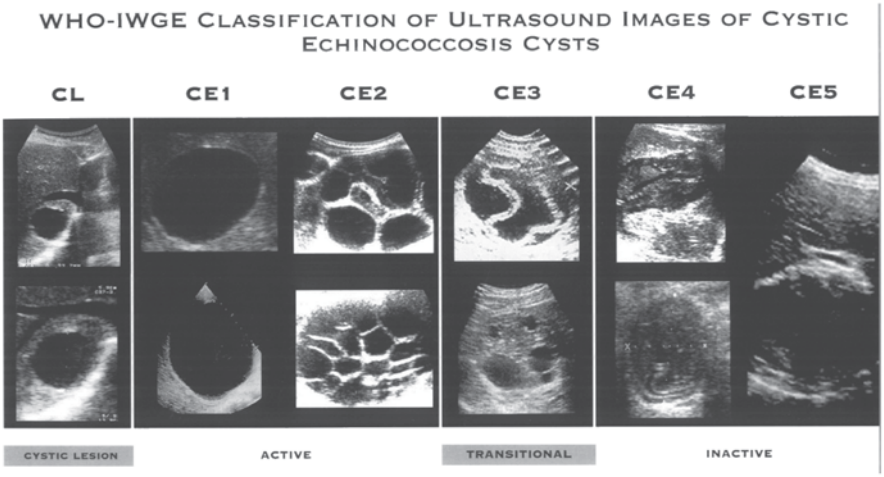

Fig. 29.4 International classification of cystic Echinococcosis cysts based on ultrasound images (according to WHO-Informal Working Group on Echinococcosis; in WHO-Informal Working Group on Echinococcosis 2003)

slow and limited growth: in more than half of the cysts there were no modifications in cyst size during the 10–12-year period of observation, in one third growth was slight (<3 cm) and in only one case (7%) the cyst grew by 4 cm. Mean cyst growth in all 14 cases with a prolonged follow-up was 0.7 cm (Frider et al. 1999; Wang et al. 2006).

Imaging Diagnosis of CE The two key procedures for indirect diagnosis are US and serology. US can be used to recognize cysts as small as 1 cm in diameter, and may be used for diagnosis in hospital settings as well as for mass screening (Macpherson et al. 2003). It usually shows one or several round masses with a well-defined contour, which may be empty or filled with echogenic structures corresponding to daughter vesicles. US can be used to recognize the detachment of the endocyst that appears as a wavy line inside the cavity. When cysts become infected, they are diffusely hyper-echogenic and no longer exhibit characteristic features. Several classifications of US images in CE have then been proposed with various degrees of complexity, after the first US characterization of CE cysts by Gharbi et al. (1981). The WHO-Informal Working Group on Echinococcosis (IWGE) has promoted a unified classification (Fig. 29.4), which is currently used as a standard to compare data from mass screenings and results of therapeutic interventions (WHO-Informal Working Group on Echinococcosis 2003). This classification groups the cysts according to their activity (Hosch et al. 2008); however, stage CE3a, characterized by the detachment of membranes may either progress to development of daughter vesicle (CE2) or to degeneration (CE4 and 5). In addition, when the classification is used to assess any therapeutic intervention, the diameter of cysts (in cm) rather than semi-quantitative values should be given (Wang et al. 2003).

On computed tomography (CT), unilocular cysts with their spherical or oval structure of near-water density are easily recognized. Conversely, multilocular cysts may have several CT patterns depending on the space occupied by daughter vesicles inside the cyst. Abscesses or necrotic tumours may mimic CE cysts. In such circumstances, with negative serological results, only aspiration cytology can establish the diagnosis of hydatid cyst (Hira et al. 1988). CE cyst aspiration with a fine needle under ultrasound guidance is currently considered as medically and ethically acceptable (WHO-Informal Working Group on Echinococcosis 2001). The appropriate procedure aimed at preventing and/or treating any anaphylactic complication and protoscolex spillage should be followed. The diagnosis-aimed puncture should be the first step of a therapeutic procedure of Puncture, Aspiration, Injection and Re-aspiration (PAIR) if the diagnosis of CE is confirmed (see below).

Magnetic Resonance Imaging (MRI) was shown recently to reproduce the ultrasound-defined features of CE better than CT, and could be of use if for any reason US is not available (Stojkovic et al. 2012). FluoroDeoxyGlucose (FDG)-Positron Emission Tomography (PET) seems to have little value for the routine diagnosis or follow-up of CE, but may be of help for differential diagnosis with malignant lung tumours (Kurt et al. 2008); however, peripheral FDG uptake was described in a case with liver cyst, with a typical "doughnut aspect" (Demir et al. 2008).

Clinical Presentation of AE Before the 1980s in the European endemic area, AE was frequently recognized at an advanced stage and misdiagnosed as liver neoplasia: jaundice was the most frequent presenting symptom, in nearly 50% of the cases; it was either progressive jaundice related to hilum involvement, associated with pruritus, or intermittent jaundice with pain and fever related to superimposed bacterial biliary infection (Bresson-Hadni et al. 2000). Hepatomegaly, generally massive, was also a possible revealing symptom in about a quarter of the cases. This is still the situation in endemic areas of China (Ayifuhan et al. 2012) except in those regions where a systematic mass screening of the population is implemented.

During the past 30 years in Europe and Japan, changes in the revealing symptoms have occurred, because of disclosure of less severe and asymptomatic cases. Less than 25% of cases are revealed by jaundice; and hepatomegaly is observed in only 15% of the cases. Discomfort in the right upper quadrant is a revealing symptom in about 30% of cases. The contrast between a hepatomegaly mimicking a liver carcinoma or advanced cirrhosis and a good clinical status raises the suspicion of AE in endemic areas. Erratic clinical signs and symptoms generally due to metastasis or extra-hepatic location of the parasite may also be observed at presentation (Bresson-Hadni et al. 2007; Ehrhardt et al. 2007), Diagnosis may be made during a surgical operation or an US, CT or PET examination for another reason, or as the result of mass screening in an endemic area. Asymptomatic cases are more frequently disclosed in immune suppressed patients, especially when organ location/dissemination of cancer, lymphoma or leukemia is looked for during patient's follow-up, when pre-treatment evaluation is performed for systemic inflammatory/autoimmune diseases, or during the follow-up of patients after organ transplantation. Such AE cases found in immunosuppressed patients appear to be more and

more frequent; evolution of AE seems faster in these patients; clinical symptoms, if any, may mimic liver abscess; and both imaging and serological diagnoses may be more difficult to interpret (Kern et al. 2011; Gruener et al. 2008; Chauchet et al. 2014).

The most frequent complications of AE are bacterial or fungal infection of the bile ducts and/or of the central necrotic area of lesions, with sepsis and septic shock (Bresson-Hadni et al. 2007; Kern 2010). Loco-regional extension or a hematogenous spread of parasitic tissue with distant metastases may cause a variety of symptoms ranging from dyspnea and bile-tinged sputum to seizures and stroke as well as skin nodules or bone pain or fractures. Unlike what happens with CE, anaphylactic reactions as revealing symptoms are extremey rare; the occurrence of such symptoms is always associated with systemic dissemination of parasitic fragments through the vessels. Bleeding from oesophagogastric varices related to portal hypertension, secondary to biliary cirrhosis or to chronic parasitic Budd-chiari syndrome or portal thrombosis have become rare because of a more systematic prevention and treatment of such varices (Bresson-Hadni et al. 2006, 2007).

Imaging Diagnosis of AE US and CT remain the basic imaging techniques in AE. US is the current screening method of choice for diagnosis and regular follow-up imaging in AE, as well as for mass screening (Bresson-Hadni et al. 2006; Macpherson et al. 2003; Bartholomot et al. 2002; Yang et al. 2006; Kantarci et al. 2012). In two third of the cases the lesion is characterized by irregular limits and heterogenous content with juxtaposition of hyperechogenic and hypoechogenic areas. The hyperechogenic fibrous tissue often contains scattered calcifications. Less typical US aspects include: (1) small haemangioma-like hyperechogenic nodules which correspond to a lesion at its earlier stage (currently more frequent in asymptomatic immunosuppressed patients; (2) pseudo-cystic lesions which correspond to huge AE lesions with massive necrosis; the surrounding hyperechogenic ring and the irregular lining should suggest the diagnosis of AE; (3) small calcified lesions which can correspond to either an abortive form of the disease or to a small-size developing AE (Bresson-Hadni et al. 2006). US combined with color Doppler can also provide useful information on biliary and vascular involvement. The limits of US examination lie in the calcification of the lesion which may prevent a proper evaluation of the lesion and its real extent. Contrast-Enhanced Ultrasonography (CEUS) is still under evaluation. Preliminary results showed that Levovist®-CEUS did not provide useful information (Ehrhardt et al. 2007); but using Sonovue® as a micro-bubble contrast agent could bring a very significant improvement both for the diagnosis and the follow-up of AE patients, by delineating the periparasitic micro-vascularized content of the lesions (Tao et al. 2011).

The typical CT aspect is a tumor-like lesion with irregular lining and heterogenous content with a mosaïc of various densities: scattered, hyperdense calcifications and hypodense areas corresponding to necrosis and/or active parasitic tissue (Didier et al. 1985; Reuter et al. 2001). No significant enhancement is observed within the lesion after bolus administration of intravenous contrast medium; however, enhancement of the periphery of the lesions is sometimes observed. An intra-hepatic

bile duct dilation in the contro-lateral lobe of the liver is the hallmark of infiltration of the hilum by the parasitic process. Hypertrophy of the contro-lateral lobe is also usual.

MRI imaging may facilitate the diagnosis in uncertain cases with non-calcified lesions. It is the best technique to characterize the different components of the parasitic lesion and to study the extension to adjacent structures (Bresson-Hadni et al. 2006; Reuter et al. 2001; Kodama et al. 2003). It shows the pathognomonic aspect of multiple small vesicles, best observed on T2-weighted images. Cholangio-MR imaging has now advantageously replaced the classical per-cutaneous cholangiography. It is an important part of the pre-operative evaluation, as it provides information on the relationship between AE lesion and the biliary tree.

PET using [18 F] FDG has been evaluated to assess the viability of the lesions, at diagnosis and in the follow-up of inoperable AE patients under long-term benzimidazoles therapy (Reuter et al. 1999). Although FDG uptake does not actually reveal the metacestode, but is mostly associated with the periparasitic infiltrate by immune cells, this indirect approach may be useful and is best correlated with parasitic activity when 'delayed' FDG uptake images are analyzed, i.e. 3 h after FDG injection (Caoduro et al. 2013).

29.5 Immunological and Molecular Diagnosis of Echinococcosis

Serological tests may confirm the diagnosis of echinococcosis, although there is currently no standardized, highly sensitive and specific as well as inexpensive test available for antibody detection of CE or AE. Cross-reactivity is observed between both cestodes, and most tests for CE can be used for the diagnosis of AE and vice versa. In those endemic countries where both diseases may be found in the same area, a differential diagnosis could theoretically be useful; but most often, distinction is made by imaging. Cellular tests have no value for the routine diagnosis of both diseases (Bresson-Hadni et al. 1989). The intra-dermal Casoni test is no longer used because of its lack of sensitivity and specificity as well as because of safety issues (risk of anaphylactic shock).

Serological Tests Detection of specific antibodies in serum uses antigens obtained from *E. granulosus* (hydatid fluid or protoscoleces) or *E. multilocularis* (protoscoleces or parasitic extracts), and/or more purified antigens or recombinant proteins from either *Echinococcus* sp. (Yang et al. 2007, 2008a, b; Zhang et al. 2012; Feng et al. 2010; Liance et al. 2000; Ito and Craig 2003). From the available literature and clinical experience, it may be stated that: (1) complement fixation tests are no longer used because of their poor sensitivity and specificity; (2) indirect haemagglutination and latex tests, using crude *Echinococcus* extracts, are both relatively inexpensive and sensitive, but poorly specific; (3) immunoelectrophoresis or immunosyneresis are specific, but poorly sensitive and time-consuming; they are no longer used;

(4) ELISA tests, using crude antigens from *E. granulosus* or *E. multilocularis*, are sensitive, but poorly specific; (5) ELISA tests using specific antigenic fractions or recombinant antigens such as antigen 5 or antigen B of *Echinococcus granulosus*, *E. multilocularis* Em2 or Em18 fractions, and recombinant Em3/10/Em 18, are more specific; (6) Western blots use either crude extracts from *E. granulosus* or *E. multilocularis* (more sensitive) or purified fractions or recombinant antigens (more specific). Combination of antigens in a single test (as the $Em2^{plus}$, commercialized by Bordier Affinity Products, Crissier, Switzerland, or the rapid DOT-immunogold assay, commercialized by Xinjiang Key Lab, Urumqi, P.R. of China) (Feng et al. 2010) or rules of interpretation for the Western blot (e.g. from *E. multilocularis* extract, as that commercialized by LDBio, Lyon, France) (Liance et al. 2000), have attempted to combine positive and differential diagnosis. By evaluating the "best" serological tests on ultrasound and/or CT-confirmed lesions, overall sensitivity reaches 80 % for liver hydatid cysts (it is lower for lung cysts, averaging 70 %) and 95 % for alveolar echinococcosis (it is lower in immune suppressed patients, averaging 85 %); specificity may reach 90 % for both (Ito and Craig 2003). However, diagnostic efficiency of serology is limited both by 1) the reduced capacity of some infected patients to develop specific antibodies (or some isotypes), for genetic or acquired reasons (such as immunosuppression) and 2) the absence of release of specific antigens by the cysts in CE, which decreases sensitivity, and by the existence of infected-non-diseased persons in endemic areas, which decreases specificity. Positive serological results in individuals at mass screening account for at least five different situations: (1) "patent", overt disease with symptoms, (2) "latent", non-apparent disease; (3) calcified dead lesions in the liver; (4) CE cyst in the lung or other organs and rare cases of isolated extra-hepatic AE despite no US lesions in the liver; and, (5) no parasitic lesions at all (Yang et al. 2007, 2008a). Negative serology with patent CE or AE lesions have been found in all mass screening surveys and is the rule in all published hospital case series (Yang et al. 2007, 2008b). Serology should thus never been used as a "first intent" test, but always combined with ultrasound imaging in mass screening, and only be used as a confirmation test for a suspected diagnosis based on imaging techniques in clinical settings.

Molecular Tests Molecular identification of both *E. granulosus* and *E. multilocularis* is mostly based on PCR using mitochondrial DNA probes, but also on nuclear sequences, including microsatellites. It may be used, if echinococcosis is suspected despite negative serology, on liver needle aspiration (for CE) and/or liver biopsy (for AE) (Dybicz et al. 2013) and it may be performed retrospectively, on paraffin-embedded samples (Grenouillet et al. 2013; Schneider et al. 2008; Simsek et al. 2011). In addition, it may be useful in epidemiological studies to identify the actual species of *E. granulosus* involved in human contamination from the animal cycle (Dybicz et al. 2013; Bardonnet et al. 2002). On the other hand, several techniques have been described to identify worms or eggs of *Echinococcus* spp. in the feces of definitive hosts: the most recent techniques aimed at avoiding the recurrent problem of RNAses and other inhibitors present in the fecal samples, while identifying the species of the animal host (Dinkel et al. 2011; Boubaker et al. 2013).

29.6 Anti-Parasite Drugs and Echinococcosis

Two Orphan Drugs for Two Neglected Diseases Compared to most of parasitic diseases and/or zoonoses, for which an appropriate and efficacious medical treatment is available, echinococcosis can rarely be cured by anti-parasite chemotherapy alone, safely and within an acceptable time schedule, and the available drugs are extremely limited (WHO-Informal Working Group on Echinococcosis 1996; Brunetti et al. 2010; Junghanss et al. 2008; Brunetti and Junghanss 2009). In addition, there have been nearly no well-designed clinical trials for any medical treatment modality in either form of echinococcosis (Junghanss et al. 2008; Kern 2006). Only two benzimidazole compounds, used since the beginning of the 1980s have proven effective against CE and AE: mebendazole (MBZ) (4.5 g/d) and albendazole (ABZ) (10–15 mg/kg/d) (WHO-Informal Working Group on Echinococcosis 1996; Brunetti et al. 2010). Both drugs have a poor bio-availability, mostly due to poor intestinal absorption; the most practical way to improve absorption is to combine ABZ with a fatty meal. ABZ (i.e. ABZ sulfoxide, the active compound originating from hepatic metabolism), however, may reach higher plasma levels than MBZ. For this reason, and because it is approved by the drug agencies of most countries, opposite to MBZ, ABZ is the most widely used drug for the treatment of echinococcosis. Both MBZ and ABZ must be given continuously, without interruption, for the period of treatment assigned to each case depending on the type of disease and treatment association. Praziquantel (PZQ) is the only other drug of use in echinococcosis, since it is the only one with a demonstrated effect on *Echinococcus* sp. oncospheres, thus of possible use for deworming definitive hosts, dogs and fox (by baiting) and interrupting the parasite cycle (Gemmell et al. 1977). PZQ also exerts a toxic effect on *E. granulosus* protoscoleces (Richards et al. 1988), and may be used as adjuvant therapy together with albendazole in CE with systemic dissemination and/or specific locations, such as bone (Taylor and Morris 1989; Yasawy et al. 1993).

Side Effects of Benzimidazoles Adverse events of benzimidazoles occur in 6–20 % of treated patients; they look more frequent when associated treatments are needed for other conditions, especially immune suppressants (Horton 1989, 1997). General complaints include headache, nausea, anorexia, vomiting, abdominal pain and itching, and weight gain; in a few cases, idiosyncratic/allergic reactions have been observed (Horton 2003). A significant, but usually reversible alopecia occurs in about 5 % of cases, favoured by cholestasis and/or portal hypertension. In the first weeks of treatment more severe side-effects may be observed, including leukopenia, as well as an increase in liver enzymes which may result from drug efficacy as well as be evidence of drug toxicity (WHO-Informal Working Group on Echinococcosis 1996; Horton 2003). A regular monitoring is therefore recommended: if aminotransferases rise over 3–4 times normal levels, discontinuation of therapy should be considered; ABZ sulfoxide plasma levels measured; and ABZ re-introduced at a lower dosage, with regular monitoring of plasma levels. ABZ has been shown to be teratogenic in rats and rabbits and, therefore, it should be avoided during pregnancy

(at least during the first trimester) and lactation (Brunetti et al. 2010). Although they are closely related drugs, MBZ may be better tolerated by patients with ABZ side-effects and vice versa; a switch to the other drug can thus be recommended before withdrawing chemotherapy definitively (Bresson-Hadni et al. 2011).

Candidate Drugs? All attempts at using other drugs for the treatment of echinococcosis have been unsuccessful until now, including promising drugs such as nitazoxanide, the effects of which were not convincing in humans after a pre-clinical trial (Stettler et al. 2004; Kern et al. 2008). Several candidates have recently been proposed, from experimental *in vitro* and *in vivo* studies (Hemphill and Muller 2009); none of them has reached phase I/II trials in humans. Amphotericin B as salvage treatment for AE patients with intolerance or resistance to benzimidazoles effectively halted parasite progression in a small series of patients, but its IV administration precludes its use for the treatment of a chronic disease (Reuter et al. 2003). Because of the exquisite sensitivity of *Echinococcus* larvae to the immune response of the host, efficacy of immune therapy may be anticipated; the only proof of concept was given a decade ago for interferon-alpha, with nearly complete prevention of *E. multilocularis* infection in experimental mice, and impressive regression of hepatic lesions in a patient with AE (Harraga et al. 1999; Godot et al. 2003) however, no clinical trials have been implemented to confirm these preliminary successes.

29.7 Treatment and Follow-up of CE Patients

Surgical Treatment of CE The objective of surgery is to remove all parasitic cysts. Hepatic resection is usually only recommended for central cysts of a left lateral segment. Controversies still exist about the preferred operating technique of cystectomy among all proposed operations. Partial cystectomy, with an appropriate management of the residual cavity, is often preferred in endemic areas, since such a technique is easier to achieve by local surgical teams. However, without concomitant treatment by ABZ, recurrence and/or dissemination of cysts is frequent despite the measures implemented to prevent spillage of protoscoleces or of germinal layer fragments, i.e. protection of the peritoneal cavity during cyst evacuation; sterilization of the cyst by injection of protoscolecidal agents, e.g. chlorehexidine, H_2O_2, 80 % alcohol, or 0.5 % cetrimide, then evacuation of the cyst content. Recurrence of hepatic cysts and peritoneal dissemination of cysts are generally underestimated (2–25 %), since very few series of patients with a prospective follow-up were ever reported in poor-resources endemic countries. In Kenya, among 663 patients with a surgical management of the disease, there were one intraoperative and one postoperative death, respectively, and 47 patients had repeated operations because of postoperative complications and/or recurrences (Cooney et al. 2004). To better avoid recurrence, currently various types of complete liver cystectomy allow surgeons to completely remove the parasite without the risks of liver resection: after the liver overlying the cyst has been incised, attempts are made to excise the

laminated membrane intact; this may be done by following the virtual gap which exists between the inner and outer fibrous layer surrounding the cyst (Peng et al. 2006). Such a 'total subadventitial cystectomy', as proposed by Chinese surgeons, should be used whenever possible, if a surgical treatment for a non-complicated cyst is necessary (Peng et al. 2002). Peritoneal dissemination and/or recurrence are difficult to treat. In any other locations (in the lung, brain, or any organ), cysts should be removed totally whenever possible (Yu et al. 2012; Duishanbai et al. 2011). For complicated liver cysts, a variety of techniques may be used, including surgical or perendoscopic biliary drainage (Dziri et al. 2009); protoscolicidal agents should never be injected in the cyst when biliary communication is suspected; formalin and hypertonic saline must no longer be used in any cases because of the risk of caustic sclerosing cholangitis. Liver transplantation (LT) has been exceptionally performed in patients with severe complications (Chomicz et al. 2003).

Non-Surgical Interventional Treatment of CE: Puncture–Aspiration–Injection–Reaspiration (PAIR) and Laparoscopy Since 1986, PAIR has been an alternative to surgery (Gargouri et al. 1990) After percutaneous puncture under ultrasonographic guidance, a complete aspiration is performed; the residual cavity is then filled with a scolicidal agent, usually ethanol, which is reaspired 10 min later. A meta-analysis has concluded to the efficiency, safety and usefulness of the procedure (Smego et al. 2003). It is indicated in medium-sized CE1 and CE3a cysts, and contraindicated for very large-sized cysts, and if there is communication of the cyst with the biliary tree, assessed using cysto-cholangiography or by checking bilirubin in the cyst content. A very limited number of anaphylactic shocks and less secondary dissemination than after surgery have been reported (Neumayr et al. 2011). Drainage may be associated with PAIR for large cysts. PAIR-like techniques, using larger tubes and vacuum aspiration have been described to treat cysts with daughter vesicles; however such procedures have very long catheter times and do not compare favourably with surgery (Schipper et al. 2002). Laparoscopic aspiration and sterilization of the cysts are also feasible but may be associated with spillage and recurrence, if the patients are not treated with ABZ before/after the procedure; per-laparoscopic cystectomy is also an option which is more and more adopted by properly trained surgical teams (Rooh-ul-Muqim et al. 2011; Koea 2012; Busic et al. 2012). Per-thoracoscopic interventions may also be performed to remove pulmonary or mediastinal cysts (Alpay et al. 2012; Aydin et al. 2012).

Anti-Parasite Treatment and Follow-up of CE Patients Benzimidazoles, which are parasiticidal on *E. granulosus in vitro*, are indicated for patients with multiple cysts in two or more organs and for patients with peritoneal cysts. ABZ may also be used to treat small-size cysts, since it was shown to be more effective on such cysts and when, given the natural history of CE, surgery might be disproportionate, especially in children. ABZ alone has a better effect on CE cysts than placebo or MBZ (Franchi et al. 1999). One prospective controlled trial of ABZ and PZQ (25 mg/kg/d) versus ABZ alone (Mohamed et al. 1998) concluded that the combined treatment was more effective than ABZ alone. However, in randomized controlled trials complete disappearance of all cysts was never reached. Treatment schedules usually

include “treatment cycles” of three month-continuous administration of ABZ, with imaging evaluation for signs of cyst degeneration after each cycle and decision to stop or prolong the treatment. Pre-and post-intervention chemotherapy with BZM may reduce the risk of recurrence; there is no optimal scheme, but the current option is to give ABZ three weeks before and during one–two months after surgery (Dziri et al. 2004). Perioperative use of PZQ to prevent protoscolex seeding, albeit logical, has not received documented evidence of efficacy. Association of ABZ with PAIR increases clinical and parasitologic efficacy (Smego et al. 2003). ABZ should not be administered when PAIR is performed during pregnancy.

It may be anticipated that the multidisciplinary approach, which is now commonly used for AE patients, will soon also become a routine for the management of CE patients. Patients treated for CE, either by combined surgery and ABZ or by ABZ alone, should have a follow-up every three months, including at least US examination. Blood cell count and amino-transferase levels should be measured every week during the first month of benzimidazole treatment. A yearly long-term follow-up, including at least US, for at least 3 years after interruption of the anti-parasite drug and/or after cystectomy is needed to detect any cyst recurrence.

Finally, long-term follow-up of patients with a hydatid cyst disclosed at mass screening has shown that a significant percentage, especially among children, had a degenerating evolution or did not change with time (Frider et al. 1999); and a “watch and wait” attitude is likely indicated in such cases with small cysts (Brunetti et al. 2010; Junghanss et al. 2008); it may also be recommended in CE4- and CE5-type cysts.

29.8 Treatment and Follow-up of AE Patients

A Multidisciplinary Approach for the Treatment of Patients with AE The therapeutic management of AE patients clearly requires a multidisciplinary approach, in which benzimidazole therapy is a common denominator (see below). A complete evaluation of the disease extension (including thoracic and brain CT) is necessary before any therapeutic decision. Depending on the size of the lesion(s), its location in the liver and vascular and biliary involvement, invasion or not of adjacent organs, presence or absence of distant metastases, the options may be a curative resection or a prolonged ABZ treatment (associated with an interventional radiological or per-endoscopic procedure, if necessary because of complications). Currently, ‘partial debulking’ liver resections followed by continuous administration of a benzimidazole must be avoided (Kadry et al. 2005; Buttenschoen et al. 2009a, b). The PNM system of classification of AE cases, designed on the model of the TNM classification of cancers, helps clinicians to choose the appropriate treatment and the clinical teams to evaluate their results comparatively (Table 29.1) (Kern et al. 2006).

Table 29.1 PNM classification and staging of alveolar echinococcosis. (According to WHO-Informal Working Group on Echinococcosis; in Kern et al. 2006)

A. PNM classification of AE cases (at presentation)
P Hepatic localisation of the parasite
P X Primary tumor cannot be assessed
P 0 No detectable tumor in the liver
P 1 Peripheral lesions without proximal vascular and/or biliary involvement
P 2 Central lesions with proximal vascular and/or biliary involvement of one lobe
P 3 Central lesions with hilum vascular or biliary involvement of both lobes and/ or with involvement of two hepatic veins
P 4 Any liver lesion with extension along the vessels and the biliary tree
N Extra hepatic involvement of neighboring organs (diaphragm, lung, pleura, pericardium, heart, gastric and duodenal wall, adrenal glands, peritoneum, retroperitoneum, parietal wall (muscles, skin, bone), pancreas, regional lymph nodes, liver ligaments, kidney)
N X Not evaluable
N 0 No regional involvement
N 1 Regional involvement of contiguous organs or tissues
M The absence or presence of distant Metastasis (lung, distant lymph nodes, spleen, CNS, orbital, bone, skin, muscle, kidney, distant peritoneum and retroperitoneum)
M X Not completely evaluated
M 0 No metastasis[c]
M 1 Metastasis
B. PNM stage grouping of alveolar echinococcosis
Stage I P1 N0 M0
Stage II P2 N0 M0
Stage III[a] P3 N0 M0
Stage III[b] P1–3 N1 M0
P4 N0 M0
Stage IV P4 N1 M0
Any P Any N and/or M1

[a] For classification, the plane projecting between the bed of the gall bladder and the inferior vena cava divides the liver in two lobes

[b] Vessels mean inferior vena cava, portal vein and arteries

[c] Chest X-ray and cerebral CT negative

Surgical Treatment of AE The only efficient treatment is partial hepatectomy when the lesions are located in liver segments accessible to resection; because the intrahepatic common bile duct is usually involved, it is often necessary to remove the bifurcation and to reconstruct the biliary tract using a Roux-en-Y loop (Kadry et al. 2005; Buttenschoen et al. 2009a, b; Crouzet et al. 2010; Ammann et al. 1998; Sato et al. 1997). Currently 1/3 of patients with AE may benefit from a curative ('radical') resection of their 'parasitic tumour'. In very severe cases, with life-threatening complications and no other options, liver transplantation may be proposed. Allogeneic liver transplantation is associated with a risk of recurrence, or of progression of extra-hepatic locations, because of immunosuppression (Koch et al. 2003). Such a risk may be alleviated by early ABZ treatment after transplantation; and unexpected long-term survival of more than 20 years has been published

in patients with residual lesions after liver transplantation (Bresson-Hadni et al. 2011). Auto-transplantation is a recent alternative which has been developed to allow easier resection of large-sized lesions with vascular involvement: long-term evaluation is however needed to make indications of such a technique more precise (Wen et al. 2011).

Non-Surgical Interventional Treatment of AE When curative resection is not possible, palliative surgery should be replaced by percutaneous or perendoscopic procedures. They consist of percutaneous radiological drainages of huge centro-parasitic abscesses or of dilated intra-hepatic bile duct above a hilum stenosis. The best option is to push the drain beyond the stenosis to obtain an external/internal biliary drainage. Such drains may be maintained for years; combined with chemotherapy, they have allowed prolonged survival in initially very severe AE cases; biliary endo-prosthesis/stent insertion is an alternative which is currently more and more frequently used, and is under evaluation (Bresson-Hadni et al. 2006).

Anti-Parasite Treatment and Follow-up of AE Patients MBZ and ABZ have only a parasitostatic effect *in vitro* and cannot kill *E. multilocularis* in most AE cases; however, their benefit for patients' survival and quality of life is now well assessed (Ammann et al. 1994; Ishizu et al. 1997). There are no comparative studies, but ABZ is currently preferred because it reduces the cost by >40 %, is easier for patients to take and is now licensed for AE in many countries (Reuter et al. 2000). In case of curative surgery, ABZ should be initiated before the operation and maintained for at least 2 years to avoid recurrence (Brunetti et al. 2010). In inoperable cases, long-term chemotherapy (i.e. for life) may significantly prolong survival (10-year survival of approximately 80 %, compared with less than 25 % in historical controls) (Brunetti et al. 2010). A more personalized medical treatment of inoperable AE patients seems now to be possible, thanks to the combination of sequential FDG-PET evaluation with delayed acquisition of images, 3 h after FDG injection, and of new serological markers, to better assess the 'activity' of the parasitic lesions (Caoduro et al. 2013; Bresson-Hadni et al. 2011; Crouzet et al. 2010; Bardonnet et al. 2013). Discontinuation of BZM after many years of treatment could be tried in selected cases when all 'activity markers' are negative (Caoduro et al. 2013; Bresson-Hadni et al. 2011; Ammann et al. 1998; Bardonnet et al. 2013; Reuter et al. 2004). In liver-transplanted patients, ABZ must be initiated before the operation, re-introduced as soon as possible after transplantation, and maintained for at least 2 years if all AE lesions were removed with the liver, and life-long in case of metacestode remnants or new AE foci discovered during follow-up (Bresson-Hadni et al. 2011).

Whatever the type of treatment, including or not surgery, all patients with AE should have a regular follow-up (every three-months, then six-months, then year, depending on the clinical status and the occurrence or not of complications). The follow-up should include US and serology, blood cell count and aminotransferase levels, and ideally FDG-PET, during the period of benzimidazole treatment; a yearly follow-up should be maintained for at least 5 years after benzimidazole withdrawal.

Monitoring of ABZ sulfoxide is also essential, both to evaluate patient's adherence to treatment and to properly adjust ABZ dosage in case of apparent resistance to treatment or of adverse effects.

29.9 Prevention and Control

As regard to the single individual, prevention of CE relies only on hygienic measures, such as washing hands before eating, avoiding contact between mouth and non-washed hands, thorough washing of raw vegetables and use of a safe water source. Chlorination does not inactivate infecting eggs.

Control programmes for CE are complex, with multiple targets, and require considerable investment of time (minimum 10 years of "attack phase" followed by a "consolidation" and "maintenance" phase) and ressources (Eckert et al. 2001; Huang et al. 2011), together with a consensual coordination of various actors (professionals and decision-makers in human and animal health, police, legislation, education, etc.). The principal points of intervention have been individuated in: (i) veterinary public health actions such as control of livestock movements and slaughter, including inspection of organs and proper disposal of infected viscera and dead animals; (ii) registration of owned dog and control of stray dog population; however, dog culling practices should be carefully evaluated (Johansen and Penrith 2009); (iii) accurate estimation of baseline epidemiological data in the animal and human population; (iv) regular treatment of dogs with praziquantel, at least every 6 weeks; (v) education of the owners about safe feeding of dogs and animal husbandry, and of the whole community about the purpose and importance of the programme; (vi) making CE a notifiable disease and introduce an appropriate supporting legislation (Torgerson et al. 2011; Barnes et al. 2012). So far, only four CE control programmes have been successful, all on islands (Iceland, New Zealand, Tasmania, Falkland Islands); and after an apparent success, one failed in Cyprus. Although purely education has not been found efficacious in reducing infection prevalence with the noticeable exception of the Iceland programme, this is a pivotal measure in all control programmes, as CE is generally not perceived as a serious condition for both animals and humans by communities and policy makers, and populations do not adhere to control measures aimed at animals to prevent a disease in humans (Barnes et al. 2012). The introduction of livestock vaccination using the highly effective EG95 vaccine could be a very useful tool to shorten control programmes length (Huang et al. 2011).

As regards the single individual, prevention of AE relies on similar measures as for CE: *E. multilocularis* eggs are extremely resistant to any chemical and to low temperatures (e.g. those reached by family-use freezers); they are only sensitive to heating, hence the advice to cook any fruit/vegetable collected in pastures/meadows or in family gardens exposed to fox and/or dog feces. Regular praziquantel treatment of dogs follows the same rule as for CE control, at family level, and was used in Alaska on highly endemic islands such as St Lawrence (Rausch et al. 1990). For

decades, it was, however, considered that *E. multilocularis*, being a parasite which circulated in wild life, was globally beyond control (Roberts and Aubert 1995). A few control programs have targeted endemic rural areas, with variable results depending on the endemic country (e.g. Alaska vs Europe vs Japan) (Hegglin and Deplazes 2008). However, most of the recent control programs have concerned urban foxes, using fox baiting with praziquantel, with results that apparently varied according to contamination pressure in the rural areas sourrounding the targeted city (Hegglin and Deplazes 2008; Comte et al. 2013). As *E. multilocularis* fox infection in cities has considerably increased, more and more countries are concerned by the problem, and as more and more immunosuppressed individuals are vulnerable to AE, more studies are needed to assess the best strategy to tackle this emerging public health problem.

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Chapter 30
Hantaviruses—Infections, Epidemiology and Hosts

Sandra S. Essbauer and Ellen Krautkrämer

Abstract Hantaviruses are pathogens of emerging importance in different areas of the world with local outbreaks as reported in 2012 in the Yosemite national park in the US or in several endemic areas in Germany. Quite recently hantaviruses have also been detected on the African continent. The knowledge on respective small mammal hosts and virus types/subtypes has rapidly increased in the last years now including also insectivores, bats and with these several associated new viruses. Usually animals are asymptomatic reservoir carriers, although a few studies show effects on rodent populations. In humans clinical symptoms of hantavirus infections are depending on the virus type causing syndromes of different severity. However, as infections are often clinically inapparent, recorded case numbers may be underestimated. The epidemiology of hantaviruses is complex as multiple biotic and abiotic factors are involved, e.g. rodent population density, corresponding virus prevalence, mast, habitats, biodiversity, rodent ecology, or climate and local weather parameters.

30.1 Introduction

The genus hantavirus belongs to the family *Bunyaviridae*. The natural hosts for hantaviruses are small mammals. For a long time rodents were thought to be the only hosts of these agents. However, in recent years the evidence of hantaviruses in insectivores and bats was confirmed in several host species (Song et al. 2007a, b; Weiss et al. 2012; Sumibcay et al. 2012). These viruses are spread through the animals' saliva, urine and feces and transmitted to humans by inhalation of virus-contaminated aerosols or in rare cases direct contact of humans and rodents (Fig. 30.1) (Heyman et al. 2012).

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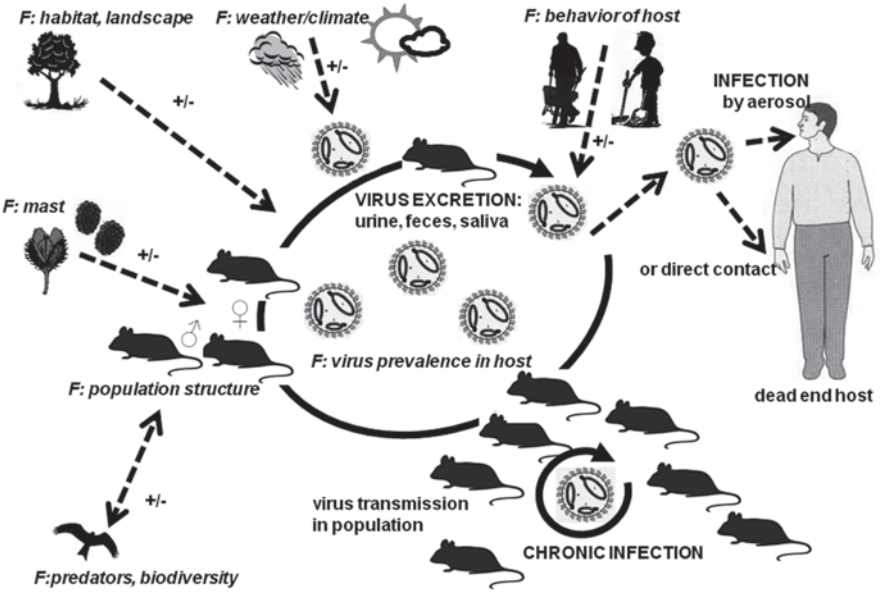

Fig. 30.1 Transmission cycle of hantaviruses and biotic and biotic factors (*F*, in italics) that may influence hantavirus incidence in rodents and humans

Despite the growing list of hantavirus species so far only several rodent-borne hantaviruses have been found to induce human disease. Moreover genetic analyses of representatives of until 2008 described Hantavirus species indicated that our present knowledge on hosts and associated hantaviruses is just on the tip of an iceberg (Henttonen et al. 2008). Thus there are compelling reasons to continue intensive studies on these agents and their hosts.

30.2 An Overview on Hantaviruses

30.2.1 Taxonomy

The actual official taxonomy list includes 24 hantavirus species with *Hantaan virus* (HTNV) being the type species of this genus (Plyusnin et al. 2011). Historically hantavirus infections have been described more than 60 years ago in the 1950's as over 3000 UN soldiers suffered from Korean hemorrhagic fever (Gajdusek 1956; Lee 1982). These infections were caused by HTNV that was the first hantavirus isolated and now is the typespecies of hantaviruses (Lee 1982). Hantaviruses have spherical or oval virions with a diameter of 80–120 nm. The genome consists of a tripartite negative sense RNA. The large (L) segment encodes the viral RNA dependent RNA polymerase (RdRp, L protein), the medium (M) segment encodes

a viral glycoprotein precursor (GPC) which is cleaved into two glycoproteins Gn and Gc and the small (S) segment codes for the viral nucleocapsid protein (N). In some hantaviruses e.g. Tula and Puumala virus the S segment further has an open reading frame (ORF) for a nonstructural protein (NSs) (Plyusnin 2002).The three viral RNA segments are complexed with N proteins and form nucleocapsids. These are packed together with the RdRp in the virus particle. The virions have a lipid envelope in which the two glycoproteins Gn and Gc are embedded (Plyusnin et al. 2011). Criteria for the taxonomy of hantavirus groups or species are based on the differences of amino acid sequences of the S and M segments (Maes et al. 2009).

30.2.2 Rodent-Associated Hantaviruses Causing Disease in Europe and Asia

In Europe several hantavirus species associated with rodents and causing disease in humans have been reported (Table 30.1): First, Puumala virus (PUUV) carried by the bank vole (*Myodes glareolus*) is the virus type with the broadest distribution in this area (Fig. 30.2a). It generally causes a mild hemorrhagic fever with renal syndrome (HFRS) (Heyman et al. 2008, 2009b, 2011). PUUV spill-over to other rodent habitat-sharing species such as *Apodemus sylvaticus* or *A. flavicollis* has been reported. However, the impact of the transmission of virus from spill-over infected rodents to humans is unknown (Heyman et al. 2009b; Klingstrom et al. 2002b; Essbauer et al. 2006; Schlegel et al. 2009). Second, Dobrava-Belgrade virus (DOBV), with its four quite recently suggested but taxonomically not yet approved subtypes Dobrava, Kurkino, Saaremaa, and Sochi (Klempa et al. 2013a) is transmitted by several *Apodemus* (A.) ssp. e.g. *A. flavicollis, A. agrarius*. DOBV causes also HFRS of different severity, from subclinical to life-threatening diseases possibly caused by some exceptionally virulent strains (Klempa et al. 2013a; Avsic-Zupanc et al. 1999; Markotic et al. 2002; Mertens et al. 2011a). It is found in some Central and several South-East European countries (Fig. 30.2b). Third, Tulavirus (TULV) is carried by several vole species of the subfamily Arvicolinae, i.e. *Microtus (Mi.). agrestis* and *Mi. arvalis* in some Central and South European countries (Plyusnin et al. 1994; Vapalahti et al. 1996; Schmidt-Chanasit et al. 2010) (Table 30.1). Its relevance to human disease is controversially discussed (Tersago et al. 2009). Only few human cases of TULV infections with mild symptoms were reported (Schultze et al. 2002; Klempa et al. 2003). Recently, in a seroepidemiological study in Germany in forest workers high TULV seroprevalences were found, so humans at least may develop specific TULV antibodies (Mertens et al. 2011a). Fourth, Seoul virus (SEOV), another hantavirus controversially discussed to be pathogenic, is carried by brown rats (*Rattus* (R.) *norvegicus*). It is suspected to be found almost worldwide. There exist some reports of SEOV detected in brown rats from Belgium (Heyman et al. 2009a; Plyusnina et al. 2012) and in the Lyon region of France (Heyman et al. 2004). SEOV sequences could be generated from two French rural *R. norvegicus* and these clustered with Indonesian/Cambodian strains. SEOV antibody reactivity of human

Table 30.1 Overview on important rodent-associated hantavirus species in Europe and Asia

Virus	Hosts		Diseases	Case fatality rate
Hantaan virus (type species) (HTNV)	Striped field mouse	*Apodemus (A.) agrarius*	HFRS, Korean HF	10–15 %
Puumala virus (PUUV)	Bank vole	*Myodes (M.) glareolus*	Mild HFRS, NE	0.03–0.4 %
Dobrava-Belgrade virus (DOBV)	Yellow-necked mouse (Dobrava)[a]	*Apodemus flavicollis*	Moderate/severe HFRS	10–12 %
	Striped field mouse (Kurkino[a])	*A. agrarius*	Moderate/severe HFRS	0.3–0.9 %
	Striped field mouse (Saarema[a])	*A. agrarius*	Subclinical?	Unknown
	Black sea field mouse (Sochi[a])	*A. ponticus*	Moderate/severe HFRS	>6 %
Tula virus (TULV)		*Microtus (Mi,.) agrestis* *Mi. arvalis* *Mi. gregalis* *Mi. rossiaemeridionalis* *Mi. (Pitymys) subterraneus* *Lagurus lagurus* *Arvicola amphibius*	No symptoms or mild HFRS	0 %
Seoul virus (SEOV)	Brown rat Black rat	*Rattus norvegicus* *Rattus rattus*	Mild HFRS, Korean HF, subclinical?	1–5 %
Amur virus	Korean field mouse	*A. peninsulae*	Mild HFRS, Korean HF	Unknown

[a] suggested, but not yet approved genotypes (Klempa et al. 2013a) *HFRS* hemorrhagic fever with renal syndrome, *HF* hemorrhagic fever, *NE* Nephropathia epidemica

sera was obtained using immunofluorescence assays, but these were not confirmed by focus reduction neutralization test (Plyusnina et al. 2012; Heyman et al. 2012). In the UK in 2012 SEOV was also detected in wild *R. norvegicus*. A genetic characterization showed that this virus resembled SEOV as described from rare mild HFRS cases of humans associated with laboratory-acquired infections by laboratory rats in the UK and Belgium (Jameson et al. 2013a, b). It was postulated that all SEOV described from different countries (Cambodia, Egypt, France, Indonesia, Japan, Singapore, South Korea, Vietnam, UK and the USA) originate from China (Lin et al. 2012).

The continent that is mostly affected by hantavirus disease is Asia. High numbers of cases were reported from China, Far East Russia and Korea (Bi et al. 2008; Liu et al. 2012; Song et al. 2006; Kariwa et al. 2007a). In contrast to Europe, where Nephropathia epidemica (NE) caused by PUUV– the milder form of HFRS- predominates, HFRS represents a severe public health problem in Asia. Cases of HFRS were reported in nearly all Chinese provinces with the northeast of China being the

Fig. 30.2 Distribution maps of **a** Puumala virus and **b** Dobrava-Belgrade virus

most severe endemic area with the highest annual HFRS incidence. Hantavirus-positive mice and rats have been found in most provinces with few exceptions in the western region (Zhang et al. 2010). Causing agents of HFRS are HTNV and SEOV. Infections with SEOV seem to demonstrate milder courses than HFRS caused by HTNV (Zhang et al. 2011). Different epidemiological patterns were observed in the

Russian Federation. About 90% of infections were reported in the Volga Federal District and are described as NE caused by PUUV (Garanina et al. 2009). Furthermore, several outbreaks were attributed to DOBV (Klempa et al. 2008). In contrast, in the Far East region HFRS cases caused by HTNV, SEOV and Amur virus were observed (Yashina et al. 2000; Miyamoto et al. 2003). The seasonality of HFRS cases in Russia also reveals geographic differences and seems to depend on the host reservoir species (Yashina et al. 2000). The seasonal pattern of reported hantavirus disease in humans in China and Korea show a pronounced peak of case numbers in winter and a second minor peak in summer (Liu et al. 2012; Song et al. 2006; Huang et al. 2012). Age and gender distribution corresponds to those seen in other hantavirus diseases (Liu et al. 2012).

Only a few HFRS cases were reported in other Asian countries, e.g. Indonesia, Sri Lanka, India, Thailand, and Vietnam (Jonsson et al. 2010; Huong et al. 2010). No human cases of HFRS were reported in Japan over the last 25 years, although cases were reported since 1960 and seropositive rodents were still present as demonstrated by several epizootiological surveys (Kariwa et al. 2007b). The list of novel hantaviruses in Europe and Asia is growing and some may be associated with HFRS cases. Further studies are necessary to provide genetic evidence for new distinct hantavirus genotypes as causing agents for HFRS.

30.2.3 Hantaviruses in the New World

Hantaviruses became prominent in the US when an outbreak of severe disease with lung manifestation (hantavirus pulmonary syndrome, HPS; hantavirus cardiopulmonary syndrome, HCPS) and death occurred in the Four-Corners Region in 1993 (Nichol et al. 1993). Sin Nombre virus (SNV) was the causative agent of this outbreak (Nichol et al. 1993; Childs et al. 1994; Duchin et al. 1994; Ksiazek et al. 1995). After the detection of SNV many hantaviruses causing HPS were detected in the North, Central and South Americas. Most prominent, two years later in 1995 Andes virus (ANDV) was described during a severe HPS outbreak in southwestern Argentina (Lopez et al. 1996). Remarkably, ANDV is the only hantavirus for which a human-to-human transmission has been demonstrated several times (Wells et al. 1997; Padula et al. 1998; Toro et al. 1998; Martinez et al. 2005; Lazaro et al. 2007; Ferres et al. 2007). An epidemic cascade suspecting nosocomial transmission was first reported from Argentina in 1996. Here several health-care personal with contact to the index patient and afterwards multiple further cases with travel history up to 1400 km occurred. Comparing genetic sequences of patients' viruses showed that 16 human cases were reliably linked in this epidemic (Wells et al. 1997; Padula et al. 1998).

So far, the two species SNV and ANDV are the most common causes of HPS in North, Central and South America, respectively. Meanwhile HPS cases and several other hantaviruses were reported from 30 US states, Canada and at least eight South and Central American States, e.g. Argentina, Bolivia, Brazil, Chile, French Guiana,

Panama, Paraguay, and Uruguay (Koma et al. 2012). In general, case numbers of HPS in Argentina, Brazil and Chile are highest (Martinez et al. 2010; MacNeil et al. 2011). In North America HPS in humans is also induced by e.g. Bayou virus (Morzunov et al. 1995; Khan et al. 1995; Hjelle et al. 1996; Ksiazek et al. 1997; Torrez-Martinez et al. 1998), Black Creek Canal virus (Rollin et al. 1995; Ravkov et al. 1995; Khan et al. 1996), New York virus (Hjelle et al. 1995; Morzunov et al. 1998) and Monongahela virus (Morzunov et al. 1998; Song et al. 1996; Rhodes et al. 2000). In 2012, ten cases of SNV-caused hantavirus infections (HPS) of which three were fatal occurred in Yosemite National Park, California, USA (Center-for-Disease-Control-and-Prevention 2012; Roehr 2012).

In summary, in Northern, Central and South America at least thirty hantavirus types or genotypes have been recognized in HPS patients or carried by sigmodontine reservoir rodents. The pattern of strains as well as the genetic diversity and the distribution are multifaceted and for many of the strains the impact on humans to cause disease is unresolved (MacNeil et al. 2011; Firth et al. 2012). Phylogeographic analyses of South American hantaviruses suggested a spread from the south-central part e.g. Paraguay, Brazil, Bolivia to the northern, southern, and eastern parts of this continent (Firth et al. 2012). New assays to distinguish New World strains by antigenetic properties using truncated N proteins were published recently (Koma et al. 2012). Table 30.2 summarizes hantaviruses in North, Central and South America that induce HPS disease in humans. Figure 30.3 gives an overview on the geographical distribution of New World hantavirus strains.

30.2.4 Hantaviruses Described in Africa—An Actual and Growing Field

The African continent was long a blank spot regarding hantavirus prevalence. Intense studies in small mammal were initiated to find viruses and hosts. Sangassou virus (SANGV) was the first reported African hantavirus detected in Muridae collected in Guinea (Klempa et al. 2006, 2012a). So far, it is the best characterized African hantavirus and also could be isolated (Klempa et al. 2012a). Since then several further hantaviruses were described in shrews from Guinea (Tanganya virus, (Klempa et al. 2007)) and Côte d'Ivoire (Azagny virus, (Kang et al. 2011b)), in Muridae from Ethiopia (Tigray virus; (Meheretu et al. 2012)), and in bats from Sierra Leone (Magboi virus, (Weiss et al. 2012)) and Côte d'Ivoire (Mouyassué virus, (Sumibcay et al. 2012)). The impact of these viruses on human health is so far almost unknown. For SANGV a role in fever of unknown origin and HFRS has been postulated (Klempa et al. 2010). A few seroepidemiological studies showed a low human seroprevalence (1–2 %) for African hantaviruses (Klempa et al. 2010, 2013b; Gonzalez et al. 1984). Further studies are needed in order to understand the impact and relevance of African hantaviruses for humans. Table 30.3 summarizes the present knowledge on these viruses.

Table 30.2 Overview on selected Hantavirus species in the New World pathogenic to humans and causing HPS

Virus	Hosts		Countries
Andes virus (ANDV)	Long-tailed pygmy rice rat	*Oligoryzomys (O.) longicaudatus*	Argentina, Chile, Brazil, Uruguay
Araraquara virus (ARQV)	Hairy-tailed bolo mouse	*Bolomys lasiurus*	Brazil
Juquitiba virus (JUQV)	Black-footed pygmy rice rat	*O. nigripes*	Brazil
Bayou virus (BAYV)	Marsh rice rat	*Oryzomys palustris*	USA (East Coast)
Bermejo virus (BMJV)	Chacoan pygmy rice rat	*O. chacoensis*	Argentina
Choclo virus (CHOV)	Northern pygmy rice rat	*O. fulvescens*	Panama
Black Creek Canal virus (BCCV)	Hispid cotton rat	*Sigmodon hispidus*	USA (South-East, Florida)
Lechiguanas virus (LECV)	Yellow pygmy rice rat	*O. flavescens*	Argentina
Oran virus (ORNV)	Long-tailed pygmy rice rat	*O. longicaudatus*	Argentina
Laguna negra virus (LANV)	Small vesper mouse	*Calomys laucha*	Paraguay, Argentina, Bolivia
New York virus (NYV)	White-footed mouse	*Peromyscus (P.) leucopus*	Canada, USA (East Coast)
Monongahela virus (MGLV)	Deer mouse	*P. maniculatus*	Canada, USA (East Coast)
Sin Nombre virus (SNV)	Deer mouse White-footed mouse	*P. maniculatus* *P. leucopus*	USA (except East Coast)
Maciel virus (MACV)	Yellow pygmy rice rat	*Necromys benefactus*	Argentinia

30.3 Epidemiology of Hantavirus Infections in Central Europe

30.3.1 Epidemiological Data on Hantaviruses in Europe

In European countries human hantavirus infections were sporadically reported since the 1970s or 1980s. A comparison of data from different countries is somewhat difficult, since the temporal onset of official reporting, the quality of official registration systems, case definitions or used assays for hantavirus detection widely differ. Since 2005 data on hantaviruses are available for 30 European countries (Heyman et al. 2008, 2009a, 2011, 2012; Essbauer et al. 2013). Infections in humans are often only diagnosed retrospectively by serological assays. However, hantavirus antibodies show high cross reactivity that makes a species diagnosis complex (Escadafal et al. 2012). Therefore homologous antigens are the method of choice for determining different hantaviral serotypes (Mertens et al. 2011a, 2011b; Koma et al. 2012; Klempa et al. 2010; Meisel et al. 2006). Currently, the focus reduction neutralization test is the accepted method for definitely distinguishing hantavirus serotypes (Heider et al. 2001).

Fig. 30.3 Hantaviruses pathogenic to humans and described in the New World. For abbreviations. (Source: see Table 30.2 Original description sites of the respective viruses are indicated)

The picture of naturally occurring hantaviruses differs in European countries, e.g. in Germany it seems to be more complex as at least three human pathogenic species PUUV, DOBV and TULV are found. Reported incidences of human PUUV infections in Finland, Norway and Sweden are highest in Europe (Tersago et al. 2009). The epidemiology of hantaviruses in France, Belgium and Germany was summarized recently (Heyman et al. 2012; Essbauer et al. 2013). In general, in the last two decades a rising trend of annually recorded numbers of clinically apparent hantavirus infections in Europe has been observed (Heyman et al. 2011). Reasons for this trend might be a higher awareness of public health authorities and improved, and/or better available diagnostic assays. On the other hand, individual behavior of humans due to spare-time activities in the nature or changes in habitats for rodents might cause higher case numbers (Heyman et al. 2011, 2012; Faber et al. 2010).

30.3.1.1 Overview on Clinically Apparent Human Hantavirus Infections: Annual and Seasonal Patterns

The annual patterns of reported human PUUV cases in North and Central Europe are different. In Scandinavia 3–4 year cycles seem to occur (Olsson et al. 2003;

Table 30.3 Overview on hantaviruses detected in reservoir hosts in Africa

Virus	Available data, assays	Evidence for disease in humans	Ref.
Sangassou virus (SANGV)	Isolated, RT-PCRs, full genome sequenced, serology (ELISA, IFA; FRNT)	4.4 % (n = 3/68) SANGV seroprevalence in patients with FUO; 1.2 % (n = 8/649) SANGV reactivity in cohort study; specific IgM and IgG antibodies in one pediatric HFRS patient in Guinea	(Klempa et al. 2006, 2010; , 2013b)
Tigray virus (TIGV)	Partial S segment sequence available	So far not investigated	(Meheretu et al. 2012)
Azagny virus (AZGV)	Partial S, M and L segment sequences available	So far not investigated	(Kang et al. 2011b)
Tanganya virus (TGNV)	Partial S, L segment sequences available	So far not investigated	(Klempa et al. 2007)
Magboi virus (MGBV)	Partial L segment sequence available	So far not investigated	(Weiss et al.2012)
Mouyassué virus (MOUV)	Partial L segment sequence available	So far not investigated	(Sumibcay et al. 2012)

FUO fever of unknown origin, *ELISA* enzyme-linked immune sorbent assay, *IFA* immunofluorescence assay, *FRNT* focus reduction neutralization assay

Vapalahti et al. 2003; Pettersson et al. 2008). Autumn and winter peaks of human cases are recorded in Fenno-Scandinavia (Rose et al. 2003; Evander and Ahlm 2009). Cycles in Central Europe do not follow a regular pattern. Varying local hantavirus outbreaks are reported in different years for Germany, France and Belgium as reviewed in (Essbauer et al. 2013). Even in these neighbouring countries seasonal differences exist and outbreaks do not occur synchronously (Essbauer et al. 2006, 2007; Faber et al. 2010; Mailles et al. 2005; Heyman et al. 2007; Ulrich et al. 2008; Koch et al. 2007; Hofmann et al. 2008; Boone et al. 2012; Ettinger et al. 2012). In France, most human infections are reported during late spring and summer (Vapalahti et al. 2003; Sauvage et al. 2002). In contrast, in Belgium and Germany seasonal peaks are quite variable: some years exclusively show summer peaks, but in other years also winter peaks occur (Heyman et al. 2012; Essbauer et al. 2013; Faber et al. 2010; Piechotowski et al. 2008). So far the seasonal pattern of hantavirus cases in Germany and also neighbouring countries seems unpredictable. In Germany, there might also exist two peaks for DOBV, one in summer and one in winter (Hautala et al. 2002). The latter might be explained by host behaviour, as late in season *Apodemus* spp. search for shelter in houses or garages and therefore humans might have enhanced contact to hosts and excreta.

In Scandinavian countries (Finland, Sweden, Norway) and Russia PUUV incidences are much higher than in Central Europe. High-endemic PUUV regions exist

in most countries, e.g. the northern counties in Sweden (Olsson et al. 2003), the French Jura (Augot et al. 2008), parts of southern Belgium (Mailles et al. 2005), and in certain regions of Germany, e.g. the Swabian Alb, Main-Spessart region, Lower Bavaria and the Osnabrück area (Ulrich et al. 2008; Hofmann et al. 2008; Ettinger et al. 2012). Additionally to the high oscillations it should be mentioned that new regions might become "occupied". For example in 2005 unusual outbreaks in the cities of Cologne, Aachen and Osnabrück were recognized (Essbauer et al. 2007; Mailles et al. 2005; Abu Sin et al. 2007). Another example are cities adjacent to the Swabian Alb, e.g. in 2010 and 2012 cases were registered in Stuttgart (Boone et al. 2012; Hautala et al. 2002).

30.3.1.2 Unapparent Hantavirus Infections: Do We See Only the Tip of an Iceberg?

In most cases officially reported PUUV infections include only clinically apparent infections. It is assumed that only 5–10 % of the infected individuals display clinical symptoms. Therefore, subclinical PUUV infections with unspecific or mild symptoms are often not diagnosed (Tersago et al. 2009). In order to get a better overview on the "true" impact of hantavirus infections on humans seroprevalence studies are conducted. For example, a general hantavirus seroprevalence of approximately 1–2 % was shown for Germany and of 0.45 % for France, respectively (Zoller et al. 1995; Mertens et al. 2009; Martens 2000; Kimmig et al. 2001; Le Guenno 1997). In endemic regions in Central Europe the seroprevalence may locally be up to 5–10 % (Mertens et al. 2009, 2011b; Zoller et al. 1995; Kimmig et al. 2001; Le Guenno 1997; Poeppl et al. 2012). In comparison, in countries with higher PUUV incidence the general hantavirus seroprevalence might be higher, e.g. in Sweden 5–8 %—with 16 % in risk groups (Ahlm et al. 1994, 1998b)—and in Finland 3–5 % (Brummer et al. 1999).

30.3.1.3 Risk Factors for Hantavirus Infections in Humans

Some professions are found to be at risk for hantavirus infections due to exposure to small mammals and their excreta, e.g. construction workers, rodent hunters, workers at horse breeding farms, farmers, lumberjacks, woods-man and soldiers (Mertens et al. 2011a; Abu Sin et al. 2007; Zoller et al. 1995; Ahlm et al. 1998; Clement et al. 1996; Rieger et al. 2005; Kramski et al. 2009; Kelt et al. 2007; Vapalahti et al. 2010, 1999). Seroprevalence in these risk groups can be as high as 15–20 %.

Risk factors are not only defined by contact to nature and rodents, but could depend on the environment of the study sites, the annual and local epidemic situation and other parameters. It was shown that staying or living close to a forest, leisure activities in a forest such as camping, jogging, noticing rodents or droppings, going on a mushroom foray might be risk factors (Abu Sin et al. 2007; Crowcroft et al. 1999; Siffczyk et al. 2006; Turcinov et al. 2013). Besides, cleaning utility rooms such as

sheds, attics, cellars and garages, or disturbing carrier nests by renovation, cleaning, etc. or other activities where the host has prolonged contact with the carrier's habitat (camping, sleeping on the ground, military exercises, etc.) were described as risk factors (Winter et al. 2009; Linard et al. 2007; Tersago et al. 2011). Smoking due to the inhalation of virus-contaminated aerosols was also discussed to be a possible risk factor for PUUV infections (Vapalahti et al. 2010).

30.3.2 Factors Driving Hantavirus Outbreaks in Humans

In summary, the reported increase of PUUV cases in Europe cannot only be explained by a better awareness of physicians or an improvement of diagnostic tools (Meisel et al. 2006; Mertens et al. 2009, 2011b; Heider et al. 2001; Zoller et al. 1995). A complex pattern of parameters drives the hantavirus epidemiology. Many biotic and abiotic factors are involved which may be regionally quite different and result in various interaction patterns. For example, data from France, Belgium and Germany show that (i) hantavirus carriers, (ii) rodents population density and PUUV prevalence in rodents, (iii) food availability such as different forms of mast, (iv) landscapes and habitats, (v) biodiversity, (vi) urbanization and host behavior, (vii) rodent ecology, (viii) features of the reservoir population such as rodents age, sex and genetics, and (ix) climate, local weather parameters and virus tenacity may influence the patterns of hantavirus outbreaks. However, there remain many open questions on the tuning of outbreaks and further longitudinal studies are needed (Heyman et al. 2012; Essbauer et al. 2013; Reusken and Heyman 2013).

30.3.3 Clinical Patterns and Pathogenesis of Human Disease

Hantavirus diseases differ in clinical picture and severity. The variations in symptoms and outcome depend mainly on the causative hantavirus species. However, individual differences were also observed: infections exhibit a broad range of severity reaching from subclinical or mild to severe disease or even fatal outcome. Young and middle-aged persons are mostly affected. Hantavirus diseases—HFRS, HCPS and NE—in children were rarely reported (Makary et al. 2010; Ramos et al. 2001; Klein et al. 2011). Men are 1.7–3.1 times more often affected than women both by Old and New World hantaviruses, respectively (Heyman et al. 2007; Klein et al. 2011).

The incubation time from hantavirus infection to the onset of first symptoms ranges from several days to a few weeks (Young et al. 2000; Vial et al. 2006). The different hantavirus diseases share the characteristic sudden onset with flu-like symptoms such as fever, headache, myalgia and gastrointestinal symptoms. After the initial phase HFRS is characterized by renal manifestation. The laboratory findings comprise thrombocytopenia, leukocytosis, and elevated levels of serum creatinine, proteinuria, hematuria and hypoalbuminemia. In the case of PUUV infections

the disease presents as acute renal failure with often massive proteinuria. Transient dialysis treatment is required in about 5% of cases (Huttunen et al. 2011; Krautkramer et al. 2012). Severe forms of HFRS leading to death due to cardiogenic shock are caused by HTNV, DOBV and SEOV (Table 30.1) (Zhang et al. 2010; Klempa et al. 2008). Renal biopsy examination of HFRS patients reveals interstitial hemorrhages, inflammatory cell infiltrates and edema. In addition, changes in the peritubular and glomerular endothelial cells were observed (Ferluga and Vizjak 2008). Glomeruli appear mostly normal in light microscopy analysis. However, proteinuria of nephrotic range indicates a possible glomerular damage and electron microscopy and immunofluorescence analysis show alterations in the morphology of glomerular endothelial cells and the glomerular cell-to-cell contacts (Ferluga and Vizjak 2008; Krautkrämer et al. 2011).

Aside from the initial phase, the clinical presentation in patients suffering from HCPS differs from that seen in most HFRS patients. HFRS is characterized by renal involvement with often massive proteinuria. In contrast, in HCPS the initial phase is followed by the cardiopulmonary phase with respiratory distress, hemodynamic alterations, pulmonary edema, hypotension and hypoxia. Tissue specimens of the lung demonstrate a mild to moderate interstitial pneumonia with mononuclear cell infiltrates, congestion and intraalveolar edema. Microscopic analysis of lung and kidney tissue shows no gross epithelial or endothelial damage despite the presence of viral antigen (Duchin et al. 1994; Zaki et al. 1995). The infection with HCPS-causing hantaviruses may result in cardiogenic shock. The case fatality rate is 30–40% (Jonsson et al. 2010; Hjelle and Torres-Perez 2010). As for HFRS, thrombocytopenia and leukocytosis were common laboratory findings. Proteinuria and a moderate elevation of serum creatinine are also observed and indicate a mild involvement of the kidney in HCPS, but apparently less prominent than observed in HFRS (MacNeil et al. 2011; Katz et al. 2001; Bayard et al. 2004; Castillo et al. 2001). Despite differences in the majority of cases, the infection with HCPS- or HFRS-causing hantavirus species share the enhanced vascular permeability that affects different anatomical sites.

Atypical courses of diseases for Old and New World hantaviruses were recognized and come actually in the focus of clinical research. Extrarenal manifestations such as complications of the central nervous system (CNS) were observed in patients infected with Old World hantaviruses (Hautala et al. 2002; Alexeyev and Morozov 1995; Ahlm et al. 1998; Hautala et al. 2010; Sariguzel et al. 2012). Furthermore, severe pulmonary and cardiovascular involvement were described for PUUV and DOBV (Linderholm et al. 1997; Connolly-Andersen et al. 2013; Mentel et al. 1999; Schutt et al. 2004). Likewise, renal manifestations in HCPS-causing viral species were observed. Several reports demonstrate cases with a predominant kidney involvement in HCPS (Passaro et al. 2001; Dara et al. 2005).

Long-term consequences of hantavirus infection are often discussed. A higher prevalence of hypertension and of increased protein excretion is observed in patients after NE disease (Miettinen et al. 2006, 2009; Rippe 2006) and several studies observed renal sequelae in patients that survived HCPS or HFRS (Pergam et al. 2009). The severity of courses, symptoms and differences in organ manifestations

are difficult to explain or to predict. Several laboratory parameters were described as predictors for severe courses and patient-specific characteristics were identified as risk factors. Genetic factors associated with severe or mild course of NE are specific human leukocyte antigen (HLA) genotypes and polymorphisms of cytokine genes (Mustonen et al. 1996, 1998; Kanerva et al. 1998; Makela et al. 2001). A predictive marker for severe course of NE and HFRS represents the number of thrombocytes (Rasche 2004; Wang et al. 2013).

Furthermore, gender-specific differences in the clinical course are known. Higher case fatality rates among women were observed in China (Klein et al. 2011), for HCPS in Argentina and during the first year after acute PUUV infection in Sweden (Martinez et al. 2010; Hjertqvist et al. 2010). However, CFRs among male and female HCPS cases in the United States were not different and a higher risk for young male patients for severe central nervous system complications was observed in Finland (MacNeil et al. 2011; Hautala et al. 2011). Multiplex analysis of the cytokine response of a Swedish cohort of PUUV infected patients revealed also gender-specific differences in the cytokine plasma levels during acute infection (Klingstrom et al. 2008). Multifaceted reasons may be responsible for the observed differences.

30.3.4 Therapeutic and Prophylactic Options

The underlying molecular mechanisms of hantavirus pathogenesis and reasons for the broad range of clinical pictures associated with different species are not completely understood. The severity of the disease depends on the extent of capillary leak. Immunological and virus-mediated effects may disturb the endothelial integrity. During acute hantaviral diseases dendritic cells (DC) and T cells become activated and levels of cytokines are elevated. Immune cells attack infected cells as demonstrated by the presence of inflammatory cells within lung and kidney of infected patients. The release of various cytokines leads also to the impairment of endothelial barrier function.

Different cell types are susceptible and permissive for hantavirus infection. Immunohistological analysis detect viral antigen in endothelial and epithelial cells of several organs (lung, kidney, heart, spleen, brain) (Zaki et al. 1995; Green et al. 1998; Settergren et al. 1997). In vitro infection studies identified also epithelial and endothelial as well as immune cells (dendritic cells, macrophages) as targets for hantavirus infection (Krautkrämer et al. 2011; Temonen et al. 1993; Raftery et al. 2002; Marsac et al. 2011; Markotic et al. 2007).

One major difference between pathogenic and non-pathogenic hantaviruses is the receptor usage. Non-pathogenic hantaviruses enter the cell via integrin β1, whereas pathogenic hantaviruses use integrin β3 and CD55/Daf (Gavrilovskaya et al. 1998, 1999; Krautkramer and Zeier 2008; Klempa et al. 2012b; Buranda et al. 2010). The analysis of the receptor usage of newly identified hantaviruses may allow drawing first conclusions in regard to the pathogenicity of the respective hantavirus species. The receptor usage of hantaviruses can be characterized by in vitro studies. Integrin β1 and integrin β3 were identified as entry receptors of the newly

described SANGV and the South American Maporal virus (MPRLV), respectively. These findings may indicate that SANGV might be non-pathogenic to humans and identifies MPRLV as possible human pathogen, although no cases of hantavirus disease caused by this virus were observed so far (Klempa et al. 2012a; Buys et al. 2011). Furthermore, MPRLV was described to induce HCPS-like symptoms in infection of Syrian Golden hamster (Milazzo et al. 2002). Therefore, animal and cell culture models are useful tools in the characterization of novel hantaviruses.

The treatment of hantavirus disease is symptomatic due to the lack of any specific antiviral therapy. The most important measure is to prevent infection by control of rodents and avoiding of contact with excreta. In Asia a specific vaccine, i.e. Hantavax, against HTNV is available. Treatment with ribavirin early in the course of infection can lower the mortality in severe courses of HFRS, but has no effect in treatment of HCPS (Huggins et al. 1986; Chapman et al. 1999; Mertz et al. 2004). Novel therapeutic strategies targeting specific steps of the viral replication cycle are under investigation and comprise small molecular inhibitors of the viral entry receptor integrin β3 that may block the entry of pathogenic Old and New World viruses (Larson et al. 2005; Hall et al. 2010).

30.4 Hantaviruses in New Hosts, in Livestock and in Pet Animals

30.4.1 New Hosts—Rodents, Insectivores and Bats

For many years voles and mice were believed to be almost the only reservoir hosts for hantaviruses. For a long time, the only non-rodent exception was the Asian house shrew or musk shrew (*Suncus murinus*), from which Thottapalayam virus (TPMV) was isolated on a single occasion in India in 1964 (Carey et al. 1971; Zeller et al. 1989). It is now the prototype shrew-borne virus. Seewis virus (SWSV) was the second shrew-associated hantavirus and seems to be prominent in several North and Central European countries (Kang et al. 2009a; Schlegel et al. 2012b). In several European and African regions intense studies on hantaviruses in insectivores such as shrews and moles are performed and many other associated hantaviruses have been discovered in the last decade.

Hantaviruses were also suspected in bats already some years ago, but as sequences resembled HTNV a laboratory contamination was supposed (Kim et al. 1994; Jung and Kim 1995). Meanwhile, several hantavirus sequences were described from at least four different bat families in Africa, South-America and also Asia. Until now, however, the impact of insectivore- and bat-associated hantaviruses on human health is not known, since there are almost no tools available to perform seroepidemiological studies. Table 30.4 summarizes hantaviruses that have been described worldwide in insectivore and bat hosts so far.

Table 30.4 Overview on Hantaviruses detected in different families of insectivores (*Eulipotyphla*) and bats (*Chiroptera*) in alphabetic order

Virus	Species		Country	Ref.
Order Eulipotyphla, Soricomorpha, Family Soricidae (shrews)				
Ash River virus (ARRV)	*Sorex* (S.) *cinereus*	Masked shrew	USA	(Arai e al. 2008a)
Azagny virus (AZGV)	*Crocidura (Cr.) obscurior*	West African pygmy shrew	Côte d'Ivoire	(Kang et al. 2011b)
Camp Ripley virus (RPLV)	*Blarina brevicauda*	Northern short-tailed shrew	USA	(Arai et al. 2007)
Cao Bang virus (CBNV)	*Anourosorex squamipes*	Chinese mole shrew	Vietnam	(Song et al. 2007b)
Imjin virus (MJNV)	*Cr. lasiura*	Ussuri white-toothed shrew	Korea	(Song et al. 2009; Gu et al. 2011)
Jeju virus (JJUV)	*Cr. shantungensis*	Asian lesser white-toothed shrew	South Korea	(Arai et al. 2012)
Jemez Springs virus (JMSV)	*S. monticolus*	Dusky shrew	USA	(Arai e al. 2008a)
Kenkeme virus (KKMV)	*S. roboratus*	Flat-skulled shrews	Far eastern Russia	(Kang et al. 2010)
Lianghe virus (LHEV)	*Anourosorex squamipes*	Chinese mole shrew	China	(Guo et al. 2013)
Qiandao lake virus (QDLV)	*S. cylindricauda*	Stripe-backed shrew	China	(Guo et al. 2013)
Seewis virus (SWSV) -including Altai virus, Artybash virus	*S. araneus* *S.minutus* *S. tundrensis* *S. daphaenodon*	Eurasian common shrew Eurasian pygmy shrew Tundra shrew Siberian large-toothed shrew	Switzerland, Czech Republic, Germany, Slovakia, Finland, Hungary, Siberia	(Song et al. 2007a; Kang et al. 2009a; Schlegel et al. 2012b; Yashina et al. 2010)
Tanganya virus (TGNV)	*Cr. theresae*	Therese's shrew	Guinea	(Klempa et al. 2007)
Thottapalayam virus (TPMV)	*Suncus murinus*	Asian house shrew	India, China, Nepal, Vietnam	(Carey et al. 1971; Zeller et al. 1989; Guo et al. 2011; Kang et al. 2011c; Luan et al. 2012)
Yakeshi virus (YKSV)	*S. isodon*		China	(Guo et al. 2013)
Order Eulipotyphla, Soricomorpha, Family Talpidae (moles)				
Asama virus (ASAV)	*Urotrichus talpoides*	Japanese shrew mole	Japan	(Arai et al. 2008a)
Oxbow virus (OXBV)	*Neurotrichus gibbsii*	American shrew mole	USA	(Kang et al. 2009b)
Nova virus (NVAV)	*Talpa europaea*	European common mole	USA	(Kang et al. 2009c)
Rockport virus (RKPV)[a]	*Scalopus aquaticus*	eastern mole	Rockport, Texas, USA	(Kang et al. 2011a)

Table 30.4 (continued)

Virus	Species		Country	Ref.
Order Chiroptera, Family Vespertilionidae				
Mouyassué virus (MOUV)	*Neoromicia nanus*	Banana pipistrelle	Côte d'Ivoire	(Sumibcay et al. 2012)
Huangpi virus (HPUV)	*Pipistrellus abramus*	Japanese house bat	China	(Guo et al. 2013)
Order Chiroptera, Family Rhinolophidae				
Longquan virus (LQUV)	*Rhinolophus (R.) spp.* *R. affinis* *R. sinicus* *R. monoceros*	Horseshoe bat Intermediate horseshoe bat Chinese rufous horseshoe bat Formosan lesser horseshoe bat	China	(Guo et al. 2013)
Order Chiroptera, Family Nycteridae				
Magboi virus (MGBV)	*Nycteris hispida*	Hairy slit-faced bat	Sierra Leone	(Weiss et al.2012)
Order Chiroptera, Family Phyllostomidae				
Araquara-like virus	*Diphylla ecaudata*	Hairy-legged vampire bat	Brazil	(de Araujo et al. 2012)
Araquara-like virus	*Anoura caudifer*	Tailed tailless bat	Brazil	(de Araujo et al. 2012)

[a] detected in archived tissue from 1986

30.5 Hantaviruses in Pet, Livestock and Laboratory Animals

Rodents are the main carriers of hantaviruses with a significant impact on human public health. Rodents may serve as prey for animals, e.g. for different birds, cats or dogs. Further, rodents live close to or in human dwellings or in buildings for livestock. Therefore, it is important to know if hantaviruses can also be transmitted to non-rodent laboratory, pet or farm animals. Pet rats have been recently reported as a possible source of SEOV infections in the UK (Jameson et al. 2013a, b; Featherstone et al. 2013). Only very limited data on serological studies of different animals e.g. for livestock such as cattle or pigs or birds of prey exist (Table 30.5). Investigations of zoo animals are sparse. Interestingly, a 3 % seroprevalence was found in 60 investigated zoo employees of the Vienna zoo without knowing the animal source for these infections (Juncker-Voss et al. 2004). Moreover, hantavirus antibodies were also detected in cattle (Danes et al. 1992). Interestingly, HTNV and PUUV were found to be able to infect bovine aortic endothelial cell (BAEC) cultures (Bahr et al. 2004; Muranyi 2004). Recent serological studies performed in Belgium gave evidence that cats have significantly higher hantavirus antibody-reactivity than dogs. A significant higher serological prevalence was also revealed in PUUV-endemic areas in southern Belgium (Dobly et al. 2012). Table 30.5 summarizes the present knowledge of hantaviruses in pet, livestock and laboratory animals.

30.5.1 Do Hantavirus Infections Have an Impact on Animals?

As shown above there exist several serological studies in animals, but so far hantaviruses could only be isolated from rodents, shrews or bats and some opossum species in Brazil (de Araujo et al. 2012). Only few studies have been performed—and mostly on rodents—in order to investigate if hantaviruses influence the hosts. Infection trials with some primates have shown different results, depending on the attenuation of the virus strains. Surprisingly, infection experiments showed that PHV, a North-American virus non-pathogenic for humans, induced disease in cynomolgus macaques and chimpanzees (Yanagihara et al. 1988). Several non-human primates species seem also to be susceptible to PUUV/TULV infection, but virus could not be isolated so far (Mertens et al. 2011c) (Table 30.5). A direct or indirect impact of hantaviruses on barn owl (*Tyto alba*) was postulated. In Belgium a monitoring over 30 years indicated that the owls' breeding success positively correlates with human hantavirus cases. These results may also reflect rodent population peaks, but unfortunately no data on hantaviruses in owls exist (Heyman et al. 2013). There is also a high variation of hantavirus prevalence in the respective rodent host. For example, the PUUV prevalence in rodents seems to be quite different depending on time, region and the local hantavirus outbreak situation (Essbauer et al. 2006; Mertens et al. 2011b; Augot et al. 2008). Hantavirus infections in rodents are chronic and may

Table 30.5 Overview on investigations of hantaviruses in pet, farm and livestock animals (except rodents, shrews, bats), modified after (Zeier et al. 2005)

Order		Animal species	Virus	Country	Percentage of seroprevalence/other evidence	Ref.
Artiodactyla	Cattle	*Bos taurus*	PUUV	Czech Republic	0.7%/-	(Danes et al. 1992)
Artiodactyla	Cattle	*Bos taurus*	HTNV	Czech Republic	0.7%/-	(Danes et al. 1992)
Artiodactyla	Deer	*Capreolus capreolus*	PUUV	Czech Republic	14.1%/-	(Danes et al. 1992)
Artiodactyla	Moose	*Alces alces*	PUUV	Sweden	1.9%/-	(Ahlm et al. 2000)
Artiodactyla	Pig	*Sus scrofa*[a]	Hantavirus	China	n.d.	(Zhang et al. 1995)
Artiodactyla	Pig	*Sus scrofa*[a]	Hantavirus	China	0–3.6%/3.3–5.6% antigen in different tissues	(Yang et al. 2004)
Carnivora	Dog	*Canis lupus*[a]	SNV	USA	3.5%/-	(Malecki et al. 1998)
Carnivora	Dog	*Canis lupus*[a]	PUUV	Belgium	4.9% (n=410)/-	(Dobly et al. 2012)
Carnivora	Red foxes	*Vulpes vulpes*	PUUV	Belgium	2.4% (n=3/124)/-	(Escutenaire et al. 2000)
Carnivora	Cat	*Felis catus*	SNV	Canada	2.9%/-	(Leighton et al. 2001)
Carnivora	Cat	*Felis catus*	SNV	USA	2.8%/-	(Malecki et al. 1998)
Carnivora	Cat	*Felis catus*	PUUV	UK	9.6–23%/-	(Bennett et al. 1990)
Carnivora	Cat	*Felis catus*	PUUV	Austria	5%/-	(Nowotny 1994, 1996)
Carnivora	Cat	*Felis catus*	PUUV	Belgium	16.9% (n=124)/-	(Dobly et al. 2012)
Ciconiiformes	Gry heron	*Ardea cinerea*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Columbiformes	Dove	*Streptopelia orientalis*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Didelphimorphia	Tate's woolly mouse opossum	*Micoureus paraguayanus*	Araquara-like virus	Brazil	-/RT-PCR, sequencing	(de Araujo et al. 2012)
Didelphimorphia	Ihering's Three-striped opossum	*Monodelphis iheringi*	Araquara-like virus	Brazil	-/RT-PCR, sequencing	(de Araujo et al. 2012)
Didelphimorphia	Big-eared opossum	*Didelphis aurita*	Araquara-like virus	Brazil	-/RT-PCR, sequencing	(de Araujo et al. 2012)

Table 30.5 (continued)

Order		Animal species	Virus	Country	Percentage of seroprevalence/other evidence	Ref.
Galliformes	Common pheasant	*Phasianus colchicus*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Lagomorpha	European hare	*Lepus sapensis*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Lagomorpha	Hare	*Lepus europaeus*	PUUV	Czech Republic	3.5%/-	(Danes et al. 1992)
Passeriformes	Daurian redstart	*Phoenicurus auroreus*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Passeriformes	European nuthatch	*Sitta europaea*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Passeriformes	Marsh tit	*Parus palustris*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Passeriformes	Yellow-throated bunting	*Emberiza elegans*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Passeriformes	Coal tit	*Parus ater*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Passseriformes	Black-faced bunting	*Emberiza spodocephala*	Hantavirus	former USSR	n.d.	(Tkachenko and Lee 1991)
Primates	Rhesus macaques	*Macaca mulatta*	?	China	n.d.	(Clement et al. 1994)
Primates	Rhesus macaques	*Macaca mulatta*	TULV	Germany	1%(n=2/209)/-	(Mertens et al. 2011c)
Primates	Rhesus macaques	*Macaca mulatta*	PUUV	Germany	6.2%(n=13/209)/-	(Mertens et al. 2011c)
Primates	Rhesus macaques	*Macaca mulatta*	PUUV/TULV	Germany	0.5%(n=1/209)/-	(Mertens et al. 2011c)
Primates	Cynomolgus macaques	*Macaca fascicularis*	PUUV	Germany	4%(n=1/25)/-	(Mertens et al. 2011c)
Primates	Cynomolgus ma-caques	*Macaca fascicularis*	PUUV/TULV	Germany	4%(n=1/25)/-	(Mertens et al. 2011c)
Primates	Olive baboons	*Papio anubis*	TULV	Germany	5.9%(n=1/17)/-	(Mertens et al. 2011c)
Primates	Olive baboons	*Papio anubis*	PUUV	Germany	17.6%(n=3/17)/-	(Mertens et al. 2011c)

Table 30.5 (continued)

Order		Animal species	Virus	Country	Percentage of seroprevalence/other evidence	Ref.
Primates	Olive baboons	*Papio anubis*	PUUV/TULV	Germany	11.8 %(n=2/17)/-	(Mertens et al. 2011ca)
Primates	Borneo orang utan	*Pongo pygmaeus*	SEOV	Taiwan	-/-	(Chen et al. 2011)
Primates	Cynomolgus macaques	*Macaca fascicularis*	PHV	–	e.i.: acute nephropathy, mild, transient proteinuria, azotemia	(Yanagihara et al. 1988)
Primates	Chimpanzee	*Pan troglodytes*	PHV	–	e.i.: acute nephropathy, mild, transient proteinuria, azotemia	(Yanagihara et al. 1988)
Primates	Cynomolgus macaques	*Macaca fascicularis*	Cell-attenuated PUUV	–	e.i.: lethargy, mild proteinuria, microhematuria	(Groen et al. 1995)
Primates	Cynomolgus macaques	*Macaca fascicularis*	Bank vole-adapted PUUV	–	e.i.: loss of appetite, apathetic behavior, fever, proteinuria, HFRS (biochemical markers, immunological characteristics)	(Klingstrom et al. 2002a; Sironen et al. 2008)

[a] forma domestica; e.i., experimental infection

have an impact on the population levels and the physiological status (Pearce-Duvet et al. 2006; Tersago et al. 2008, 2012; Luis et al. 2012).

30.6 Molecular Typing of Hantaviruses

Due to the late onset of specific symptoms the hantaviral antigen can only very rarely be found in human patients. Therefore, often the rodent carriers are used to study the molecular epidemiology of these viruses. Despite the long knowledge of human cases, first viral genome data were only available in the early 1990s (Antic et al. 1992; Avsic-Zupanc et al. 1992; Xiao et al. 1993a, b; Horling et al. 1995; Pilaski et al. 1994).

For PUUV there exist different geographic clusters. Several Northern European (e.g. North-/South-Scandinavian, Finish, Danish) PUUV strains are separated from those found in Central Europe (e.g. from Belgium, France, Germany, Slovakia) or the Alpe-Adrian area (e.g. from Austria, Slovenia, Croatia) (Ettinger et al. 2012; Cvetko et al. 2005; Avsic-Zupanc et al. 2007; Plyusnina et al. 2007, 2009; Nemirov et al. 2010; Razzauti et al. 2009, 2012). Natural reassortment of fragments was shown for PUUV (Razzauti et al. 2008, 2009). In Germany, the picture seems to be even more complex as at least eight geographically and phylogenetically distinct PUUV subclusters can be found (Mertens et al. 2011b; Essbauer et al. 2007; Ulrich et al. 2008; Hofmann et al. 2008; Ettinger et al. 2012).

A strict coevolution of rodent host and virus species was previously postulated for hantaviruses as these viruses group into three main clades that correspond to their rodent host subfamilies (Arvicolinae, Murinae, Sigmodontinae) (Morzunov et al. 1998; Plyusnin et al. 1996; Hughes and Friedman 2000; Plyusnin and Morzunov 2001). Meanwhile there are several findings that do not completely support this hypothesis. One example is the detection of hantaviruses in non-rodent hosts that put this theory in question (Ramsden et al. 2009). Phylogenetic analyses of TULV showed that the evolution seems to be not host-related, although different genetic geographic subclusters were shown in Europe (Schmidt-Chanasit et al. 2010; Plyusnina et al. 2007; Schlegel et al. 2012a). In South-America, a recently performed comprehensive analysis of all so far described hantaviruses revealed that there exist three phylogenetic clades, the Andes/-like viruses, the Laguna Negra/-like viruses, and the Rio Mamore/-like viruses. A long coevolution of host and virus seems also to be present and viruses of one clade are found in several host species (Firth et al. 2012). A detailed phylogenetic analysis of entire coding regions of hantavirus genomes of all available hantaviruses showed that there exist four phylogroups in mammalian hosts and an ancient reassortment between these was postulated (Guo et al. 2013). Divergence of viruses and hosts differs for some hantaviruses indicating a cross-species transmission during hantavirus evolution (Firth et al. 2012; Guo et al. 2013). Furthermore, hantaviruses in Chiroptera and Soricomorpha (shrews and moles) may be ancient as similarly shown for paramyxoviruses in bats (Henttonen et al. 2008; Guo et al. 2013; Drexler et al. 2012).

30.7 Discussion of Unresolved Issues—Outlook

The steadily increasing knowledge of circulating hantavirus strains and of natural occurring reassortment raises questions on basic virological questions such as taxonomy and definition of strains. Further possible coinfections of reassortants might bear so far unknown natural melting vessels for more pathogenic variants. As for many of the herein new described hantaviruses of insectivores and bats the impact on humans is unknown, virus isolates and diagnostic tools to investigate these in humans have insistently to be developed. As these also have been described in new countries, there might be an increasing risk for travelers to get infected. Concerning the variable clinical patterns of hantavirus infections research should focus to understand the severe symptoms and the gender-dependent differences in clinical outcome. For vaccine and treatment research further reliable animal models are essentially needed. As hantaviral infections in hosts in contrast to humans seem to be almost asymptomatic, it would also be important to understand the underlying factors, immune regulation or in vivo infection patterns.

In conclusion, despite 20 years of intense studies and rapidly growing awareness on hantaviruses, there is a broad field of open questions that should be addressed in the next years of hantavirus research.

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Chapter 31
Human African Trypanosomiasis: The Smoldering Scourge of Africa

August Stich

Abstract Human African trypanosomiasis (HAT) or sleeping sickness is one of Africa's classic tropical diseases. First case reports go back to the fourteenth century. Until recently the impact of HAT on health in Africa has been devastating. Many areas had long been rendered uninhabitable for people and livestock. Millions may have died in Central Africa around Lake Victoria and in the Congo basin within the last 100 years.

31.1 Historic Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is one of Africa's classic tropical diseases. First case reports go back to the fourteenth century. Until recently the impact of HAT on health in Africa has been devastating. Many areas had long been rendered uninhabitable for people and livestock. Millions may have died in Central Africa around Lake Victoria and in the Congo basin within the last 100 years.

In 1895, the British scientist Sir David Bruce (1855–1931) suggested an association between trypanosomes and the 'cattle fly fever', a major problem for livestock in southern Africa. In 1902, Robert M. Forde and Everett Dutton from the Liverpool School of Tropical Medicine identified trypanosomes in the blood of a patient during a research expedition in The Gambia, and in 1903, the Italian Aldo Castellani isolated trypanosomes from the cerebrospinal fluid. In the same year, tsetse flies were identified as the vector.

Up to the 1960s control programmes had been quite successful. However, recent epidemics in the Democratic Republic of Congo, Northern Angola, Sudan, Uganda, and other countries resulted in a major resurgence of HAT. At the turn of the twentieth century, the achievements in sleeping sickness control during colonial times had been nearly completely reversed. Fortunately, recent successes of programmes run by national institutions and various dedicated non-governmental organisations finally succeeded in a reduction of transmission in many accessible areas of central

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Africa. However, nobody knows the true prevalence of HAT in Africa, as many countries are still in a state of war and civil unrest with a desperate population that cannot be reached by health interventions.

Today about 60 million people in 36 African countries are exposed to the potential risk of HAT. In some 300 currently existing active foci, up to 100,000 people are still believed to be infected, almost all with *Trypanosoma brucei gambiense*. They are doomed if left untreated. For expatriates and international travellers, sleeping sickness has always been a rare disease, although occasional clusters of cases in tourists to Tanzania, Zambia and Malawi are regularly reported.

There is hardly any other tropical disease which demonstrates more clearly the hypocrisy characterizing our time. On one side, trypanosomes are kept in culture and studied extensively in numerous research laboratories. Their genome is sequenced, and many molecular, biochemical, and immunological phenomena have been discovered as a result of basic science research; scientific interest in the disease is usually restricted to its research aspects. On the other hand, diagnostic and especially therapeutic tools are increasingly unavailable, because patients in rural Africa are not commercially viable consumers. Global concern about the crisis of HAT in Africa is a question of scientific ethics and international solidarity.

31.2 Taxonomic Classification

HAT is caused by subspecies of the protozoan haemoflagellate *Trypanosoma brucei* which is transmitted to man and animals by tsetse flies (*Glossina spp.*). The distribution of the vector restricts sleeping sickness to the African continent between 14° North and 29° South.

Human disease occurs in two clinically and epidemiologically distinct forms, West African and East African sleeping sickness (Table 31.1). A third subspecies of the parasite, *T.b. brucei*, causes disease in cattle, but is non-pathogenic in humans. In Uganda, the only country where all three forms occur, the areas of *gambiense* and *rhodesiense* sleeping sickness are currently about to overlap.

Trypanosoma brucei (phylum Sacromastigophora, order Kinetoplastida) is an extracellular protozoan parasite. Like *Leishmania*, it possesses a centrally placed nucleus and a kinetoplast, a distinct organelle containing extranuclear DNA. The kinetoplast is the insertion site of an undulating membrane, which extends over nearly the whole cell length and ends as a free flagellum.

The three subspecies of *T. brucei* are morphologically indistinguishable. However, they differ considerably in their interaction with their mammalian host and the epidemiological pattern of the diseases they cause. Formerly, *T.b. gambiense* and *T.b. rhodesiense* isolates were characterized either by isoenzyme analysis or by animal inoculation. The advent of molecular techniques created expectations of more reliable tools for their differentiation. However, genomic characterization has revealed several more subdivisions rather than the three expected. Whereas West African isolates proved relatively homogeneous, East African isolates from humans

Table 31.1 The principal features of West and East African sleeping sickness

Disease	West African sleeping sickness	East African sleeping sickness
Parasite	*Trypanosoma brucei gambiense*	*Trypanosoma brucei rhodesiense*
Vector	Transmitted by riverine tsetse flies (*palpalis* group)	Transmitted by savannah tsetse flies (*morsitans* group)
Clinical course	Insidious onset, slow progression, death in stage II after many months or years	Acute onset, chancre frequent, rapid course, death frequently in stage I (cardiac failure)
Diagnosis	Parasitaemia scanty, winterbottom's sign, serology	Parasitaemia usually higher and easily detectable, serological tests unreliable
Treatment	See Table 31.3	
Epidemiology	Tendency for endemicity, man as main reservoir with evidence for several other mammal species, severe public health problem in many West and Central African countries	Wild (antelopes e.g. bushbuck) and occasionally domestic animals as reservoir and source of case clusters and epidemic outbreaks

and animals did not simply conform to what is still called *T.b. rhodesiense* and *T.b. brucei*, but showed a complex relationship with evidence of so far undiscovered genetic exchange in the vector. Further molecular research may soon lead to a comprehensive phylogenetic tree and a deeper insight into trypanosomal evolution and biology.

31.3 Biology

Although congenital, blood-borne, and mechanical transmission have been reported and may play an occasional role, the main mode of transmission is through the bite of infected tsetse flies (*Glossina spp.*, order Diptera). These are biologically unique insects, which occur only in Africa in 31 distinct species and subspecies. Less than half are potential vectors of HAT. Their distinctive behaviour, ecology, and chosen habitat explain many epidemiological features of sleeping sickness. Tsetse flies can live for many months in the wild, but give birth to only about eight larvae per lifetime. Both sexes feed on blood. They require warm temperatures, shade, and humidity for resting and larviposition which makes their distribution highly localized. Recently, the mapping and monitoring of possible HAT transmission foci has become possible with the use of satellite imaging techniques.

During the blood meal on an infected mammalian host, the tsetse fly takes up trypanosomes ('short-stumpy form') into its mid-gut, where they develop into procyclic forms and multiply. After about 2 weeks, they migrate to the salivary glands as epimastigotes where they finally develop into infective metacyclic forms. At the next blood meal, they are injected into a new vertebrate host where they appear as 'long-slender' trypomastigotes and multiply by binary fission. In contrast to *Leishmania* and *T. cruzi*, *T. brucei* is an exclusively extracellular parasite.

The cyclic changes of the trypanosome into different developmental stages are accompanied by variations in morphology, metabolism, and antigenicity. Several unique metabolic pathways have been described in trypanosomes, distinct from their host and thus qualifying as potential drug targets.

The blood stream forms of *T. brucei* are covered with a dense coat of identical glycoproteins, numbering up to about 500 aminoacids per molecule. Being highly immunogenic, they stimulate the production of specific antibodies, mainly of the IgM subclass. Once the surface glycoproteins have been recognized by host antibodies, the parasite will be attacked and destroyed through complement activation and cytokine release, giving rise to local and systemic inflammatory reactions.

However, about 2 % of *T. brucei* in each new generation change the expression of their specific surface glycoprotein. The 'coat' will then be different in the new clone (thus: 'variant' surface glycoprotein: VSG). This phenotypic switch is done mainly by programmed DNA-rearrangements, moving a transcriptionally silent VSG gene into an active, telomerically located expression site. Each *T. brucei* parasite already has the information for hundreds of different VSG genes, and within a whole trypanosome population, the potential repertoire for such different VSG copies seems to be virtually infinite.

Every new VSG copy is antigenically different, thus stimulating the production of a new IgM population. This antigenic variation is the major immune evasion strategy of the parasite, enabling the trypanosome to persist in its vertebrate host. It also reduces parasite load and prolongs the infection. But the inevitable outcome is immune exhaustion of the host (supported by additional immunosuppressive metabolites of the parasites), penetration of trypanosomes into immune-privileged sites such as the central nervous system, and finally death.

31.4 Pathogenicity and Pathology

HAT progresses much more rapidly (over weeks or months) in the East African or *rhodesiense* variety compared with a more insidious onset and protracted course that can last years in *gambiense* (West African) infection. Generally, there are many more circulating organisms in *rhodesiense* compared with *gambiense* infections, making direct parasitological diagnosis easier in East African sleeping sickness. The number of organisms in the blood also varies widely depending on the immunological response to infection. These numbers come and go in waves, because antibodies that develop to one antigenic type of trypanosome can lyse them, but are not effective in stopping multiplication of a smaller number of organisms that have a different antigenic type, and it takes time for immune systems to respond to this new variant. Host inflammatory responses mediated by cytokines are prominent and, together with immunological responses, may contribute to pathophysiology. There are two stages of infection, which are important to distinguish because stage determines the choice of treatment and subsequent risks.

A few days after an infective tsetse bite, a small papule may develop, more often in *rhodesiense* than in *gambiense* infections. As organisms multiply locally, they

excite inflammatory responses, an erythematous tissue reaction with oedema and lymphadenopathy can follow. This "trypanosomal chancre" is commonly found in *rhodesiense*, but rarely in *gambiense* infections. Organisms then invade quickly the haemolymphatic system where they multiply. Symptoms of fever, rigors, headaches and arthralgias follow. These may be less prominent in *gambiense* infections. Lymphadenopathy is detected frequently, especially in the posterior triangle of the neck. This "Winterbottom's sign" is diagnostically useful, because lymph juices from these glands can show parasites and help to establish a diagnosis.

After local multiplication at the site of inoculation, the trypanosomes invade the haemolymphatic system, where they can be detected after 7–10 days. During this period of spread, they are exposed to vigorous host defence mechanisms, which they evade by antigenic variation. This continuous battle between antigenic switches and humoral defence results in a fluctuating parasitaemia with parasites frequently becoming undetectable, especially in *gambiense* HAT. The cyclic release of cytokines during periods of increased cell lysis results in intermittent, non-specific symptoms: fever, chills, rigors, headache, and joint pains. These can easily be misdiagnosed as malaria, viral infection, typhoid fever, or many other conditions. Hepatosplenomegaly and generalized lymphadenopathy are common, indicating activation and hyperplasia of the reticuloendothelial system.

Trypanosomes cross the blood brain barrier within weeks after infection in *rhodesiense* and months in *gambiense* infections. This indicates the beginning of the "sleeping sickness" phase, which is associated with personality changes, headaches, withdrawal from the environment and other signs of neurological involvement. Patients find it harder to carry out any but the simplest of tasks and show a kind of "mental tunnel vision". There are changes in circadian rhythm as well, such as nocturnal insomnia and daytime somnolence. Unless treated, these symptoms progress and are associated with apathy, inanition, and eventually secondary infections such as pneumonias that precede death.

31.5 Clinical Manifestations

Sleeping sickness is a dreadful disease, causing great suffering to patients, their families, and the affected community. The infection often has an insidious onset, but *T. brucei*, whether the East or West African subspecies, will invariably kill if the patient is not treated in time. The natural course of HAT can be divided into different and distinct stages. Their recognition and differentiation is important for the clinical management of the patient.

31.5.1 The Trypanosomal Chancre

Tsetse bites can be quite painful, usually leaving a small and self-healing mark. In the case of a trypanosomal infection, the local reaction can be quite pronounced and longer lasting. A small raised papule will develop after about 5 days. It increases

rapidly in size, surrounded by an intense erythematous tissue reaction with local oedema and regional lymphadenopathy. Although some chancres have a very nasty appearance, they are usually not very painful unless they become ulcerated and superinfected. They heal without treatment after 2–4 weeks, leaving a permanent, hyperpigmented spot.

Trypanosomal chancres occur in more than half the cases of *T.b. rhodesiense*. In *T.b. gambiense*, they are much less common and often go undetected in endemic populations.

31.5.2 Haemolymphatic Stage (HAT stage I)

A reliable sign, particularly in *T.b. gambiense* infection, is the enlargement of lymph nodes in the posterior triangle of the neck (Winterbottom's sign). Other typical signs are a fugitive patchy or circinate rash, a myxoedematous infiltration of connective tissue ('puffy face syndrome'), and an inconspicuous periostitis of the tibia with delayed hyperaesthesia (Kérandel's sign).

In *T.b. rhodesiense* infection, this haemolymphatic stage is very pronounced with severe symptoms, clinically often resembling *falciparum* malaria or septicaemia. Frequently, patients die within the first weeks after the onset of symptoms, mostly through cardiac involvement (myocarditis). In the early stage of *T.b. gambiense* infection, symptoms are usually infrequent and mild. Febrile episodes become less severe as the disease progresses.

Patients presenting with an appropriate history of travel in endemic areas, perhaps a history of being bitten by tsetse flies and systemic symptoms of fever should raise suspicion of HAT.

31.5.3 Meningoencephalitic Stage (HAT stage II)

Within weeks in *T.b. rhodesiense* and months (sometimes years) in *T.b. gambiense* infection, cerebral involvement will invariably follow; trypanosomes cross the blood–brain barrier.

The onset of stage II is insidious. The exact time of central nervous system involvement cannot be determined clinically. On histology, perivascular infiltration of inflammatory cells ('cuffing') and glial proliferation can be detected, resembling cerebral endarteriitis. As the disease progresses, patients complain of increasing headache and their families may detect a marked change in behaviour and personality. Neurological symptoms, which follow gradually, can be focal or generalized, depending on the site of cellular damage in the central nervous system. Convulsions are common, usually indicating a poor prognosis. Periods of confusion and agitation slowly evolve towards a stage of distinct perplexity when patients lose interest in their surroundings and their own situation. Inflammatory reactions in the hypo-

thalamic structures lead to a dysfunction in circadian rhythms and sleep regulatory systems. Sleep pattern become fragmented and finally result in a somnolent and comatose state. Progressive wasting and dehydration follows the inability to eat and drink.

In children, HAT progresses even more rapidly towards this meningoencephalitic stage. Parents often notice insomnia and behavioural changes long before the diagnosis is established.

There is no pathognomonic clinical sign of late HAT, a wide range of possible neurological and psychiatric differential diagnoses is opened instead. However, the appearance of the patient with apathy, the typical expressionless face and swollen lymph nodes at the posterior triangle of the neck, is very suggestive for HAT in endemic areas.

31.6 Laboratory Diagnosis

HAT can never be diagnosed with certainty purely on clinical grounds. Definitive diagnosis requires the detection of the parasite in chancre aspirate, blood, lymph juice, or cerebrospinal fluid using various parasitological techniques.

31.6.1 Lymph Node Aspirate

Lymph node aspiration is widely used, especially for the diagnosis of *gambiense* HAT. Fluid of enlarged lymph nodes, preferably of the posterior triangle of the neck (Winterbottom's sign), is aspirated and examined immediately at × 400 magnification without additional staining. Mobile trypanosomes can be detected for a few minutes between lymphocytes.

31.6.2 Wet Preparation, Thin and Thick Blood Film

During all stages of the disease, trypanosomes may appear in the blood stream where they can be detected in unstained wet or in stained preparations. The yield of detection is highest in the thick blood film, a technique widely used for the diagnosis of blood parasites such as *Plasmodia* or microfilaria. Giemsa or Field staining techniques are appropriate.

Especially in *gambiense* HAT, parasitaemia is usually low and fluctuating, often even undetectable. Repeated examinations on successive days are sometimes necessary until trypanosomes can be documented.

31.6.3 Concentration Methods

To increase the sensitivity of blood examinations, various concentration assays have been developed. Trypanosomes tend to accumulate in the buffy coat layer after centrifugation of a blood sample. The best results have been obtained with the mAECT (mini anion exchange column technique), where trypanosomes are concentrated after passage through a cellulose column, the QBC method (quantitative buffy coat), which was originally developed for the diagnosis of malaria, or the CTC method (capillary tube centrifugation), which is widely used in the field.

31.6.4 Nucleic Amplification Techniques

Several specific primers have been described to detect trypanosomal DNA using the polymerase chain reaction (PCR). They had been successfully applied to samples from blood, lymph juice and CSF, mostly under conditions of a research laboratory. Until today PCR assays are still inferior to conventional parasitological techniques.

31.6.5 Serological Assays

Serology is a useful tool to detect antibodies against trypanosomes. Various test methods have been described; some of them are now commercially available. They are mainly based on ELISA technique or immunofluorescence, but provide reliable results only in *gambiense* HAT.

For rapid screening under field conditions, the CATT (card agglutination test for trypanosomiasis) is an excellent tool in areas of *T.b. gambiense* infestation. It is easy to perform and delivers results within 5 min. A visible agglutination in the CATT suggests the existence of antibodies, but does not necessarily imply overt disease. Still, any positive serological result always requires parasitological confirmation before the initiation of treatment.

Recently novel diagnostic tests similar to the rapid detection tests (RDT) for the diagnosis of malaria have been developed and are currently undergoing evaluation under field conditions.

31.6.6 Non-Specific Laboratory Findings

Anaemia and thrombocytopenia are caused by systemic effects of cytokine release, especially of TNF-α. Hypergammaglobinaemia can reach extreme levels as a result of polyclonal activation of plasma cells. IgM levels detected in HAT are among the highest observed in any infectious disease.

31.6.7 Diagnosis of Stage II

Stage determination is crucial for the correct management of a patient. This cannot be done on clinical grounds alone. Therefore, cerebrospinal fluid must be examined in every patient found positive for trypanosomes in blood or lymph aspirate. In addition a lumbar puncture should also be performed in all patients in whom HAT is suspected clinically even if peripheral examinations had proved negative. A minimum of 5 ml of cerebrospinal fluid is required to examine for:

- *Leucocytes*: cerebral involvement in HAT stage II is accompanied by pleocytosis, mostly lymphocytes, in the cerebrospinal fluid. By convention a number of five cells or more per mm^3 cerebrospinal fluid defines central nervous system involvement even if the patient does not (yet) have neurological symptoms. Pathognomonic for HAT is the appearance of activated plasma cells with eosinophilic inclusions in the cerebrospinal fluid, the morular cells of Mott
- *Trypanosomes*: the chances of detecting trypanosomes in the cerebrospinal fluid increase with the level of pleocytosis and the technique used. The highest yield is obtained by cerebrospinal fluid double centrifugation and rapid microscopy at the bedside
- *Protein*: in patients with HAT, a level of 37 mg of protein per 100 ml cerebrospinal fluid (dye-binding protein assay) or more is highly suggestive of the advanced stage. Stage II HAT is characterized by an autochthonous production of IgM antibodies in the cerebrospinal fluid, which can be selectively detected if suitable laboratory facilities exist (e.g. latex IgM test)

31.7 Treatment

31.7.1 General Considerations

HAT is curable, especially if the diagnosis is made at an early stage of the disease. In the harsh reality of the African situation, however, there are many major obstacles to successful patient management:

1. Sleeping sickness is a disease of rural, remote places. The active foci of sleeping sickness are usually in far-away and insecure places, which are difficult to reach. Many treatment centres work under emergency conditions with extremely restricted resources. Numerous patients, without proper access to health care, are left unattended
2. The diagnosis is difficult. Initial diagnosis and exact staging of trypanosomiasis requires sophisticated methods that are potentially harmful to the patient and thus justified only in the hands of experienced personnel. Repetitive training programmes, constant supervision, and continuous quality control are necessary but in reality rarely available

Table 31.2 The choice of drugs in the treatment of sleeping sickness

	Gambiense sleeping sicknes		*Rhodesiense* sleeping sickness	
HAT Stage I	Pentamidine		Suramin	
HAT Stage II	1st line	NECT (nifurtimox eflornithine combination therapy)	1st line	Melarsoprol
	2nd line	Melarsoprol	2nd line	+ Nifurtimox

3. The treatment of trypanosomiasis is extremely costly although the drugs themselves are now covered by a donation programme. Invariably, demand exceeds the locally available resources. External funding and sustainable donor commitments for rural Africa is generally decreasing
4. The treatment is complicated. Treatment of HAT is dangerous, prolonged, and usually requires hospitalization. Most patients with late stage trypanosomiasis are severely ill and malnourished. Adverse drug reactions during treatment are difficult to assess due to concomitant pathologies. Their management requires considerable medical skill and good nursing care. Hospitals in rural Africa are often inadequately equipped and staffed to accomplish good patient care
5. Many drugs are not easily available. Trypanosomicidal agents were on the verge of disappearance despite increasing demand. The range of drugs is diminishing, and hardly any new treatments are in sight. This is especially worrying in view of the reported spread of drug resistance
6. HAT treatment is not standardized. Trypanosomiasis treatment regimens vary considerably between countries and treatment centres. Results from different centres are comparable to only a very limited extent. Few properly conducted and sufficiently powered clinical trials are available to evaluate duration, dosage, and possible combinations of drugs. Sufficient infrastructure for carrying out clinical research exists in only a handful of places
7. The price for cure of HAT is high: dangerous drugs with limited availability and prolonged treatment schedules administered in many places by poorly trained personnel in rudimentary medical facilities. Little progress has been achieved in the last 30 years

The treatment of HAT depends on the trypanosome subspecies and the stage of the disease (Table 31.2).

31.7.2 Stage I Drugs

31.7.2.1 Pentamidine

Since its introduction in 1937, pentamidine has become the drug of choice for *gambiense* HAT stage I, achieving cure rates as high as 98%. However, there are frequent failures in *rhodesiense* HAT. Lower rates of cellular pentamidine uptake in *T.b. rhodesiense* may explain these differences. Some cures of stage II infections

Table 31.3 Dosage and principal adverse reactions of antitrypanosomal agents

	Dosage regimen	Adverse drug reactions
Pentamidine	4 mg/kg body weight intramuscular daily or on alternate days for 7–10 injections	Hypotensive reaction (common) with tachycardia, dizziness, even collapse and shock, especially after intravenous administration, close monitoring of pulse rate and blood pressure after injection is mandatory Inflammatory reactions at the site of injection (sterile abscesses, necrosis) Renal, hepatic, and pancreatic dysfunction Neurotoxicity: peripheral polyneuropathy Bone marrow depression
Suramin	Day 1: Test dose of 4–5 mg/kg body weight Day 3, 10, 17, 24, and 31: 20 mg/kg body weight, maximum dose per injection 1 g	Pyrexia (very common) Early hypersensitivity reactions such as nausea, circulatory collapse, urticaria Late hypersensitivity reactions: skin reactions (exfoliative dermatitis), haemolytic anaemia Renal impairment: albuminuria, cylinduria, haematuria (high renal tissue concentrations); regular urine checks during treatment are mandatory Neurotoxicity: peripheral neuropathy Bone marrow toxicity: agranulocytosis, thrombocytopenia
Melarsoprol	New regimen Day 1–10: 2.2 mg/kg body weight (not evaluated for *T.b. rhodesiense*)	Treatment-induced encephalopathy Pyrexia Neurotoxicity: peripheral motor or sensory polyneuropathy Dermatological reactions: pruritus, urticaria, Exfoliative dermatitis Cardiotoxicity Renal and hepatic dysfunction
Eflornithine	Most commonly used dosage regimen 100 mg/kg body weight at 6-hourly intervals for 14 days	Gastrointestinal symptoms such as nausea, vomiting and diarrhoea Bone marrow toxicity: anaemia, leucopenia, thrombocytopenia Alopecia, usually towards the end of the treatment cycle Neurological symptoms such as convulsions
Nifurtimox	5 mg/kg body weight 3 times daily for 30 days	Abdominal discomfort such as nausea, pains, and vomiting in half of the treated patients, often leading to a disruption of the treatment course Neurological complications: convulsions, Impairment of cerebellar function, Polyneuropathy Skin reactions
NECT	Nifurimox 5 mg/kg every 8 h over 10 days + Eflornithine 200 mg i.v. every 12 h over 7 days	As above, but less as compared to monotherapy

have also been reported, but cerebrospinal fluid drug levels are usually not sufficiently high to guarantee a reliable trypanosomicidal effect in the central nervous system.

Pentamidine is usually given by deep intramuscular injection, often on an outpatient basis. If hospital care and reasonable monitoring conditions are available, an intravenous infusion, given in normal saline over 2 h, might be used instead. The main advantage of pentamidine over other drugs is the short treatment course and ease of administration. Adverse effects are related to the route of administration or its dose and are usually reversible.

In clinical medicine, pentamidine is also used as second-line therapy for visceral leishmaniasis and especially in the prophylaxis and treatment of opportunistic *Pneumocystis jirovecii* pneumonia in AIDS. Since the start of the HIV pandemic, the price of pentamidine was increased more than tenfold by producers, making it unaffordable by health institutions in low-income countries. After an intervention led by WHO, a limited amount of pentamidine is now made available for use in HAT.

31.7.2.2 Suramin

In the early twentieth century, the development of suramin, resulting from German research on the trypanosomicidal activity of various dyes (‘Bayer 205’), was a major break-through in the field of tropical medicine. For the first time, African trypanosomiasis, at least in its early stages, became treatable without causing major harm.

Suramin is still used to treat stage I HAT, especially *rhodesiense*. Like pentamidine it does not reach therapeutic levels in cerebrospinal fluid. Suramin is injected intravenously after dilution in distilled water.

Adverse effects depend on nutritional status, concomitant illnesses (especially onchocerciasis) and the patient’s clinical condition. Although life-threatening reactions have been described, serious adverse effects are rare.

31.7.3 Stage II drugs

31.7.3.1 Melarsoprol

Until the systematic introduction of the arsenical compound melarsoprol in 1949, late stage trypanosomiasis was virtually untreatable. Since then, it has remained the most widely used stage II antitrypanosomal drug both for *T.b. gambiense* and *rhodesiense* infections. It has saved many lives, but has a high rate of dangerous adverse effects. Increasing frequency of relapses and resistance has been reported in some parts of Congo, Angola, Sudan and Uganda.

Melarsoprol clears trypanosomes rapidly from the blood, lymph, and cerebrospinal fluid. Its toxicity usually restricts its use to late-stage disease. It is given by slow intravenous injection; extravascular leakage must be avoided.

A new, simpler regimen is based on recently acquired knowledge of the drug's pharmacokinetics. The most important adverse effect is an acute encephalopathy, provoked around day 5–8 of the treatment course in 5–14 % of all patients. There is severe headache, convulsions, rapid neurological deterioration, or deepening of coma. Characteristically, the comatose patient's eyes remain open. Most probably, this is an immune-mediated reaction precipitated by release of parasite antigens in the first days of treatment. The overall case fatality under treatment ranges between 2 and 12 %, depending on the stage of disease and the quality of medical and nursing care. Simultaneous administration of glucocorticosteroids (prednisolone 1 mg/kg body weight; maximum 40 mg daily) might reduce mortality, especially in cases with high cerebrospinal fluid pleocytosis. However, in areas where tuberculosis, amoebiasis, or strongyloidiasis are highly prevalent, corticosteroids have dangers of their own!

31.7.3.2 Eflornithine (DFMO)

Initially developed as antitumour agent, eflornithine (alpha-difluoro-methylornithine) was introduced in 1980 as an antitrypanosomal drug, in the hope that it might replace melarsoprol for treatment of stage II trypanosomiasis. However, exorbitant costs and limited availability have restricted its use mostly to melarsoprol-refractory cases of *gambiense* sleeping sickness. *T.b. rhodesiense* is much less sensitive due to a much higher turn-over rate of the target enzyme ornithine-decarboxylase and therefore, cannot be treated with Eflornithine.

It can be taken orally, but intravenous administration is preferred as it achieves a much higher bioavailability and success rate. Eflornithine should be administered slowly over a period of at least 30 min. Continuous 24-h administration is preferable if facilities allow.

The range of adverse reactions to eflornithine is wide, as with other cytotoxic drugs in cancer treatment. Their occurrence and intensity increase with the duration of treatment and the severity of the patient's general condition.

In the late 90ies of the last century, no pharmaceutical company has produced eflornithine for use against HAT, despite demands by WHO. The discovery of its therapeutic effect in cosmetic creams against facial hair helped to restimulate production and thus had a beneficial 'spin-off effect' for HAT. In 2001 agreements were signed between WHO and two major drug producing companies which led to a "Public-Private-Partnership" (PPP) and helped to assure a sufficient supply of eflornithine and other drugs essential for the treatment of HAT. The agreement was prolonged several times on a 5 year basis.

31.7.3.3 Nifurtimox

Ten years after its introduction for the treatment of American trypanosomiasis in 1967, nifurtimox was found to be effective in the treatment of *gambiense* sleeping sickness. It has a place as second line treatment in melarsoprol-refractory cases or in combination chemotherapy.

Nifurtimox is generally not well tolerated, but adverse effects are usually not severe. They are dose-related and rapidly reversible after discontinuation of the drug.

31.7.3.4 Combination Treatments in HAT

Melarsoprol, eflornithine, and nifurtimox interfere with trypanothione synthesis and activity at different stages. There is also experimental evidence that combinations of suramin and stage II drugs might also be beneficial. Therefore, by reducing the overall dosage of each individual component, drug combinations could perhaps reduce the frequency of serious side-effects, and the development of resistance, which are such common problems in the treatment of sleeping sickness.

In 2009 a large multi-center clinical trial was published demonstrating the advantages of a nifurtimox eflornithine combination therapy (NECT) over eflornithine monotherapy in *gambiense* HAT. NECT is now considered to be the treatment of choice in the late stage of *T.b. gambiense* infection.

31.8 Preventive Measures

31.8.1 Individual Protection

Tsetse flies have a very patchy distribution. Infested strips of land are often well known to the local population and should be avoided as far as possible. HAT among tourists and occasional visitors to endemic areas is a rare but regularly reported event. Pentamidine or suramin chemoprophylaxis is historical, and can no longer be recommended.

Insect repellents are of limited use. Long-sleeved, bright clothing (avoidance of black or dark blue) can decrease attractiveness to insects and is the best defence against attacking tsetse flies.

31.8.2 Control in Endemic Areas

In the past, tremendous efforts were undertaken to control the threat posed by trypanosomiasis to humans, livestock and economic development in rural Africa. Control programmes are based on the five complementary pillars given in Table 31.4.

Table 31.4 Control of HAT

1	Diagnosis and treatment of patients
2	Active case finding
3	Vector control
4	Implementation and continuation of a surveillance system
5	Training, health education and community participation

The most important strategy is active case finding. This requires mobile teams, which regularly visit villages in endemic areas. Mostly based on the results of CATT screening, patients, preferably in the early stage of the disease, are identified and treated. Gradually, the parasite reservoir is depleted. As *Glossina* is a relatively incompetent vector with infectivity rates usually below 0.1% and susceptible to control measures such as insecticide application or trapping, the combination of various approaches can lead to a complete break of the transmission cycle. This was achieved in the past in many places. However, the recent resurgence of sleeping sickness in areas ridden by war and civil unrest, in combination with the decreasing availability of drugs on the international market and the general loss of interest in health in Africa, gives rise to the fear that HAT will soon be again out of control.

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Part VI
Waterborne Zoonoses

Chapter 32
Waterborne Zoonoses: *Cryptosporidium* and Cryptosporidiosis: A Small Parasite that Makes a Big Splash

Lucy J. Robertson

Abstract Cryptosporidiosis usually manifests as gastrointestinal infection and is associated with considerable morbidity and, in some circumstances, mortality. Effective treatment that is suitable for all patients, including those that are particularly affected by infection, children and the immunocompromised, is lacking.

There are several species of *Cryptosporidium*, some of which are zoonotic. The most important of these is *C. parvum*, but others, including *C. cuniculus* (predominantly associated with rabbits), *C. meleagridis* (predominantly associated with poultry), and *C. ubiquitum* (predominantly associated with sheep and cervids), are also of public health importance. *C. hominis* is generally only infective to humans. *Cryptosporidium* is particularly suited to waterborne transmission. In this chapter various waterborne outbreaks are reviewed, particularly those associated with zoonotic transmission, and also Standard Methods for analysing water samples for contamination with these parasites. Although most waterborne outbreaks of cryptosporidiosis are due to *C. hominis* (and are therefore not zoonotic), most foodborne outbreaks are apparently zoonotic. Zoonotic transmission also occurs when there is close contact between infected animals and humans, particularly veterinary students and young children on petting farms. Thus, cryptosporidiosis is an important zoonosis and with the potential for causing community-wide outbreaks of disease due to both waterborne and foodborne transmission.

32.1 Introduction

Cryptosporidium spp. are protozoan parasites that have been reported from a large variety of different hosts globally, including humans. To date, approximately 25 different species of *Cryptosporidium* have been identified and described, and of

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these around 50% have been reported as being infectious to humans. However, most reported human infections involve *Cryptosporidium parvum* (an important zoonotic species, particularly associated with infections in calves) or *C. hominis*, which is found primarily in humans. Other species that are considered important as human pathogens include *C. meleagridis*, a zoonotic infection primarily associated with infections in turkeys, but also relatively commonly identified in children in South America, and *C. cuniculus*, another zoonotic species particularly associated with infections in rabbits (Table 32.1). The other species of *Cryptosporidium* with zoonotic potential tend to be associated only with sporadic cases of human infection (e.g. *C. ubiquitum* infections that are usually associated with infections in sheep and cervids) or are particularly associated with infections in immunocompromised patients (e.g. *C. suis* infections, more commonly associated with infection in pigs, and *C. felis* infections, more commonly associated with infections in cats). Although prevalence data are patchy, *Cryptosporidium* infection has emerged as a global public health problem, and has been reported from over 100 countries.

Waterborne transmission is important in cryptosporidiosis; numerous waterborne outbreaks of cryptosporidiosis have occurred worldwide due to oocyst contamination of drinking water sources. The largest waterborne illness outbreak of any kind in the U.S. occurred in 1993 when over 400,000 people acquired cryptosporidiosis in Milwaukee (Mac Kenzie et al. 1994). More recently, around 27,000 people suffered from waterborne cryptosporidiosis in Östersund, Sweden in 2010.

In this chapter, emphasis will be primarily directed towards those species of *Cryptosporidium* that are predominantly zoonotic (that is, not *C. hominis*, as this is associated almost exclusively with human infections), and that have also been associated with waterborne infection. Thus, *C. parvum* will be the main focus of the chapter, but also *C. cuniculus*. In addition, more general information will be provided including on the lifecycle, clinical symptoms, diagnosis and treatment of *Cryptosporidium* infections, both from a medical and a veterinary perspective, transmission routes and the epidemiology of infection, waterborne and foodborne outbreaks of zoonotic cryptosporidiosis, and the detection of these parasites in water.

32.2 General Information on *Cryptosporidium* and Cryptosporidiosis: Lifecycle, Clinical Presentation, Diagnosis, and Treatment

For all *Cryptosporidium* species, the lifecycle is direct (no intermediate host). When viable oocysts are ingested by an appropriate susceptible host, they usually excyst in the small intestine (*C. baileyi* can be associated with intra-tracheal infection in chickens) where the resultant sporozoites invade epithelial cells—and locate epicellularly (within the cell, but not within the cytoplasm). Repeated cycles of asexual reproduction result in destruction of these cells and the production of enormous quantities of meronts. A sexual cycle, gametogony, results in oocyst

Table 32.1 Overview of different domestic animals and their major relevant species of *Cryptosporidium*, including clinical presentation and zoonotic potential. (Information in the table derived and adapted from Santín and Trout 2008; Robertson and Fayer 2012; Robertson et al. 2014)

Host animal	Species of *Cryptosporidium*	Clinical notes for host animal species	Zoonotic potential
Cats	*C. felis*	May be associated with persistent diarrhoea	Yes: sporadic cases diagnosed in both immunocompetent & immunocompromised patients
Cattle	*C. parvum*	Common in pre-weaned calves—acute onset diarrhoea. Intestinal location	Yes: considerable
	C. bovis	Common in post-weaned calves—less pathogenic than *C. parvum*	No
	C. andersoni	Older post-weaned calves, yearlings and adults-some failure to thrive. Infects abomasal gastric glands	Yes
	C. ryanae	Common in post-weaned calves	No
Cervids	*C. parvum*	Information on species detected amongst farmed deer is lacking; diarrhoea in young calves, possibly severe, but can also be asymptomatic	Yes: considerable
	C. ubiquitum		Yes: sporadic cases diagnosed in both immunocompetent & immunocompromised patients
Dogs	*C. parvum*	Often asymptomatic: may be associated with diarrhoea	Yes: considerable
	C. canis	Often asymptomatic: may be associated with diarrhoea	Yes: sporadic cases diagnosed in both immunocompetent and immunocompromised patients
Horses	*C. parvum*	Foals seem more susceptible; both asymptomatic cases and clinical infections (diarrhoea) reported	Yes: considerable
Pigs	*C. parvum*	Less common than in bovines and small ruminants; diarrhoea and vomiting	Yes: considerable
	C. suis	Relatively common, mild symptoms	A few reports from immunocompromised patients
	C. scrofarum	Relatively common, mild symptoms	No

Table 32.1 (continued)

Host animal	Species of *Cryptosporidium*	Clinical notes for host animal species	Zoonotic potential
Poultry	*C. meleagridis*	Mostly intestinal and generally associated with mild symptoms	Yes: cases in both immunocompetent and immunocompromised patients reported. Particularly associated with infection in Peru
	C. baileyi	Detected in many different anatomical sites including digestive tract, respiratory tract, and urinary tract. Has been associated with high morbidity and mortality	A few reports from immunocompromised patients
	C. galli	Infects the proventriculus, and has been associated with acute diarrhoeal disease	No
Rabbits	*C. cuniculus*	Clinical symptoms in rabbits are apparently mild or lacking	Yes: first identified as pathogenic to human during a waterborne outbreak
Small ruminants (sheep, goats)	*C. parvum*	Relatively common in pre-weaned lambs, associated with diarrhoea	Yes: considerable
	C. xiaoi	Common in older lambs and sheep, often apparently asymptomatic	No
	C. ubiquitum	Common in older lambs and sheep, often apparently asymptomatic	Yes: sporadic cases diagnosed in both immunocompetent & immunocompromised

production. The oocysts sporulate whilst within the host (unlike other genera of Apicomplexa such as *Eimeria* spp. or *Toxoplasma gondii*) and may excyst within the same host, resulting in re-invasion of the epithelial cells and continuation of the infection. Alternatively, the oocysts are excreted in the faeces, and are immediately infectious for the next host. These small oocysts (for most species they are approximately spherical and around 3–5 μm in diameter, although there is inter-species variation) are also very robust and can survive for long periods under cool, moist conditions.

In the human host, cryptosporidiosis is an enteric disease, generally characterized by watery diarrhoea, abdominal pain, nausea and related symptoms. However, other symptoms have been reported, including low grade fever and headache. Immunocompromised individuals and children in developing countries are most affected by cryptosporidiosis, and the relationship of infection with growth faltering, malnutrition, and diarrhoeal mortality is in need of further exploration (Shirley et al. 2012). In some individuals, however, infection may be largely asymptomatic.

The spectrum of symptoms depends not only on the host (age, nutritional status, immunity), but also on parasite factors including the number ingested and species. While cryptosporidiosis is self-limiting in immunocompetent individuals, a high relapse rate has been reported in some studies, as well as post-infection sequelae, that can be both gastrointestinal and non-gastrointestinal. However, in immunocompromised patients the symptoms are often more severe, and infection may become chronic, debilitating and potentially life-threatening, with high volumes of diarrhoea, spreading of infection beyond the primary site, and severe weight loss.

In animals, the symptoms of *Cryptosporidium* infection appear to depend highly on parasite adaptation to the host and host age/immunological status, although results from different studies vary, and for many species infections are largely asymptomatic (see Table 32.1). Nevertheless, infections of some domestic animals with some species of *Cryptosporidium* may result in severe infection, usually with acute diarrhoea as the main symptom. In some animals, especially young animals and particularly in association with concomitant infections or conditions, this may even be fatal.

For both human and animal infections, diagnosis usually depends on the demonstration of oocysts (or their antigens or DNA) in faecal samples. As the oocysts are very small, the use of a staining technique, particularly using antibodies labelled with a fluorochrome and screening with fluorescence microscopy (immunofluorescent antibody testing; IFAT), is recommended. IFAT is considered to be a gold standard, although other techniques such as modified Ziehl-Neelsen or auramine phenol staining may also be used successfully. A pre-screening concentration stage (formol-ether or flotation on sucrose or salt) may enhance sensitivity. Immunochromatographic kits are rapid and simple to use, but may have limited sensitivity in light infections. While molecular methods such as PCR are not yet the mainstay of the general diagnostic laboratory, and detection is limited only to those species that are appropriate to the primers and probes selected, multiplex assays are beginning to be more widely used, particularly in medical diagnostics.

Successful treatments for cryptosporidiosis in both humans and animals remain elusive, although one treatment (nitazoxanide) has been FDA-approved for symptom alleviation in immunocompetent humans and has shown promise for treating cryptosporidiosis in animals. Failure of therapeutic agents being evaluated is often considered to reflect the specific taxonomic position of *Cryptosporidium*, which indicates that it is more closely related to organisms such as greganines rather than to classical coccidia. For immuncompromised patients with HIV-infection, the development of effective antiretroviral therapies has been of more value for decreasing mortality due to cryptosporidiosis than any parasite-targeted treatment, and this has perhaps reduced the urgency for developing an effective treatment. In developing countries, however, antiretroviral therapy coverage is often limited and thus cryptosporidiosis in HIV patients, particularly in association with other insults to health, may prove fatal.

The identification of *Cryptosporidium* as one of the four main aetiological agents associated with serious childhood diarrhoea (Kotloff et al. 2013) indicates that there is still a need to develop an effective chemotherapy targeting this parasite.

32.3 Transmission Routes and Epidemiology of *Cryptosporidium* Infection

As *Cryptosporidium* oocysts are immediately infectious upon excretion (unlike the majority of classical coccidial oocysts), direct faecal oral transmission (animal-to-animal, animal-to-human, human-to-animal, and human-to-human), as well as re-infection of the excretor is relatively common and has been well documented. Although it is often impossible to exclude entirely the possibility that transmission has occurred via a contaminated vehicle, person-to-person spread is particularly well described within families (often secondary cases after a primary outbreak infection), in institutions such as childcare nurseries, and in hospitals. With respect to zoonotic transmission, infection of veterinary students examining diarrhoeic calves has, in particular, been well-documented and also of children visiting petting farms where they have direct contact with young animals. However, again infection from environmental contamination (that is, from oocyst contamination of a transmission vehicle such as food) often cannot be entirely excluded in such settings.

However, as well as being immediately infectious, *Cryptosporidium* oocysts are also notoriously robust, being able to survive in damp, cool environments for weeks or months, and being resistant against a range of commonly used disinfectants, including chlorination at levels used by the water industry. This means that transmission via a vehicle, such as water or food, is also well-documented, particularly as this may lead to an outbreak situation in which hundreds or thousands of individuals may be infected. It should, however, be noted that oocysts do not survive prolonged freezing at below −15 °C, freeze-thawing, or desiccation and that they are also killed by cooking.

In general, clinical cryptosporidiosis occurs most often in the toddler age group (1 and 2 year olds), presumably due to lack of previous infection (and hence immunity) and due to the unhygienic behaviour in this age group, especially mouthing of myriad objects. Although more commonly diagnosed in male children, possibly due to greater exposure to contaminated objects or perhaps an artefact associated with medical consultation, in adults it is women who tend to be more frequently diagnosed with clinical cryptosporidiosis than men (Nichols 2008). This is probably due to greater exposure of women than men to children with cryptosporidiosis, who may be excreting infective oocysts, but might also reflect that drinking water consumption is often higher among women. A range of studies have explored risk factors for infection. Although these vary according to study conditions, particularly whether associated with an outbreak or with sporadic infections and whether the study has focussed on a population in an industrialised or developing country, in general, the key risk factors identified include: ingestion of contaminated drinking or recreational water; contact with infected persons or animals, particularly calves and lambs; travel to areas where the disease is considered endemic; contact with children under 6 years of age (particularly, but not exclusively, children with diarrhoea) (Bouzid et al. 2013). Interestingly, several investigations have indicated a negative association between cryptosporidiosis and eating raw vegetables, while, in contrast, various outbreaks of cryptosporidiosis have been associated with consumption of fresh produce. It has been proposed that this apparently protective effect may be due to repeated exposure to low numbers of *Cryptosporidium* oocysts on raw vegetables thus allowing the development of protective immunity (Bouzid et al. 2013).

Associations have also been made between season and cryptosporidiosis (Lal et al. 2012), with infection peaks in spring and late summer/early autumn and least cases reported in winter, regardless of the country of study. It is suggested that the spring peak may reflect agricultural practices (calving and other young livestock), as well as greater potential for water contamination due to heavy rainfalls and spring snowmelt (Lal et al. 2012). In some instances the late-summer peak may represent the return home of tourists who have been vacationing in more endemic areas, and perhaps associated with behaviours resulting in greater exposure risks, such as increased contact with recreational water.

The development and use of tools for molecular epidemiological studies have provided useful insights into the transmission of cryptosporidiosis. Small subunit (SSU) rRNA-based tools are now commonly used for genotyping *Cryptosporidium* identified in both human and animal infections, and also for oocysts isolated from water and other environmental samples (Xiao 2010). At the sub-genotyping level, one of the popular tools is the DNA sequence analysis of the 60 kDa glycoprotein (gp60) gene. This gene contains tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at its 5′ end, along with extensive sequence differences in the non-repeat regions, and has been used to categorise *C. parvum* and *C. hominis* (the most common *Cryptosporidium* spp. causing infections in humans) to subtype families (Xiao 2010). Besides the sequence heterogeneity of the gp60 gene which make it useful as a *Cryptosporidium* subtyping tool (it is one of the most polymorphic markers identified in the *Cryptosporidium* genome), it is also of biological

relevance. The gp60 gene codes for a protein that is located on the surface of the apical region of invasive stages of the parasite, and thus provides a biological possibility of associating parasite characteristics, including clinical presentation, with subtype family.

The use of these and other molecular tools has revealed that, although cryptosporidiosis is generally considered an infection of global importance, there is a clear geographical distribution of *C. parvum* and *C. hominis* in human infections. Although both *C. parvum* and *C. hominis* are common in European countries, *C. parvum* is the dominant species in humans in the Middle East, while in the rest of the world, particularly in developing countries, *C. hominis* is usually the predominant species in humans (Xiao 2010). Furthermore, within-country geographic variations have also been noted, with *C. parvum* infections being more common than *C. hominis* in rural regions of the United States and Ireland (Xiao 2010). These data suggest that zoonotic cryptosporidiosis is more common in the Middle East and Europe than in developing countries, at least at present; this is also supported by some of the data from surveys of calves in some developing countries, where infection rates have sometimes been unexpectedly low (Chang'a et al. 2011). This may reflect both climatic effects (oocysts are vulnerable to desiccation and UV exposure) and also farm management, with a large proportion of farming in developing countries being conducted by small-holders with just a couple of cattle.

32.4 Waterborne Transmission of Zoonotic *Cryptosporidium* Species

Cryptosporidium lends itself to waterborne transmission due to various factors in its biology. These factors include the low infective dose (theoretically as low as a single oocyst, although human volunteer studies suggest tens of oocysts), the large numbers of oocysts excreted during infection (calves infected with *C. parvum* may produce as many as 6×10^7 oocysts per gram of faeces, and a single infected calf may excrete 4×10^{10} oocysts during its 2nd week of life and 6×10^{11} oocysts during its first month of life (Uga et al. 2000; Nydam et al. 2001), and the robustness and longevity of the oocysts in damp, cool environments, including upon exposure to drinking water chlorination regimes. In addition, the small size of oocysts enables them to penetrate some of the physical barriers in water treatment. Together, these factors have resulted in numerous outbreaks of waterborne cryptosporidiosis, both associated with drinking water and also with recreational water. A review of 325 outbreaks of human disease attributed to the waterborne transmission of pathogenic protozoa (from the beginning of records up until around 2003) indicated that the majority of them (approximately 51%) were caused by *Cryptosporidium* infection (Karanis et al. 2007). Although the majority of these are suggested to be *C. parvum* infections, the lack of molecular characterisation methods at the time of most of these outbreaks coupled with the fact that the majority of different species of oocysts are morphologically indistinguishable, mean that it is likely that

a large proportion of these outbreaks were actually due to *C. hominis* infections (and possibly some other species of *Cryptosporidium*). A follow-up review of more recent outbreaks (Baldursson and Karanis 2011) indicated that *Cryptosporidium* spp. continued to be the dominant aetiological agent of waterborne outbreaks of protozoan disease between 2004–2010, with more than 60 % of the 199 documented outbreaks due to *Cryptosporidium* infection. Although the review does not provide a species overview for these outbreaks (*C. hominis* or *C. parvum*), reference to the original papers cited in the review demonstrates that the majority of these outbreaks for which species information was available, were due to *C. hominis*. However, even for outbreaks with *C. parvum*, this knowledge is insufficient to determine whether zoonotic transmission has occurred (from animals to humans, via water as the transmission vehicle), since contamination can also occur from human sewage even with *C. parvum* infections, and thus traditional epidemiological data analysis is needed in addition to molecular epidemiological data in order to determine the source of contamination. However, when the aetiological agent is *C. hominis*, then zoonotic transmission is much less likely to have occurred (Nydam et al. 2005).

Nevertheless, some waterborne outbreaks of cryptosporidiosis have often been attributed to contamination of water catchments by animals, and although sometimes the indications implicating zoonotic transmission have been speculative (and later proven to be incorrect or unlikely), on other occasions the evidence has been substantial.

For example, grazing cattle or slaughterhouse effluent contaminating Lake Michigan were mentioned as two possible sources of *Cryptosporidium* oocysts in the large outbreak in Milwaukee, Wisconsin in 1993 (Mac Kenzie et al. 1994). But retrospective analysis of clinical isolates revealed that the outbreak was caused by the anthroponotic *C. hominis* (Sulaiman et al. 1998), and it is worth noting that among the most recent waterborne outbreaks in UK (compiled by Chalmers 2012), the majority do not indicate zoonotic transmission as *C. hominis* infection predominates.

However, during an outbreak in Scotland in 1988 substantial testing of water and environmental samples was conducted (Smith et al. 1989)—albeit with the less sophisticated methods available at that time—and that are less sensitive and specific than those currently developed (sucrose flotation was used, for example, rather than immunomagnetic separation (IMS)), indicated that irregular seepage of oocyst-containing water into a break-pressure tank was the most likely cause of the outbreak. This seepage was considered to have been exacerbated by heavy rains, and the occurrence of muck spreading and the spraying of cattle slurry prior to the outbreak in the vicinity of a fire clay pipe draining into the break-pressure tank indicated that this was the likely zoonotic source of oocysts (Smith et al. 1989). Another outbreak in UK in 1992 was again associated with heavy rainfall and, in this instance, flooding of a field in which livestock (species not mentioned) grazed and thereafter drainage into a shaft associated with a groundwater drinking water supply (Bridgman et al. 1995) suggests again waterborne zoonotic transmission. It should be noted that although the traditional epidemiological investigations associated with both these outbreaks was extensive, they occurred before the introduction

of reliable molecular tools that could be used to determine whether the aetiological agent was potentially zoonotic, and, moreover, before *C. parvum* and *C. hominis* had been recognised as separate species.

Although zoonotic transmission of cryptosporidiosis from lambs has been definitively shown in various outbreaks associated with direct contact such as petting farms (Gormley et al. 2011; Robertson et al. 2014), and a range of waterborne outbreaks of cryptosporidiosis in UK have implicated sheep as the source of the infection, a lack of studies in which oocysts from both grazing lambs or sheep and oocysts in water and in patient samples have been characterised has resulted in a lack of clarity over whether these really are the source of contamination (Robertson 2009). This is unfortunate, as sometimes the assumption that lambs or sheep are the source of contamination has resulted in measures being implemented that may have been unnecessary. These have included extensive boil water notices, relocation of sheep during the lambing season, and even contributed to the closure of some sheep farms, all measures that may result in anxiety and frustration for local communities (Robertson 2009). Thus, now that we have tools to help in deciding sources of contamination of water supplies, it is important to ensure that they are appropriately used.

While the focus of zoonotic transmission, including by the waterborne route, tends to focus on *C. parvum*, it should be noted that sheep are also frequently infected with *C. ubiquitum*, and this species has also been detected in a number of sporadic human cases globally (Cieloszyk et al. 2012; Elwin et al. 2012; Feltus et al. 2006; Leoni et al. 2006; Ong et al. 2002; Soba et al. 2006; Trotz-Williams et al. 2006). As this species has also been identified in storm water, wastewater, raw water and drinking water (Jiang et al. 2005; Liu et al. 2011; Nichols et al. 2010; Van Dyke et al. 2012) and was the third most common species in raw water in Scotland, as well as the most common species identified in drinking water (Nichols et al. 2010), there is a clear potential for zoonotic waterborne transmission. Given that no such waterborne outbreaks have been described to date, it is possible that infectivity to humans is relatively limited. Likewise deer also are relatively commonly infected with both *C. parvum* and *C. ubiquitum*, but there have been no documented cases of proven zoonotic transmission from deer. A study from Australia suggested that deer were not likely to be a threat for zoonotic transmission of cryptosporidiosis in a specific protected drinking water supply watershed (Cinque et al. 2008); a study from USA, however, reached the opposite conclusion and stated that deer in a particular watershed posed a potential threat regarding zoonotic cryptosporidiosis and therefore were appropriate targets for source water protection (Jellison et al. 2009).

Another zoonotic species of *Cryptosporidium, C. cuniculus,* has, however, been associated with waterborne transmission. *C. cuniculus* is rarely, but sporadically, identified in human infections (e.g. of 3030 *Cryptosporidium*-positive faecal samples submitted for routine typing in UK between 2007–2008, only 37 were diagnosed as *C. cuniculus* (1.2%); Chalmers et al. 2011) and transmission of *Cryptosporidium* to humans from farmed rabbits has not been recorded (Robertson et al. 2014). Indeed, an investigation exploring associations between farm animals and

human patients with cryptosporidiosis did not indicate rabbits as a particular source of infection among farmed animals (Smith et al. 2010). However, a waterborne outbreak of cryptosporidiosis in England in 2008 affecting 29 people was identified to the species level in eight patients as *C.cuniculus*, and to the subtype level as subtype VaA18 (Chalmers et al. 2009). Furthermore, *Cryptosporidium* oocysts of the same species and sub-genotype were identified in the colon of a carcass of a rabbit (presumably wild) that was found in a tank at the implicated water treatment works. Thus, this outbreak that was investigated using both traditional and molecular epidemiological tools demonstrates convincingly a waterborne outbreak of zoonotic cryptosporidiosis.

Another of the most important zoonotic species of *Cryptosporidium* is *C. meleagridis*. This species is the third most common species in human cryptosporidiosis worldwide (Robertson et al. 2014). Studies on the prevalence and species distribution for cryptosporidiosis in humans in South America have identified that the prevalence of *C. meleagridis* infection is very similar to that of *C. parvum* (Cama et al. 2008; Cama et al. 2003; Cama et al. 2007). Although *C. meleagridis* infection is usually associated with infection in turkeys, the only documented case of zoonotic transmission from a bird source demonstrates that chickens rather than turkeys were the source of infection (Silverlås et al. 2012). Moreover, no waterborne transmission has been documented. However, as not all patients in epidemiological investigations that have been diagnosed with *C. meleagridis* infections have had contact with birds or even animals (Elwin et al. 2012), transmission via a contaminated vehicle (such as food or water) seems possible, or—alternatively—direct infection from another person with that infection.

As well as adequate water treatment, including sufficient barriers to ensure acceptable removal and/or inactivation of pathogens such as *Cryptosporidium*, one of the major mechanisms for ensuring safe water supply is implementing an appropriate catchment control policy. With respect to zoonotic *Cryptosporidium* infection, this may mean limiting or restricting access of domestic animals and wildlife to vulnerable sites, particularly watercourses, associated with drinking water supply and possibly recreational use. Such restrictions might be of particular validity during periods of heavy rain, when wash-off contamination is likely to be highest. Environmental studies have demonstrated that contamination of surface waters with *Cryptosporidium* oocysts are significantly affected by land use, such as cattle husbandry and manure management practices, as well as seasonal and weather characteristics (Keeley and Faulkner 2008). However, such restrictions regarding grazing of domestic animals, for example, may have little impact if there is a large reservoir of infection in wild animals, and will also serve little purpose if the restricted animals are not infected with zoonotic pathogens. For example, a study in Canada that sought to investigate the contribution of dairy cattle to protozoan contamination of water sources (Budu-Amoako et al. 2012), concluded that the *Cryptosporidium* oocysts being shed were predominantly non-zoonotic *C. bovis* and *C. andersoni*, and therefore of little significance to public health. Given that pre-weaned calves are the most likely age group and species to shed *C. parvum* oocysts, it could be argued that measures to protect water catchments from young ruminants and their

faeces, including spreading of calf manure on fields where runoff cannot occur, should be directed towards this animal group (Robertson et al. 2014). However, it should be emphasized that water courses should be subjected to individual risk assessment and measures implemented accordingly; it should not be forgotten that even with adequate catchment protection and water treatment, unexpected events may occur that nevertheless may result in contamination (e.g. the entry of a rabbit with *C. cuniculus* infection into water treatment works). Therefore, it is necessary to follow outbreaks of diarrhoea in the community, and—where appropriate—to analyse water samples for contamination.

32.5 Detection of Zoonotic *Cryptosporidium* Oocysts in Water Supplies

Our understanding of the importance of the waterborne route of infection for *Cryptosporidium*, particularly exacerbated by the occurrence of communitywide outbreaks with hundreds or thousands of individuals infected, led to a need for analysis of water samples for these parasites. Such information provides a handle on the extent of contamination in different water sources and how this may be affected by factors such as weather patterns and season. Such data are also used as input for risk assessments, to evaluate whether water supplies are likely sources of infection in outbreak situations, to determine the efficacy of different removal or inactivation measures, and to have a basis for recommending the type and extent of water treatments and interventions that are necessary for a particular water supply. However, analysis of water samples for *Cryptosporidium* oocysts is not easy. Unlike bacteria they cannot be readily cultivated, and there are no simple, easy-to-quantify indicators or appropriate surrogates. Thus, in order to detect and quantify *Cryptosporidium* contamination of water, the individual oocysts must be isolated and enumerated or their DNA (or some other marker) must be isolated and the quantity measured. In order to determine if the oocysts detected are zoonotic, molecular analyses must also be conducted, either downstream from detection or as a component of the detection method.

Over the past 30 years or so, different approaches and equipment have been investigated for their suitability at conducting this task. Method requirements include reproducibility, specificity, and sensitivity (given the low concentrations of these parasites usually occurring in water). Additionally, it is preferable for the method to be cheap, user-friendly, rapid, and with the potential to be implemented in a standard analytical laboratory.

The first documented Standard Methods for analysis of water for *Cryptosporidium* oocysts were probably the US EPA ICR (United States Environment Protection Agency Information Collection Rule) Method (US EPA 1996) and the "SCA Blue book method" (Anonymous 1990). These methods included collection of a water sample by filtration through a yarn-wound polypropylene filter; removal of all particulates from the filter using detergents and mechanical extraction; concentration of

the particles by centrifugation (or settling); purification/isolation of putative parasites by buoyant density gradient centrifugation (usually using Percoll-sucrose, specific gravity of 1.10); detection of the parasites using immunofluorescent antibody testing (IFAT) in which the sample concentrate is incubated with fluorochrome-labelled monoclonal antibodies against the oocyst walls.

These methods have been improved over time, particularly with respect to the production of improved sampling techniques (different filter types and continuous-flow centrifugation) and the use of IMS for isolation of oocysts from the sample concentrate prior to detection. Interlaboratory trials have been used for validation work for different techniques, and now the most commonly followed Standard Methods are probably US EPA Methods 1622 and 1623.1 (both of which are regularly updated and available for downloading from the US EPA homepage), and ISO Method 15553 (Anonymous 2006). In addition, individual countries or regions have also produced versions of these methods or considered them.

32.6 Foodborne Transmission of Zoonotic *Cryptosporidium*

Although cryptosporidiosis is generally considered a waterborne, rather than foodborne, disease, the potential for foodborne transmission is widely acknowledged. Food contamination with *Cryptosporidium* oocysts may occur during production, processing, or preparation, and the longevity of the oocysts means that they can survive various processing treatments, including chlorine baths and blast freezing (Duhain et al. 2012). In addition, washing of fresh produce may fail to remove contaminating oocysts, since they not only adhere to surfaces but may also infiltrate into leafy vegetables via stomatal openings (Macarisin et al. 2010a, b).

Fewer foodborne cryptosporidiosis outbreaks have been documented than waterborne outbreaks. This could be because fewer foodborne outbreaks have occurred (because, for example, the oocysts survive less readily on food due to potential for desiccation, freezing or heating, or because contamination of food is less likely to occur than contamination of water). Alternatively, this could reflect that fewer people tend to be infected in foodborne outbreaks (due to more restricted distribution of the contaminated product), and therefore the event may be less likely to be identified. One interesting aspect of comparing foodborne outbreaks and waterborne outbreaks is that whereas waterborne outbreaks of cryptosporidiosis tend to be most frequently caused by the anthroponotic species *C. hominis*, foodborne outbreaks tend to be most frequently caused by the zoonotic species *C. parvum*. In a review considering geographical distribution of foodborne cryptosporidiosis (Robertson and Chalmers 2013), seven of the eight outbreaks in which molecular analyses were conducted on samples report *C. parvum* as the aetiological agent, and only one *C. hominis*. A further outbreak of foodborne cryptosporidiosis from May 2012 in the UK (Anonymous 2013) was primarily epidemiologically associated with consumption of ready-to-eat pre-cut mixed leaves from a major supermarket

chain and was also found to be due to *C. parvum*. However, infection with *C. parvum* does not necessarily indicate zoonotic transmission from an animal source, since humans can also transmit *C. parvum*. However, epidemiological investigations into at least some of these outbreaks suggest that an animal source of *Cryptosporidium* is probable. Moreover, zoonotic transmission also seems likely for some of the outbreaks in which molecular analyses were not conducted, as evinced from traditional epidemiological investigations. For example, an outbreak in USA in 2003, in which ozonated apple cider was the infection vehicle was demonstrated to be largely due to infection with two similar sub-types of *C. parvum* (for one patient, infection with *C. ubiqutum* was recorded) (Blackburn et al. 2006). However, in two earlier outbreaks of cryptosporidiosis associated with consumption of apple cider, species identification had not been conducted (Millard 1994; Anonymous 1997), although evidence from one of these outbreaks strongly suggested that animals were the probable source of infection, with calves with cryptosporidiosis grazing in the orchard from which the apples had been obtained.

Another foodborne outbreak of cryptosporidiosis was suspected to have been transmitted via the consumption of vegetables that had been sprayed with water possibly contaminated with cattle faeces, with both human cases and implicated animals infected with the same subtype of *C. parvum* (Collier et al. 2011). Furthermore, it would seem likely that the outbreaks of cryptosporidiosis that have been postulated to have been caused by the consumption of inadequately pasteurized cow milk (Gelletlie et al. 1997; Harper et al. 2002) are due to zoonotic transmission, although this cannot be proven in the absence of insufficient information.

Why foodborne cryptosporidiosis should be more likely to be due to zoonotic transmission and waterborne cryptosporidiosis should be more likely to be due to anthroponotic transmission is not entirely clear. However, it would seem probable that greater effort is made to keep a barrier between human sewage and food production areas, but it is simply not possible to maintain this barrier between human sewage and water catchment areas. Furthermore, along the farm-to-fork food production chain there are various opportunities for contact between food products and animal faeces from potentially infected animals, but relatively few opportunities for contact between human sewage and food products.

32.7 Conclusion

Cryptosporidiosis is an important protozoan disease that is associated with considerable morbidity and, in some circumstances, mortality on a global level. The main symptom is diarrhoea. One of the most important facets of this infection from a clinical perspective is the absence of an effective treatment that is suitable for all patients, including children and the immunocompromised, two patient groups that are particularly affected by infection.

There are several species of *Cryptosporidium*, some of which have some degree of host-specificity, but many of which have the potential to infect humans and are

considered zoonotic. The most important of these is *C. parvum*, but other species, including *C. cuniculus* (predominantly associated with rabbits), *C. meleagridis* (predominantly associated with poultry), and *C. ubiquitum* (predominantly associated with sheep and cervids) are also of relevance to public health. In addition, *C. hominis* affects predominantly humans, but occasional infections in other animals have been reported. Various characteristics of *Cryptosporidium* mean that it lends itself to waterborne transmission, and many waterborne outbreaks have been documented, often affecting hundreds or thousands of individuals. The importance of the waterborne transmission route has instigated the development of Standard Methods for analysing water samples for contamination with these parasites. Although the majority of documented waterborne outbreaks of cryptosporidiosis are due to infection with *C. hominis* (and are therefore not zoonotic), the majority of foodborne outbreaks of cryptosporidiosis are apparently zoonotic, as evidenced by both traditional and molecular epidemiological investigations. In addition, zoonotic transmission has frequently been recorded when there is close contact between infected animals and humans, particularly veterinary students and young children on petting farms. Thus cryptosporidiosis is an important zoonosis and with the potential for causing community-wide outbreaks of disease due to both waterborne and foodborne transmission.

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Chapter 33
Giardiasis: A Zoonotic Infection or Not?

Simone M. Cacciò

Abstract The flagellated protozoan *Giardia duodenalis* is the etiologic agent of giardiasis, a very common gastrointestinal infection of humans and mammals, with a global distribution. Infection is initiated by the ingestion of cysts. Multiple transmission routes are possible, including human-to-human, animal-to-human, and animal-to-animal contacts, and through consumption of contaminated water and food. The parasite has caused a large number of waterborne outbreaks worldwide. The clinical manifestations of giardiasis are quite variable and range from the absence of symptoms to acute or chronic diarrhoea, characterized by dehydration, abdominal pain, nausea, vomiting and weight loss. The role played by animals in the epidemiology of human infection has attracted the interest of researchers since many years. Livestock and wildlife have been considered as an important source of environmental contamination and thus as potential reservoirs for waterborne transmission, whereas companion animals have been associated with direct transmission to humans. Assessing the zoonotic transmission of the infection requires molecular characterization as there is considerable genetic variation within *G. duodenalis*. To date eight major genetic groups (or assemblages) have been identified, two of which (A and B) infect both humans and animals, whereas the remaining six (C to H) are host-specific and not transmittable to humans. Molecular characterization of genotypes within assemblages A and B has shown that, in most cases, animals do not share identical genotypes with humans, therefore providing little support to zoonotic transmission. Carefully designed studies, particularly in endemic countries, are needed to understand the occurrence of, and the factors associated with, zoonotic transmission.

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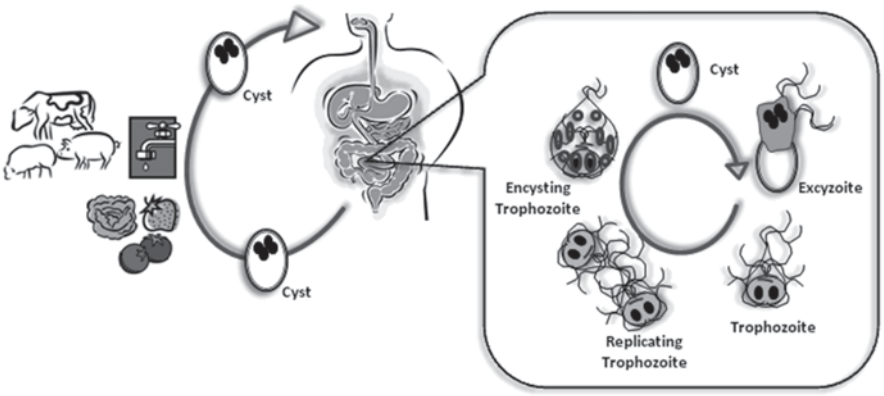

Fig. 33.1 Schematic representation of the life cycle of *Giardia*

33.1 An Introduction to the Organism

Species within the genus *Giardia* are unicellular flagellates that infect the gut of different classes of vertebrates. There are currently six recognised species in the genus, including *Giardia agilis* in amphibians, *G. ardeae* and *G. psittaci* in birds, *G. microti* and *G. muris* in rodents, and *G. duodenalis* (syn. *G. lamblia*, *G. intestinalis*) in mammals including humans (Thompson and Monis 2012). The life cycle of *Giardia* is direct and involves only two stages, the trophozoite, which is the replicative stage, and the cyst, which is the infective stage (Fig. 33.1). Infection is initiated by ingestion of cysts either by the faecal-oral route (direct human-to-human, animal-to-human or animal-to-animal contacts) or indirectly through consumption of contaminated food or water. Exposure to the acidic environment of the stomach provides the necessary stimuli for the excystation of the trophozoite from the cyst in the duodenum (Gardner and Hill 2001). Trophozoites undergo repeated mitotic division and are eventually triggered to develop into environmentally resistant cysts in response to the bile conditions of the small intestine. Cysts shed with faeces are immediately infectious and are able to survive for weeks to months in the environment and, as a result, environmental contamination can lead to the contamination of water and food (Feng and Xiao 2011). In humans, the infective dose is about 10–100 cysts (Rendtorff 1954). Most outbreaks of giardiasis have been linked to the consumption of contaminated drinking water. In a recent review it has been reported that of the 199 recorded outbreaks caused by protozoa worldwide during the period 2004–2010, 70 (35 %) were caused by *Giardia* (Baldursson and Karanis 2011).

33.2 Taxonomy of *Giardia*

On the basis of morphologicalfeatures, organisms in the genus *Giardia* are classified in the phylum Metamonada, subphylum Trichozoa, superclass Eopharyngia, class Trepomonadea, subclass Diplozoa and order Giardiida (Thompson and Monis 2012). The taxonomy of *Giardia* species has been, and still is, a subject of intense debate and controversy, and this has resulted in a confusing nomenclature with different names being used for the same species (Monis et al. 2009). Early taxonomy was based on the assumption of strict host specificity (i.e., a different species for each host) and a total of 51 species of *Giardia* have been described, including 30 from mammals (of which 2 are from humans), 14 from birds, 4 from amphibians, 2 from reptiles and 1 from fish (Thompson and Monis 2012). It was not until the seminal work of Filice (1952), however, that the taxonomy of *Giardia* species was reconsidered. Filice concluded that no experimental evidence supported the validity of species named on the basis of host specificity, and considered as valid only three morphologically distinct species, namely *Giardia muris*, *G. agilis* and *G. duodenalis*, on the basis of the shape of internal median bodies as well as body shape and length (Filice 1952). This scheme rapidly became accepted, albeit, as acknowledged by Filice himself, many species were put under the *G. duodenalis* 'umbrella', due to the lack of tools to discriminate reliably between variants. With the development of advanced microscopic techniques, ultrastructural description of trophozoites allowed the description of two new species from birds, *Giardia ardeae* and *Giardia psittaci*, whereas *Giardia microti*, a parasite infecting various rodents, was recognized as a separate species due to the unique presence of fully differentiated trophozoites in the cysts. Therefore, six species are recognized in the genus (Table 33.1).

The development of techniques for the in vitro propagation of trophozoites in axenic conditions (Farthing et al. 1983) opened the possibility to characterize *Giardia* isolates from different hosts using genetic techniques. The first important series of experiments aimed at comparing strains on the basis of polymorphisms of many isoenzymes, and revealed a large amount of genetic variability among *Giardia duodenalis* strains (Andrews et al. 1989). Importantly, clustering analysis of the isoenzyme data identified strongly supported groups of genetically related strains that, in most cases, were derived from specific hosts (Monis et al. 2003). DNA sequence analyses further confirmed the validity of these genetic groups (Monis et al. 1999), which are referred to as assemblages. To date, eight assemblages are recognized, which have different host distribution: assemblages A and B are found in humans and other mammals, assemblages C and D are specific for dogs and other canids, assemblage E is found in livestock, assemblage F in cats, assemblage G in rats, and assemblage H in marine mammals (Table 33.1). These assemblages are separated by very large genetic distances and are likely to represent distinct species, a conclusion reinforced by comparisons at the whole genome level of assemblages A, B and E (Franzen et al. 2009; Jerlström-Hultqvist et al. 2010). A revision of the taxonomy and a new nomenclature at the species level has been proposed (Monis et al. 2009)

Table 33.1 The species currently recognized within the *Giardia* genus, their host distribution and distinctive morphological characteristics. The eight assemblages comprised within *G. duodenalis*, and their host distribution, are also indicated

Species	Host	Distinctive morphologic features	Length/width of the trophozoite (μm)
Giardia agilis	Amphibians	Long and narrow trophozoites with club-shaped median bodies	20–30/4–5
Giardia ardeae	Birds	Rounded trophozoites, prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies round-oval to claw-shaped	~10/~6
Giardia psittaci	Birds	Pear-shaped trophozoites, with no ventro-lateral flange. Claw-shaped median bodies	~14/~6
Giardia muris	Rodents	Rounded trophozoites with small, round median bodies	9–12/5–7
Giardia microti	Rodents	Trophozoites similar to G. duodenalis. Cysts contain fully differentiated trophozoites	12–15/6–8
Giardia duodenalis		Pear-shapedtrophozoites with claw-shaped median bodies.	12-15/6-8
Assemblage A	Mammals, including humans		
Assemblage B	Mammals, including humans		
Assemblage C	Domestic and wild canines		
Assemblage D	Domestic and wild canines		
Assemblage E	Ruminants, pigs		
Assemblage F	Cats		
Assemblage G	Rats		
Assemblage H	Marine mammals		

but, as controversies persisted, it has not been formally adopted. Therefore, the term assemblage will be used in this chapter.

It is important to recall that further genetic variation exists within assemblages (intra-assemblage variability), and that a number of sub-assemblages have been identified and referred to as AI, AII, BIII, and BIV (that is to say, using a Roman number as suffix; Monis et al. 2003). Isolates that belong to sub-assemblages are genetically close, but not identical: i.e., sub-assemblages are clusters of closely related isolates (Monis et al. 2003). However, the lack of a standardized nomenclature for the genetic variants found within sub-assemblages has created confusion, with different names (A1, A2, A3 or S1, S2, S3) being given in different studies (Lalle et al. 2005; Sulaiman et al. 2003). These genetic variants should be described as genotypes, and, when multiple loci are investigated, as multi-locus genotypes (MLGs). To avoid further confusion, the names assigned in the original publications will not be used here, and genotypes will be identified at the level of the sub-assemblage to which they belong (e.g., AI, BIV etc).

33.3 Tools for the Molecular Typing of *Giardia duodenalis*

The introduction of molecular techniques, in particular those based on the in vitro amplification of nucleic acids (i.e., Polymerase Chain Reaction and related methodologies), has revolutionized the study of the epidemiology of giardiasis. Molecular tools are thought to provide higher sensitivity and specificity compared to both microscopic or immunologic assays, and offer the possibility to identify *Giardia* isolates at the level of species, assemblage, sub-assemblage and genotypes (Cacciò and Ryan 2008).

The first PCR assays targeted fragments of conserved eukaryotic genes, sometimes using degenerated primers (18S rRNA, glutamate dehydrogenase, elongation factor 1-alfa, triose phosphate isomerase; Monis et al. 1999), or genes uniquely associated with the parasite (e.g., beta-giardin; Lalle et al. 2005). These markers are still the most widely used for genotyping parasite isolates (Feng and Xiao 2011). In more recent studies, a number of other markers have been tested for their applicability in the detection and genotyping of *Giardia duodenalis*. All of these markers correspond to single-copy genes, with the exception of ribosomal sequences.

It is important to note that these markers differ widely in terms of genetic variability (Wielinga and Thompson 2007); indeed, some, like the 18S rRNA and the elongation factor 1-alfa, are strongly conserved and can be used to identify *G. duodenalis* assemblages, but are of little use for studies where genetic variation within assemblages needs to be determined. Nevertheless, due to the multicopy nature of the 18S rRNA, the PCR that targets this locus has a high sensitivity and is often used for detection of *Giardia* from different matrices (e.g., human and animal faeces, water samples). Some researchers considered *Giardia* has a clonal organism, and argued that the use of a single marker with high genetic heterogeneity will provide a resolution as high as multilocus sequence typing (Sulaiman et al. 2003). In more recent studies, however, the use of a multilocus typing scheme has been shown to represent a more informative approach for genotyping this parasite (Cacciò et al. 2008; Lebbad et al. 2010).

Current MLST-schemes are predominantly based on housekeeping genes and the variation found in these markers seems sufficient for genotyping. Highly variable molecular markers, such as microsatellites and other repeated DNA sequences, will be useful for sub-typing, but it appears that these sequences are rarely represented in the genome.

33.4 The Complex Genetics of *Giardia duodenalis*

With the increasing application of PCR-based techniques for the characterization of field isolates (Cacciò and Ryan 2008), and, more recently, the application of next generation sequencing for the analysis of whole genomes (Franzen et al. 2009), it has become clear that the genetics of *Giardia* is more complex than previously thought. It should be recalled that *Giardia* is functionally a tetraploid organism

(it has 2 diploid nuclei) and has been considered as an asexually replicating organism (Adam 2001). The replication is equational rather than reductional, which means that nuclear asymmetry is maintained throughout the replication cycle (Yu et al. 2002). Therefore, mutations are expected to be fixed independently in each nucleus and, if genetic exchanges do not occur, allelic heterozygosity should accumulate, as observed in other asexual organisms (Gladyshev et al. 2010).

Opposite to this prediction, an extremely low level of allelic sequence heterozygosity (ASH) was found when the genome of an assemblage A isolate was sequenced (Morrison et al. 2007). While this was a surprising finding, sequencing of an assemblage B isolate revealed 50 times more ASH in this genome than in the assemblage A genome (Franzen et al. 2009).

Furthermore, ASH is often visualized as "double peaks" in chromatograms obtained from direct sequencing of PCR products, and this have been mainly observed with assemblage B isolates (Gelanew et al. 2007; Lebbad et al. 2008), in agreement with genomic data. Formally, these sequencing profiles can be generated by the genuine presence of different parasites (mixed infections) and/or by ASH of a single population of parasites, but it is difficult to distinguish between the two situations when direct sequencing of PCR products is used. An important and elegant contribution on this issue was recently published (Ankarklev et al. 2012). The authors used micromanipulation to isolate single trophozoites from the assemblage B (GS isolate) of *Giardia duodenalis*, as well as single cysts from human patients. They showed that ASH is present in single trophozoites from the GS lineage. Furthermore, ASH was demonstrated at the level of single *Giardia* cysts of assemblage B from clinical samples. Additionally, alignment of sequence data from several different cysts that originated from the same patient yielded different sequence patterns, thus suggesting the presence of multiple sub-assemblage infections in congruence with ASH within the same patient (Ankarklev et al. 2012).

A second level of complexity was put into focus when multi-locus typing revealed a lack of concordance between loci, resulting in the amplification or lack of amplification of particular loci from a given isolate, or in the detection of different assemblages (or species) when isolates were typed at more than one locus (Sprong et al. 2009). Of note, earlier studies using allozymes (Andrews et al. 1989) already demonstrated that some isolates had multiple banding patterns consistent with those expected from a diploid heterozygote. The non-concordance in typing results has been observed in both human and animal (particularly dogs, but also cattle) isolates of *G. duodenalis* (Sprong et al. 2009). There are two different explanations for this lack of concordance: 1) the presence of genetically different cysts in a faecal sample coupled with a preferential amplification of one assemblage at a particular locus and of another assemblage at another locus (i.e., a true mixed infection followed by PCR bias); or, 2) the existence of recombinant isolates in which genetic exchanges have occurred at the gene(s) targeted by the PCR assay(s).

Mixed infections with genetically different isolates are not uncommon, as demonstrated by the use of assemblage-specific PCR assays, which are now available as both conventional PCR (Geurden et al. 2008; Vanni et al. 2012) or real-time PCR (Almeida et al. 2010). The percentage of mixed infections is higher in infected

individuals living in developing countries (e.g., Gelanew et al. 2007; Cooper et al. 2010; Table 33.2). Thus, mixed infections must be considered in the context of molecular epidemiology, and this calls for a systematic use of a multilocus genotyping approach (Cacciò et al. 2008).

On the other hand, recombination between isolates of sub-assemblage AII was demonstrated in human isolates from an endemic country (Cooper et al. 2007, 2010), and, similarly, evidence from several studies suggest that recombination occurs among assemblage B isolates (Lasek-Nesselquist et al. 2009; Siripattanapipong et al. 2011). Whether a true meiotic process occurs in *G. duodenalis* remains to be established, and other mechanisms of genetic exchanges should be considered. Indeed, even if the *Giardia* genome contains many (21 of 29) of the genes required for meiosis (Ramesh et al. 2005), their exact role is unknown, and they can be involved in other functions, such as DNA repair. Therefore, at present, it is difficult to assess the extent and impact on recombination on molecular epidemiologic studies, as the mechanism(s) involved remain elusive (Cacciò and Sprong 2010). In any case, a better understanding of the genetics of *G. duodenalis* is essential to improve current genotyping schemes, particularly for assemblage B.

33.5 Epidemiology and Molecular Epidemiology of Giardiasis in Humans

Giardiasis is a very common gastro-intestinal infection of humans; it has been estimated that about 200 million people in Asia, Africa, and Latin America have symptomatic infections (WHO 1996; Yason and Rivera 2007). The parasite has a global distribution, but the prevalence of infection is clearly higher in developing regions of the world, where *Giardia* is common in both children and adults (Cacciò and Sprong 2011). In recognition of the burden of disease caused by the parasite and to underline its link to poverty, the WHO has included giardiasis in the list of neglected diseases since 2004 (Savioli et al. 2006).

Infection rates have been reported in both developing countries (range 8–30 %) and industrialised countries (range 1–8 %) (Feng and Xiao 2011; Table 33.2). Those rates are probably higher in individuals with diarrhea, but the current epidemiological scenario is largely influenced by the fact that many countries did not report any data, by the lack of monitoring programs, and by the high rate of asymptomatic carriage of *Giardia* in humans (Cacciò and Sprong 2011). This suggests that giardiasis is strongly underdiagnosed and underreported.

In humans, giardiasis is mainly a paediatric infection, with the highest prevalence observed in children aged 1–4 years. This pattern is found both in industrialized and developing countries, and is thought to be due to lower hygiene and higher susceptibility of children at the first exposure to the parasite (Cacciò and Sprong 2011). A secondary peak is observed in adults aged 30–40; in this case, women represent the risk category, likely because of direct transmission of *Giardia* from children to their mothers. Other risk groups include institutionalized children,

Table 33.2 Prevalence of *Giardia duodenalis* assemblages A, B, and mixed infections in humans in different regions of the world[a]

Location	Number of samples	Prevalence (%)	Samples genotyped	Assemblage A	Assemblage B	Mixed infections
Europe						
Albania	125	17.6	22	10	12	
Belgium	373	4.0	72	18	54	
France			25	9	16	
Germany	202	1.5	3	3		
Italy			120	65	39	16 (A+B)
The Netherlands			98	34	64	
The Netherlands	892	2.0	18	9	9	
Norway			21		21	
Norway			63	3	60	
Poland	232	1.3	3	2	1	
Portugal	190	3.7	7	7		
Portugal			25	25		
Spain	327	3.0	7	2	4	1 (A+B)
Spain			108	43	61	4 (A+B)
Sweden			207	73	128	6 (A+B)
United Kingdom			199	48	145	6 (A+B)
United Kingdom			33	9	21	3 (A+B)
			1031	360	635	36
North America						
Canada	658	0.5	3	3		
Canada	52	28.9	15	3	9	3 (A+B)
Canada			6	6		
United States			14	14		
United States			2		2	
			40	26	11	3
Oceania						
Australia			12		11	1 (A+B)
Australia	353	7.6	23	7	16	
Australia			124	31	93	
New Zealand	66	7.6	5	1	4	
New Zealand			30	23	7	
			194	62	131	1
Central and Southern America						
Argentina			43	40	3	
Brazil	245	51.8	30		30	
Brazil	366	23.8	62	62		
Brazil			37	29	8	
Cuba			20	9	11	
Guatemala	645	5.4	20	7	12	1 (A+B)

Table 33.2 (continued)

Location	Number of samples	Prevalence (%)	Samples genotyped	Assemblage A	Assemblage B	Mixed infections
Mexico			19	19		
Mexico			9	9		
Mexico			12	12		
Nicaragua			119	25	94	
Peru	1531	20.4	167	66	81	20 (A+B)
Peru	845	23.8	16	10	6	
Peru			25	6	19	
			579	294	264	21
Africa						
Egypt			15	1	13	1 (A+C)
Egypt	52	34.6	18	1	14	1 (B+E), 2 (E)
Ethiopia			59	31	13	8 (A+B), 7 (A+F)
Guinea Bissau	50	56	25	3	22	
Ivory Coast	307	19.8	61	25	36	
Nigeria	157	3.2	5	5		
Sahrawi	120	34.2	32	12	18	2 (A+B)
Uganda	427	20.1	34	5	25	4 (A+B)
Uganda	62	5.0	3	3		
			252	86	141	25
Asia						
Bangladesh	2534	12.7	267	20	231	16 (A+B)
China			8	4	4	
China			18	12	6	
India	51	27.4	14	6	8	
India			16	5	8	3 (A+B)
India			19	6	9	4 (A+B)
Japan			3	2	1	
Laos			5		5	
Malaysia	321	23.7	42	1	41	
Nepal	1096	4.1	35	7	26	2 (A+B)
Saudi Arabia	1500	6.5	40	23	15	2 (A+B)
South Korea			5	5		
Taiwan	209	3.8	8	8	-	
Thailand	6967	0.9	61	5	31	25 (A+B)
Thailand	204	20.3	35	25	1	2 (A+B), 6 (A/B +C/D), 1 (C)
Thailand	531	6.2	12	5	7	
Thailand	189	5.8	10	3	7	
Turkey			44	19	25	
Yemen	503	17.6	65	43	22	
			707	199	447	61
All data			2803	1027 (37%)	1629 (58%)	147 (5%)

[a] Data have been compiled by the author. References are available upon request

returning travellers, immigrant/refugees and adopted children (Cacciò and Sprong 2011; Escobedo et al 2010).

A strong seasonality has been observed in industrialized countries. In the USA a twofold increase in transmission of giardiasis occurs during the summer, and coincides with increased outdoor activities (e.g., swimming and camping) (Yoder et al. 2012). Similarly, a study in New Zealand (period 1996–2000) showed peaks in the late summer and early autumn (Hoque et al. 2004). In Europe, the average monthly incidence is ~950 cases, but an autumn peak of ~1350 cases is observed (European Centre for Disease Prevention and Control: Annual Epidemiological Report on Communicable Diseases in Europe 2008. Stockholm, European Centre for Disease Prevention and Control, 2008). Bearing in mind the delay between infection, development of symptoms and submission of specimens which may amount to 5 weeks or more, this late summer/autumn peak probably represents an increase in infection during late summer months, resulting from travel and out-door recreation activities.

Information on risk factors for giardiasis is mainly derived from outbreak investigations, whereas few studies have focused on endemic or sporadic giardiasis. Outbreaks are most frequently waterborne, are caused by drinking or recreational water contamination, although other transmission routes have also been implicated (Adam 2001; Eisenberg et al. 2002).

The routes of transmission for sporadic cases are largely unknown, but recent case-control studies in Europe and New Zealand have identified person-to-person contact, contact with livestock, travel, and exposure to both potable and recreational water as important risk factors for sporadic disease (Chute et al. 1987; Hoque et al. 2001; Stuart et al. 2003; Espelage et al. 2010).

Humans are infected by two *G. duodenalis* assemblages, namely assemblage A and B (Mayrhofer et al. 1995). Initial studies on protein polymorphisms have shown that isolates of assemblage A can be divided into four sub-assemblages (AI, AII, AIII and AIV), and the host distribution indicated that human isolates belonged to AI and AII, while animal isolates belonged to AI, AIII and AIV (Monis et al. 2003). Similarly, sub-assemblages BI, BII, BIII and BIV were described in assemblage B, and, as it is the case for assemblage A, human isolates appeared to form 2 clusters (BIII and BIV), whereas animal isolates (monkeys and a dog) belonged to sub-assemblages BI and BII (Monis et al. 2003).

More recent studies at the DNA level confirmed that humans are infected with genotypes belonging to assemblages A and B, worldwide (Table 33.2). Only in a few studies, mostly from developing countries, other assemblages have been identified in human stool samples (Sprong et al. 2009). The geographical distribution of assemblages largely overlaps, but globally assemblage B is more prevalent than assemblage A (Table 33.2). As mentioned above, genetic characterization of isolates of assemblage A is relatively straightforward. The large majority of human isolates belong to sub-assemblages AII, whereas sub-assemblages AI is found less often, and sub-assemblage AIII is not found at all (Sprong et al. 2009; Feng and Xiao 2011). When assemblage A isolates have been typed at multiple loci, these sub-assemblages appear to form strongly supported monophyletic groups (Cacciò et al. 2008), although the level of polymorphisms of the investigated genes is rather low.

The situation is less clear for assemblage B. Many genotypes within sub-assemblages BIII and BIV have been described at various loci, but insightful comparisons are hampered by the higher genetic heterogeneity, presence of ASH and possible recombination events among field isolates (Cacciò et al. 2008; Sprong et al. 2009; Lebbad et al. 2008).

33.6 Epidemiology and Molecular Epidemiology of Giardiasis in Animals

33.6.1 Farmed Animals

The prevalence of *Giardia* in farmed animals has been investigated mainly in cattle, and to a lesser extent in other ruminants and pigs (Geurden et al. 2010; Feng and Xiao 2011). Prevalence varies considerably among studies, and is clearly influenced by the age of the animals tested, study design, geographical and climatological parameters as well as management practices (Geurden et al. 2010). Moreover, the lack of a gold standard reference test for the diagnosis of *Giardia* makes the comparison between studies problematic, as it is well know that different diagnostic techniques can yield different estimates of prevalence (Geurden et al. 2004), and that molecular techniques have higher sensitivity than microscopic techniques (Trout et al. 2005). Finally, the sensitivity of diagnostic methods is also influenced by the intermittent nature of the shedding of cysts.

In cattle, the animal prevalence ranges from 9 to 73 % and the farm prevalence ranges from 45 to 100 %; in sheep, the animal prevalence ranges from 1.5 to 36 % and the farm prevalence ranges from 10 to 100 %; in goats, the animal prevalence ranges from 4 to 53 % and the farm prevalence ranges from 66 to 95 %; in pigs, the animal prevalence ranges from 1.5 to 38 % and the farm prevalence ranges from 10 to 84 % (Geurden et al. 2010). Notably, the prevalence peaked in animals aged between 1 and 6 months, and decreased from the age of 6 months onwards, independently from the species considered. Despite the large variability, the high farm prevalence can be taken as an indication that a large proportion of animals will get infected (Xiao and Herd 1994).

From a public health perspective, prevalence data must be integrated by a genetic characterization of the parasite, in order to identify the assemblage(s) present in animals and determine their zoonotic potential (Cacciò and Ryan 2008). Molecular surveys have shown that assemblage E generally predominates in cattle (Feng and Xiao 2011), but assemblage A is increasingly being detected and is probably more widespread in the bovine population than initially assumed (Table 33.3). For example, a recent multicenter trial examined 2072 *Giardia* isolates from cattle in Germany, UK, France and Italy, identified 45.4 % as positive for *Giardia*, and found a high overall prevalence of assemblage A (43 %) (Geurden et al. 2012). The prevalence of assemblage A ranged from 61 % for France to 41 % for Germany, 29 % for

Table 33.3 Distribution of *G. duodenalis* assemblages in farm and companion animals[a]

		Assemblages						
Host	N of samples typed	A	B	C	D	E	F	Mixed infections
Farm animals								
Cattle	2126	291	73			1697		65
Sheep	367	66	22			261		18
Goat	76	6				60		10
Pigs	226	29	76			116		5
Horse	97	22	49			26		
Companion animals								
Dog	1102	237	63	272	460			70
Cat	189	73	5	2	12	2	63	2

[a] Data have been compiled by the author. References are available upon request

the UK and 28% for Italy. Importantly, 32% of the samples had a mixed assemblage A and E infection, indicating that prevalence is more correctly estimated using assemblage-specific assays (Geurden et al. 2012).

Assemblage B seems to be less common in cattle. However, a recent study in China identified an overall prevalence of 5.2% for *Giardia* by microscopy, of which 43.8% (7/16) were genotypes of assemblage B (Liu et al. 2012). In New Zealand, assemblages A and B have been commonly identified in cattle, whereas assemblage E was not detected at all (Hunt et al. 2000; Learmonth et al. 2003; Winkworth et al. 2008). However, a more recent study in New Zealand identified both assemblage A and E in cattle isolates, with a higher prevalence of assemblage E (Abeywardena et al. 2012). Thus, the significance of New Zealand's dairy herd as a potential reservoir of zoonotic *Giardia* should be further investigated.

As with cattle, sheep and goats are mainly infected with assemblage E (Ruiz et al. 2008; Robertson 2009, Feng and Xiao 2011; Table 33.3), but assemblage A has also been reported in several countries. In goats, of 36 isolates that were typed at 3 loci, 78% belonged to sub-assemblage AI, 22% to sub-assemblage AII, whereas sub-assemblage AIII was not detected (Sprong et al. 2009). Using the same 3 loci, Lebbad et al. (2010) found several genotypes in 7 sheep isolates, all of which clustered with sub-assemblage AI. The same authors detected a mixed A and E infection in 3 sheep samples. Similarly, a multi-locus study of 120 lambs in Spain revealed that the detection of assemblage E and A, and of mixed infections, is strongly influenced by the marker used (Gómez-Muñoz et al. (2012). Indeed, typing at the 18S rRNA assigned 55% of the isolates to assemblage A and 25% as mixed A+E infection, but when other loci (*bg*, *tpi*, and *gdh*) were investigated, the percentage of sequences belonging to genotype A or mixed A/E was much lower (6.5% at *bg*, 10.8% at the *gdh* and 15.4% at the *tpi*). Assemblages A and E differ by a single nucleotide substitution in the 18S rRNA gene fragment commonly used for genotyping, and is therefore essential to confirm data obtained at this locus by further genotyping (Gómez-Muñoz et al. 2012). Assemblage B is not common in sheep and

goats, but has been reported in sheep in China, Norway, Italy and Spain (Aloisio et al. 2006; Castro-Hermida et al. 2007; Castro-Hermida et al. 2011; Robertson et al. 2010; Zhang et al. 2012).

Assemblage E is commonly found in pigs, but assemblages A and B have also been identified (Table 33.3). Genotypes of sub-assemblage AI also appear to predominate: indeed, one study reported sub-assemblage AI in 12 of 14 isolates that were typed at 3 loci (Sprong et al. 2009). On the contrary, assemblage B has been rarely detected in pigs (Sprong et al. 2009). However, a recent study that examined swine manure samples from ten farms in Ontario, Canada, identified assemblage B in 92% of the isolates typed: the authors concluded that there is a potential for transmission between swine and humans by means of water or foods contaminated with *Giardia* cysts (Farzan et al. 2011).

In horses, the prevalence ranges from 0 to 35% among surveys, being usually higher in foals than in adults (Santin et al. 2013). Few molecular studies have been conducted on equine giardiasis; however, about 50% of the isolates tested belonged to assemblage B, whereas assemblages A and E had both a prevalence of about 25% (Table 33.3).

33.6.2 Companion Animals

Giardiasis is considered to be a common infection of dogs and cats (Feng and Xiao 2011). As commented above for farmed animals, several diagnostic techniques with different sensitivities have been used to estimate the prevalence of infection, including microscopic examination of fecal smears after flotation, ELISA and PCR. Prevalence estimates are influenced by other factors, in particular the nature of the population studied (household, shelter, stray), animal age, breed, the dog's spay/neuter status and geographic location (Ballwebber et al. 2010; Mohamed et al. 2013).

In the USA, a nation-wide study of intestinal parasites of dogs was conducted using flotation and microscopy (Little et al. 2009). Among the 1,199,293 dogs tested, *Giardia* was detected in 47,972 (4%). The fact that most dogs were asymptomatic and adult (>1 year of age), and the poor sensitivity of the diagnostic technique used, may account for this low prevalence. Indeed, another large survey (Carlin et al. 2006) examined 6114 symptomatic dogs and 4978 symptomatic cats and, using a more sensitive test that detects soluble *Giardia* cyst wall antigens (IDEXX SNAP test), estimated a prevalence of 15.6% among dogs, and of 10.8% among cats (Carlin et al. 2006). Another very recent study (Mohamed et al. 2013) examined a total of 2,468,359 dogs visiting 777 Banfield hospitals in 43 states from 2003 to 2009. Using a fecal flotation test, 10,843 dogs (0.44%) were positive for *Giardia* spp., with higher prevalence in young dogs and significant differences among states (from 4% in Maine, to 2.63% in Colorado, to 0.10% in 11 different states; Mohamed et al. 2013).

In Germany, a retrospective study was conducted on faecal samples from 8560 cats and 24,677 dogs collected between 2003 and 2010 (Barutzki and Schaper,

2011). Samples were from privately owned dogs and cats presented at local veterinary surgeons for either gastrointestinal disorders, routine examination and animal vaccination, general health check or without specified reasons. *Giardia* was identified by microscopy in 4890 dogs (18.6%) and in 1,078 cats (12.6%). The prevalence was higher in dogs aged up to 3 months (37.5%) and in dogs of 3 to 6 months of age (38.2%) compared to older animals, and the same pattern was observed for cats (19.5 and 24% in the same age groups, respectively). In another large European survey, Epe et al (2010) analysed fecal samples from symptomatic and asymptomatic dogs and cats collected in France Germany, Italy, Spain and the UK. Using the IDEXX SNAP test, the estimated prevalence was 24.78% (2147 of 8685) in dogs and 20.3% (855 of 4214) in cats. In agreement with other studies, younger animals and symptomatic animals had a higher prevalence of *Giardia* (e.g., Mark-Carew et al. 2013). Molecular analysis of dog isolates has highlighted a complex picture, with a remarkable number of animals showing mixed infections and the identification of both host-specific assemblages (C and D) and zoonotic assemblages (A and B). In a systematic analysis of published data, Sprong et al. (2009) showed that about 25% of the 600 dog isolates genotypes were classified as assemblage A, whereas the rest of the isolates were assemblage C or D, with the latter being more common overall. Assemblage B appears to be rare, and has been detected only in a limited number of dogs in Europe, Asia, and Australia (Ballweber et al. 2010; Feng and Xiao 2011; Table 33.3). Importantly, it was noticed that 34% of the dog isolates that were typed at more than two loci showed a mixture of assemblages (Sprong et al. 2009), and the occurrence of mixed infections in dogs was corroborated by the use of assemblage-specific primers in subsequent studies (Beck et al. 2012; Scorza et al. 2012). It is however difficult to distinguish between mixed infections and the occurrence of recombinants in field isolates, and opinions differ regarding the mechanisms and the extent of genetic exchanges in *Giardia*, and of their impact on genotyping (Sprong and Cacciò 2010). In any case, until a more robust genotyping scheme is developed, caution should be used in interpreting data from dog isolates of *Giardia*, particularly when only single loci are investigated, as previously commented (Beck et al. 2012).

Comparatively less is known on the occurrence of *G. duodenalis* assemblages in cats. The available data (Table 33.3) show a similar prevalence of the host-specific assemblage F and of the zoonotic assemblage A, whereas assemblage B, C and D have been very rarely found in this host (Sprong et al. 2009).

33.6.3 Wild Animals

As a matter of fact, it was the association between infected beavers and waterborne outbreaks of human giardiasis that led the World Health Organization to classify *Giardia* as a zoonotic parasite (WHO 1979). Despite this, the information currently available on *Giardia* in wild animals is still rather scarce (summarized in Table 33.4). From these studies, it appears that assemblages A and B are common

Table 33.4 Prevalence of zoonotic *G. duodenalis* assemblages in wildlife. Data from captive animals are not included

Host (common name)	Origin	N of samples	Prevalence	Assemblage	Reference
Wild rodents					
Castor canadensis (beaver)	USA	62	1.5%	B	Feng and Xiao (2011)[a]
Castor canadensis (beaver)	USA	7		B	Feng and Xiao (2011)[a]
Ondatra zibethicus (muskrat)	USA	8		B	Feng and Xiao (2011)[a]
Rattus norvegicus (rat)	Sweden	8		B	Feng and Xiao (2011)[a]
Wild ungulates					
Alces alces (wild moose)	Norway	455	12.3%	A	Feng and Xiao (2011)[a]
Cervus elaphus (red deer)	Croatia	374	1.1%	A	Beck et al. (2011)
Capreolus capreolus (roe deer)	Croatia	21	24%	A	Beck et al. (2011)
Dama dama (fallow deer)	Italy	139	11.5%	A	Feng and Xiao (2011)[a]
Ovibos muschatus (muskox)	Canadian Arctic	72	21%	A	Kutz et al. (2008)
Odocoileus virginiasus (white tailed deer)	USA	26	3.8%	A	Feng and Xiao (2011)[a]
Rangifer tarandus (reindeer)	Norway	188	7.3%	A	Feng and Xiao (2011)[a]
Sus scrofa (wild boar)	Croatia	144	1.4%	A	Beck et al. (2011)
Wild carnivores					
Canis aureus (jackal)	Croatia	8	12.5%	A, B	Beck et al. (2011)
Canis latrans (coyote)	USA	22	32%	B	Feng and Xiao (2011)[a]
Canis latrans (coyote)	Canada	70	18.6%	A	Feng and Xiao (2011)[a]
Canis lupus (wolf)	Croatia	127	10.2%	A	Beck et al. (2011)
Lycaon pictus (African painted dog)	Africa	71	27%	A, B	Feng and Xiao (2011)[a]
Vulpes vulpes (red fox)	Norway	269	4.8 %	A, B	Feng and Xiao (2011)[a]
Vulpes vulpes (red fox)	Australia	1		A	McCarthy et al. (2008)
Vulpes vulpes (red fox)	Croatia	66	4.6%	A	Beck et al. (2011)

Table 33.4 (continued)

Host (common name)	Origin	N of samples	Prevalence	Assemblage	Reference
Non-human primates					
Alouatta pigra (black howler monkey)	Belize, Mexico	66	31 %	A, B	Vitazkova and Wade (2006)
Gorilla gorilla beringei (mountain gorilla)	Uganda	100	2 %	A	Feng and Xiao (2011) [a]
Macaca fuscata (Japanese macaque)	Japan	3		B	Feng and Xiao (2011) [a]
Marsupials					
Macropus fuliginosus (western grey kangaroo)	Australia	72	4.2%	A	McCarthy et al. (2008)
Macropus fuliginosus(western grey kangaroo)	Australia	105	4.8%	A, B	Feng and Xiao (2011) [a]
Isoodon obesulus (quenda)	Australia	72	1.5%	Novel genotype	Feng and Xiao (2011) [a]
Trichosurus cunninghami and T. *vulpecula* (mountain brushtail and silver grey brushtail possum)	Australia	76	25%	A	Feng and Xiao (2011) [a]
Phascolarctos cinereus (Koala)	Australia	11	27.3%	A	Feng and Xiao (2011) [a]
Wallabia bicolor (swamp wallaby)	Australia	16	12.5%	A	Feng and Xiao (2011) [a]
Marine mammals					
Phoca groenlandica (harp seal)	USA	1		A, B	Feng and Xiao (2011) [a]
Phoca vitulina (harbor seal)	USA	1		B	Feng and Xiao (2011) [a]
Delphinus delphis (common dolphin)	USA	4		A, B	Feng and Xiao (2011) [a]
Lagenorhynchus acutus (white sided dolphin)	USA	3		B	Feng and Xiao (2011) [a]
Grampus grisou (Risso's dolphin)	USA	1		B	Feng and Xiao (2011) [a]
Phocoena phocoena (harbor porpoise)	USA	3		A, B	Feng and Xiao (2011) [a]

[a] the original reference can be found in Feng and Xiao (2011)

in wild animals, supporting the notion that these assemblages have a very wide host range (Feng and Xiao 2011).

In non-human primates (NHP), the large majority of data has been obtained from captive animals (Feng and Xiao 2011), but since this does not reflect the natural transmission of the parasite in these hosts, these data are not considered here. In the few studies carried out on wild NHP, the prevalence ranged from 2 to 31 %, and only assemblages A and B have been found, like in humans (Table 33.4).

In wild hoofed animals, the prevalence of infection varies between 1 and 21 % depending on the host and geographic origin (Table 33.4). Interestingly, a new sub-assemblage, AIII, has been described using multi-locus genotyping (Lalle et al. 2007; Cacciò et al. 2008) and appears to be largely restricted to this group of animals, albeit it has been found in a cat (Lebbad et al. 2010), but never in humans. Assemblage E, which is highly prevalent in livestock, is very rarely observed in wild hoofed animals (Table 33.4).

In wild carnivores, the prevalence ranges from 4.6 to 32 % (Table 33.4). Molecular analyses have shown that the host-adapted assemblages C and D are prevalent in coyotes and wolves, but assemblages A and B have been found in all wild carnivores tested (McCarthy et al. 2008; Ash et al. 2010; Beck et al. 2011; Feng and Xiao 2011). The most common sub-assemblage found in these hosts is AI.

Wild rodents are commonly infected with *Giardia microti* or with the non-zoonotic assemblage G (Feng and Xiao 2011). However, zoonotic assemblages, particularly assemblage B, have been identified in rats, muskrats, and beavers (Table 33.4; Fayer et al. 2006; Sulaiman et al. 2003; Lebbad et al. 2010).

In marsupials, the prevalence of *Giardia* has been poorly investigated, despite marsupials being one of the dominant mammalian groups within watersheds in Australia (Power et al. 2005). From the limited information available, the prevalence ranges from 1.5 to 27.3 % (Table 33.4). A novel host-adapted genotype, the quenda genotype, has been identified in southern brown bandicoots (or quendas) in Western Australia (Adams et al. 2004). Assemblage A seems to predominate in most marsupial species, but assemblage B is also present. A recent study has suggested that human interaction may have been a source of infection in marsupials, as a particular genotype was identified in captive marsupials from wildlife parks, but not in wild marsupials (Thompson et al. 2008).

In marine mammals, a genetically distinct genetic group, known as assemblage H, has been identified in species of seals, dolphins and porpoise (Lasek-Nesselquist et al. 2010). This assemblage has not been found in other mammals, including humans. However, the same species can also harbor assemblages A and B (Lasek-Nesselquist et al. 2008; Table 33.4).

33.6.4 Zoonotic Transmission

As summarized above, animals can harbor both zoonotic and host-specific *G. duodenalis* assemblages that are morphologically identical; therefore molecular typing tools are required to track transmission (Feng and Xiao 2011).

Zoonotic transmission between cattle and humans has been proposed in numerous studies (e.g Uehlinger et al. 2011; Kahn et al. 2011). In addition to direct animal to human contact, transmission of zoonotic assemblages from cattle may occur through the contamination of ground and surface water (Feng and Xiao 2011; Budu-Amoako et al. 2012). In this scenario, calves are thought to play an important role because of the large number of cysts (10^5–10^6 cysts per gram of faeces) they can shed. It must be kept in mind, however, that cattle are more commonly infected with assemblage E, and that infection with assemblage A seems to occur only in young animals and only transiently (Mark-Carew et al. 2011). According to these data, no evidence incriminating infected cattle in any of the investigated outbreak has been provided until now.

In a recent study in India, genotype A1 was identified at the *bg* locus in cattle and in dairy farm workers from the same farms (Kahn et al. 2011). As cattle are more commonly infected with genotypes in sub-assemblage AI (mostly genotype A1), whereas humans are more commonly infected with genotypes in sub-assemblage AII (Xiao and Fayer 2008), the finding of genotype A1 in the farms workers supported zoonotic transmission of the infection to farm workers (Kahn et al. 2011).

The role of companion animals has also been a subject of intense investigation, particularly in dogs living in urban areas of developed countries (Ballweber et al. 2010). As reported above, molecular surveys of *Giardia* from dogs and cats have shown a higher frequency of host-adapted, non-zoonotic assemblages (Sprong et al. 2009; Feng and Xiao 2011). However, dogs and cats are also infected with assemblages A and B, but without data from owners or known handlers, information on the frequency of zoonotic transmission cannot be obtained.

More interesting observations on zoonotic transmission were obtained in studies conducted in defined endemic foci in which both humans and dogs have been sampled and characterized genetically. The first study (Traub et al. 2004) involved tea-growing communities in Assam (India) and provided evidence for the circulation of identical zoonotic genotypes in humans and dogs. While the molecular evidence of this study were not fully convincing, zoonotic transmission was supported by strong epidemiological data showing a highly significant association between the prevalence of *Giardia* in humans and the presence of a *Giardia* positive dog in the same household (Traub et al. 2004). The presence of zoonotic species of *Giardia* in dogs and their owners sharing the same living area have been also reported in Temple communities in Bangkok (Inpankaew et al. 2007; Traub et al. 2009) and northern Canadian indigenous communities (Salb et al. 2008). In contrast with these findings, a study in a high endemic region of Peru, where infected dogs and humans constantly commingle, found that dogs and humans are infected with different genotypes of *Giardia*, suggesting that zoonotic transmission is very uncommon (Cooper et al. 2010). Therefore, the role of dogs as reservoirs of cysts infectious to humans deserve further studies.

Wild animals, particularly those living in aquatic environments, have been implicated as a source of infection to humans, mostly on the basis of indirect evidence (Appelbee et al. 2002). In Canada, giardiasis is also known as “beaver fever”, as beavers have been implicated in outbreaks of infection that occurred in people after

consumption of municipal water (Lopez et al. 1980; Dykes et al. 1980). It was then shown that cysts isolated from beavers were infectious to gerbils and could be grown under in vitro laboratory conditions (Wallis et al. 1984). Likewise, cysts of human origin were infectious to beavers, albeit large number of cysts were needed to cause infection (Erlandsen et al. 1988). These studies demonstrated the potential for cross-transmission between different hosts, as well as the fact that various hosts, including beavers, were susceptible to genetically different parasites (Proctor et al. 1989). However, what has remained unclear is whether beavers were the actual source of water contamination or if, on the contrary, beavers got infected with cysts of human origin (reverse zoonosis or "spill back") and just acted as multipliers of cysts, leading to environmental contamination (Thompson et al. 2009).

A recent study on *G. duodenalis* in rhesus monkeys (*Macaca mulatta*) living in a large public park in China (Ye et al. 2012) found assemblages A and B in these animals. Interestingly, the genotypes of assemblage A belonged to sub-assemblage AII, which is the main type found in humans, and those of assemblage B were previously found in humans in China. Considering the close contact between human and monkeys in this setting, direct or indirect (through water) transmission from monkeys to humans appears possible.

Another study highlighted the impact of habitat disturbance on the transmission of *G. duodenalis* in people, livestock and wild primates in the Kibale National Park, Uganda (Johnston et al. 2010). Indeed, people and primates were found to be infected with sub-assemblage BIV, whereas livestock and primates were infected with assemblage E (first report of assemblage E in primates). The study shows that cross-species transmission of multiple *G. duodenalis* assemblages may occur in locations where people, livestock, and primates overlap in their use of habitat. Reverse zoonotic transmission may be frequent in tropical settings where habitat disturbance forces people and livestock to interact at high rates with wildlife, and this could have negative consequences for wildlife conservation (Johnston et al. 2010).

33.6.5 Clinical Giardiasis in Animals and Humans

The adverse consequences of infection and its pathogenic potential have been best recognised in humans. The clinical spectrum of the infection is wide, and ranges from the absence of overt symptoms (asymptomatic giardiasis) to acute or chronic giardiasis (Farthing 1997). Symptoms appear one or two weeks after ingestion of cysts, and usually persist for 3–4 days; the main symptom is diarrhoea, but flatulence, epigastric cramps, nausea, vomiting, weight loss, itch and urticaria can also be observed (Farthing 1997). All these symptoms has been correlated to dysfunctions of the small intestine, including a decrease of the surface area of brush border, atrophy of microvillus and villus, enterocyte immaturity, deficiencies of luminal enzymes, malabsorption of electrolytes, fats, D-xylose, lactose, vitamin A, and vitamin B12 (Adam 2001). Malabsorption of nutrients and electrolytes creates an osmotic gradient that draws water into the small intestinal lumen, and that results in small intestinal distension, rapid peristalsis, and, finally, diarrhoea.

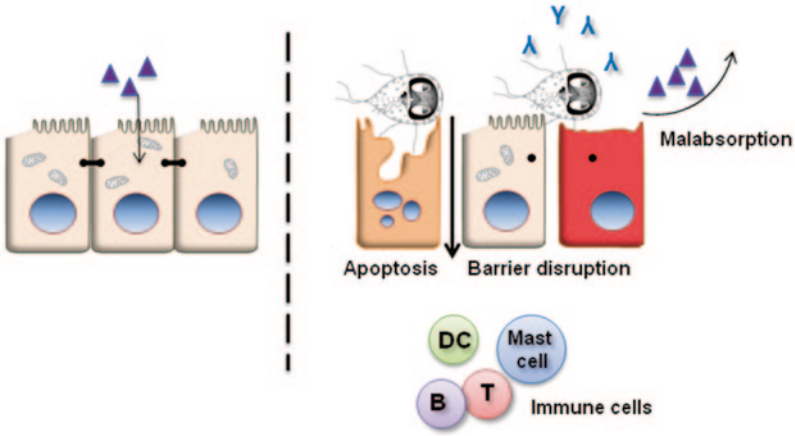

Fig. 33.2 Schematic representation of key pathogenic events occurring during *Giardia* infection

Exacerbation of symptoms has been observed in infants and children, especially in developing countries, where giardiasis has been associated with the failure-to-thrive syndrome, retarded growth and development, poor cognitive function and detrimental effects on nutritional status (Berkman et al. 2002) Giardiasis may be responsible for long-term consequences and sequelae in a naïve population. In Norway, investigations after a large waterborne outbreak have shown that about 10% of infected individuals had persistent symptoms, with mean disease duration of 7 months, and that at least 5% of them developed persistent post-infectious fatigue syndrome (Naess et al. 2012). Furthermore, case reports and epidemiologic studies have associated giardiasis with the development of irritable bowel syndrome, allergies, and reactive arthritis (Wensaas et al. 2012).

The mechanisms that contribute to disease are only partially understood, but are clearly multifactorial. Figure 33.2 provides a schematic representation of the major events involved in disease progress. Both host and parasite factors contribute to the pathogenesis of giardiasis, and the existence of genotype/assemblage-specific pathogenic mechanisms is conceivable (Cotton et al. 2011).

The establishment of the infection depends on the high mobility and strong attachment of the trophozoite to enterocytes in the upper small intestine of the host. This is accomplished by a unique structure of *Giardia*, the ventral adhesive disc, and by the flagella, and avoids parasite elimination by peristalsis. The trophozoite must survive in the very hostile environment of the small intestine, where it is readily exposed to proteases, lipases, bile salts and products of the host's immune response. *Giardia* is the only gut-dwelling eukaryotic organism known to possess a mechanism of antigenic variation (Carranza and Lujan 2010). The surface of trophozoites is covered by a dense coat of variable surface proteins (VSP), and a single VSP is normally dominating in a population of parasites. The on–off switching in the expression of genes encoding VSP allows the parasite to replace the expressed

VSP during infection, possibly to escape the host immune reaction. Notably, the VSP repertoire of different assemblages is totally different and no identical VSP are found in the genome of the three strains sequenced to date (Franzen et al. 2009; Jerlstrom-Hultqvist et al. 2010).

The involvement of secreted proteins with toxin-like activities has been hypothesized, but no giardial toxin has been identified so far. However, four parasite proteins, arginine deiminase (ADI), ornithine carbamoyl transferase (OCT), α-enolase and elongation factor 1-α, have been found in the supernatant of trophozoites cocultured with human intestinal epithelial cells (Skarin et al. 2011). In particular, the secretion of ADI and OCT seems to be involved in the inhibition of the host´s innate immune response via interference with the synthesis of nitric oxide (NO), that is cytostatic to *Giardia* parasites. The underlying molecular mechanism is the subtraction of L-arginine which is needed for NO synthesis by the host epithelial cell nitric oxide synthetase (Ropolo and Touz 2010).

A key component in the pathogenesis of *Giardia* infection is damage to the enterocyte epithelium. An increase in the rate of enterocyte apoptosis has been observed in in vitro experiments (Chin et al. 2002), shortly after colonization of the small intestine, and has been reported also in patients with chronic giardiasis (Troeger et al. 2007). Apoptosis appears to be mediated by activation of pro-apoptotic caspase-3 and -9, increased expression of pro-apoptotic Bax, decreased expression of anti-apoptotic Bcl-2 and induced proteolytic cleavage of poly(-ADP-ribose) polymerase (PARP). Following the induction of apoptosis, *Giardia* trophozoites can also induce alterations in the enterocyte tight junctions by the breakdown/relocalisation of proteins associated with these structures. Indeed, F-actin, zonula-occludens-1, claudin-1 and α-actinin are relocated from the cell periphery to the cytosol (Cotton et al. 2011). Inhibition of caspase-3 prevents relocalisation of F-actin and ZO-1, suggesting a direct cause-effect relationship between *Giardia*-induced apoptosis and small intestinal barrier function (Chin et al. 2002). The breakdown of epithelial barrier allows macromolecules and electrolytes to pass into the sub-mucosa, bypassing normal uptake by epithelial cells. The paracellular flow of nutrients and electrolytes can contribute to nutrient malabsorption by reducing electrochemical gradients needed for proper uptake, and can cause inflammation in some individuals, probably through activation of innate immune effectors like macrophages (Solaymani-Mohammadi and Singer 2010). Little is known about the host factors directly involved in *Giardia* infection, albeit host responses clearly play a key role in the pathogenesis of giardiasis. All the parasite secreted proteins, the VSP and the major disk proteins are recognized by sera of *Giardia*-infected humans as well as by sera from infected mice, indicating their importance in antibody-mediated *Giardia* immunity. Results obtained from *Giardia* infection in a murine model have suggested that IgA antibodies contribute to protective immunity against giardiasis (Solaymani-Mohammadi and Singer 2010). However, resolution of the infection occurs even in mice that are unable to produce antibodies (Singer and Nash 2000). A relevant role of T-cell responses for the control of *Giardia* infections has been demonstrated. In fact, T-cell–deficient mice develop chronic giardiasis (Singer and Nash 2000) and patients with common variable immunodeficiencies inlcuding

Bruton's X-linked agammaglobulinemia have been associated with a predisposition to chronic giardiasis (Faubert 2000). Furthermore, a decreased mucosal surface area for the absorption of nutrients, electrolytes and water has been associated with a CD8+lymphocyte-dependent shortening of microvilli in a murine model (Scott et al. 2004).

In comparison, less is known on the clinical manifestations and impact of giardiasis in animals. However, data from experimental infection of calves, goat kids and lambs (Olson et al. 1995; Koudela and Vitovec 1998; Ruest et al. 1997) indicate that giardiasis essentially leads to microvillus alterations, including a decreased crypt to villus ratio and brush border enzyme deficiencies, similar to what is described above for humans. The effects of severe infection, including malabsorptive diarrhea and lower weight gain, are therefore possible in animals.

The main clinical sign, as in humans, is diarrhea. Acute diarrhea can occur in infected calves, but chronic and intermittent symptoms are more often observed (St. Jean 1987; Geurden et al. 2006). In goat kids and lambs (Olson et al. 1995; Koudela and Vitovec 1998) as well as in lambs naturally infected with assemblage B (Aloisio et al. 2006), excretion of formless faeces has been observed after experimental infection.

The potential economical impact in production animals due to giardiasis is less clear. In goat kids and lambs, experimental infection resulted in a decreased feed efficiency and subsequently, a decreased weight gain (Olson et al. 1995; Koudela and Vitovec 1998). However, whether the same phenomenon occurs in calves or pigs is unknown, as experimental data for those species are not available (Geurden et al. 2010). Therefore, *Giardia* can be considered a potential cause of diarrhoea in production animals, although further experimental data are needed to clarify the full pathogenic role of this parasite (Geurden et al. 2010).

Infection in adult dogs and cats is usually asymptomatic, with immature animals being more susceptible to clinical disease (Kirkpatrick 1987). Acute diarrhoea, when seen, tends to occur in very young dogs and cats; in older animals, diarrhea may be acute, intermittent, or chronic (Barr 2006). Clinical disease in cats is particularly uncommon.

33.6.6 Open Issues and Future Prospects

The issue of zoonotic transmission has dominated the debate around *Giardia* for about 30 years, since the WHO recognized giardiasis as a zoonosis. In recent years, there have been substantial advances in our understanding of the biology, genetics and taxonomy of *Giardia*, and a wealth of data has been generated through the molecular characterization of isolates from many hosts. As a result of this large effort, and of several genome sequencing projects, it has become clear that the genetics of *Giardia* is complex, and that more complexity is found among isolates of assemblage B, possibly due to more frequent genetic exchanges. Molecular epidemiologic surveys have demonstrated that animals are more commonly infected

with assemblage-specific *Giardia* parasites, which are not infectious to humans. However, animals are also infected with assemblage A and, to a lesser extent, assemblage B, which are zoonotic.

Sub-assemblages AI and AII are found in both humans and animals. Sub-assemblage AI is preferentially found in livestock and pets, whereas sub-assemblage AII is predominantly found in humans. Sub-assemblage AIII is almost exclusively found in wild hoofed animals, and is likely to be non-zoonotic. Assemblage B predominantly affects human and non-human primates and to a much lesser extent wildlife and dogs. Therefore, while the potential for zoonotic transmission exists, studies in well defined epidemiologic settings are needed to confirm which animals can act as reservoirs and under which conditions zoonotic transmission may occur. Further, the extent of reverse zoonosis transmission from humans to animals should also be evaluated, in view of its importance for conservation management. Future studies, in particular those aimed at further comparative genomics of assemblages, should lead to a more comprehensive understanding of the host-specificity and transmission cycles of *Giardia*.

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Chapter 34
Leptospirosis and Leptospires—The Silent Assassins

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Abstract Leptospirosis is one of the most common yet under reported zoonoses. Leptospires, the etiological agents of leptospirosis are ubiquitous pathogens, with a world-wide distribution, causing a spectrum of disease ranging from a mild influenza-like illness to Weil's disease, which manifests itself in multi-organ failure. The following chapter reports on the epidemiology and transmission of the disease in humans and animals. The chapter will also delineate the symptoms observed in humans and animals and in concluding outline unresolved and evolving issues for microbiologists, epidemiologists and public health officials.

34.1 Introduction

Leptospires are 6–20 μm in length and 0.1–0.2 μm in diameter and have optimal growth at 30°C (Levett 2001). In the genus there are some 20 leptospiral species (nine pathogenic, six saprophytic/environmental and five intermediate species). Serologically there are more than 300 serovars and leptospirosis has been reported in over 150 mammalian species (Picardeau 2013; Ko et al. 2009). The main animal reservoirs include rodents, dogs, cattle, horses and pigs. These animals may act as maintenance hosts for adapted serovers such as serovar Canicola in dogs or serovars Ballum, Icterohaemorrhagiae or Copenhageni in rodents. Renal colonization and shedding of leptospires in the urine of infected animals sets the scene for the

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transmission of the organism to infect humans and other animals who are incidental hosts (Adler and de la Pena Moctezuma 2010; Faine et al. 1999).

34.2 Epidemiology in Animals

Epidemiological studies in animal populations, particularly in endemic regions have relied on the use of serosurveys. Roqueplo et al. (2013) conducted a cross sectional survey to estimate the prevalence of leptospiral antibodies in wild and domestic animals in New Caledonia. This study reported that 43 % of cattle, 72 % of Rusa deer, 80 % of horses, 43 % of dogs and 100 % of cats ($n=8$) had reactive leptospiral titres. Interestingly, members from the serogroups Icterohaemorrhagiae, Australis, Canicola, Ballum and Cynopteri appeared to circulate in the majority of hosts investigated.

Similarly, Desvars et al. (2013) conducted a serosurvey of 574 animals belonging to 12 species on Reunion Island and reported reactive leptospiral titres in approximately 80 % of rats, 34 % of cattle, 48 % of pigs, 26 % of cats and 47 % of dogs. This study also investigated the renal carriage (leptospires in kidney tissues) of ten animal species by qPCR and reported renal carriage in approximately 18 % of cattle, 16 % of pigs, 66 % of rats, 85 % of mice and 30 % in cats and dogs. leptospiral DNA was also detected in two bat urines echoing previous suggestions of a rodent-bat infection cycle (Matthias et al. 2005).

In a meta review of leptospiral serosurveys undertaken over a 20 year period in Rio de Janeiro, Brazil, Martins and Lilenbaum (2013) reported a seroprevalence rates of approximately 36 % of rats, 73 % of dogs, 38 % of cattle, 40 % of horses, 66 % of pigs, 40 % of wild animals excluding felines, 15 % of wild felines and tamarins. The predominant infecting serogroups were Icterohaemorrhagiae, Sejroe, Australis and Pomona. Another study from Brazil also reported high seropositivity (71 %) in 119 racehorses to serovar Copenhageni (Hamond et al. 2012). Leptospirosis in horses is of interest because of the association with post infection recurrent uveitis which has been postulated to cost the United States equine industry in the vicinity of 100–250 million dollars per year (Verma and Stevenson 2012). The high prevalence of reactive antibody titres and carriage of leptospires in rodents is well known in the Leptospirosis community. Recently in an ecological study of *Leptospira interrogans* in rats in Vancouver it was noted that an increase in weight or body fat and bite wounds increased the probability of infection in rodents (Himsworth et al. 2013).

34.3 Epidemiology in Humans

The global burden of human leptospirosis is currently unknown, however, estimates of the annual incidence range from 0.1 to 1 case/100,000 people in temperate areas to 100 cases/100,000 during epidemics in tropical regions (Everard and Everard

1993; Levett 2001). In addition, the incidence of leptospirosios is also higher in those environments prone to flooding (Lau et al. 2010a). An estimated 300,000–500,000 severe cases occur each year, with case fatality reports of up to 30 % (WHO 2003; Hartskeerl 2006). In an attempt to develop a better understanding of the burden of leptospiral disease, the WHO (2011) estimates that the global incidence in endemic areas exceeds five severe cases per 100,000. Given the lack of reporting in many developing areas, misdiagnosis, lack of awareness, patients failing to present for treatment and those with subclinical infections, it is almost impossible to determine the true incidence. The source of infection in humans is usually through either direct or indirect contact with the urine of an infected animal. Further, the usual portal of entry is via compromised cutaneous or mucosal membranes (Levett 2001). Occupation is a significant risk factor as dairy and cattle farmers, veterinarians, abattoir workers, meat inspectors, rodent control workers and other occupations where intermit contact with animals is required, all have a greater chance of direct contact with the urine of infected animals. Occupations that bring humans into indirect contact with animal urine are also at risk of infection, e.g. sewer workers, miners, soldiers, septic tank cleaners, fish farmers, gamekeepers, canal workers, rice field workers, taro farmers, banana farmers and sugar cane workers (Faine et al. 1999; Levett 2001; Tulsiani et al. 2011). Recreational activities while travelling are also considered a risk factor for the disease (Lau et al. 2010b).

In Europe as a whole, the overall incidence rate in 2010 was 0.13 per 100,000 inhabitants (Dupouey et al. 2014). In Germany, there were 2694 reported cases of leptospirosis from 1962 to 2003. During this time period, the highest mean annual incidence was 0.11 per 100,000 in 1962–1967. The lowest mean annual incidence was 0.04 per 100,000 between 1992 and 1997 (Jansen et al. 2005). In the Netherlands, there were 2553 (mainly severe) cases of leptospirosis reported from 1925 to 2008 although the average incidence was 0.25 per 100,000 population. The incidence in the Netherlands showed a small decrease over the 84 year period and as in Germany male patients accounted for the majority of infections (Goris et al. 2013a, b; Jansen et al. 2005). In France, approximately 600 cases per year are diagnosed, however, half of them are from French overseas principalities. The incidence of 0.5 per 100,000 in mainland France is similar to that seen in Germany and the Netherlands. However, in the overseas territories, an average incidence of 1060 per 100,000 was reported between 2007 and 2009. In French Polynesia, the average incidence is 39 per 100,000 and in New Caledonia, the average incidence is 45 per 100,000 (Picardeau 2013).

The Asia Pacific region has some of the highest incidence rates for leptospirosis since high population densities are potentially a risk factor for leptospirosis (Victoriano et al. 2009). This is not surprising given the frequent climatic calamities, overcrowding, poor sanitation, proximity of domestic and wild animals and occupational risks. In China over the past two decades, the average annual incidence was 0.7 per 100,000 inhabitants. Major outbreaks have occurred following flooding and heavy rainfall. Interestingly, 60 % of cases in China are due to infection with *L. interrogans* serovar Lai. The principle vector for transmission are rats such as *Apodemus agrarius* (Zhang et al. 2012). In South Korea, the predominate infecting

serovar and vector are similar to that seen in China however, the incidence is lower. Between 1998 and 2011 in South Korea there were 1528 reported cases of leptospirosis giving rise to an incidence rate of 0.22 per 100,000 inhabitants (Kim 2013).

In Australia, the annual incidence is 8.9 cases per million (Pappas et al. 2008). In North Queensland, leptospirosis is endemic as agriculture such as banana and sugar cane farming, which are common to these areas, are high-risk industries. Seasonal changes have a direct impact on the incidence of the disease as the frequency of infection is highest during the wet season (January–April) whilst being relatively low during the dry season (June–December). Although the organism was only identified in the late 1990s, *L. borgpetersenii* serovar Arborea has emerged as the most predominate infecting serovar in Queensland (Tulsiani et al. 2011; Wynwood et al. 2014).

Leptospirosis is endemic in the Caribbean Islands and in many parts of Central and Southern America. Pappas et al. (2008) reported that the incidence in Trinidad and Tobago is 120.4 per million, Barbados 100.3 per million and Jamaica 78 per million. In El Salvador, Brazil and Argentina the incidence is 358, 12.8 and 9.5 per million respectively.

34.4 Evidence of Human-to-Human Transmission

Currently, reported evidence of human-to-human transmission is scarce. However, diagnosis of such transmission has been confirmed by serological testing. Bolin and Koellner (1988) reported the case of a 29 year old breast feeding mother who worked as a veterinarian and had a confirmed *L. interrogans* serovar Hardjo infection. The mother continued to breast feed during her illness and 21 days post-onset of symptoms, the infant displayed clinical signs consistent with leptospirosis. A positive result was confirmed by the microscopic agglutination test (MAT). In another report detailing possible human-to-human transmission, Harrison and Fitzgerald (1988) discussed the advent of possible sexual transmission of *L. interrogans* serovar Icterohaemorrhagie. The diagnosis of this condition was also confirmed serologically by MAT.

34.5 Evidence of Animal-to-Human Transmission

Reports of human-to-human transmission are rare and as such Adler and de la Pena Moctezuma (2010, p. 289) submit that ‘human to human transmission for practical purposes is non-existent and that leptospirosis is recognised globally as a zoonosis.’ Since cases and outbreaks of leptospirosis are either unreported or misdiagnosed, it is not surprising that reports, which attempt to identify and track the course of leptospirosis outbreaks, are rare. Recently, Li et al. (2013a, b) used molecular methods such as multi locus sequence typing and multiple locus variable-number tandem

repeat analysis to type isolates recovered from rodents in the Guizhou province in China. The authors found that the newly sequenced strains were consistent with serological investigations undertaken in leptospirosis patients from Guizhou province. Desvars et al. (2013) reported that 16S RNA gene sequencing identified four pathogenic genomospecies, which are responsible for human leptospirosis, have also been isolated in Mayotte rats.

34.6 Disease Symptoms in Humans

At present, the minimum infecting dose leading to leptospirosis is unknown, however, the incubation period is assumed to be inversely correlated with the size of the inoculum. For example, a high infecting dose may engender a short incubation period when compared to a low infecting dose. Conversely, small doses may result in prolonged incubation times which may extend into the immune phase. It is anticipated that these small infecting doses might be responsible for mild or even sub clinical infection (Faine et al. 1999). Once in the blood, leptospires are capable of circulating to all tissues. Leptospires that evade phagocytic cells of the recticuloendothelial system grow in an exponential manner doubling every eight hours (Faine et al. 1999). There is evidence to suggest that phagocytosed leptospires do not survive long within the interior of the phagocyte (Vinh et al. 1982; Wang et al. 1984). Virulent strains have the ability to attenuate phagocytic responses by activating apoptosis in the macrophage (Merien et al. 1998). Moreover, Adler and de la Pena Moctezuma (2010) write that the ability to resist complement and death by neutrophilic destruction may be a feature of virulent leptospires in non-immune hosts. Central to the pathology observed in leptospirosis is the damage caused to the endothelium of small blood vessels. This engenders ischaemia in target organs thus resulting in renal, hepatic and pulmonary damage and thrombocytopenia. A number of leptospiral virulence factors such as haemolysins, fibronectin binding proteins and numerous surface proteins such as LipL32, Lig A, Lig B, lipoprotein Loa22 and the 6 Len proteins (LenABCDEF) are postulated to play a role in pathogenesis (Adler and de la Pena Moctezuma 2010; Bulach et al. 2006; Hoke et al. 2008; Matsunaga et al. 2003; Merien et al. 2000; Picardeau et al. 2008; Ristow et al. 2007; Stevenson et al. 2007). Recently, *L. interrogans* catalase KatE and HtpG (high-temperature protein G is the bacterial homolog to the highly conserved molecular chaperone Hsp90) have also been shown to be virulence factors in leptospirosis (Eshghi et al. 2012; King et al. 2014).

Following the initial incubation period, the infection enters the acute phase of the disease which can last up to ten days (Tulsiani et al. 2011). Clinically, during the acute phase, patients typically present with headache, fever, excruciating myalgia and arthralgia and sometimes rigiours, vomiting, photophobia and a mucosal rash (Faine et al. 1999). Haemoptysis, hypotension and bradycardia are also common presentations. These symptoms are considered non-specific thereby making the diagnosis of leptospirosis difficult. Hepatosplenomegaly, jaundice (produced as a

result of hepatocellular damage, increased erythrocyte destruction and the resulting increase in circulating haemoglobin and bilirubin), renal failure, liver failure and acute respiratory distress are common features of the more acute form of the disease (Sutliff et al. 1953; Solbrig et al. 1987; Faine et al. 1999, Levett 2001). Host factors, or more specifically, the activation of the innate immune system in which a myriad of cytokines are released (cytokine storm) in response to the invading pathogen also play a central role in the clinical outcome (Reis et al. 2013). Following the acute phase, patients enter the immune phase where immunoglobulins, specific for the destruction of leptospires, are produced to resolve the infection (Levett 2001).

34.7 Disease Symptoms in Animals

34.7.1 Canines

The severity of leptospirosis in canines may be dependent on the size of the infecting dose, infecting serovar, age and health of the dog as well as vaccination status. Clinical signs may vary from the sub-clinical or asymptomatic infection with infections due to *L. interrogans* sv Canicola to chronic infection characterised by chronic hepatitis and uveitis, sub-acute disease accompanied by pyrexia, anorexia, vomiting, renal failure and petechiae (Prescott 2008; Sykes et al. 2011). The acute and peracute disease while uncommon, may result in coagulopathy, vascular injury and death (Prescott 2008). Other clinical signs include arched back, swollen tender kidneys, depression, melena and blood stained urine. Death can occur 36 hours to four days after the onset of symptoms. Serovars causing the more sinister clinical picture include serovars Australis, Grippotyphosa, Icterohaemorrhagiae, Autumnalis and Pomona. Regular vaccinations may assist to prevent severe disease however, the vaccines are serovar specific and do not engender protection against all possible infecting serovars (Faine et al. 1999).

34.7.2 Felines

Given the predatory activities of cats towards rodents, it is reasonable to conclude that cats are at high risk for contracting leptospirosis. Surprisingly, the clinical presentation of diseased cats is rare even though there is greater seroprevalence of leptospiral antibodies in cats than in dogs (Roqueplo et al. 2013). In addition, renal insufficiency and hepatic inflammation may be evident in those animals that present with leptospirosis (Arbour et al. 2012; Bryson and Ellis 1976; Lapointe et al. 2013).

34.7.3 Equines

Clinical features of leptospirosis disease in horses include fever, anorexia, jaundice, mucosal petechiae and depression. Severe forms of the disease (respiratory failure) are more likely to occur in foals than adult horses. Reproductive catastrophies are common in infected mares. Antibodies directed towards leptospiral LruA and LruB proteins have been shown to cross-react with structures in the eye, thus resulting in an auto-immune basis for equine recurrent uveitis or moon blindness (Verma and Stevenson 2012; Verma et al. 2013).

34.7.4 Bovines

Cattle infected with serovars for which they are not maintenance hosts are more likely to display clinical disease, especially if the host is young, i.e. a calf. Clinical signs in cattle with acute disease include fever, pulmonary congestion, jaundice, haemoglobinuria and anaemia. Renal lesions may be observed at slaughter. In cows, the milk drop syndrome has also been observed. Chronic infections may also engender reproductive catastrophies (Faine et al. 1999; Pearson et al. 1980).

34.7.5 Swine

Younger pigs are more likely to display acute leptospirosis compared with more mature ovines. Clinical features are similar to those observed in other animals and include jaundice, weakness, haematuria, anorexia, renal failure and convulsions. Adult pigs are usually asymptomatic, however, may have renal lesions. Again reproductive catastrophies (abortions and stillbirths) occur as a result of maternal infection (Baker et al. 1989; Faine et al. 1999).

34.7.6 Unresolved Issues

There are numerous unresolved issues which the Leptospirosis community needs to address. Firstly, for nearly a century, culture and serology have underpinned the diagnostic practices of laboratories with an interest in this field (Martin and Pettit 1918). With the dawn and rapid advances in molecular diagnostics, there is now a plethora of molecular techniques available for laboratories to undertake primary diagnostic and reference services. However, as Goarant (2014) eloquently argued, reconciling historical serological knowledge with modern molecular epidemiological practices remains a challenge as does identifying the most appropriate DNA targets and techniques for *Leptospira* spp. typing. As whole genome sequencing becomes less costly, less time consuming and less technically demanding, we are hopeful of gaining consensus and resolving these issues.

Secondly, the issue of chronic illness and the occurrence of post-leptospirosis symptoms in patients need a more comprehensive investigation. While neuropathies and mental illness may be considered persistent sequelae, 10 % of patients may complain of uveitis and headaches for years (Faine et al. 1999; Shpilberg et al. 1990). In an interesting and much needed attempt to add framework to understand the burden of human leptospirosis, Goris et al. (2013b) reported that 21.1 % of their patient cohort frequently reported complaints such as myalgia and headache beyond 24 months post infection.

Finally, governments around the world are encouraged to invest more in public health initiatives centring on surveillance and reporting structures for Leptospirosis and educating medical officers and the public of the disease. Until such initiatives are universal, leptospires will remain silent assassins and serious attempts to understand and prevent leptospirosis will be futile.

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Chapter 35
Glanders & Melioidosis: A Zoonosis and a Sapronosis—"Same Same, but Different"

Caoimhe Nic Fhogartaigh and David A. B. Dance

Abstract Glanders, caused by infection with *Burkholderia mallei*, primarily causes infection in equines, but may be transmitted to humans, and thus qualifies as a true zoonosis. Melioidosis is caused by *B. pseudomallei*, genetically very similar to *B. mallei*, but which is an environmental saprophyte capable of infecting humans and a wide range of other animals. Good evidence of animal-to-human, or even human-to-human, transmission of melioidosis is lacking, and so it most appropriately referred to as a sapronosis, or at most a sapro-zoonosis. Although rare in Western countries, both micro-organisms have recently gained much interest because of their potential use as bioterrorism agents. The increasing recognition of melioidosis in humans and recent outbreaks of glanders in animals have led to their description as emerging or re-emerging diseases. Laboratory-associated infections with both organisms have also occurred, resulting in their categorisation as Hazard Group 3 pathogens. In this chapter we review the epidemiology of animal and human cases of glanders and melioidosis, the evidence for different modes of transmission, pathogenesis and clinical features, diagnosis and treatment, as well as public health and disease control issues.

35.1 Introduction

Glanders, caused by infection with *Burkholderia mallei*, primarily causes infection in equines, but may be transmitted to humans, and thus qualifies as a true zoonosis. Melioidosis is caused by *B. pseudomallei*, genetically very similar to *B. mallei*, but which is an environmental saprophyte capable of infecting humans and a wide

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35.2 Glanders

35.2.1 History and Epidemiology

Symptoms of glanders in equines were reported as early as 425 BC by Hippocrates, however it was Aristotle who first described it as "μηλις" (malis in Latin, from which *B. mallei* takes its name) in approximately 350 BC (Sharrer 1995). *B. mallei* was first isolated from the liver of an infected horse in 1882 (Boerner 1882). Infection resulted in significant morbidity and mortality in equines worldwide, and was occasionally transmitted to humans in prolonged close contact with horses, such as grooms, coachmen, veterinarians and butchers; or to other animals through direct or indirect contact.

Glanders has since been eradicated from Western Europe, USA and Canada due mainly to the reduction in the use of horses in everyday life, but also to improved animal husbandry and hygiene and strict programmes enforcing statutory testing and slaughter of infected animals (Blancou 1994; Derbyshire 2002), however the disease persists in the Middle-East, parts of Asia, and South America. Within the last 20 years, increasing numbers of equine cases have been reported from countries including Pakistan, India, Brazil, Turkey, Iran, Iraq, Afghanistan, Kuwait, Bahrain, UAE, Lebanon, Latvia, Belarus, Mongolia and China (Office International des Épizooties -World Organisation for Animal Health 2013; Khan et al. 2012). These are usually sporadic involving single or small numbers of animals, although occasionally larger outbreaks have occurred, such as that in India between 2006 and 2010 involving eight states and 164 equines (Khurana et al. 2012). Sporadic human cases have also been reported from Cameroon, Curacao, Sri Lanka, and Turkey (Office International des Épizooties - World Organisation for Animal Health 2011). Laboratory associated human cases, such as that which occurred in a military research microbiologist in USA in 2000 (Srinivasan et al. 2001), the first case in the USA for over 50 years, have also been reported occasionally.

Due to its high fatality rates and transmissibility of the disease in animals and humans, glanders has long been considered as a potential biological weapon. When horses were widely used for military purposes, devastating natural outbreaks occurred, for example during the American Civil War (Sharrer 1995). *B. mallei* was actually used by the Germans in sabotage attempts during World War I (Christopher et al. 1997), and in human experiments by the notorious Japanese Unit 731 in Manchuria in the period leading up to World War II (Darling and Woods 2004). It is reported to have been weaponized by the Soviet Union, and used against the Mujaheddin in Afghanistan in the 1980s (Alibek and Handelman 1999). With resurgent bioterrorism concerns, *B. mallei*, listed as a category B bioterrorism agent by the Centers for Disease Control and Prevention and a Tier 1 agent by the US Department of Agriculture, is now being studied in many laboratories throughout the world.

35.2.1.1 Modes of Transmission

It was initially thought that glanders was transmitted through the air, however in the early eighteenth century it was proposed that transmission took place through direct contact with infected horses, or indirectly through contaminated harnesses and water troughs (Khan et al. 2012; Kinsley 1911). Inoculation or ingestion of infected clinical material was demonstrated to cause infection in horses and other animals in experiments conducted in the late nineteenth and early twentieth centuries, which also confirmed that 'glanders' and 'farcy' were different manifestations of the same disease (Schutz 1898; O'Leary 1908). Later, nasal discharge and skin exudate from infected animals was shown to contain large numbers of bacteria that could be readily cultured, and it was shown that viable bacteria could survive for at least four weeks suspended in water (Miller et al. 1947).

Once it has contaminated harnesses, grooming tools, hoof trimming equipment, water troughs or hands, *B. mallei* may transmit to new hosts through skin abrasions, mucous membranes, ingestion of water containing infective material, or inhalation of dried infected particles (Carr Gregory et al. 2007). The disease spreads quickly in overcrowded, poorly hygienic, and humid environments (Khan et al. 2012). Occasional cases have been reported in carnivores fed on infected meat (Alibasoglu et al. 1986; Khaki et al. 2012). Vertical transmission has occurred naturally from mare to foal, and from laboratory infected guinea pig sows to their offspring (Loeffler 1886; Rutherford 1906).

Zoonotic transmission to humans appears to be relatively uncommon. During World War II, human infections were rare despite a 30% prevalence in horses in China (Darling and Woods 2004). Disease has occurred almost exclusively in individuals whose occupation involves close and prolonged contact with horses, but there is often no history or clinical evidence of inoculation or path of infection (Bernstein and Carling 1909). As is the case for melioidosis, diabetes may place humans at greater risk of infection after exposure, although reports of this are remarkably rare, perhaps because of the rarity of human glanders since diabetes became readily

treatable (Srinivasan et al. 2001). Human infection by ingestion has not been definitively reported, in fact even where there is known to have been consumption of glanderous meat, human infection has not occurred (Loeffler 1886). Human-to-human transmission is also rare, but has been reported, and has usually involved close contact with the infected individual either as a family member, a carer, during medical procedures or post-mortem examination (Loeffler 1886; Robins 1906). In a review of 156 reported cases at the turn of the last century, around 10% were believed to have been acquired from human contact (Robins 1906). In the present day, improved living conditions, universal precautions, disinfection and available treatment make human-to-human transmission much less likely to occur.

In developed countries, laboratory exposure seems to be a greater threat than animal contact, and anecdotal observations suggest that *B. mallei* may be more infectious in this setting than *B. pseudomallei* (Howe and Miller 1947). Some cases have occurred following obvious aerosol exposures during spillage of culture material, or direct inoculation injuries (Howe and Miller 1947), but many did not recall a particular exposure (Srinivasan et al. 2001). It is suspected that most laboratory-acquired cases are a result of inhalation (Carr Gregory et al. 2007).

Although outbreaks continue to occur in the endemic regions listed above, little is known about the ecology and population dynamics of *B. mallei*. A recent study investigated the molecular epidemiology of glanders in Pakistan. Isolates from 15 glanderous horses in the Punjab between 1999 and 2007 underwent variable number of tandem repeat (VNTR) analysis, phylogenetic analysis and comparison to 10 whole-genome-sequenced strains of *B. mallei*. The results confirmed the Punjab strains to be genetically distinct from the sequenced strains, and to form three distinct clades, with the majority belonging to a single clade temporally and geographically spread, suggesting that this is ecologically established in the Punjab region (Hornstra et al. 2009). Together with additional epidemiological data, the authors concluded that human movement of equines contributed to the dispersal of *B. mallei* genotypes and that strains could persist for at least 1.5 years. Similarly, glanders infection in a dromedary in Bahrain was shown to be genetically similar to the Dubai 7 strain which caused an outbreak in horses in United Arab Emirates in 2004 (Wernery et al. 2011), and it was suggested that the strain was introduced from that region through international horse trade.

35.2.2 Microbiology

B. mallei is a facultative intracellular, aerobic, non-motile Gram-negative bacillus. The results of DNA-DNA hybridisation (Rogul et al. 1970), multilocus sequence typing (MLST) (Godoy et al. 2003), and whole genome sequencing (Nierman et al. 2004) have demonstrated unequivocally that *B. mallei* is actually a clone of *B. pseudomallei* which has undergone a substantial reduction in the size of its genome during the process of adaptation as an equine pathogen (Nierman et al. 2004), differing at only a single nucleotide site in one of seven housekeeping genes studied. Based

on these data it should not taxonomically be considered a separate species, however it retains species status due to its specific clinical and epidemiological behaviour.

Very few recent clinical isolates are available for study, so knowledge of the characteristics of *B. mallei* is based on historical descriptions and archived strains, which may be laboratory-adapted to varying degrees. The organism often has an irregularly stained appearance on Gram's stain due to the presence of 'lipoid granules' (Worley and Young 1945). Miller noted that when the organism was stained in clinical specimens, there was an impression of a capsule, however this is not apparent using common capsule stains (Miller et al. 1947). It is nutritionally versatile, being able to use a wide range of organic compounds as a carbon source, and can oxidise glucose and usually mannitol. It is able to grow on most laboratory media, but requires glycerol for optimum growth (Miller et al. 1947), initially forming shiny and translucent colonies which tend to become mucoid with age. Most strains are oxidase positive. The optimal temperature for growth is 37 °C; many strains grow poorly below 25 °C but all will grow at 41 °C. *B. mallei* is unable to survive in dried pus for longer than a few days, or for 24 h when exposed to sunlight, although it can survive in tap water for at least 4 weeks (Miller et al. 1947; Howe and Miller 1947). It is resistant to colistin and polymyxin B.

It grows less luxuriantly on laboratory media than *B. pseudomallei*, from which it may be distinguished by its susceptibility to aminoglycosides and lack of motility. *B. mallei* is non-flagellated, despite retaining flagellar genes that are not expressed (Song et al. 2010), whereas *B. pseudomallei* has 2–4 polar flagella per cell.

35.2.3 Pathogenesis

B. mallei has the ability to invade and replicate inside epithelial and phagocytic cells and evade host immune mechanisms, resulting in an acute and fatal course, or a more chronic persistent infection state. In vivo animal models of glanders, in particular hamster and mice models, have provided important data on various pathogenic mechanisms. The *B. mallei* ATCC 23344 genome contains at least two *luxI* and four *luxR* homologues, which are quorum sensing (cell-density) based regulators of virulence factor expression. When inactivated, reduced bacterial virulence was observed in mice (Ulrich et al. 2004). The genome also encodes a *virAG* two-component regulatory system that is required for virulence in hamsters (Nierman et al. 2004), and overexpression results in transcription of approximately 60 genes (Schell et al. 2007).

Like many pathogenic Gram-negative bacteria, *Burkholderia* spp. use a Type III secretion system (T3SS) to interact with and invade host cells. This system involves secretion of a protein onto the membrane of a host cell, to which the bacteria can subsequently bind, form a pore, and insert effector proteins directly into the host cell cytosol. *B. mallei* contains two T3SS, one of which is the animal pathogen-like Bsa T3SS (T3SSAP) which is required for rupture of endocytic vacuoles, escape into the host cell cytoplasm (Ribot and Ulrich 2006) and actin-based motility to

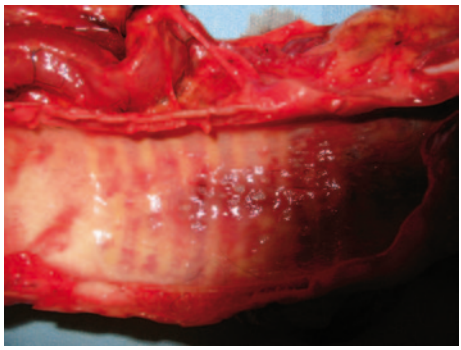

Fig. 35.1 Tracheal ulcers noted on post-mortem of a glanderous horse. Copyright Prof. D.E. Woods

promote spread within and between cells (Ulrich and DeShazer 2004). A type VI secretion system, T6SS-1, part of the *VirAG* regulon, is essential for *B. mallei* virulence in the hamster model of glanders (Schell et al. 2007), and has an important role in growth and actin-based motility following uptake of *B. mallei* by murine macrophages (Burtnick et al. 2010). *B. mallei* also exhibits Bim-A dependent intracellular actin-based motility, similar to that discussed later for *B. pseudomallei*.

In laboratory infected guinea pigs, *B. mallei* has been shown to produce a thick carbohydrate capsule (Popov et al. 1991), the coding sequence of which is 99% identical to that of the *B. pseudomallei* capsule (DeShazer et al. 2001). This enables *B. mallei* to resist macrophage and complement-mediated killing, promoting survival in serum (Burtnick et al. 2002). Furthermore, mutated strains lacking a capsule appear non-pathogenic in mice and hamsters (DeShazer et al. 2001). Lipopolysaccharide has also been shown to be a potent activator of Toll-like receptor-4 in vitro (Brett et al. 2007).

Through the various modes of transmission outlined previously, using these pathogenic mechanisms, *B. mallei* is able to penetrate mucosae, invade lymphatics, and spread haematogenously. Post-mortem examinations of glanderous animals have revealed nodules and ulcers of the nasal passages, larynx, lip and other tissues (Fig. 35.1); sero-sanguinous fluid in the nasal cavity, paranasal sinuses and trachea; sub-pleural lung nodules; diffuse, miliary granulomatous nodules with caseo-necrotic centres; pulmonary oedema or severe bronchopneumonia; and less frequently, muscle abscesses (Khan et al. 2012). Some ulcerating lesions are believed to be endotoxin mediated (Carr Gregory et al. 2007).

35.2.4 Clinical Presentation in Animals

In addition to the normal equine hosts, glanders has been confirmed in camels, bears, wolves, dogs and felines (Office International des Épizooties -World Organisation for Animal Health 2013; Wernery et al. 2011) and in laboratory experiments guinea-pigs and hamsters appear to be susceptible, whereas cattle, fowl, rats

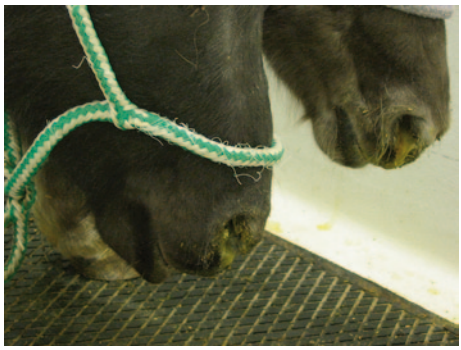

Fig. 35.2 Typical purulent nasal exudate due to glanders infection in horses. Copyright Prof. D.E. Woods

and pigs appear to be more resistant (Hu et al. 1958; Minnet 1959). Donkeys are particularly susceptible and develop an acute fatal form of infection, whereas horses tend to develop a chronic, more insidious, yet eventually fatal illness. The clinical presentation of equine glanders may be acute or chronic, and it typically manifests as a respiratory illness with pulmonary and nasal involvement ("glanders"; Fig. 35.2) or with cutaneous nodules and lymphangitis ("farcy", Fig. 35.3), although these forms often coexist, and pulmonary involvement is almost invariably found at post-mortem. The incubation period varies from a few days to several months (Office International des Épizooties -World Organisation for Animal Health 2013). The clinical presentation in other susceptible animals appears similar to that in equines.

Acute infection in donkeys begins with fever, anorexia, loss of stamina, and respiratory symptoms such as nasal discharge and cough. This is shortly followed by swelling of the nostrils, nodules and ulceration of the nasal septum, mucopurulent nasal discharge, submaxillary lymphadenopathy (often with suppuration), and increasing shortness of breath (Office International des Épizooties -World Organisation for Animal Health 2013; Wernery et al. 2011; Minnet 1959). Death occurs within a few days to weeks as a result of respiratory failure and sepsis.

In horses, glanders generally follows a more chronic course with episodic exacerbations followed by improvement in symptoms. The animal may have intermittent, low grade fever, and mild respiratory symptoms, however the disease may remain latent for months to years without significant symptoms or signs. As disease progresses, cough, weakness and signs of wasting develop, and nasal and cutaneous forms ensue with inflammatory nodules and ulceration of the nasal cavity and upper respiratory tract (see Fig. 35.1), purulent nasal discharge (see Fig. 35.2), and lymphangitis or nodular lymphadenitis particularly affecting the limbs (Fig. 3.2). The skin nodules may also ulcerate, and deep lesions are often associated with joint swelling and oedema of the hind quarters resulting in lameness. Shortness of breath progresses as lung nodules and abscesses develop, and nodules are often found in the liver and spleen. Neurological involvement has been noted but is rare (Dobberstein 1935). Although chronic cases may survive for many years, the animal will usually become increasingly debilitated and eventually die (Office International des

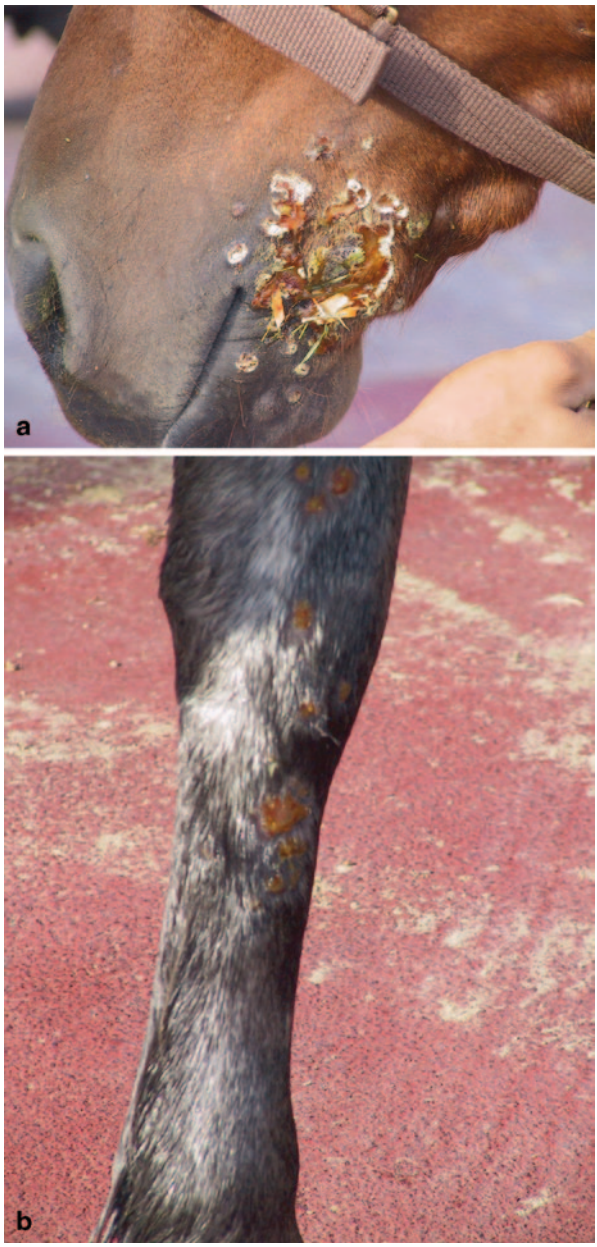

Fig. 35.3 **a** Cutaneous nodules around the jaw and **b** lymphangitis of the lower limb representing "farcy" due to glanders in a horse. Copyright of the Central Veterinary Research Laboratory, Dubai, United Arab Emirates

Épizooties -World Organisation for Animal Health 2013; Khan et al. 2012; Minnet 1959; Saquib et al. 2012). Chronic and subclinical cases are potential sources of transmission to other animals or humans through shedding of bacteria in respiratory secretions and skin exudate (Wittig et al. 2006).

35.2.5 Clinical Presentation in Humans

Knowledge of the clinical features of glanders in humans is based on a relatively narrow window in the literature of just over 100 years between the early nineteenth and the early twentieth centuries, and a few more recently published laboratory-acquired cases (Srinivasan et al. 2001; Robins 1906; Howe and Miller 1947). The clinical manifestations appear to relate to the route of infection, and whether the disease remains localised or disseminates. Localised infection typically produces pus-forming, ulcerating nodules and abscesses of the skin, subcutaneous tissues or mucous membranes, with associated lymphangitis or regional lymphadenopathy. Depending on the site affected, there may be swelling and increased discharge from nasal, ocular, or respiratory mucous membranes. Fever, malaise, headache, myalgia and gastrointestinal upset are common accompanying features.

Cutaneous inoculation or entry of *B. mallei* via mucous membranes typically results in a localised infection at the site of entry within 1–5 days. Although involvement of the nasal or oral mucosa has been well described, this is by no means invariable and certainly not as prominent as it is in horses, but pustular lesions around the face appear to be common. If untreated, lymphatic or haematogenous spread takes place after one to four weeks, resulting in pulmonary, septicaemic or disseminated infection with abscesses in many organs, but particularly the spleen, liver and lungs (Carr Gregory et al. 2007). Multiple, painful skin and soft tissue nodules and abscesses may be a particularly prominent feature, and these often contain a characteristic oily pus ("farcy oil"). Pulmonary involvement, secondary to aerosol inhalation or as part of disseminated infection, may present with cough, purulent sputum, shortness of breath and chest pain as a result of pneumonia, lung abscess, or pleural effusion. Pneumonia, abscesses with cavitation and miliary nodules have been seen on chest radiographs (Carr Gregory et al. 2007). Septicaemia may develop immediately or up to 2 weeks after initial exposure or recurrence, and has a poor prognosis.

Although the infection was generally fatal over days to weeks before antibiotics were available, a more protracted course of disseminated infection interrupted by latent periods has also been described (Bartlett 1988), as well as cases of localised abscesses which responded to incision and drainage only (Bernstein and Carling 1909). The laboratory-acquired cases all survived with antibiotic treatment despite delays in making the diagnosis.

35.2.6 Diagnosis

A definitive diagnosis of glanders, in animals or humans, generally requires isolation and identification of *B. mallei* from clinical samples, although seroconversion following known exposure would also be highly suggestive of infection. Specimens from suspected or confirmed cases should be handled in a Containment Level 3 facility. All suspected cases should have blood and urine culture, together with

sputum, pus, exudate from superficial lesions and other samples as available or appropriate. Guidelines for culture and identification of *B. mallei* have been developed (American Society for Microbiology 2008). Gram's stain of clinical samples may demonstrate the irregularly stained Gram-negative bacilli. The organisms are difficult to demonstrate in tissue sections where they may have a beaded or encapsulated appearance (Miller et al. 1947). Isolation from non-sterile sites may be optimised by using a selective medium such as *Burkholderia cepacia* agar, although selective media containing aminoglycosides designed for *B. pseudomallei*, such as Ashdown's agar, are inhibitory to the aminoglycoside-susceptible *B. mallei* (Glass 2009). *B. mallei* is often not correctly identified by API 20NE (Amornchai et al. 2007) and other commercial identification systems (Glass 2005). All suspected *B. mallei* isolates should be referred to the relevant national reference laboratory for molecular confirmation.

Molecular techniques have been developed to identify *B. mallei* in laboratory culture (Lee et al. 2005; Thibault et al. 2004; U'Ren et al. 2005) and although their use is currently restricted to research and reference laboratories, they could potentially be used to detect the organism in clinical specimens. PCR targeting the flagellin gene of *B. mallei* (fli-P) was used successfully to detect the organism in clinical samples taken during a glanders outbreak in horses (Scholz et al. 2006).

In suspected cases of glanders in animals, the mallein skin test was historically used for diagnostic purposes. The test is based on a hypersensitivity reaction to a protein fraction (mallein) of *B. mallei* following intrapalpebral or subcutaneous injection or administration in eyedrops, leading to marked eyelid swelling, a painful raised lesion, or conjunctivitis respectively after one to two days, often accompanied by fever (Office International des Épizooties -World Organisation for Animal Health 2013). Mallein testing can, however, lead to subsequent false positive results in other serological tests (Hagebock et al. 1993), and may be falsely negative in animals with acute glanders or in the late stages of chronic disease (Neubauer et al. 2005). It is no longer recommended due to animal welfare concerns, but may be used in remote endemic regions where storage and transport of samples for serological testing is problematic (Office International des Épizooties -World Organisation for Animal Health 2013).

Numerous serological tests for the diagnosis of glanders in horses exist including complement fixation test (CFT), competitive enzyme-linked immunosorbent assay (cELISA), immunoblot (IB), Rose Bengal Test (RBT), indirect haemagglutination assay (IHA), agar-gel immunodiffusion (AGID), indirect fluorescent assay test (IFAT), counter immunoelectrophoresis (CIE) and dot ELISA. Many of these methods are not widely validated, cross react with other *Burkholderia* species, and like the mallein test may give false negative results in acute cases or very debilitated animals. CFT represents the current method of choice for the diagnosis of glanders and is required before international horse trade (Office International des Épizooties -World Organisation for Animal Health 2013). It is 90–95% sensitive, becoming positive within one week of infection, and remaining positive in chronic cases and exacerbations of latent cases (Office International des Épizooties - World Organisation for Animal Health 2013). A cELISA has been developed

using an uncharacterised anti-lipopolysaccharide monoclonal antibody, and shown to have similar performance characteristics to the CFT (Katz et al. 2000). However, both methods have suboptimal specificity (Neubauer et al. 2005), particularly when testing serum samples from animals in glanders-endemic areas (Khan et al. 2011). False positive tests can have serious consequences in terms of animal slaughter and financial losses. Performing an immunoblot as a confirmatory test for all positive CFT results has been suggested as a means of overcoming the sensitivity issues (Khan et al. 2011). The serodiagnosis of glanders in animals should only be undertaken and interpreted by specialists with relevant expertise. Ideally, the diagnosis should be confirmed by culture if possible.

No validated serological test is currently available for the diagnosis of human glanders, although numerous melioidosis serodiagnostic tests are in use around the world and, given the serological cross-reactivity between *B. mallei* and *B. pseudomallei*, it is likely that these would become positive in many cases of human glanders. Recent molecular and immunological research may lead to the identification of more specific and immunogenic *B. mallei* antigens which could be purified to optimise serological diagnosis.

35.2.7 Treatment

B. mallei is intrinsically resistant to a range of antimicrobial agents, including early beta-lactams, but unlike *B. pseudomallei*, *B. mallei* remains susceptible to aminoglycosides and macrolides (Kenny et al. 1999; Thibault et al. 2004). Most strains are susceptible to carbapenems, ceftazidime, amoxicillin–clavulanic acid, piperacillin, doxycycline and trimethoprim-sulfamethoxazole (Kenny et al. 1999; Thibault et al. 2004; Al Izzi and Al Bassam 1989; Heine et al. 2001). Despite low mean inhibitory concentrations in vitro, certain antimicrobials, including aminoglycosides, may not be effective due to the intracellular nature of the infection. Doxycycline and fluoroquinolones, such as ciprofloxacin and ofloxacin, with good intracellular and tissue penetration, have demonstrated efficacy when used to treat experimental infection in animals (Batmanov 1991; Iliukhin et al. 1994; Russell et al. 2000), however in one study relapse occurred in some doxycycline treated animals (Russell et al. 2000). Doxycycline has also been shown to have some efficacy as post-exposure prophylaxis following aerosol and intraperitoneal challenge in animals (Iliukhin et al. 1994; Russell et al. 2000).

Trials to determine optimal treatment for animal and human glanders are lacking. Until recently, glanderous animals, including those that are asymptomatic with positive serological tests, have been euthanized according to strict veterinary public health policies in order to prevent spread to other domestic animals or humans. In the case of high value animals, such as those in equestrian sport, an expensive treatment regimen may be justified. During an outbreak of culture confirmed glanders in 23 horses at the Lahore Polo Club in Pakistan, a combination of intravenous enrofloxacin and trimethoprim-sulphadiazine for three weeks, followed by oral doxycycline for a total of 12 weeks, successfully treated all infections (Saquib et al. 2012).

Recommendations for treatment of human glanders adopt the same antimicrobial regimens as those validated for melioidosis, which are based on clinical trial evidence. This consists of an intensive phase of intravenous antimicrobial therapy (ceftazidime or a carbapenem) for a minimum of 10–14 days, followed by an eradication phase of oral antimicrobial (trimethoprim-sulfamethoxazole with or without doxycycline) for 12–20 weeks, or longer if there is widespread visceral disease (Lipsitz et al. 2010). Without the latter phase, there is likely to be a high risk of relapse, particularly in those with disseminated infection. Drainage of abscesses where possible is an important adjunct to antimicrobial therapy.

35.2.8 Prevention and Control

Attempts to develop vaccines against *B. mallei* have so far been unsuccessful, and so control and eradication of glanders has depended on the detection and elimination of infected animals to prevent onward transmission. A requirement for serological testing of animals prior to international transport in order to prevent the introduction of glanders into glanders-free regions has been recommended by the World Organisation for Animal Health (Office International des Épizooties - World Organisation for Animal Health 2003).

If animal or human glanders is suspected, the case should be isolated and personal protective clothing worn by any person who must come into contact. Local and national public health and veterinary authorities must be notified immediately, and confirmed cases in animals reported to the World Organization for Animal Health. Any confirmed human glanders case occurring without equine exposure should prompt investigation into a deliberate release of the organism. In human cases, isolation and appropriate infection control precautions (according to the site of infection) should be taken until the patient is culture negative.

Confirmed animal cases and serologically positive animal contacts should be destroyed humanely, with the provision of adequate compensation to owners. Reasonable compensation schemes helped to eradicate glanders in Canada (Derbyshire 2002). In contrast, in some developing countries as little as $1.1 US dollars is paid in compensation for slaughter of a glanderous animal which may be the basis of the owner's livelihood, thus forcing them to sell the animal and risk onward transmission to other animals and regions (Khan et al. 2012; Saquib et al. 2012). Premises and facilities of infected animals should be quarantined, cleaned and disinfected. Carcases as well as contaminated bedding, feed, manure and equipment in the vicinity should be buried or incinerated.

Prevention of laboratory-acquired human infection depends on appropriate handling using biosafety level 3 practices, and the institution of appropriate guidelines in the event of accidental laboratory exposure (Lipsitz et al. 2010).

35.3 Melioidosis

35.3.1 History and Epidemiology

Melioidosis was first described by Whitmore and Krishnaswami as a "glanders-like…pyaemic or septicaemic" illness occurring in morphia addicts in Rangoon in 1911 (Whitmore and Krishnaswami 1912) and was documented in 5% of post-mortem examinations in Myanmar around this time (Cheng and Currie 2005). The name originates from the Greek "μηλις" (distemper of asses) and "ειδος" (resemblance), and the name was suggested by Stanton and Fletcher in 1921 (Stanton and Fletcher 1921), who went on to report a number of human and animal cases around Kuala Lumpur (Stanton and Fletcher 1932). It was later demonstrated that the causative bacterium, now known as *B. pseudomallei*, was saprophytic and could be cultured from soil and surface water in Vietnam (Chambon 1955), and subsequently from many other parts of southeast Asia and northern Australia. In Australia, *B. pseudomallei* was first identified in sheep in 1949 (Cottew et al. 1952), and the first human case occurred in a diabetic patient who died of septicaemic melioidosis in north Queensland in 1950 (Rimington 1962). It was suggested that the emergence of melioidosis in Australia might be due to spread of isolates from southeast Asia, but historical isolates from Thailand and Australia have recently been shown to be distinct by MLST, with Australian isolates appearing ancestral to those found in southeast Asia (Currie et al. 2007).

Melioidosis is endemic in many tropical regions, mainly between latitudes 20°N and 20°S, although *B. pseudomallei* is unevenly distributed in the environment in these areas and the true distribution has not been accurately defined (Dance 2000). The highest isolation rates have been found in rice paddies, rubber plantations and other cleared and cultivated areas (Nachaingmai et al. 1985; Strauss et al. 1969) but high rates have also been seen in urban sports fields in Singapore (Thin et al. 1971), and grazing sites of animals with melioidosis in Australia (Thomas et al. 1979). Factors that may influence environmental distribution include temperature, humidity, rainfall, ultra-violet exposure, soil composition, vegetation, fertilisers and soil disturbance such as excavation or ploughing (Inglis et al. 2001). Warm climates favour the persistence of *B. pseudomallei* in the environment, however when introduced to a non-endemic area the organism may persist for several years in soil. This apparently occurred during a prolonged outbreak in France in the 1970s, which was thought to have followed the importation of an infected animal (Mollaret 1988). With increasing migration of humans and animals around the world, new endemic foci may become established. Sporadic cases have been reported in the Americas, the Caribbean and sub-Saharan Africa (Cheng and Currie 2005; Dance 1991), although the true incidence in these areas is unclear because of a lack of laboratory facilities and clinical awareness. Ongoing mapping of the distribution of *B. pseudomallei* and melioidosis is available at http://www.melioidosis.info/map.aspx.

Molecular tools have demonstrated that environmental isolates are often identical to epidemiologically related human or animal strains, that there is considerable

diversity among isolates persisting in a particular region, and that clonal outbreaks have occurred when the organism is introduced to a non-endemic region (Cheng and Currie 2005; Currie et al. 1994).

The relatively small numbers of cases reported in endemic areas during the latter half of the twentieth century probably represented the "tip of the iceberg" due to limited culture facilities in many rural, high risk regions (Dance 1991). This suggestion is supported by the fact that western armed forces, with access to high quality laboratory diagnostics, reported at least 100 confirmed cases of melioidosis amongst French (Rubin et al. 1963) and American (Sanford 1985) soldiers respectively during the conflicts in Vietnam. In Thailand, very few cases were reported until the improvement of district microbiology laboratories and increased clinical awareness in the 1980s, which led to in around 800 case reports by 1986 (Leelarasamee and Bovornkitti 1989).

By the year 2000, melioidosis was regarded as an emerging infection due to increasing reports of confirmed cases in endemic regions, particularly Thailand, where it is estimated that more than 2500 culture-positive cases of human melioidosis occur annually, increasing reports of cases from regions where the disease was not known to be endemic, and concerns that it could be spread to non-endemic regions by infected animals (Dance 2000). Much of the increase was probably due to improved diagnostics and clinical awareness, but the increasing prevalence of predisposing medical conditions such as diabetes in populations of endemic areas (Dance 2000) and possibly climate change and increasing travel and migration, may also have impacted on melioidosis epidemiology.

Currently, the greatest burden of melioidosis is seen in Thailand (especially the northeast) and Northern Australia where annual incidence rates are up to 21.3/100,000/year (Limmathurotsakul et al. 2010) and 16.5/100,000 (Currie et al. 2000) respectively. Melioidosis is now the third most common cause of death from infectious disease in northeast Thailand (Limmathurotsakul et al. 2010), and is the commonest cause of fatal community-acquired bacteraemic pneumonia in the Northern Territory of Australia (Currie et al. 2000). The disease is highly seasonal, with 75–85% of cases presenting during the rainy season (Suputtamongkol et al. 1994; Currie et al. 2010) and incidence rates as high as 41.7/100,000 have been recorded during severe rains (Currie et al. 2004). Large case series have identified occupational exposure to soil and water, male sex, Aboriginal Australians, diabetes, alcoholism, chronic lung disease, chronic renal disease, thalassaemia and kava and steroid use to be risk factors for melioidosis (Cheng and Currie 2005; Suputtamongkol et al. 1994; Currie et al. 2010). The majority of cases have a predisposition, but in around 20% none is identified (Currie et al. 2010).

With respect to animal infection, *B. pseudomallei* appears to affect a broader range of animal hosts than glanders, with infection in equines being relatively rare, although it may occasionally cause severe infections in horses. Species that have been infected include terrestrial and aquatic mammals, birds and fish. Goats, sheep, pigs and camels appear particularly susceptible, whereas dogs, cats and cattle appear more resistant, but may develop disease if they become immunocompromised (Choy et al. 2000). Sporadic cases or small outbreaks have been reported in various

primates, marsupials, deer, buffalo, camels, llamas, zebras, horses, mules, rabbits, rodents, parrots, crocodiles, dolphins, and seals (Sprague and Neubauer 2004) . Animal cases have also been reported in other regions, such as southern and western Australia (Currie et al. 1994; Ketterer et al. 1986), China (Li et al. 1994), Iran (Baharesefat and Amjadi 1970), Saudi Arabia (Barbour et al. 1997), United Arab Emirates (Wernery et al. 1997), South Africa (Van der Lugt 1995), and Spain (Galimand and Dodin 1982). Epizootics have been reported after importation of animals from areas of endemicity. This was believed to be the source of a cluster of infections in sheep, goats and pigs in Aruba in 1957 (Fournier 1965), an outbreak in a Paris zoo which spread to other zoos and equestrian clubs in France in the 1970s (Mollaret 1988), and an outbreak in primates in the UK in the 1990s (Dance 1992). More detail on confirmed cases of melioidosis in different animal species worldwide can be found in a relatively recent review (Sprague and Neubauer 2004).

35.3.2 Modes of Transmission

Whitmore's early observations of melioidosis in guinea pigs led him to believe that the infection was transmitted by consumption of food and drink contaminated by urine, sputum, or other secretions containing viable bacteria, from infected persons or animals (Whitmore 1913). In the 1930s, Stanton and Fletcher also proposed that infection occurred by ingestion, although they believed that rodents were a zoonotic reservoir (Stanton and Fletcher 1932). It was subsequently observed that human infections commonly followed exposure to mud and water, and that *B. pseudomallei* could be isolated from soil and surface water (Chambon 1955), leading to the current knowledge that it is an environmental saprophyte. Well documented modes of transmission include inoculation and aspiration of water during near drowning (such as during the Asian tsunami in 2004), and laboratory-acquired infection (although only two such instances have been reported in the literature). Epidemiological evidence and animal studies also suggest a role for inhalation and ingestion, although it is often impossible to define precisely how and when infection occurred. Although sporadic cases have been anecdotally associated with infection in animals, there is no solid evidence for zoonotic or person-to-person spread (Dance 2000), and it is equally likely that both humans and animals have acquired infection from the same environmental source.

Inoculation of organisms through penetrating injuries or pre-existing skin lesions appears to be the major mode of acquisition, particularly in farmers who are continually exposed whilst working in the mud and surface water of paddy fields (Suputtamongkol et al. 1994). Twenty five percent of patients in one case series recalled a previous inoculation injury, but often there is no such history (Currie et al. 2000). Inoculation is the method most frequently used to induce infection in animal models, and natural infection in animals occurs in this way by entry of bacteria through minor skin trauma, bite wounds and scratch injuries.

Infection after inhalation has been demonstrated in laboratory animals (Jeddeloh et al. 2003), and this may be an important mode of acquisition in humans. During periods of very heavy rainfall, increases in pneumonic cases of melioidosis occur, probably as a result of aerosolisation of the bacteria (Currie and Jacups 2003). Inhalation was previously thought to be the primary mode of transmission due to the high incidence of melioidosis in U.S. military helicopter crews during and after the Vietnam war.

Ingestion has also been proposed as a mode of infection in both humans and animals. Contaminated water supplies have been implicated by PFGE as the point source of melioidosis outbreaks in Australia (Currie et al. 2001; Inglis et al. 1999). Suppurative parotitis, a common presentation in children with melioidosis in south-east Asia, is believed to be due to the ingestion of contaminated water or soil, resulting in the ascent of bacteria from the mouth to the parotid gland (Stoesser et al. 2012). Although not confirmed, an untreated river water supply was implicated in melioidosis outbreaks occurring in intensive piggeries (Ketterer et al. 1986). In these outbreaks, an oral mode of transmission was suspected due to the common finding of infected gastro-hepatic nodes. Faeco-oral transmission was felt to be unlikely due to the fact that *B. pseudomallei* was infrequently isolated from faecal samples of infected pigs.

Despite early theories, melioidosis does not appear to be a true zoonosis. Three anecdotal cases of possible zoonotic infection in Australia were described by Choy et al. (2000). In one case, *B. pseudomallei* was cultured from a wrist lesion of a meat worker in Darwin; secondly, a vet in rural Queensland developed abscesses on the arm, but this does not appear to have been confirmed as melioidosis by culture; and similarly, a goat farmer had a lesion on his hand resembling a "milker's lesion" for 2 months preceding a diagnosis of melioidosis, which again does not appear to have been culture-confirmed. In Malaysia, a case of suspected sheep-to-human-transmission was reported in a 10 year old boy (Idris et al. 1998). The evidence for this was entirely circumstantial, and it is more likely that he contracted the illness from soil and water in the environment (from which *B. pseudomallei* was also isolated). Earlier anecdotal evidence of animal-to-human transmission of melioidosis was reported during "L'affaire du Jardin des Plantes", an outbreak of melioidosis that started in a Paris zoo and spread to other zoos and equestrian clubs in France through transport of infected animals and contaminated manure. At least two fatal human cases were said to have occurred during this outbreak, although details were never published (Mollaret 1988; Dodin and Galimand 1986). In none of these cases has there ever been genotypic evidence of the relationship between the human and animal isolates, and so the case for animal-to-human transmission remains unproven.

There have been concerns that goats, which appear to be particularly susceptible to melioidosis and often develop mastitis as a manifestation of the infection (Fig. 35.4), could transmit the disease via infected milk. However, small studies of infected goats have found that the organism is only isolated from body fluids in a minority of cases (Thomas et al. 1988). Furthermore, a recent literature review of bacterial infections following animal bites world-wide, did not identify any cases

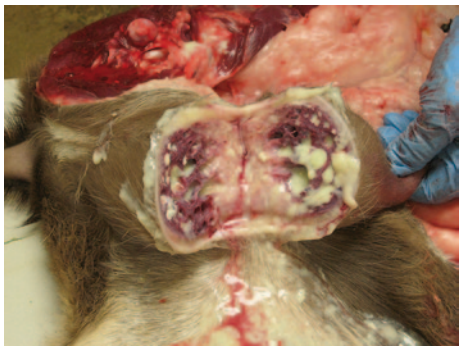

Fig. 35.4 Mastitis found on post-mortem of a goat who died of melioidosis. Copyright Dr Carl Soffler

of melioidosis, supporting the fact that transmission from body fluids is unlikely (Abrahamanian and Goldstein 2011). However, clearly it makes sense in public health terms to avoid drinking milk or eating meat from infected animals.

Human-to-human transmission is also extremely rare. The diabetic sister of a confirmed case in Thailand with septicaemia, pneumonia and wound infection subsequently developed septicaemic melioidosis after a period of close contact with him in hospital. However, she was from an endemic area in northeast Thailand and may have acquired disease there with coincidental presentation or reactivation at the stressful time of her brother's illness (Kunakorn et al. 1991). Similarly, transmission between two siblings with cystic fibrosis from an endemic region in Australia may have occurred when identical isolates of *B. pseudomallei* were cultured from their sputa (Holland et al. 2002). The authors propose that person-to-person transmission may be facilitated by underlying cystic fibrosis, as occurs with *B. cepacia*, however, the siblings may also have acquired the infection from the same environmental source. Another possible human-to-human transmission was reported in the wife of a Vietnam veteran with chronic prostatitis, who had significantly raised antibody titres to *B. pseudomallei*, presumably due to sexual exposure as she had never visited an endemic area (McCormick et al. 1975). Several neonatal cases of melioidosis have been reported suggesting transplacental or perinatal transmission (Cheng and Currie 2005), and further cases have implicated breast milk from mothers with mastitis (Ralph et al. 2004). Intrauterine infection has also been described in animals (Choy et al. 2000).

35.3.3 Microbiology

Like *B. mallei, B. pseudomallei* is an irregularly staining, oxidase-positive, motile Gram-negative bacillus which sometimes exhibits marked bipolarity microscopically. It can be distinguished from *B. mallei* by its motility and usually its resistance to aminoglycosides. It grows readily on most routine culture media, initially forming smooth colonies which become dry and wrinkled on prolonged incubation (Fig. 35.5). Important characteristics include arginine dihydrolase and gelatinase

Fig. 35.5 Typical dry, wrinkled colonies of Burkholderia pseudomallei after 48 hours culture on Ashdown's media

activity, the inability to assimilate arabinose (distinguishing *B. pseudomallei* from the closely related avirulent *B. thailandensis*), and growth at 42 °C. Its intrinsic resistance to aminoglycosides, polymyxins and the early beta-lactams, but susceptibility to co-amoxiclav, is particularly characteristic, and any oxidase positive Gram negative bacillus with these characteristics should be assumed to be *B. pseudomallei* until proved otherwise. The species is antigenically homogeneous, but a number of molecular techniques, most usefully MLST, can distinguish between isolates.

35.3.4 Pathogenesis

A range of bacterial factors have been associated with virulence, but the relative contributions of individual virulence factors to the disease process have not been well characterized. A variety of adhesins, in particular type 4 pili, appear to be involved in attachment of bacteria to different eukaryotic cell types, and expression is regulated by the *pilA* gene (Allwood et al. 2011). Like *B. mallei, B. pseudomallei* utilizes up to three T3SS, including Bsa T3SS. In vitro experiments have demonstrated the importance of this system, and its individual components, in host cell invasion, escape from endosomes and intracytoplasmic survival (Stevens 2002). Mutations in components of the T3SS in *B. pseudomallei* have reduced ability to cause disease in animal models (Stevens et al. 2004). Cell-to-cell spread takes place by actin-based motility which is dependent on the BimA protein (Stevens et al. 2005). The antiphagocytic polysaccharide capsule, quorum sensing mechanisms, and bacterial components such as lipopolysaccharide, flagella, secreted products (protease, lipase, lecithinase, various toxins) and a siderophore ('malleobactin') also have important roles in environmental protection and adaptation, and host immune system evasion (Cheng and Currie 2005). The ability of the organism to sur-

vive and grow intracellularly or become metabolically inactive within granulomas probably contributes to the persistent nature of the infection and the risk of relapse.

The clinical outcome after exposure to *B. pseudomallei* in the environment varies from person to person, ranging from asymptomatic seroconversion (the commonest outcome) to fulminant sepsis and death, and is dependent on the size and route of the inoculum, the virulence of the infecting strain, and host immune factors. On the host side, innate immune mechanisms, macrophage and neutrophil function, and both cellular and humoral responses probably all play a role in defence against the organism, hence the strong associations with immune-suppressing conditions such as diabetes, thalassaemia, renal impairment and alcohol excess (Cheng and Currie 2005; Currie et al. 2004). Interestingly, HIV does not appear to be a risk factor. An exaggerated host response with high levels of pro-inflammatory cytokines such as TNF-alpha may also have a pathogenic role (Nuntayanuwat et al. 1999).

35.3.5 Clinical Presentation in Humans

The majority of infections appear to be subclinical with 60–70 % of populations in endemic areas acquiring antibodies to *B. pseudomallei* by the age of 4 years without clinically apparent disease (Wuthiekanun et al. 2006). When disease manifests, it may be localised or disseminated with septicaemia. The incubation period varies depending on the mode of acquisition and infecting dose, with most cases occurring within 3 weeks after an inoculation injury, and as soon as 24 h after a near-drowning event (Currie et al. 2000; Suputtamongkol et al. 1994). Pneumonia is the commonest presentation, and is evident in around half of all cases (Currie et al. 2010). Cavitation may occur in the upper zones mimicking tuberculosis. Localised abscesses may occur in any other organ including the skin and soft tissues, lymph nodes, liver, spleen, genitourinary tract (especially the prostate gland in males), parotid gland, bone or joint, and nervous system. Localised disease without bacteraemia generally has a good outcome and low mortality. However, in over 50 % of cases the patient is bacteraemic, and just over one third of these are in septic shock at presentation, which has a mortality approaching 50 % even with optimal treatment (Suputtamongkol et al. 1994; Currie et al. 2010). There appear to be geographical variations in manifestations, with hepatosplenic abscesses more common in Asian populations and suppurative parotitis in Asian children, and higher rates of prostatic and neurological melioidosis seen in Australia (Cheng and Currie 2005), although this could be biased by better access to imaging. Recrudescent melioidosis after treatment occurs in up to 5 % of cases. This is due to reactivation of the original strain (relapse) in approximately 75 % of cases, which is usually associated with a failure to sterilise deep-seated foci of infection in disseminated disease, but may also be associated with poor adherence to therapy or an insufficient duration of eradication therapy (Currie et al. 2000). Reinfection with a different strain accounts for 25 % of recurrent infections.

35.3.6 Clinical Presentation in Animals

As outlined above, a wide range of animal species may be affected by melioidosis, with a range of clinical manifestations and severity. In fact, the disease in animals is usually similar to that in humans, with subclinical infections common and abscesses occurring in any organ, particularly lungs, liver, spleen, and associated lymphatics. The acute form presents as fulminant sepsis with haematogenous dissemination and high mortality, often associated with respiratory distress and diarrhoea, and tends to occur in younger animals of susceptible species. The chronic form presents as a more non-specific illness in older animals, with low grade fever, anorexia, cough, progressive emaciation and lameness (Choy et al. 2000). In sheep, goats, and horses, nasal and ocular discharge (similar to that seen in glanders) is common, and central nervous system involvement may contribute to paralysis, convulsions, nystagmus and blindness (Sprague and Neubauer 2004). Mastitis appears to be a particular feature in goats (Fig. 35.4), orchitis has been described in rams and boars, and skin lesions, limb oedema, lymphangitis and meningoencephalitis in horses (Sprague and Neubauer 2004). Monkeys are affected in a similar way to horses, but neurological involvement is more unusual (Sprague and Neubauer 2004) .

35.3.7 Diagnosis

Melioidosis should be considered in any person or animal who has visited or migrated from an endemic area presenting with septicaemia and/or abscesses, especially if they have a predisposing condition such as diabetes. Confirmation of the diagnosis relies on culture of the organism from blood, sputum, pus, or other body fluid indicated by the clinical presentation. Liaison with the microbiology laboratory is of utmost importance if melioidosis is suspected. Firstly, the organism is a hazard group 3 pathogen and must be handled in appropriate laboratory containment in case of transmission to laboratory staff. Secondly, selective media such as Ashdown's or *B. cepacia* media may be used to optimise the isolation of the organism. And thirdly, if not aware of the clinical context, growth in cultures may be dismissed as a contaminant by the unwary.

Culture may take several days, and meanwhile microscopy of pus, sputum or urine may reveal bipolar or unevenly staining Gram-negative rods, although this appearance is not specific. Immunofluorescent staining of such samples is a useful rapid diagnostic tool but is not widely available (Wuthiekanun et al. 2005). Once cultured, commercial identification kits such as the API 20NE usually identify the organism correctly but may give misleading results (Amornchai et al. 2007), so presumptive isolates should be sent to a Reference Laboratory if in doubt as to the identity. A latex agglutination test using a monoclonal antibody to the 200 kDa extracellular polysaccharide is also useful for screening suspect colonies or positive blood culture fluid, with 95% sensitivity and 99.7% specificity (Anuntagool et al. 2000).

There is no standard serological test for melioidosis. An indirect haemagglutination (IHA) test, using a crude mixture of poorly characterized antigens, is most widely used, but it lacks sensitivity and specificity in humans, particularly in endemic areas where background seropositivity rates are high. A rapid immunochromatographic test for IgG appears to be more specific than IHA in populations of endemic areas (Wuthiekanun et al. 2004). Until improved kits using more refined standardised antigens are available, the utility of serological tests is largely limited to non-endemic regions. Despite the limitations, serology continues to be used in veterinary medicine, and a two-step method by screening with IHA followed by confirmation with a complement fixation test was shown to be sensitive and specific in caprine melioidosis (Thomas et al. 1988). Molecular methods have also been developed but are not yet used for routine diagnostic purposes.

Radiology is an important adjunct to microbiological diagnosis, and may demonstrate diffuse nodular infiltrates, abscess, or cavitating pneumonia on chest radiograph, and abdomino-pelvic abscesses on ultrasound or CT.

35.3.8 Treatment

B. pseudomallei is intrinsically resistant to many classes of antibiotics, including some third generation cephalosporins, early penicillins, aminoglycosides, colistin and polymyxin, and exhibits relative resistance to quinolones and macrolides (Cheng and Currie 2005). The treatment of melioidosis may be classified into acute and eradication phases. In the acute phase the aim is to kill bacteria in the circulation and prevent patients dying of overwhelming sepsis, and in the eradication phase the aim is to kill any residual bacteria in abscesses or tissues and prevent relapse of infection. Currently, ceftazidime or a carbapenem for 2 weeks is advised for the acute phase, and co-trimoxazole with or without doxycycline, or monotherapy with co-amoxiclav for 12–20 weeks for eradication.

In a trial of 161 patients in Thailand (65 with confirmed melioidosis, 54 of these septicaemic), ceftazidime (120 mg/kg/day) in the acute phase reduced mortality from 74 to 37 %, compared with the conventional combination regimen of chloramphenicol, doxycycline and co-trimoxazole (White et al. 1989). Other cephalosporins, such as cefotaxime and ceftriaxone, were associated with significantly greater mortality compared with ceftazidime in retrospective analyses (Chaowagul et al. 1999). Subsequent trials assessed ceftazidime with and without the addition of co-trimoxazole in the acute phase of melioidosis, and failed to demonstrate any difference in mortality between the monotherapy and combination groups (Chierakul et al. 2005).

Carbapenems are the most active drugs in vitro against *B. pseudomallei*, and are more rapidly bactericidal (Smith et al. 1996). A randomised trial comparing ceftazidime (120 mg/kg/day) with imipenem/cilastatin (50 mg/kg/day) for a minimum of 10 days, was unfortunately terminated early and therefore underpowered. It showed no difference in mortality between the two groups, but higher rates of treatment

failure in the ceftazidime group (41.3 versus 20.3%) (Simpson et al. 1999). Co-amoxiclav is considered second line therapy for the acute phase.

The conventional combination of chloramphenicol, doxycycline and co-trimoxazole for eradication therapy was extremely poorly tolerated leading to reduced compliance and increased rates of relapse. Omitting chloramphenicol was shown to be beneficial in terms of side effect profile, with no adverse treatment outcomes (Chaowagul et al. 2005). Years of clinical experience in Australia (Cheng and Currie 2005), however, suggest that co-trimoxazole monotherapy for 12–20 weeks is probably adequate to prevent relapse. A trial conducted in Thailand (Chetchotisakd et al. 2014), supports the use of co-trimoxazole monotherapy. In the rare cases of co-trimoxazole resistance (determined by MIC as disk diffusion tests are unreliable), and where co-trimoxazole is contraindicated, co-amoxiclav is the preferred eradication therapy, although this is associated with increased rates of relapse (Rajchanuvong et al. 1995). As mentioned previously, poor compliance also contributes to relapse, and so it is crucial that each patient is counselled in the importance of completing the full treatment course regardless of symptomatic improvement.

Apart from appropriate antibiotic therapy, the management of melioidosis must also incorporate optimal supportive treatment for sepsis, including maintenance of blood pressure, adequate glycaemic control, and management of respiratory and acute renal failure. Around one quarter of cases require admission to intensive care (Currie et al. 2010). The drainage of abscesses should also take place where possible. Adjunctive granulocyte colony stimulating factor (G-CSF) has been used to boost host neutrophils in an attempt to control infection, but despite promising outcomes in a retrospective study, G-CSF did not significantly reduce mortality in a randomised controlled trial (Cheng et al. 2004, 2007).

Even with appropriate antimicrobial and supportive therapy, mortality remains high for septicaemic cases. Poor prognostic factors include shock, absence of fever, leucopenia, abnormal liver function, renal impairment, high level or persistent bacteraemia, hypoglycaemia and acidosis (Cheng and Currie 2005; Limmathurotsakul et al. 2011).

As is the case for glanders, the long duration of treatment of melioidosis in animals can be expensive and ineffective. In cases where treatment is deemed necessary, such as in animals of economic value, treatment regimens are as for human cases.

35.3.9 Prevention and Control

As yet, there is no effective human or animal vaccine for prevention of melioidosis, but potential candidates are under investigation. For example, immunisation with a *B. thailandensis* strain possessing a similar antigenic capsule to *B. pseudomallei* induced significant protection against a lethal intraperitoneal *B. pseudomallei* challenge in a mouse model (Scott et al. 2013).

Preventive measures must therefore focus on avoidance of contact with *B. pseudomallei* in the environment. A matched case-control study carried out in northeast Thailand found that working in rice fields, walking barefoot, bathing in pond water, exposure to rain, water inhalation, and having an open wound all significantly increased the odds of acquiring melioidosis (Limmathorotsakul et al. 2013). A lower risk of melioidosis was associated with wearing protective clothing such as long trousers and rubber boots, and washing with clean water after working in the fields. The authors, therefore, recommended avoidance of direct contact with soil and environmental water whenever possible, but wearing protective clothing and washing after exposure if this is unavoidable. Wounds should be kept covered until they have completely healed, and the application of herbal remedies to wounds should be avoided, as this was also associated with an increased risk of melioidosis. Since there was a small but significant risk observed with drinking untreated water, and since *B. pseudomallei* was found in water drunk by 7 % of cases and 3 % of controls, including borehole, wells and piped supplies, it was also recommended that only treated water should be drunk in endemic areas (Limmathorotsakul et al. 2013). It has also been recommended that goat's milk be pasteurised to avoid potential zoonotic transmission by ingestion (Choy et al. 2000), although this has never been reported, but this makes sense in general public health terms. Due to the low but theoretical risk of person-to-person transmission, human cases should be nursed in isolation with contact precautions and care taken when handling any body fluids. People with strongly associated predisposing conditions, such as diabetes, should be informed of their increased risk of melioidosis, and advised to avoid the above high risk activities. Unfortunately in rural areas, and during heavy rain and winds, exposure may be unavoidable for many.

It has been recommended that animals be removed from contaminated sources, such as soil or water in endemic regions, to prevent melioidosis outbreaks in herds (Choy et al. 2000), however infections have still occurred when pigs were reared on artificial, hard surfaces such as concrete (Thomas et al. 1981). Chlorination of water has been shown to eliminate *B. pseudomallei* (Howard and Inglis 2005), but only if pH and concentrations of organic substrates are carefully controlled, and this could prove difficult in water troughs which may become highly contaminated (Choy et al. 2000). When an animal becomes infected in an endemic area, it has been suggested that strict maintenance of a hygienic environment may prevent a larger outbreak, although supporting evidence is lacking. Regular disinfection with potassium hypochlorite and cresol (to include all surfaces and the lower limbs of the animal), removal of infected excrement several times per day, and the avoidance of large quantities of water were used in an effort to curtail the outbreak in Paris zoos and equestrian clubs in the 1970s (Sprague and Neubauer 2004). Infected carcasses of animals must be condemned and destroyed. Guidelines for handling and disposal are available in the Manual for Meat Inspection in Developing Countries (http://www.fao.org/docrep/003/t0756e/T0756E05.htm#ch4.2.9). There are no mandatory requirements for serological screening for melioidosis in animals that are transported internationally, although it is possible that such animals might react in serological or skin tests to the closely related *B. mallei*. Serological testing of

imported primates for melioidosis was used following an outbreak amongst Cynomolgus monkeys in the United Kingdom in the 1990s but has never been used routinely (Dance et al. 1992).

35.4 Conclusion

Both glanders and melioidosis may be regarded as re-emerging infections with the ability to infect both animals and humans, although only glanders is a true zoonosis. Glanders has been eradicated from many countries, and melioidosis is largely restricted to southeast Asia and tropical North Australia, but various factors have contributed to their emergence such as increasing awareness and diagnostic capability, increasing prevalence of underlying predisposing conditions, increased transport of animals (and associated contaminated waste and equipment) internationally, human migration patterns, and adventure travel to tropical regions. Sub-clinically infected human and animal carriers risk further transmission of infection in the case of glanders, as well as persistence in a new environment under the right physical conditions. Global warming may extend the current geographic limitations of melioidosis and place a greater population at risk of exposure. This is particularly of concern as the prevalence of diabetes and other immunosuppressive states increases in many developing countries. Finally, we must be alert to the possible use of these agents in bioterrorism. Increased awareness of these pathogens is important so that early recognition, treatment, and public health action occurs, and so that organisms are handled at the appropriate level of containment to prevent laboratory-associated cases. Further research is required to develop effective vaccines and optimise prevention strategies.

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Part VII
Emerging and Re-emerging Zoonoses

Chapter 36
Zoonotic Aspects of Tuberculosis: Disease of the Past or Re-emerging Zoonosis?

Anita Luise Michel

Abstract Tuberculosis is a serious chronic disease of humans and animals caused by members of the *Mycobacterium tuberculosis* complex. While *M. tuberculosis* affects primarily humans, *Mycobacterium bovis* has a wide range of host species including wild and domestic animals and humans. This chapter investigates the role of different host species in the transmission of tuberculosis at various animal/human interfaces. While drivers and modes of zoonotic TB transmission are reviewed, a broad spectrum of complexities hampering the eradication of this disease are highlighted. The most important constraints, next to factors related to pathogen-host interactions such as HIV co-infection and multidrug resistance of MTBC strains, are socio-economic shortcomings in the control of bovine tuberculosis in the cattle population as well as traditional customs and habits related to the consumption of milk and other animal products.

> Theodor Smith (1898):
> …the virulence of sputum bacilli to bovine bacilli is as 1–20, or 1–30 when tested on rabbits. No one will venture to assert, with these figures in mind, that when these races are implanted in the human body this great difference will be wiped out and the resulting disease be the same. Such differences signify certain unknown differences in behavior in the human body which must be studied by the clinician and the pathologist before they can become available as positive knowledge.

36.1 Introduction

Tuberculosis is an ancient human disease which developed in Africa and colonized the world via migrating modern humans approximately 70,000 years ago (Gagneux 2012; Hershberg et al. 2008). Throughout the millennia tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) has remained a major human health problem. While initially driven by socio-economic and environmental determinants, drug resistance, HIV/AIDS and adaptive evolutionary changes in the tubercle bacillus have appeared more recently as powerful new drivers with a devastating effect

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on the host-pathogen relationship in tuberculosis (Gagneux 2012; Donoghue et al. 2004; Borrell and Gagneux 2009; Kwan and Ernst 2011; World Health Organization 2011).

Tuberculosis in animals was known before biblical times, but it was not until the isolation of the tubercle bacillus and early comparative studies between *M. bovis* and *M. tuberculosis* by Koch and others late in the nineteenth century that distinguished between strains from bovine and human sources (Smith 1898, Koch 2013). Today *M. bovis* as the main cause of tuberculosis in cattle and other domestic and wild mammals has been well studied. Due to its zoonotic nature and to differentiate its causal relationship from that of *M. tuberculosis* in humans, *M. bovis* (and exceptionally *M. caprae*) infections in humans are referred to as zoonotic tuberculosis (TB) (Cosivi et al. 1998). The epidemiological link between *M. bovis* infected cattle and zoonotic TB in humans has long been recognised and many reports highlighting established and possible routes of transmission have been published in the literature, but nevertheless arereliable estimates of global prevalence and burden of zoonotic TB are still lacking.

A complete account of TB as a zoonosis should include the spillover of *M. tuberculosis* from humans to domestic and wild animals, as well as the transmission of other members of the MTBC between humans and animals. An effort has been made to give an overview of the current status of the combined body of veterinary and medical published knowledge in this chapter.

36.2 Causative Agents of Tuberculosis in Human and Animal Hosts

The genus *Mycobacterium* encompasses more than 150 currently fully or partially described species most of which are living as saprophytes and never or only very rarely cause disease in humans and animals. All known pathogenic mycobacteria which cause tuberculosis in humans and animals are very closely related (>99% identical at DNA level) and belong to the *Mycobacterium tuberculosis* complex (MTBC) (Brosch et al. 2002; Covert et al. 1999; Bercovier and Vincent 2001).

While until the beginning of the twenty-first century the M. tuberculosis complex only comprised *M. tuberculosis, M. africanum, M. bovis, M. bovis BCG, M. microti* and *M. cannettii*, improved characterisation of *M. bovis* subtypes and characterisation of "novel" species led to the expansion of the *M. tuberculosis* complex, which now also includes *M. pinnipedii, M. caprae, dassie bacillus* (a MTBC member isolated from the South African rock hyrax, *Procavia capensis* or 'dassie', *M. orygis and M. mungi* (Gagneux 2012; Aranaz et al. 2003; Cousins et al. 1994; Cousins et al. 2003; van Ingen et al. 2012; Alexander et al. 2010).

Recognised as the *M. tuberculosis* complex member with the widest host range, *Mycobacterium bovis* (*M. bovis*) was previously assumed to be the progenitor of the closely related *Mycobacterium tuberculosis* (*M. tuberculosis*) which was believed to have specialised in infecting and causing disease in humans. To the contrary, full

genome sequencing revealed that the genome of *M. tuberculosis* is significantly larger than that of *M. bovis* and that the reduction in genome size must have been accompanied by a loss in specialisation. Analysis of ancient DNA samples suggests a time scale for the evolution of the *M. tuberculosis* complex of 20,000–40,000 years during which *M. bovis* and other members of the complex evolved (Djelouadji et al. 2011). The early spillover event to domesticated cattle resulted in the emergence of a pathogen able to cause tuberculosis in a wider range of hosts on the one hand and to undergo further genetic modifications on the other hand, which prompted their establishment in certain host families and species (Brosch et al. 2002).

M. caprae, M. pinnipedii, M. orygis, M. mungi and dassie bacillus have only recently been characterised and found to belong to different branches of the *M. tuberculosis* complex phylogenetic tree. They were named after the animal species they were initially isolated from which implied a certain degree of host specificity. *M. microti* and *M. cannettii* have been described much earlier, but were for a long time believed to be pathogenic in murines and humans, respectively. Recent reports have shown that some of these organisms can infect and cause disease in other species as well and the full host range still needs to be defined (Aranaz et al. 2003; van Ingen et al. 2012; Alexander et al. 2010; Dawson et al. 2012; Hansen et al. 2012; Pate et al. 2006; Kiers et al. 2008; Goh et al. 2006; Mostowy et al. 2004).

36.3 Clinical Signs

In both animals and humans TB is a chronic, debilitating disease; therefore, clinical signs in the early stages are usually inapparent. During later stages manifestation of clinical signs depends on the organs and organ systems that are affected and is determined by the route of infection on the one hand and the subsequent dissemination to other organs on the other hand (Wedlock et al. 2002; Grange 2001).

36.3.1 Animals

In most animal species granulomatous lesions can be found primarily in the lymph nodes of the head and thorax. In herbivores, pulmonary tuberculosis is the most frequently encountered form of TB which develops following aerosol transmission by inhalation. While the affected animal may live for years without showing clinical disease, a painful cough may develop during advanced stages accompanied by hyperpnoea and dyspnoea. Animals can be observed standing with the elbows adducted, neck extended and lowered head to facilitate breathing. The animal's body condition is initially unchanged but deteriorates after prolonged disease, becoming gradually emaciated with a dull hair coat and sunken eyes (Cousins et al. 2004).

Infection of the bovine udder commonly presents as chronic organ tuberculosis and less frequently as military tuberculous mastitis, caseous tuberculous mastitis or tuberculous galactophoritis. Tuberculous mastitis is of major importance because

of the public health threat and the potential spread of the disease to the offspring (Cousins et al. 2004; Palmer et al. 2002).

Generalised tuberculosis, characterised by extensive miliary tuberculous lesions in numerous organs can be mostly seen in wildlife and in cattle, especially in countries where no bovine tuberculosis (BTB) control measures are applied. In developed countries a higher frequency of non-visible but culture positive lesions is encountered in tuberculous cattle due to the effects of the test and removal strategy (Neill et al. 2001).

Apart from pulmonary TB predators are often seen to develop intestinal TB, but may also show military TB as well as discharge from non-healing wounds and abscessed peripheral lymph nodes inflicted by bite wounds during acts of intra-species aggression.

In non-human primates the disease is usually pulmonary and may run a fulminating course, frequently with miliary lesions (Keet et al. 1996; Michel et al. 2006).

36.3.2 Humans

Humans acquire zoonotic TB either as an aerosol or as a food-borne infection. Very rarely mucous membranes and broken skin can be portals of entry. The direct health related consequences therefore involve both pulmonary and extra-pulmonary manifestations. The clinical signs elicited by zoonotic TB are indistinguishable from TB caused by *M. tuberculosis*. In the past extra-pulmonary TB following ingestion of the tubercle bacilli outweighed pulmonary manifestation and presented primarily as cervical lymphadenopathy (scrofula) and in some cases as chronic skin tuberculosis (lupus vulgaris). A variety of other extra-pulmonary manifestations including intestinal, meningeal, genitourinary as well as TB of the bones, joints and generalised disease have also been reported (Grange 2001; Akkerman et al. 2012; Cordova et al. 2012; Torres-Gonzalez et al. 2013; Ridaura-Sanz et al. 2012; Mechai et al. 2011; Gonzalez-Duarte et al. 2011; Jain 2011; Albrecht et al. 1995; Schubel et al. 2006). In rare cases pulmonary and extra-pulmonary localisation of lesions can be present in the same patient (Ahmad et al. 2011).

36.4 Epidemiology of the *Mycobacterium tuberculosis* Complex at the Animal/Human Interface

The susceptibility, pathogenicity, transmissibility and wider impact of tuberculosis caused by members of the *M. tuberculosis* complex largely depends on and varies significantly between the affected host species (LoBue et al. 2010). Despite improved diagnostic tools and intensified control measures the number of reports on tuberculosis caused by *M. bovis* or *M. tuberculosis* in new and known host species has increased considerably over the past two decades. This is partly due to advanced

and more sensitive molecular tools to characterise MTBC organisms, but mostly due to increased spillover of these pathogens from their respective reservoir species, domestic cattle and humans, to other species.

The type of interaction between the pathogen and the infected hosts can vary significantly, both regarding the prospects for the pathogen in terms of replication and dissemination as well as for the host population. For some host species the potential to serve as maintenance hosts for *M. bovis* has been demonstrated. These species are able to harbour and circulate the infection within their population allowing persistence of the infection and horizontal transmission between species (Fitzgerald and Kaneene 2013; Palmer et al. 2012; Renwick et al. 2007). In contrast, spillover or dead-end host species experience occasional tuberculosis outbreaks which usually die out due to the lack of sustainable pathogen amplification, dissemination and/or transmission at population level. Often the consequences for the affected individual(s) result in severe disease and death, but the transmission to other individuals may be limited to those living in very close and frequent contact (De Klerk-Lorist et al. 2012).

While humans are considered the only reservoir species for *M. tuberculosis* they are dead-end hosts for *M. bovis*, with sporadic cases of human-to-human and human-to-cattle transmission have been reported (Jain 2011; Stone et al. 2012). Non-human primates—especially, but not exclusively—those living in captivity, have also shown a high susceptibility for the disease (Michel et al. 2013).

36.5 *Mycobacterium bovis* Infections at the Animal/Human Interface

Tuberculosis caused by *M. bovis* in animals was primarily known as a disease of cattle, hence its name bovine tuberculosis. Despite the fact that *M. bovis*-associated tuberculosis has been confirmed in many mammalian species, it is still widely referred to under the same name, mainly to disclose the identity of and distinguish the causative agent from other MTBC members.

To differentiate between classical human tuberculosis (*M. tuberculosis*) and the disease caused by *M. bovis* in humans the latter was referred to as zoonotic tuberculosis, a name which has been retained and sometimes expanded to include infections in humans caused by the closely related *M. caprae* (Muller et al. 2013).

(i) Host species of *M. bovis* *M. bovis* is best described as a multi-host pathogen with the ability to establish itself successfully in multi-species ecosystems around the world. The extremely broad host spectrum of *M. bovis* includes both domestic and wild animals as well as humans, and has led to the establishment of a number of maintenance host species in a range of countries with wildlife populations. As a consequence of its negative impacts on many mammal species *M. bovis* has been recognised as a One Health determinant at the livestock/wildlife and animal/human interfaces (Palmer et al. 2012; Bengis et al. 2002; Michel et al. 2010; Maas et al. 2013; Miller and Olea-Popelka 2013).

M. bovis is most frequently transmitted to humans from infected cattle which are the main reservoir and maintenance host, either via direct and close contact with infected animals or by ingestion of contaminated products such as milk (Cosivi et al. 1998; Torres-Gonzalez et al. 2013). Zoonotic tuberculosis hence constitutes a spillover infection. Similarly, cases or outbreaks of *M. bovis* in other animal species are the consequence of spillover from cattle. They occur sporadically and disease levels usually reflect those in cattle. Given favourable conditions for transmission any infected spillover species can, in turn, pose a risk to human health given favourable conditions for transmission. Domestic animal species implied in the epidemiology of zoonotic tuberculosis include goats, sheep, pigs, farmed deer and camelids, while cases in horses, cats and dogs appear to be comparatively rare (Cousins 2001).

The occurrence and importance of *M. bovis* infection in goats as well as in sheep is low on a global scale but varies greatly depending on the country and the husbandry system. In most affected flocks the infection is linked to co-grazing with infected cattle herds (Cousins 2001; Lall 1969; Cadmus et al. 2009). In Spain, tuberculosis in sheep and goats is primarily caused by *M. caprae* (Rodriguez-Campos et al. 2011) which poses a public health threat comparable to *M. bovis* (Gutierrez et al. 1997; Oh et al. 2002b).

The prevalence of *M. bovis* infection in pigs has declined sharply in countries with effective bovine tuberculosis control in cattle, supporting the assumption that they are dead-end hosts (Cousins 2001; Pesciaroli et al. 2012). Exceptions to this trend are reported from developing countries. In the widespread absence of control schemes for *M. bovis*, an increasing small scale pig production may be accompanied by increased transmission of zoonoses at a highly diffuse animal/human interface where pigs share their habitat with humans (Muwonge et al. 2012). Likewise, the presence of a wildlife reservoir for *M. bovis* can effectively drive pathogen transmission to domestic or feral pigs, as shown for wild boar in Spain and possum in New Zealand (Nugent et al. 2012; Parra et al. 2003).

Camelid species comprise camels (bactrian and dromedary) from the Old World and alpaca and llama from the New World, which are all susceptible to tuberculosis. In pastoral communities of East and North Africa, the Sahel region as well as the Middle East, camels provide milk, meat and draft power for transportation of goods. The close association between pastoralists and their animals facilitates the bi-directional transmission of tuberculosis. Although it is largely unknown how widespread and significant the disease is in camel populations, published reports document its presence in Egypt, Kenya, Ethiopia Somalia, Mauritania, Niger, Pakistan and the United Arab Emirates (Cousins 2001; Wernery; Kinne 2012; Alvarez et al. 2012; Boukary et al. 2012; Gumi et al. 2012b; Mamo et al. 2011).

Alpacas and llamas are South American camelids which are imported into the European Union and other countries as pets and for natural fibre. Unlike cattle, these species are considered exotic and usually no regular tuberculosis testing programmes exist. Several outbreaks of *M. bovis* including human cases have caused public health concern (Rhodes et al. 2012; Dinkla et al. 1991).

Reports on *M. bovis* infection in domestic carnivores are in general very rare, whereby cats are considered more susceptible to *M. bovis* than dogs. Depending on

the extent of the disease in cattle in a high prevalence setting, cats were considered potential reservoirs of infection for cattle and possibly humans. Recent studies to investigate the role of cats in the epidemiology of *M. bovis* on infected farms could not find any evidence in the US in support of feline involvement, while Zumarraga et al (2009) could establish that stray cats in Buenos Aires, Argentina, shared *M. bovis* strains with cattle and possibly with humans (Aranaz et al. 1996; Zumarraga et al. 2009; Wilkins et al. 2008; Shrikrishna et al. 2009).

Farmed cervids, deer and elk represent a significant alternative livestock industry in New Zealand, China, the US, Russia, Canada and Mauritius (Jori, unpublished information). Farmed deer and farmed elk develop high levels of *M. bovis* infection within the farmed population with spillover to cattle and humans hence representing a significant animal and public health concern for farmers and downstream handlers (Waters et al. 2011; Whiting and Tessaro 1994).

Free-ranging wildlife can contract *M. bovis* from cattle as demonstrated in a number of countries for a range of wildlife species, some of which have shown maintenance host potential including African buffalo, Kafue lechwe (*Kobus leche kafuensis*, an African antelope species), Eurasian badger, brush-tailed possum, wild boar and white-tailed deer (Palmer et al. 2012; de Lisle et al. 2001; De Garine-Wichatitsky et al. 2013). The zoonotic risk of infection for humans is low as they normally do not get into close enough contact with infected wildlife. An exception are hunters, wildlife veterinarians, ecologists and other professionals who may be subjected to occupational exposure.

(ii) Drivers and Modes of *Mycobacterium bovis* Transmission to Humans Zoonotic TB is nowadays often regarded as a disease of the past, especially in the developed world. This is mainly due to its sharp decline after the implementation of control measures for BTB in the cattle population in developed countries during the twentieth century. The few human cases recorded in recent decades could be mostly linked to a history of living in a country lacking BTB control measures. This not only illustrates the connection between zoonotic TB in humans and BTB in cattle, but it further implies that the risk of exposure to zoonotic TB is positively correlated with the BTB prevalence and thus indirectly to the presence and efficacy of disease control measures in cattle.

It is a well-accepted fact that historically contaminated milk and dairy products constituted the biggest risk factor in human infection with *M. bovis*. Numerous published reports from around the world have provided ample evidence for the dissemination of *M. bovis* in milk. In severely affected herds up to 50% of the cattle shed bacilli, but milk producers are generally unaware of the infection in their herds as the appearance of raw milk containing *M. bovis* is initially indistinguishable from uninfected milk and infected dairy cattle in most cases do not show obvious or typical clinical signs. Highly sensitive PCR based detection methods have been able to demonstrate that shedding of *M. bovis* in milk occurs not only in skin test positive, but even in skin test-negative cows, questioning the suitability of standard skin testing for the identification of infected cattle (Miller and Olea-Popelka 2013; Zarden et al. 2013; Srivastava et al. 2008; Zumarraga et al. 2012; Schwedler 1982; Aydin et al. 2012; Ben Kahla et al. 2011; Franco et al. 2013).

In processed milk samples such as boiled and fermented milk the survival of *M. bovis* and *M. tuberculosis* depends on the infectious dose which is greatly reduced and ultimately destroyed under the influence of lactobacilli and possibly temperature and pH. Different investigators showed that spiked naturally soured milk contains viable *M. tuberculosis* and *M. bovis* after a storage period of 5–14 days (Dormer et al. 1953; Michel et al. 2011).

Within the European Union the percentage of BTB positive herds per country ranged from 0 to a maximum of 9% in the United Kingdom in 2011. The rate of zoonotic TB, however, was not clearly associated with the BTB status in the same countries. This could at least partially be attributed to the fact that over half of the infected humans diagnosed in BTB free countries originated from another country where they contracted *M. bovis* prior to immigration. In other cases zoonotic TB could have come from infected cattle or other species present in officially tuberculosis free countries (European Food Safety Authority 2013; Schoning et al. 2013).

In contrast, the UK had the highest percentage of existing skin test positive herds in the EU during 2011, yet the case load in humans was low compared to other countries in the EU. Based on the premises that the occurrence of *M. bovis* in humans essentially depends on the presence of the pathogen in the associated cattle population, one might expect that a high disease burden in cattle would fuel the zoonotic transmission to humans. This has indeed been illustrated in Mexico where the high prevalence of latent tuberculosis infection among dairy farm workers in some parts of the country was attributed to the persistently high *M. bovis* infection rates in dairy cattle. Several studies have furthermore shown an epidemiological link between the consumption of Mexican cheese produced from unpasteurised milk and the case load of zoonotic TB among persons of Hispanic ethnicity living in the US. On the other hand, the infection rates found among non-Hispanic US-born TB patients and US cattle herds have been very low. (Portillo-Gomez and Sosa-Iglesias 2011; Rodwell et al. 2010; Milian-Suazo et al. 2010; Hlavsa et al. 2008).

Against this background and considering the soaring impact of zoonotic tuberculosis evident during the pre-pasteurisation age in Europe, it may be concluded that the large scale elimination of contaminated milk from the human food chain by pasteurisation is the most effective preventive measure for zoonotic tuberculosis (Myers and Steele 1969). It does not mean that either repeated or incidental occupational exposure to infectious aerosols from tuberculous animals, whether dead or alive, should be ignored. The zoonotic risk from these sources for the individual is difficult to assess as both severely and persistently or early and intermittently excreting animals may transmit a large dose of bacteria at a given time. It is, however, assumed that infected animals with severe disease are more effective shedders of *M. bovis* than early cases with small microscopic lesions (Kao et al. 2007; Costello et al. 1998; Cross et al. 2000; de la Rua-Domenech 2006).

Several studies identified occupational exposure as the highest risk factor for *M. bovis* infection among TB cases in humans. Occupational zoonotic TB via direct contact has been most frequently reported in livestock owners and traders, dairy farm and abattoir workers and less frequently in veterinary personnel and animal handlers in zoological collections (Cordova et al. 2012; Boukary et al. 2012; Dinkla

et al. 1991; Robert et al. 1999; Twomey et al. 2010; Adesokan et al. 2012; Gumi et al. 2012a; Dalovisio et al. 1991; Stetter et al. 1995; Firdessa et al. 2012). The risk of transmission appears to be higher where livestock and humans share poorly ventilated air space or (Torres-Gonzalez et al. 2013; Mfinanga et al. 2003). Once infected, humans can, in rare cases, transmit the disease back to cattle (Fritsche et al. 2004).

Alternatively, aerosol-derived *M. bovis* infection can be acquired through close contact with infected companion animals, whereby cats are considered to have a higher risk of contracting *M. bovis* from ingesting contaminated milk, raw meat or viscerals, and also because they often live and sleep in the same rooms with their owners. It should be noted, however, that this risk is hypothetical and only very little evidence is available in the literature (Wilkins et al. 2008; Shrikrishna et al. 2009; Une and Mori 2007).

An equally low risk of transmission has been associated with hunting activities both directly when carcasses are opened up and indirectly when meat of wild animals is consumed (Michel et al. 2010; Wilson et al. 2009).

Overall the zoonotic risk of *M. bovis* transmission during incidental occupational or recreational exposure to aerosols from infected animals is presumably very low in countries where generalised tuberculosis disease in cattle is effectively minimised through a functioning BTB control or eradication scheme.

The situation is profoundly different in developing countries which are mostly unable to afford expensive test and slaughter schemes. Consequently 85% of the cattle and 82% of the human population in Africa co-exist in areas where BTB is either only partly controlled or not controlled at all (Cosivi et al. 1998; Michel et al. 2010). As a result BTB prevalences in these countries are high or at best unknown with many adverse implications on human health and human livelihoods on a broader scale (WHO 2009). For this reason the consumption of unpasteurised milk is regarded as the most significant health risk in countries where no BTB control is applied (Torres-Gonzalez et al. 2013; Michel et al. 2010; Miller and Olea-Popelka 2013; Boukary et al. 2012; Ayele et al. 2004). This situation cannot easily be changed as for some communities pasteurised milk may either not be available, accessible or too expensive. Other reasons stem from socio-cultural practices guiding firm traditions associated with the consumption and processing of fresh milk, but also of meat including high risk organs, and blood. Taste preferences and misperceptions about the nutritional characteristics of pasteurised milk are other widespread reasons (Cosivi et al. 1998; Hegarty et al. 2002; Mfinanga et al. 2004; Hambolu et al. 2013; Dlamini 2013).

The advent of the HIV/AIDS pandemic has raised questions among public health specialists and researchers about the potential negative impact of the widespread immunosuppression on the transmission of *M. bovis* to and between humans. This is of particular concern in many African countries which have among the highest HIV and *M. tuberculosis* infection and co-infection rates and where no BTB control measures in cattle are applied (WHO. http://www.afro.who.int/en/clusters-a-programmes/dpc/acquired-immune-deficiency-syndrome/aids-country-profiles.html and http://www.afro.who.int/en/clusters-a-programmes/dpc/tuberculosis/tub-country-profiles.html).

Some of these countries have a further BTB source in wildlife and thus an added risk from bushmeat hunting and consumption (WHO) (Cosivi et al. 1998; Amanfu 2006; Etter et al. 2006). No detailed assessment of the burden of zoonotic TB has been done and there is uncertainty about its role in the global human TB epidemic. Preliminary investigations point towards a higher relative risk for HIV co-infected patients in some countries, while other studies could not find confirmation for an association between zoonotic TB and the HIV status (Muller et al. 2013; Park et al. 2010).

A true assessment of the *M. bovis* related burden of disease in humans is potentially flawed by the limitations of diagnostic tools. Sputum microscopy and conventional culture systems without supplementation to enhance growth of *M. bovis* are diagnostic approaches well suited for *M. tuberculosis* but not for differentiation of zoonotic tuberculosis. The recently demonstrated negative effect of HIV co-infection on the diagnosis of *M. tuberculosis* patients by interferon gamma release assays could also pose a challenge for data collection in surveys on zoonotic TB (Michel et al. 2010, Oni et al. 2012).

(iii) Public Health Impact of *Mycobacterium bovis* Historically the impact assessment and control of zoonotic TB has been influenced by different entities such as physicians (mainly paediatricians), veterinarians, consumers, milk producers and regulatory authorities at municipal and national levels. Before World War I milk-borne tuberculosis was often seen as a bacteriological and environmental problem which called for better hygiene and sanitation. Some critics therefore mistrusted the early plans for pasteurisation as control measure (Jones 2004).

Before the introduction of BTB eradication programmes in cattle and milk pasteurisation a significant percentage of human tuberculosis cases in Europe and other affected regions were caused by *M. bovis*. Myers and Steele (1969) reported that 66 % of tuberculosis in children was caused by *M. bovis* (Myers and Steele 1969).

Following the implementation of BTB control schemes the sharp decline in bovine cases diminished the previously devastating public health problem to a rare disease in humans. The near disappearance of zoonotic TB caused physicians to lose the awareness and vigilence for the disease.

Despite the successful interventions in developed countries zoonotic tuberculosis has re-emerged in a number of them for various reasons. Most importantly, the spillover of *M. bovis* from wildlife reservoirs, namely badger, wild boar, brushtailed possum and deer has triggered an upward trend in the BTB prevalence in cattle accompanied by a small, but noticeable increase in human cases in affected European countries, New Zealand and the US (Michigan) (Palmer et al. 2012; European Food Safety Authority 2013; de la Rua-Domenech 2006; Wilson et al. 2009; Baker et al. 2006; Rodriguez et al. 2010; Food Safety Authority of Ireland 2008).

In 2012 and 2013, officially bovine tuberculosis free countries such as Belgium, Switzerland, Austria and Germany have experienced sporadic outbreaks of BTB in cattle which may have been introduced and persisted under relaxed surveillance activities. Although the risk for zoonotic transmission is very low, undiagnosed bovine cases progressing to advanced stages of disease may present a source of

infection in isolated cases as reported in Germany, The Netherlands, Austria and Switzerland (Schoning et al. 2013), (www.promed.org).

On the other hand, the bulk of the zoonotic TB load in low incidence or BTB free countries has been attributed to immigrants from countries with a high BTB burden in cattle or to the re-activation of latent *M. bovis* in older patients who have contracted the infection before the implementation of effective BTB control measures (European Food Safety Authority 2013; Larsen et al. 2008; Jalava et al. 2007; Majoor et al. 2011; Kolditz et al. 2010). The localisation of lesions in patients with tuberculosis following reactivation of latent TB is different from the primary manifestation of disease decades earlier. Partially as a result of this the proportion of pulmonary compared to extra-pulmonary cases of zoonotic TB has increased in recent decades. Although these findings are certainly also a reflection of the low incidence of foodborne *M. bovis* infections in those countries, the difficulties and challenges in diagnosing extra-pulmonary TB should not be underestimated (Cordova et al. 2012; Robert et al. 1999).

Overall it has been stated that zoonotic TB is a rare disease in the EU with a total number of 132 cases reported in the year 2011 and no observed trend. The proportion of zoonotic TB cases among the total number of confirmed TB cases in human ranged from 0 to 1.9%.

Outside Europe the situation is much more variable. The reported percentage of zoonotic TB among human TB cases was 0.34% in China, between 0 and 2.5% in Latin America, between 0.4% and 6.4% in Egypt (Grange) and 5.3% in a first investigation in Turkey. Some regionally based studies in Africa found that up to 30% of human TB cases were caused by *M. bovis*, while the overall median value for 13 African countries was 2.8%. Based on these figures a recent study estimated the burden of zoonotic TB in these African countries at 7/100,000 population per year. Small sample sizes and associated errors, bias in sample selection, underdiagnosis of zoonotic TB and general scarcity of laboratory data do not allow a more accurate assessment of the zoonotic TB incidence at present. Nevertheless, the currently available data indicate that on a global level zoonotic TB is probably negligible in humans compared to *M. tuberculosis*. However, in certain settings and population groups the consequences of *M. bovis* infection are substantial and must not be underestimated. Among the most serious implications is an apparently higher mortality rate among TB patients suffering from *M. bovis* than among those infected with *M. tuberculosis* which was found by several investigators in different countries (Cordova et al. 2012; Muller et al. 2013; Portillo-Gomez and Sosa-Iglesias 2011; Rodwell et al. 2008).

In poor and marginalised communities with limited access to veterinary and health services the implications of BTB and zoonotic TB go far beyond losses in animal production and disease diagnosis in humans. The added societal implications center around the role of livestock in daily livelihoods and wellbeing. For this reason BTB was declared a neglected zoonosis by the WHO and other international organisations concerned with human health and wellbeing (WHO 2009).

36.5.1 Epidemiology of Mycobacterium tuberculosis *Infections at the Animal/Human Interface*

Humans serve as reservoir host for *M. tuberculosis* from which spillover to a wide range of animal species, both domesticated and wild, has been reported. Experience has shown that the risk for spillover in wildlife increases with their proximity to humans and their civilisation. In captive wild animals the extremely close proximity and the opportunity for repeated exposure to *M. tuberculosis* from humans under often poor hygienic conditions have led to devastating losses among exotic animals in zoos and non-human primate colonies in medical research facilities worldwide. Today *M. tuberculosis* continues to be a significant cause of disease and death among zoo animals and domesticated Asian elephants (Michel et al. 2003, 2013; Montali et al. 2001; Griffith 1928; Angkawanish et al. 2010).

In developing countries with a high human TB burden not only captive, but free-ranging wildlife are under increasing pressure of *M. tuberculosis* spillover due to ecologic changes of anthropogenic origin. For example, vervet monkeys and chacma baboons are becoming increasingly habituated in the vicinity of human settlements in South Africa and were shown to contract *M. tuberculosis* possibly through their scavenging behaviour (Michel et al. 2013). Recently, the infection was diagnosed in a free-ranging African elephant in Kenya, whereby the source of infection remained unkown as it had spent part of its life in an orphanage (Obanda et al. 2013).

The zoonotic risk through spillback of *M. tuberculosis* infection from animals has not been systemically investigated, but information available in the literature provides circumstantial evidence which justifies a public health concern. Currently reports of spillback from wild animals to humans involve primarily captive elephants in the US, whereby the global situation may be biased by difficulties experienced in high incidence countries to prove the direction of transmission (Oh et al. 2002a; Murphree et al. 2011; Michalak et al. 1998). It should be noted that in cases of occupational exposure or where game is regularly harvested for human consumption the risk of *M. tuberculosis* transmission to humans may be considerably higher than for *M. bovis*.

The potential for spillback from cattle has not received much attention in the past for obvious reasons. At the turn of the nineteenth century Robert Koch and others first investigated the transmissibility of *M. tuberculosis* to cattle and found that cattle could be infected by various routes, but did not show any pathological signs. They subsequently concluded that *M. tuberculosis* was avirulent for cattle. This view has been confirmed by numerous authors who reported immune responses and isolation of the causative organism in the absence of tuberculous lesions (Koch 2013; Lesslie 1967; Krajewska et al. 2012). Nevertheless, the isolation of a significant number of *M. tuberculosis* from cattle can be used as a sentinel for human infection and warrants investigation (Srivastava et al. 2008; Food Safety Authority of Ireland 2008).

In the more recent past, granulomatous lesions indistinguishable from those caused by *M. bovis* have been reported especially in countries with a high human TB burden (Chen et al. 2009; Sulieman and Hamid 2002). The prevalence of *M. tuberculosis* in herds also appears to be higher in developing countries than in Europe (Pavlik et al. 2003).

It has been suggested that the genotype of the *M. tuberculosis* strain, the immune status and response of the animals as well as the TB burden in the human population may determine the virulence and outcome of *M. tuberculosis* infections in cattle (Whelan et al. 2010; Ocepek et al. 2005). Host-mycobacterial co-adaptations resulting in host-pathogen specificity for tuberculosis as reported by Di Pietrantonio and Schurr, may further contribute to the disease outcome (Di Pietrantonio and Schurr 2013).

Multidrug resistant *M. tuberculosis* strains currently pose an insurmountable challenge in the fight against human tuberculosis. Drug and multidrug resistance has also been reported in *M. bovis* strains isolated both from humans and cattle (Cordova et al. 2012; Kolditz et al. 2010; Cavirani et al. 2003; McLaughlin et al. 2012; Silaigwana et al. 2012). Transmission of such strains (*M. tuberculosis* and *M. bovis*) to humans opens the possibility of their dissemination into the human food chain via unpasteurised milk, namely in developing countries where milk is largely consumed untreated (Sechi et al. 2001). If this trend is widespread and resistance studies are not performed, there is a potential to mistreat patients and propagate multidrug resistant MTBC strains.

Mixed infections in humans with both *M. bovis* and *M. tuberculosis* have been reported and denote the prospect of zoonotic transmission (Jain 2011; Silva et al. 2013). Co-infections are caused by regular contact with a person suffering from active tuberculosis. In most of these cases the prevalence of *M. bovis* was found to be higher than that of *M. tuberculosis*. This may indicate that animal-to-animal transmission is more effective for *M. bovis* than for *M. tuberculosis*, while the opposite is true for person-to-person transmission (Srivastava et al. 2008; Majoor et al. 2011; Romero et al. 2011; Prasad et al. 2005; Bilal et al. 2010; Thakur et al. 2012; Chu et al. 2012).

In other domestic animals, *M. tuberculosis* has been reported as a sporadic infection with potential, but very rare, spillback to humans in dogs, cats (Aranaz et al. 1996; Erwin et al. 2004; Posthaus et al. 2011; Parsons et al. 2012), camels (Gumi et al. 2012a), goats (Wernery and Kinne 2012), pigs, (Arega et al. 2013) deer (Chu et al. 2012),

36.5.2 Epidemiology of Other Members of the Mycobacterium tuberculosis *Complex at the Animal/Human Interface*

M. caprae has been reported as a cause of tuberculosis in humans in several countries (Hansen et al. 2012; Goh et al. 2006; Mostowy et al. 2004; Muller et al. 2013; Bayraktar et al. 2011a; Bayraktar et al. 2011b). Other animal adapted MTBC

members are less frequently involved in the development of zoonotic tuberculosis. The general scarcity of data has led to a misperception that these organisms are host specific.

M. orygis has been mostly isolated from wild bovids and recently human-to-cow transmission was reported. The organism may be more common than currently anticipated, but misdiagnosed in humans and animals, depending on the diagnostic approach used for identification, which warrants careful investigations of *M. tuberculosis* complex isolates at the wildlife/livestock/interface (van Ingen et al. 2012; Dawson et al. 2012; Gey van Pittius et al. 2012).

M. microti occurs widespread in small rodents and sporadic in other mammals, including pigs, cats, dogs and camels, but the true host spectrum and prevalence is unknown. Human infections and disease are considered rare and of low virulence (Frank et al. 2009; Emmanuel et al. 2007; Taylor et al. 2006; Geiss et al. 2005; Deforges et al. 2004; Jahans et al. 2004; Oevermann et al. 2004).

Several animal keepers contracted *M. pinnipedii*, presumably by aerosol, from sea lions in a zoo in The Netherlands (Kiers et al. 2008).

36.5.3 Molecular Tools in Epidemiological Investigations

The development of genetic typing techniques for *M. bovis* and *M. tuberculosis* strains has opened the opportunity to study the molecular epidemiology of tuberculosis in both humans and animals. Techniques most frequently used are spoligotyping, restriction fragment length polymorphism (RFLP) and mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing (Supply et al. 2000; Frothingham and Meeker-O'Connell 1998; Durr et al. 2000).

Trace-back and trace- forward investigations using the genetic fingerprint of isolates have been very successfully used in human and veterinary medicine to control tuberculosis, resepctively. In the veterinary field outbreak investigations with stamping out of infected herds form the backbone of BTB control and were greatly facilitated by genetic typing technologies. Especially the possibility to trace animals, to determine the source of an outbreak in obscure cases and to follow epidemiologically linked herd contacts has boosted the efficacy of disease control and prevention strategies worldwide.

Where human/livestock/wildlife interactions led to the transmission and spread of MTBC to multiple species including zoonotic TB, genetic typing provided a valuable tool to generate evidence for and study inter-species and transboundary transmission of MTBC strains (Dawson et al. 2012; Michel et al. 2013; Milian-Suazo et al. 2010; Adesokan et al. 2012; Vosloo et al. 2001; de Garine-Wichatitsky et al. 2010; Michel et al. 2009; Deresa et al. 2013; Etchechoury et al. 2010; Milian-Suazo et al. 2010b; Moonan et al. 2009; Kazwala et al. 2006; Gibson et al. 2004; Michel and Huchzermeyer 1998).

36.6 Conclusions

According to the Global Tuberculosis Control WHO report 2009, there were 9.27 million new cases of tuberculosis in 2007 and tuberculosis in humans has been declared a global emergency. In view of this staggering health situation zoonotic tuberculosis caused by animal-adapted members of the MTBC seem to play a negligible role today.

However, it must not be forgotten that the achievements in reducing zoonotic tuberculosis to a rare disease in previously endemic countries are the result of radical, long-term BTB control and public health strategies which required extraordinary financial investments and human resources. In countries unable to commit to these requirements BTB in animals and zoonotic tuberculosis in humans remain veterinary and public health burdens. The risk of zoonotic tuberculosis is likely to increase with the level at which traditional behaviour governs consumption of untreated milk, meat including bush meat and other animal products from animals infected with *M. bovis*, *M. tuberculosis* or other members of the MTBC.

In addition, zoonotic TB can be the result of exposure to aerosols from *M. bovis* infected animals or rarely humans. Lastly, *M. tuberculosis* spillover to animals has the potential to become an increasing cause of tuberculosis in domestic and wild animals in high TB burden countries and spillback to humans cannot be ruled out in the long term.

In summary, tuberculosis is a multi-species infection which can cross species barriers with relative ease. From a public health perspective it warrants control in all species in order to prevent a negative impact on human health.

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Chapter 37
Hepatitis E: The Commonest Viral Zoonosis Worldwide?

Harry R Dalton, Jacques Izopet, Malcolm Banks, Richard Bendall and Nassim Kamar

Abstract Until recently, HEV was thought not to occur in developed countries. It is now clear that locally acquired HEV is common in many developed countries. HEV infection acquired in these areas differs from that in developing countries in a number of important aspects: it is caused by genotype 3 (and 4 in China and Japan); it mainly affects middle-aged/elderly males; it is zoonotic with a porcine primary host. Pig herds worldwide are infected with HEV genotype 3 and HEV has been found in the human food chain in a number of developed countries. However, the route of transmission is not fully understood, since most cases are not obviously associated with pigs/pig products. HEV can be transmitted by blood transfusion and surprisingly high numbers of asymptomatic blood donors are viraemic at the time of donation.

Our understanding of the clinical phenotype of HEV infection in humans has undergone a sea-change in recent years. Previously, HEV was thought to cause only acute self-limiting hepatitis. However, HEV may cause persistent disease in the immunocompromised. Patients with chronic HEV infection have no symptoms, but some develop rapidly progressive liver cirrhosis. The full clinical spectrum of HEV is still emerging. HEV has important extra-hepatic manifestations, which deserve further investigation. For example, HEV can cause a wide range of neurological illness. In particular, very recent data suggests that Guillain-Barré syndrome and neuralgic amyotrophy are associated with locally acquired HEV in approximately 5% and 10% of cases respectively.

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37.1 Overview of Virology of HEV

HEV is a small non-enveloped virus with an icosahedral capsid and a size of 27–34 nm. The virus has a positive-sense, single-stranded RNA 7.2-kilobases-long genome which is capped and polyadenylated at the 5' and 3'-termini, respectively (Reyes et al. 1990; Tam et al. 1991). The HEV genome contains three open reading frames (ORF). ORF1 encodes a protein of 1693 amino acids containing functional domains present in the non-structural proteins of other positive-stranded RNA viruses (Koonin et al. 1992). These functional domains include methyltransferase, cysteine protease, RNA helicase and RNA-dependent RNA polymerase. ORF2 encodes the viral capsid protein of 660 amino acids that is responsible for virion assembly (Li et al. 1997), interaction with target cells (He et al. 2008; Kalia et al. 2009), and immunogenicity (Xing et al. 2011). The ORF2 protein consists of three linear domains, the shell domain (S) (amino acids 129–319), the middle domain (M) (amino acids 320–455), and protruding domain (P) (amino acids 456–606) harbouring the neutralizing epitope(s) (Guu et al. 2009; Tang et al. 2011; Xing et al. 2010; Yamashita et al. 2009). ORF3, which overlaps ORF2, encodes a small protein of 113 or 114 amino acids involved in virion morphogenesis and release (Emerson et al. 2010; Yamada et al. 2009). HEV replicates in the cytoplasm, with a subgenomic RNA producing OFR2 and OFR3 proteins and the full genomic RNA encoding non-structural proteins and serving as a template for replication (Fig. 37.1). Current data indicate that ORF1 protein is not subject to proteolytic processing (Perttila et al. 2013).

HEV belongs to the genus *Hepevirus* in the *Hepeviridae* family (King et al. 2011). This family contains HEV variants infecting humans and several animals including mammals, birds and fish. While avian strains and cutthroat trout virus cannot be transmitted to humans, several HEV variants infecting mammals such as domestic pigs, wild boar, deer and rabbit are causative agents of zoonoses (Izopet et al. 2012; Meng et al. 1997; Sonoda et al. 2004; Tei et al. 2003). Other HEV variants infecting mammals such as rats (Johne et al. 2010), ferrets (Raj et al. 2012) and bats (Drexler et al. 2012) have not been found in humans.

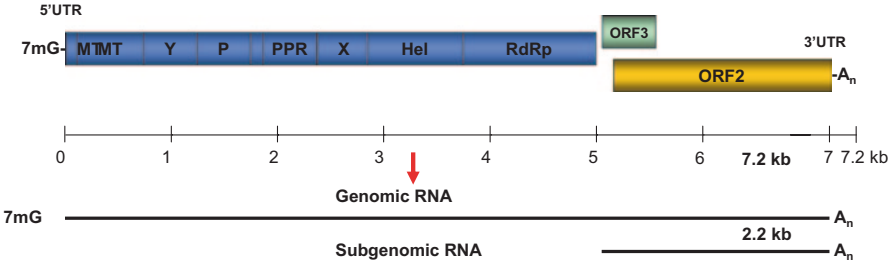

Fig. 37.1 HEV genome. HEV particles contain a positive-sense genomic RNA of 7.2-kilobases that is capped and polyadenylated. During genome replication a full *genomic RNA* encoding non-structural proteins and a *subgenomic RNA* producing *ORF2* and *ORF3* proteins are produced. Abbreviations: *MT* methyltransferase, *Y* domain, *PPR* polyproline region, *X* domain, *Hel* Helicase, *RdRp* polymerase

Based on complete genome and ORF1/ORF2 amino acid sequences, 3 groups of mammalian HEV have been described: HEV variants infecting humans, pigs, wild-boar, deer and rabbit, those infecting rats and ferrets, and those from bats (Smith et al. 2013). The first group contains the four major HEV genotypes (genotype 1–4) and new genotypes from wild-boar and rabbit. HEV genotype 1 and genotype 2 are restricted to humans and transmitted via contaminated water in developing countries from Africa, Asia, and in Mexico. Both HEV genotypes 3 and 4 infect humans, pigs and other mammalian species and are responsible for sporadic cases of autochthonous hepatitis E in both developing countries and industrialized countries. HEV genotype 3 has a worldwide distribution (Dalton et al. 2008a). In contrast, HEV genotype 4 is largely found in China and Japan (Lu et al. 2006), but has recently been isolated in Europe both in pigs (Hakze et al. 2011) and in humans (Colson et al. 2012; Garbuglia et al. 2013; Tesse et al. 2012).

Phylogenetic analyses indicate that HEV subgenotypes circulating in human and animals in the same area are closely related, supporting zoonotic transmission (Bouquet et al. 2011; Legrand-Abravanel et al. 2009). Studies of the evolutionary history and population dynamics of HEV indicate that HEV has evolved through a series of steps, in which the ancestors of HEV may have adapted to a variety of different animal hosts including humans during evolution (Purdy and Khudyakov 2010).

37.2 Epidemiology in Animals

Anthroponotic transmission was considered the sole route of human infection with HEV until the 1990s. The notion that hepatitis E might have a zoonotic source was suggested by the infection of pigs with human HEV by Balayan et al. (1990) and significantly strengthened by the detection of a high degree of sequence homology between HEV genotype 3 strains from humans and pigs in the USA (Meng et al. 1997; Schlauder et al. 1998). Further suspicion was aroused by the observation of autochthonously acquired cases of genotype 3 hepatitis E in developed countries without the risk factor of contaminated water present in developing regions.

Despite the absence of overt disease in pigs, reports of a high to very high HEV seroprevalence in many countries have now been published, indicating that HEV genotypes 3 and 4 are globally ubiquitous in these animals. Evidence for zoonotic transmission from pigs is now overwhelming and includes the general phylogenetic intercalation of pig and human HEV strains of genotypes 3 and 4. However, the relatively long incubation period for clinical hepatitis E means that direct proof of transmission via comparison of nucleotide sequences obtained from consumed pig products and patient samples may be achieved only very rarely.

Transmission in pigs is considered to be faecal-oral, and serological evidence indicates that most pigs are infected at or around 10 weeks of age, presumably coincident with loss of passive immunity. In strict epidemiological terms, only domestic pigs have been proven to be a true reservoir of HEV; the work of Bouwknegt and colleagues (Bouwknegt et al. 2007) demonstrated natural transmission between

pigs in a controlled environment and showed a reproduction ratio (R_0) of 8.8 (95% CI 4–19). Estimations by others of HEV R_0 in pigs have varied from 4 to 5 using seroconversion (Satou and Nishiura 2007) and 2–4 by extrapolation from field data, respectively (Backer et al. 2012). These data together indicate that HEV genotype 3 is capable of epidemic spread within naïve pig herds. Infected pig herds shed huge quantities of HEV in faeces, at least some of which reach watercourses (Rutjes et al. 2010), where it may be used as source for drinking water, recreational water use or for irrigation of crops (Dalton et al. 2008a).

Following the development of hepatitis E in a patient who ate raw wild boar liver (Matsuda et al. 2003) wild boar populations in many European, Asian, N America have been tested and shown to carry HEV genotype 3 closely aligned to human and domestic pig strains. More recently a divergent HEV line was detected in wild boar in Japan (Takahashi et al. 2011); these strains may represent a novel genotype and their zoonotic significance is as yet unclear.

The first and still one of the most compelling cases of foodborne zoonotic transmission involved the consumption of infected Sika deer meat in Japan (Tei et al. 2003). In this case frozen portions from deer meat that had been consumed by the affected family contained a HEV genotype 3 RNA sequence identical to that from the patients. Further extensive studies in Japan and in the USA, however, have indicated that the prevalence in Sika deer is so low that it is not considered to be a significant reservoir (Matsuura et al. 2007; Yu et al. 2007), but conflicting studies to some extent contradict this assumption (Tomiyama et al. 2009). Several reports of HEV genotype 3 in deer (Forgach et al. 2010; Rutjes et al. 2009; Boadella et al. 2010), the growing populations of deer in Europe, Asia and Northern America and the increasing consumption of deer meat indicate that cervids may represent the second most important reservoir for zoonotic HEV transmission after suids.

Rats have long been speculated to be potential reservoirs of HEV, largely because of their omnipresence near humans and the detection of antibodies against HEV in earlier studies (Kabrane-Lazizi et al. 1999). However, details of RNA sequence were lacking until recently when Johne et al. (2010) demonstrated HEV RNA from Norway rat tissues from Germany. The rat HEV was related to, but divergent from genotype 3 human/pig strains, indicating a lower potential for zoonotic transmission.

The first reports of rabbits naturally infected with HEV emerged from farmed rabbits in Gansu province, China in 2009 (Zhao et al. 2009). Subsequently, there have been reports of HEV antibody and RNA from farmed and feral rabbits in China, the USA (Cossaboom et al. 2012) and France (Izopet et al. 2012). In all cases reported to date, the genotype of the rabbit strains has < 80% homology to that of the established genotypes 1–4, leading to the suggestion that these strains should be assigned to a new genotype (Ma et al. 2010; Geng et al. 2011). Although clearly in a separate clade from genotypes 1–4, the rabbit strains identified to date show considerable genetic heterogeneity, and there is evidence of sympatric clustering. In terms of clinical disease, there have been no reports of significant gross pathology in rabbits infected with HEV, but, as with pigs and other non-human mammals, there is histopathological evidence of mild to moderate focal hepatitis, with some elevations in alanine transaminase (ALT) plasma levels.

The zoonotic potential of the rabbit HEV was suggested by significant antigenic cross-reactivity with genotypes 1–4 and by the work of Geng et al. (2011), who showed putative zoonotic markers in the rabbit HEV genome. This was strengthened by experimental cross-infection of pigs with rabbit HEV (Cossaboom et al. 2012). However, more recent studies (Geng et al. 2013) showed that there was no evidence of cross-species circulation of HEV in a study of wild rabbits and pigs living in close proximity in Hebei province, China. A single HEV strain isolated from a human with elevated ALT has been shown to cluster with the rabbit HEV strains (Izopet et al. 2012) and, whilst there is yet no evidence to support significant circulation of rabbit HEV in humans, continued surveillance is recommended.

Other mammalian species including horse (Saad et al. 2007), mongoose (Li et al. 2006), ferret (Raj et al. 2012) and bats (Drexler et al. 2012) have been shown to carry HEV, but at this point in time they are not considered of major significance or potential in the zoonotic transmission of HEV. Likewise sheep, cattle, goats, dogs and cats may have antibodies against HEV, but again are considered of minor significance in HEV epidemiology. Avian HEV, although sharing epitopes with mammalian HEV, is clearly distinct genetically (Payne et al. 1999), with approximately 50–60 % homology to HEV genotypes 1–4, and is not perceived to represent a reservoir for zoonotic transmission of HEV. Shellfish in sewage deposition areas have been implicated as physical vectors of HEV genotype 3 (Pina et al. 1998), but the cut-throat trout HEV described recently (Batts et al. 2011) is highly divergent from the zoonotic HEV genotypes.

37.3 Epidemiology in Humans

In developing countries, hepatitis E is a waterborne, non-zoonotic, infection transmitted between humans and caused by HEV genotypes 1 and 2. It causes sporadic cases with occasional and dramatic outbreaks in areas with poor sanitary infrastructure, including conflict zones and refugee camps. Outbreaks of hepatitis E are more likely at times of flooding, for example during the monsoon season. Cases typically occur in the 15–30 years age range. Seroprevalence studies show that in endemic areas the seroprevalence in children < 15 years is very low. It rapidly increases between the ages of 15–30 years and then plateaus (Labrique et al. 2010). These observations are unexplained and are in contrast to HAV seroprevalence which shows evidence of near universal infection during childhood. In developing countries zoonotic hepatitis E probably does occur, since HEV genotypes 3 and 4 are present in pigs and other mammals in these areas. However, the presence of zoonotic disease is probably obscured by the large numbers of infections due to HEV genotypes 1 and 2 circulating in humans in these communities. In developing countries that have seen significant improvements in sanitary infrastructure in recent years the epidemiology of hepatitis E is changing, and zoonotic infections are starting to emerge. A good example of this is eastern China, which has seen a shift from HEV genotype 1 to zoonotic genotype 4 as the predominant cause of human infections over the last 10–15 years (Kamar et al. 2012b).

The published literature regarding anti-HEV IgG seroprevalence is problematic. Early studies showed very low seroprevalence estimates in the 1–4% range (Bendall et al. 2010). These studies are flawed, because they employed assays of poor sensitivity and therefore may have underestimated the seroprevalence. For example, in a study using an insensitive anti-HEV IgG assay the seroprevalence in southwest England was 3.6%, but rose to 16% when a more sensitive assay was employed (Bendall et al. 2010). As more sensitive assays have been used, a clearer picture of seroprevalence is starting to emerge, with much higher estimates than previously. Thus seroprevalence rates of 25% in the Netherlands (Hans Zaaijer, personal communication), 29% in Germany (Wenzel et al. 2013), and 52% in southwest France (Mansuy et al. 2011) have now been recorded. The latter results suggest that HEV is hyperendemic in the Toulouse area of France. This is consistent with the large number of cases documented in the region and the high incidence of infection documented by molecular techniques in the Toulouse transplant population (Legrand-Abravanel et al. 2011).

The seroprevalence and incidence of hepatitis E vary according to geographical location. For example, in the UK the seroprevalence is 16% in south west England (Dalton et al. 2008b), 12% in the rest of England (Beale et al. 2011), and 4.6% in Scotland (Juraj Petrik, personal communication). In addition, the seroprevalence in the south of France is four times higher than that found in the north of France (Boutrouille et al. 2007). Why seroprevalence varies in this way is not well understood. In the UK the annual incidence has been estimated to be 0.2% (Beale et al. 2011) and in the USA (Faramawi et al. 2011) it is calculated to be 0.7%. The latter figure suggests that in the USA each year 2.1 million infections with HEV occur. This figure contrasts with the very small number of autochthonous cases that are detected in the USA (Drobeniuc et al. 2013). One important reason for the discrepancy is that currently in the USA there are no diagnostic assays that are approved for use in humans, therefore symptomatic cases are not diagnosed.

The source and route of infection of most cases of autochthonous hepatitis E in developed countries remains unknown, even after rigorous investigation. Possible routes of infection include (Fig. 37.2):

a. Direct transmission: via contact with infected animals and/or their faeces.
b. Direct transmission: via consumption of food products derived from infected animals, which contain viable HEV.
c. Waterborne transmission: either (i) directly via ingestion of water contaminated with faeces from infected animals; or (ii) indirectly through consumption of foods contaminated with faecally contaminated water.

Route A occurs in individuals who are occupationally exposed to pigs, as several studies have shown higher prevalence of anti-HEV IgG antibodies amongst pig farmers and veterinarians (Drobeniuc et al. 2001; Meng et al. 2002). However, only a small minority of patients with hepatitis E report a history of pig contact, and the number of people in developed countries who work with pigs is too small to explain the very high HEV seroprevalence recently documented in many of these populations.

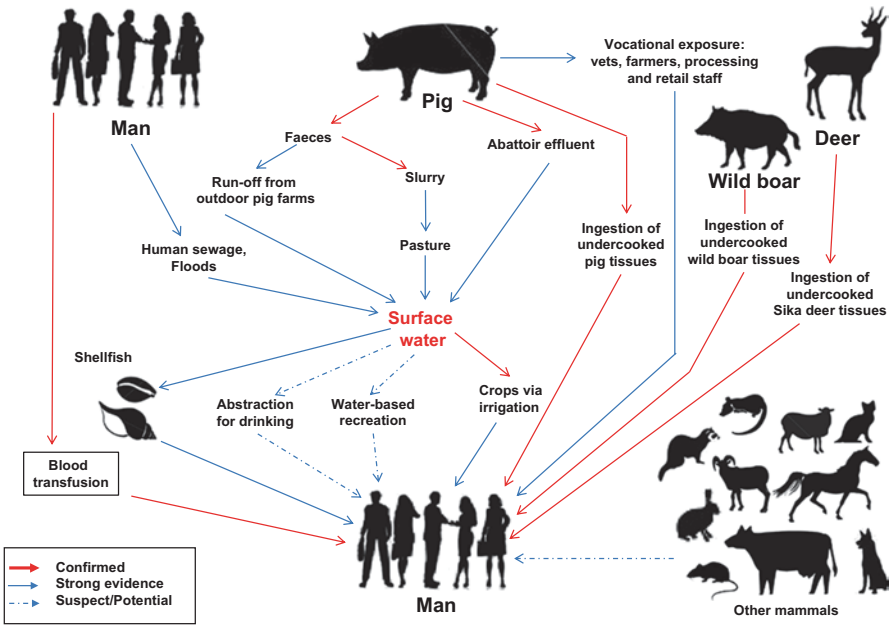

Fig. 37.2 Transmission routes of autochthonous hepatitis E in developed regions

Route B is an important and well documented route of infection, as HEV has been found in retail meat and meat products (Berto et al. 2012; Colson et al. 2010; Feagins et al. 2007; Banks et al. 2010). Small clusters of hepatitis E have occurred following consumption of raw/undercooked meat from infected animals (Matsuda et al. 2003), and some epidemiological studies have identified consumption of game or pork products as risk factors for HEV infection (Mansuy et al. 2011; Wichmann et al. 2008). Dietary differences could explain many of the differences in the incidence/prevalence of hepatitis E in different populations. However, in most individual cases a clear food source cannot be established.

Route C is, perhaps, the most interesting. In developing countries that are endemic for HEV genotypes 1 and 2, waterborne infection with HEV derived from humans is the main route of transmission. In developed countries this route is blocked because human excrement is generally separated from watercourses by sewage treatment. However, in these locations agricultural run-off containing animal faeces enters watercourses without treatment and eventually enters the sea. HEV has been documented in environmental waters, including rivers and the sea (Cossaboom et al. 2012; Ishida et al. 2012). This means that direct waterborne transmission could occur through exposure to contaminated water. Two studies from the UK have shown that cases of hepatitis E cluster in coastal areas (Ijaz et al. 2005; Madden et al. 2012). Provisional data from a case control study in Cornwall (a maritime peninsula in southwest Britain) show that, compared to controls, cases of hepatitis E are more

likely to live within 2 km of the coast. The evidence supporting indirect waterborne infection is stronger as HEV has been detected in strawberries irrigated with contaminated water (Brassard et al. 2012), HEV genotype 3 has been detected in filter-feeding shellfish (Crossan et al. 2012; Donia et al. 2012) and some human cases have been epidemiologically linked to shellfish consumption (Said et al. 2009).

37.4 Acute Hepatitis E in Humans

As previously noted, acute hepatitis E due to infection with HEV genotypes 3 and 4 is thought to be of zoonotic origin. Infection due to the zoonotic genotypes differs in several ways from the "classical" anthroponotic disease caused by HEV genotype 1 (see Table 37.1 for details). It may therefore not be possible to generalise the findings from studies in one geographic area where one genotype predominates to another where different genotypes predominate. As far as possible, the following description relates to infection with the zoonotic genotypes. The main route of acquisition for hepatitis E is thought to be oral. Many studies have demonstrated clear evidence of infection from contaminated meat (Tei et al. 2003; Colson et al. 2010; Tamada et al. 2004) and as documented elsewhere, HEV genotype 3 is widespread in wild and domestic pigs (Meng et al. 1999). Bloodborne infection through transfusion is also recognised (Matsubayashi et al. 2004; Boxall et al. 2006; Matsubayashi et al. 2008) and seroprevalence is high among those working with pigs (Drobeniuc et al. 2001; Meng et al. 2002) suggesting that direct zoonotic infection by the faeco-oral route occurs. Risk factors for acute HEV genotype 3 infection include male sex, increasing age, alcohol consumption and pre-existing liver disease (Dalton et al. 2008b; Ijaz et al. 2005; Turner et al. 2010). The extent to which these risk factors relate to acquisition of infection rather than expression of disease (symptomatic vs asymptomatic infection) is not well understood and is the subject of on-going research. In developed countries autochthonous hepatitis E has a clear predilection for middle-aged and elderly men (mean age ~ 63 years, M:F > 3:1) (Dalton et al. 2008b; Drobeniuc et al. 2013; Mansuy et al. 2004; Dalton et al. 2007a; Mitsui et al. 2004). This is a consistent finding, but remains unexplained. Two studies have shown that hepatitis E occurs more frequently in individuals who drink excessively alcohol. One hypothesis is that subclinical hepatitis and steatosis/fibrosis (which is common in older male drinkers) might be the key host factors that lead to clinical disease expression on HEV exposure (Said et al. 2009; Dalton et al. 2011). Symptomatic hepatitis is probably not the usual outcome of acute HEV3 infection (Shata and Navaneethan 2008; Renou et al. 2011), though the evidence is indirect. For instance, during an outbreak on a cruise ship only 33 % of infections had clinical hepatitis (Said et al. 2009) and asymptomatic infection has been detected in blood donors (Guo et al. 2010; Ijaz et al. 2012). Data from the cruise ship outbreak suggests that the incubation period is around 25–40 days for acute HEV genotype 3 infection (Said et al. 2009). A prodromal illness may occur with malaise, anorexia, nausea, abdominal pain, fever and myalgia (Dalton et al. 2008b; Borgen et al. 2008).

Table 37.1 Epidemiology of hepatitis E in developing and developed countries

	HEV in developing countries	HEV in developed countries
Genotype	1 and 2[a]	3 and 4
Source of infection	Human	Zoonotic; pigs are primary host[b]
Route of infection	Faeco-oral; via infected water	Faeco-oral Direct transmission: via contact with infected animals Direct transmission: via consumption of food products which contain viable HEV. Waterborne transmission: Direct Indirect
Seroprevalence	Low in children <15 years increases rapidly 15-30 years	Steady increase throughout age groups. High seroprevalence in pig handlers and veterinarians
Incidence	Variable: 64/1,000 patient years, Bangladesh	Variable: 7/1,000 patient years, USA
Outbreaks	Yes. Can involve thousands of cases	No. Occasional small case clusters from a point food source
Attack rate	about 50 %	67–98 % are asymptomatic
Demography of cases	Young adults	Middle aged/elderly males
Infectivity	High	? Low
Person to person spread	Very limited	No
Transfusion related infection	No	Yes
Seasonality	Yes. Outbreaks occur at times of flooding/monsoon	No
Outcome in pregnancy	Mortality of 20–25 %, usually in 3rd trimester	Excess mortality has not been documented
Disease in travellers returning from endemic areas	Well described	Beginning to emerge as high risk areas become defined
Chronic infection in immunocompromised	No	Yes. Genotype 3 only

[a] In some developing countries such as southeast China, zoonotic infection with HEV genotype 4 is emerging as the main cause of hepatitis E in humans. This may be due to improvements in sanitary infrastructure which has asserted a negative ecological pressure on waterborne HEV genotype 1
[b] HEV genotype 3 and 4 have also been transmitted from human to human via infected blood products

Jaundice ensues in a proportion of patients (75 % in one study (Dalton et al. 2008b)) associated with abnormal liver function tests. The serum concentration of alanine transaminase is usually significantly elevated (mean values 1382–2021 iu/l) (Dalton et al. 2008b; Turner et al. 2010) and normalises within a month. Recovery is usually complete, without recurrence and in immunocompetent individuals chronic infection does not occur. Large outbreaks have not been seen in developed countries and intra-household spread of infection is uncommon suggesting that hepatitis E is much less contagious than hepatitis A.

The main exception to this benign pattern of infection occurs when a patient with pre-existing chronic liver disease contracts hepatitis E. In this group, the infection may precipitate liver failure and death. In a small study, 2 of 3 of such cases died (Dalton et al. 2007b). Unlike HEV genotype 1, which causes severe, often fatal infection in pregnant women, HEV-3 has not been reported to cause obstetric problems (Andersson et al. 2008). Acute infection may be associated with neurological and other extra-hepatic complications, these are detailed later. In uncomplicated infection the mortality is low, but rises with age. In two UK series (Dalton et al. 2008b; Turner et al. 2010) 4.0–7.5 % of cases died. This is almost certainly an overestimate of overall mortality, since the studies were hospital-based, the patients were older and some of the fatal cases had pre-existing liver disease. Given the preponderance of asymptomatic infections, zoonotic hepatitis E could reasonably be described as generally a mild, self-limiting illness with the risk of more serious disease in certain risk groups.

37.5 Chronic Hepatitis E in Humans

In addition to acute resolving hepatitis and fulminant hepatitis it has been shown, within the last few years, that HEV may also cause chronic hepatitis in immunosuppressed patients, i.e., in solid-organ-transplant and stem-cell-transplant recipients, hematological patients receiving chemotherapy, and individuals infected by the human immunodeficiency virus (HIV) who have a low CD4 lymphocyte count (Kamar et al. 2008; Tavitian et al. 2010; Dalton et al. 2009; Ollier et al. 2009). All cases of chronic HEV infection are related to HEV genotype 3: no case of chronic infection with genotypes 1, 2 and 4 has been reported so far. Until very recently, a diagnosis of chronic hepatitis was defined as persisting HEV replication lasting for at least 6 months. However, new data from infected organ-transplant recipients show that no spontaneous clearance of HEV occurs between 3 and 6 months after acute infection. Thus the working definition of chronic infection, at least in the transplant population, is persisting HEV viraemia for more than 3 months (Kamar et al. 2013).

37.5.1 HEV Infection in Solid-Organ-Transplant Patients

The routes of HEV transmission are similar to those observed in non-transplant patients (Legrand-Abravanel et al. 2010). Solid-organ-transplant patients often receive blood products; however, no case of HEV transmission via transfusion has been reported in this setting (Legrand-Abravanel et al. 2011). Only one case of HEV transmission via a liver-transplant has been published so far (Schlosser et al. 2012). Because of the high variability of serological assays, the seroprevalence of HEV infection among solid-organ-transplant patients cannot currently be determined

Table 37.2 Chronic HEV infection in transplant recipients

Study	Year	Country	*n*	Type of transplant	Prevalence of chronic infection (PCR + ve >6 months) (%)	HEV IgG seroprevalence (%)	HEV IgG assay employed
Abravanel et al. (2011)	2011	France	700	Liver, kidney, and kidney/pancreas	3.2	12.7	Adaltis
Abravanel et al. (Submitted)	2013	France	171	Kidney, liver	3.5	43.9	Wantai
Moal et al. (2012)	2012	France	1350	Kidney	1.2	NA	Adaltis
Pischke et al. (2010)	2010	Germany	226	Liver	0.9	4.4	Abbot
Pischke et al. (2012)	2012	Germany	274	Heart	1.5	11.3	Genelabs
Haagsma et al. (2009)	2009	Netherlands	285	Liver	1.75	3.5	Genelabs
Pas et al. (2012)	2012	Netherlands	1200	Solid organ	1	NA	Wantai
Buti et al. (2010)	2009	Spain	108	Liver, kidney	NA	2.3	Biokits

(Bendall et al. 2010) (Drobeniuc et al. 2010; Baylis et al. 2011; Carpentier et al. 2012). However, detection of HEV RNA in this population ranges from 0.9 to 3.5 % (Table 37.2).

After transplantation, the clinical and biological presentations of HEV infection differ from those in non-transplant patients: the majority of transplant patients are asymptomatic, very few patients present with jaundice, ALT levels are moderately increased (around 300 IU/L), anti-HEV IgM and IgG may be negative, and some patients may never seroconvert (Kamar et al. 2011b). Because of the latter issue, it is recommended that HEV RNA is assessed in serum and/or the stools, if HEV infection is suspected in immunosuppressed patients. In a large European multicenter study, it was observed that 60 % of solid-organ-transplant recipients infected by HEV developed chronic hepatitis and 9 % developed cirrhosis (Kamar et al. 2011b). Strikingly, in this population HEV induces very rapid progression of liver fibrosis, leading to cirrhosis (Kamar et al. 2010b, 2011b). If a liver transplant is performed in a patient with detectable HEV RNA, HEV infection can occur in the liver transplant, which may also lead to cirrhosis (Haagsma et al. 2008).

A low platelet count when HEV infection is diagnosed and the use of tacrolimus, rather than cyclosporine A, have been identified as independent predictive factors for chronic HEV infection (Kamar et al. 2011b). A specific anti-HEV response to T cells was found to be significantly lower in solid-organ-transplant patients that developed chronic HEV infection compared to those with resolving hepatitis (Suneetha et al. 2012). In addition, lower serum concentrations of IL-1 receptor

antagonist and IL-2 receptor during the acute phase of HEV infection, as well as increased concentrations of serum chemokines (implicated in leukocyte recruitment to the liver: RANTES, MCP-1, CXCL-8), have been associated with persistent infection (Lhomme et al. 2012). In addition, greater heterogeneity of HEV quasispecies has been found during the acute phase in patients who go on to develop chronic HEV infection (Lhomme et al. 2012).

37.5.2 HEV Infection in Non-Transplant Patients

A few cases of chronic genotype-3 HEV infection have been reported in HIV-positive patients (Kamar et al. 2012b), of which some have developed cirrhosis (Dalton et al. 2009). In addition, a strain that was obtained from an HIV-positive patient with chronic HEV infection was a recombinant human hepatitis E virus (Shukla et al. 2011). Chronic HEV infection has also been reported in patients with haematological malignancies (Kamar et al. 2012b).

37.5.3 Management of Chronic HEV Infection

Chronic infection with HEV should be treated in a stepwise fashion. The first step is to reduce immunosuppressive therapy that targets T-cells, as this achieves clearance of HEV in one-third of chronically infected solid-organ-transplant patients (Kamar et al. 2010b, 2011b). The second step is to consider anti-viral therapy. In a few liver-transplant patients and one hemodialysis patient pegylated-interferon-α induced a sustained virological response, i.e. undetectable serum HEV RNA for at least 6 months after anti-viral therapy had been completed (Kamar et al. 2010c; Haagsma et al. 2010; Kamar et al. 2010a). In addition, in a few small case series, a 3–6-month course of ribavirin therapy induced a sustained virological response in more than two-thirds of solid-organ-transplant patients (Mallet et al. 2010; Kamar et al. 2010d; Chaillon et al. 2011; Pischke et al. 2012; de Niet et al. 2012; Del Bello et al. 2012; Pischke et al. 2013). Interestingly, in patients who clear the virus, no HEV reactivation has been observed after retransplantation (Kamar et al. 2012a).

In hematological and HIV-positive patients with chronic HEV infection, a few case reports show that pegylated interferon-α and ribavirin (used as monotherapy or in combination) can efficiently eradicate HEV (Alric et al. 2010, 2011; Dalton et al. 2011).

37.6 HEV-Induced Extra-Hepatic Manifestations

An increasing number of HEV-induced extra-hepatic manifestations have been reported during both acute and chronic infection. Neurological manifestations, such as Guillain–Barré syndrome, Bell's palsy, neuralgic amyotrophy, acute transverse

myelitis, brachial plexopathy, and acute meningoencephalitis, have been observed during acute and chronic phases (Tse et al. 2012). They are estimated to occur in 5.5% of HEV patients (Kamar et al. 2011a).

In solid-organ-transplant patients, HEV-induced membranoproliferative glomerulonephritis and membranous glomerulonephritis, as well as cryoglobulinemia, have been observed in acute and chronic HEV infection (Kamar et al. 2012c). Severe myositis (Del Bello et al. 2012) and severe thrombocytopenia (Colson et al. 2008; Fourquet et al. 2010) have been also observed in HEV-infected patients. These manifestations have been observed with HEV genotypes 1 and 3. In addition, several cases of pancreatitis have been reported following infection with HEV genotype 1 (Sinha et al. 2003; Bhagat et al. 2008; Thapa et al. 2009; Deniel et al. 2011).

37.7 Unresolved Issues

HEV is an emerging pathogen. Though it has probably caused human disease for over 200 years (Teo 2012), it was identified only 30 years ago. Our knowledge and understanding of the virus have expanded greatly since then, but there remain many unresolved issues (see Table 37.3). For instance, the zoonotic nature of genotypes 3 and 4 has only recently become apparent, and the clinical impact in humans is still emerging. The existence of chronic infection and neurological manifestations of HEV are unexpected findings. Chronic infection has so far only been detected with zoonotic HEV infection among immunocompromised patients; we do not know, however, the extent of the problem and what the long term consequences are. When HEV presents with neurological injury, the neurological illness dominates the clinical picture, and patients frequently are anicteric. Consequently, the range and incidence of neurological illness associated with HEV may be much greater than we currently know.

Our understanding of the mechanisms of transmission of zoonotic HEV is poorly developed, so preventative measures are hard to devise. There is, however, a safe and effective vaccine available for HEV prevention (Zhu et al. 2010). It is currently only licensed for use in China. If the vaccine were approved for use in other countries it is not clear how a vaccination programme might be funded, since the countries in greatest need are amongst the poorest in the world. Control of zoonotic infection is an even more complex issue because of the very wide host range of these viruses and their considerable diversity.

If the pace of discovery that we have seen in hepatitis E research over the last 10 years is maintained, it is likely that the number of clinical syndromes attributable to HEV in humans will grow, that the range of animals infected by the virus and the effect of the virus on those animals will expand and that our appreciation of the impact of this previously obscure pathogen will deepen. It may well prove to be the commonest zoonotic viral infection worldwide.

Table 37.3 HEV: unresolved issues

Current knowledge	Unresolved Issues
Epidemiology	
HEV is associated with a range of neurological illnesses	How common is neurological injury in HEV infection?
HEV genotype 3 is hyperendemic in southern France	Are there any other areas of hyper-endemicity in developed countries?
Genotype 3 and 4 infection is more commonly seen in older men	Why?
Many seroprevalence studies have used assays of poor sensitivity and may have underestimated the seroprevalence	What are the accurate seroprevalence figures in differing geographical locations using sensitive, specific and validated assays?
Accurate estimates of incidence are few	How does the incidence of infection vary in differing geographical areas?
The incidence of asymptomatic infection is poorly documented	How common is asymptomatic infection, and what factors predispose to this?
Transmission	
HEV has been found in the environment, e.g watercourses and the sea	How widespread is the environmental contamination, and how long does HEV survive?
Several animals are reservoirs of infection	What other animals are reservoirs?
HEV genotypes 3 and 4 can be transmitted by consumption of infected meat.	Are other routes of infection important?
HEV can be transmitted by transfusion	What is the risk of transfusion, and how does this vary from country to country?
Re-infection with HEV has been documented	How commonly does this occur, and what are the consequences?
Chronic Hepatitis	
Only HEV genotype 3 causes chronic infection	Can the other genotypes cause chronic infection?
HEV causes chronic infection in transplant recipients, patients with HIV and haematological malignancy	Does chronic infection occur in individuals with more subtle defects of immunity?

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Chapter 38
West Nile Virus: From Africa to Europe, America, and Beyond

Lyle R. Petersen

Abstract Since the mid-1990s, West Nile virus (WNV) outbreaks of increased severity first appeared in Africa, then in Europe, and finally in North America. These outbreaks were due to related lineage one viruses of apparent pathogenicity. More recently lineage 2 strains also of apparent increased pathogenicity have caused outbreaks in Europe. Some of the implicated lineage 1 strains and the lineage 2 strains have a mutation in the NS3 helicase gene, which confers increased viral pathogenesis in birds. The recent pattern of sporadic cases and outbreaks of WNV that has emerged in Europe and North America shows no signs of abating. While broad areas of high risk can be identified, the sporadic, local and regional outbreaks that occur within these areas remain elusively unpredictable. Fewer than one percent of persons infected develop neuroinvasive disease, characterized by meningitis, encephalitis, or acute flaccid paralysis. Increasing age is a risk factor for neuroinvasive disease, both in humans and horses. Four WNV vaccines are currently marketed for horses and clinical trials have been conducted for two human vaccines. Treatment is supportive.

38.1 Virology

West Nile virus (WNV) is one of more than seventy viruses of the family *Flaviviridae* of the genus Flavivirus (Heinz et al. 2000). The flaviviruses comprise some of the most medically important arthropod-borne viruses, including the yellow fever, Japanese encephalitis, dengue, and tick-borne encephalitis viruses. Like other flaviviruses, WNV is an enveloped spherical-shaped virion encompassing a single-stranded RNA molecule of positive polarity of approximately 11-kb. The genomic RNA of WNV encodes three 5' structural proteins (C-prM-E) and seven nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) at the 3' end of the genome that are flanked by 5' and 3' untranslated regions involved in transcription and translation (Fig. 38.1). Both structural and nonstructural proteins are translated as a

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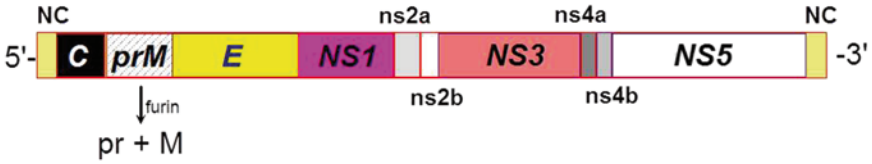

Fig. 38.1 WNV genome

single polyprotein that is cleaved into the respective viral proteins by host and viral proteases both co- and post-translationally (Chambers et al. 1990).

Serologically, WNV is a member of the Japanese encephalitis serocomplex, which includes the Japanese encephalitis, Murray Valley encephalitis and the St. Louis encephalitis viruses (Calisher 1988). Sequence analyses suggest that WNVs can be categorized into at least five phylogenetic lineages (May et al. 2011), although up to seven genetic lineages have been suggested (Vazquez et al. 2010; De Filette et al. 2012). Only lineage 1 and 2 WNVs have been associated with significant disease outbreaks in humans.

The lineage one viruses can be further subdivided into three sublineages (a–c): isolates from the Western Hemisphere, Africa, the Middle East, and Europe constitute lineage 1a; Kunjin virus from Australasia represents lineage 1b; and lineage 1c consists of viruses from India (Bondre et al. 2007; May et al. 2011). Distribution of lineage 1 most likely occurred during the past 300 years, possibly resulting from increased trade between Africa and India and Australia. The lineage 1a viruses are the most widely dispersed and epidemiologically important, having caused large outbreaks in Europe, Russia and North America during the past two decades. Lineage 1a can be further subdivided phylogenetically into several clusters, each with a relatively distinct geographic focus of circulation (May et al. 2011). Nevertheless, all but one cluster contains isolates from Africa, suggesting frequent gene flow from Africa to Europe and Russia, most likely by migrating birds.

WNV was first discovered in the Western Hemisphere during simultaneous human epidemics and avian epizootics in the New York City area in 1999 (Mostashari et al. 2001). The means and origin of the WNV brought into North America are unknown. A Middle Eastern origin was first hypothesized based on the fact that the initial North American isolates (East Coast genotype) from 1999 were most closely related to a lineage 1a WNV isolated from Israel in 1998 (Lanciotti et al. 1999). However, more recent analysis suggests that the Israel outbreak was not the progenitor of the North American outbreak, but rather that both outbreaks were initiated by the introduction of strains from the same independent location, probably Africa (May et al. 2011). The WNV strain imported into North America contain a single nucleotide change in the NS3 gene (T249P), which confers avian virulence in otherwise nonvirulent strains (Brault et al. 2007). Since approximately 2002, the East Coast genotype has largely been displaced by a new genotype (WN02) encompassing several conserved amino acid substitutions (Davis et al. 2005). This displacement may have resulted from a temperature-dependent increased efficiency

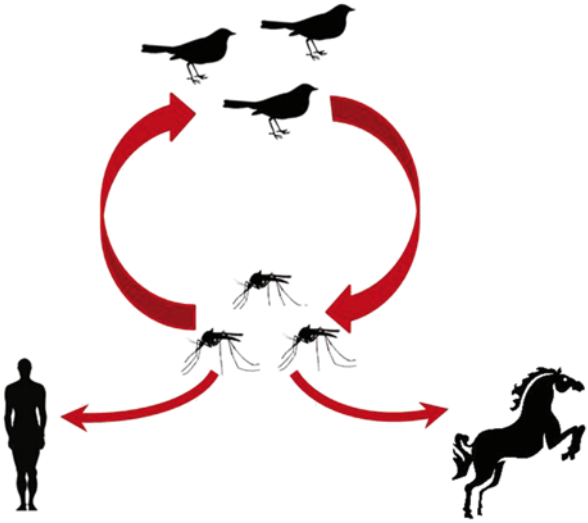

Fig. 38.2 WNV transmission cycle

and rapidity of dissemination and transmission of the WN02 virus in North American mosquito vectors (Ebel et al. 2004; Kilpatrick et al. 2008).

Only lineage 1 and 3 WNV strains had been found in Europe until 2004, when a lineage 2 strain pathogenic to birds of prey was identified in Hungary (Bakonyi et al. 2006). Before then, lineage 2 WNVs were isolated almost exclusively from African transmission cycles and were considered to be of low pathogenicity (Murgue et al. 2002). Subsequently, highly pathogenic lineage 2 WNV strains have caused both human and animal disease in South Africa (Venter 2009; Zaayman and Venter 2012) and Europe (Papa 2012; Bakonyi et al. 2013). Isolates from lineage two viruses have histidine at the 249 locus of the NS3 gene; however, isolates that have caused large outbreaks in Greece since 2010 contain a proline substitution at this locus (Papa et al. 2011a; Barzon et al. 2013b; Papa et al. 2013). Like lineage one viruses, phylogenetic analysis suggests multiple introductions of lineage two viruses into Europe from Africa (Ciccozzi et al. 2013; McMullen et al. 2013).

38.2 Transmission Cycle

WNV exists in a bird-mosquito-bird transmission cycle (Fig. 38.2). Following mating, female *Culex spp*. mosquitoes obtain a blood meal from a vertebrate host in order to obtain protein to make eggs. She may become infected from a blood meal from a vertebrate host with sufficient viremia levels to efficiently infect mosquitoes. Viremia in vertebrate hosts generally is about 5–7 days in duration (Komar et al. 2003). After depositing her eggs, she seeks another vertebrate host to feed upon. If sufficient time has elapsed since the initial blood meal for the virus to replicate within the mosquito and spread to her salivary glands (extrinsic incubation period), she will transmit the virus to this and subsequent vertebrate hosts during feeding.

38.2.1 Mosquitoes

Although WNV has been detected in 65 different mosquito species in the United States (Centers for Disease Control and Prevention), only a few ornithophilic *Culex* mosquito species drive epizootic and epidemic transmission: *Culex pipiens* in the northern half of the country; the closely-related species *Culex quinquefasciatus* in the southern and western states; and *Culex tarsalis* in many areas that overlap with the distribution of *Culex pipiens* and *Culex quinquefasciatus* (Andreadis et al. 2004; Kilpatrick et al. 2006b; Andreadis 2012; Godsey et al. 2012). *Culex pipiens* and *Cx. quinquefasciatus* typically utilize man-made habitats for laying eggs (oviposition) and larval development, including peridomestic containers and above- and below-ground waste water systems (Reisen et al. 1990; Reisen 2012). *Culex tarsalis* is often associated with irrigated farmland (Reisen 2012), but may use diverse sources for breeding such as abandoned swimming pools (Reisen et al. 2008). The ubiquitous sources for *Culex* mosquito breeding make larval control a formidable challenge. In Europe, *Culex modestus* and *Culex pipiens* are important vectors (Reiter 2010). The major mosquito vector in Africa and the Middle East is *Cx. univittatus*, with other *Culex* species important in some areas (Hubalek and Halouzka 1999; Solomon 2004; Ochieng et al. 2013).

Several other mosquito species may play important roles in certain circumstances. For example, *Culex restuans*, an ornithophilic mosquito present in high numbers early in the transmission season and commonly found infected with WNV, may play an important role in early amplification of enzootic transmission in the northeastern United States (Andreadis and Armstrong 2007). *Culex salinarius*, a mosquito found in high numbers during August and September in coastal areas of the northeastern United States, is frequently found to have high WNV infection rates. Since this mosquito feeds indiscriminantly on both birds and mammals and readily bites humans, it may be an important bridge vector to humans (Andreadis 2012).

In temperate climates, WNV overwinters in hibernating (diapause) adult female *Culex* mosquitoes, probably in some birds and possibly in rodents (Nasci et al. 2001; Reisen et al. 2006a; Platt et al. 2008). Although the means by which pre-hibernating *Culex* females become infected is not entirely clear, it has been conclusively demonstrated that vertically infected female *Culex pipiens* that enter diapause in the fall are able to initiate infection the spring (Anderson and Main 2006). In semi-tropical environments, such as found in the southeastern United States, transmission occurs throughout the year, albeit at a very low level during cooler periods (Tesh et al. 2004).

38.2.2 Vertebrate Hosts

The extensive distribution of WNV throughout Africa, the Middle East, southern Europe, western Russia, southwestern Asia, and Australia (Kunjin subtype) derives in part from its ability to infect numerous bird species. Infection has been documented in at least 326 bird species in the United States alone (Centers for Disease

Control and Prevention). Numerous passerine birds develop extremely high viremias and thus are competent amplifier hosts (Komar et al. 2003; Weingartl et al. 2004). Nevertheless, a relatively small subset of the bird community may significantly influence WNV transmission dynamics (McKenzie and Goulet 2010); high avian species diversity, particularly if it is rich in non-passerine species, can dampen WNV transmission (Ezenwa et al. 2006). For example, the American robin (*Turdus migratorius*) may be an important amplifier host despite its low abundance relative to other WNV susceptible birds (Kilpatrick et al. 2006a; Savage et al. 2007). Kilpatrick has hypothesized that WNV outbreaks are promoted by mosquito feeding shifts from American robins to humans coincident with late-season robin dispersal (Kilpatrick et al. 2006b); however, others have failed to demonstrate a shift of feeding preference from robins to mammals (Savage et al. 2007).

WNV transmission may be influenced by the spatial interactions of birds in several ways. One study examining American robins suggested that communal roosts may form important WNV amplification foci (Diuk-Wasser et al. 2010); however, another study showed that roosts are not necessarily important for WNV amplification (Benson et al. 2012). Cloacal and oral shedding of WNV is common in infected birds (Komar et al. 2003) and direct bird-to-bird transmission has been documented in birds housed together (Banet-Noach et al. 2003; Komar et al. 2003; Weingartl et al. 2004). The influence of non-mosquito, direct bird-to-bird transmission on the amplification cycle is unknown, although one mathematical model suggests that it may play an important role in some circumstances (Naowarat and Tang 2004).

The role of non-avian vertebrate hosts for maintaining or amplifying the virus is unknown. Humans and horses generally develop insufficient WNV titers in the blood to infect mosquitoes, but squirrels, chipmunks and rabbits may develop sufficiently high viremia to infect mosquitoes, raising the possibility that small mammals might contribute to WNV transmission cycles (Platt et al. 2007; Reisen and Brault 2007; Platt et al. 2008). Alligators may also serve as competent reservoirs in the southeastern United States (Klenk et al. 2004).

38.2.3 Determinants of Human Disease Incidence and Outbreaks

As with most mosquito-borne arboviral diseases, WNV incidence in humans exhibits considerable annual and geographic variation. Nevertheless, outbreaks in temperate climates tend to occur during mid- to late summer after sufficient viral amplification in the bird-mosquito-bird transmission cycle has produced enough infected mosquitoes to present a human infection risk (Fig. 38.3). Certain areas also tend to have consistently higher human disease incidence, suggesting an underlying permissive ecology that promotes WNV amplification. Factors such as urban and agricultural land covers (Bowden et al. 2011), rural irrigated landscapes (DeGroote and Sugumaran 2012), orchard habitats (Crowder et al. 2013), farming activities as determined by total crop sales (Miramontes et al. 2006) and several socioeconomic factors such as housing age and community drainage patterns (Ruiz et al. 2007), per capita income (DeGroote and Sugumaran 2012), and even neglected swimming pool density (Reisen et al. 2008; Harrigan et al. 2010) relate to higher WNV

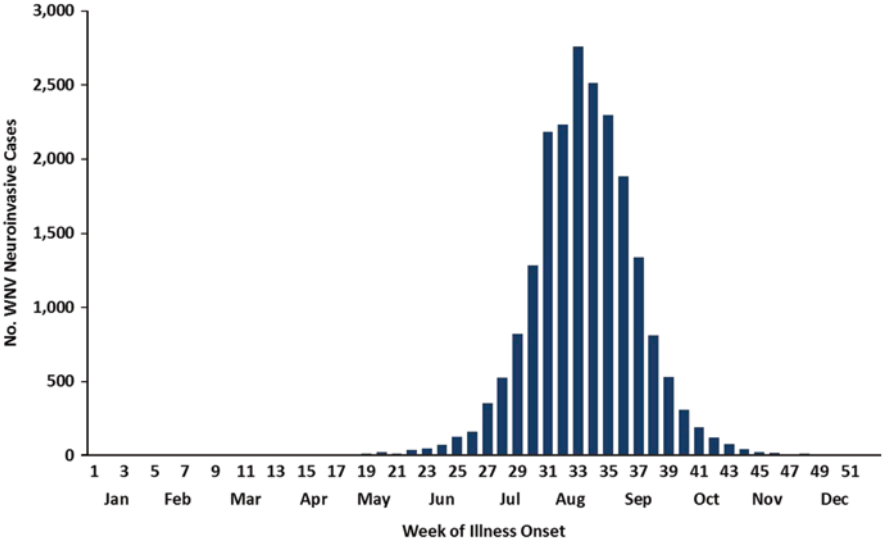

Fig. 38.3 Cumulative incidence of WNV neuroinvasive disease, by week of symptom onset, United States 1999–2012

incidence in some locations. Overall, persons living in rural areas in the United States and Canada seem to be at higher risk of acquiring WNV infection than those living in urban settings. A United States nationwide study comparing viremic to uninfected blood donors showed that residents of rural areas were 3.4 times more likely to be infected than residents of surburban or urban locations (Orton et al. 2006). In Saskatchewan, Canada, residents of rural areas were approximately six times more likely than urban dwellers to have WNV antibodies (Schellenberg et al. 2006).

Weather has an enormous impact on WNV amplification in the bird-mosquito-bird transmission cycle. Increasing ambient temperature shortens the viral extrinsic incubation period and increases viral replication in mosquitoes, both of which greatly increase the probability of viral transmission to susceptible hosts (Cornel et al. 1993; Dohm et al. 2002; Reisen et al. 2006b; Richards et al. 2007; Kilpatrick et al. 2008). However, changes in ambient temperature influence the abundance of vector mosquitoes, host survival and distribution, and human behavior in unpredictable ways differentially influenced by the underlying local ecology. Rainfall and humidity variably influence mosquito abundance and survival, distribution of hosts, and human behavior. Given the infinite possible combinations of temperature and rainfall events and their variable effects on arboviral transmission parameters, combined with other parameters that influence transmission, such as underlying immunity in birds, no models have been able to predict when and where WNV outbreaks will occur (Petersen and Fischer 2012). Nevertheless, human disease incidence over broad areas in temperate climates has been correlated with increased temperatures (Wimberly et al. 2008; Soverow et al. 2009; Hartley et al. 2012) as well as increased

(Landesman et al. 2007; Soverow et al. 2009) or decreased rainfall (Landesman et al. 2007; Wang et al. 2010).

38.3 Transmission to Humans

Nearly all humans acquire WNV infection from mosquito bites. Persons with extensive outdoor exposure might be expected to be at higher risk for infection. One study in the United States found that children were more likely to spend time outdoors and were more likely to become infected with WNV (LaBeaud et al. 2007). People of lower socioeconomic status and particularly those who are homeless may be at higher risk of WNV infection (Gujral et al. 2007; Meyer et al. 2007).

Transfusion-associated WNV transmission was first detected during the 2002 WNV epidemic in the United States when 23 transfusion recipients were infected through receipt of platelets, red blood cells, or fresh frozen plasma from 16 viremic blood donors (Pealer et al. 2003). Mathematical models indicated that the risk of transfusion-associated WNV transmission during the 2002 epidemic ranged from 2.1 to 4.7 per 10,000 donors in high incidence areas (Biggerstaff and Petersen 2003). These findings prompted screening of the United States and Canadian blood supplies using WNV nucleic acid amplification (NAT) tests since 2003. Blood centers conduct NAT testing on minipools of 6 to 16 specimens, depending on test kit manufacturer. While universal pooled blood donation screening has nearly eliminated WNV transfusion transmission, some "breakthrough" transmissions have occurred from donations with virus levels below the limit of detection by minipool screening (Petersen and Epstein 2005; Centers for Disease Control and Prevention 2007). To minimize the risk of "breakthrough" transmissions, blood centers switch to individual donation testing in areas experiencing outbreaks; however, one transmission occurred from a donor with viremia below levels detected by individual unit testing (Centers for Disease and Prevention 2013)

In 2002, transmission via donated organs was first documented when four recipients of organs from a common donor developed WNV infection (Iwamoto et al. 2003). Since then, five additional transmission clusters in the United States (Nett et al. 2012) and one in Italy (Inojosa et al. 2012) have been documented and published. Interestingly, serum from the donors of three of these seven clusters had negative NAT results, suggesting that viable WNV can be sequestered in organs for a short time after viremia has cleared. The urgency of procuring and transplanting organs has precluded routine WNV screening of organ donors (Nett et al. 2012).

Intrauterine transmission of WNV was first documented in 2002 when a woman with WNV encephalitis during the 27th week of pregnancy delivered a term infant with chorioretinitis, cerebral lesions, and laboratory evidence of congenitally acquired WNV infection (Centers for Disease Control and Prevention 2002a; Alpert et al. 2003). Fortunately, intrauterine transmission appears uncommon. A follow-up study of 71 women infected with WNV during pregnancy showed that none of their 72 live infants had malformations linked to WNV infection or had conclusive

laboratory evidence of congenital WNV infection (O'Leary et al. 2006). However, three infants born to women with illness occurring within 3 weeks prepartum had evidence of WNV infection that could have been congenitally acquired or acquired at the time of birth: one had WNV meningitis at 10 days of age, one had a neonatal rash and was positive for anti-WNV IgM at 1 month of age, and one had WNV encephalitis with underlying lissencephaly detected at 17 days of age (O'Leary et al. 2006). A study of 549 infants born to mothers who were pregnant during a community WNV outbreak found evidence of WNV infection in 4% of the mothers and none of the infants (Paisley et al. 2006). Similar birth outcomes were noted among infants born to mothers with and without evidence of WNV infection during pregnancy (Paisley et al. 2006).

WNV transmission has also been reported through percutaneous exposure and inhalation in laboratories, conjunctival exposure while handling dead birds, in a dialysis center by unidentified means, and at a turkey farm, possibly by aerosol (Nir et al. 1965; Centers for Disease Control and Prevention 2002b, 2003, 2004; Fonseca et al. 2005). Transmission via breast milk has also been reported, but appears uncommon (Hinckley et al. 2007).

38.4 Global Epidemiology

38.4.1 Africa

Genetic sequencing of WNV isolates suggests that Africa was the source of the virus' introduction to other areas (May et al. 2011). Consistent with this hypothesis, serological studies in humans and animals conducted in several countries indicate widespread viral circulation (Table 38.1) (Murgue et al. 2002). Seroprevalence ranges markedly by location, even within the same country. For example, a serological study of Egyptian university students showed that WNV antibody prevalence ranged from 28% in Cairo to 74% in Upper Egypt (Darwish and Ibrahim 1971). Similarly, a serological survey of randomly selected household members demonstrated WNV antibody prevalences ranging from 2% in the Northern Sinai to 35% in Upper Egypt (Soliman et al. 2010). Consistent with an endemic pattern, antibody increased by age. A similar pattern of increasing seroprevalence with age was noted in a serological survey of humans in Kenya (Sutherland et al. 2011). While serological data suggest frequent WNV exposure in Africa, these data must be interpreted cautiously because of differences in testing methodology and the considerable serological cross-reactivity among the flaviviruses.

In contrast to the apparent frequent exposure to WNV throughout much of Africa, human and animal illness has been reported infrequently. Nevertheless, sporadic cases of mild illness undoubtedly occur much more commonly than reports indicate. The virus was first recognized in a febrile woman in Uganda in 1937 (Smithburn et al. 1940) and the virus has been occasionally isolated from the blood of febrile children in Egypt. The most notable outbreak occurred in South Africa

Table 38.1 Serological studies of WNV antibodies in Africa

Location	Year	Species	Seroprevalence (%)	Reference
Camaroon	2000–2003	Humans	7	(Kuniholm et al. 2006)
Chad	2002–2005	Horses	97	(Cabre et al. 2006)
Congo	1939–1940	Humans	1–45	(Smithburn and Jacobs 1942)
Democratic Republic of Congo	1992–1999	African elephants	100	(Kading et al. 2013)
Democratic Republic of Congo	1994–1995	African forest buffalo	6	(Kading et al. 2013)
Chad	2002	African elephants	100	
Egypt	1951–1954	Humans	24–84	(Taylor et al. 1956)
Egypt	1951–1954	Mammals-multiple species	2–86	(Taylor et al. 1956)
Egypt	1951–1954	Birds-multiple species	13–65	(Taylor et al. 1956)
Egypt	1953	Hooded crows	0–100	(Work et al. 1955)
Egypt	1969	Humans	50	(Darwish and Ibrahim 1971)
Egypt	1999–2002	Humans	24	(Soliman et al. 2010)
Kenya	1939–1940	Humans	9	(Smithburn and Jacobs 1942)
Kenya	1987	Human	1	(Morrill et al. 1991)
Madagascar	2004	Ring-tailed lemurs	94	(Sondgeroth et al. 2007)
Morocco	2003	Camels	10	(Touil et al. 2012)
Morocco	2008	Birds-multiple species	4	(Figuerola et al. 2009)
Morocco	2009	Camels	14	(Touil et al. 2012)
Morocco	2009	Camels	29	(El-Harrak et al. 2011)
Morocco	2011	Humans	12	(El Rhaffouli et al. 2012)
Senegal	2005	Horses	85	(Chevalier et al. 2010)
Senegal	2002–2005	Horses	92	(Cabre et al. 2006)
Senegal	2007	Horses	78	(Chevalier et al. 2006)
South Africa	1962–1965	Birds-multiple species	12	(McIntosh et al. 1968)
Sudan	1939–1940	Humans	13–46	(Smithburn and Jacobs 1942)
Sudan	1951–1954	Humans	40	(Taylor et al. 1956)
Tunisia	2003	Humans	4–14	(Riabi et al. 2010)
Uganda	1939–1940	Humans	3–14	(Smithburn and Jacobs 1942)

in 1974 following heavy rains and above normal temperatures in early summer. Despite an estimated 18,000 cases, neuroinvasive disease or mortality was not recorded (Jupp 2001). More recently, a new pattern of outbreaks of unusual severity in northern Africa has occurred. An outbreak of approximately 50 human cases with neurological disease occurred in Algeria in 1994 (Le Guenno et al. 1996), which was followed in 1997 by an outbreak involving 173 patients (Triki et al. 2001). An outbreak associated with severe neurological disease was noted in a military camp in the Democratic Republic of Congo was noted in 1998 (Nur et al. 1999). In Sudan in 2002, at least 31 cases of encephalitis occurred during an outbreak in children (Depoortere et al. 2004). In addition, outbreaks of severe neurological disease in equines in Morocco in 1996 and 2003 were associated with 94 and 7 equines, respectively (Schuffenecker et al. 2005). These outbreaks were associated with viral strains of apparent increased virulence and were closely related to the lineage 1 WNV strains that caused large human outbreaks in Romania, Russia, Israel, and the United States (Lanciotti et al. 1999; Schuffenecker et al. 2005). In recent years, sporadic cases of neurologic disease from lineage 2 WNV strains have been identified in South Africa (Venter 2009; Venter and Swanepoel 2010; Zaayman and Venter 2012).

38.4.2 Europe

WNV strains are likely transported between sub-Saharan Africa and Europe by migratory birds. This hypothesis is supported by the relatively high prevalence of WNV antibodies in migratory birds noted in several Russia, Israel, and several European countries (Lelli et al. 2012; Valiakos et al. 2012), as well as by phylogenetic analyses of WNV strains suggesting multiple WNV introductions to Europe in recent years (Parreira et al. 2007). Thus, the likely long co-evolution of virus and hosts in Europe may account for the relative paucity of widespread mortality in European birds compared to what has occurred among many North American bird species (Komar et al. 2003; LaDeau et al. 2007). Nevertheless, lineage I WNV strains genetically related to the strain imported into New York City in 1999 (East Coast strain) resulted in clinical illness in white storks (*Cicona ciconia*) migrating from central Europe through Israel in 1998 (Malkinson et al. 2002) and an epizootic of encephalitis in Hungarian geese (*Anser anser domesticus*) in 2003 (Bakonyi et al. 2006). In 2004 and 2005, several deaths in goshawks (*Accipiter gentilis*) and a sparrow hawk (*Accipiter nisus*) from the same region of Hungary were attributed to a central African lineage two virus, which was the first report of a lineage two virus outside of Africa (Erdelyi et al. 2007).

From the 1960s through the 1980s, WNV isolates were obtained infrequently in southern and central Europe from mosquitoes, humans, birds, and horses, although serological surveys conducted in humans, birds, and other animals suggested more widespread viral exposure (Hubalek and Halouzka 1999; Linke et al. 2007). Human or equine illness was sporadic, with isolated human cases of WN fever identified

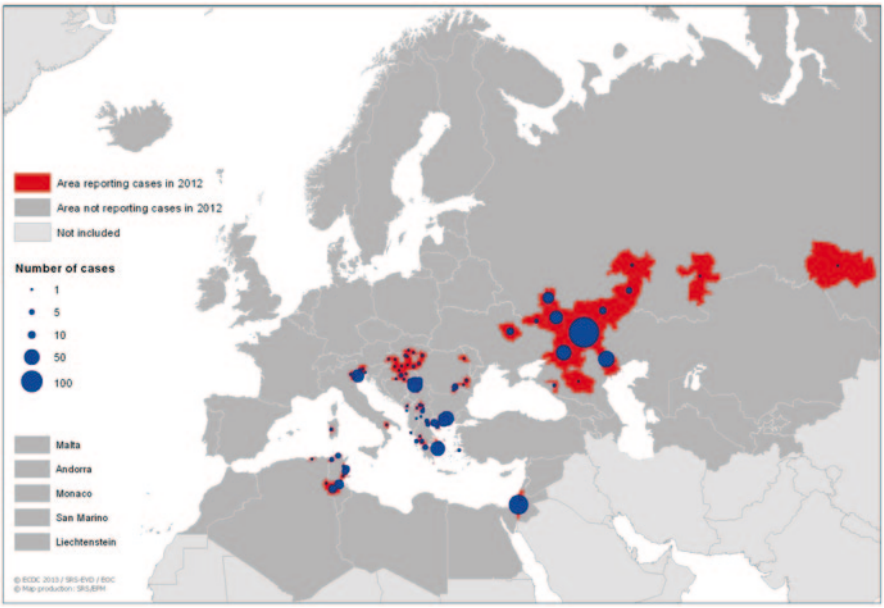

Fig. 38.4 WNV disease cases in the European Union and neighboring countries 2012. (Source: European Centers for Disease Control)

in France, Spain, Romania, Belarus, and Czechland (Hubalek and Halouzka 1999). An outbreak in the Camargue region of southern France from 1962 to 1965 resulted in 15 virologically confirmed human cases with one death and approximately 80 equine cases with 25–30 % mortality (Murgue et al. 2001a).

The first large human outbreak in Europe occurred in Romania with 352 cases of neuroinvasive disease in 1996 (Tsai et al. 1998). An equine outbreak with 14 cases occurred in Italy in 1998 and another with 76 cases in the Camargue region in 2000 (Murgue et al. 2001b; Autorino et al. 2002). Four humans with WNV-related illness were noted in the Camargue region in 2003, which represented the first human WNV-related illnesses documented there since 1965 (Del Giudice et al. 2004).

WNV outbreaks and sporadic cases subsequently have increased in frequency and geographic distribution in Europe (Fig. 38.4). Human cases and dead birds have been associated with both lineage one and two WNV strains (Barzon et al. 2012a, b, c; Magurano et al. 2012; Savini et al. 2012) and strains from both virus lineages have been identified in *Culex pipiens* mosquitoes (Savini et al. 2012), indicating co-circulation of both viruses. In Italy, human cases have been reported annually since 2008, first in the north-eastern part of the country and later elsewhere, including the island of Sardinia beginning in 2011 (Rizzo et al. 2012). A major WNV outbreak in Greece in 2010 caused by a lineage two strain resulted in 262 laboratory-confirmed cases and 35 deaths. Serological investigation indicated that wild birds had been extensively exposed before the outbreak (Valiakos et al. 2012).

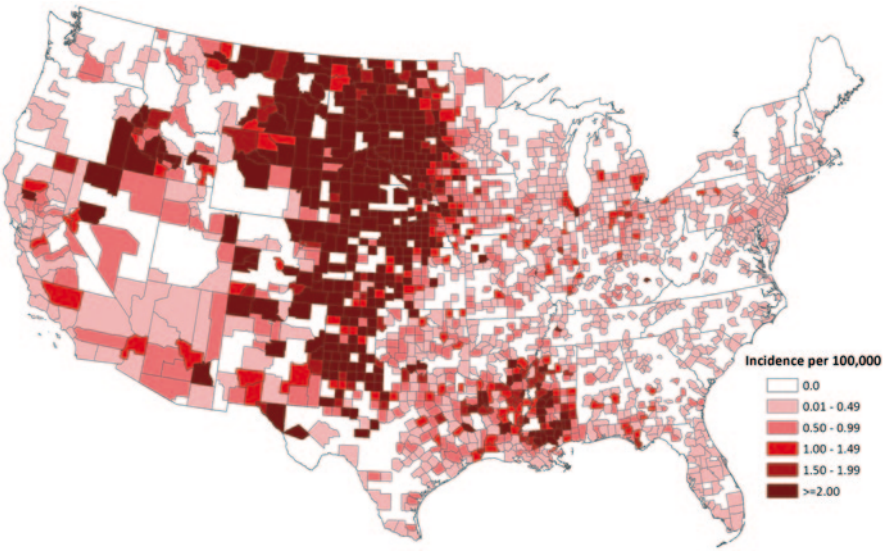

Fig. 38.5 Average annual incidence of WNV neuroinvasive disease, by county, United States, 1999–2012

38.4.3 Americas

WNV spread rapidly throughout the Americas following its discovery in the New York City area in 1999 (Nash et al. 2001). It spread across the United States and reached the Pacific Coast by 2003 (Lindsey et al. 2010). The virus was first detected in Canada in 2001. By 2001 WNV had spread south to islands in the Caribbean Sea (Komar and Clark 2006); by 2003 to El Salvador, Guatemala, and Belize; by 2004 to Colombia and Venezuela (Mattar et al. 2005; Bosch et al. 2007); and by 2005 to Argentina (Adrian Diaz et al. 2008). Despite the virus' apparent extensive distribution in Latin America and the Caribbean, only isolated instances of human illness have been reported (Komar and Clark 2006; Pupo et al. 2006; Gubler 2007; Hunsperger et al. 2009). The explanation for this discrepancy is unknown; however, it is noteworthy that this same pattern is observed in tropical Africa. Perhaps the continuous avian host availability for ornithophilic mosquitoes in tropical areas might decrease the likelihood that infected mosquitoes would feed on humans.

The virus is now endemic throughout much of the United States and southern Canada (Lindsey et al. 2010; Petersen et al. 2013). Through 2012, 16196 patients with neuroinvasive disease and 1549 deaths were reported in the United States; 975 patients with neuroinvasive disease were reported in Canada. In the United States, sporadic human cases occur each year throughout the country along with focal or regional outbreaks of varying sizes. Large outbreaks, each involving nearly 3000 patients with neuroinvasive disease, occurred in 2002, 2003 and 2012. Despite the sporadic nature of outbreaks, certain areas appear to be at higher overall risk than

others (Fig. 38.5). More than 90% of cases have onsets from July through September (Fig. 38.3) (Lindsey et al. 2008).

38.4.4 Israel, Asia, Russia

The first reported WNV human epidemic occurred in Israel in 1951–1952 (Bernkopf et al. 1953), and was followed by a series of human outbreaks throughout the 1950s and in 1980. Illnesses were generally mild, although neuroinvasive disease was prominent during an outbreak among the elderly in 1957 (Murgue et al. 2002). Little subsequent WNV activity occurred in Israel until 1997 and 1998 when WNV was identified from dying migrating storks from Europe as well as other bird species, including domestic geese (Malkinson and Banet 2002; Malkinson et al. 2002). Two human fatalities occurred in 1999, followed by an outbreak with 417 cases and 35 deaths the following year (Chowers et al. 2001; Weinberger et al. 2001). Sporadic cases and small outbreaks have continued in subsequent years.

Since 1963, WNV has been isolated from ticks, birds, and mosquitoes in the southern area of European Russia and western Siberia, and in adjacent republics of the former Soviet Union. Serological surveys of healthy humans indicated up to 8% anti-WNV IgG antibody seroprevalence (Platonov 2001). Yet outbreaks were uncommon until 1999 when a large outbreak of severe neurological disease involved 318 cases and 40 deaths in Volgograd (Platonov et al. 2001). This outbreak occurred during an unusually hot and dry summer. The lineage 1 virus that caused the 1999 outbreak was genetically related to that which caused the 1996 Romanian outbreak (Lanciotti et al. 2002). Sporadic cases and outbreaks have subsequently occurred from both lineage one and two viruses, mainly in areas near the Volga River (Fig. 38.4). Nevertheless, the distribution of the virus may be extensive in Russia, as suggested by a 2004 report of WNV in patients in Novosibirsk in southwestern Siberia and by a serosurvey showing a 15% seroprevalence of WNV neutralizing antibodies in birds in far-Eastern Siberia (Murata et al. 2011).

Although WNV illness is infrequent elsewhere in the Middle East and South Asia, Turkey began to experience large numbers of human cases throughout the country in 2010 and 2011, concurrent with many cases occurring in Greece, Russia, and Israel (Kalaycioglu et al. 2012). Serological surveys in humans and horses conducted in Iran since 1967 indicate circulation of the virus, although human illness has been uncommonly reported (Naficy and Saidi 1970; Chinikar et al. 2012, 2013). Serological evidence of WNV antibodies in humans in India was first identified in 1952 and the virus was identified since then in mosquitoes and in ill humans in several areas (Paul et al. 1970; George et al. 1984). A study of samples collected throughout India from 1992 to 2001 identified 88 ill persons with WNV-specific antibodies, suggesting that WNV-related illness incidence may be higher than that currently recognized (Thakare et al. 2002). Several recent outbreaks occurring concurrently with other mosquito-borne illness have been noted in India (Khan et al. 2011; Shukla et al. 2012). A WNV outbreak occurred in Xinjiang, China in 2004 (Li et al. 2013).

38.4.5 Australia

Kunjin virus, a lineage I WNV variant (1b) (May et al. 2011), is endemic throughout most of tropical Australia and eastern Queensland. Phylogenetic analysis suggests a single successful introduction of viruses originating from Africa sometime within the last 300 years (May et al. 2011). Documented illness is rare, and cases occur in infrequent small outbreaks or sporadically. Illness is typically mild, non-encephalogenic, and non-life threatening (Knope et al. 2013).

38.5 Clinical Aspects

38.5.1 Humans

Approximately 25 % of humans infected with WNV become ill (Zou et al. 2010). The incubation period for clinical illness generally ranges from 2 to 14 days, but immunocompromised patients may experience prolonged incubation periods up to 21 days (Pealer et al. 2003; Rhee et al. 2011). Most of those who become ill develop West Nile fever, characterized by the sudden onset of symptoms such as headache, malaise, fever, myalgia, chills, vomiting, rash, and eye pain (Zou et al. 2010). Fever may be low-grade or absent. A rash, which often appears around the time of defervescence, tends to be morbilliform, maculopapular, and non-pruritic, and predominates over the torso and extremities, sparing the palms and soles (Anderson et al. 2004; Del Giudice et al. 2005; Ferguson et al. 2005; Gorsche and Tilley 2005). Persistent fatigue, headaches, and difficulties concentrating may continue for weeks or months and can be quite debilitating.

Risk factors for developing West Nile fever are not well defined. A follow-up study of asymptomatic, viremic blood donors indicated that increasing viral load and female gender, but not age, subsequently increased the risk of developing West Nile fever (Zou et al. 2010). A smaller follow-up study of viremic blood donors suggested that younger persons were more likely to develop West Nile fever (Brown et al. 2007).

Approximately one in 150 to 250 persons infected with WNV develop neuroinvasive disease (Mostashari et al. 2001; Petersen et al. 2012), which is manifested by meningitis, encephalitis, or acute flaccid paralysis. Advancing age profoundly increases the risk of neuroinvasive disease, particularly encephalitis (Lindsey et al. 2010; Carson et al. 2012). The risk may approach one in fifty among persons aged at least 65 years, a rate 16 times higher than that for persons aged 16 to 24 years (Carson et al. 2012). In addition, a history of cancer, diabetes, hypertension, alcohol abuse, renal disease and *CCR5* deficiency as well as male gender may increase the risk of neuroinvasive disease (Bode et al. 2006; Murray et al. 2006; Lindsey et al. 2010; Carson et al. 2012; Cho and Diamond 2012; Lindsey et al. 2012b). Persons infected through transplantation of infected organs are at extreme risk of developing

neuroinvasive disease (Nett et al. 2012); however, conflicting data exist regarding risk among previous organ recipients infected via a mosquito bite (Kumar et al. 2004; Freifeld et al. 2010).

West Nile meningitis is clinically similar to that of other viral meningitides with abrupt onset of fever and headache along with meningeal signs and photophobia. Headache may be severe, requiring hospitalization for pain control; associated gastrointestinal disturbance may result in dehydration (Sejvar et al. 2008). West Nile encephalitis ranges in severity from a mild, self-limited confusional state to severe encephalopathy, coma, and death. Extrapyramidal disorders are frequently observed (Pepperell et al. 2003; Sejvar et al. 2003a; Burton et al. 2004; Sayao et al. 2004). Increased intracranial pressure, cerebral edema, and seizures are infrequent (Doron et al. 2003). Patients with West Nile encephalitis frequently develop a coarse tremor, particularly in the upper extremities. The tremor tends to be postural, and may have a kinetic component (Sejvar et al. 2003a; Burton et al. 2004; Emig and Apple 2004; Sayao et al. 2004). Myoclonus, predominantly of the upper extremities and facial muscles, may occur, and may be present during sleep. Features of Parkinsonism may be seen (Robinson et al. 2003; Sejvar et al. 2003a) and cerebellar ataxia has been described (Kanagarajan et al. 2003; Burton et al. 2004; Sayao et al. 2004).

WNV-associated paralysis most commonly results from destruction of the anterior horn cells of the spinal cord (Glass et al. 2002; Leis et al. 2002; Jeha et al. 2003; Li et al. 2003; Sejvar et al. 2003b, 2005). Asymmetric weakness usually develops rapidly within the first 48 h after symptom onset, although patients with extensive spinal cord involvement develop a more symmetric dense quadriplegia. Central facial weakness, frequently bilateral, may occur (Jeha et al. 2003). Respiratory failure requiring emergent endotracheal intubation may result from diaphragmatic and intercostal muscle paralysis (Fan et al. 2004; Sejvar et al. 2005). Sensory loss or numbness is generally absent though some patients experience intense pain in the affected limbs just before or during the onset of weakness (Sejvar et al. 2005). Other causes of weakness associated with WNV infection include Guillian-Barré syndrome and other demyelinating neuropathies, motor axonopathy, axonal polyneuropathy, involvement of ventral spinal roots, myasthenia gravis, and brachial plexopathies (Leis and Stokic 2012).

Other manifestations described with WNV infection include mulitfocal choroiditis, vitritis, myocarditis, pancreatitis, fulminant hepatitis, rhabdomyolysis, stiff-person syndrome, and autonomic instability (Petersen and Hayes 2008).

Full recovery is the norm for patients with uncomplicated West Nile fever or meningitis; however, initial symptoms, particularly extreme fatigue, may be prolonged (Watson et al. 2004). West Nile fever may precipitate death among a few persons of advanced age or with underlying medical conditions (Sejvar et al. 2011). Outcomes of West Nile encephalitis are variable and may not correlate with severity of initial illness. Patients hospitalized with WNV encephalitis frequently require assistance with daily activities following acute care discharge (Pepperell et al. 2003; Emig and Apple 2004) and often report substantial functional and cognitive difficulties for up to a year following acute infection. Only 37% of patients in the 1999 New York City outbreak achieved full recovery at 1 year (Klee et al. 2004) and 53%

of patients in Idaho reported symptoms lasting at least 6 months, mostly fatigue, muscle aches, and difficulties with memory and concentration (Cook et al. 2010).

While some studies have documented neurocognitive deficits on standardized testing as long as 1 year after acute illness (Haaland 2006; Sadek et al. 2010), others have failed to confirm this finding (Sejvar et al. 2008). Nevertheless, self-reported fatigue, somatic, and cognitive complaints lasting months or years are common among persons recovering from WNV illness (Klee et al. 2004; Carson et al. 2006; Anastasiadou et al. 2013). Neuropsychiatric symptoms, including depression, anxiety, and apathy, have been reported (Sejvar et al. 2003a; Murray et al. 2007; Loeb et al. 2008; Berg et al. 2010). One investigator found WNV RNA in urine in patients up to 7 years following acute illness and implied an association with chronic renal failure (Murray et al. 2010); however, three other studies failed to substantiate persistent WNV RNA in urine (Gibney et al. 2011; Baty et al. 2012; Barzon et al. 2013a). Among patients with acute flaccid paralysis due to poliomyelitis-like syndrome, one-third recover regarding their strength to near baseline, one-third have some improvement, and one-third have little or no improvement (Sejvar et al. 2006).

Case fatality rates among patients with neuroinvasive disease generally are around 10% (Lindsey et al. 2010). Advanced age is the most important risk factor for death, ranging from 0.8% among those aged less than 40 years to 17% aged at least 70 years (Lindsey et al. 2010). Encephalitis with severe muscle weakness, change in the level of consciousness, diabetes, cardiovascular disease, hepatitis C virus infection, and immunosuppression are possible risk factors for death (Nash et al. 2001; Murray et al. 2006; Lindsey et al. 2010). Long-term, all-cause mortality is two to three times higher among survivors of acute WNV illness (Green et al. 2005; Lindsey et al. 2012a).

38.5.2 Birds

Serologic studies demonstrate that numerous bird species are exposed to WNV during outbreaks, with antibody prevalences as high as 70% noted in some species (Savage et al. 1999; Komar et al. 2001a, b; Ringia et al. 2004; Gibbs et al. 2006; Valiakos et al. 2012). In the United States, avian mortality has been noted in more than 300 species (http://www.cdc.gov/westnile/faq/deadBirds.html), with corvids particularly impacted (LaDeau et al. 2007; Nemeth et al. 2007; Foppa et al. 2011). Consistent with these findings, mortality following experimental infection of eight species of North American birds ranged from 33–100% (Komar et al. 2003). However, surveillance in recent years in the United States has recorded fewer WNV-related dead bird reports. Whether this reflects surveillance fatigue, decreases in susceptible populations, or decreased avian mortality following infection is not known. One analysis suggested increasing survival in American crows (*Corvus brachyrhynchos*) following WNV infection (Reed et al. 2009).

One particular area of concern has been the impact of WNV on raptors (Gancz et al. 2006; Harris and Sleeman 2007; Saito et al. 2007). Experimental infection via the oral route of several raptor species, including great horned owls

(*Bubo virginianus*) and American kestrels (*Falco sparverius*), has been recorded (Nemeth et al. 2006), suggesting that oral ingestion in addition to mosquito exposure may be important routes of infection in at least some raptor species.

While considerably less avian mortality has been noted in Europe compared to the United States, avian mortality has been occasionally reported in several species (Malkinson et al. 2002; Glavits et al. 2005; Bakonyi et al. 2013). The reason for this difference is unknown, but may be related to the long-standing exposure of European birds to WNV strains imported from Africa. Nevertheless, experimental infection of several European species, including red-legged partridges (Sotelo et al. 2011), falcons (Ziegler et al. 2013), and Carrion crows (*Corvus corone*) (Dridi et al. 2013), with WNV strains circulating in Europe in recent years result in considerable mortality.

38.5.3 Equines

Serologic studies demonstrate that a high proportion of horses living in endemic areas may be exposed to WNV (Schmidt and Elmansoury 1963; Epp et al. 2007; Gardner et al. 2007). However, studies of experimentally infected horses confirm that most infections are clinically unapparent and produce a transient low-level viremia of approximately 1 week in duration (Schmidt and Elmansoury 1963; Bunning et al. 2002). These low-level viremias are inadequate to infect mosquitoes, confirming that horses are incidental hosts (Bunning et al. 2002). Reported symptoms and signs include fever, anorexia, incoordination, weakness or ataxia, muscle rigidity, fasciculations, tremors, and cranial nerve dysfunction, depression, and recumbency (Cantile et al. 2000; Porter et al. 2003; Ward et al. 2004). The mean duration of illness is approximately 22 days (Salazar et al. 2004) and reported survival rates are in the range of 20–30 % (Porter et al. 2003; Salazar et al. 2004; Ward et al. 2004). Animals that become recumbent and unable to rise are nearly 80 times more likely to die than those able to rise (Salazar et al. 2004). Residual symptoms are common among surviving horses.

38.5.4 Other Animals

Numerous other animals may become infected with WNV, mostly resulting in minimal or no symptoms. Species without reported clinical disease but with WNV antibodies detected during serologic surveys include bats, deer, raccoons, opossums, and small rodents. Experimental infection of several species as diverse as fox squirrels (*Sciurus niger*) (Root et al. 2006), American alligators (*Alligator mississippiensis*) (Klenk et al. 2004), dogs (Blackburn et al. 1989), cats (Austgen et al. 2004), Eastern chipmunks (*Tamias striatus*) (Platt et al. 2007), monkeys (*Macaca mulatta*) (Pogodina et al. 1983), and common garter snakes (*Thamnophis sirtalis*) (Steinman et al. 2006) exhibit transient viremia and some morbidity and mortality. Viremia in

some species such as American alligators and Eastern chipmunks was high enough to infect mosquitoes, suggesting a possible role in WNV transmission in some settings. Isolated reports of morbidity and mortality due to WNV in numerous wild or captive animal species exist. These include squirrels, harbor seals, macaques, elk, sheep, and alpacas. Experimental evidence suggests that WNV infection can persist in some species. Virus could be isolated from the organs of rhesus monkeys at least 5 months after inoculation, and interestingly, virus isolates obtained at least 2 months after inoculation were non-pathogenic when inoculated into white mice (Pogodina et al. 1983). WNV could be cultured from urine in hamsters up to 242 days following inoculation (Tesh et al. 2005).

38.6 Pathogenesis

Mosquito salivary components introduced at the site of infection modulate the initial immune response by target cells including keratinocytes (Lim et al. 2011) and dendritic cells through several mechanisms including focalized suppression of immune effector cell trafficking to the site of inoculation (Schneider and Higgs 2008; Styer et al. 2011). Infected dendritic cells or keratinocytes migrate to the draining lymph node from which a serum viremia is generated that relays infection to visceral organs and potentially to the central nervous system (CNS). Additional mosquitoes that feed on avian amplification hosts exhibiting high-level viremias during this viremic phase become infected. Given the low-level serum viremias observed in humans and horses, they are unlikely to infect probing mosquitoes and as such are considered "dead end" hosts despite the potential for development of severe neurological disease.

West Nile virus is capable of replicating and eliciting pathology in the brain (i.e., neurovirulent); however, a critical perquisite to generate neurological disease manifestations in humans is the virus' capacity to gain access to the CNS (i.e., neuroinvasiveness). Postulated WNV neuroinvasive mechanisms based on small rodent models include: (i) direct viral crossing of the blood-brain barrier due to cytokine-mediated increased vascular permeability (Wang et al. 2004; Kong et al. 2008), (ii) a Trojan horse mechanism in which infected tissue macrophages are trafficked across the blood brain barrier (Verma et al. 2009; Bai et al. 2010), (iii) direct infection and passage through the endothelium of the blood brain barrier (Dropulic and Masters 1990) and (iv) retrograde axonal transport of the virus to the CNS via the infection of olfactory or peripheral neurons (Hunsperger and Roehrig 2006; Wang et al. 2009). Regardless of how WNV enters the CNS, additional studies in murine models have indicated that viral replication can persist in various tissues, including the CNS, thus shedding additional light on the potential etiology for long term neurological sequelae observed in neuroinvasive disease patients (Appler et al. 2010).

Many of the clinical features of CNS infection in both humans and animals are accounted for by the predilection of WNV to certain areas of the CNS, such as the basal ganglia, thalami, brain stem, cerebellum, and anterior horn cells (Cantile et al.

2000, 2001; Kelley et al. 2003; Leis et al. 2003; Li et al. 2003; Guarner et al. 2004). For example, asymmetrical paralysis is associated with destruction of anterior horn cells and Parkinsonian symptoms with involvement of the basal ganglia (Sejvar and Marfin 2006). Histopathologic changes include microglial nodules composed of lymphocytes and histiocytes, perivascular inflammation consisting predominantly of CD8 T-lymphocytes, and leptomeningeal mononuclear inflammatory infiltrates when meningitis is present (Cantile et al. 2000; Sejvar and Marfin 2006).

38.7 Diagnosis

Numerous experimental studies in animals as well as observations in humans indicate that viremia usually develops in 1 to 2 days following infection, which then lasts approximately 1 week. Viremia is cleared about the time WNV-specific IgM antibodies can be detected, with IgG antibodies developing shortly thereafter (Busch et al. 2008). One unusual feature is that IgM antibodies persist in many humans for 1 year or longer (Roehrig et al. 2003; Busch et al. 2008). WNV can persist in organs and tissues of humans and animals after acute viremia has cleared. Immunocompromised patients can exhibit prolonged viremia with delayed or absent development of IgM and IgG antibodies (Pealer et al. 2003). IgG and neutralizing antibodies probably can be detected for life in immunocompetent individuals following natural infection.

38.7.1 Diagnosis in Humans

Detection of IgM antibodies in CSF or serum forms the cornerstone of diagnosis in most clinical settings. Several commercial IgM antibody-capture immunosorbent assay (MAC-ELISA) kits are available; microsphere-based immunoassays have also been developed (Johnson et al. 2005a). Because IgM antibody does not pass the blood-brain barrier, presence of IgM antibodies in CSF is indicative of CNS infection. IgM antibodies are present in at least 90% of patients with encephalitis or meningitis within 8 days of symptom onset. IgM or IgG antibodies may not be present at clinical presentation, particularly among patients with West Nile fever (Anastasiadou et al. 2011; Papa et al. 2011c). One study showed that only 58% of patients with West Nile fever had a positive MAC-ELISA result (Tilley et al. 2006). Nevertheless, MAC-ELISA testing of acute- and convalescent-phase sera should provide a definitive diagnosis.

Considerable cross-reactivity of serologic tests among the flaviviruses can complicate serological diagnosis. Recent vaccination with yellow fever or Japanese encephalitis vaccines or recent infection with a related flavivirus such as St. Louis encephalitis or dengue viruses can produce a false-positive WNV MAC-ELISA result. The plaque reduction neutralization test can help distinguish the cross-reactions among the flaviviruses when the infecting flavivirus is the first flavivirus

exposure. However, neutralization test results for WNV infected patients with previous flavivirus exposure are usually inconclusive; often the highest neutralizing antibody titer is to the first infecting flavivirus rather than to WNV ("original antigenic sin" phenomenon) (Johnson et al. 2005b). In addition, the persistence of detectable IgM antibodies (Papa et al. 2011b)—in one study in 17 % of patients after 1 year (Busch et al. 2008)—means that a positive WNV MAC-ELISA result may be unrelated to the current illness.

Identification of WNV RNA by RT-PCR in human cerebrospinal fluid, serum, or other tissues has diagnostic utility in certain clinical settings as an adjunct to the MAC-ELISA. A combined approach using nucleic acid testing and MAC-ELISA increased the sensitivity of testing from 58 % using serology alone to 94 % among patients with West Nile fever (Tilley et al. 2006).

38.7.2 Diagnosis in Non-Human Vertebrates

MAC-ELISA has been developed for use in horses, and can be readily adapted to other animal species where anti-IgM antibody reagents are commercially available. Alternatively, seroconversion for IgG, neutralizing antibodies, and haemagglutinin inhibiting (HAI) assays in acute- and convalescent-phase serum samples collected 2–3 weeks apart can be used as screening assays. The latter two approaches do not require species-specific reagents and thus have broad applicability. The ELISA format may be used when employed as inhibition or competition ELISAs, which avoid the use of species-specific reagents. A blocking ELISA has been applied to a variety of vertebrate species with very high specificity and sensitivity, reducing the necessity of a second confirmatory test (Blitvich et al. 2003). Similarly, the microsphere immunoassay, when used comparatively with WNV antigen-coated beads and SLEV antigen-coated beads, performs with high specificity and sensitivity (Johnson et al. 2005a). As with human diagnostics, the PRNT is used to confirm serology in non-human vertebrates. The same caveats regarding the "original antigenic sin" phenomenon apply both to human and non-human vertebrate diagnostics.

38.8 Prevention

WNV prevention is accomplished by vaccination or by preventing infected mosquitoes from biting people or other susceptible vertebrates. Preventing bites from infected mosquitoes is accomplished by reducing mosquito numbers using an integrated pest management approach and by use of personal protection measures such as application of mosquito repellents. The integrated pest management approach is guided by timely surveillance to monitor the level of risk to humans.

38.8.1 Surveillance

Integrated pest management is guided by regular monitoring of vector mosquito populations and WNV activity levels in humans and other vertebrates to determine if, when, and where interventions are needed to keep mosquito numbers below levels that produce risk of human disease. Mosquito surveillance is accomplished by either larval or adult monitoring. Larval surveillance identifies and samples aquatic habitats where vector mosquitoes may breed. Adult mosquito surveillance identifies the abundance of adult vector mosquitoes and monitors viral infection rates in mosquitoes. Adult mosquito surveillance for WNV is mostly accomplished using CO_2-baited light traps or gravid traps. Light traps monitor host-seeking mosquitoes, whereas gravid traps capture females seeking a location to deposit eggs. *Culex* species are differentially attracted to each of these types of traps. Gravid traps are most likely to capture infected mosquitoes, since they have already taken a blood meal. A consistent approach is required from year to year in order to establish levels of mosquito activity that equate to increased human risk. The vector index, roughly defined as the number of infected vector mosquitoes times the infection rate in those mosquitoes, may be the best measure of impending human risk in urban settings (Chung et al. 2013).

WNV activity in non-human vertebrates can be measured by WNV-related avian mortality, seroconversion to WNV in sentinel chickens or other sentinel animals, seroprevalence in wild birds, and cases of WNV illness in animals, primarily horses. Unfortunately, all of these approaches have significant limitations: there is little avian mortality in Europe and Africa, avian mortality appears to be decreasing in North America, sentinel chickens are expensive to maintain, seroprevalence in wild birds is difficult to monitor consistently and may not correlate highly with future risk of transmission, and mortality in horses has been dramatically reduced due to equine vaccination (Gardner et al. 2007). It is also difficult to correlate activity in non-human vertebrates with human risk.

Monitoring of human neuroinvasive disease cases provides the best measure of the overall scope of a WNV outbreak. While these cases represent a small fraction of the total number of human infections, they are usually hospitalized and more completely captured by surveillance (Zou et al. 2010; Weber et al. 2011). The total number of human infections can be estimated by multiplying the number of neuroinvasive disease cases by the ratio of infections to neuroinvasive disease cases. Only a small fraction of West Nile fever cases are captured by surveillance; however, they can be the first indication of the occurrence of human infections in an area. Infections in blood donors can be monitored in areas where blood donor screening is conducted. The main limitation of human surveillance is that several weeks may occur between the date of infection and when illness is reported to health authorities (Chung et al. 2013).

38.8.2 Mosquito Control

Community-based mosquito control programs utilizing integrated pest management principles use several methods to reduce mosquito populations below levels that increase human risk (Reisen and Brault 2007). These include eliminating mosquito breeding sites (source reduction) and applying larvicides to aquatic habitats where mosquitoes may breed. When adult mosquito density becomes high, adulticides are applied through ultra-low volume (ULV) spraying applied by ground-based or aerial mounted sprayers.

Although the effectiveness of this approach cannot be readily assessed due to the highly focal and sporadic nature of WNV illness, a few well controlled studies do exist. In response to surveillance findings indicating increasing human risk, early-season control of adult mosquitoes using ULV applications of insecticides in a populated agricultural area in California decreased subsequent WNV transmission (Lothrop et al. 2008). In addition, ULV pesticide application decreased WNV infected mosquito abundance and reduced human WNV case incidence during a WNV outbreak in another populated area of California (Carney et al. 2008; Macedo et al. 2010). Human health risks associated with ULV organophosphate or synthetic pyrethroid pesticide use appear to be negligible, largely because the timing of application and low volume of pesticide used result in minimal human exposure (Centers for Disease Control and Prevention 2005; Duprey et al. 2008; Chung et al. 2013).

38.8.3 Personal Protection

Personal protection measures include application of mosquito repellents and wearing permethrin-treated clothing. However the effectiveness of these measures is difficult to assess. A Canadian study comparing people who practiced at least two personal protective strategies (wearing repellent, wearing protective clothing, or avoiding outdoor exposure to mosquitoes) with those who did not showed that personal protective strategies halved the risk of WNV infection (Loeb et al. 2005). A study comparing two adjacent communities in the United States found that incidence of WNV disease was better correlated ecologically with the practice of personal protection strategies than with the level of local mosquito control efforts (Gujral et al. 2007).

Commercially available insect repellents containing DEET, IR3535, oil of lemon eucalyptus, and picaradin are effective in reducing or preventing mosquito biting (Fradin and Day 2002). Unfortunately, few people report regular repellent use even during well publicized WNV outbreaks (McCarthy et al. 2001; Gibney et al. 2012).

38.8.4 Vaccines

The introduction of a vaccine against WNV for use in horses has substantially reduced the incidence of equine WNV disease in the United States (Gardner et al. 2007;

Table 38.2 West Nile virus vaccines for horses approved in the United States and the European Union

Vaccine	Viral antigen	Status	Comment
West Nile Innovator® in U.S. and Equip® WNV (Pfizer) in E.U. (Zoetis)	Formalin inactivated whole virus	Approved in U.S./E.U.	2 doses, booster annually
Vetera® WNV vaccine (Boehringer Ingelheim)	Inactivated virus	Approved in U.S.	2 doses, booster annually
Recombitek® (Merial)	prM and E proteins expressed in canarypox virus	Approved in U.S./E.U.	2 doses, booster annually
Equi-Nile® (Merck)	Inactivated chimeric virus, WNV prM/E proteins in yellow fever 17D backbone	Approved in U.S.	2 doses, booster annually
PreveNile® (Intervet)	Live chimeric virus, WNV prM/E proteins in yellow fever 17D backbone	Approved in U.S.; recalled in 2010 because of adverse events	
West Nile-Innovator DNA® (Fort Dodge Animal Health)	Plasmid DNA coding for prM and E proteins	Approved in U.S.; currently not commercially available	

Petersen and Roehrig 2007). Two licensed live, attenuated vaccines and two licensed inactivated vaccines are available in the United States or the European Union (Siger et al. 2006; Long et al. 2007a, b; Seino et al. 2007; De Filette et al. 2012); a DNA vaccine has also been licensed in the United States, but is not commercially available (Davis et al. 2001) (Table 38.2). Several human WNV vaccine constructs employing various strategies have been developed, some of which have reached human clinical trials (De Filette et al. 2012). Chimeric vaccines inserting WNV PrM and E protein genes into attenuated yellow fever and dengue four virus backbones have undergone successful phase 2 and 1 clinical trials, respectively (Biedenbender et al. 2011; Dayan et al. 2012; Durbin et al. 2013). A successful phase 1 WNV DNA vaccine trial has been completed (Martin et al. 2007). Phase-3 efficacy trials have not been attempted due the unknown market potential of a WNV vaccine and logistical difficulties in conducting phase-3 clinical trials for this sporadic and widely dispersed disease (Martina et al. 2010; Beasley 2011). While universal vaccination against WNV is not likely to result in societal financial savings, further evaluation is required to determine whether vaccination of groups at high-risk of developing neuroinvasive disease is warranted (Zohrabian et al. 2006; Lindsey et al. 2008; Martina et al. 2010).

38.9 Treatment

Treatment is supportive. Several investigated therapeutic approaches in humans include immune γ-globulin, WNV-specific neutralizing monoclonal antibodies, corticosteroids, ribavirin, interferon α-2b, and anti-sense oligomers (Diamond 2009;

Beasley 2011). No study has documented efficacy, largely due to difficulty in recruiting sufficient numbers of patients. Case reports suggesting efficacy should be interpreted with extreme caution due to WNV's highly variable clinical course. No clinical studies have been conducted in horses.

38.10 Future Directions

The recent pattern of sporadic cases and outbreaks of WNV that has emerged in Europe and North America shows no signs of abating. While broad areas of high risk can be identified, the sporadic, local and regional outbreaks that occur within these areas remain elusively unpredictable. In populated area within high-risk zones, establishing effective surveillance and response capacity, and assessing the effectiveness of these activities are priorities. Further evaluation of target populations and cost-efficacy of a human WNV vaccine will help determine the need for continued human vaccine development. Practical regulatory pathways and paradigms for testing and approval of WNV vaccines and therapeutics adapted to the sporadic outbreak nature of WNV are required.

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Chapter 39
Crimean-Congo Haemorrhagic Fever Virus, an Emerging and Re-Emerging Pathogen

Felicity Jane Burt and Dominique Goedhals

Abstract Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne viral zoonosis distributed in Africa, Asia, eastern Europe and the Balkans. The broad geographic range correlates with that of the principal vector of the virus, ticks belonging to the genus Hyalomma. Humans acquire infection through tick-bite or exposure to infected blood or tissues of animals or humans. Human infection is characterized by febrile illness with headache, myalgia and petechial rash, frequently followed by a hemorrhagic state and in some cases a fatal outcome. The importance of the tick-vertebrate-tick cycle in maintaining virus transmission is well established. Small mammals are considered important amplifying hosts of the virus. Domestic livestock show mild or no clinical signs of illness but develop a short period of viremia, during which the virus can be transmitted to humans, hence there is an occupational risk associated with employment in the livestock industry among farmers, farm workers, abattoir workers and veterinarians. The emergence of CCHF from 2002 in several countries in the Balkans raises concerns that this virus could expand its current geographic distribution and establish new endemic foci. Monitoring and control of the spread will require increased diagnostic capacity as well as surveillance and novel approaches to development of vaccines and drugs and vector control.

39.1 History of the Virus

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne viral zoonosis distributed in Africa, Asia, eastern Europe and the Balkans. The broad geographic range correlates with that of the principal vector of the virus, ticks belonging to the genus *Hyalomma*. The virus is a member of the *Nairovirus* genus of the family *Bunyaviridae* (Hoogstraal 1979). A disease with symptoms suggestive of CCHF infection was described as early as the twelfth century in regions of eastern Europe and Asia. In 1944 a disease was described among peasants that became ill following exposure to tick-bites while harvesting crops on the Crimean Peninsula and was given the

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name Crimean haemorrhagic fever (CHF). The disease was subsequently shown to be caused by a filterable agent present in suspensions prepared from certain tick species and in human blood samples collected from patients during the early stages of disease. It was only in 1967 that the aetiologic agent was isolated in a laboratory using newborn mice. The availability of a laboratory host made it possible to characterize the virus, and it was shown in 1969 that isolates of CHF were antigenically identical to an African virus, named Congo virus, isolated from a febrile child in Stanleyville in the Belgian Congo (now referred to as Kisangani in the Democratic Republic of the Congo). Studies on physicochemical characteristics, morphology and morphogenesis showed that the viruses were indistinguishable (Casals 1969; Chumakov et al. 1970; Hoogstraal 1979; Korolev et al. 1976; Simpson et al. 1967). The names were subsequently combined and the virus is now referred to as Crimean-Congo haemorrhagic fever virus (CCHFV).

Humans become infected by tick-bite or from contact with infected blood or other tissues of livestock or human patients. Human infection is usually characterized by febrile illness with headache, myalgia and petechial rash which can progress to a hemorrhagic state with a fatal outcome. In livestock, including ostriches, and in potential reservoir hosts, usually small mammals such as hares and ground frequenting birds, infection is either asymptomatic or causes mild fever which is frequently associated with a short period of viraemia playing a role in transmission as well as amplification of the virus in nature.

39.2 The Virus

CCHFV is classified as a member of the genus *Nairovirus*, of the family *Bunyaviridae* (Karabatsos 1985; Calisher 1991; Casals 1969). The genus, consisting of 34 viruses, is divided into seven serogroups on the basis of serological analysis using complement fixation, haemagglutination, neutralization, immunoprecipitation and immunofluorescence techniques (Casals and Tignor 1980; Calisher and Karabatsos 1989). Although the classification of the nairoviruses was originally based on antigenic relationships, the groupings have subsequently been substantiated using molecular analyses to determine genetic relationships between the viruses. CCHFV belongs to the serogroup of the same name. Other members of the CCHFV serogroup include Hazara virus, isolated from *Ixodes redikorzevi* ticks in Pakistan and the closest known antigenic relative of CCHFV, and Khasan virus isolated from *Haemaphysalis longicornis* ticks from the former USSR (Begum 1970; Smirnova 1979). CCHFV is the only member of the group known to cause disease in humans.

Members of the genus *Nairovirus* are spherical structures, 90–120 nm in diameter (Donets et al. 1977). As for all the bunyaviruses, the genome of CCHFV is comprised of three single-stranded RNA segments in a negative-sense orientation. The segments, designated small (S), medium (M) and large (L), encode for the viral nucleoprotein (NP), glycoprotein precursor (GPC) and RNA dependent RNA polymerase (RdRp) proteins, respectively (Schmaljohn and Patterson 1990). Each

of the three RNA segments is encapsidated in the viral encoded nucleoprotein to form ribonucleoprotein particles (RNP) within the virion (Clerx et al. 1981). Each segment comprises a non-translated region (NTR) at the 3′ and 5′ termini flanking a single transcriptional unit. The NTRs have genus specific sequences at the termini, with cis-acting signals for RNA synthesis and segment packaging, and internal regions that are neither genus specific nor conserved between segments within a species, which are likely associated with genome replication (Barr et al. 2003). The 3′ and 5′ NTRs have significant nucleotide complementarity and base pairing of these regions leads to formation of panhandles resulting in circular conformation of each segment. The base-pairing structure likely provides a functional promotor region for the viral polymerase, RdRp (Flick et al. 2002).

The L segment of CCHFV is approximately 12,000 bases in length and has a single ORF encoding for a large, approximately 450 kDa, protein (Honig et al. 2004; Kinsella et al. 2004). The L protein of CCHFV, and other nairoviruses, is signific antly larger than the RdRps of other bunyaviruses and comparison of sequence data led to the identification of an ovarian tumour (OTU) like protease motif in the amino terminal region of the L protein (L-OTU) of CCHFV followed by a zinc finger motif and helicase domain (Honig et al. 2004). The OTU domains are a superfamily of proteases. Viral OTU domains specifically have ubiquitin (UB) deconjugating activity. Conjugation of UB and ubiquitin like (UBL) molecules to specifically targeted proteins plays a role in the regulation of innate immune responses (Frias-Staheli et al. 2007). Deconjugation of these molecules has an inhibitory effect on antiviral pathways dependent on UB and UBL activation. The size of the polyprotein and the identification of amino terminal domains led to the proposal that the L protein is potentially a polyprotein that is cleaved autoproteolytically. Although in the absence of L-OTU activity the RdRp has been shown to function, and viral replication is unaffected, to date there is no evidence of proteolytic processing of the L protein and the function of the L-OTU has yet to be fully clarified (Bergeron et al. 2010). The protease could likely have a role in evasion of the host immune response by suppression of the innate immunity activated by UB and UBL molecules (Frias-Staheli et al. 2007; Bergeron et al. 2010).

The synthesis of nairovirus glycoproteins, encoded on M segment RNA, appears to involve a precursor polypeptide, a coding strategy which is quite distinct from that used by other genera of bunyaviruses. The M segment of CCHFV is approximately 5400 bases and has one ORF which encodes a precursor polypeptide, with a highly variable amino-terminal domain and a fairly conserved carboxyl-terminal region. The two mature glycoproteins, Gn (37 kDa) and Gc (75 kDa), are derived by signalase cleavage of two precursors, designated preGn (140 kDa) and preGc (85 kDa) (Sanchez et al. 2002). Post translational cleavage of the precursors by SKI-1 and SKI-1 like proteases generates mature Gn and Gc and a mucin-like and GP38 domain (Sanchez et al. 2006). Formation of infectious virus has been shown to be dependent on the presence of cellular serine endoprotease (Bergeron et al. 2007). Further processing of the mucin like GP38 domain by furin/proprotein convertases generates three secreted glycoproteins GP160, GP85 and GP38 of unknown function. More recently a non-structural (NSm) protein has been identified although its function is currently unknown (Altamura et al. 2007).

By analogy with other genera of the *Bunyaviridae*, it can be assumed that glycoproteins are responsible for recognition of receptor sites on susceptible cells and consequently cell tropism and pathogenicity of the virus in humans, for the induction of protective immune response, and probably play a role in tick host selection. Monoclonal antibodies directed against Gc have been shown to prevent CCHFV infection using *in vitro* neutralization assays, although not all protected mice against lethal infection in passive immunization experiments (Bertolotti-Ciarlet et al. 2005). In contrast, non-neutralizing monoclonal antibodies directed against Gn protected mice against lethal challenge suggesting a role for antibody dependent cell-mediated cytotoxicity in viral clearance (Bertolotti-Ciarlet et al. 2005). The exact immune correlates of protection have yet to be determined.

The CCHFV S segment is approximately 1600 bases in length with a single open reading frame which encodes a 482 amino acid NP (approximately 54 kDa), the major structural protein of the virus (Marriott et al. 1992). Viral RNA is encapsidated by NP to form the ribonucleoprotein (RNP) complex. The exact mechanism of this interaction is unknown; complex formation, however, is essential for RNA synthesis and segment packaging.

CCHFV is classified as a class four pathogen because it has the propensity for human-to-human transmission, can be responsible for laboratory infections and causes severe human disease with possible fatal outcome. This dictates that culture of the virus is permitted only in biosafety level four, maximum-security, laboratories.

39.3 CCHFV in Nature

The importance of the tick-vertebrate-tick cycle in maintaining CCHFV transmission is well established. Although the virus has been isolated from at least 31 species of ticks of 7 genera, including 29 ixodids and 2 argasids, in most instances the isolations likely resulted from recent ingestion of a blood meal from a viraemic host and are therefore no definite proof that these ticks can act as competent vectors in all these cases (Hoogstraal 1979; Watts et al. 1989). However, the distribution of human cases, serological evidence and virus isolations from ticks correlate exactly with that of the ticks belonging to the genus *Hyalomma*, providing strong evidence that these ticks are the principal vectors associated with CCHFV (Hoogstraal 1979; Watts et al. 1989). Transtadial transmission has been shown for members of three genera of ixodid ticks, *Hyalomma*, *Dermacentor* and *Rhipicephalus* (Hoogstraal 1979; Watts et al. 1989). Similarly, transovarial transmission has been shown to occur within some species from these genera (Gonzalez et al. 1992; Gordon et al. 1993; Logan et al. 1989; Wilson et al. 1991).

Hyalomma ticks are referred to as two-host-ticks with regards to their life cycle in nature. Each stage in the tick life cycle attaches to a vertebrate host and takes a blood meal before molting to the next instar. The immature ticks, larvae and nymphs, attach to small vertebrate hosts and feed before molting to the next instar. Infection acquired by the larvae feeding on a viraemic host can be transmitted after

the molt to the next instar. Similarly, virus acquired by the immature stages can be transmitted to adult ticks. Transovarial transmission of CCHFV to larvae, and the ability of larvae to transmit infection to vertebrates, was found to occur but at low levels considered below a threshold that would be adequate to perpetuate the virus in the absence of amplification in mammalian hosts (Hoogstraal 1979; Watts et al. 1989).

39.4 Zoonotic Hosts and Their Role in Transmission

Small mammals are considered important amplifying hosts of CCHFV. Viraemia has been demonstrated in several small vertebrates such as little susliks, hedgehogs and scrub hares (Shepherd et al. 1989a; Watts et al. 1989) and in some instances it has been shown that these hosts are capable of infecting ticks (Hoogstraal 1979; Shepherd et al. 1989a, 1991; Watts et al. 1989). Domestic livestock show mild or no clinical signs of illness but develop a short period of viraemia, lasting up to a week, during which the virus can be transmitted to humans and to naïve ticks. Humans acquire infection through broken skin from infected blood or tissues while performing procedures on animals such as castration or during slaughtering. Hence there is an occupational risk associated with employment in the livestock industry among farmers, farm workers, abattoir workers and veterinarians. Within abattoirs infection tends to occur among workers involved in handling animals during the initial bleeding stages of the slaughter process or the workers handling animal hides that are likely tick infested. Virus infectivity is likely reduced as the pH of the tissues decreases after time reducing the risk to workers at later stages in the slaughter process and to meat consumers (Swanepoel et al. 1998).

Immature *Hyalomma* ticks feed on small mammals and ground-frequenting birds and this plays a significant role in maintenance of the virus in nature (Hoogstraal 1979; Watts et al. 1989). The role of large vertebrates in the cycle of CCHFV in nature is limited by the low frequency of transovarial transmission from adult ticks. Acquisition of infection by immature ticks on small vertebrates likely constitutes the most important amplifying mechanism which ensures perpetuation of the virus (Watts et al. 1989).

Birds tend to be refractory to CCHF infection, with the exception of ostriches, which, in the absence of clinical disease, develop a high level viraemia demonstrable for 4 days and a strong antibody response following experimental infection (Hoogstraal 1979; Shepherd et al. 1987; Swanepoel et al. 1998). However, an additional mechanism of transmission referred to as “non-viraemic” transmission has been demonstrated in birds in which infected ticks are able to transfer infection to uninfected ticks during feeding on a non-viraemic host. Non-viraemic transmission of infection between ticks is believed to be facilitated by factors present in tick saliva (Jones et al. 1987) and has been demonstrated for CCHFV using infected adult and non-infected immature *H. truncatum* and *H. impeltatum* ticks fed together on non-viraemic mammals.

39.5 Epidemiology and Genetic Diversity

CCHFV has the most extensive worldwide distribution of any of the arboviruses. Prior to 2002, cases of naturally acquired human infection had been documented in the former Soviet Union, China, Bulgaria, Yugoslavia, Albania, Kosovo (formerly Yugoslavia), Pakistan, Iran, Iraq, United Arab Emirates, Saudi Arabia, Oman, Tanzania, Central African Republic, Democratic Republic of the Congo (formerly Zaire), Uganda, Kenya, Mauritania, Burkina Faso, South Africa and Namibia (Al Tikriti et al. 1981; Burney et al. 1980; Dunster et al. 2002; El Azazy et al. 1997; Gear et al. 1982; Hassanein et al. 1997; Hoogstraal 1979; Papa et al. 2002b; Saluzzo et al. 1984, 1985; Schwarz et al. 1995; Suleiman et al. 1980; Tantawi et al. 1980; Watts et al. 1989).

In addition, evidence for the presence of CCHFV either from isolation of the virus or detection of viral nucleic acid from ticks or non-human mammals has been documented in Madagascar, Senegal, Nigeria, Central African Republic, Ethiopia, Afghanistan and, more recently, Morocco (Watts et al. 1989; Palomar et al. 2013). Serological evidence alone has been reported from Egypt, Zimbabwe, Benin and Kuwait (Watts et al. 1989).

More recently CCHFV emerged in Turkey with more than 7000 human cases of CCHF reported since it was first identified in the country in 2002 (Mazeltou et al. 2010). The first human case in India was reported in 2011 with subsequent nosocomial infections occurring among health care workers after contact with the index patient (Mishra et al. 2011) and the first human infection in Greece was confirmed in 2008 (Papa et al. 2008). Serological evidence of CCHFV was recently detected in Romania (Ceianu et al. 2012) and in 2010 virus was detected in adult *Hyalomma lusitanicum* ticks collected from red deer (*Cervus elaphus*) in Spain (Estrada-Peña et al. 2012). Phylogenetic analysis showed the virus was genetically similar to strains circulating in Africa. CCHFV detected in ticks collected from migratory birds in Morocco showed 100% identity with isolates from Sudan and Mauritania and 98.9% identity with the isolate from Spain (Palomar et al. 2013). Although to date no autochthonous cases of CCHF infection have been reported in southwestern Europe, there is concern that the virus could emerge in currently non endemic regions of Europe where competent vector species are present.

The emergence of CCHFV from 2002 in several countries in the Balkans and re-emergence in south-western regions of the Russian Federation in 1999 after a 27 year absence raises concerns that this virus could expand its current geographic distribution and establish new endemic foci (Maltezou et al. 2010). The reasons for re-emergence are likely multi-factorial and include global warming with changes in weather patterns that influence tick populations, increased animal movement as a result of livestock trade as well as human activities such as changes in farming practices and land development (Maltezou et al. 2010; Maltezou and Papa 2010; Randolph and Rigers 2007).

Molecular methods have facilitated the identification and differentiation of genotypes of CCHFV. Partial and complete genome sequence data have been

used to determine the genetic relationship between strains of CCHFV within specific geographic locations and between geographically distinct regions (Burt et al. 2005; Drosten et al. 2002; Chinikar et al. 2004; Deyde et al. 2006; Seregin et al. 2004; Yashina et al. 2003; Hewson et al. 2004a, b; Papa et al. 2002a, b, c, 2004, 2005; Tonbak et al. 2006). The studies concur that a high degree of nucleotide diversity exists although amino acid diversity is less, particularly within the NP, which could account for the serological cross reactivity between geographically distinct isolates of the virus. Analysis of global diversity has shown that CCHFV isolates group within seven genetically distinct lineages: Africa 1 (Senegal), Africa 2 (Democratic Republic of the Congo and southern Africa), Africa 3 (southern and West Africa), Europe 1 and 2 (Greece), Asia 1 (Middle East, Pakistan and Iran) and Asia 2. Nucleotide sequence divergence and amino acid sequence divergence determined using complete genome data was 20 and 8 % for the S segment, 31 and 27 % for the M segment and 22 and 10 % for the L segment, respectively (Deyde et al. 2006). Analysis of tree topologies for each segment has shown incongruencies in groupings, particularly for the M segment, providing evidence for the natural occurrence of segment reassortment (Burt et al. 2009; Deyde et al. 2006; Hewson et al. 2004b). There appears to be a higher frequency of reassortment events for the M segment. Alternatively, M segment switching may result in a more viable virus compared with other segment reassortment. RNA viruses have the ability to reassort when dual infection occurs. It has been proposed that these events are more likely to occur within vectors rather than vertebrate hosts as ticks remain infected for longer periods and are exposed to multiple hosts potentially infected by different strains of CCHFV (Hewson et al. 2004b; Deyde et al. 2006; Morikawa et al. 2007). Reassortment events provide a mechanism for genetic diversity and, although relatively rare, genetic recombination events have also been shown to occur for CCHFV contributing to genetic variability (Deyde et al. 2006).

To date, the most comprehensive analysis of genetic relationships, with regard to the number of sequences included in the analysis, is based on 168 partial S segments (Mild et al. 2010). Maximum likelihood phylogenies showed that CCHFV strains can be divided into seven genotypes. Based on nomenclature assigned by Mild et al., predominant genotypes were located within distinct regions. Genotype 1 isolates were found in Asia and the Middle East, Genotype 2 isolates were found in Asia, Genotype 3 were identified in Africa, Genotype 4 in European countries, Genotypes 5 and 7 in regions of Africa and Genotype 7 in Europe. In addition, multiple genotypes were identified in some regions as illustrated in Fig. 39.1. The same genotypes can be found in geographically distinct locations and different genotypes can be located in similar regions. CCHFV appears to circulate within and between continents with phylogeny studies supporting the proposed mechanisms for dispersal of the virus. Genetic diversity within regions has likely resulted from movement and trade in livestock and bird migration with resultant introduction of multiple lineages from carriage of infected ticks. In addition reassortment and recombination provide additional mechanisms for the generation of genetic diversity.

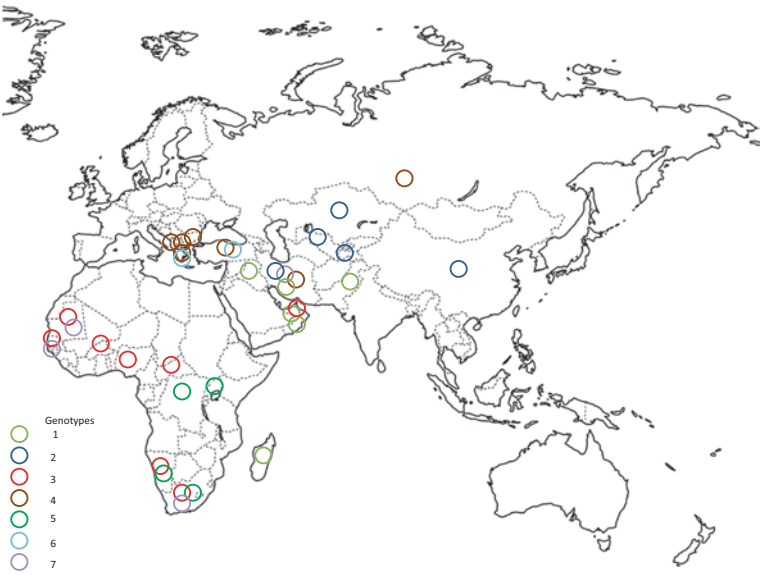

Fig. 39.1 Crimean-Congo haemorrhagic fever patient in South Africa

39.6 Infection of Humans

The incubation period of CCHFV in humans following infection from a tick bite is usually 1–3 days (maximum 9) and 3–6 days (maximum 13) from exposure to infected blood or tissues of animals or humans including secondary infections in a nosocomial setting. The onset of illness is often sudden, with non-specific symptoms including headache, dizziness, sore throat, sore eyes and photophobia. Myalgia and malaise are also common with backache and leg pains. High fever and rigors often become apparent at this stage and the fever may be intermittent. Gastrointestinal symptoms may include nausea, vomiting, diarrhoea and abdominal pain. During the early stages of illness, lassitude, depression and somnolence may be noted as well as neuropsychiatric changes such as confusion and aggression. Other signs include hyperaemia of the face, neck and chest, injected conjunctivae and chemosis. Hepatomegaly with right hypochondrial pain, tachycardia and hypovolaemia are often present. On days 3–6 of illness, a petechial rash often appears especially on the trunk and limbs. This may progress to large ecchymoses and bruising as illustrated in Fig. 39.2 (Hoogstraal 1979; Swanepoel et al. 1987). Other less common skin presentations include a macular or maculopapular rash (Ergönül et al. 2004; Akyol et al. 2010). When present, haemorrhagic manifestions appear at day 4–5 including haematemesis, melaena, haematuria, epistaxis, vaginal and gingival bleeding. Subconjunctival and retinal haemorrhages have also been described (Engin et al. 2009). In other cases, the bleeding tendency may be limited to leakage or oozing from injection or venipuncture sites. Jaundice may be present during the

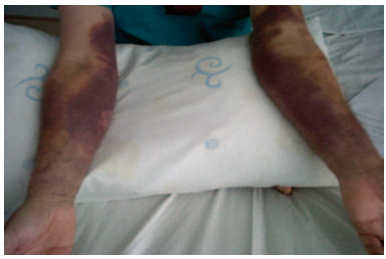

Fig. 39.2 Global distribution of Crimean-Congo haemorrhagic fever virus genotypes

second week of illness (Hoogstraal 1979; Swanepoel et al. 1987). Isolated cases of epididymo-orchitis, parotitis, peritoneal and pleural effusions, acalculous cholecystitis and intraabdominal abscesses have been described during the acute stage of disease (Aksoy et al. 2010; Kaya et al. 2012; Şensoy et al. 2011; Guner et al. 2011).

Fatalities are usually due to multi-organ failure or haemorrhagic complications such as intracranial haemorrhage, occurring mostly on days 5–14 of illness. The mortality rate is reported to be 5–50% with higher mortality rates following nosocomial infections than for infection via tick bites (Gozalan et al. 2007; Hoogstraal 1979). Distinct geographical variations in mortality rates are noted, with rates in Turkey described at 1–5% compared to approximately 30% in South Africa (Ergonul et al. 2006a; Swanepoel et al. 1987). Differences in pathogenicity of strains are yet to be identified and the differences observed in mortality rates may be influenced by the significantly higher numbers of cases occurring in eastern Europe compared to South Africa. Recovery usually begins by day 9 or 10 of illness, although conjunctivitis, weakness, confusion and amnesia may persist beyond a month. Other residual symptoms and signs may include polyneuritis, headache, dizziness, nausea, anorexia, alopecia, vision and hearing loss, and poor memory (Hoogstraal 1979; Swanepoel et al. 1987). Post traumatic stress disorder and impaired health related quality of life have been described in the long term follow up of CCHF patients, with intensive care admission, bleeding, and administration of blood products identified as risk factors (Gul et al. 2012). Similar findings have been described in survivors of other acute, life-threatening conditions and it seems likely that this is related to the severity of the illness rather than the infectious organism.

The clinical picture of CCHFV infection in children is similar to that in adults, although tonsillopharyngitis and abdominal complaints are more common in this age group. The relatively small number of cases identified in children suggests a tendency towards milder disease with a mortality rate of less than 5% (Dilber et al. 2009; Tezer et al. 2010). Intrauterine or perinatal infection of infants has been described following maternal infection with CCHFV during pregnancy and resulting in abortion or haemorrhagic manifestations at birth, although CCHFV infection was not laboratory-confirmed in all cases (Ergonul et al. 2010). Transmission by breastfeeding has not been detected in exposed infants (Erbay et al. 2008).

A single case of clinical infection due to the AP92-like strain, not previously known to cause disease, has been described in a 6 year old boy from Turkey following a tick bite. The child presented with fever, raised liver enzymes, and prolonged

prothrombin time and activated partial thromplastin time. The child made an uneventful recovery with haemorrhagic manifestations and laboratory findings returning to normal by day 10 of illness (Elevli et al. 2010; Midilli et al. 2009).

Abnormal clinical pathology values in patients with CCHF include elevated aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatise (ALP), γ-glutamyltransferase (GGT), lactic dehydrogenase (LDH), creatine kinase (CK), bilirubin and creatinine levels, respectively. These elevations are marked in patients with fatal infections. Leukocyte levels may be elevated or decreased, with leukocytosis more common in fatal cases. Thrombocytopaenia is found in all patients with CCHF; low thrombocyte counts at an early stage of illness are associated with an increased mortality. In addition, markedly abnormal prothrombin ratio (PR), activated partial thromboplastin time (APPT), thrombin time (TT), fibrinogen and fibrin degradation products (FDP) are found early in patients with a fatal outcome, with milder abnormalities in nonfatal cases. The haemoglobin levels often decline even in the absence of overt bleeding (Swanepoel et al. 1989; Ergonul et al. 2006a).

39.7 Pathogenesis

Many similarities exist between the pathogenesis of CCHF and other viral haemorrhagic fevers. Following inoculation, the virus replicates in local cells before spreading to regional lymph nodes and then disseminates haematogenously to various tissues and organs both in lymph and blood monocytes. High levels of viral replication in organs such as the liver and adrenal glands contribute to the clinical picture by decreasing synthesis of coagulation and plasma proteins and dysregulation of blood pressure homeostasis (Geisbert and Jahrling 2004). This is supported by histopatholgical findings of coagulative necrosis in the liver, kidneys and adrenal glands (Burt et al. 1997). The effect of CCHFV on endothelial cells resulting in capillary leakage appears to be exerted chiefly by immunologically mediated mechanisms including immune complex deposition and complement activation, although direct viral replication in endothelial cells has been demonstrated (Joubert et al. 1985; Connolly-Andersen et al. 2011). Release of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) also increases endothelial permeability. Increased levels of TNF-α are significantly associated with severe CCHF disease (Papa et al. 2006; Ergonul et al. 2006b). TNF-α is known to be associated with macrophage activation leading to haemophagocytosis and also stimulates vasodilating substances and antifibrinolytic activity. Interleukin-6 (IL-6), which is released by Kupffer cells due to liver injury, is increased in all patients with CCHF including both mild and severe disease presentations (Papa et al. 2006; Ergonul et al. 2006b). Both TNF-α and IL-6 are Th1 cytokines which stimulate activation of monocytes and contribute to haemophagocytosis. Haemophagocytosis has been described in both adults and children with CCHFV infection and may contribute to the cytopenias observed (Karti et al. 2004; Fisgin et al.

2008; Dilber et al. 2009). High levels of interleukin-10 (IL-10) and interferon gamma (IFN-γ) are also associated with a fatal outcome (Ergonul et al. 2006b). It seems likely that high levels of IL-10 released early in infection result in a degree of immunosuppression which allows high levels of viral replication. This in turn stimulates release of IFN-γ and TNF-α (Saksida et al. 2010). These findings are also supported by studies in mouse models (Bente et al. 2010). The pathogenesis of CCHF also includes disseminated intravascular coagulopathy (DIC) early in infection (Swanepoel et al. 1989).

39.8 Laboratory Diagnosis

The recent emergence and re-emergence of CCHFV in eastern Europe and the Balkans emphasizes the importance of increasing diagnostic capacity and developing standardized, rapid and sensitive assays. Although human cases of infection with CCHFV can be identified based on clinical and laboratory criteria, laboratory confirmation is essential for distinguishing CCHF from conditions with similar clinical features (Swanepoel et al. 1987, 1989; Ergonul 2006c). Classification of the virus as a biosafety level four pathogen determines that the virus can only be cultured within the confines of a biosafety level four laboratory and that laboratories with less sophisticated biosafety levels must inactivate clinical samples prior to testing.

Virus can be isolated in a variety of susceptible mammalian cell cultures, although Vero cells are most frequently used. The virus seldom induces cytopathic effects and infection must be confirmed using immunofluorescent tests. Alternatively, the virus can be isolated by intracerebral inoculation of day-old mice (Hoogstraaal 1979; Clerx et al. 1981; Watts et al. 1989). Isolation of the virus in cell cultures can take 1–6 days whereas mice need 5–10 days to succumb to infection (Shepherd et al. 1986). Although isolation in cells is more rapid, mouse inoculation is a more sensitive technique.

During the acute stage of illness viral nucleic acid can be readily amplified and detected using reverse transcription polymerase chain reaction (RT-PCR) assays. Viral RNA is extracted from clinical samples and as negative sense RNA is not infectious, the amplification can be performed without the requirement of a biosafety level four laboratory. Diagnostic RT-PCR is based on amplification of a conserved region of the genome.

The first diagnostic RT-PCR for CCHFV was based on two nested primer pairs designed by an alignment of the S segment from seven geographically distinct isolates (Schwarz et al. 1995; Rodriguez et al. 1997; Burt et al. 1998). Subsequently, there has been significant development and implementation of diagnostic real time RT-PCR assays. Amplicons can be detected using an intercalating dye and the use of a melt curve analysis to detect specific amplified products or sequence specific probes in which probes are hybridized to complementary regions of the genome (Schwarz et al. 1995; Rodriguez et al. 1997; Burt et al. 1998; Drosten et al. 2002;

Yapar et al. 2005; Duh et al. 2006, 2007; Papa et al. 2007; Garrison et al. 2007; Wolfel et al. 2007, 2009; Kondiah et al. 2010). Real time molecular assays can be designed to determine viral load. Quantification of viral load using real time RT-PCR has been used as a prognostic indicator with reports that a viral load greater than 1×10^8 RNA copies/ml plasma can be considered to predict a fatal outcome (Duh et al. 2006; Garrison et al. 2007; Wolfel et al. 2007; Kondiah et al. 2010; Cevik et al. 2007).

ELISA have been described for detection of viral antigen. Although having the advantage that sophisticated laboratory equipment is not required they lack the sensitivity of molecular amplification or viral isolation and are not frequently used for routine diagnosis (Shepherd et al. 1988; Saijo et al. 2005a).

Serological assays have an important diagnostic role during the convalescent stage of infection. Infection is confirmed based on demonstration of seroconversion, a four-fold or greater increase in IgG antibody activity in paired serum samples, or IgM activity in a single specimen. In contrast, indirect IF and ELISA are frequently employed as diagnostic tools. These assays can distinguish between IgG and IgM responses and are rapid and sensitive techniques for detecting an early immune response (Shepherd et al. 1989b; Burt et al. 1994). Although traditionally most reagents were prepared in house requiring culture of the virus in maximum containment laboratories with subsequent inactivation of the reagents, there are now commercially available ELISA and IF assays. In addition, recombinant antigens have been developed for use as diagnostic and surveillance tools (Samudzi et al. 2012).

In fatal cases antigen and viral nucleic acid can be detected in post mortem tissues. Histopathologic features are not pathognomonic and definitive diagnosis requires virological assays or antigen detection in formalin fixed tissue samples using immunohistochemistry (Burt et al. 1997).

39.9 Kinetics of Viraemia and Antibody Responses

CCHFV is most frequently isolated from sera collected from patients on days 1–6 after onset of illness when virus titers are highest, although virus has also been isolated from samples collected from days 1–12 (Shepherd et al. 1986). Viral nucleic acid has been detected in samples collected up to 18 days after onset of illness; the diagnostic sensitivity, however, decreases with the presence of an antibody response. The development of molecular assays has significantly improved diagnostic capability during the acute stage of illness when patients lack immune markers and virus isolation is dependent on severity of illness and levels of viraemia. In non-fatal infections the ability to isolate virus decreases from days 7 to 12. Although IgG and IgM antibodies have been detected as early as day 3 of illness, they are more frequently detected from day 5 onwards (Shepherd et al. 1989b; Burt et al. 1994; Saijo et al. 2005b; Tang et al. 2003). Patients with a fatal outcome frequently do not develop a detectable antibody response. IgG antibodies remain detectable at least 10–12 years after illness and possibly for longer, whereas IgM antibodies decline

to undetectable levels in most patients 4–6 months post-infection (Shepherd et al. 1989b; Burt et al. 1994).

To date it is unclear what facilitates clearance of the virus. Appearance of a humoral antibody response does not always correlate with clearance of CCHFV. In an investigation to try and elucidate the effect of antibodies on viral load, it was deduced that the detection of IgM had no influence on survival or viral load. While IgG levels appeared to be inversely related to viral load, the virus titers decreased in non-fatal infections independent of detectable antibodies (Duh et al. 2007; Wolfel et al. 2007). The results indicate a role for innate or cellular immune responses in viral clearance.

In summary, during the acute phase of illness confirmation of infection is achieved by isolation of the virus, detection of viral nucleic acid using molecular techniques or detection of viral antigen in ELISA. During convalescent stages antibody responses are detected using ELISA or indirect immunofluorescence assays. Accurate interpretation of the results is facilitated by an accurate history of date of onset of illness and consideration of the kinetics of viraemia and antibody responses.

39.10 Differential Diagnosis

A number of conditions must be considered in the differential diagnosis of CCHFV infection. The geographic location and travel history of patients presenting with a compatible clinical picture can assist in excluding unlikely infectious conditions. Conditions which should be considered include other tick-borne infections such as rickettsiosis (especially by *Rickettsia conorii* and *Rickettsia africae*), Q fever (*Coxiella burnetti*), ehrlichiosis, babesiosis, borreliosis, and severe fever with thrombocytopaenia syndrome virus. Other viral haemorrhagic fevers must be considered dependent on the geographic location, e.g. Ebola virus, Marburg virus, Lassa virus, and Lujo virus in Africa, Rift Valley fever virus in Africa and the Middle East, yellow fever virus in Africa, South and Central America, and dengue virus in tropical and sub-tropical regions. In addition, other common infectious conditions should be ruled out such as bacterial sepsis, malaria, leptospirosis, viral hepatitis, and disseminated herpes simplex virus infection (Burt 2011). Non-infectious conditions may include leukaemia, lymphoma, drugs and auto-immune diseases (van Eeden et al. 1985).

39.11 Treatment

Strict barrier nursing should be implemented when managing CCHF patients to prevent nosocomial transmission. Supportive therapy should include maintenance of fluid and electrolyte balance and administration of platelets, fresh frozen plasma and red cell preparations as needed (Ergonul 2008).

Limited information is available regarding the use of specific CCHFV immunoglobulin or monoclonal antibody preparations. Randomised controlled trials demonstrating efficacy in a clinical setting for both treatment and post-exposure prophylaxis are lacking (Keshtkar-Jahromi et al. 2011).

Similarly, the use of oral or intravenous ribavirin for the treatment of CCHF remains controversial. Although two systematic reviews and meta-analyses of the available randomised trial and observational studies showed insufficient evidence for a clear benefit to using ribavirin in the treatment of CCHFV infection, this antiviral agent has been approved by the World Health Organisation for CCHFV infection based on *in vitro* data (Soares-Weiser et al. 2010; Ascioglu et al. 2011). Adverse drug effects are uncommon, but may include mild haemolytic anaemia and thrombocytosis (Fisher-Hoch et al. 1995; Ergönül et al. 2004; Ozkurt et al. 2006)

39.12 Prevention and Control

Widespread control of ticks using acaricides is an impractical approach to infection control. Prevention of infection and awareness of the disease is a more practical method of reducing the risk of infection and the number of cases.

Limited attempts have been undertaken to develop DNA based vaccines (Spik et al. 2006). Currently, there is one vaccine for human use that is only available in Bulgaria. The vaccine is an inactivated suckling mouse brain derived preparation (Papa et al. 2011). The identification of mouse models for CCHFV should help to understand mechanisms of pathogenesis and immune correlates of protection which will assist the development of novel antiviral drugs or efficacious vaccines.

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Chapter 40
A Review of Hendra Virus and Nipah Virus Infections in Man and Other Animals

Kim Halpin and Paul Rota

Abstract Hendra virus (HeV) and Nipah virus (NiV) emerged in the last decade of the twentieth century. They were the cause of a number of outbreaks of respiratory and neurological disease infecting horses and pigs respectively. Transmission from infected domestic animal species resulted in human infections as well, with high case fatality rates a feature. Today they continue to cause outbreaks of human and animal disease. NiV causes yearly disease outbreaks in humans in Bangladesh, and HeV causes sporadic disease outbreaks in horses in north eastern Australia. Due to their zoonotic nature, they have been ideal candidates for collaborative projects in the One Health space, bringing public health and animal health professionals together. This has lead to insightful epidemiological studies, which has resulted in practical disease prevention solutions including a horse vaccine for HeV and NiV spill-over prevention interventions in the field. As more surveillance is undertaken, their known distributions have expanded, as has the range of reservoir host species. The majority of bat species for which there is evidence of henipavirus infection belong to the group known as the Old World family of fruit and nectar feeding bats (Family *Pteropodidae,* Suborder *Megachiroptera*). This review of the bat borne henipaviruses discusses the epidemiology, pathology, transmission and disease symptoms in these closely related viruses which belong to the Genus *Henipavirus,* Family *Paramyxoviridae.*

40.1 Epidemiology of Hendra Virus in Animals

On the 1st August 1994, a heavily pregnant 10 year old thoroughbred brood mare died suddenly in a paddock in northern coastal Australia. Ironically the first of August is deemed the birthday of all thoroughbred horses in the southern hemisphere[1].

[1] All thoroughbreds have the same birthday so that their ages can be standardized for comparison. In the southern hemisphere the date is the 1st August.

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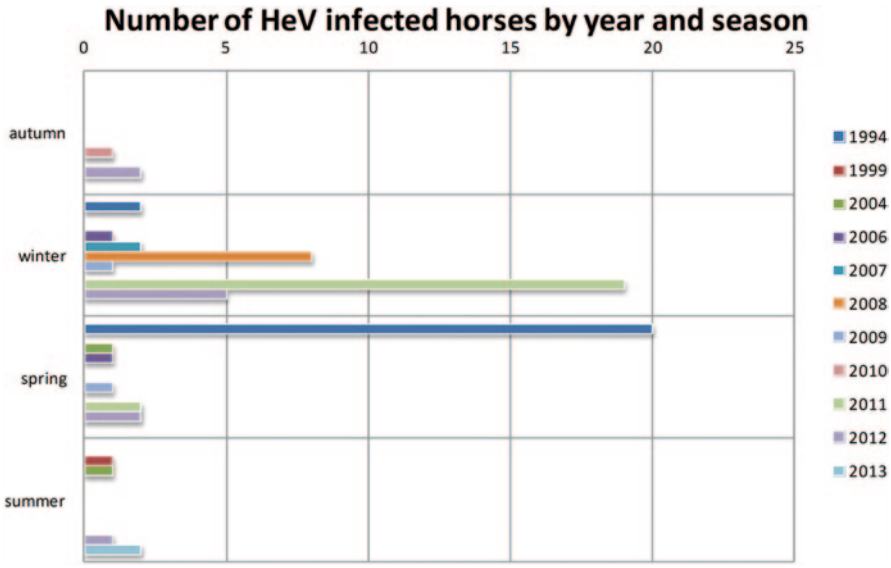

Fig. 40.1 Total number of HeV infected horses by year and season. Winter and spring are the months when three Australian species of pteropus bats are either pregnant or giving birth

However this date in 1994 would go down in history as the day Hendra virus (HeV) emerged.

Hendra virus was first discovered in horses, and horses remain by far the most commonly infected domestic species. The reservoir host of this virus are bats of the genus *Pteropus*. Finding the reservoir host for this virus resulted in the first reported isolation of a zoonotic paramyxovirus from bats (Halpin et al. 2000). To date, only horses have become directly infected from bats. Horses act as HeV amplifying hosts.

As of April 2013, there have been 42 spill-over events between horses and bats with most events involving only one infected horse. In total, 73 horses have been infected (Fig. 40.1). Only two outbreaks have involved more than three horses, and the spread of the virus between the stabled horses in these outbreaks was a result of close contact and assisted mechanical transmission of the virus. Aerosol transmission is unlikely as sneezing and coughing were not features of the syndrome and the spatial distribution of cases in the stables was not consistent with this form of spread (Baldock et al. 1996; Field et al. 2010).

The precise mortality rate in horses has not been possible to calculate as all live horses diagnosed with Hendra virus infection are euthanized according to national policy[2], presumably to prevent relapsing infection and possible further transmission. Experimentally we know that some horses can survive infection. In the outbreaks where there have been numerous horses infected this has also been the case.

[2] Australian veterinary emergency plan. Version 3.3; 2009 [cited 2013 Mar 28] http://www.animalhealthaustralia.com.au/wp-content/uploads/2011/04/RPB3.3-05FINAL27Jul11.pdf.

In the 1994 outbreak in Hendra, seven out of twenty horses apparently survived a lethal infection, and seroconverted before being euthanized (Murray et al. 1995) [refer to clarification]. In the 2008 Redlands outbreak, one horse survived for 42 days after clinical signs abated before being euthanized (Field et al. 2010).

Experimentally, cats can be infected and succumb to the virus (Westbury et al. 1995; Williamson et al. 1998). Anecdotally there was a story about a cat that became sick in the suburb of Hendra at the time of the first recognized outbreak. The cat was never identified and a diagnosis of the cat's condition was never elucidated. It perhaps remains an urban myth, but given the experimental evidence it seems likely that a cat could become infected if it came into close contact with high titres of virus which horses are able to generate (Williamson et al. 1998).

In one of the 2011 outbreaks, a dog on an infected property was found to have antibodies to the virus. It most likely had close contact with HeV laden material from an infected horse, but was clinically well and remained so until it was euthanized in accordance with national policy[2]. Since this time, experimental infections in dogs have been conducted at the Australian Animal Health Laboratory, to better understand the role that dogs might play in the epidemiology of the disease. In these studies dogs could be reliably infected with HeV. Consistent with the field observation, few if any clinical signs were noted during the acute stage of infection. Viral shedding from the oral cavity occurred for a relatively short period of time, and oral secretions collected from dogs during this period were capable of transmitting infection to naïve ferrets (Middleton 2013). Neutralising antibody titres generated in these dogs were similar to that observed in the single recorded canine field case of HeV infection. In dogs, the key site of virus replication within the oral cavity was the tonsil. Middleton (2013) concludes that it is feasible for Hendra virus to be transmitted to people from acutely infected dogs. Further studies are planned to test the repeatability of these observations as well as to assess the duration of the period of maximum transmission risk.

Infection in horses most likely occurs after close contact with bat urine, spats and birthing material which contain sufficiently high enough titres of virus to infect a horse. Luckily for horses, these bat samples rarely contain high titres of virus. The risk of transmission to horses was found to be increased during pteropus bat reproductive periods (especially late pregnancy) and at times when the colonies were undergoing nutritional stress (such as during lactation), presumably leading to higher viral loads (Plowright et al. 2008; Breed et al. 2011). The reproductive cycle in other bat species has been linked to seropositivity and viral activity of other viruses including filoviruses, coronaviruses, lyssaviruses and astroviruses (Pourrut et al. 2007; Drexler et al. 2011; Turmelle et al. 2010).

Hendra virus is present in all four mainland *Pteropus* species of bats in Australia, namely the black flying fox (*Pteropus alecto*), the grey headed flying fox (*P. poliocephalus*), the spectacled flying fox (*P. conspicillatus*) and the little red flying fox (*P.scapulatus*).

It appears that the reservoir host co-exists with this virus in complete harmony. The virus spreads quite easily amongst bats, with the HeV seroprevalence in bat colonies fluctuating over time and geographical spread. In one bat colony,

seroprevalence steadily increased from 45 to 69% over a 2 year period supporting a model of endemic infection in the population (Breed et al. 2011). Absence of disease attributable to HeV infection is supported by experimental observations (Halpin et al. 2011). This is consistent with the observation that many viruses do not cause disease in their reservoir host. The long-term coexistence of viruses and their reservoir hosts has given co-evolution a good chance to reach a relative equilibrium (Domingo 2010). The theory of viral co-evolution with chiropteran hosts has been previously suggested, and all field observations and experimental evidence to date supports this (Halpin et al. 2007).

40.2 Epidemiology of Nipah Virus in Animals

Since HeV was detected in fruit bats of the *Pteropus* genus, these bats were among the first species investigated as possible reservoirs for Nipah virus (NiV) after its emergence in 1999 (Halpin et al. 2000). Neutralizing antibodies to NiV were detected in *Pteropus hypomenalus* and *Pteropus vampyrus* during wildlife surveillance following the initial NiV outbreak in Malaysia in 1999, but NiV was not isolated at this time (Yob et al. 2001). The first NiV isolates from bats were obtained from colonies of *Pteropus hypomenalus* on Tioman Island, Malaysia (Chua et al. 2002). Since then, antibodies to NiV have been detected in other *Pteropus* species (*Pteropus lylei*, *Pteropus giganteus*) and less frequently in other species of bats including *Hipposideros larvatus* and *Scotophiilus kuhli* from Cambodia, Thailand, Indonesia, and Bangladesh (Hsu et al. 2004; Reynes et al. 2005; Sendow et al. 2006; Wacharapluesadee et al. 2005). In 2000, NiV was isolated from a urine sample collected underneath the roost of *Pteropus lylei* bats in Cambodia (Reynes et al. 2005). More recently, Nipah virus was isolated from *Pteropus vampyrus* in Malaysia (Sohayati et al. 2011). Serologic evidence of Nipah infection was also obtained from *Rousettus leschen* and *Cynoptera sphinx* in Vietnam (Hasebe et al. 2012). Several species of Chinese bats also contained antibodies to Nipah or Nipah-like viruses (Li et al. 2008). A very thorough study of the presence of henipaviruses in Australasia indicated that NiV was present in East Timor and that non-NiV, non-HeV henipaviruses were present in Sumba, Sulawesi, and possibly Papua New Guinea (Breed et al. 2013). The authors suggested that NiV can be detected in areas where *Pteropus vampyrus* is present. In Madagascar, seropositive *Pteropus rufus* and *Eidolon dupreahum* bats have been found, and 39% of *Eidolon helvum* from Ghana had NiV reactive antibodies (Hayman et al. 2008; Iehle et al. 2007). Henipavirus-like sequences were obtained from *Eidolon helvum* in Ghana (Drexler et al. 2009). The detection of antibodies to and sequences of henipaviruses in African bats suggests that the range of potential NiV infections may be wider than previously thought, though no human cases of NiV have been reported from any region other than Southeast Asia.

Experimentally infected *Pteropus* bats develop subclinical NiV infection with only sporadic viral excretion in urine. Some animals seroconvert and some show evidence of infection by detection of viral antigen in tissues (Middleton et al. 2007; Halpin et al. 2011).

With regard to domestic species affected by NiV, pigs featured in the first outbreak in Malaysia (Chua et al. 2000). Pigs presumably became infected from bats, and the disease spread throughout piggeries with pigs serving as an amplifying host. Most of the human infections occurred in people with direct contact to sick pigs. Serologic studies demonstrated evidence of infection among other domestic species of animals in Malaysia, including horses, dogs and cats (Chua et al. 2000; Hooper and Williamson 2000). In the outbreak in Meherpur, Bangladesh in 2001, close contact to both infected patients as well as to sick cows was associated with NiV infection in humans, although samples from cows were not available for testing (Hsu et al. 2004).

40.3 Epidemiology of Hendra Virus in Humans

It has been estimated that over 700 people have come into contact with Hendra virus infected horses, however, to date it has only been those who have had very close contact with infected bodily fluids through performing invasive procedures, and/or have not worn fully protective gear who have become infected with HeV.

There has been no human-to-human spread of the virus. The first person to become infected and die from Hendra virus was assisting his wife, a veterinarian, to perform an autopsy on a horse that had died suddenly in a paddock (Rogers et al. 1996). This patient recovered from a short illness, but went on to die 13 months later after a relapse with encephalitis (O'Sullivan et al. 1997). In the second outbreak of Hendra virus, the horse trainer and a strapper, who both had very close contact to infected horses in their racing stables, became infected (Selvey et al. 1995). The next person to become infected was a veterinarian who had performed an autopsy on a horse who had died from colic-like symptoms. At the time, colic-like symptoms had never been associated with HeV infection in horses. The veterinarian came down with a flu-like illness, but recovered and to this day has neutralizing antibodies to the virus (Taylor et al. 2012). The next two people to become infected were a male veterinarian who had performed a nasal lavage on a horse which had respiratory symptoms, and the veterinary nurse who had assisted with the procedure (Playford et al. 2010). The veterinarian went on to die. The last person to become infected with Hendra virus was a male veterinarian who cared for a horse which was also diagnosed with Hendra virus (Field et al. 2010).

The human case fatality rate stands at 57%, with four deaths and three survivors. Interestingly to date only male patients have died, however with such a small sample size this should not be over-interpreted.

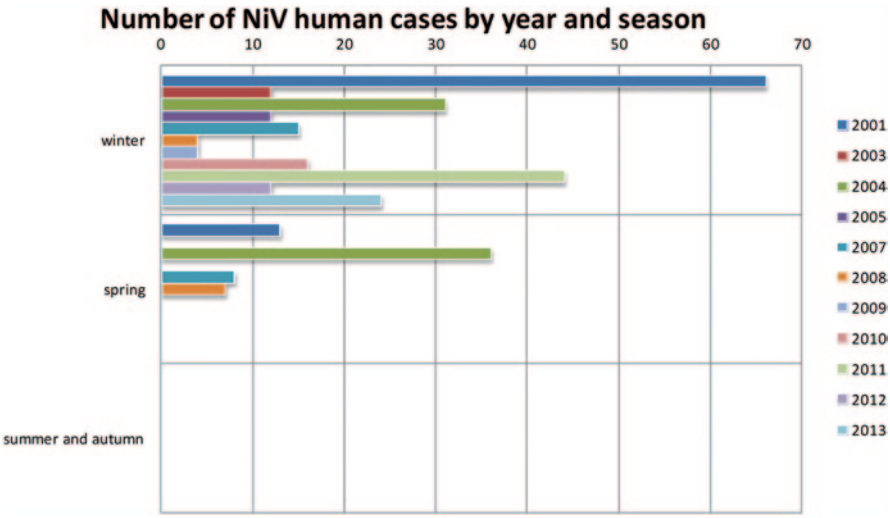

Fig. 40.2 Total number of NiV infected people by year and season in Bangladesh and India. Winter and spring are the seasons when date palm sap is harvested in the region where NiV infections occur. (Source: Luby et al. 2006, Data taken from http://www.iedcr.org and http://www.searo.who.int/entity/emerging_diseases/links/nipah_virus_outbreaks_sear/en/index.html)

40.4 Epidemiology of Nipah Virus in Humans

The first detected outbreak of NiV occurred in Malaysia and lasted 9 months. Overall, 276 cases were reported which included 106 deaths (Chua et al. 2000). Nipah virus was transmitted to pigs and spread rapidly among swine herds causing primarily respiratory symptoms in pigs. Pig-to-human transmission resulted in acute febrile encephalitis mostly among adult males who worked in the pig industry. The outbreak spread to Singapore via the transport of infected pigs (Chua et al. 2000; Paton et al. 1999). Culling of more than 1 million pigs was undertaken in an attempt to control the outbreak (Chua et al. 2000).

Since the outbreak in Malaysia, outbreaks have been reported almost annually in Bangladesh and India from 2001 (Fig. 40.2) (Chadha et al. 2006; Luby and Gurley 2012; Luby et al. 2009a). The epidemiologic characteristics of the outbreaks in Bangladesh differed from the Malaysia outbreaks in several respects. Most notably, the case fatality rate in Bangladesh (2001–2010) ranged from 38 % to as high as 100 %, with an average mortality rate of 73 %, while the mortality rate for Malaysia was approximately 38 % (Luby and Gurley 2012). Infected individuals in Bangladesh were more likely to have respiratory symptoms, and there was evidence of human-to-human spread.

Luby et al. 2009b showed in their study that Nipah case-patients who had difficulty breathing were more likely than those without respiratory difficulty to transmit Nipah. Although a small minority of infected patients transmit Nipah virus,

more than half of identified cases result from person-to-person transmission. In these cases, virus was spread during close contact while caring for sick individuals or preparing bodies for burial (Blum et al. 2009; Chadha et al. 2006; Luby et al. 2009b).

All confirmed Nipah outbreaks in Bangladesh have occurred in the same central and northwestern regions (Luby et al. 2009b). Notably, the only two outbreaks that have been reported from India have been in regions within 50 km of the border with Bangladesh and immediately contiguous with the affected areas in Bangladesh (Chadha et al. 2006).

40.5 Evidence of Animal to Human Transmission of Hendra Virus

While bats are the reservoir host of the virus, humans have only become infected after close contact to infected horses. In Australia there are many bat carers who have close contact to sick and injured bats. They get bitten and scratched and come into contact with urine and faecal material, as well as placenta and birthing fluids. However, no bat carer has ever been diagnosed with infection. An extensive serological survey of bat carers in Queensland was performed in the mid 1990s, and there was no serological evidence of exposure to the virus (Selvey et al. 1996). However, bat carers are at risk of becoming infected with Australian bat lyssavirus.

In one HeV experiment, a small amount of viral RNA was detected in the nasal secretions of HeV infected horses two days after exposure to the virus and at least 2 days before the onset of clinical signs, suggesting that transmission of the virus from the infected horse may be possible before it is obviously unwell (Marsh et al. 2011). However at this early stage of infection, the amount of viral genome detected was very low and it is unlikely that this would be enough to infect another host. The findings also supported the observation in experimentally infected pteropus bats that a local mucosal infection, from days two to approximately six post exposure precedes a systemic infection (Halpin et al. 2011). Only after the systemic infection has been established does it become possible to isolate infectious virus from urine and blood.

Sequence analysis of different isolates from both horses and pteropus bats reveals extreme conservation at the genome and protein levels (Marsh et al. 2010; Smith et al. 2011). In one study comparing five horse isolates from five locations which spanned almost 2000 km, across three time points, to the original 1994 isolate, less than 1 % variation at both the nucleotide and amino acid levels was shown across the 18.2-kb genome (Marsh et al. 2010). This genetic stability supports the theory of co-evolution where HeV is well adapted to its host resulting in minimal pressure to change over time (Halpin et al. 2007; Smith et al. 2011).

40.6 Evidence of Animal-to-Human Transmission of Nipah Virus

In contrast to Malaysia where pigs clearly served as the amplifying host that facilitated spread of the virus from bats to humans, no intermediate animal host was identified in Bangladesh. Several routes of transmission of NiV from bats to humans have been identified by studying the nearly annual outbreaks in Bangladesh and the single outbreak in India. Consumption of contaminated date palm sap or contaminated fruit has been linked to a number of cases and outbreaks in Bangladesh (Rahman et al. 2012). Case-patients reported no history of physical contact with bats, though community members often reported seeing bats. Infrared camera photographs have shown that *Pteropus* bats frequently visited date palm trees in those communities where sap was collected for human consumption. This provided an opportunity for intervention in an attempt to prevent NiV spillover to humans. It has been shown that skirts (made from bamboo, dhoincha, jute stick and/or polythene) covering the sap producing areas of a tree effectively prevented bat-sap contact (Khan et al. 2012).

Genetic analysis of NiV isolates and sequences obtained from clinical samples indicated that the outbreaks in Bangladesh were the result of multiple, independent introductions of virus into the human population (Harcourt et al. 2005; Luby et al. 2009b). Sequences of NiV isolates from human outbreaks in India and Bangladesh showed more heterogeneity than the sequences obtained in the initial Malaysian outbreak (AbuBakar et al. 2004; Arankalle et al. 2011; Chadha et al. 2006; Harcourt et al. 2005; Lo et al. 2012) and phylogenetic analysis indicated there are at least two distinct lineages of NiV circulating in Southeast Asia. Sequences obtained from Malaysia and Cambodia are designated as genotype M, while sequences obtained from Bangladesh and India are designated genotype B. Genotypes can be assigned based on the sequence of a 729 nucleotide window in the N-terminal region of the N gene ORF. Levels of nucleotide variation among full-length ORFs between genotypes M and B ranged from 6 to 9% and between the complete genomes nucleotide variation is approximately 8% (Lo et al. 2012). It is not clear if there are biologic differences between the genotypes and this question is the subject of ongoing investigation (Clayton et al. 2012; DeBuysscher et al. 2013).

40.7 Principles of Pathogenesis

These two paramyxoviruses, NiV and HeV, enter cells by binding to the receptor Ephrin-B2, which is expressed on neurons, smooth muscle, and endothelial cells surrounding small arteries (Bonaparte et al. 2005; Negrete et al. 2005). Ephrin-B3 serves as an alternative receptor for NiV, but not HeV (Negrete et al. 2006). After receptor binding by the attachment protein, G, the fusion protein (F) which is cleaved to create two linked polypeptides, F_1 and F_2, fuses to the host cell mem-

brane, initiating endocytosis (Wang et al. 2001). Following fusion between the viral envelope and the host cell membrane, the viral ribonucleocapsid is released into the cytoplasm (Lamb and Parks 2007). The polymerase complex composed of the polymerase (L) and phosphoprotein (P) initiates transcription of viral mRNAs. As translation of viral mRNA occurs, viral proteins accumulate in the cell, and the polymerase switches from transcription to genome replication.

Newly made genomes are encapsidated by the nucleoprotein (N) and polymerase complexes become associated with packaged nucleocapsids. The glycoproteins are synthesized in the endoplasmic reticulum (ER), mature through the Golgi network and are transported to the cell membrane. The processing of the fusion (F) glycoprotein occurs in the endosome (Diederich et al. 2005). The cytoplasmic tails of the F and G glycoproteins play a role in the interaction with the matrix (M) protein, which initiates virus maturation and budding (Ciancanelli and Basler 2006; Lamb and Parks 2007; Ong et al. 2009; Patch et al. 2007, 2008).

This tropism for endothelial cells results in a pathology characterized by vasculitis, thrombosis, ischaemia, necrosis and CNS parenchymal infection (Wong et al. 2002, 2009; Weingartl et al. 2009).

A post-mortem study of human NiV infection determined that a systemic multiorgan vasculitis associated with infection of endothelial cells was the main pathologic feature, with infection being most pronounced in the central nervous system (CNS) (Wong et al. 2002). In the CNS vascular endothelium, immunohistochemical analysis showed intense staining of endothelial, parenchymal, and multinucleate giant cells which are characteristic of paramyxovirus infection. Evidence of endothelial infection and vasculitis was also observed in other organs, including lung, heart, spleen, and kidney. NiV has been isolated from cerebrospinal fluid, tracheal secretions, throat swabs, nasal swabs, and urine specimens of patients (Chua et al. 2001; Goh et al. 2000; Wong et al. 2002) and detection of viral RNA by RT-PCR in urine and throat swabs samples is routinely used to confirm NiV infection.

40.8 Disease Symptoms in Humans and Animals

Early cases of Hendra virus infection in horses had clinical signs of an acute respiratory disease (Murray et al. 1995). However, as more cases appeared, the spectrum of clinical signs widened to include colic-like symptoms and neurological manifestations. The incubation period is between 4 and 16 days (Baldock et al. 1996), after which time clinical signs such as fever, tachycardia, inappetence, depression, dyspnea and restlessness may be observed (Marsh et al. 2011). Associated with the laboured breathing, a nasal discharge which may be frothy or blood- tinged, develops. Ataxia and myoclonus may also be seen (Rogers et al. 1996).

The first fatal human cases of Hendra virus infection died of an acute respiratory illness (Selvey et al. 1995). The second fatal human case suffered from relapsing encephalitis (O'Sullivan et al. 1997) with the third and fourth cases succumbing to encephalitis (Field et al. 2010; Playford et al. 2010). Two of the surviving human

cases suffered from a self-limited influenza-like illness at the time of Hendra virus infection (Hanna et al. 2006). The third survivor showed development of an influenza-like illness that progressed to acute encephalitis and suffered a long and debilitating neurological illness which to this day has not fully resolved (Playford et al. 2010). To date, this patient remains seropositive (Taylor et al. 2012).

The incubation period for NiV ranges from 6 days to 2 weeks; after symptom onset patients deteriorated rapidly usually requiring hospitalization (Eaton et al. 2007; Hossain et al. 2008). In a subset of 14 secondary patients who had well defined exposure to another case, the incubation period was 6–11 days (Luby et al. 2009b). In humans, NiV causes acute febrile encephalitis including fever, headache, drowsiness, dizziness, myalgia, and vomiting with reduced consciousness and evidence of brainstem involvement being a poor prognostic factor. Some patients with NiV initially present with pulmonary symptoms such as cough, atypical pneumonia and acute respiratory distress. The percentage of NiV patients presenting with respiratory disease was higher in Bangladesh (69 %) than in Malaysia (25 %) (Luby et al. 2009b; Tee et al. 2009). Some NiV cases experienced relapse of disease or late onset encephalitis after initial infection, which occurred on average approximately 8 months after initial infection (range: 9 days—22 months) and both syndromes have similar clinical manifestations (Goh et al. 2000; Tan et al. 2002; Tyler 2009). Upon post mortem examination viral antigen was found in the brains of patients with relapse and late onset encephalitis indicating viral replication took place in these tissues. Unlike acute NiV encephalitis cases, relapse and late onset encephalitis cases did not show vasculitis in the CNS (Tan and Chua 2008; Tan et al. 2002; Tyler 2009). A number of NiV infected individuals also experienced residual neurological symptoms that ranged from mild cognitive or cerebellar disabilities to more severe cognitive impairment, with some remaining in a vegetative state (Goh et al. 2000).

In the NiV outbreak in Malaysia, a newly identified porcine respiratory and neurologic syndrome developed in some pigs infected with NiV. This syndrome was characterized by fever, barking cough, behavioral changes, uncoordinated gait, spasms, and myoclonus (Mohd Nor et al. 2000).

40.9 Unresolved Issues

Both viruses are designated biosafety level (BSL) 4 agents which makes it difficult for researchers to work with these viruses. Furthermore, diagnostic tests requiring the use of live virus are restricted. However these tests are very important because of the nonspecific nature of clinical signs associated with henipavirus infections. Molecular detection of viral genome is currently the central arm of henipavirus infection diagnosis. Expanding the surveillance and laboratory capacity for rapid diagnosis of encephalitis outbreaks is crucial to early detection and containment in areas at risk for NiV and HeV.

There are currently no vaccines, passive immunoprophylaxes, or antiviral chemoprophylaxes approved for human henipavirus infections and it will difficult to interrupt the transmission of viruses from the natural reservoir to horses, pigs, or other amplifying hosts. Numerous studies have identified potentially valuable vaccines and antiviral compounds (Broder et al. 2012). At present, human infections with NiV can potentially be prevented by early recognition of NiV infection of animals and the use of appropriate barrier precautions when exposed to potentially infectious material or persons.

Clearly HeV poses a serious threat to the veterinary profession. Five of the seven (71%) people infected with Hendra virus were associated with this profession. Fortunately for horse owners and breeders, there have been no infections in this cohort to date. However, any horse which is infected with Hendra virus poses a serious threat to all who come in contact with the animal, and this includes dogs, cats, ferrets and possibly other animals. This situation prompted the development of a vaccine for horses which was released at the end of 2012 (Broder et al. 2013). It remains to be seen what the uptake of this vaccine will be like, and how it will impact the epidemiology of the disease. A successful equine vaccination program has the potential to reverse the current trend of veterinarians exiting equine practice in HeV-endemic regions due to the perceived personal risk and workplace liability (Mendez et al. 2012).

The discovery of HeV in pteropus bats in 1996 (Halpin et al. 2000) marked the beginning of a new wave of research activities, which led to the association of bats with some of the most notable viral pathogens to emerge in recent history, including NiV (Chua et al. 2002), severe acute respiratory syndrome–like coronaviruses (Li et al. 2005), Ebola virus (Leroy et al. 2005), and Marburg virus (Towner et al. 2009). Two recently published studies from Papua New Guinea identified an increased henipavirus seroprevalence from less than 10% in the period 1996–1999 to 55% in 2010, suggesting that the dynamics of HeV or NiV or another closely related virus is changing (Breed et al. 2010; Field et al. 2013). Additional studies are needed to determine the ecologic, environmental and epidemiologic circumstances that favor transmission of NiV and HeV from their natural reservoir host to humans and other domestic species. Pteropid bat cell lines have recently been developed (Crameri et al. 2009) and future studies should help improve our understanding of how NiV and HeV persist in various bat species.

We know at least one other henipavirus exists in south east Asia. Cedar virus is the most recently discovered henipavirus (Marsh et al. 2012). It remains to be seen if Cedar virus has the capacity to spill over from its reservoir host, the pteropus bat, to other species and cause disease. The question is not, will other henipaviruses be discovered? The question is, how many other henipaviruses will be discovered and which ones will pose a threat to the health of humans and other animals?

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Part VIII
Nature is the Greatest Bioterrorist: Zoonotic Pathogens as Bioterroristic Agents

Chapter 41
Dangerous Viral Pathogens of Animal Origin: Risk and Biosecurity

Zoonotic Select Agents

Jean-Paul Gonzalez and Gavin Macgregor-Skinner

Abstract Most of emerging infectious diseases affecting humans are of animal origin and transmitted under natural circumstances from either, wild or domestic vertebrate animals giving the way of zoonotic infection or epidemics. Zoonotic diseases carry a common ancient history between human and animals as a result of pathogen exchanges involving transgression of the species barrier. Nowadays, several agents have been targeted for their potential to be a major risk for human and animal populations and, have been characterized by their potential to be highly pathogenic and/or transmissible, and lacking of any means of protection. Those agents have been listed as "Select Agents" having the potential to pose a severe threat to both human and animal health, as well as to animal and plant products. Several of the most dangerous agents responsible of viral hemorrhagic fever are review in this chapter including: Ebola virus, Marburg virus, Rift valley fever virus, Kyasanur forest virus, Omsk hemorrhagic fever virus, Alkhurma hemorrhagic fever virus.

41.1 Introduction

More than 75% of recently emerging infectious diseases affecting humans are of animal origin; about two third of all human pathogens have an animal source as a natural reservoir (Taylor et al. 2001). The nosologic term of "Zoonosis" has been crafted to gather all transmissible diseases harboring a potential to infect both human and animal (Palmer et al. 2001). Zoonosis (i.e. zoonotic diseases) are transmissible diseases between animals and man with an infectious (microbes and

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prions) or parasitic origin. In another term, a zoonotic disease represents any animal disease communicable to human and/or vice versa. Ultimately, zoonosis can be transmitted from animals to humans, directly or indirectly, sometimes by a vector or an intermediate host, or also from humans to other animals. This is considered as reverse zoonosis and called anthroponotic disease, or zooanthroponosis. Zoonoses can be of viral (Yellow Fever, HIV, hantavirus), bacterial (tularemia, leptospirosis, lyme disease), rickettsial (Q-fever), fungal (aspergillosis, histoplasmosis), parasitic (giardiasis, cryptosporidiasis), or prions (Creutzfeldt–Jakob disease) origins. Also the mechanisms of transmission are the main factors driving the risk of human infection. Infectious agents are transmissible under natural circumstances from wild or domestic vertebrate animals to humans. They can also be transmitted from animal products causing foodborne diseases, e.g. *Escherichia coli* O157:H7, Campylobacter, Calicivirus, or Salmonella.

The origin of zoonotic diseases occurred probably when humans came in close contact (scavenging or hunting) with wild animals. Indeed, several zoonoses have been known since early prehistoric times. The first hominids were in direct contact with animal groups which previously appeared on Earth some 540 million years ago (ya.). The history of mankind, starting with Australopithecus, begins about 5 million ya. and coincides with the first contact and potential of microbe exchanges between fauna and this human precursor. Also, one of the most ancient hominids, Australopithecus, was not hunter, but a pretty game (!) hunted by large and powerful carnivorous. Also sick and infected individuals were eaten by such large predators, and human epidemics turned short (Debré and Gonzalez 2013). Earlier *Homo* species from the Pleistocene era (2.6 million–11,700 ya.) utilized larger animals for subsistence (Rabinovich et al. 2008) including mammoths, long horned bisons, saber-toothed cats, giant ground sloths, among others mammals of North America, Asia, and Europe. It is quite acceptable that these creatures were able to exchange their parasites, e.g. intestinal and blood parasites or fur ectoparasites, with humans.

Hunting remained a crucial component of hunter-gatherer societies before the domestication of livestock and the dawn of agriculture 11,000 ya. First attempts to domesticate dogs, goats, and sheep, occurred as early as 15,000–9,000 ya., giving rise to domestic zoonotic parasitic disease. Ultimately, about 1000 ya., 22 species were domesticated including dog, goat, sheep, cattle, camel, pigs, and chicken. Later, during the Neolithic period, when agricultural practices appeared, domestication was well under way supporting the appearance of e.g. flea-or louse-transmitted bacterial zoonoses or pyogenic infections after contact to wild and domestic animals. In fact, in prehistoric times, when human populations were organized in small tribes with a limited number of 100–200 individuals, the human population was actually an accidental victim of infectious diseases, developing rapidly an herd immunity and leaving the pathogens to infect and survive in the more abundant animal populations (e.g. anthrax, rabies, tularemia, cysticercosis) (Debré and Gonzalez 2013).

Indeed, zoonotic diseases carry a common history between human and animals as a result of pathogen exchanges involving a transgression of the species barrier. Altogether, such events occur in a variety of situations involving different hosts,

vectors, the pathogens natural cycle's, and the ability of a pathogen to target specific host cells or organs sharing some structural identity between taxonomically distant species (i.e.: human to non-human mammal species).

41.1.1 Zoonotic risk

Essentially, a zoonotic risk exists and increases with the frequency of contact between infected animals and uninfected permissive human hosts, as well as with the capacity of a pathogen to infect both.

Transgressing the Species Barrier The pathogen species-jumping ability is relevant from wild as well as domestic animal species that can transmit their own microbes to human. The species barrier can easily be violated when species are sympatric and/or taxonomically closely related (e.g.: Arenavirus and different rodent species). Although some pathogens have a high infectious specificity and are usually restricted to infect one host species, some of them can pass the species barrier after a mutation or genetic re-assortment (e.g.: the SARS coronavirus from chiropteran to Palm civet, avian influenza from bird to pig) and/or after an alteration of the permissive host (e.g. due to immunodeficiency). Ultimately, zoonotic diseases result from parasites, *sensu lato,* that can live apparently harmlessly in a natural host while producing disease upon entry into a different host. Some prominent examples are e.g. HIV having a non-human primate origin and influenza viruses generated from pig and bird viruses after genetic re-assortment, both subsequently evolving to be adapted to a human-to-human virus transmission.

Disease Emergence in Humans A variety of classical human viral diseases are suspected to be the consequence of such a virus jump from animal to human. The origin of such species-jumping leading to disease emergence in the human population takes place in different situations generally associated with human behavior. As mentioned above, the first pathogen exchanges between humans and animals probably occurred sequentially from hunting wild animals to animal domestication.

For example, it is hypothesized that the following diseases originated from either domestic and wild animals: smallpox from rodents more than 10,000 ya., common cold rhinovirus from cattle more than 4,000 ya., influenza from pigs more than 8,000 ya., measles from cattle plague 300 ya., HIV from non-human primates (NHP) less than 100 ya. (Hughes 2010).

Human Population at Risk While many of the zoonotic microbial agents (e.g. the bacteria causing tuberculosis or diphtheria) are resident in domestic mammals and birds, farmers, breeders and all those involved in food animal production are at risk, since the growing contact between humans and wildlife clearly increases the zoonotic risk (e.g. the example of Ebola fever) (Daszak et al. 2000). This can be caused either by encroachment of human activity into wilderness areas or by movement of wild animals into areas of human activity (Artsob 2004).

There are undoubtedly many zoonotic agents waiting in Nature that have the potential to be introduced into humans. Among animal reservoirs with a high and manifest risk for zoonotic transmission are the NHP because of their genetic closeness to humans (Gonzalez et al. 2013) and pigs because of the similarity of their digestive, respiratory and immune systems with the human ones (Martien et al. 2012).

Besides the "natural" risk of an emergence of a certain zoonosis that is directly linked to pathogen evolution (i.e.: change in pathogenicity) and ecology (e.g., extreme weather events, natural catastrophies, climate change), more cryptic threats exist and are a cause of concern: the possibility of zoonotic emergence from xenotransplantation from an infected animal biological product (Allan 1996) and the deliberate release of infectious agents into human or animal populations by people (Atlas 2001).

Altogether, most of the factors involved in zoonotic emergence are of human origin, e.g. occupational (poaching, hunting, butchering), due to individual behavior (pets, eating bush meat), by man-made environmental changes (landscape fragmentation, protected area parks and recreational activities), or through social behavior (migration).

41.1.2 Biosecurity

Biosecurity is a set of preventive measures designed to reduce the risk of infection by multiple actions (quarantined pests, contain invasive alien species, master viable genetically modified organisms [GMO], identify pathogen genetic shift, etc.) modulated by the foundations of risk in line with the assessment of biological risk. To this end, scientific research became the principal actor in a complex process aimed at understanding and mastering the emergence of pathologies (Gonzalez and Fair 2013).

Risk Assessment The biological risk can be either of natural (i.e.: the random encounter of the pathogen, the natural host and human), accidental (i.e.: unexpected "spill over" of the pathogen that infect another host including human), or deliberate origin (i.e.: an individual—criminal—or a group—terrorist—undertaking taking action to infect human or animals). Preventive measure needs a risk assessment with respect to the identified pathogen and its potential to target human and animal (or vector) populations. Several pathogens have been identified as particularly dangerous in that matter regarding their intrinsic characteristics. Ultimately, human and animal populations can consequently be identified concerning their vulnerability to the agent (i.e.: pathogenicity and occurrence in the same environment) (Table 41.1).

Select Agents Several classes of diseases and agents have been identified as presenting a particular high level of danger including hemorrhagic fever of viral or bacterial origin, infectious neurological syndromes, severe respiratory syndromes among others. Also, regarding the pathogenicity of infectious agents (virulence) and infectiousness (potential to spread) with respect to the risk for either the general

Table 41.1 Common human and animal highly pathogenic viruses

Virus family	Virus name	Geographical origin	Hosts: Main/ secondary	Vector	Transmission and public health issues	Risk assessment	Human pathogenic	Reference
Arenavirus	Barmah forest	Australia	Possums, kangaroos and wallabies	Mosquito	HT: by bites from infected mosquitos	Z	Mild illness, ILS (pain) recovery	Marshall et al. (1982)
	Venezuelan equine encephalits	America	Horse/zebra, donkey		HT: Zoonosis fever epizootics	SA/P3/	ILS/ENC	Gardner et al. (2008)
	Chapare[a]	America	Rodent, potentially but no evidence yet/-?		HT: ?	SA/P4	H, HF: ILS + vomiting + hemorrhagic signs	Delgado et al. (2008)
Arenavirus	Junin[a]	Argentina	*Calomys musculinus* (dry lands vesper mouse or corn mouse)		HT: rodent biological fluid	SA/P4/	H, HF: neurological signs; mortality 20–30%	Maiztegui (1975)
	Lassa fever[a]	Africa	*Mastomys (Praomys) natalensis* (natal multimammate mouse)		HT: rodent biological fluid	SA/P4/	H, HF: + neurological signs; mortality 30% during epidemics	Buckley et al. (1970)
	Lujo virus	Africa	Rodent, potentially but no evidence yet?		HT: ?	SA/P4/	H/HF: 80% fatalities	Paweska et al. (2009)
	Lymphocytic Choriomeningitis	WW	Mice with weight loss, retardation of growth and hair development, often lethal		HT: mice	Z	H: two phase, ILS and meningitis- encephalitis; E: neurological damage	Armstrong and Lillie (1934)
	Machupo[a]	Bolivia	*Calomys callosus* (large vesper mouse)		HT: rodent	SA/P4/	H/HF: slow onset with influenza like fever + Petechial; 30% mortality	Johnson et al. (1966)

Table 41.1 (continued)

Virus family	Virus name	Geographical origin	Hosts: Main/ secondary	Vector	Transmission and public health issues	Risk assessment	Human pathogenic	Reference
	Sabia	Brazil	Rodent ?		HT: aerosol	SA/P4/	H/HF one fatal case among 3 known; E: 1 death/3	Coimbra et al. (1994)
	Guanarito[a]	Brazil	*Zygodontomys brevicauda* (short-tailed cane mouse)		HT: rodent	SA/P4/	H/HF 23.1% mortality	Salas et al. (1991)
Bornavirus	Borna	WW	Horse/cattle, sheep, dog		HT: rodent ?	Z	P/NS	Lipkin (2007)
Bunyavirus	Oropouche fever	Brazil	Sloth	Mosquito	HT: mosquito bite	Z	P/meningitis	Anderson et al. (1961)
Coronavirus	SARS- coronavirus (SARS-CoV)	Asia/pandemic	*Panguma lar-vata*/bats? And		HT: Civets direct contact	SA/P3-4 /	H/acute RS, 10% mortality	Peiris et al. (2003)
Filovirus	Ebola[a]	Central Africa	Bats fruit eating		HT: Bats	SA/P4 /	H/HF 70% mortality	Yun (2012)
	Marburg[a]	Central Africa	Bats		HT: Bats	SA/P4/	H/HF 25+% mortality	Yun (2012)
Flavivirus	Dengue fever	Asia, Africa, Americas	Monkey?	*Aedes aeypti, A. albopictus*	HT: Mosquito	P2	H/Fever to HF	Petersen and Gubler (2003)
	Eastern equine encephalitis	Americas	Horse	Ticks	HT: Mosquito	SA/P3-4/	H/ENC 25% + mortality	Zacks and Paessler (2010)
	Kyasanur forest disease	Southeast Asia	Monkey	Ticks	HT: ticks	SA/P4/	H/HF ENC 3–5% mortality	Work et al. (1959)
	Omsk hemorrhagic fever	Siberia	Rodents	Ticks	HT: tick	SA/P4/	H/ILS to HF, mortality 1–10%	Chumakov et al. (1948)
	Tick-borne encepha-litis, TBE							Barrett et al. (2008)

Table 41.1 (continued)

Virus family	Virus name	Geographical origin	Hosts: Main/ secondary	Vector	Transmission and public health issues	Risk assessment	Human pathogenic	Reference
	TBE far eastern subtype	Europe	Game	Ticks	HT + tick bite	SA/P4/	H/ENC, mortality 1–2 %	
	TBE Siberian subtype	Siberia		Ticks	HT + tick bite	SA/P4/	H/ENC, mortality 1–2 %	
	West Nile virus	WW	Horse/bird	Mosquito	HT: Mosquito bite	Z	H/ENC	
	Western equine encephalitis	Americas	Horse		HT: Mosquito bite	Z	H/ENC mortality 35 %	Zacks and Paessler (2010)
	Yellow fever	Africa, South America	Monkeys	Mosquito	HT: Mosquito bite	Z/P3	H/HF, ENC	Monath et al. (2008)
Hantavirus	Hantaviruses (exept KHF)	WW	Rodents		HT: rodent feces, urine	Z/P3 P4	H/HF—ReS	Lee (1989)
	Korean hemorrhagic fever, KHF[a]	Asia	Rodent		HT: rodent feces, urine; S and S:, E:	Z	H/HF	Lee et al. (1978)
	Puumala	Europe	Rodents		HT: rodent feces, urine	Z	P/ReS	Brummer et al. (1980)
Henipavirus	Hendra	Australia	Bats	Horses	HT: + bat urine, animals ?	SA/P4/	H/NS—RS	Field et al. (2009)
	Nipah	Asia (Africa?)	Bats		HT: + bat urine, human ?	SA/P4/	H/RS	Halpin et al. (2000)
Herpesvirus	Cytomegalovirus (B Herpes)	WW	Primates		HT: direct contact	?	+ mild (fever)	Michaels et al. (2001)
	Herpes simian B	WW	Monkey macaque		HT: direct contact	Z	H/ENC	Gay and Holden (1933)
	Lymphocryptovirus (LCVs) gammaherpesvirus	WW	Primates (Old world and new world)		Direct contact, saliva; Epstein-Barr virus (EBV), human LCV		?	Ablashi et al. (1978)

Table 41.1 (continued)

Virus family	Virus name	Geographical origin	Hosts: Main/ secondary	Vector	Transmission and public health issues	Risk assessment	Human pathogenic	Reference
Nairovirus	Crimean-Congo haemorrhagic fever[a]	Africa, Asia	Cattle	Ticks	HT: tick bite	SA/P4	HF	Chumakov et al. (1968)
Orhtomyxovirus	Influenza A virus H1N1	WW	Pigs/birds		HT: Influenza syndrome	Z	H/ILS + RS	Suarez et al. (2000)
	Influenza A virus: Highly Pathogenic Avian influenza (HPAIV)[a]	WW	Ducks, shore birds, gulls (natural reservoirs of AIV[c]		Several AI strains infect human (H5N1, H7N2, H7N3, H7N, H9 H9N2, H9N and, H10N7)	USDA SA	H/RS	Chan PK (2002)
Paramyxovirus	Newcastle disease	WW	Avian		HT: direct contact	USDA SA	P/conjunctivitis	Nelson et al. (1952)
Phlebovirus	Rift Valley fever	Africa	Cattle	Mosquito	HT: mosquito bite	SA/P3-4 /	H/HF	
Poxvirus	Monkeypox	Central Africa	Monkeys		HT: direct contact, bite	SA/P4/	Skin lesions	Ladnyj et al. (1972)
	Orf	WW	Sheep goat		HT: direct contact (skin wound)	Z	P/MD	Geraut (2006)
Retrovirus	Human Immunodeficiency, HIV (ref. to SIV)	WW	NHP		HT (Historical: see Hahn et al.)	Z	HP/P3	Hahn BH, et al. (2000)
	Simian Foamy (SFV)	Africa	Primates		HT: monkey bite	Z	Asymptomatic	Wolfe et al. (2004)
	Simian Immundeficiency (SIV)	Africa	Primates		HT: ?	S	Asymptomatic (?)	Switzer et al. (2010)

Table 41.1 (continued)

Virus family	Virus name	Geographical origin	Hosts: Main/ secondary	Vector	Transmission and public health issues	Risk assessment	Human pathogenic	Reference
	Simian T-cell Leukemia (STLV)	Africa	Primates		HT: ?	P	Asymptomatic (?)	Mahieux and Gessain (2011)
Rhabdovirus	Rabies	WW	Canids (lethal)	Feline	HT: bite	Z	H Neurological syndrome; 100% fatal/NS	
	Vesicular stomatitis (VS)[a]	Americas	Horses, cattle, pigs		HT: aerosolization or direct exposure	USDA SA	H/IL	

WW worldwide, *HT* human transmission (+ : positive, ? : unknown), *SA* national institute of allergy and infectious diseases select agent (see ref.), *SA USDA* select agent of veterinary importance (see ref. and Table 41.2), *P* (*3-4*) p level of security = (ref. CDC), *H* highly pathogenic, *ILS* influenza like syndrome, *P* potentially pathogenic, *S* suspected, *MD* mild disease, *NS* neurological syndrome, *ReS* renal syndrome, *RS* respiratory syndrome, *WW* world wide, *HF* hemorrhagic fever, *SA* select agent, *USDA SA* USDA select agents, *Z* recognized as zoonotic

[a] potential biological weapon

[b] Select Agents Regulations (42 CFR Part 73, 7 CFR Part 331, 9 CFR Part 121) in the Federal Register on March 18, 2005

[c] domestic and wild avian species (including chickens, turkeys, ducks, domestic geese, quail, pheasants, partridge, parrots, gulls, shorebirds, seabirds, emu, eagles, and others). cause disease in horses, pigs, whales, and seals; expanding to others mammalian species, i.e. cats, dogs, foxes, leopards, tigers, civets, pigs, raccoons

human population or laboratory workers, they have been classified as P3–4 level of containment agents (Richmond and McKinney 1999).

For practical reasons, several agents have been targeted for their potential to be a risk for human and animal populations and characterized according their potential to be highly pathogenic or to be highly transmissible—in particular by aerosols—and the lack of any means of protection, e.g. by a vaccine. Those agents have been listed by HHS and USDA as "Select Agents" having the potential to pose a severe threat to both human and animal health, (potentially plant health), or to animal and plant products. Among these 45 Select Agents (33 viruses and 12 bacteria) 31 (69 %) are zoonotic, while the remaining are known to infect only animals (Table 41.2)[1].

Risk Mitigation and International Perspective Major factors have to be taken in account in order to reduce the risk of transmission between animals and humans. Besides reducing the direct contact among the two populations, tools and strategies to fight zoonoses has to be specifically developed. Select Agents have to be surveyed for their emergence, circulation and evolution. Highly pathogenic agents, as well as Select Agents, have to be diagnosed and handled by well-trained workers in certified appropriate laboratory structures (P3 and P4 laboratories, etc.) and their circulation controlled (i.e.: shipping, transferring from one laboratory to another, etc.).

41.2 Highly Pathogenic Viral Zoonoses

41.2.1 Viral Hemorrhagic Fevers (VHF)

Viral Hemorrhagic Fevers (VHF) appear as a whole clinical entity characterized by (high) fever and bleeding that can progress to shock and death. The first severe VHF identified was the Ebola Hemorrhagic Fever (1976), although the Marburg virus was isolated and characterized earlier in 1967; Marburg virus, however, appears in the medical literature as part of the nosocomial framework of VHF only in 1977 when published aside with the Ebola virus (Bowen et al. 1977). Later, several already known VHF joined the concept including: the Hemorrhagic Fevers with Renal failure (known since 1951), the Hantavirus in 1978 (Lee et al. 1978); the Lassa fever and Bolivian and Argentine HF, Yellow Fever, Rift Valley Fever, Crimean Congo Hemorrhagic fever (CCHF), and others. The group of VHF was identified as a nosologic entity associated with viruses belonging essentially to five distinct families of RNA viruses: the four Arenaviridae, Filoviridae, Bunyaviridae, and Flaviviridae. Only recently in September 2012 scientists reported the isolation of a member of the Rhabdoviridae family responsible for VHF in the Bas-Congo district of the Democratic Republic of Congo (Grard et al. 2012). Several VHFs share many important features: (1) many of them may be transmitted by arthropod-borne agents

[1] http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20Exclusions.html.

Table 41.2 Common human and animal highly pathogenic viruses

HHS select agents (zoonotic)	USDA select agents (not zoonotic)		
Virus	Bacteria/Rickettsia	Virus	Bacteria/Rickettsia
Chapare	*Bacillus anthracis*[a]	African horse sickness	*Mycoplasma capricolum*
Crimean-Congo haemorrhagic fever	*Brucella abortus*	African swine fever	*Mycoplasma mycoides*
Eastern equine encephalitis	*Brucella melitensis*	Avian influenza	
Ebola[a]	*Brucella suis*	Classical swine fever	
Guanarito	*Burkholderia mallei*[a]	Foot-and-mouth disease[a]	
Hendra	*Burkholderia pseudomallei*[a]	Goat pox	
Junin	*Coxiella burnetii*	Lumpy skin disease	
Lassa fever	*Francisella tularensis*[a]	Newcastle disease virus	
Lujo	*Rickettsia prowazekii*	Peste des petits ruminants	
Machupo	*Yersinia pestis*[a]	Rinderpest virus[a]	
Marburg[a]		Sheep pox	
Monkeypox		Swine vesicular disease	
Nipah			
Kyasanur forest disease			
Omsk hemorrhagic fever			
Rift valley fever			
Sabia			
Tick-borne encephalitis complex			
Variola major (Smallpox)[a]			
Variola minor (Alastrim)[a]			
Venezuelan equine encephalitis			

HHS (US department of) health and human Services, *USDA* US department of agriculture

[a] Tier 1 Agent

(usually mosquito vector), (2) person-to–person transmission is possible through direct contact with infected patients, their blood or other body fluids; (3) natural animal reservoirs are mainly rats and mice, but also domestic livestock, monkeys or other NHP may serve as intermediate hosts. Moreover, with the increasing international travel, these mainly tropical viruses may now be imported into non-endemic countries thus posing a major global risk for human public health. Furthermore, several of these agents have been associated with nosocomial outbreaks involving health care and laboratory workers.

Due to special biosecurity concerns, we will mainly focus in the following on Filoviruses, RVFV, other flavivirus responsible of hemorrhagic fevers, Kyasanur Forest disease and Omsk HF. Alkhurma HF virus is cited in cursory detail because its limited geographic distribution.

41.2.1.1 Filoviruses (Ebola and Marburg)

Filoviruses

Ebola and Marburg viruses are the only members of the genus Filovirus in the *Filoviridae* family and can cause severe hemorrhagic fever in humans and NHP.

The genus *Marburgvirus* consists of a single species, *Marburg marburgvirus*, with 2 member viruses, Marburg virus (MARV) and Ravn virus (RAVV).

The genus *Ebolavirus* contains five species: *Bundibugyo ebolavirus*, *Zaire ebolavirus*, *Reston ebolavirus*, *Sudan ebolavirus*, and *Taï Forest ebolavirus*, whose members are Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), and Taï Forest virus (TAFV), respectively (Kuhn et al. 2010). Ebola-Reston is the only known Filovirus that does not cause severe disease in humans; however, it can still be fatal in monkeys and it has been recently recovered from infected pigs in South-East Asia. A third, tentative genus ("*Cuevavirus"*) has been suggested for a novel filovirus, Lloviu virus (LLOV; species "*Lloviu cuevavirus*"), which has not yet been isolated in culture. With the exception of RESTV and possibly LLOV, all of these viruses cause severe and often fatal viral hemorrhagic fever (VHF) upon infection in humans (Negredo et al. 2011).

The Pathogen

Ebola and Marburg viruses are elongated filamentous molecules, highly variable in length, and are typically between 800–1000 nm long, and can be up to 1400 nm long due to concatamerization, with a uniform diameter of 80 nm. The viral fragment is pleomorphic, and may appear in the shape of a "6", a "U", or a circle, and it is contained within a lipid membrane. Each virion contains one molecule of single-stranded, negative-sense viral genomic RNA, complexed with the proteins *NP*, *VP35*, *VP30*, and *L* (Kiley et al. 1982; Sanchez et al. 1992; Geisbert and Jahrling 1995; Mwanatambwe et al. 2001; Pringle 2005).

Pathogenesis

Two independent studies reported that Ebola virus cell entry and replication requires the cholesterol transporter protein Niemann-Pick C1 (NPC1). The studies described that when cells from Niemann Pick Type C1 patients were exposed to Ebola virus in the laboratory, the cells survived and appeared immune to the virus, further indicating that Ebola relies on NPC1 to enter cells. The same studies described similar results with Ebola's cousin in the filovirus group, Marburg virus, showing that it too needs NPC1 to enter cells (Carette et al. 2011; Côté et al. 2011). Furthermore, NPC1 was shown to be critical to filovirus entry because it mediates infection by binding directly to the viral envelope glycoprotein (Côté et al. 2011). Miller et al. (2012) confirmed the findings that NPC1 is a critical filovirus receptor that mediates infection by binding directly to the viral envelope glycoprotein and that the second lysosomal domain of NPC1 mediates this binding. Carette et al. (2011) showed mice that were heterozygous for NPC1 were protected from lethal challenge with mouse adapted Ebola virus. Together, these studies suggest NPC1 may be a potential therapeutic target for an Ebola anti-viral drug.

Clinical Signs

Ebola and Marburg virions enter the host cells through endocytosis and replication occurs in the cytoplasm. Upon infection, the virus targets the host blood coagulative and immune defense system and leads to severe immunosuppression (Harcourt et al. 1999).

Ebola virus disease is clinically indistinguishable from Marburg virus disease, and both are similar to many other diseases prevalent in Equatorial Africa (Grolla et al. 2005).

Early signs of infection are non-specific and flu-like, and may include sudden onset of fever, asthenia, diarrhea, headache, myalgia, arthralgia, vomiting, and abdominal pains (Bwaka et al. 1999). Less common early symptoms such as conjunctival injection, sore throat, rashes, and bleeding may also appear. Shock, cerebral oedema, coagulation disorders, and secondary bacterial infection may co-occur with onset of infection (Feldmann 2010). Hemorrhagic symptoms begin 4–5 days after onset, which includes hemorrhagic conjunctivitis, pharyngitis, bleeding gums, oral/lip ulceration, hematemesis, melena, hematuria, epistaxis, and vaginal bleeding. Hepatocellular damage, marrow depression (such as thrombocytopenia and leucopenia), serum transaminase elevation, and proteinuria may also occur. Persons that are terminally ill typically present with obtundation, anuria, shock, tachypnea, normothermia, arthralgia, and ocular diseases. Hemorrhagic diathesis is often accompanied by hepatic damage and renal failure, central nervous system involvement, and terminal shock with multi-organ failure. Contact with the virus may also result in symptoms such as severe acute viral illness, malaise, and maculopapular rash. Pregnant women will usually abort their foetuses and experience copious bleeding. Fatality rates range between 50 and 100 %, with most dying of dehydration caused by gastric problems (Casillas et al. 2003).

Diagnosis can be confirmed by virus isolation, ELISA to detect viral antigens or patient antibodies in serum or organ homogenates, RT-PCR, immunohistochemistry, and electron microscopy of tissue sections and/or biopsies (Grolla et al. 2005).

Ebola and Marburg virus are morphologically indistinguishable; laboratory studies are extremely hazardous and should be performed in a Biosafety Level 4-equivalent containment Level 4 facility. Laboratory researchers have to be properly trained in BSL-4 practices and wear proper personal protective equipment.

Ebola Virus Epidemiology

Occurrence of Ebola and Marburg virus disease has been primarily limited to countries in sub-Saharan Africa. The name, Ebola, comes from the Ebola River in the Democratic Republic of the Congo, where it was first found in 1976. Marburg virus was first discovered in 1967 and is named after the German city of Marburg.

Ebola virus disease (EVD) was first described after almost simultaneous viral hemorrhagic fever outbreaks occurred in Zaire and Sudan in 1976 (WHO 1978a). EVD is believed to occur after an ebolavirus is transmitted to a human index case via contact with an infected animal host. Human-to-human transmission occurs via direct contact with blood or bodily fluids from an infected person (including embalming of a deceased victim) or by contact with contaminated medical equipment such as needles. In the past, explosive nosocomial transmission has occurred in underequipped African hospitals due to the reuse of needles and lack of implementation of universal precautions. Aerosol transmission has not been observed during natural EVD outbreaks, although there are reports suggesting or suspecting aerosol transmission between NHP or in humans based on epidemiological observations (Dalgard et al. 1992; Jaax et al. 1995; Johnson et al. 1995; Roels et al. 1999). The potential for widespread EVD epidemics is considered low due to the high case-fatality rate, the rapidity of demise of patients, and the remote rural areas where infections occur.

Marburg Virus Epidemiology

In 1967, simultaneous outbreaks occurred in laboratory workers handling African green monkeys imported from Uganda in Marburg, Frankfurt (Germany), and Belgrade (Yugoslavia, now Serbia). There were 25 reported primary laboratory-acquired cases with seven deaths. The 25 cases arose from contact and accidents with blood and tissues from infected African green monkeys and six secondary cases (medical personnel, one spouse) developed from the primary cases (Siegert 1972). Between 1975 and 1987, isolated cases were reported in South Africa (originating from Zimbabwe), Kenya, Zimbabwe, Kenya, and the Democratic Republic of Congo (Gear 1977; Smith et al. 1982). A large long running outbreak occurred between 1998 and 2000 in the Democratic Republic of Congo, resulting in 154 cases and 128 deaths, and two different Marburg viruses, MARV and RAVV,

co-circulated and caused disease (Bausch et al. 2006). The largest outbreak to date occurred in 2004 and 2005 centered in Uige, Angola where 374 cases were reported with 329 deaths (Roddy et al. 2010). Since 2007, a number of cases have been reported in Uganda, some of which have been diagnosed into other countries (i.e. USA, The Netherlands) in individuals returning from Uganda (CDC 2003; Timen et al. 2009). Marburg virus has been isolated from blood; serum; secretions, including respiratory and throat secretions; semen; urine; and various tissues and organs from human or animal hosts, or their homogenates (Fisher-Hoch 2005).

Crossing the Species Barrier and Transmission—Ebola Virus

Between 1976 and 1998, from 30,000 mammals, birds, reptiles, amphibians, and arthropods sampled from outbreak regions, no *Ebolavirus* was detected apart from some genetic traces found in six rodents (*Mus setulosus* and *Praomys sp*.) collected from the Central African Republic (Pourrut et al. 2005). Traces of EBOV were detected in the carcasses of gorillas and chimpanzees during outbreaks in 2001 and 2003, which later became the source of human infections. However, the high lethality from infection in these species makes them unlikely as natural reservoir (Pourrut et al. 2005). Plants, arthropods, and birds have also been considered as possible reservoirs; however, bats are considered the most likely candidate. Bats were known to reside in the cotton factory in which the index cases for the 1976 and 1979 outbreaks were employed, and they have also been implicated in Marburg virus infections in 1975 and 1980 (Pourrut et al. 2005). Of 24 plant species and 19 vertebrate species experimentally inoculated with EBOV, only bats became infected (Swanepoel 1996). The absence of clinical signs in these bats is characteristic of a reservoir species. In a 2002–2003 survey of 1030 animals that included 679 bats from Gabon and the Republic of the Congo, 13 fruit bats were found to contain EBOV RNA fragments (Leroy et al. 2005). As of 2005, three types of fruit bats (*Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*) have been identified as being in contact with EBOV. They are suspected to represent the EBOV reservoir hosts (Pourrut et al. 2007).

The existence of integrated genes of filoviruses in some genomes of small rodents, insectivorous bats, shrews, tenrecs (insectivora from Madagascar), and marsupials indicates a history of infection with filoviruses in these groups as well. However, it has to be stressed that infectious Ebola virus have not yet been isolated from any nonhuman animal (Taylor et al. 2010).

Transmission between natural reservoirs and humans are rare, and outbreaks are usually traceable to a single index case where an individual has handled the carcass of a gorilla, chimpanzee, or duiker (a small antelope species) (Peterson et al. 2004). The virus then spreads person-to-person, especially within families, hospitals, and during some mortuary rituals where contact among individuals becomes more likely (Hewlett and Amolat 2003).

The virus can be transmitted through body fluids. Transmission through oral or conjunctiva exposure is likely and has been confirmed in NHP (Jaax et al. 1995).

Filoviruses are not naturally transmitted by aerosol. They are, however, highly infectious as breathable 0.8–1.2 μm droplets in laboratory conditions; because of this potential route of infection, these viruses have been classified as Category "A" biological weapons (Johnson et al. 1995; Leffel and Reed 2004)[2].

Crossing the Species Barrier and Transmission—Marburg Virus

The natural reservoirs of Marburg viruses remain to be identified unequivocally. However, the isolation of both MARV and RAVV from bats and the association of several MVD outbreaks with bat-infested mines or caves strongly suggest that bats are involved in Marburg virus transmission to humans. Avoidance of contact with bats and abstaining from visits to caves is highly recommended, but may not be possible for those working in mines or people dependent on bats as a food source. Monkeys are susceptible but are incidental hosts and individuals handling infected monkeys or their fluids and cell cultures of Marburg virus have become ill (Towner et al. 2009; Timen et al. 2009; Swanepoel et al. 2007).

In 2009, the isolation of infectious MARV was reported from healthy Egyptian rousettes (*Rousettus aegyptiacus* or Egyptian fruit bat) (Towner et al. 2009). This isolation, together with the isolation of infectious RAVV, strongly suggests that Old World fruit bats are involved in the natural maintenance of marburgviruses. Further studies are necessary to establish whether Egyptian rousettes are the actual hosts of MARV and RAVV or whether they get infected via contact with another animal and therefore serve only as intermediate hosts.

The first experimental infection study of *Rousettus aegyptiacus* with MARV provided further insight into the possible involvement of these bats in MARV ecology. Experimentally infected bats developed relatively low viremia lasting at least five days, but remained healthy and did not develop any notable gross pathology. The virus also replicated to high titers in major organs (liver and spleen), and organs that might possibly be involved in virus transmission (lung, intestine, reproductive organ, salivary gland, kidney, bladder and mammary gland). The relatively long period of viremia noted in this experiment could possibly also facilitate mechanical transmission by blood sucking arthropods or infection of susceptible vertebrate hosts by direct contact with infected blood (Paweska et al. 2012).

Biosecurity of Filoviruses

Filoviruses (Ebola viruses and Marburg viruses) are listed as World Health Organization Risk Group 4 Pathogens, National Institute of Allergy and Infectious Diseases (NIAID) Category A Priority Pathogens, Select Agents, and Centers for

[2] National Institutes of Health, National Institute of Allergy and Infectious Diseases. Category A, B & C Priority Pathogens. 2013. http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx Accessed May 27, 2013.

Disease Control and Prevention (CDC) Category "A" Bioterrorism Agents due to the absence of prophylaxis or treatment regimens, their high lethality (up to 90 % in larger outbreaks), their high infectivity (LD_{50} = 1 virion in rodent models), and their stability in artificial aerosols. Research on infectious filoviruses requires Biosafety Level 4 (BSL-4) laboratories.

Filoviruses can survive up to 4–5 days on contaminated surfaces, and can survive in liquid or dried material for a number of days (Belanov et al. 1996; Bray 2003). They are susceptible to sodium hypochlorite, beta-propiolactone, 3 % acetic acid (pH 2.5), phenolic disinfectants, formaldehyde and paraformaldehyde, 1 % glutaraldehyde, formalin, lipid solvents, and detergents such as SDS. They are physically inactivated by heating for 30–60 min at 60 °C, boiling for 5 min, gamma irradiation (1.2×10–1.27×10 rad), and UV radiation (Elliott et al. 1982; Kurata et al. 1983; Mitchell and McCormick 1984; Mahanty et al. 1999).

Ebola Vaccine

Most of the Ebola virus VP proteins are capable of eliciting protective immune responses and therefore are important to consider as potential components of a vaccine to protect humans from Ebola hemorrhagic fever. An "Ebola ΔVP30" strain replication incompetent virus as been generated with a lack of the gene encoding for the VP30 protein, therefore it cannot replicate and do not form infectious progeny in wild-type cells. The genome is stable, without a single event of virus replication; experimental infection of animals did not cause disease in infected animals (Halfmann et al. 2008, 2009).

41.2.1.2 Arenavirus

Arenaviruses are negative stranded RNA viruses of the *Arenaviridae* family. They naturally and chronically infect asymptomatic rodent host-reservoirs. Each rodent species is persistently infected by a specific virus and represents a model of virus-host coevolution (Gonzalez et al. 2007). One exception is made with the Tacaribe virus that has been isolated from naturally infected chiropteran (Downs et al. 1963).

Clinical Signs Several arenaviruses can accidentally infect humans and are responsible for mild to severe zoonotic diseases. Although the arenavirus prototype species, Lymphocytic Choriomeningitis Virus of mice (LCMV) is responsible for a neurological syndrome in humans, at least seven out of the 24 arenavirus species are known to be highly pathogenic for humans and responsible of Viral Hemorrhagic Fever (VHF). Six of them are classified as Select Agents ([3]) including the South American Arenaviruses (Guanarito from Venezuela, Junín from Argentina,

[3] http://www.selectagents.gov/resources/List_of_Select_Agents_and_Toxins_2012–12-4-English.pdf.

Machupo and Chapare from Bolivia and Sabía from Brazil) and the African one, Lassa Fever Virus (from Guinea, Nigeria and Sierra Leone). Also the Lujo virus, not yet a Select Agent, has been recently described in AustralAfrica and represents an emerging potential threat for the region (Paweska et al. 2009).

Although bleeding tendencies are often recorded but not always life threatening, a high mortality of 30 % of infected patients can occur during epidemics. Four others arenaviruses including Flexal (Brazil), Pichínde (Columbia), Tacaribe (Trinidad and Tobago) and White Water Arroyo (California) viruses have been found to potentially infect humans and potentially represent also highly dangerous agents (for a review, Gonzalez et al. 2007).

Epidemiology Asymptomatic infections of rodents are generally suspected to be associated with an insufficient or inappropriate host immune response (Hayes and Salvato 2012) resulting in chronic viremia and/or viruria which leads to shedding of the virus into the environment via urine or faeces.

Exceptionally, chronic infection may have a deleterious effect on their reservoir's fitness, which reduces rodent host fertility (Webb et al. 1975). NHP can be experimentally infected, but there is no evidence that these viruses are pathogenic for domestic animals (e.g.: livestock, cats, dogs), while exotic pets (hamster, mice, etc.) represent a potential source of infection.

Besides the specific association between "arenavirus species—rodent species", the geographic range of an arenavirus ecologic niche appears to be more restricted than the one of its rodent reservoir-host with a more circumscribed enzootic domain, which is often limited by natural barriers (e.g. rivers, elevations, climate, food access). This appears as one of the major characteristics of the epidemiological and dispersion patterns of arenaviruses and therefore VHFs associated with them (Salazar-Bravo et al. 2002).

Argentine HF (Junín virus) was identified in the early 1940s in Argentina and described in the 1950s in the rural area of Buenos Aires province, while the virus was characterized only in 1958. Today the virus distribution expend to 150,000 km² of the Pampa. The Vesper mouse (*Calomys* spp.) is the natural host and direct rodent-to-human transmission occurs via ingestion of contaminated food or water, inhalation of rodent urine infested particles or via direct contact of broken skin with rodent excrements. Currently, Argentine HF remains a major and severe enzootic disease of public importance in Argentina with an endemic risk of crossing the natural barrier of the Rio Paraná and spill over to the closest neighboring countries of Uruguay (Polop et al. 2008).

Bolivian HF (BHF) (Machupo virus) was identified after several outbreaks of BHF in 1963 in the Beni province of Bolivia. Although BHF incidence increases late during the rainy season, small outbreaks are a dominant feature of the epidemiological pattern with several years of dormancy thereafter. The natural host *Calomys callosus* invades houses during floods of the rainy season resulting in close contact and human infection (Kilgore et al. 1997).

Chapare virus was isolated once from a fatal human case of hemorrhagic fever during a unique reported outbreak of HF that occurred in 2003 in the Chapare River

region close to Cochabamba in Bolivia, the original setting of Machupo virus responsible of the BHF (Delgado et al. 2008). There is no information concerning an eventual natural rodent host.

Venezuela HF emerged in 1989, with several cases that occurred in the central plains of Venezuela. A new Guanarito virus was isolated and named after the region where the first outbreak occurred (Salas et al. 1991). The main affected populations are settlers moving into cleared forest areas to practice small agriculture. *Zygodontomys brevicauda* appears to be the principal host (i.e.: reservoir) of the virus.

Lassa fever (LF) was described in 1956 in the eponym village of Lassa. LF occurs in rural West Africa, and appears to be hyper-endemic in Sierra Leone with an antibody prevalence of 8–52%, Guinea (4–55%) and Nigeria (21%). Natural transmission of Lassa virus (LASV) occurs from its domestic, ubiquitous, prolific and common multimammate rodent virus reservoir, *Mastomys natalensis*. As for other Arenaviruses it is transmitted to humans directly through rodent urine and faeces or indirectly by contaminated food. Person-to-person transmission has been described posing a risk for healthcare workers. The virus can also be contracted by an airborne route or by direct contact with infected human blood, urine, or semen, up to three months after clinical recovery. LF is a prominent threat outside the endemic area with several imported cases in Germany (Gunther et al. 2000), Japan (Hirabayashi et al. 1988), the United States (Holmes et al. 1990), the United Kingdom (Kitching et al. 2009) among others. About 80% of patients experience a mild or asymptomatic infection. LF has a relatively low mortality rate up to 5%. Among the endemic countries, it is estimated that LF is responsible for about 5000 deaths a year. Pregnant women have the greatest risk of fatality. After an incubation period of 1–3 weeks an acute illness develops while the virus infects every tissue from the mucosa (e.g., intestine, lungs and urinary system) and subsequently progresses to the vascular system. Initial non-specific symptoms include fever, facial swelling, muscle fatigue, conjunctivitis and mucosal bleeding. Later on there might develop gastrointestinal tract bleeding, bloody vomiting, dysphagia, melena, accompanied with cough, dyspnea worsening to cardiovascular system dysfunctions (pericarditis, tachycardia) and hepatitis; finally hearing deficit, meningo-encephalitis and seizures occur. Death is due to multiorgan failure. With respect to this multiple organ infection and accompanying HF signs differential diagnoses include other VHFs such as Ebola or Marburg, malaria or influenza (Yun and Walker 2012; for a review[4]).

After LASV, Lujo virus is the second known to date human pathogenic arenavirus of Africa. Among the five identified cases in 2008, four died; the fifth case was treated with ribavirin early after onset of clinical disease and survived. It has been only reported from a few patients from Zambia and from a subsequent nosocomial outbreak in South Africa (Briese et al. 2009). A natural reservoir has not yet been identified.

[4] http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/lassaf.htm; http://www.pasteur.fr/ip/easysite/pasteur/fr/presse/fiches-sur-les-maladies-infectieuses/fievre-de-lassa.

Sabía virus was first isolated from a fatal case of Brazilian HF (BrHF) in the village of Sabía, outside of Sao Paulo, Brazil in 1990 (Lisieux et al. 1994). Two other non-fatal accidental infections were later recorded (Gandsman et al. 1997). Chapare virus infected patients were also clinically considered as BrHF cases. Both viruses do not have an identified reservoir, however, like the other arenaviruses, they naturally appear to have only a limited geographical distribution.

Crossing the Species Barrier Transmission Virus transmission within rodent populations occurs through vertical (mother to progeny), or horizontal routes (directly through bites or indirectly by contacts with urine or feces). Arenavirus transmission from natural rodent hosts to humans occurs through contacts with infected rodent biological fluids (i.e. blood, saliva or urine), when people (through rodent bites, trapping or eating rodents) are directly exposed to the infected rodent, or indirectly, when exposed to food contaminated with rodent urine and/or by inhalation of infested rodent excreta. Also, human-to-human transmission may occur and arenaviruses can be transmitted trough aerosolized particles and sperm fluid. Moreover, transmission to humans may occur by accidental inoculation with infected body fluids and through tissue transplantation (Emonet et al. 2006; Paweska et al. 2009).

Biosecurity, Therapy and Prevention

Prevention of arenavirus infection consists of interrupting virus transmission from rodents to humans, and from humans to humans. Rodent control seems to be efficient only in certain conditions (i.e.: urban settings). Hospital based nursing barrier appears highly efficient, including personal protective measures (gloves, masks and gowns), good hygiene and appropriate sterilization of equipment. The highest-risk of infection occurs during unprotected contact with body fluids from an infected person. Linens should be handled per CDC guidelines[5]. Environmental surfaces and contaminated equipment are properly disinfected by 1:10–1:100 dilution of sodium hypochlorite or other EPA-registered disinfectants. The viruses can also be inactivated by ultraviolet, gamma irradiation, temperatures of 56 °C for 20 min and, by a pH less than 5.5 or greater than 8.5.

One anti-virus drug against arenavirus infection has been identified: Ribavirin® is an anti-viral drug that interferes with RNA viral replication. It has been proved to be an efficient treatment against LASV if administered early and might in some cases also be effective against other arenaviruses including BHF, Sabía virus or Lujo virus. Also it has been shown to be effective in advanced stages of LASV infection by reducing the virus load (McCormick et al. 1986; Barry et al. 1995; Enria et al. 1994; Kilgore et al. 1997, Briese et al. 2009).

Several antiviral molecules are under development with the most promising one directed to interfere with arenavirus cell entry (Larson et al. 2008; York et al. 2008; Charrel et al. 2011). Although hyperimmune serum has been effectively used in

[5] http://www.cdc.gov/mmwr/preview/mmwrhtml/00037085.htm.

several instances, clinical experiences are limited and only circumstantial reports are available. Hyperimmune serum treatment has been used successfully for AHF patients and a plasma bank was established in Argentina (Maiztegui et al. 1979). Also, neutralizing antibodies contained in Human immune plasma appear to be effective in patients with BHF by reducing viremia. However, LASV infection only leads to a limited neutralizing antibody reaction and hyperimmune serum treatment is not applicable.

Among all arenaviruses, only one vaccine, i.e. the live attenuated Junín virus vaccine Candid #1, has been conclusively developed and produced: its immunogenicity and efficacy in humans was proven to be greater than 84 % without causing any serious adverse effects (Maiztegui et al. 1998). Other vaccines tested in animal models include: an attenuated recombinant LASV vaccine using vesicular stomatitis virus as vector that causes a protective immune response in NHP against a lethal LASV challenge (Geisbert et al. 2005); an attenuated Lassa/Mopeia construct ML-29 virus demonstrated protection against LASV challenge in guinea pigs and Rhesus macaques (Lukashevich et al. 2008); a yellow fever 17D vaccine expressing LASV glycoprotein precursor protected also guinea pigs against LASV challenge (Bredenbeek et al. 2006; Charrel and de Lamballerie 2010 for review).

41.2.1.3 Rift Valley Fever

Rift Valley Fever (RVF) is a viral zoonosis that primarily affects domestic livestock and also humans in Africa. RVF present a clinical spectrum from mild fever to fatal hemorrhagic syndrome. RVF virus is spread by infected *Aedes* spp. or *Culex* spp. mosquitoes. RVF virus is a member of the Phlebovirus genus of the Bunyaviridae family.

Clinical Signs Only a small percentage of patients develops a severe form of the disease including: ocular disease with retinal lesions (0.5–2 % of patients); meningo-encephalitis (< 1 %) with headache, loss of memory, confusion, convulsions, and coma; hemorrhagic fever (< 1 %) starting with severe liver impairment, jaundice, followed by hemorrhage, vomiting blood, melena, purpuric rash, nose and gums bleedings, or menorrhagia. Hemorrhagic forms have a case-fatality as high as 50 %. The virus may be detected in blood for up to ten days.

RVFV is also able to infect many animal species causing particularly severe disease in domesticated animals including cattle, sheep, camels and goats. Sheep are very sensitive to infection: 90 % of infected lambs die, and abortion occurs in up to 100 % of infected pregnant ewes.

Epidemiology Human infections can result from direct contact with infected animal biological products, by handling of animal tissue during slaughtering or butchering, conducting veterinary procedures, or from the disposal of carcasses or fetuses. Consequently, herders, slaughterhouse workers, farmers and veterinarians are at high risk of infection. The virus can infect humans through inoculation (i.e.: wound), inhalation of aerosols, by ingesting unpasteurized or uncooked milk or

from mosquito bites. To date, no human-to-human transmission of RVF has been documented. Outbreaks of RVF occur essentially in rural environment (see WHO[6] for review).

RVF may occur as large outbreaks when heavy rains favor intense breeding of mosquito vectors. Deaths of newborn animals and abortion in pregnant sheep, goats, and cattle may happen and humans can become infected by contact with infected animal tissues or by mosquito bites. The active circulation of RVFV in Africa and the Arabian Peninsula constitutes a threat for human and animal health all over the African continent and beyond (Grobbelaar et al. 2011).

Biosecurity and Prevention Rift Valley fever belongs to the Select Agent list. It is a potential biological weapon particularly because of its high pathogenicity and its potential to be airborne transmitted (Borio et al. 2002)

Basic nursing barrier and standard infection control precautions are recommended to avoid RVFV transmission to health care workers.

A live-attenuated MP-12 RVFV strain has been developed as a vaccine; the vaccine has been shown to protect bovine and ovine dams against RVFV challenge and is safe and efficacious for use in neonatal calves and lambs (Morril et al. 1997)**.** Another live attenuated RVFV vaccine lacking the NSs and NSm genes cannot be transmitted by mosquitoes (Bird et al. 2011; Crabtree et al. 2012).

41.2.1.4 Kyasanur Forest Disease

The Kyasanur Forest Disease (KFD) is a tick-borne VHF endemic to and geographically limited to Karnataka State of Central-West India (Work and Trapido 1957). The KFD virus belongs to the Flaviviridae family.

In the early 1990s a new and close related highly pathogenic virus (more than 30 % mortality rate), the Alkhurma virus, was isolated in Saudi Arabia and represents another threat for the local population (Charrel et al. 2001).

Clinical Signs

After an incubation period of 3–8 days, KFD starts with a sudden onset of fever, headache, severe muscle pain, cough and dehydration: later on a gastrointestinal syndrome and bleeding occurs. 10 % of the patients develop low blood pressure and pancytopenia. Some patients show a biphasic form and experience after 2 weeks a second phase of fever and neurological syndrome leading to a case fatality rate (CFR) of 3–5 %. Approximately 400–500 cases of KFD occur in India per year.

[6] http://www.who.int/mediacentre/factsheets/fs207/en/.

Epidemiology[7]

Although the main hosts of KFDV are rodents, shrews, bats, and monkeys may also carry the virus. Cattle, goats and sheep may become infected without playing a role in the transmission of the disease. KFDV is transmitted from the bite of an infected tick, principally *Haemaphysalis spinigera* (Work et al. 1959).

Crossing the Species Barrier

Humans can get infected from tick bites or by contact with an infected animal (often sick monkeys: *Presbytis entellus* or *Macaca radiata*). KFDV is common in young adults exposed during the dry season in the forest.

Biosecurity and Prevention

A formalin-inactivated tissue-culture vaccine has been used for vaccination campaigns since the early 1990s in the endemic area of India with an efficacy of 79.3–93.5 % after respectively one or two doses (Dandawate et al. 1994).

41.2.1.5 Omsk Hemorrhagic Fever

The tick-borne arbovirus Omsk Hemorrhagic Fever Virus (OHFV) is a member of the Flaviviridae family and classified as a biosafety level 4 virus. Several tick species can transmit the virus including *Dermacentor reticulatus*, *D. marginatus* and *Ixodes persulcatus*.

Clinical Signs As for KHFD, after a one week-incubation period, a first clinical phase of infection, begins with several symptoms including fever, chills, headache, muscular pain, rash, and cervical adenopathy. After two weeks a neurological syndrome appears sometimes accompanied by a hemorrhagic syndrome with severe platelet loss and leucopenia. A third of patients develops pneumonia, nephritis, meningitis, or a combination of these complications. The CFR ranges from 1 to 10 %, surviving patients acquire life-long immunity

Epidemiology

The geographic distribution of the OHFV appears restricted to western Siberia (Kharitonova and Leonov 1985) in Omsk, Novosibirsk, Kurgan, and Tyumen oblasts. The main hosts of OHFV are rodents and in particular the non–native

[7] http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/kyasanur-eng.php#note10.

muskrat (*Ondatra zibethica*) as a natural OHFV reservoir. Muskrat was imported to Siberia from Canada in the 1920s and the virus finds a particular receptive host to replicate and spread efficiently.

The sylvatic cycle of OHFV involves rodents and in particular the non–native muskrat as a natural OHFV reservoir, but also water voles (*Arvicola terrestris*), while most animals within endemic areas can be infected and bitten by the tick vectors. OHFV survives in water and is transferred to humans via contaminated water or an infected tick.

Crossing the Species Barrier Humans become infected through tick bites or contact with blood, feces or urine of infected muskrats (and other hosts). Gamasid mites are also thought to play a minor role in transmission within the sylvatic cycle. OHFV can also spread through milk from infected goats or sheep.

Prevention

Preventing OHF consists of avoiding tick exposure; consequently persons engaged in farming, forestry, and hunting (i.e.: Siberian muskrat) are at highest risk of infection.

41.2.2 Viral Encephalitis

41.2.2.1 Eastern Equine Encephalitis

The Pathogen Eastern equine encephalitis virus (EEEV) is a member of the genus Alphavirus, family Togaviridae. Other medically important alphaviruses found in the Americas include Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV). EEEV has a single-stranded, positive-sense RNA genome. The virus particles are spherical and have a diameter of 60–65 nm (Snyder et al. 2009). Of the four lineages of EEEV, Group I is endemic in North America and the Caribbean and causes most human disease cases; the other three groups (IIA, IIB, and III) cause primarily equine illness in Central and South America (Zacks and Paessler 2010).

Clinical Signs The incubation period for Eastern equine encephalitis virus (EEEV) disease ranges from 4 to 10 days. EEEV infection can result in one of two types of illness, systemic or encephalitic (involving swelling of the brain, referred to as EEE). The type of illness will depend on the age of the person and other host factors. It is possible that some people who become infected with EEEV may be asymptomatic.

Systemic infection has an abrupt onset and is characterized by chills, fever, malaise, arthralgia and myalgia. The illness lasts 1–2 weeks, and recovery is complete when there is no central nervous system involvement. In infants, the encephalitic

form is characterized by abrupt onset; in older children and adults, encephalitis is manifested after a few days of systemic illness. Signs and symptoms in encephalitic patients are fever, headache, irritability, restlessness, drowsiness, anorexia, vomiting, diarrhea, cyanosis, convulsions, and coma.

EEE is the most severe of the arboviral encephalitis entities and has a mortality of 50–75% (Petersen and Gubler 2003). Death usually occurs 2–10 days after onset of symptoms, but can occur much later. Of those who recover, 15–50% are left with disabling and progressive mental and physical sequelae, which can range from minimal brain dysfunction to severe intellectual impairment, personality disorders, seizures, paralysis, and cranial nerve dysfunction. Many patients with severe sequelae die within a few years (Zacks and Paessler 2010).

No human vaccine against EEEV infection or specific antiviral treatment for clinical EEEV infections is available. Patients with suspected EEE should be evaluated by a healthcare provider, appropriate serologic and other diagnostic tests ordered, and supportive treatment provided.

Epidemiology EEEV is transmitted to humans through the bite of an infected mosquito. Human EEEV cases occur relatively infrequently, largely because the primary transmission cycle takes place in and around swampy areas where human populations tend to be limited. Overall, only about 4–5% of human EEEV infections result in EEE. EEEV infection is thought to confer life-long immunity against re-infection. It does not confer significant cross-immunity against other alphaviruses (e.g., Western Equine Encephalitis Virus), and it confers no cross-immunity against flaviviruses (e.g., West Nile Virus) or bunyaviruses (e.g., La Crosse Virus)

In the United States, about six human cases of EEE are reported annually. Most cases of EEE have been reported from Florida, Georgia, Massachusetts, and New Jersey. EEEV transmission is most common in and around freshwater hardwood swamps in the Atlantic and Gulf Coast states and the Great Lakes region. Between 1964 and 2010, there were 270 confirmed cases of EEE in the US. Several states in the northeastern USA have seen increased virus activity since 2004. Between 2004 and 2006, there were 17 equine cases and at least 13 human cases of EEE reported in Massachusetts. In 2006, approximately 500,000 acres (2000 km^2) in southeastern Massachusetts were treated with mosquito adulticides to reduce the risk of humans contracting EEE. Subsequently, between 2007 and 2010, there were two confirmed human cases and six equine cases reported to CDC and USDA respectively.

In October 2007, a citizen of Livingston, West Lothian, Scotland became the first European victim of this disease. The man had visited New Hampshire during the summer of 2007 on a fishing vacation, and was diagnosed as having EEEV on 13 September 2007. He fell ill with the disease on 31 August 2007, just one day after flying home.[5]

In 2012, 209 equine cases of EEE were reported from 19 US States, and 15 human cases of EEE reported from six US States. In 2012, two residents of Vermont were confirmed to have EEE, and this was the first time the illness had been reported in this state.

Crossing the Species Barrier Eastern equine encephalitis virus (EEEV) is maintained in a cycle between Culiseta melanura mosquitoes and avian hosts in freshwater hardwood swamps. Cs. melanura is not considered to be an important vector of EEEV to humans, because it feeds almost exclusively on birds. Transmission to humans requires mosquito species capable of creating a "bridge" between infected birds and uninfected mammals such as some Aedes, Coquillettidia, and Culex species.

Wild birds are the main reservoir for transmission of EEEV. Humans, horses, and other animals (domestic fowl, feral pigs, cattle and rodents) are not significant reservoir hosts (Zacks and Paessler 2010). Amphibians and reptiles are a possible reservoir for the virus to overwinter. Mosquitoes and infected eggs are also a reservoir for the viruses (Pfeffer and Dobler 2010).

Person-to-person transmission has not been reported for EEEV viruses. Direct bird-to-human infection can occur, although humans and horses are not amplifying hosts as virus titers in their bodies are insufficient to infect mosquitoes. Eggs of mosquitoes can be infected by the female (Pfeffer and Dobler 2010).

Horses are susceptible to EEEV infection and some cases are fatal. EEEV infections in horses, however, are not a significant risk factor for human infection, because horses (like humans) are considered to be "dead-end" hosts for the virus (i.e., the concentration of virus in their bloodstreams is usually insufficient to infect mosquitoes). (Zacks and Paessler 2010).

Biosecurity and Prevention All residents of and visitors to areas where virus activity has been identified are at risk of infection with EEEV, particularly persons who engage in outdoor work and recreational activities in these areas. Persons over age 50 and younger than age 15 are at greatest risk for severe disease (encephalitis) following infection. EEEV infection is thought to confer life-long immunity against re-infection.

EEEV is difficult to isolate from clinical samples; almost all isolates (and positive PCR results) have come from brain tissue or CSF. Laboratory acquired infections have been reported, and accidental parenteral inoculation, contact of the virus with broken skin or mucous membranes, and bites from infected laboratory arthropods or rodents are the primary hazards associated while working with these viruses.

EEEV do not persist in the environment, and are susceptible to many common disinfectants including 1 % sodium hypochlorite, 70 % ethanol, 2 % glutaraldehyde and formaldehyde. EEEV can be inactivated by exposure to 50 % ethanol at concentration for 60 min, also by moist or dry heat, or by drying, or by UV rays (Aguilar et al. 2005).

EEEV was one of more than a dozen agents that the United States researched as potential biological weapons before the nation suspended its biological weapons program. Samples taken from people and animals with suspected EEEV infection should be handled by trained staff working in Biosafety Level 3 (BSL-3/ABSL-3) **containment** laboratories (CDC 2007).

41.2.2.2 Venezuelan Equine Encephalitis

The Pathogen Venezuelan equine encephalitis virus (VEEV) is a spherical arbovirus that belongs to the Togaviridae family and is an alphavirus (Atasheva et al. 2010). It is 70 nm in diameter and has an enveloped single stranded RNA genome (Gardner et al. 2008).

The Venezuelan equine encephalomyelitis complex contains at least six viral subtypes, I–VI. Subtype I, the Venezuelan equine encephalomyelitis virus (VEEV), is divided into five antigenic variants or serovars, AB to F. Some of the other five subtypes also have official species names; subtype II is known as Everglades virus, subtype III as Mucambo virus, and subtype IV as Pixuna virus.

VEE complex viruses are divided into epizootic (or epidemic) and enzootic (or endemic) groups. The epizootic viruses, which are amplified in equines and are responsible for most epidemics, are found in VEEV subtypes I-AB and I-C. The remaining viruses, including VEEV I-D, VEEV I-E and variants in subtypes II-VI are enzootic (sylvatic) subtypes. These viruses are generally found in limited geographic areas, where they usually occur in natural cycles between rodents and mosquitoes. The enzootic subtypes are typically non-pathogenic for horses and are not amplified in this host; however, in 1993 an enzootic I-E variant was responsible for an outbreak of VEE among horses in Mexico (Weaver et al. 2004).

Clinical Signs In humans, VEEV usually causes mild to severe influenza-like symptoms; 4–14 % of cases, however, develop neurological complications (Gardner et al. 2008). Children and young adults are more likely to develop encephalitis; however, fatalities in humans are rare reaching about 1 % of all reported cases (de la Monte et al. 1985). Usually, flu-like symptoms such as headache, myalgia, fatigue, vomiting, nausea, diarrhoea, pharyngitis and fever appear abruptly, 2–5 days after exposure to the virus. The VEE virus can also cause retro-orbital and occipital headaches as well as leucopenia and tachycardia. Symptoms of encephalitis, only appearing in a minority of cases, occur 4–10 days after exposure and include somnolence, convulsions, confusion, photophobia, and coma. Fatal human cases are usually caused by encephalitis as well as brain, lung and gastrointestinal bleeding (Weaver et al. 2004). Long-term neurological damage can be caused by this virus and it can infect the foetus in pregnant women causing birth defects and stillbirths (de la Monte et al. 1985). Generally, the symptoms last between 3 and 8 days and can be biphasic, recurring 4–8 days after the initial symptoms (Sidwell et al. 1967).

Enzootic VEEV usually infects horses sub-clinically or cause mild symptoms. Epizootic subtypes may cause a generalized acute febrile disease with or without neurologic signs. Asymptomatic infections also occur.

Fatal VEE has been reported in various mammals including rabbits, goats, dogs and sheep during epizootics. Some VEE viruses also kill laboratory rodents including hamsters, guinea pigs and mice; however, natural reservoir hosts for enzootic strains usually remain asymptomatic. Experimentally infected, NHP develop a non-specific febrile illness similar to human disease.

Epidemiology Epizootic VEE viruses (VEEV I-AB and I-C) are found in South and Central America. Most VEE epidemics occur in northern and western South America, but some may spread into adjacent countries, including the US. Enzootic VEE viruses have been found in Mexico, parts of the US, and South and Central America.

The virus was first observed in horses in 1935 after outbreaks in Columbia, Venezuela and Trinidad, and was isolated in 1938. In the 1960s, over 200,000 human cases and 100,000 equine deaths were reported in Colombia and smaller epidemics occurred in Venezuela and Mexico. Between 75,000 and 100,000 infections were reported in Venezuela and Colombia in 1995. The outbreaks usually occur after a season of heavy rains, due to increases in the mosquito population (Weaver et al. 2004).

VEE can be widespread in human populations during epidemics; more than 10% of the population in an area may be affected. Between epidemics, sporadic cases of VEE are caused by enzootic viruses. Humans are highly susceptible to VEE; approximately 90–100% of exposed individuals become infected, and nearly 100% develop clinical signs. However, most infections are mild. Less than 1% of adults develop encephalitis, with approximately 10% of these cases ending in death; the overall CFR in adults is less than 1%. Very young or elderly patients are more likely to develop severe infections. Encephalitis, with a CFR of 35%, occurs in approximately 4% of children less than 15 years of age. More severe disease, with a higher incidence of neurologic signs, might occur in both children and adults after a biological attack with aerosolized virus.

Instances of person-to-person transmission have not been reported for the VEE virus, although an infected individual can transmit the virus to mosquitoes. Generally, humans and equines become infected by mosquitoes of the **Psorophora** and **Ochlerotatus** genus. Equines can spread the virus to each other through aerosols and to mosquitoes via bites (Pfeffer and Dobler 2010)

Crossing the Species Barrier There are two types of cycles involved in the VEE virus. The enzootic cycle is maintained by rodents and mosquitoes. The epizootic cycle implicates horses, mosquitoes and humans, although there is the potential for the virus to affect many other animal species (Pfeffer and Dobler 2010). Horses are the amplifying host in the cycle and are necessary for a larger outbreak of VEE (de la Monte et al. 1985).

VEEV is typically spread by mosquitoes, although certain types of ticks and mites can spread the virus as well (Weaver et al. 2004). The **Culex (Melanoconion)** mosquito is normally responsible for the dispersal of the enzootic strain of the VEE virus (Zacks and Paessler 2010). **Ochlerotatus taeniorhynchus, Psorophora confinnis, Psorophora columbiae, Ochleratus sollicitans, Mansonia titillans** and **Anophilis aquasalis** are some of the species of mosquitoes known to carry the epizootic varieties of the VEEV (Weaver et al. 2004).

VEE epidemics typically begin in horses, with human cases developing weeks later: Unlike EEE outbreaks, which usually end with the onset of colder temperatures, VEE epidemics can last for several years. Epizootic subtypes of VEEV can

cause significant morbidity and mortality in equids; the infection rate can be as high as 90%, and the morbidity rate varies from 10–40% in some areas to 50–100% in others. The CFR in horses is 38–90%. Fatal infections have also been reported in goats, rabbits, dogs and sheep during epizootics, as well as in laboratory rodents infected with some isolates.

Most enzootic VEEV subtypes do not result in serious disease or deaths in horses, but limited outbreaks of encephalitis have been reported with some variants.

Rodents are usually the natural hosts for enzootic VEEV, but birds are involved in a few cycles. The maintenance host for epizootic VEEV between outbreaks is unknown; during epidemics, these viruses are amplified mainly in equids.

Epidemic VEEV can cause serious disease in horses, mules, burros, donkeys and zebras. During epizootics, fatal cases have also been reported in domesticated rabbits, dogs, goats and sheep. Cattle, pigs, bats and opossums can also be infected. Experimental infections have been reported in NHP, guinea pigs, mice and hamsters; some isolates are fatal for laboratory rodents, although they are usually asymptomatic in their normal rodent hosts.

Biosecurity and Prevention VEEV can be found in the body fluids of horses, and transmission by direct contact or aerosols is theoretically possible in this species. However, natural transmission of VEEV between horses or from horses to humans has not been seen. Infected laboratory rodents can also shed this virus, and people have been infected after exposure to aerosolized debris from cages.

Vaccinations of equines with the TC-83 vaccine and protection against mosquitoes (protective clothing, insecticides) are some of the proposed ways to reduce VEE outbreaks. While the TC-83 vaccine is recommended for laboratory workers, there is no licensed vaccine available for the general population (Weaver et al. 2004).

Arboviruses may be present in blood, cerebrospinal fluid, urine and exudates. The virus may be found in nasal, eye and mouth secretions of infected animals as well as in contaminated animal bedding. The greatest risks when working with VEEV are exposure to infected aerosols, accidental subcutaneous inoculation, and contact with broken skin or contaminated animal bedding. VEEV is stable in dried blood and exudates as well as in freeze dried materials (aerosols) (Chosewood and Wilson 2009). One viral infectious particle injected subcutaneously is enough to infect an individual with VEEV (Collins and Kennedy 1983).

Like other enveloped viruses, VEEV virus is susceptible to disinfectants such as 1% sodium hypochlorite, 4% formaldehyde, 2% gluteraldehyde, 70% ethanol, 3–6% hydrogen peroxide, 2% and peracetic acid (Collins and Kennedy 1983). Microbial inactivation is possible using moist or dry heat (Block 2001). Togaviruses can be inactivated by 15 min of heat at 65 °C (Lelie et al. 1987).

During the Cold War, both the United States biological weapons program and the Soviet biological weapons program researched and weaponized VEEV. In April 2009, the U.S. Army Medical Research Institute of Infectious Diseases at Fort Detrick reported that samples of VEEV were discovered missing during an inventory of

a group of samples left by a departed researcher. The report stated the samples were likely among those destroyed when a freezer malfunctioned.

41.2.2.3 Tick-Borne Encephalitis

The Pathogen Tick-borne encephalitis virus (TBEV) is a single-stranded RNA virus that belongs to the genus *Flavivirus*, and was initially isolated in 1937. TBEV has three subtypes: European, Siberian, and Far Eastern, and is the most important arthropod-borne virus in Europe (Ramelow et al. 1993; Barrett et al. 2008).

The family Flaviviridae includes other tick-borne viruses affecting humans and these viruses are closely related to TBEV and Russian Spring Summer encephalitis, such as Omsk hemorrhagic fever virus in Siberia, Al Khumra virus in Saudi Arabia, and Kyasanur Forest disease virus in India. Louping ill virus (United Kingdom) is a member of this family; it causes disease primarily in sheep and has been reported as a cause of a TBE-like illness in laboratory workers and persons at risk for contact with sick sheep (e.g.: veterinarians, butchers) (see above paragraphs 5.2.1.4 and 5.2.1.5).

Clinical Signs Tick-borne encephalitis (TBE) is a human viral infectious disease involving the central nervous system. The disease most often manifests as meningitis, encephalitis or meningoencephalitis. Although TBE is most commonly recognized as a neurologic disease, mild febrile illnesses can also occur. Long-lasting or permanent neuropsychiatric sequelae are observed in 10–20 % of infected patients. Approximately two thirds of infections are asymptomatic. The median incubation period for TBE is 8 days (range, 4–28 days). The incubation period for milkborne exposure is usually shorter (3–4 days). Hemmer et al. (2005) recommended that tickborne encephalitis should be included in the differential diagnosis of meningoencephalitis in northeastern Germany, even if the patient has not been in tickborne encephalitis–endemic areas.

Among patients with central nervous system involvement, approximately 10 % require intensive care and 5 % need mechanical ventilation. Clinical course and long-term outcome vary by subtype of TBEV. The European subtype is associated with milder disease, a case-fatality ratio of < 2 %, and neurologic sequelae in up to 30 % of patients. The Far Eastern subtype is often associated with a more severe disease course, including a case-fatality ratio of 20–40 % and higher rates of severe neurologic sequelae. The Siberian subtype is more frequently associated with chronic or progressive disease and has a case-fatality ratio of 2–3 %.

Epidemiology Tick-borne encephalitis (TBE) has become a considerable public health risk in several European countries, and on average, between 1990 and 2009, nearly 8500 cases of TBE were reported annually in Europe including Russia, although with considerable variability in incidence from year to year (Suss 2011). Many factors contribute to this increase: expanding tick populations due to climatic factors (Randolph 2009; Randolph 2010), social and behavioral changes (Kriz et al. 2004), as well as changes in land use and leisure activities (Sumilo et al. 2007).

Reporting of TBE cases has improved as it is a notifiable disease in 16 European countries, including 13 European Union (EU) Member States (Austria, Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Latvia, Lithuania, Poland, Slovak Republic, Slovenia, Sweden) and three non-EU Member States (Norway, Russia and Switzerland) (Donoso et al. 2008).

TBE is endemic in temperate regions of Europe and Asia (from eastern France to northern Japan and from northern Russia to Albania) and up to about 4921 ft (1500 m) in altitude. Russia has the highest number of reported TBE cases, and western Siberia has the highest incidence of TBE in the world. Other countries where the incidence is high include the Czech Republic, Estonia, Germany, Hungary, Latvia, Lithuania, Poland, Slovenia, Sweden, and Switzerland. High vaccination rates in Austria have reduced the incidence of TBE; however, unvaccinated travelers to this country are still at risk. European countries with no reported cases are Belgium, Iceland, Ireland, Luxembourg, the Netherlands, Portugal, Spain, and the United Kingdom (Suss 2008). Asian countries known to be endemic for TBE include China, Japan, Mongolia, and South Korea (Lu et al. 2008; Walder et al. 2006).

Crossing the Species Barrier TBEV is transmitted to humans through the bite of an infected tick of the Ixodes species, primarily *I. ricinus* (European subtype) or *I. persulcatus* (Siberian and Far Eastern subtypes). The virus is maintained in discrete areas of deciduous forests. Ticks act as both vector and virus reservoir, and small rodents are the primary amplifying host. Tickborne encephalitis (TBE) can also be acquired by ingesting unpasteurized dairy products (such as milk and cheese) from infected goats, sheep or cows, and reports of this route of infections come from Slovakia, Poland, the Baltic States, and other Eastern European countries (Kerbo et al. 2005; Vaisviliene et al. 2002; Balogh et al. 2010). TBEV transmission has infrequently been reported through laboratory exposure and by slaughtering viremic animals. Direct person-to-person spread of TBEV occurs only rarely, through blood transfusion or breastfeeding (Dumpis et al. 1999).

TBE is also emerging in Europe's canine population, and the numbers of clinical cases in dogs are expected to increase (Leschnik et al. 2002; Beugnet and Marié 2009). Humans are accidental dead-end hosts for ticks and for TBEV as, humans do not transmit the disease despite showing noticeable viremia (Heinz 2008)[8].

Biosecurity and Prevention Reducing exposure to ticks is the best method to prevent TBE in humans. It is also recommended to avoid consuming unpasteurized dairy products (Rendi-Wagner 2004). Repellents or insecticides provide unreliable protection against tick bites, and there is no specific antiviral treatment for TBE; therapy consists of supportive care and management of complications (Ginsberg and Stafford 2005).

Being a zoonosis, TBE cannot be easily eliminated from endemic areas. However, the introduction of large-scale vaccination campaigns has proven to be highly effective in reducing the burden of disease. In Austria, where the vaccination coverage in the general population has reached approximately 90%, the number of

[8] http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18848.

clinical cases could be reduced to about 10 %, as compared to the prevaccination era (Heinz et al. 2007). In most highly TBE-endemic countries, large-scale vaccination campaigns are not implemented (Heinz 2008). The risk of acquiring TBE in a highly endemic area in Austria was calculated at approximately 1/10,000 per person-month (Rendi-Wagner 2004). WHO (WHO 2012) recommends tick bite prevention in endemic areas during the summer months; only at-risk travellers should be offered vaccination. Travellers are considered to be at risk when hiking or camping in rural and forested areas up to altitudes of 1400 m (WHO 2012).

41.2.3 Other Severe Clinical Syndromes

41.2.3.1 Monkeypox

The Pathogen *Monkeypox* is a viral disease caused by the Monkeypox virus, an orthopoxvirus. Human cases have been reported from nine countries in central and western Africa where the disease is endemic—Democratic Republic of Congo, People's Republic of Congo, Central African Republic, Gabon, Cameroon, Nigeria, Cote d'Ivoire, Liberia, and Sierra Leone.

The virus was first identified in the State Serum Institute in Copenhagen, Denmark, in 1958 during an investigation into a pox-like disease among monkeys. Monkeypox virus is pathogenic for both animals and humans: Human monkeypox infection was first identified in 1970 in a 9 month old child in the town of Basankusu, Equateur Province, Democratic Republic of Congo and initially NHP were suspected as the source of outbreaks (Ladnyj et al. 1972; Marrennikova et al. 1972).

Over the next year, six further human cases of monkeypox infection were reported in Liberia, Sierra Leone and Nigeria (Foster et al. 1972). From 1970 to 1979, 47 human cases of monkeypox were identified, 38 of which were from Zaire, and the majority were in close proximity to the tropical rainforest (Nalca et al. 2005). A total of 79 cases were subsequently reported over the next 12 years. In 1996–1997 a major outbreak involving 88 cases occurred; between 2001 and 2002 51 human cases were reported in the Democratic Republic of Congo (Hutin et al. 2001; Heymann et al. 2008).

During May and June 2003, the first cases of human monkey pox disease outside of the African continent were reported in an outbreak in Midwestern United States (Illinois, Indiana, Kansas, Missouri, Ohio and Wisconsin) due to direct contact with ill prairie dogs that were kept or sold as pets and which had been recently exposed to imported Monkeypox virus-infected West African rodents from Ghana (Reed et al. 2004).

There were ten confirmed cases and nine probable cases of monkeypox between September and December of 2005 reported in Unity, Sudan (now South Sudan). The particularly intriguing aspect of this outbreak is the evidence of possible human-to-human transmission. In this case, a traditional healer was linked to three of the four transmission chains in the outbreak. The healer had a confirmed case of monkeypox, and a number of the monkeypox patients were either children whom

the healer had recently treated for illnesses or young adults who had gone to him for a tooth extraction procedure (removal of the incisors to signify passage into adulthood is a cultural tradition in this part of Sudan) (Nakazawa et al. 2013).

Clinical Signs Monkeypox disease is characterized by the onset of non-specific symptoms which can include fever, headache, backache, and fatigue during a prodromal period of 2–3 days (Reynolds et al. 2006). This is followed by a 2–4 week period in which a rash develops and progresses from macules, to papules, to vesicles, and then to pustules, followed by umbilication, scabbing and desquamation (CDC 2003). The rash is usually confined to the trunk, but can spread to the palms and soles of the feet, occurring in a centrifugal distribution (Parker et al. 2007). Lesions can also develop on mucous membranes, in the mouth, on the tongue, and on the genitalia (Nalca et al. 2005). The pathogenicity of monkeypox is similar to that of smallpox except for the pronounced lymphadenopathy associated with monkeypox and generally milder symptoms (Heymann 2008). Lymphadenopathy is thus considered to be a key distinguishing feature of monkeypox (Weber and Rutala 2001). The CFR is approximately 1–10% in Africa, with higher death rates among young children (Parker et al. 2007). In children unvaccinated against smallpox, the case-fatality rate ranges from 1 to 14% (Heymann 2008). In addition, children may be more susceptible to monkeypox due to the termination of regular smallpox vaccinations following the worldwide eradication of the disease in 1980.

The incubation period varies from 6 to 16 days. The number of lesions varies from a few to several thousands, affecting oral mucous membranes (in 70% of cases), genitalia (30%), and conjunctivae (20%), as well as the cornea.

There are no drugs or vaccines available for monkeypox, although vaccination against smallpox has been proven to be 85% effective in preventing monkeypox in the past (Parker et al. 2007). Prophylactic vaccination with the smallpox vaccine may be useful within 4 days and up to 14 days after initial contact with a confirmed monkeypox case (CDC 2007).

Epidemiology Monkeypox affect all age groups; however, children under age of 16 have constituted the greatest proportion of cases (Heymann 2008).

Infections of index cases result from direct contact with blood, bodily fluids, or rashes of infected animals. In Africa, human infections have been documented through handling of infected monkeys, Gambian rats or squirrels.

Secondary transmission is human-to-human, resulting from close contact with infected respiratory tract excretions, with skin lesions of an infected person or with recently contaminated objects. Transmission via droplet respiratory particles has also been documented. Transmission can also occur by inoculation or via the placenta (congenital monkeypox). There is no evidence to date that person-to-person transmission alone can sustain monkeypox in the human population.

The differential diagnoses include usually smallpox, chickenpox, measles, bacterial skin infections, scabies, medicamentous allergies and syphilis.

Monkeypox can be definitively confirmed by a number of different tests (ELISA, antigen detection tests, PCR, virus isolation).

Crossing of the Species Barrier In Africa, monkeypox infection has been found in many animal species: rope squirrels, tree squirrels, Gambian rats, striped mice, door-mice and NHP. Doubts persist on the natural history of the virus and further studies are needed to identify the exact reservoir of the monkeypox virus and how it is maintained in nature.

In the USA, the virus is thought to have been transmitted from African animals to a number of susceptible non-African species (like prairie dogs) with which they were co-housed.

Multiple events of human-to-human transmission have been reported, but sustained Monkeypox virus infection cycles among humans have not been documented (Damon et al. 2006; Formenty et al. 2010).

Likos et al. (2005) investigated phylogenetic relationships between Monkeypox virus isolates by examining five whole-genome sequences and confirmed the existence of two distinct groups: the first group contained isolates from the Congo Basin (Congo Basin clade), and the second group included isolates from countries in western Africa. Differences in epidemiologic and clinical features between Monkeypox virus isolates (e.g., higher morbidity and CFR caused by the Congo Basin clade) support the differentiation between these two clades.

Biosecurity and Prevention During monkeypox outbreaks, close contact with other patients is the most significant risk factor for monkeypox virus infection. In the absence of specific treatment and a vaccine, the only way to reduce infection in people is by raising awareness of the risk factors and educating people about the measures they can take to reduce exposure to the virus.

Public health educational messages should focus on the following risks.

- Reducing the risk of human-to-human transmission. Close physical contact with monkeypox infected people should be avoided. Gloves and protective equipment should be worn when taking care of sick people. Regular hand washing should be carried out after caring for or visiting patients.
- Reducing the risk of animal-to-human transmission. Efforts to prevent transmission in endemic regions should focus on thoroughly cooking all animal products (blood, meat) before eating. Gloves and other appropriate protective clothing should be worn while handling sick animals or their infected tissues, and during slaughtering procedures.

Restricting or banning the movement of small African mammals and monkeys may be effective in slowing the expansion of the virus outside Africa.

Captive animals should not be inoculated with smallpox. Instead, infected animals should be isolated from other animals and placed into immediate quarantine. Any animals that might have come into contact with an infected animal should be quarantined and observed for monkeypox symptoms for 30 days.

Health-care workers caring for patients with suspected or confirmed monkeypox virus infection, or handling specimens from them, should implement standard infection control precautions. Healthcare workers and those treating or exposed to patients with monkeypox or their samples should consider being immunized against

smallpox. However, the smallpox vaccination should not be administered to people with comprised immune systems.

Samples taken from people and animals with suspected monkeypox virus infection should be handled by trained staff working in Biosafety Level 3 (BSL-3/ABSL-3) **containment** laboratories (CDC 2007). Orthopoxviruses are susceptible to 0.5% sodium hypochlorite, chloroxylenol-based household disinfectants, glutaraldehyde, formaldehyde, and paraformaldehyde; and are inactivated by heat (autoclaving and incineration) (Butcher and Ulaeto 2005). Orthopoxviruses are stable at ambient temperatures when dried (CDC 2007).

41.2.3.2 Severe Acute Respiratory Syndrome

The Severe Acute Respiratory Syndrome (SARS) Coronavirus (SARS-CoV) is responsible for an acute and often fatal respiratory syndrome that was identified for the first time in the Guangdong province of South China in 2003 (Peiris et al. 2003). SARS-CoV consequently expended encompassing 37 countries and created the first emerging pandemic of the twenty-first century.

Clinical Signs SARS-CoV may cause an often-severe illness marked initially by systemic symptoms of muscle pain, headache, and fever, followed in 2–10 days by a respiratory symptoms (cough, dyspnea, and pneumonia) and a marked lymphocytopenia. Increased respiratory distress led to a CFR of 9.6% (Smith 2006).

Epidemiology SARS emerged as a unique pandemic starting as an epidemic in Guangdong Province, China in November 2002. It further expanded from person to person worldwide as a pandemic in less than 9 months and ultimately infected more than 8000 persons killing more than 700. The pandemic ended in May 2004.

The virus is supposed to have originated from its natural host, a horseshoe bat (*Rhinolophussinicus*). Subsequently, it is thought to have been transmitted to and mutated within a secondary host, the palm civet (*Panguma larvata*) serving also as an amplification host, before it was passed into humans as a new human-pathogenic virus, the SARS-CoV (Zhong et al. 2003). SARS-CoV was found to infect also raccoon dogs (*Nyctereuteus* sp.), ferret badgers (*Melogale* spp.) and domestic cats. SARS-CoV emerged several times from the same intermediate host, the palm civet, to transgress the species barrier and infect humans. Nevertheless, SARS-CoV seems to have also emerged several times in the past in the province of Guangdong, but remained unnoticed as potential epidemic risk. The conclusion was that bats acted as a reservoir of SARS-CoV with the potential to infect other mammals including humans (Li et al. 2005).

Likewise but surprisingly, ten years after the SARS-pandemic, a novel human coronavirus (HCoV-EMC) emerged in the Middle East in 2012 (Berminghan et al. 2012). The HCoV-EMC was identified following respiratory infections with a clinical presentation of severe acute respiratory syndrome of a Qatari man in a British hospital and, a woman who died in Saudi Arabia. The virus consequently caused 12 other confirmed cases and five deaths worldwide (Saudi Arabia, Jordan, and

Britain). HCoV-EMC, that appears distant genetically from the former SARS-CoV, seems to have a zoonotic origin naturally infecting chiropteran species (Kelland 2013; Kindler et al. 2013).

Crossing of the Species Barrier SARS-CoV appears to have transgressed efficiently and successively two species barrier from bat to carnivores to humans and, ultimately, be highly pathogenic for the later with the potential to infect human pulmonary and intestinal epithelium (Sims et al. 2008).

Interestingly, HCoV-EMC appears genetically in the same phylogenetic clade as other bat coronaviruses (Chan and Poon 2013).

In the past decade chiropterans have been confirmed as hosts or reservoirs of several emerging diseases including SARS, nipah, hendra, Ebola, Marburg and rabies viruses posing a zoonotic risk (Gonzalez et al. 2008).

Prevention Because SARS-CoV may be be transmitted by aerosol (i.e. aerosolized droplets from coughing), and due to its physical stability in the environment, the low or absent protective immunity in the human population, and the lack of effective antivirals or vaccines, infection control against SARS relied primarily on the prevention of person-to-person transmission (see for review Cheng et al. 2007).

41.3 Conclusion and Perspectives

Humans and animals did host, share and exchange their pathogens since prehistoric times.

A literature review by Olival, Bogich, Karesh et al. (pers. comm. 2013) on virus isolation from different animal hosts shows that NHP, primates and small domestic ungulates are the mammals that share the most virus species with humans; when corrected for the number of species and by the respective sampling/research methods, monkeys, rodents and bats are the most important reservoirs for zoonotic agents. Moreover, if we focus on known viruses and correct for the number of species and sampling per taxonomic order, chiropterans appear to potentially harbor three and six time more different virus species than rodents and NHP, respectively. Also Rodent and Chiropteran are one of the most species richness among the vertebrate orders, they harbor a variety of viruses that can be potentially infectious for human. Moreover, apes share a so close relationship by nature with human, i.e. >90% of genomic identity, that they theoretically can easily exchange pathogens and pass such "thin" inter species barrier form NHP to Human Primates.

There is no more *terra incognita* on Earth. Humans, by migratory habits, professional or recreation occupations explored already the entire natural environments on the planet, stepping into the immense variety of its ecosystems. While the vast ocean is still open for discovery, zoonotic risk is not out of the scope. As an example, humans are more likely to interact with pinnipeds, than with any other marine mammals and a newly described influenza from seals may potentially infect humans (White et al. 2013). Influenza B virus as well as measles can be shared by

human and seals. Also it is well documented that transmission occurs from human to animals like *Coxiella burneti* found infecting seals in Alaska. Moreover, *Streptococcus agalactiae*, a member of human gastrointestinal normal flora, is known to infect sea mammals as well as other marine fauna including fishes (!) among others (Delannoy et al. 2013; Duncan et al. 2012).

Understanding the fundamentals of virus emergence from an animal reservoir and its transmission to humans—but also from one animal species to another—as well as mastering the territories at risk with regard to their environments—including biological and physical environmental components (i.e. increase of the human population, climate change and exceptional weather or natural events)—are essential for controlling and preventing zoonoses and potentially emerging zoonoses.

Viruses will continue to pass the species barrier without geographical borders and acquire new abilities to survive within new hosts without losing their intrinsic pathogenic potential.

More than 60 % of 335 emerging infectious diseases identified since 1940 have a zoonotic origin. Among them more than two third are from wildlife animal (Jones et al. 2008). Furthermore, specific territories or domains of emergence, within a given environment, where people, livestock and wildlife encounter each other, have been identified and characterized. An analysis of all documented events has led to develop a spatial and temporal approach for a better understanding of dynamic risk factors (so-called drivers) associated with disease emergence (Souris et al. 2010). By understanding these variable drivers of different scales (e.g. from molecular to spatial, including environmental factors) using computing assisted analysis and mathematical models we might finally be able to predict and hopefully prevent emerging zoonotic infections (Morse et al. 2012). Obviously, theoretical models will have always to be sustained by accurate survey networks coupled with multidisciplinary research. Several of these drivers have to be carefully monitored, e.g. human expansion and its propensity to invade animal territories (i.e. protected area), the emergence of new pathogens from the natural fauna, ecological and environmental conditions, human and animal behaviors, socioeconomic changes, etc.

Biodiversity plays a role in both directions, favoring the risk of exposure to new potentially pathogenic agents and protecting the host against unknown microbes. On one hand, biodiversity exists for the microorganisms as well as for all the other animals, such increasing the variety of potential human pathogens that have not yet "jumped" from animals to humans. On the other hand, the biodiversity of the human major histocompatibility complex, MCH, helps to prevent infection by new pathogens. Eventually, new pathogens may adapt to a new human host (humanization) and ultimately resist to disappearance (i.e. drug resistance) (Maillard and Gonzalez 2006).

Climate change and societal behavior favor the encounters of hosts, vectors and pathogens that never "met" before: Human and animal populations are highly reacting to climate change (e.g.: mosquitoes) and move or expand towards new territories. Human density, i.e. risk of encounter/transmission from animals to humans, and changes in behavior (pets, hunting) are the driver of emerging zoonoses.

Survey and networking, connected to research, molecular biology and/or virus discovery are the strategic key to predict and prevent the emergence of new zoonoses as well as the next pandemic zoonosis (Gonzalez et al. 2011). Moreover technological advances in molecular diagnostics, mathematical modeling, communication, and informatics enable a targeted global surveillance of emerging and previously unknown infections in both human beings and other species (Morse et al. 2012).

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Chapter 42
Bacterial Zoonotic Pathogens as Bioterroristic Agents

Stefan Hörmansdorfer

Abstract Bioterrorism means the deliberate release of viruses, bacteria or other agents used to cause illness or death in people, animals or plants. Infectious agents or their toxins, often known for long as the cause of classical infectious diseases may be used for bioterroristic purposes, but not all of them are equally suited as biological weapons.

Anthrax, caused by *Bacillus (B.)* anthracis, a gram-positive spore-forming rod, is a very long known animal disease with zoonotic potential. The ability to form endospores makes *B. anthracis* especially suitable for bioterroristic use as these endospores are highly resistant to environmental influences, disinfectants, heat or radiation and can easily be aerosolized. *B. anthracis* possesses two main virulence factors, the anthrax toxin and the ability to form a capsule. Both virulence factors are plasmid-encoded. Human anthrax manifests itself as cutaneous anthrax, alimentary anthrax, inhalational anthrax or injectional anthrax.

Tularemia is a zoonosis with a broad host range. Wildlife animals are the main reservoir for humans. It is especially a disease of hares, rabbits and other rodents. As humans are highly susceptible for tularemia, its agent, *Francisella (F.)* tularensis, which can be transmitted by aerosol, is supposed to have a bioterroristic potential, although no attempts of bioterroristic misuse have been known so far. While the most virulent subspecies *F. tularensis ssp. tularensis* is confined to North America, a less virulent subspecies, *F. tularensis ssp. holarctica*, is widely distributed over the Northern hemisphere, predominantly over North America, Scandinavia, Russia and Japan. Human tularemia is a febrile, inflammatory disease, which starts with unspecific symptoms like headache, growing pains, fever, chills and weakness. The further course of disease depends on the agent's virulence and its route of entry.

Bioterrorism means the deliberate release of viruses, bacteria or other agents used to cause illness or death in people, animals or plants (http://emergency.cdc.gov/bioterrorism/overview.asp). Infectious agents or their toxins, often known for long as the cause of classical infectious diseases may be used for bioterroristic purposes, but not all of them are equally suited as biological weapons.

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The CDC discerns three categories of organisms or toxins according to their easiness of spread, the severity of the illness caused and the public and social aspects following their release (http://emergency.cdc.gov/bioterrorism/overview.asp). Category A comprises biological agents and toxins which can be easily disseminated or transmitted from person to person; may result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption and require special action for public health preparedness such as *Bacillus anthracis* (anthrax), *Clostridium botulinum* toxin (botulism), *Yersinia pestis* (plague), Variola major virus (smallpox), *Francisella tularensis* (tularemia) or filoviruses, arenaviruses and other hemorrhagic fever viruses (Viral hemorrhagic fevers) (Maidhof 2007).

Successful attempts of biological warfare with bubonic plague or small pox are known from medieval times. During World War I and II some countries had scientific programs for developing biological weapons, but an applicable weapon has never been delivered. In the recent past *B. anthracis* spores were used in a bioterroristic raid in the USA ("Amerithrax") during autumn 2001 shortly after the attack of 9–11, when letters containing highly concentrated anthrax spores were sent by mail. 22 cases were reported, 11 of them suffered from inhalational anthrax with five fatalities and the remaining 11 cases were cutaneous anthrax (Leonard 2010). Bioterrorism does not only mean an attack with microorganisms or their toxins against humans, but also against farm animals or plants. During World War I the German Reich tried to infect animals in neutral countries with anthrax or glanders, especially to hinder the delivery of horses for military use (Mogridge et al. 2010).

In the following, anthrax and tularemia will be presented as examples for zoonotic bacterial agents with an indwelling bioterroristic potential.

42.1 Anthrax

Anthrax is a very old disease which is supposed to be mentioned in the Holy Bible as the fifth plague of Egypt. It has been reported since about 1500 B.C., depicted by the oldest hebrew, greek and roman authors (Böhm 1995; Koch 1885). The etiology of anthrax was proven by Robert Koch in 1876 (Selbitz 2011). Anthrax is primarily an animal disease, but with a broad host range including humans. Especially in former times lots of people fell ill and often died from gastrointestinal anthrax (Koch 1885). At that time, eating anthrax infected animals was a major source of human infection besides inhalational and cutaneous anthrax.

42.1.1 Etiology

Anthrax is caused by *Bacillus anthracis*, a large, facultatively anaerobic, gram-positive rod, which is non-motile and non-hemolytic on blood agar (see Fig. 42.1), although a few strains may show a weak hemolysis under the colonies. The bacilli

Fig. 42.1 *Bacillus anthracis* on sheep-blood agar (incubation at 37 °C for 18 h)

appear as single rods or in chains, especially in host tissue. *B. anthracis* grows with flat, dry, rough, greyish colonies with curved and curled peripheral projections, which give them a "medusa head"-like appearance. As a non-fastidious organism, *B. anthracis* grows well on a broad range of non-selective nutrient media. There is a near relationship between *B. anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis*, which are summarized as *Bacillus cereus* group. It is difficult to discern the four species by mere biochemical means, so that besides differentiation by molecular methods further criteria like motility, penicillin susceptibility, gelatin liquefaction or capsule formation under 10 % CO_2 have to be determined (Böhm 1995; Selbitz 2011; Quinn et al. 2011; Markey et al. 2013).

B. anthracis possesses the ability to form endospores. These endospores have a remarkable tenacity towards environmental factors such as heat, cold, dryness, radiation and a great range of desinfectants. To reduce anthrax spores by 90 % within a minute, temperatures up to 110 °C are necessary (Böhm 1995). The endospore formation is enhanced by oxygen and starts, when *B. anthracis* is set free from an infected host by bleeding or opening of the carcass. The first endospores can be detected after 4–10 h and the sporulation process is finished after 24–48 h (Mogridge et al. 2010). Those endospores remain viable in soil for decades. Under laboratory conditions a viability of up to 70 years is proven (Böhm 1995).

42.1.2 Global Distribution

Anthrax was once known all over the world, but national reduction programs have confined anthrax to some endemic regions in the Mediterranean region, central and south America, Africa, Asia and the far and middle East. Sporadic cases may despite occur in nearly all countries (OIE: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home; WHO 2008).

42.1.3 Virulence Factors

B. anthracis expresses different minor virulence factors such as phospholipases, proteases, iron uptaking proteins, siderophores and anthrolysin O, which are encoded chromosomally or on plasmids (Mogridge et al. 2010). Moreover, virulent strains of *B. anthracis* possess two major plasmids, pX01 and pX02. The 182 kb plasmid pX01 contains 217 genes including the three components of anthrax toxin, *pagA, cya* (edema factor) and *lef* (lethal factor). Plasmid pX02 consists of 95 kb with 113 genes including the capsule forming genes (Mogridge et al. 2010). *B. anthracis* strains which bear only one of those two virulence plasmids are non-virulent or significantly attenuated (Markey et al. 2013). Those attenuated strains may be used as live vaccines (Böhm 1995).

42.1.4 Capsule

The capsule consists of anionic polypeptides, i. e. linear chains of poly-γ-D-glutamic acid linked to the peptidoglycan of the bacterial cell wall. These long chains exceed a molecular weight of 400 kDa. They are protease-resistant and confer protection against phagocytosis being poorly recognized by phagocytes due to their anionic charge. By masking immunogenic surface structures they prevent the humoral immune response. The capsule is expressed especially within the host. These conditions can be mimicked during culture, growing the bacteria on bicarbonate agar under enhanced CO_2 partial pressure (5–10 %). This yields very moist and even slimy colonies—a phenomenon unique to *B. anthracis* compared to other members of the *B. cereus* group (Böhm 1995; Quinn et al. 2011; Markey et al. 2013; Mogridge et al. 2010).

42.1.5 Anthrax Toxin

The production of anthrax toxin is also enhanced by a higher CO_2 pressure (5–10 %) and temperatures of about 37 °C. The highest toxin production occurs during the late logarithmic growth phase. Anthrax toxin consists of two binary components, lethal toxin and edema toxin. Both toxins are formed with lethal factor (LF, 90 kDa) or edema factor (EF, 89 kDa) as the enzymatic component being each linked to the protective antigen (PA, 83 kDa), which is the receptor binding subunit, responsible for internalization of the toxic components into the cytosol of the host cells (Mogridge et al. 2010).

Edema toxin is a calcium- and calmodulin-dependent adenylate cyclase. Its catalytic rate exceeds this of mammalian adenylate cyclases by 100-fold. By disturbing cellular signal pathways the edema toxin has numerous effects like impairing phagocytes in their phagocytic abilities and altering their cytokine production, caus-

ing necrosis in some tissues, leading to intraluminal fluid accumulation or decrease of the circulating lymphocyte population (Mogridge et al. 2010).

Lethal toxin is a zinc dependent metalloprotease which interferes with cellular signaling pathways impairing numerous cellular functions of immune cells. Lethal toxin induces cell death in macrophages, dendritic cells and neutrophils as well as in lung epithelial cells and endothelial cells (Mogridge et al. 2010).

42.1.6 Host Range

Anthrax is a disease with a broad host range. It is primarily an animal disease with ruminants, especially cattle, sheep and goats being highly susceptible. Reports of anthrax in buffaloes, horses, camels, elephants, rein deer and other domesticated or wild living herbivores are not seldom either. Carnivores and humans are moderately susceptible, while pigs exhibit only low susceptibility. Most birds are nearly unsusceptible, but rare reports on the disease in ostrichs, ducks or birds of prey exist (Böhm 1995; Selbitz 2011).

42.1.7 Epidemiology

Soil is the natural reservoir of endospores being formed during the decomposition of animal carcasses having died of anthrax. The endospores remain soilbound and infectious for decades due to their high tenacity towards environmental factors. Endospores from buried animal carcasses can reach the surface during heavy rain and flooding of pastures after years and even decades thus becoming a source of infection again. Herbivores may catch the infection by inhalation of aerosolized endospores or their ingestion during grazing. Carnivores are infected by devouring infected animals or their carcasses. Anthrax is not a highly contagious disease. It can barely be transmitted from an infected animal to another except by direct contact with body fluids, especially blood which is shed by animals near agony. Biting insects or flies are discussed as vectors and are made responsible for explosive outbreaks. Transfer to different regions is supported by human activities such as trade with animal products as hides, wool, bone meal or meat meal (Selbitz 2011; Mogridge et al. 2010; WHO 2008).

42.1.8 Infectious Dose

LD_{50} ranges from <10 spores in susceptible herbivores to 10^7 spores in less susceptible animals, when administered parenterally. The normal way of infection is inhalation or ingestion of endospores, hereby the infectious dose even in susceptible animals is in the order of ten of thousands endospores (WHO 2008).

42.1.9 Disease in Animals

The incubation period is 3–5 days with a range between 1 and 14 days.

Ruminants predominantly fall ill with septicemic anthrax, often suffering from sudden death within minutes or hours. The acute illness is highly febrile (fever over 40 °C) and shows signs of severe disease like apathy, dyspnea, tremor, colic, cramps and cyanosis. Near agony there is shedding of dark, non-coagulable blood from mouth, nose, anus and with the urine. This blood contains lots of sporulating bacilli and is highly infectious. Death occurs within about 12 h. In rare cases prolonged forms occur with edema, colic, dyspnea and indigestion (Böhm 1995; WHO 2008).

Horses normally show an acute disease with high fever (40–41 °C), dyspnea, cyanosis and colic. Hemorrhages, bloody diarrhea and local, necrotizing swellings are described. Near agony bleeding from different orifices may be seen. Death occurs within 1–3 days (Böhm 1995; WHO 2008).

Septicemic anthrax with an incubation period of 2–4 days is seldom in pigs. Usually, aching, pharyngeal swellings with fever, pharyngitis, dyspnea and edema develop. Black, necrotizing nodules may be seen in the mucosa and the skin (pharyngeal form). Icterus, vomiting and diarrhea are signs of intestinal anthrax. Lethality is much lower in pigs compared to ruminants and horses. Chronic, localized anthrax with no clinical signs is possible and the infection is detected in the slaughterhouse (Böhm 1995; WHO 2008).

Carnivores may fall ill with septicemic anthrax with an incubation period of 2–6 days and fever up to 40 °C. They die within hours or a few days with symptoms similar to those of ruminants. Dogs are considered much more resistant than cats. Subacute cases show diarrhea, edema predominantly in the laryngeal area or the mouth with central necrosis. Death occurs within 3–5 days. Minks are very susceptible to anthrax, showing septicemic anthrax with a mortality up to 100 %, depending on the level of feed contamination (Böhm 1995).

42.1.10 Human Anthrax

Humans are much less susceptible than herbivores. The infectious dose (ID_{50}) is estimated to range between thousands and tens of thousands of endospores (WHO 2008). Infection occurs by contact to infected animals or contaminated animal products (WHO 2008; Bauerfeind et al. 2013). Thus, human anthrax is more or less an occupational disease of farmers, veterinarians, butchers, slaughterhouse workers or workers in the fur, leather or wool industry, but also in transport or dock workers (Bauerfeind et al. 2013). Endospores may penetrate through small wounds or by inhalation. Ingestion by eating infected meat is of little importance nowadays, but was a major route of transmission in former times. Human anthrax manifests itself as cutaneous anthrax, alimentary anthrax or inhalational anthrax (Koch 1885; WHO 2008; Bauerfeind et al. 2013).

More than 95 % of all cases are *cutaneous anthrax*. The incubation period is 2–5 days with a range from 12 h to 17 days. The endospores penetrate through little skin

lesions or wounds and germinate. The disease starts with local reddening, followed by the formation of a papule, which becomes a vesicle within 12–24 h, containing serous or putrid liquid. After a week there is a marked edema with swollen edges while the center necrotizes and a characteristic blue-black eschar is formed. The local process is painless, but pain and fever occur when the regional lymph nodes are affected (WHO 2008; Bauerfeind et al. 2013).

Alimentary anthrax has an incubation period of 1–7 days. Symptoms are loss of appetite, vomiting and nausea, later abdominal cramps, hematemesis and bloody diarrhea with gut necrosis, ulceration and ascites. The mesenterial lymphnodes are heavily involved. The clinical picture is that of an acute abdomen (WHO 2008; Bauerfeind et al. 2013).

Ingestion of endospores may cause *oropharyngeal anthrax* with sore throat, dysphagia, regional lymphadenopathy and a marked edema of the throat together with a pseudomembranaceous inflammation of the tongue and the tonsils (WHO 2008; Bauerfeind et al. 2013).

Inhalational anthrax is the most severe form of human anthrax. The ID_{50} is estimated to be 8000–50,000 endospores. The incubation period is 4–6 days. There is a preset of unspecific, influenza-like symptoms like malaise, cough, sore throat and fever, followed by worsening dyspnea, headache, sweating, high fever, tachypnea, tachycardia, pleural effusion, cyanosis, disorientation and finally coma and shock. There is massive involvement of the pleural lymphatic system leading to lung congestion and interstitial edema aggravated by toxin-mediated endothelial damage of lung capillaries (WHO 2008; Bauerfeind et al. 2013).

Since 2009 a special form of human anthrax—*injectional anthrax*—was seen in intravenous drug addicts. It is supposed that the injection of heroin contaminated with anthrax endospores sets a severe soft tissue infection of muscle, fasciae and connective tissues around the injection site. The patients develop a severe local inflammation with swelling, reddening, edema, abscessation, necrotizing fasciitis and compartment syndrome with nausea and dyspnea. The local infection may generalize to septicemic anthrax with a very poor prognosis (Bauerfeind et al. 2013; Holzmann et al. 2012; Ramsay et al. 2010). The first cases were detected in Scotland. From December 2009 to December 2010 there were 119 cases of injectional anthrax in intravenous drug users in Scotland of whom 14 people died. Another five cases with four fatalities were reported from England and two cases from Germany (Health Protection Scotland 2011). Since June 2012 there were 14 further cases: four in Germany, two in Denmark, one in France, and seven in the UK (one in Scotland, five in England and one in Wales). Six of these cases were fatal (ECDC 2014).

All known manifestations may lead to septicemic dissemination and anthrax meningitis with fever, somnolence, myalgia, nausea, vomiting, cramps, delirium and toxic shock (WHO 2008; Bauerfeind et al. 2013).

42.1.11 Diagnosis

The diagnosis of anthrax comprises clinical symptoms and clinical tests together with laboratory tests. Besides the microscopic detection of gram-positive, spore-

and capsule forming rods in chains directly from infected tissue or blood, the most important test is the isolation of the agent by culture. Culture from infected tissue should always be combined with a PCR for both virulence plasmids as an antibiotic pretreatment may effectively kill the bacteria so that culture alone may yield false negative results. Isolates may be identified and further characterized by biochemical differentiation in combination with other morphological parameters, MALDI-TOF, immunofluorescence or other antibody techniques and molecular methods to detect anthrax specific chromosomal sequences and plasmid-located toxin and capsule genes or reveal the clonal descent of the isolated strain. A special bacteriophage (γ-phage) may be used to identify anthrax strains (WHO 2008; Bauerfeind et al. 2013).

42.1.12 Therapy

B. anthracis is susceptible to a variety of antibiotics. β-lactam-antibiotics, streptomycin, tetracyclins, erythromycin, clindamycin or ciprofloxacin are recommended for treatment (Selbitz 2011; WHO 2008; Bauerfeind et al. 2013).

42.2 Tularemia

Tularemia is a zoonosis with a broad host range. Wildlife animals are the main reservoir for humans. It is especially a disease of hares, rabbits and other rodents. As humans are highly susceptible for tularemia, its agent, *F. tularensis*, which can be transmitted by aerosol, is supposed to have a bioterroristic potential, although no attempts of bioterroristic misuse have been known so far.

42.2.1 Etiology

F. tularensis are small, coccoid, pleomorphic, gramnegative rods. They are strictly aerobic, oxidase-negative, weakly catalase positive and non-motile. Their cell wall is rich of lipids and gives them a high tenacity in the environment, so that *F. tularensis* may survive in soil, mud or water for weeks and even months (Markey et al. 2013; Bauerfeind 2011).

As fastidious organisms they need rich, cysteine supplemented media, e. g. cysteine-heart agar with chocolatized blood, chocolate agar with Iso-VitaleX, Thayer-Martin-agar or Martin-Lewis agar (see Fig. 42.2). Antibiotics should be added to suppress the contaminant flora. The incubation under 5 % CO_2 supports the growth of *Francisella spp.* The incubation period should be at least 48 h and be extended to 10 days (Markey et al. 2013; Müller et al. 2013; Bauerfeind 2011).

Fig. 42.2 *Francisella tularensis* ssp. *holarctica*, isolated from a European hare

Francisella (F.) is the only genus of the family *Francisellaceae*. It comprises the species *F. tularensis, F. hispaniensis, F. noatunensis, F. philomiragia* and *F. halioticida*. The last three species are linked to water and sometimes isolated from fish. *F. tularensis* consists of four subspecies, i. e. *F. tularensis* ssp. *tularensis*, *F. tularensis* ssp. *holarctica*, *F. tularensis* ssp. *mediasiatica* and *F. tularensis* ssp. *novicida*. The four subspecies differ especially in geographical distribution, but also in biochemical activities and host specificity (Markey et al. 2013). For *F. tularensis* ssp. *holarctica* 3 biovars are proposed: biovar I (erythromycin sensitive), biovar II (erythromycin resistant) and biovar japonica, which can ferment glycerol (Müller et al. 2013; Bauerfeind 2011). *F. tularensis* ssp. *tularensis* is the most virulent strain and therefore classified into risk group 3, while the other subspecies belong to risk group 2.

42.2.2 Virulence Factors

The virulence factors of *F. tularensis* are poorly understood yet. *F. tularensis* form a capsule which is responsible for serum resistance. In the host they live inside macrophages. They can manipulate the host immune response by phase variation of their lipopolysaccharide (LPS), concerning the O antigen and the lipid A moiety. The LPS of *F. tularensis* differs from that of other gramnegative bacteria and does not induce interleukin 1-release from mononuclear cells and poorly induces tumor necrosis factor. As other intracellular pathogens they modulate the phagosome biogenesis, hinder them from fusion with lysosomes and escape to the cytoplasm (Markey et al. 2013).

42.2.3 Epidemiology and Geographical Distribution

F. tularensis is widely distributed over the Northern hemisphere, predominantly over North America, Scandinavia, Russia and Japan (Markey et al. 2013). *F. tularensis*

ssp. *tularensis* mainly occurs in North America with some findings of probably imported cases in Austria and Slovakia, whereas *F. tularensis* ssp. *holarctica*—the less virulent variant—is distributed over North America, Europe, Siberia, Israel, Iran and Asia. *F. tularensis* ssp. *mediasiatica* is confined to Central Asia, while *F. tularensis* ssp. *novicida* can be found in North America, Spain and Australia (Markey et al. 2013; Bauerfeind et al. 2013).

F. tularensis have been isolated from about 250 wildlife animal species, which serve as natural reservoir for man. The most important host animals are hares, rabbits, squirrels, voles, mice, rats, beavers and other rodents, but also deer, foxes, bears and other carnivores and birds as well as reptiles and amphibians or fish may be infected (Markey et al. 2013; Bauerfeind et al. 2013). Biting arthropods play a vital role in the transmission of *F. tularensis*. These include flies, mites, mosquitoes, lice, fleas and especially ticks, of which some species may transmit the agent transovarially and are thus a natural reservoir themselves (Markey et al. 2013; Bauerfeind et al. 2013; Bauerfeind 2011). Aquatic protozoae are discussed as reservoirs, too (Bauerfeind et al. 2013; Bauerfeind 2011).

Tularemia is transmitted to humans by direct contact with infected animals, their excretions and organs or by bites. Further sources of infection are contaminated water or food and biting arthropods. Aerosol transmission may occur by processing agricultural products. The agent invades the host through small lesions of the skin or through the conjunctivae or the mucosae of the upper gut or respiratory tract (Markey et al. 2013; Bauerfeind et al. 2013; Bauerfeind 2011).

Tularemia is an occupational disease for humans, especially for hunters, butchers, cooks or agricultural and forest workers. Carnivores, especially cats may transmit the disease to man, while dogs are relatively resistant (Markey et al. 2013; Bauerfeind et al. 2013; Bauerfeind 2011). Most infections of man occur while treating and preparing hunted hares. A direct transmission from human to human has not been described yet. (Bauerfeind 2011).

42.2.4 Infectious Dose

The infectious dose for inoculation into wounds is with ten bacteria very low, followed by an infectious dose of 10–50 bacteria for inhalation and about 10^8 bacteria for ingestion (Bauerfeind et al. 2013).

42.2.5 Disease in Animals

Animals develop an acute or chronic general disease with a wide range of symptoms from mild, regional lymphadenopathy to acute septicemia. Further signs are depression, anorexia, fever, vomiting, diarrhea, lymphadenomegaly, ulcers and hemorrhage (Markey et al. 2013; Bauerfeind 2011). Outbreaks with high morbidity and mortalitiy are seen in hares. The main organs infected are lymphnodes, lung,

pleura, spleen, liver and kidneys, where miliary, whitish to yellowish necroses are formed, which resemble pseudotuberculous lesions. In sheep late abortions or the birth of weak lambs are described (Bauerfeind 2011).

42.2.6 Human Tularemia

The incubation period is 3–10 days with a range of 1–21 days. The disease lasts for 2–3 weeks without treatment and is followed by a long phase of reconvalescence. The rate of lethality is 4–6 %, with sepsis up to 30 % despite treatment. Infections with *F. tularensis* ssp. *holarctica* take a lighter course than those with *F. tularensis* ssp. *tularensis*. The disease normally leaves life-long immunity (Bauerfeind 2011).

The disease starts with unspecific symptoms like headache, growing pains, fever, chills and weakness. The further course of disease depends on the agent's virulence and its route of entry.

The *ulceroglandular form* starts with a red papule at the site of entry, which undergoes necrosis and finally forms an ulcus. The regionary lymphnodes (often axillary or inguinal lymphnodes) are swelling and develop purulent inflammation and abscessation. If the local skin necrosis is missing, the disease is known as *glandular form*.

Invasion through the conjunctivae results in a heavy conjunctivitis with lymphadenopathy (*oculoglandular form*).

The *oropharyngeal form* manifests with stomatitis, pharyngitis, tonsillitis and otitis with involvement of the cervical lymphnodes.

Pneumonia and pleuritis with retrosternal pain is characteristic for the *pneumonic form*, while nausea, vomiting, diarrhea, intestinal pain and gastrointestinal hemorrhage are symptoms of the *gastrointestinal form*.

The *typhoidal or septic form* shows high, continuous or intermittent fever, chills, headache, meningitis, myalgia, apathy, diarrhea, intestinal pain, hepatosplenomegaly, renal failure and shock with multiple organ failure (Markey et al. 2013; Bauerfeind 2011).

42.2.7 Diagnosis

Tularemia should be suspected when the above mentioned clinical symptoms meet a history of wildlife animal contact. The diagnosis is proven by bacteriological examination or serological tests. Clinical specimens like swabs from ulcers, wounds or conjunctivae, pus, bioptates or sputum are cultured. It must be regarded, that *F. tularensis* does not grow on normal media, but needs special, cysteine supplemented media. The culture is not always successful, therefore PCR from clinical specimens should be undertaken in parallel, if tularemia is suspected. Isolates are differentiated by immunofluorescence or by PCR. Molecular methods including 16S rRNA sequencing are used to determine species and subspecies (Markey et al. 2013; Bauerfeind et al. 2013; Bauerfeind 2011).

42.2.8 Therapy

F. tularensis may be resistant to β-lactam antibiotics, azithromycin and macrolides. They are sensible to aminoglycosides, fluorchinolones tetracyclines and chloramphenicol. For therapy of humans gentamicin, streptomycin, ciprofloxacin or doxycycline are recommended (Markey et al. 2013; Bauerfeind et al. 2013; Bauerfeind 2011).

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Part IX
Controversial or Non-resolved Issues

Chapter 43
Bovine Paratuberculosis and Human Crohn's Disease—Is There a Zoonotic Linkage?

Erdmute Neuendorf and Nikolaus Ackermann

Abstract *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is an acid-fast bacterium, which causes paratuberculosis, an infectious enteric disease of ruminants, also called Johne's disease (JD). Since the publication by Thomas Kennedy Dalziel in the year 1913 MAP has been discussed as probable causative agent of Morbus Crohn (syn. Crohn's Disease (CD)), an inflammatory disease of the human intestinal tract. Here we describe the history, etiology, diagnosis, clinical and epidemiological aspects of paratuberculosis and CD to elucidate the role of MAP in the pathogenesis of CD. The theory still remains open for controversial discussion and future studies are needed to find a final conclusion. At the moment, however, there is not enough evidence to convincingly demonstrate that MAP is the etiological agent for CD.

43.1 Introduction

Morbus Crohn (syn. Crohn's Disease (CD)) is an inflammatory disease that has the potential to involve any part of the human intestinal tract anywhere from the mouth to the anus. The disease is generally located at the terminal ileum and the proximal colon. A linkage between CD and *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is discussed since the publication by Thomas Kennedy Dalziel in the year 1913 (Dalziel 1989).

43.2 Johne's Disease/Paratuberculosis

Paratuberculosis, also called Johne's disease (JD) is an infectious disease of ruminants caused by MAP. Paratuberculosis can be found worldwide. Only Sweden and some states in Australia are proven to be free of this disease (http://www.cfsph.iastate.edu/Factsheets/pdfs/paratuberculosis.pdf).

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Primary susceptible species are cattle, sheep, goats, deers including wild ruminant species, and other ruminants like camels (Kennedy and Benedictus 2001; Tiwari et al. 2006). The host range of JD is wide. Infections of non-ruminants like wild rabbits, foxes, stoats (a weasel species), mandrills or macaques are described (Beard et al. 2001; McClure et al. 1987; Zwick et al. 2002). Calves inoculated with MAP from a free-living rabbit developed typical histological lesions consistent with JD, demonstrating that wild animals other than ruminants may also contribute to the spread of the disease. But the main source of infection for calves is the exposure to feces of infected mature cattle (Beard et al. 2001).

43.3 History

Since the middle of the nineteenth century both the clinical signs and pathological anatomy of paratuberculosis are known as chronic enteritis with marked transformation of the intestinal mucosa. Johne and Frothingham demonstrated acid-fast bacilli in altered tissues and described for the first time this disease as a singular case of tuberculosis in cattle (Johne and Frothingham 1895).

Due to the different biological properties of the infectious agent and due to the different pathological patterns Bang proposed in the year 1906 to separate the illness from tuberculosis. Since then bovine paratuberculosis is classified as a separate disease (Bang 1906).

In 1910/1912 Twort published the isolation and cultivation of the infectious agent for the first time. Finally, in 1933 the experimental infection of cattle with MAP succeeded. By that the Henle-Koch postulates were fulfilled. Since then it is proven that MAP is the monocausal agent of bovine paratuberculosis (Twort and Ingram 1912).

43.4 Etiology

43.4.1 Infectious Agent

MAP belongs to the family of *Mycobacteriaceae*, gram-positive bacteria, which comprise more than 100 species. They proliferate intracellularly and are characterized by their acid-stability and lipid-enriched cell wall. The subspecies MAP is clearly distinct from other pathogens of the family like *Mycobacterium tuberculosis*, the infectious agent of tuberculosis or *Mycobacterium leprae*, the infectious agent of leprosy. MAP is a mycobacterial subspecies with a slow replication rate and a generation time of over 20 h. Its cultural growth is mycobactin dependent (Rowe and Grant 2006). MAP is a subspecies member of the *M. avium* complex. While members of the *M. avium* complex typically cause disease in immunocom-

promised hosts, MAP has been elucidated as causative agent of JD in immunocompetent ruminants (Behr and Kapur 2008).

43.4.2 Pathogenesis of Johne's Disease

MAP can be transmitted directly, most commonly fecal-orally or orally by colostrum. Infections by the intrauterine route or semen are also reported (Uzoigwe et al. 2007).

85 % of the animals get infected in the first days of their life, 5 % during the first year and 10 % in utero. Commonly the infection starts in the first 30 days of life, later on an increasing age-resistance is acquired. Calves are mainly infected orally by fecally contaminated drugs or by colostrum, older animals by contaminated food, water or pastures. The incubation time is reported to range from 2 to 10 years (Chiodini et al. 1984).

The disease is characterized by a profuse and intractable diarrhea that ends up in severe weight loss and death.

The disease develops chronically and intermittently. Over a very long period infected animals show no signs of illness. Therefore, JD usually stays undetected for a long time. However, the spreading of bacteria starts before the onset of clinical symptoms.

Hence mainly animals in a late subclinical stage play an important role in the propagation of paratuberculosis. Whitlock and Buergelt described that one animal with clinical symptoms represents only the tip of an iceberg. Every cow with symptoms may indicate 15–25 more affected animals in the herd (Whitlock and Buergelt 1996).

Furthermore the intermittent spread is another cornerstone of the maintenance of MAP in the herd. While one clinically affected cow releases up to 10 million infectious particles per gram feces (Whitlock et al. 2005), the excretion dose of a subclinically diseased cow ranges between 10 and 100 infectious agents per gram.

The infection dose is reported to be 10,000 agents for the infection of one calf (Gerlach 2002).

43.4.3 Ability to Survive

The thick, waxy cell wall renders the organism highly resistant. The pathogen survives in soil up to 11 months. In running water a survival time up to 163 days is reported. In standing water or bovine feces the organism was detectable for at least 35 weeks (Lovell et al. 1944; Whittington et al. 2005; Whittington et al. 2004).

Beside the high environmental survival time of MAP also the high resistance to heat is significant. MAP in milk was stated to survive pasteurization (Grant 2003; Millar et al. 1996).

43.4.4 Time Line of Paratuberculosis

The course of disease can be divided into four phases of different immunology, clinical signs and pathomorphology (Whitlock and Buergelt 1996):

1. the silent early stage of infection
2. the subclinical stage
3. the clinical stage
4. the advanced clinical stage

1. The Silent Early Stage of Infection After oral ingestion MAP is taken up by M cells, mainly in the ileum, and transported into the Peyer's patches. There the organism is phagocytized by resident macrophages and persists in their phagosomes, thereby eliciting a cell mediated immune reaction. In this stage there is no detectable spread of the agent by feces.
The animal shows no signs of disease. When examining the tissue acid-fast bacilli can be seen histologically in intestinal lymph nodes or intestinal sections and MAP can be cultivated (Whitlock and Buergelt 1996).

2. The Subclinical Stage The intracellular proliferation of the pathogen in macrophages results ultimately in cell death and release of the agent into surrounding tissues and the gut lumen. By the liberation of MAP the humoral immune response is initiated. Acid-fast bacilli can be (intermittently) detected in the feces (Coussens 2001; Sweeney et al. 1992).
The animal still shows no signs of disease. Stages 1 and 2 correspond to the incubation time of the disease and can range between 2 to 10 years.
Pathomorphologically a moderate hypertrophy of the mucosa and an enlargement of the mesenterial lymph nodes is accompanied by infiltration of epitheloid and Langhans giant cells and foamy macrophages containing phagocytized acid-fast bacilli.

3./4. The Clinical Stage/Advanced Clinical Stage Initial clinical signs follow the subclinical stage. The first apparent sign is gradual weight loss. Congruent with the weight loss the manure consistency becomes more fluid. The clinical symptoms can be seen for months with intermittent times of amelioration. Later, clinically affected animals become increasingly lethargic, weak and emaciated. "Water-hose" or "pipe stream" diarrhea, hypoproteinemia and intermandibular edema (bottle jaw) characterize the advanced stage of the disease (Tiwari et al. 2006).
Morphological changes in JD include chronic inflammation involving all layers of the intestinal wall (transmural involvement), thickening of involved segments, with narrowing of the lumen, linear ulceration of the mucosa, and a submucosal edema with elevation of the surviving mucosa, producing a characteristic cobblestone appearance.

According to Clarke (Clarke 1997), the histopathology of JD ranges from the more common pluribacillary or lepromatous form to the less common paucibacillary or

paucimicrobial tuberculoid form comparable with leprosy in humans. Due to the histopathological features of CD which closely resemble those found in animals with the paucibacillary form of JD, it has been suggested that the two diseases may share the same etiology (Grant 2003, Collins et al. 2000; Greenstein 2003; Moss et al. 1992).

43.5 Diagnosis of Bovine Paratuberculosis

For microbiological diagnosis of MAP direct and indirect methods are distinguished. While the first detect the agent or parts of the agent itself, the latter are based on specific immune responses that occur after contact with the pathogen (exposure, infection, immunization).

43.5.1 Direct Detection of MAP in Feces or Organs

The gold standard is the cultural detection of the pathogen. It is considered as 100 % specific, but its sensitivity is not higher than 35 %. For improving sensitivity the culture can be combined with PCR. The main disadvantage of MAP culture is the slow growth of the pathogen with an incubation period of up to 18 weeks in primary culture (Whan et al. 2005).

However, a positive result is proof for the presence of living reproductive MAP in the animal.

The fecal culture is able to detect most animals in advanced stages of the disease, but identifies only a few animals in early stages of infection. It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100 %.

Furthermore, the intermittent shedding of bacteria contributes also to the relative low sensitivity cited above.

In case of massive shedding MAP can be detected directly in the feces by light microscopy after Ziehl-Neelsen staining. PCR assays using IS*900*, *f57* or other target sequences are used for the direct detection of MAP. Since 2012 a commercial real-time PCR assay is authorized in Germany for the detection of MAP in feces (Life Technologies S.A.S.; Carlsbad, CA, USA).

43.5.2 Indirect Detection of MAP in Serum or Milk

The serological tests commonly used for detection of a humoral response to paratuberculosis in cattle are complement fixation (CF), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID).

At present the ELISA is the most sensitive and specific test for serum antibodies to *M. avium* ssp. *paratuberculosis* in cattle. According to the OIE manual (http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/) the ELISA detects in total about 30–40 % cattle identified as infected by fecal culture.

In Germany several different commercial ELISA assays are licensed for the detection of paratuberculosis specific antibodies (http://www.fli.bund.de/en/startseite/institutes/institute-of-molecular-pathogenesis/reference-laboratories/nrl-for-paratuberculosis.html). The sensitivity of these methods, however, is also very limited. As cited above: for an early diagnosis of paratuberculosis in young animals all detection methods are not effective enough. Because of the limited sensitivity antibody tests are useful to determine the health state in a herd, but are not an adequate tool for determining the disease in individual animals.

There are two tests for detection of a cell-mediated immunity: the gamma interferon release assay for blood samples and the skin test for delayed-type hypersensitivity. According to the OIE manual both have limited value in the field at present and further research is needed with respect to the interpretation criteria.

In summary, at present there are various diagnostic methods suitable for the detection of animals with JD in a progressive stage. But in spite of intense research they still have considerable sensitivity problems. A reliable diagnosis of the early stages of the disease is still missing. The fact that MAP is shed intermittently in feces and milk lead to post mortem screening attempts like testing of lymph nodes in the slaughterhouse (Munster et al. 2011), as mesenteric lymph nodes are generally accepted to be the main locus of MAP colonization.

Nevertheless, according to the guidelines for handling of paratuberculosis in ruminant populations (Federal Gazette No. 28 from 10.02.2005, p. 2165) there is currently no approval for *intra vitam* tests that are appropriate for a comprehensive over-all monitoring in Germany.

After all, the time consuming culture is still presumed to be the most significant tool to identify MAP.

43.5.3 Therapy and Vaccination

It is not possible to cure MAP infected, clinically ill animals. Antibiotics, although showing growth inhibition *in vitro* do not lead to permanent treatment success in animals. A short-term alleviation of clinical symptoms by use of various antibiotics or antihistamines has been described in the literature. The MAP excretion, however, could not be prevented.

In Germany no vaccine is currently approved. However, in principle vaccinations are possible. The first vaccine was developed in 1926. Many authors describe the use of vaccines in terms of advantages and disadvantages.

The excretion of the pathogen and thus the spread of infection can be reduced by vaccination. Available vaccines, based on whole killed or live-attenuated bacteria

will not protect against infection and disease and the use of serological methods for animal disease control in vaccinated herds is no longer suitable. Furthermore interference with the tuberculin skin test used for the control of bovine tuberculosis is described (Bastida and Juste 2011; Juste 2012; Patton 2011; Rosseels and Huygen 2008).

43.6 Animal Disease Control Measures

43.6.1 Economic Importance of the Disease

The economic impacts on dairy and beef industry as a result of decreased milk production, increased susceptibility to other disease, progressive weight loss and veterinary costs are considerable.

Calculations from The Netherlands came to the conclusion that the damage for a farm with 100 dairy cows over a period of 20 years may cost 6800 € per year. In farms with clinically diseased animals the damage is estimated to be about 900 € per animal showing clinical symptoms (von Schloss 2000).

43.6.2 Control Measures in Other Countries

Since 1970 control measures are implemented in The Netherlands, but according to the OIE (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasetimelines), The Netherlands are not yet free of paratuberculosis infection.

Only Sweden is free of paratuberculosis, because the most restrictive measures were applied. Stocks with animals tested positive in cultural and/or serological tests were completely culled.

Rehabilitation programs were performed amongst others in the USA, the Czech Republic, Italy and some regions of Germany (voluntary programs). This demonstrates that the fight against paratuberculosis is a very complex and a long lasting approach (Köhler et al. 2003). Over 10 years may be necessary to achieve a JD-free status in a herd.

In Germany different sanitary programs are published for example the "Ratgeber Paratuberkulose" of the Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health) (http://www.bmelv.de/SharedDocs/Downloads/Landwirtschaft/Tier/Tiergesundheit/Tierseuchen/Paratuberkulose_Ratgeber.html) or hygiene measures of the German Farmers' Federation (DBV) (http://media.repro-mayr.de/61/101361.pdf).

In essence, they are all based on a strict hygiene management emphasizing on a paratuberculosis free young stock and the elimination of MAP positive animals.

43.7 MAP in Milk and Cheese

43.7.1 MAP in Milk

In principle there are two possibilities for MAP to get into raw milk: directly by the mammary gland (only in case of clinically infected animals leading to a suspected bacterial load of about two to eight bacteria per 50 ml milk), and indirectly through fecal contamination. Compared to the secretory route the fecal contamination is much more important, because significant amounts of MAP can enter into the milk from infected animals including also the subclinical infected population.

In the dairy industry different heating methods are used. The most important is the high-temperature, short-time (HTST) pasteurized milk process (72–75 °C, 15–30 s), with a market share of approximately 40 % (pasteurization) and the ultra-high temperature (UHT) process (≥ 135°C, ≥ 1 s), with a market share of about 60 % (UHT milk) (Protocol of the German Federal Institute for Risk Assessment, 2001: http://www.bfr.bund.de/cm/343/protokollmcpara.pdf). Dairy products are mainly produced on the bases of HTST milk.

To investigate the thermal resistance of MAP in milk, numerous experimental investigations were performed. After the UHT process, no surviving MAP were found in milk.

In HTST treated milk, experimentally infected with 10^2–10^3 colony forming units (CFU)/ml of milk, viable MAP could be detected. However, there was a reduction of microbial count to five orders of magnitude. This corresponds to a reduction of 99.999 %. After experimental contamination of milk with lower bacterial counts (10 CFU/ml) no viable pathogen could be detected (Publication of the Bavarian Health and Food Safety Agency, 2005: http://www.lgl.bayern.de/tiergesundheit/tierkrankheiten/bakterielle_pilzinfektionen/paratuberkulose/doc/paratuberkulose_ag.pdf).

For interpretation of these data it is important to consider which methodology of thermal inactivation was applied. In terms: was the experiment conducted under commercial-type pasteurizers with continuous turbulent flow for heat distribution comparable to the ones used in the dairy industry? This has been neglected in many studies and could be the cause of conflicting results (Chiodini and Hermon-Taylor 1993; Grant et al. 1996; Meylan et al. 1996; Robertson et al. 2012).

However, experiments performed in the Max Rubner Institute (Federal Research Institute of Nutrition and Food) in Germany between 2006 and 2010 demonstrated that the common pasteurization process leads to a profound reduction of MAP in milk. Due to the fact that MAP can only replicate in the host and that the dilution process starts immediately after its entry into the food chain, experts state that if at all only minimal amounts of viable MAP may reach the consumer by milk.

Furthermore there is no scientific evidence that CD is driven by the uptake of MAP by food (Study of Germany's national Public Health Institute (RKI), 2003: http://www.bfr.bund.de/cm/343/morbus_crohn_und_mycobacterium_avium_ssp_paratuberculosis_literaturstudie.pdf; Research report of the Federal Ministry of Food,

Agriculture and Consumer Protection: (https://openagrar.bmelv-forschung.de/servlets/MCRFileNodeServlet/Document_derivate_00000093/FoRep_2_2000_14-17_hammer.pdf).

43.7.2 MAP in Cheese

Only limited data are available on the occurrence of MAP in raw milk cheeses: experimental production of cheese from artificially contaminated raw milk showed that MAP are also greatly reduced during the ripening process (Donaghy et al. 2004; Spahr and Schafroth 2001; Sung and Collins 2000).

43.7.3 MAP in Retail Milk

To address the occurrence of MAP in HTST-treated milk from the market field, studies in the US (Ellingson et al. 2005), Great Britain (GB) (Food Standards Agency UK: http://www.foodstandards.gov.uk/multimedia/pdfs/milksurvey.pdf;) (Grant 2003) and Ireland (O'Reilly et al. 2004) were conducted. MAP in low numbers and frequency has been demonstrated in the US and UK: In UK 1.8 % of 567 tested packages contained MAP, in the US 2.8 % of 702 packs. In an Irish study, which was published in September 2004, no viable MAP was detected in any of the 357 examined milk cartons. In the Irish study 56 % of the investigated manufacturing firms treated the milk at least at 75 °C for 25 s. Poor stable hygiene is discussed as main reason for positive milk, which may explain regional differences in the studies.

43.8 Crohn's Disease

CD is a chronic relapsing inflammatory bowel disease (IBD) affecting the human gastrointestinal tract with preference for the terminal ileum and colon, but possible involvement of all its other parts (Baumgart and Sandborn 2012). All age groups and both genders can be affected, with the main peak for disease onset between the ages of 17–40 years (Thia et al. 2010). Patients are often febrile and suffer from painful abdominal cramps and chronic diarrhea; their stool is bloody or mucous. The Montreal classification was established to categorize the different phenotypic behavior of CD, with the majority of patients being affected by the non-stricturing non-penetrating phenotype, the remainder by the more aggressive stricturing or penetrating phenotypes which are characterized by gut stenoses or fistulas, respectively (Satsangi et al. 2006). The etiology of the disease is unknown, but genome wide association studies have identified an enormous amount of susceptibility loci (Franke et al. 2010; Jostins et al. 2012). Besides a genetic susceptibility to the disease, environmental factors also play a very important role in its development. A

whole lot of such lifestyle factors have been found to be associated with CD, like e.g., a reduction in women breastfeeding, air pollution, tobacco use, increased hygiene conditions, or the consumption of Western diet. Interestingly, CD is frequently triggered or exacerbated after an infectious gastroenteritis (Garcia Rodriguez et al. 2006). In an animal model, a virus infection was able to induce a CD-like phenotype in genetically susceptible individuals (Cadwell et al. 2010). It has been tempting to speculate from the first discovery of CD up to now, that a pathogen might be the etiological agent for development of CD.

43.8.1 Theories and Attempted Methods to Elucidate the Role of MAP in CD

As early as 1913 Thomas Kennedy Dalziel suggested that the histological characters of CD and JD are so similar as to justify the proposition that they might be the same, even though, as he also stated, the absence of the acid-fast bacillus would suggest a clear distinction (Dalziel 1989). Since then, the hypothesis of MAP as etiological agent for CD has been tried to be verified by multiple approaches, like immunohistochemistry, attempts to cultivate the bacterium, experiments to transmit CD to animals, by serological tests, molecular methods, and treatment programs of CD with antimycobacterial antibiotics.

43.8.2 Microbiological Approach

It is amazing that up to now no final conclusion about the role of MAP in CD could be drawn. The reason for this most probably lies in the elusive behavior of MAP in the human body. Histological immunostaining of resected granuloma tissue of CD patients against MAP antigen could up to now only confirm the primary statement of Dalziel that the acid-fast bacillus is absent, even though it has been performed repeatedly by several work groups (Van Kruiningen 2011). Cultural growth of MAP requires extremely long culture times, special selective cultural media and experienced lab personnel (Turenne et al. 2007). Additionally, because of the difficult handling procedures laboratory cross-contamination in mycobacterial laboratories is not rare, which should always be regarded when interpreting mycobacterial culture results (Van Kruiningen 2011). This might in some cases explain the discrepancies between different studies, where in a few smaller studies MAP could be detected in very small patient collectives, while it was undetectable in major study groups. MAP was detected in breast milk samples of two patients with CD, but not in five controls (Naser et al. 2000a), in another study it could be cultivated from four out of ten biopsies of children with early onset CD, but not in two ulcerative colitis or four non-IBD patients (Kirkwood et al. 2009). The group of Naser also reported the detection of MAP from blood samples of CD patients; however, there was also growth of MAP in samples from ulcerative colitis and healthy patients (Naser et al.

2004). Conversely, in a major culturing attempt on IBD samples from 191 patients, including 79 CD patients, from US and Denmark, not one of 3985 cultures had been positive (Collins et al. 2000).

43.8.3 Epidemiological Approach

If MAP infection would really be an etiological agent for CD, one would imagine that cattle farmers who are exposed to animals with JD are more often afflicted by CD than farmers with healthy animals. However, studies from the US and UK could not show a higher prevalence of CD in farmers handling JD animals (Jones et al. 2006; Qual et al. 2010).

43.8.4 Infection Experiments

Also all inoculation experiments of animals with triturated intestinal material from CD patients were up to now unsuccessful in induction of a JD-like infection, even though also susceptible animals, like goats or rabbits, were infected (Van Kruiningen 2011).

Because of these above-mentioned problems in detecting MAP in the tissue, but the striking similarity of the pathology of CD with JD, other hypotheses concerning the role of MAP in CD have arisen. A plausible explanation for the limited detectability of MAP could be that the pathogen is ingested at a young age and stimulates the immune system to create a chronic intestinal inflammatory disease but cannot consistently be found in the mucosa years after its ingestion (Bernstein et al. 2004). According to this hypothesis, the pathogen itself might only be present in very small numbers in the human body or might already have completely disappeared, but its former or residual presence would still be detectable by MAP-specific antibody measurements or nucleic acid tests (NAT).

43.8.5 Serological Approach

Therefore many studies compared antibody titers against MAP in serum of CD patients and controls. In this context different capture antigens in the assays with expectably different specificity for MAP antibodies were used (Van Kruiningen 2011). The results gained by these antibody measurements were often inconclusive; in some cases, there were significantly higher antibody responses for CD than for control patients (Collins et al. 2000; Naser et al. 2000b), in other studies no significant differences could be found (Bernstein et al. 2004; Cho et al. 1986; Kobayashi et al. 1988). This even sometimes occurred when using the identical test in different countries (Collins et al. 2000). As atypical mycobacteria comprise a huge group of

different species, many of them existing ubiquitously in the environment, e.g. in tap water or in the soil, and with probably also a considerable amount of still undiscovered species, it is tempting to speculate that cross-reactivity of MAP-"specific" antibodies towards antigens of different atypical mycobacteria is highly probable (Osterstock et al. 2007). Therefore, it is very difficult to interpret the meaning of positive serological test results regarding their specificity for previous or present MAP colonization, infection or immunity.

43.8.6 Molecular Biological Approach

Comparably, an enormous amount of studies has been published with focus on MAP-DNA detection in intestinal tissue, granulomas, or peripheral blood mononuclear cells (PBMC). In most studies, the main target gene was the IS element IS*900*, which has been postulated to be specific for MAP. Most of these studies were based on classical PCR or nested PCR on biopsies, buffy coat of blood or PBMC (Kirkwood et al. 2009; Naser et al. 2004; Autschbach et al. 2005; Bernstein et al. 2003; Bull et al. 2003; Juste et al. 2009; Suenaga et al. 1995), one study used laser-microdissected tissue (Ryan et al. 2002), another performed in-situ labeling on paraffin-embedded tissues (Hulten et al. 2001). Also—similar to the serological analyses- there were many conflicting results between these studies, with some studies showing a significantly higher presence of IS*900* DNA in CD samples and others showing no difference to controls. However, a meta-analysis of NAT-based techniques detected an association between MAP and CD (Abubakar et al. 2008). Given that the IS*900* element would be exclusively present in MAP and therefore indeed highly specific, then one possible explanation might be a variable sensitivity of the applied NAT in the study-specific analyzed tissues. Alternatively, as NAT are extremely sensitive methods, sample contamination could be a major issue in some of these studies. Even the water used during endoscopy for taking the biopsies could be contaminated with mycobacteria (Van Kruiningen 2011). Moreover, it becomes increasingly clear that IS*900* is indeed also present in other mycobacteria species; it could be detected by NAT in mycobacterial isolates related to *M. cookii*, *M. scrofulaceum*, and the *M. avium-intracellulare* complex (Van Kruiningen 2011; Cousins et al. 1999; Englund et al. 2002; Motiwala et al. 2004), which questions the specificity of the IS*900*-PCR method and heightens the contamination risk.

43.8.7 Therapeutical Approach

As MAP obviously is an easily detectable pathogen in cattle with JD, but very elusive and highly debated in humans, other researchers tried to demonstrate the implication of MAP or mycobacteria in CD indirectly by studying the effect of antimycobacterial antibiotic therapy on CD patients. By this approach the ultimate proof for the role of MAP in CD can never be achieved, as the application of antibiotics can

influence many factors other than MAP, e.g., by their antiphlogistic effects on the immune system or maybe by elimination of other unknown causative bacteria, or even sometimes by their curative effect on an existing but unrecognized gut tuberculosis. Even more problematic for this approach is the actual possibility that MAP disease might not even be curable by antibiotics, as it is the case with its hypothetical animal counterpart JD. Besides different uncontrolled clinical studies, a five-year follow-up study and a randomized prospective, parallel, placebo-controlled, double-blind study have tried to find a benefit of antimycobacterial therapy for CD patients (Borody et al. 2002; Selby et al. 2007; Thomas et al. 1998) with variable success. Selby's study had the statistically best set-up and found no evidence for a beneficial effect over a time period of 2 years; however, the study was criticized for low dosage and suboptimal application of the antibiotics.

43.9 Summary

In conclusion, the scientific community is still divided into supporters and critics for a possible role of MAP in CD. While the supporters bring forward the detection of MAP in blood, intestine and even milk samples of human CD patients, the opponents still miss the final convincing evidence that the presence of MAP in the human body can really initiate CD. One of the main arguments of critics still stands fast, namely that in all of recorded medical and veterinary medical history there are no published accounts of the transmission of JD to humans (Van Kruiningen et al. 2011). Novel data from a meta-analysis of CD genome-wide association scans detected considerable overlap between susceptibility loci for IBD and mycobacterial infection (Jostins et al. 2012), which could indeed mean that mycobacteria are involved in the development of IBD or that the associated changes in these susceptibility loci necessary for mycobacterial control somehow auto-induce autoimmune processes with subsequent pathogen-free granuloma formation. Therefore, the theory still remains open for controversial discussion and future studies will hopefully lead to a final conclusion. At the moment, however, there is not enough evidence to convincingly demonstrate that MAP is a possible etiological agent for CD.

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Chapter 44
Clostridia: *Clostridium botulinum* and *Clostridium difficile*: Ubiquitous Spore-Forming Bacteria as New Zoonotic Pathogens?

Ute Messelhäußer

Abstract *Clostridium* spp., anaerobic spore-forming bacteria, are not commonly counted among the classical zoonotic agents. They are regularly found in the environment, e. g. in soil, dust or sludge, and also in the intestine of healthy humans and animals without causing any symptoms. Botulism is a typical intoxication, normally caused by the ingestion of contaminated food or animal feeding stuff or in rare cases by the bacterial contamination of deep wounds. Based on the classical foodborne transmission route, the European Union classified botulism as a zoonotic intoxication. During the last few years some scientists thought to have indications for a zoonotic transmission of an infective form of botulism, the so-called "chronic botulism". However, currently there is no resilient evidence for this theory. An infection with *Clostridium difficile* can result in heavy diarrhea in humans and is supposed to infect also animals, but the zoonotic potential of these bacteria, e. g. the transmission via food or the classical way of zoonotic infection, the direct transmission between human and animal, is discussed controversially. However, scientific data suggest that the zoonotic potential of the organism might not be very high and the contact with contaminated food or animals, carrying *C. difficile*, is not a decisive risk factor for an infection.

44.1 *Clostridium botulinum*

44.1.1 Clostridium botulinum—The Organism and the Toxin

Clostridium (C.) botulinum is a strictly anaerobic, spore-forming, rod-shaped, gram-positive bacterium, which mainly occurs in the soil of both terrestrial and aquatic environments. Spores of the organism can also be found in the intestine of healthy humans and animals. The species *C. botulinum* comprises four genetically and physiologically different groups of bacteria, which are connected through

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their ability to produce Botulinum Neurotoxin (BoNT). Therefore the scientific world does not speak of "*Clostridium botulinum*" any longer, but of "Botulinum-Neurotoxin-producing clostridia". BoNT exists in seven different antigenic types (BoNT A to G), of which BoNT E and F are known to be toxic for humans, BoNT C and D mainly for different animals and BoNT A and B for both animals and humans. The toxicity of BoNT G is largely unknown so far. BoNT A, B, E and F are simply constructed polypeptides, consisting of a 100 kDa "heavy" chain and a 50 kDa "light" chain linked through a disulfide bond. The light chain is a zinc-containing endopeptidase which blocks the release of acetylcholine. This is the reason of the atonic paralysis, the classical symptom of BoNT intoxication. BoNT is one of the most lethal biological toxins; the lethal dosis for a healthy adult person is as low as 0.1 to 1 µg.

The formation of neurotoxin occurs under strict anaerobic conditions after germination of the spores in the last growth phase and the toxin is released after lysis of the bacterium. Growth and toxin production can take place in nearly every food matrix or tissue excepting those with a low pH < 4.6 or low a_w < 0.94 (EFSA 2005). The temperature for optimal growth and toxin formation differs between the groups of BoNT-producing clostridia, proteolytic strains are normally not able to grow and produce toxin below 10 °C. In contrast non-proteolytic strains, also called "psychotrophic" strains can grow at temperatures as low as 3 °C (Graham et al. 1997). BoNT belongs to the group of heat-labile toxins, normal cooking temperatures can destroy (pre)formed toxin, but not the spores. Spores are safely eliminated using a temperature-time-combination of 3 min at 121 °C for proteolytic strains or of 10 min at 90 °C for non-proteolytic strains (ACMSF 1992).

44.1.2 The Four "Classical" Forms of Botulism

44.1.2.1 Foodborne Botulism

Foodborne botulism is the most common form of botulism in animals and humans. Illness is caused by the ingestion of food or animal feeding stuff like silage containing (pre)formed toxin. In both animals and humans the classical symptoms of botulism are atonic paralysis of extremities and at an advanced state respiratory paralysis. In humans the intoxication manifests itself after 12 to 48 h first with the classical symptoms of a foodborne illness like nausea, vomiting and acute abdominal pain followed by double vision, dysphagia and atonic paralysis of extremities. Without early treatment (antitoxin therapy and artificial respiration, sometimes over months) botulism mostly leads to death. In cattle and horses the first symptoms are an atonic paralysis of the mastication muscles with a prolapse of the tongue also followed by atonic paralysis of the extremities. Death occurs as a result of respiratory paralysis. Poultry and waterfowl show after an incubation time of a few hours to three days paralysis of the wings, weakness and death within 10 days, waterfowl drown with clear sensorium. Foodborne botulism can also be acquired by minks and ferrets. Dogs, cats and pigs are much less susceptible for the toxin; foodborne botulism is rarely described in these animals.

Sources of human foodborne botulism are insufficiently heated, mostly homemade, canned foods, such as meat, sausages and vegetables or cured meat products with low salt concentrations. In animals foodborne botulism mainly occurs in cases of deficient ensiling or of contamination of hay or silage with cadavers. Via cadavers the spores and the toxin can also reach the environment. Waterfowl is mostly poisoned in the summer months by ingestion of toxin-containing slurry.

44.1.2.2 Wound Botulism

In rare cases spores of BoNT-producing clostridia can contaminate deep wounds, e. g. through drug abuse or iatrogenic contamination. The spores can germinate under anaerobic conditions, grow and produce the toxin directly in the tissue. Four to 14 days after the entry of the spores the affected person shows the classical symptoms of botulism as described above.

44.1.2.3 Infant Botulism (Infant Intestinal Toxemia Botulism)

Infant botulism is a toxicoinfection in children under 6 months, in rare cases also of older children up to 12 months of age. Spores of BoNT-producing clostridia are ingested with contaminated food, e. g. honey or infant formula, or from the environment. Due to a less developed and diversified intestinal microflora the spores can colonize and germinate in the large intestine of the infant. The produced toxin leads to the classical botulism symptoms, which are preceded at first by constipation, weakness and poor feeding. Later a typical atonical paralysis occurs. Fortunately, today the toxiconifection leads only in very rare cases to the death of the patient. Normally, treated patients recover fully from infant botulism (Arnon 1995).

44.1.2.4 Infective Botulism (Adult and Toddler Intestinal Toxemia Botulism)

Approximately a dozen cases of intestinal toxemia botulism have been described in toddlers and adults so far. In most cases there are predisposing factors, which affect the intestinal microflora negatively and allow spores of BoNT-producing clostridia to colonize an adult intestine. Possible factors can be a longstanding treatment with broad-spectrum antibiotics and illnesses, which affect the intestinal anatomy and physiology, e. g. inflammatory bowel diseases, vagotomy or considerable decreased intestinal motility (Arnon 1995). The patients develop the classical symptoms of botulism described above. Importantly, intestinal toxemia botulism without the typical atonic paralysis has not been described in the scientific literature so far.

In the veterinary literature a similar disease is reported, the equine grass sickness. The disease results in a partial or complete paralysis of the gastrointestinal tract and normally ends with the death of the horse (Hunter et al. 1999). However, a link between equine grass sickness and BoNT has not been scientifically verified until now.

44.1.2.5 Occurrence of Classical Botulism Cases

In summary, botulism is a very rare disease in humans and animals. In Europe the confirmed case rate of human botulism constantly ranges between 0.02 and 0.03 cases per 100.000 population. The most frequent reported form of botulism in Europe is foodborne botulism; infant botulism and wound botulism is only seen in single cases (ECDC 2013) The mainly foodborne transmission route is one of the reasons why the European Union classified botulism as a bacterial zoonotic intoxication which has to be monitored in the different member states depending on the epidemiological situation according to Annex I B no. 1 of Directive 2003/99/EC (EU 2003).

In the USA CDC reported for the year 2011 140 confirmed human botulism cases, of which 104 (73 %) cases were due to infant botulism (CDC 2013).

44.1.3 "Chronic or Visceral Botulism"—A New, Emerging Zoonotic Disease?

Since the mid-1990s some veterinary research groups postulate - besides the classical forms of botulism- the existence of a chronic disease entity in cattle called "chronic" or "visceral botulism" (Böhnel et al. 2001). This disease is said to originate from the ingestion of spores of BoNT-producing clostridia from the feed or the environment. The path of infection and intoxication is compared by these research groups to the adult intestinal toxemia botulism; however, the chronic form of botulism would not lead to an acute and life-threatening intoxication, but to a chronic intake of minimal dose rates of BoNT. This chronic contamination is hypothesized to cause unspecific symptoms in the individual cow, e. g. apathy, indigestion, edemas, chronic lameness and sudden unexpected death (Böhnel et al. 2001). On farms affected by this so-called chronic botulism the groups claim that the milk yield and the fertility rate decrease significantly. Medical symptoms simultaneously and coincidentally found among a few farmers on affected farms such as weakness of the extremities and dysfunction of vegetative nerve system, e. g. pupillary motoric, were claimed by one medical research group to be linked to a chronic intake of BoNT (Dressler and Saberi 2009). The story of a new, emerging zoonotic disease, the "chronic" or "visceral botulism", which affects farmers, veterinarians and cattle and can be transmitted, alternating between humans and animals, was born. Until today, more than ten years later, scientifically sound evidence for the existence of a chronic form of botulism and also for the transmission of spores or vegetative cells of BoNT-producing clostridia between humans and animals in terms of a zoonosis is still missing. The symptoms of chronic botulism are not really correlated to the presence of toxin; the link is still postulated by only a few research groups in Germany (Krüger et al. 2012; Rodloff and Krüger 2012). In other countries all over the world, cases of human or animal chronic botulism have not been described so far. Research in the next few years will show if there might be a possible link

between the symptoms described on the different farms and BoNT-producing clostridia, maybe in form of a multifactorial disease. At the moment there is no reliable scientific indication for botulism as a zoonotic emerging infection transmissible between humans and animals.

44.2 *Clostridium difficile*

44.2.1 Clostridium difficile—The Causative Agent of Nosocomial Pseudomembranous Colitis in Humans

Clostridium (C.) difficile is also a gram-positive, rod-sharped, strictly anaerobic bacterium, which was first described by Hall and O'Toole in 1935 as part of the microbial intestinal flora of infants (Hall and O'Toole 1935). *C. difficile* is counted to the group of facultative pathogenic bacteria, because it can be detected also in the intestine of healthy persons without any characteristic symptoms. Predominantly the organism causes nosocomial disease linked to antibiotic treatment; however there are also community-acquired *C. difficile* infections without any correlation to hospitalization (CDC 2005). The pathogenicity of *C. difficile* is based on the ability to produce different toxins, an enterotoxin A, a cytotoxin B and in some strains (ribotype 027) also a binary toxin, which is linked to more severe symptoms (Schroeder 2005).

Usually a *C. difficile*-associated diarrhea (CDAD) develops after an intensive therapy with broad spectrum antibiotics, which destroy the normal microbial intestinal flora. In such a situation *C. difficile* spores can colonize the intestine, germinate and overgrow the remaining normal microbial flora. The severity of the symptoms depends on the immune status of the patient and can vary from mild diarrhea to a pseudomembranous colitis with a case fatality rate of 6 to 30 %. Most patients who develop severe symptoms like a pseudomembranous colitis get infected in the hospital, only a small part carries the causative organism already before hospitalization (Schroeder 2005). Hygienic deficiencies in hospitals and healthcare facilities can also cause transmission of *C. difficile* between patients and lead to outbreaks with a major number of deaths, especially if hypervirulant strains of increasing importance like such as ribotype 027 are involved.

44.2.2 Clostridium difficile in Animals

The relevance of *C. difficile* as a pathogenic agent in animals is not really clarified until now. It is known that *C. difficile* can be isolated from feces of foals, pigs, dogs or cats with clinical signs of diarrhea (Schneeberg et al. 2013; Songer 2010), but also from healthy animals (Rodriguez-Palacios et al. 2013). *C. difficile* can also be detected in different processed and non-processed food of animal origin

(de Boer et al. 2011). These are the reasons, why scientists all over the world discuss intensively the zoonotic potential of the organism.

44.2.3 Community-Acquired C. difficile Infections—A Sign of its Zoonotic Potential?

Against the background of an increasing number of community-acquired *C. difficile* infections in healthy people without the classical risk factors for CDAD (e. g. old age, prolonged hospital stay, a recent surgical procedure) a zoonotic potential of *C. difficile* is widely discussed. Numerous publications indicate the presence of *C. difficile* in nearly every food matrix of animal origin; *C. difficile* is also detected in feces of healthy pets, e. g. dogs and cats. However, recent studies have identified the exposition to antimicrobial drugs as a major risk factor also for symptomatic community-acquired *C. difficile* infections. Active sentinel surveillance in the State of New York over a period of 6 month showed for example that 76 % of patients with a community-acquired CDAD had used antimicrobial drugs within 12 weeks before infection. Strong evidence that certain kinds of food or animal exposure were involved was not found (Dumyati et al. 2012). Similar studies were also done in other countries and the results indicate that even though the same ribotypes can be found in animals, food and humans, the contact with healthy animals shedding *C. difficile* or with contaminated food is not a major risk factor for a community acquired *C. difficile* infection (Hensgens et al. 2012). Based on the ubiquitous nature of this spore-performing organism it seems to be impossible to prevent humans getting in contact with *C. difficile*. In intensive care or healthcare units with critically ill persons it should only be tried to prevent the spreading of CDAD between the patients and to contain the dominance of these bacteria by antibiotic treatment. In summary, the zoonotic potential of *C. difficile* is not really clear and for normal immunocompetent persons the contact with the organism should not lead to a symptomatic infection.

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Part X
Economic and Ecological Aspects of Zoonoses

Chapter 45
Economic Aspects of Zoonoses: Impact of Zoonoses on the Food Industry

Impact of Zoonoses on the Food Industry

Sara Babo Martins, Barbara Häsler and Jonathan Rushton

Abstract The increase of complexity of livestock production and the associated value chains has led to changes in the food systems that feed us, which in turn carry new challenges from zoonotic diseases in particular their impact, and the costs of surveillance, control and prevention. Direct losses to the animal and public health sectors, connected mainly to value losses due to morbidity and mortality in humans and animals, and indirect losses, such as the economic cost caused by the reaction to disease and the limiting of its negative effects, all contribute to this negative impact. Its full assessment can be challenging, but economic tools and frameworks can be used to estimate zoonotic disease impact and the economic efficiency of possible technical ways of dealing with these diseases. In this chapter, we review the impact of zoonoses across sectors, also in the context of an increasingly complex value chain, address the economic concepts behind the balance between losses due to direct costs of disease and expenditures in reaction to disease presence, and identify possible economic tools and frameworks to assess the impact of zoonoses and interventions.

45.1 Introduction

The initial successes in control of infectious diseases in animals were the control and eradication of rinderpest and contagious bovine pleuropneumonia in the late 1800s (Fisher 1998). The distribution of livestock diseases began to change more rapidly in the 1960s and 1970s as European and North American countries and Japan began to make serious inroads into the control of a range of both transboundary

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and endemic animal diseases[1]. This was achieved through significant investments in human skills, building on previous investments in veterinary organisations, education and infrastructure from the mid nineteenth century onwards. In the human health side attempts to manage diseases that have affected societies appear to have been in place in Europe from around the 1500s through the problems caused by the plague (Harrison 2004). The period of enlightenment expanded the knowledge of causal agents of disease (Hays 2009) and there were major breakthroughs in the control of diseases such as smallpox with the use of vaccines leading ultimately to the official eradication of the disease in 1979 (Harrison 2004). In terms of overall service delivery much work has gone into the management of human health and most countries recognise the need for coordinated health services, ultimately culminating in the recognition of the need for the International Health Regulations in 2005. These initiatives have been prompted largely by disease shocks and the core contagious diseases that spread between humans.

It is only recently that attention has been drawn towards the insidious nature of zoonoses in the food system. In part this relates to investments in rigorous and organised programmes that use epidemiology and economics research to assist in decision-making. Similar to the disease and response processes of the major diseases in animal and humans, major foodborne diseases have created a need to invest and investigate further the impact of zoonoses in the food system. Of greatest note in recent times are:

- the emergence of new zoonotic diseases such as bovine spongiform encephalopathy (BSE) and highly pathogenic avian influenza H5N1; and
- the impact of foodborne pathogens such as *E.coli* O157, salmonella and campylobacter.

New problems relating to zoonotic pathogens mean that the major impacts of livestock diseases are related to human health and welfare (Shaw 2009). In developed countries these impacts can be enormous, dwarfing the production losses due to disease. Some of these diseases also create large impacts due to food scares and trade restrictions, and implications in other larger aspects of the rural economy.

45.2 Context

The increasing complexity of livestock production and their associated value chains had a background of changes in the political and institutional environment. From the late 1940s to the 1970s, state action was accepted to be important in economic and agricultural development. However, during the 1980s there was a change in thinking that stressed the market as a way to organise economic activity, supported by a small or even a minimal role for the state.

[1] It is recognised that some diseases were controlled well before this period (see Fisher 1980), however major breakthroughs were mainly made in the period suggested.

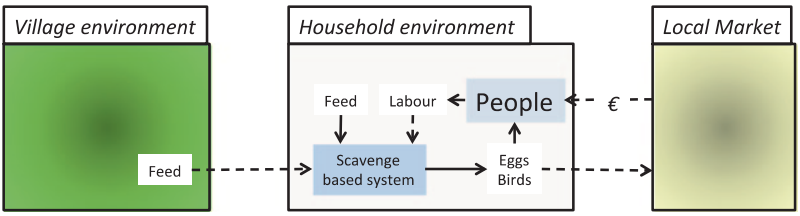

Fig. 45.1 Scavenge based poultry system

Different elements of these changes in public policy have had varying impacts on zoonoses management and public health in general. Until the 1980s animal health was seen as a public good and involved government services. However, pressure on public budgets and an increasing role for the private sector saw privatisation of many former governmental veterinary services and activities. The public health systems suffered less, but zoonoses control often fell between weakened veterinary systems and relatively powerful human health institutions and agencies with a focus on diseases and health problems that affect largely people.

In addition to the dynamics of the changes in the public funding of health systems, the food systems have evolved creating more difficulties in the management of pathogens.

45.2.1 Changing Context of the Food Systems that Feed us

Over a period of around 200 years the world has moved from relatively simple livestock value chains to increasingly complex ones. The pace of this change has accelerated in the livestock sector with the increasing use of intensive systems where animals are housed and fed and no longer allowed to scavenge or graze. In the case of poultry this has been particularly dramatic, scavenge based systems for poultry production were common and used mainly local resources, with the household consumption of products and the infrequent sale to local markets (see Fig. 45.1). The presence of these systems in a local environment allows people to observe the health of the animals, and it is also probable that they will see the slaughter of the animals. The information on production and processing allows people to make individual judgements of how best to prepare and eat their food.

In the complex food value chains, that are now dominant in many parts of the world, primary production has complex relationships with consumers through processing and marketing companies. The links in the chain are maintained by middle men, transport companies and finance groups. Where the value chains become integrated, i.e. owned and controlled by one company, the middlemen disappear. In addition, consumer demands have become more sophisticated for processed food and food with zero risk of foodborne diseases (Rushton 2009). For the intensive poultry systems that are increasingly dominant in the provision of meat across the world,

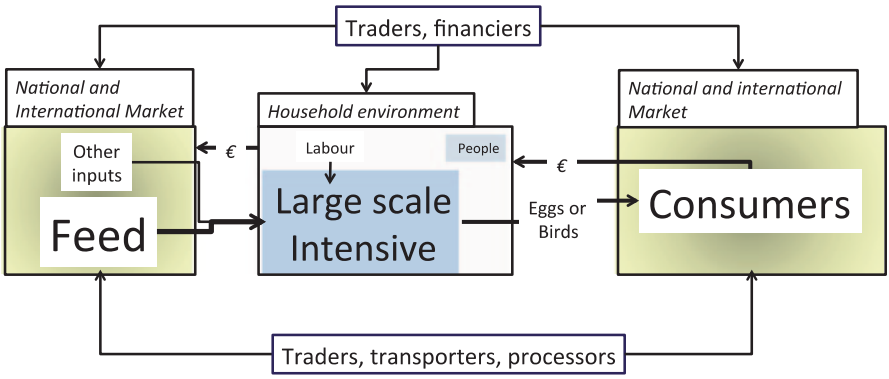

Fig. 45.2 A schematic diagram of the complex intensive poultry food systems. (Rushton 2009)

the system is global. Day-old birds and feed are produced in different parts of the world, the fattening of birds and their slaughter take place some distance from the families that eat the meat (see Fig. 45.2). These systems do not allow an individual to gain much insight in or information on the origin of the meat eaten, the consumer has to be given that information with labelling and/or systems of trust on the quality, which includes food safety, of the product.

The changes in the livestock food systems have not been gradual, there have been jumps associated with major technological changes. These have come at different points in the food system. For example the use of vaccines against Gumboro disease in poultry has allowed birds to be housed in large numbers and changed the way they have been fattened. The ability to freeze meat allows the slaughter and transport of carcasses from distant places to points of consumption. These changes have also been stimulated by the social and economic change in society, the growth in human populations and the greater proportion of people found in urban rather than rural areas. The urban based people require food to be produced and processed for them and the tendency across the world is for food systems to become more technologically and economic efficient in order to provide food that is relatively cheap in comparison to other goods in society. Overall this has lead to fewer and fewer people to been involved in primary production; for livestock this means that animals are managed in larger herds or flocks and more animals per person handling them. The economic activity of the food system is found in the processing, catering and retailing areas where food preparation that would have traditionally been done in the home is now carried out by third parties. The shape of the food system in terms of numbers of people is therefore like a pyramid with a large number of consumers being supplied with animal source proteins produced by fewer and fewer farmers, who are selling to complex food processing, retailing and catering companies (see Fig. 45.3).

In the UK it is estimated that only 336,000 people (0.6% of the working popualtion) are involved in primary agriculture, yet the food processing, retailing and

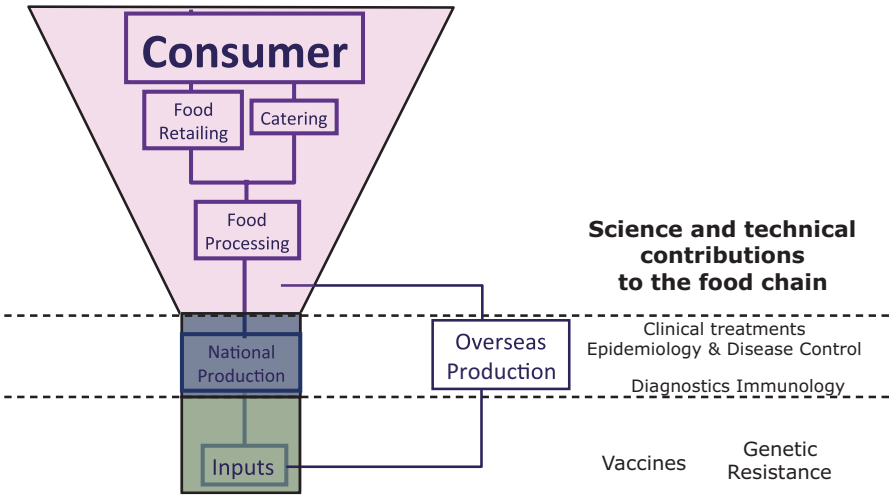

Fig. 45.3 A schematic diagram of the food systems. (Rushton et al. 2012)

catering part of the food system employs around 12 % of the working population and generates 7–8 % of the Gross Domestic Product (GDP). This food system relies on imports from other countries and feeds on a daily basis for more than 60 million people. In short, never have so many been fed by so few.

The scale of the current food systems and the additional complexity given by the increased number of people and links involved in the supply chain implies that the presence of infectious diseases in these system, including zoonoses, impact a wider number and variety of stakeholders, sectors and people in the chain, bringing also new challenges to disease control.

45.2.2 Classification of Zoonoses

Zoonoses are diseases and infections that are naturally transmitted between vertebrate animals and humans (WHO 2013). This group of diseases is generally classified according to the zoonotic agent itself, the degree of human-to-human transmissibility or through the route of transmission of the disease from animals to humans (Karesh et al. 2012; Lloyd-Smith et al. 2009). The classification according to the route of transmission groups zoonoses into diseases mainly transmitted through food or foodborne zoonoses, such as salmonellosis or campylobacteriosis (EFSA 2013); diseases mainly transmitted to humans through other means than food, i.e. by vectors, or through direct contact or close proximity with infected animals, such as avian influenza and Q fever (EFSA 2013), and zoonoses with multiple routes of transmission, including through food and direct contact, such as brucellosis.

While foodborne zoonoses are particularly important in terms of direct impact for the food industry, indirect costs of zoonoses transmitted by other means than

food can also produce significant impact through indirect costs to the food industry (Rassy and Smith 2012; McLeod et al. n. d.).

45.2.3 Measuring Impact

The impact of zoonotic diseases is challenging to assess. The greater complexity of measuring the impact of zoonoses relates to the frequent underreporting of these diseases and to the multiple sources of losses that are associated with zoonoses. In general they cause problems in different sectors such as the public health, the animal health and the environment, with no particular sector being affected strongly, but with an overall large impact (Grace et al. 2012; Keusch et al. 2009).

Direct and indirect costs in the animal sector, on the public health sector, on the environment and at their interfaces, and both on the public and on the private levels, contribute to the overall impact of zoonotic diseases. However, current estimates and tools available to measure these economic impacts tend to be specific to each sector. While some estimates have incorporated aspects of animal costs and human costs (Bennett and IJpelaar 2005; Choudhury et al. 2013), the different components of impact are usually considered individually rather than in an integrated way (Grace et al. 2012), meaning that the full extension of impact associated with zoonotic disease is seldom captured. While the distinction between direct and indirect costs might not be always straightforward, the following sections will reflect in more detail on the direct and indirect costs of zoonoses.

a. Direct costs of zoonoses

- **Direct costs of zoonoses on the animal sector**

Direct costs of zoonoses associated with livestock production relate to losses due to morbidity and mortality in food producing animals, which lead to a decrease in the expected output due to disease, treatment costs, and the increase in expenditures on non-veterinary resources (Bennett and IJpelaar 2005).

Morbidity and mortality of animals due to zoonotic diseases carry also other losses related to the wider social, cultural and economic value of animals and their health and welfare to people. Animals can be a source of income and employment, provide draught power and organic fertilizer, serve as means of transport, particularly in low income settings, serve as guardians of livestock and households and companions to people (Meslin 2006; Torgerson 2013), and also serve as a form of insurance and status.

Generally, morbidity and mortality effects in animals are measurable in monetary units. Frequently used tools include cost-analysis, incorporating the losses mentioned above (Bennett and IJpelaar 2005), and decision tree analysis, which might model different scenarios of production (Carabin et al. 2005; Choudhury et al. 2013). A key constraint identified in these analyses relates to the scarcity of disease-related data available to populate the economic models developed (Bennett and IJpelaar 2005). Other methods used include the elicitation of expert opinions to provide a rank of zoonoses with highest impact and to inform prioritization (Grace et al. 2012).

– **Direct costs of zoonoses on the Public Health sector**

Likewise, direct costs of zoonotic disease for the public health sector are also related to mortality and morbidity in the human population.

Currently, the most widely used methodology in the estimation of the impact of disease in the human population is through the use of disability-adjusted life years (DALYs), the method currently used to measure the global burden of disease estimates regularly produced by the World Health Organization (WHO 2004). DALYs provide a summary measure that allows to combine both mortality and morbidity effects and indicators, by presenting the present value of future years lost due to premature death or due to poor health (Grace et al. 2012). Its use allows the comparison of results across a multitude of illnesses and populations. However, DALYs only estimate the disutility to the individual of being ill, and do not capture medical costs of illness to the individual or society (Grace et al. 2012). Other summary measures available include the quality-adjusted life-years (QALY) that while calculated using a different methodology, also combine mortality and morbidity effects. QALYs have been more used on the evaluation of medical interventions as a health outcome for cost-utility analysis (Gold et al. 2012).

Recent estimates of the burden of zoonotic disease indicate that zoonoses contribute to 26% of the DALYs lost to infectious disease and 10% of the total DALYs lost in low income countries, respectively, and to 1% of DALYs lost to infectious disease and to 0.02% of the total disease burden in high income countries (Grace et al. 2012). Particularly in low income countries, this burden is amplified by losses associated with malnutrition, also closely linked to zoonotic disease (Grace et al. 2012).

For foodborne diseases, estimates of the impact on public health, produced by the UK Food Standards Agency, are available for the UK (Table 45.1).

– **Costs of zoonoses on the environment**

Zoonotic diseases impact on the ecosystems by threatening the diversity of species as well as the number of connections between them, affecting the stability of the ecosystems (Grace et al. 2012). Linked to the complexity associated with valuation of the ecosystems and its measurement, tools for the assessment of the costs of zoonotic disease to the ecosystems are less developed, but comprehensive assessment frameworks considering the various burdens of zoonotic disease in human, animal and ecosystem health of zoonotic diseases have been proposed under the Ecohealth approach (Grace et al. 2012).

b. **Indirect costs of zoonoses**

Indirect costs of disease are those derived from the human reaction to the presence of or to the risk of presence of a disease (Meslin et al. 2000). These include costs associated with disease prevention and establishment of control measures, market impacts, such as trade and travel regulations and restrictions, and those derived from consumer reaction and changes in consumer confidence in the food chain. They can further include costs accrued from unproductive downtime on farms and organizational costs related to disease response from the veterinary, food and public health services (Keusch et al. 2009).

Table 45.1 Foodborne pathogen problems in the UK in 2007. (Jones 2009)

Pathogens	Total Cost		Death		Hospitalisation		Cases	
	%	Rank	%	Rank	%	Rank	%	Rank
Campylobacter spp.	33	1	18	3	83	1	36	1
Salmonella non-typhoidal	15	2	21	2	6	2	3	5
VTEC O157	4	5	5	5	2	5	0	10
Listeria monocytogenes	12	3	37	1	2	3	0	11
Norovirus	6	4	7	4	1	8	19	2
Total	£ 1520 million		443		18,906		925,766	

– **Prevention, control and surveillance**

To avoid the losses due to disease mentioned above, another economic cost accrues from efforts to prevent, control or eradicate a disease. Examples illustrating this are provided by control and prevention measures carried out to tackle some zoonotic events, such as BSE (see Box 1), Highly Pathogenic Avian Influenza (HPAI, see Box 2) or bovine tuberculosis, where control and surveillance activities are estimated to cost £ 74 to 99 million per year in the UK (Torgerson and Torgerson 2008).

Box 1. Economic Impact of Zoonoses—*The BSE Example*

The impact of the BSE crisis has been the subject of numerous assessments in several of the countries affected (reviewed by Keusch et al. 2009; The World Bank 2010). The disease has led to important direct and indirect losses. Before the link between BSE in cattle to Creutzfeldt–Jakob disease (CJD) in humans was established, the disease losses were mainly linked to the loss in value of infected carcasses and to the costs of establishing control measures, namely the disposal of specified risk material (Atkinson 2007). The establishment of the link between the two diseases meant the additional emergence of important indirect costs linked to market impacts, including the contraction in domestic demand of beef products, loss of export markets and a fall of beef cattle prices (Atkinson 2007). Beef consumption and domestic prices of cattle, beef and beef products were reported to have substantial drop-offs in many countries (Probst et al. 2013; Serra 2011).

To respond to the crisis, a series of preventive and control measures have been implemented in the countries affected. In the UK, the estimation of the costs associated with control and regulation compliance in the years of 1996/1997 indicated additional costs of around £ 25–50 million, to which added costs associated with slaughtering and culling of £ 220 million (Atkinson 2007). In Germany, the total costs associated with prevention, control and surveillance of BSE were estimated to range between € 1.8 and 2.0 billion, with approximately 54% of the costs being incurred by the extension of the feed ban for animal protein to all farmed livestock and 21% to active surveillance

(Probst et al. 2013). An analysis of the cost-effectiveness of these measures in the Netherlands indicated a cost of € 4.3–17.7 million, from 2002 to 2005, per life year saved (Benedictus et al. 2009).

Exports were also strongly impacted by trade restrictions put in place. For the UK, the export market of beef and trade in live calves, worth £ 670 million in 1995, was lost with the trade ban imposed in 1996 (Atkinson 2007). These losses associated to a loss in output from beef and related products, were offset, in some instances, by general equilibrium outcomes (Wigle et al. 2007). In Europe, for example, poultry, pork, vegetables and milk products benefited from the BSE crisis (Benedictus et al. 2009). In May 2003 the Canadian government reported the detection of a single case of BSE in a national cattle population of nearly 13.5 million animals (FAO 2006). This led to 40 countries banning the import of a large range of live animals and livestock products from Canada. Mitura and De Piétro (2004) estimated that the impact of the international livestock trade ban was significant for Canada. In 2003, Canadian farm cash receipts from cattle and calves were estimated at $ 5.2 billion, a sharp drop of $ 2.5 billion (33 %) from 2002. At farm-level it was estimated that on average a family farm with an unincorporated beef unit would have lost $ 20,000. The more wide-ranging impact of the trade ban was the movement of cattle from Mexico to the USA to fill the demand for store cattle that would have come from Canada. While this has created a positive impact for cattle producers in Mexico, it has meant that beef prices in Mexico have risen affecting Mexican consumers, and that the USA is potentially importing animals from areas with low tuberculosis status (Ayala and Velasco 2005). Later in 2003 the USA also declared the discovery of a single animal with BSE (out of an estimated cattle population of 96 million, FAO 2006) which led to 53 countries banning the imports of American beef. Coffey et al. (2005) estimated that the losses associated with this trade ban were between $ 3.2 and 4.7 billion. These authors also estimated that BSE has had considerable costs in terms of increased needs for surveillance at a farm and slaughterhouse level. On an international level the use of BSE cases in Canada and USA as a trade barrier can have a negative impact on disease reporting. Livestock exporting countries, whose economies have far less ability to absorb rapid changes in export demand for livestock products, are unlikely to report minor levels of animal disease where there is a risk of exaggerated and rapid trade bans.

Estimations of losses on the public health sector are not as readily available in the literature as for the animal health sector and food chain. An estimate from the UK points to running costs associated to staff time and expert committees of 2.5 million GBP, in 1988–1996. The cost of patient care for CJD patients were considered too uncertain to be quantified (The BSE Inquiry Report 2000).

- **Market impacts: from trade and travel regulations to consumer reaction and product recalls**

The indirect impact of zoonoses, associated with losses due to changes in consumption patterns and/or trade and travel restrictions imposed as a consequence of zoonotic disease outbreaks, can be extensive and last beyond the duration of an outbreak, depending on the risk perception of trade partners and tourists.

The example of the Severe acute respiratory syndrome (SARS) epidemic demonstrated the economic impacts of travel restrictions affecting tourism and its contribution to reductions in GDP growth in some countries, through reductions on service exports, particularly tourism related exports (Xiaoqin Fan 2003; Keusch et al. 2009). For China, Taiwan, Hong Kong and Singapore, this impact has been estimated to be $ 13 million, or 0.5–1.1 % of the GDP (Keusch et al. 2009). The 1994 plague outbreak in India also led to economic losses due to internal and external travel restrictions (Keusch et al. 2009). More recently, the H1N1 emergence in Mexico resulted in a reduction of almost a million overseas visitors and losses of around $ 2.8 billion for the country's economy (Rassy and Smith 2012).

Losses due to changes in consumption patterns and trade disruption can also be highly visible. In the European Union, and following the BSE food scare, more than half of the consumers were fearful of BSE and unsure as to whether beef meat was safe for consumption (Bánáti 2011). This led to important losses explained in further detail in Box 1. While BSE has led to a significant drop in consumption and a market shock, other scares have been relatively short-lived with consumption returning back to previous levels, not causing an overall loss, but a disruption in the food system which may make some businesses go bankrupt, leading to loss of employment and /or restructuring of the industry.

Product recalls have become an important component of the food system as the value chains have lengthened. Of the recent major food recall incidents, many have been related to zoonotic pathogens and contaminants, such as *E.coli* in beef in the USA in 1997 (with a cost estimate of $ 25 million on recalls plus significant loss in company value), salmonella on vegetables and vegetable products such as peanut butter (cost of incidents ranging between $ 70 and 250 million) and in Europe some incidences around the detection of dioxins in meat (for Ireland, the cost estimate of the recall of pork meat was € 200 million in 2008).

Box 2. Economic Impact of Zoonoses—Highly Pathogenic Avian Influenza in Chile

In 2002, Chile reported for the first time an outbreak of HPAI. This was also the first isolation of avian influenza virus in South America (Lupiani and Reddy 2009). The poultry industry in the country produced, at that time, 400,000 tons per year of fresh poultry meat, with exports, mainly to Mexico and the European Union, following an increasing trend ($ 69 million in 2001, $ 44 million in 2002 and $ 72 million in 2003) (Orozco 2005). Following the outbreak, the access to export markets was closed (Orozco 2005).

As a response, the national authorities put in place a series of mitigation measures, including stamping out of affected farms, setting up of surveillance, pre-diagnosis quarantine, depopulation, movement control, and increased biosecurity (Max et al. 2007) and to regain access to the export markets as soon as possible, a zooning strategy was adopted (Orozco 2005; Max et al. 2007). The culling of the two infected farms to stamp out the disease resulted in the destruction of 560,000 breeding chicken and turkeys (Rojas 2009). Within 7 months, Chile was declared free from HPAI (Max et al. 2007).
The initial financial impact of the disease was calculated by Verdugo et al. (2006, cited Rojas 2009) to be $ 31.7 million, with costs largely borne by the private sector. An economic impact assessment of HPAI in Chile estimated that over the whole economy losses reached $ 250 million (Wright 2004)

In Europe, the costs of recall are strongly related to the traceability systems in place. If traceability is poor, then large amounts of product have to be recalled that are likely to be unaffected by the problem.

45.2.4 Balancing Direct Costs of Disease and Reaction

In summary, zoonotic disease in food systems creates two sources of economic cost. First, there are the value losses caused by the negative effects of disease itself when people are ill or die leading to reduced productivity, lost income, and people suffering, including family members or friends who take on the role as unpaid caregivers. Similar losses occur in animal populations when animals are ill or die leading to production losses and negative impacts on animal welfare. The second economic cost is caused by people's reaction to disease to limit the negative effects of hazards. The additional resource costs incurred in the attempt to offset the output value losses have a positive opportunity cost, as they might have been used to generate other outputs which are valued elsewhere in the economy.

In assessing the rationality of any resource-using decision, the key criterion is whether the value of outputs consequently recovered is at least sufficient to cover the additional resource costs. As a basic rule, to achieve the maximum net benefit, the total impact of disease should be as small as possible. In other words, a balance should be found between the level of disease and the associated losses as well as the expenditures made to tackle the disease in animal and human populations. Frequently, the economic cost of disease is presented as an aggregate figure that comprises both the impact caused by the disease and the impact caused by the reaction to the disease (Rushton 2013). To find the right balance between losses and expenditures, it is important to understand the relationship between the two.

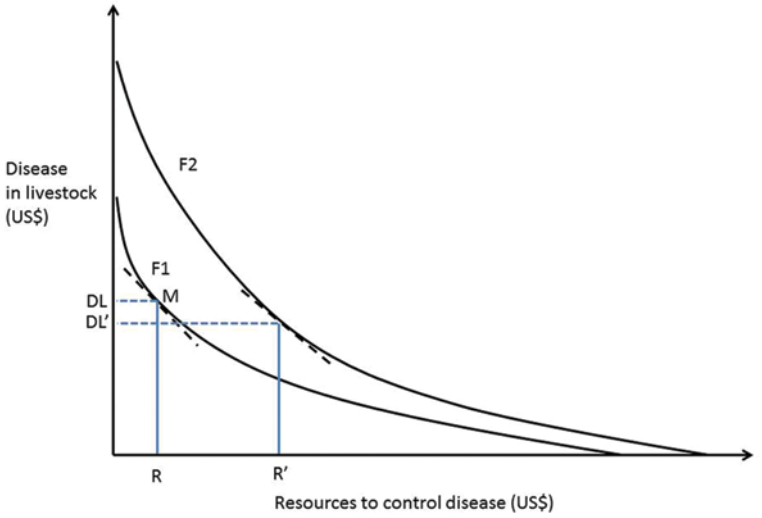

Fig. 45.4 The relationship between output losses and control expenditures with externalities. (Anon 2012) (Note: F1 Is the situation where externalities are not taken into account whilst F2 takes externalities into account)

At societal level, the cost of resources committed to mitigation should at least be compensated by the value of the resulting recovered outputs for a policy to be acceptable; and, ideally, the net benefits to society should be maximised (Mclnerney et al. 1992). Because the "external costs" of zoonotic disease are commonly not taken into account in markets, outcomes result that are not socially optimal, i.e. social welfare is lower than it might otherwise be.

Consequently, the loss-expenditure curve can be expanded to take into account the negative externality resulting from zoonotic disease, as illustrated in a recent OECD report (Anon 2012). In Fig. 45.4 the economically optimal balance of expenditure and disease losses is shown at point "M", where the level of resource expenditure is R and disease loss is DL. When the value of disease losses is higher because of the externalities due to zoonotic disease, the loss-expenditure curve might be as shown by curve F2. In such a case, the optimum level of resource expenditure should be at a higher level (R'), and disease losses at a lower level (DL'). In this example, the curve cuts the x-axis which means that the disease could be eradicated (although it would not be the least-cost economic optimum).

Identification of the economic optimum for zoonotic disease mitigation depends on the price ratio between mitigation resources and mitigation outcome (= loss avoidance). The optimal level can be influenced by two key factors, namely technical changes that lead to an improvement of disease control measures with no price changes and changes in the value of losses avoided relative to the costs of mitigation (Howe et al. 2013). Consequently, when the value of unpriced negative externalities are taken into account, the total losses increase, which impacts on the optimal point of mitigation as illustrated in Fig. 45.4. To be able to add the negative externalities,

their value must be added to the total losses. Often their value cannot be directly deducted from prices in the conventional sense; instead they must be valued using indirect estimation methods, such as the DALY approach described above. Similarly, the value people place on the welfare of their companion and recreational animals can be estimated for example from how much they spend on veterinary services and medicines. Freedom from fear of infection is more problematical to quantify, but may be revealed by changes in people's normal consumption behaviour.

Disease mitigation programmes at national level generally comprise two important components, namely surveillance and intervention. Surveillance is defined as the "systematic, continuous or repeated, measurement, collection, collation, analysis, interpretation and timely dissemination of animal health and welfare related data from defined populations, essential for describing health hazard occurrence and to contribute to the planning, implementation, and evaluation of risk mitigation measures" (based on Hoinville et al. 2013). In other words, surveillance provides information for interventions directed at loss avoidance. Surveillance and intervention are often inextricably linked; they can be technical substitutes or complements (Howe et al. 2013). The above conceptual basis to define the optimal level for mitigation can then be extended to identify the optimal combination of surveillance and intervention, namely the point where the marginal cost of surveillance and intervention (combined at least cost) equal the marginal benefit (= loss avoidance) on the expansion path (Howe et al. 2013). The expansion path (Fig. 45.5) links all identical tangent points on iso-mitigation curves (along such a curve, the losses avoided are the same for all combinations of surveillance and intervention) with the gradient corresponding to the price ratio for surveillance and intervention. Each point of tangency is a least-cost combination for surveillance and intervention for the given price ratio. If the iso-cost line represents a budget constraint, the surveillance and intervention resources cannot be increased above this level. In Fig. 45.5, the optimal location on budget line BB' would be at H for A3 avoided losses, although H' would also be a feasible, albeit inferior use of resources. If adding externalities such as negative effects of disease in people, the point H would no longer be optimal and a case would need to be made to increase investment to be able to reach point K (Häsler 2011; Howe et al. 2013) (45.5).

In the examples used so far, it was assumed that there would be diminishing marginal benefits (= loss avoidance) with increasing expenditures for disease mitigation. In theory, it may well be the case that there are disease mitigation programmes, where this is different. For example, it may be that no loss avoidance is achieved at low levels of expenditure (marginal benefit = zero), but that expenditures need to reach a certain threshold for an effect to become noticeable, or that there are increasing marginal benefits at first followed by diminishing marginal benefits (Tisdell 1995). Such scenarios obviously would lead to different optimal points of disease mitigation, as illustrated by Tisdell (1995).

Importantly, only by understanding these relationships it is possible to make the best use of the resources available for disease mitigation and reduce the economic cost of zoonotic disease to a minimum. Empirical data allow demonstrating whether a disease mitigation programme is subject to increasing or diminishing returns. Only

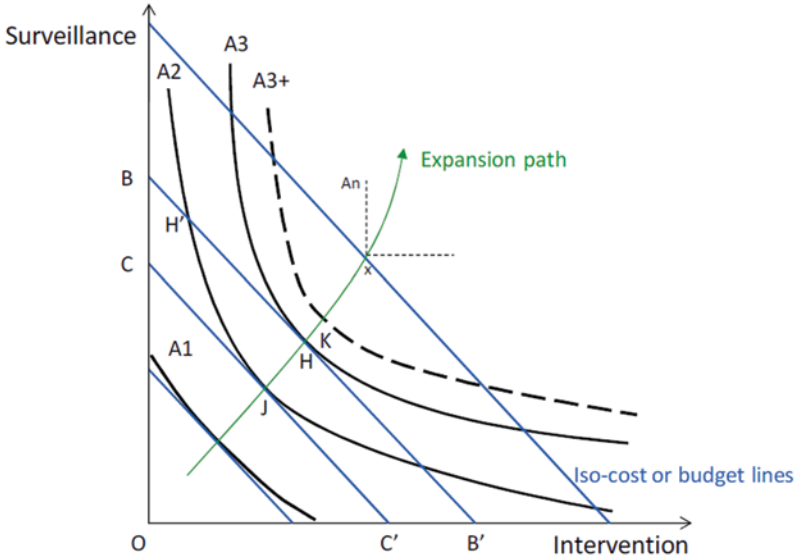

Fig. 45.5 An iso-mitigation map with an expansion path to identify the optimal balance between loss avoidance, surveillance and intervention resources. A1, A2, A3, A3+ and An are distinct levels of losses avoided. J, H, K and x mark least-cost combinations of surveillance and intervention corresponding to distinct levels of loss avoidance. (Häsler 2011)

with information about the relationships described, economic analysis of zoonotic disease mitigation can move towards an optimal level of investments; otherwise the analysis is limited to the assessment of acceptability criteria.

45.3 Economic Tools to Assess the Impact of Zoonoses and Interventions

When there is a choice to be made about the allocation of resources to zoonotic disease mitigation, it should be ensured that "the value of what is gained from an activity outweighs the value of what is being sacrificed' (Williams 1983). In other words, for an investment to be justifiable the benefits should outweigh the costs; an acceptability criterion commonly found in cost-benefit analyses that compare the costs and benefits of potential programmes in monetary terms.

In a nutshell, cost-benefit analyses compare the total discounted benefits of a project in monetary units with its total discounted costs in monetary units and recommend the implementation of the project if the benefits exceed the costs. It includes the definition of the useful life of the project, the estimate in physical units of benefits (losses avoided) and costs (mitigation resources used), translation of the physical units into economic values, the conversion of future values into present values by discounting, and finally the calculation of the net benefit (net present

value = total discounted costs—total discounted benefits). Because the benefit-cost ratios as choice criteria can be misleading when multiple options are compared, some authors recommended to use the net present value instead of the benefit-cost ratio (McInerney 1991; Tisdell 1995; Howe et al. 2013). In any assessment of this nature it is therefore important to review all measures of project worth, namely the net present value, the benefit-cost ratio, and internal rate of return and timing permitting an examination of the estimation of the benefit and cost streams.

A similar basic principle is seen in cost-effectiveness analysis, which is commonly used to assess human health interventions. To date, it has rarely been applied to animal health decision-making problems (Babo Martins and Rushton 2014). Cost-effectiveness analysis aims to assess the effect of a programme in non-monetary units in relation to its cost. In human health economics the effect often refers to the avoidance of illness or death, but the outcome of any objective can—in theory—be measured in various technical terms, for example reduction of CO_2 emissions or detection of cases of disease. However, it is important that the value of the effect in question reflects a (non-monetary or monetary) benefit. A special case to cost-effectiveness analysis is the least-cost analysis where two or more programmes or projects achieve the same effect. The economic assessment then aims to identify the cheaper option.

Importantly, all these fundamental concepts explained above only provide an estimation of the economic efficiency (optimal balance, acceptable combination, least-cost option) of technical feasible ways of dealing with zoonotic disease. Some authors have proposed frameworks that take into account wider issues, including risk management options and the understanding of the factors impacting those options (Narrod et al. 2012; Grace et al. 2012). It is the case of the framework proposed by Narrod et al., consisting of a modified risk analysis framework to enhance reduction of zoonotic disease burden, including the analysis outputs of animal and human disease transmission models and economic impact models (Narrod et al. 2012).

Independent of which type of economic efficiency criterion is to be assessed, there is a need to attribute value to the different impacts mentioned above. Value can be defined as the importance, worth, or usefulness people attach to something, reflecting their judgment of what is important in life, and which in economic activity determines the choices made about the use of scarce resources (pers. comm. Keith Howe). A short summary is provided here:

- When dealing with morbidity and mortality in animals due to zoonotic disease, units of physical losses (e.g. number of animals that died, number of animals suffering from milk loss, rate of milk loss) can be combined with data on production (e.g. milk yield) and/or market prices (e.g. market value of the animal, milk price). Illustrations of how such losses can be calculated can for example be found in Mcdermott et al. (2013) and Herrera et al. (2008). An overview of various techniques suitable to assess the economics of animal disease can be found in Rushton et al. (1999).
- The negative impact of disease on human health is commonly measured using DALYs or QALYs, (see details above). Further, the cost-of-illness (COI) methodology attempts to measure all the costs associated with a particular disease or

condition. It takes into account the direct costs of illness (e.g. visit to a physician, laboratory or treatment expenses), indirect costs (e.g. value of lost productivity from time off work due to illness), and intangible costs (e.g. psychological costs due to pain or suffering).

- Impacts on the ecosystem can either lead to production losses (e.g. when pollinators such as bees are affected by disease in turn causing harvest losses) or the reduction of ecosystem services to people. For example, if an area cannot be accessed anymore, because of a risk of zoonotic disease transmission, a value loss occurs to people in that they cannot use the area as they usually would. There are several techniques available in environmental economics to attribute a value to the personal use of ecosystem services. Techniques based on revealed willingness-to-pay (WTP) for an ecosystem service focus on prices people are willing to pay to make use of the environmental good; they include hedonic pricing, market price method, productivity method, and travel cost method (King and Mazzotta 2013). The travel cost method is used to explain the underlying principle of the revealed WTP approach: it is a popular method to estimate economic use values associated with ecosystems people use for their recreation, such as recreational fishing or hiking. The method can be used to assess changes in access costs for or quality of a site or elimination or addition of a site. The fundamental principle of this method is that the travel costs people need to cover to visit the site reflect its price and the number of trips in relation to the price allows estimating a demand curve as it is done for goods that have a market price. An example of an application of this method can be seen in the paper by Shrestha et al. (2002) who measured the value of recreational fishing in the Pantanal in Brazil. When dealing with ecosystem services that are not reflected in market prices, people cannot reveal what they are willing to pay for those services. In such cases, methods are used that ask people directly what they are willing to pay for having a service or what they are willing to accept in terms of compensation for losing a service.
- The willingness-to-pay or contingent valuation approach is also used in animal health to attribute a value to food safety or animal welfare. The approach is based on the assumption that the maximum amount an individual is willing to pay for a commodity reflects the value it has for this person. Miller and Unnevehr (2001) for example conducted a household survey to investigate consumers' WTP for enhanced pork meat safety. They found that roughly 80 % of the consumers were willing to pay at least $ 0.10 more per pound of certified safer pork. Another study used a hypothetical market scenario in the UK to investigate people's WTP to support legislation to phase out the use of battery cages in egg production in the EU by 2005 (Bennett 1998). The survey showed a mean WTP of £ 0.43 increase in price per dozen eggs (with a market price of around £ 1.40 per dozen), purporting to indicate the value respondents attributed to improved animal welfare. The main criticism of the WTP is that it does not give reliable valuations, since the choices are often hypothetical and people tend to overestimate their willingness to pay. Another drawback is that non-users of a good or service might find it difficult to attribute a value to it because their knowledge of it is very limited.

- Various outbreaks of zoonotic disease in the past showed that food safety scares can alarm consumers to the extent that they reject consuming certain products. Such a reduction in demand caused by loss in consumer confidence can lead to a reduction in market prices and is reflected in a drop in quantity demanded and the associated changes in market prices. Similarly, zoonotic disease outbreaks can lead to movement or export bans, which impacts on the quantity of product placed on the market and consequently affects prices. The value of these changes in supply and demand can be assessed using surplus models to measure producer, processor and consumers surplus changes, as illustrated for avian influenza outbreaks in South East Asia and the United States, respectively (Hall et al. 2006; Paarlberg et al. 2007) and BSE (Weerahewa et al. 2007).
- In countries where there are no substitution possibilities for foods potentially contaminated with pathogens, consumers may put themselves at risk for foodborne disease when consuming the food due to the lack of alternatives or they may increase the risk of malnutrition by excluding nutritious foods from their diets. Foodborne disease leading to diarrhoea reduces appetite and/or the absorption of nutrients in the body. Malnutrition on the other hand impairs the immune response and predisposes affected people to infection thereby creating a vicious cycle of malnutrition and infectious disease. Zoonotic disease can impact on nutrition in three ways: (1) through the avoidance of food because of food scares (as described above); (2) market shocks that impact on the availability of livestock and fish products (due to reduced production and/or control measures such as movement or export bans), or (3) the direct impact of infectious pathogens on health. There is a wide range of different methods available to assess food and nutrition security indicators; a review has recently been published by Pangaribowo et al. (2013).

These valuation approaches can be used to measure the impact of zoonotic disease, and/or form part of an economic assessment to inform resource allocation for zoonotic disease mitigation.

45.4 Conclusions

Economics provides several tools and frameworks that can be used in the measurement of the impacts and to inform resource allocation for zoonotic disease mitigation. However, the measurement of the impact of zoonotic diseases presents several challenges. The impacts of zoonoses are felt in multiple sectors, in multiple links of increasingly complex value chains, and in the interface between sectors and jurisdictions. These multiple dimensions are generally complex to capture as whole, with many studies focusing on impact assessments per sector and failing to capture the entire realm of effects. In addition to the need of addressing impacts in a wider context, aspects such as unused human, financial and capital capacity in the food system, reduced confidence in the market place, particularly for export markers and important lags created in terms of confidence and investment—often taking years to

recover capacities, skills, markets—should be incorporated in future, more refined, impact assessments of zoonoses. Data availability and quality to populate assessments represents a further challenge. Underreporting of cases of zoonotic disease, particularly in low-income settings, are for example believed to be contributing to the underestimation of the burden of these diseases in the human and animal populations (Keusch et al. 2009; DFID 2011), therefore influencing disease mitigation decisions and contributing to the perpetuation of their impact.

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Chapter 46
Zoonoses of Poverty: Measuring and Managing the Multiple Burdens of Zoonoses and Poverty

Delia Grace

Abstract The greatest health burden of zoonoses falls on poor people in developing countries. Poor people have greater exposure to zoonoses through livestock keeping; living in agricultural communities; greater exposure to peri-domestic and wild animals; and less access to clean water. Although their consumption of animal source products is low, the quality of these products is poor.

In addition to human health burdens, zoonoses reduce livestock productivity and are important barriers to trade in livestock products, as well as causing more difficult to quantify harms such as spillover to wildlife populations. These additional impacts also contribute to poverty in developing countries.

Assessing the impacts of zoonoses helps prioritize management. Among the most important zoonoses in developing countries are gastro-intestinal pathogens, leptospirosis, cysticercosis, brucellosis, tuberculosis and rabies.

Poverty, and investment and innovation is urgently needed to tackle zoonoses in developing countries where they currently impose massive burdens on human, animal and ecosystem health.

46.1 Poverty and Zoonoses

46.1.1 Zoonoses

Zoonoses are diseases transmissible between animals (livestock and wildlife) and humans. Around 60 % of all human diseases and around 75 % of emerging infectious diseases are zoonotic (Taylor et al. 2001; Woolhouse et al. 2005). The first section of the chapter provides an overview of zoonoses and poverty and the second section provides more details on zoonoses, which have been identified as priorities in poor countries.

Zoonoses threaten human health in different ways.

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Many diseases which are now transmitted between people originated in animals: they can be considered ‘souvenirs’ rather than hierlooms” (meaning they have been acquired rather than passed down from ancestors (Grace and McDermott 2011). These diseases originally came from animals: hence the name ‘old zoonoses’. They include HIV-AIDS, influenza, malaria, measles and dengue. Their current order of magnitude is about similar to that of all endemic zoonoses (almost all of this due to HIV-AIDS) and their impacts are mostly felt in poor people.

- Endemic zoonoses are continually present to a greater or lesser degree in certain populations. Examples are cysticercosis, brucellosis, bovine tuberculosis, leptospirosis and food-borne zoonoses. They are common in poor populations but often neglected by the international donor, standard setting, and research communities.
- Outbreak or epidemic zoonoses typically occur intermittently. Examples are anthrax, rabies, Rift Valley fever, and leishmaniasis. Epidemic zoonoses may occur as outbreaks in naïve populations or when triggered by events such as climate changes, flooding, waning immunity or concomitant hunger or disease. Their overall health impact is much less than endemic zoonoses but because they can ‘shock’ systems they reduce resilience.
- Emerging zoonoses are those that newly appear in a population or have existed previously but are now rapidly increasing in incidence or geographical range. They are relatively rare, around 300 events in the last 70 years (Jones et al. 2008). Most are of minimal impact. Donors and decision-makers are often concerned about emerging diseases, whose impacts on poor farmers are orders of magnitude less than the impacts of endemic zoonoses. However, the potential impact (e.g. a new HIV-AIDS) is at least of similar magnitude to endemic zoonoses. Zoonoses do not just threaten human health; they also reduce livestock productivity and impose costs on domestic and international trade (McDermott et al. 2013). Some zoonoses impose a large morbidity and mortality burden in the livestock host (e.g. brucellosis). In other cases, disease impacts in animals may be few (e.g. types of salmonellosis in chicken) but there may be high costs for the food sector from inspection and intermittent food recalls. A study in Mongolia incorporated both private and public costs of human illness and costs borne by the livestock sector (Roth et al. 2003). This found that only 10 % of the benefits of control accrued to the public sector. Other costs of zoonoses may be more difficult to quantify, for example the cost of lost biodiversity when animal diseases spill over to wildlife (as when rabies in Ethiopia threatens survival of the Ethipian wolf, the rarest canid in the world).

46.1.2 Poverty Context

Poverty can be defined as a pronounced deprivation in wellbeing. No single indicator exists to measure all dimensions of poverty simultaneously, however, internationally comparable metrics, such as the US$ 1 a day ($ 1.25), are useful for spatial and temporal comparisons. Estimates of poverty are probably reasonably accurate.

The proportion of people living in poverty (<$ 1.25 per day[1]) dropped by half between 1990 and 2010, but 1.3 billion people still live on less than $ 1.25 a day and around 2.5 billion on less than two dollars a day (World Bank 2013).

In the past three decades, dramatic drops in poverty are mainly due to development in China: in Africa and South Asia numbers of people in poverty or stable or increasing. More than 75% of the people living in poverty live in nine countries and 80% of poor people in 12 countries. In terms of intensity of poverty, 17 countries have more than 50% of the population living on less than $ 1.25 per day. Whereas in 1990, nine tenths of the poor lived in poor countries, presently three quarters live in middle-income countries (mainly India, China and Brazil) (WorldBank 2013).

46.1.3 Poverty and Zoonoses

On a national scale, the greatest burden of zoonotic disease is borne by poor countries. This is evident in the major evaluation of human disease, the Global Burden of Disease Study (GBD). This was commissioned by the World Bank in 1991 to provide a comprehensive assessment of the burden of 107 diseases and injuries and ten selected risk factors for the world. Burden of disease is calculated using the disability-adjusted life year (DALY). This time-based measure combines years of life lost due to premature mortality and years of life lost due to time lived in states of less than full health. The GBD represents the most authoritative source of information on human illness. The study was updated in 2004 and again in 2010 (GBD 2012).

There are some challenges in using the GBD to assess zoonoses. Firstly, zoonoses (especially in poor countries) are widely unreported, and under-reporting is relatively greater for zoonoses than for non-zoonotic diseases of comparable prevalence (Schelling et al. 2007). Secondly, several zoonoses with considerable burdens are not included in the GBD. For example rabies, echinococcosis, cysticercosis, leptospirosis and brucellosis Thirdly, the GBD is organized around diseases and not pathogens so assumptions need to be made about the proportion of a disease that is due to zoonotic pathogens: for example, gastro-intestinal disease is one category in the GBD but the proportion of this due to zoonotic diseases is not recorded.

Even with these assumptions, it is clear that poor countries bear the greatest burdens of zoonotic disease. In rich countries only 4% of the total disease burden is from infectious diseases and around one two hundredth of the burden from zoonoses or diseases recently emerged from animals. Only one thousandth of the burden is from zoonotic disease. In poor countries, nearly 40% of the disease burden is attributable to infectious diseases, one twentieth of the total burden attributable to zoonoses or diseases emerged from animals and one fortieth of the total burden zoonotic (Grace et al. 2012a). We next discuss why people in poor countries may be more vulnerable to zoonotic infections, and why the most poor may be the most vulnerable.

[1] 2005 international prices

46.1.4 Poverty and Exposure to Zoonoses

There are several reasons why poor people might bear a greater burden of zoonoses. Firstly, they may be exposed to higher levels of pathogens, secondly they may be more vulnerable to disease, and thirdly they may be less able to prevent or treat zoonoses.

The most obvious way poor people are more at risk, is because they have much greater exposure to pathogens harbored by animals.

- Poor people are much more likely to keep or be in contact with animals than rich people. Recent estimates suggest nearly one billion people living on less than two dollars a day are dependent to some extent on livestock (Staal et al. 2009). Over 600 million are found in South Asia, mostly in India. Sub-Saharan Africa has over 300 million poor livestock keepers, concentrated in East and West Africa, with fewer in southern and central Africa. Poorer households are more likely to keep small ruminants and richer to keep large ruminants. Poultry keeping tends to be evenly distributed across wealth groups. However, poorer people are more likely to keep livestock in the house or close at hand and biosecurity and hygiene practices may be lower.
- Within poor countries, the poorest of the poor often keep less livestock. However, the less poor may keep more livestock than the relatively better off. A recent 12-country study supports this, finding that on average, around 68% of rural households in the bottom 40% as regards expenditure kept some farm animal compared to 65–58% of those in the top 40%; in urban areas 22–26% of the poor kept livestock, and 8–12% of the well-off (Pica-Ciamarra et al. 2011). Consumption of bushmeat is also much more common in developing countries.
- Because of poor sanitation and waste disposal there is greater contact with peridomestic animals such as bats and rodents in poor communities. There are also large numbers of community owned dogs and cats, which are often semi-scavenging though they may return to one household. Poor people are also more exposed to vectors, both through work in agriculture and because they are unable to afford bed nets and other prevention measures.
- Food borne transmission is a common route for exposure to zoonoses. Overall, livestock consumption is much lower in poor countries than in rich countries. The better off in poor countries have higher consumption of meat, milk, fish and eggs which are indirect routes for zoonoses transmission and so would be more at risk from infection through this route than poor people. Studies in developing countries typically find that food contains high levels of pathogens, often the majority of samples will exceed international standards (Grace et al. 2010). Water borne transmission is important from some zoonoses, notably *Cryptosporidium parvum, Eschericia coli* O157, *Salmonella*, *Campylobacter* and *Taenia solium* (Atwill et al. 2012). Because poverty is strongly linked to lack of access to clean water, the poor are more at risk by transmission through this pathway.

People in poor countries may also be more biologically vulnerable. Compared to rich countries, the proportion of infants, pregnant or lactating women and immune-

suppressed people is higher. Moreover, high levels of malnutrition and high exposure to toxins (especially mycotoxins) increase vulnerability to infection.

In addition, private and public health services are often under-resourced and under-performing. Common problems include: lack of diagnostic facilities; lack of trained personnel; lack of appropriate drugs; high prevalence of fake drugs; expenses associated with obtaining treatment.

In summary, poor people have greater exposure to zoonoses through livestock keeping; living in agricultural communities; greater exposure to peri-domestic and wild animals; and less access to clean water. Although their consumption of animal source products is low, the quality of these products is poor. Poor people also have less access to medical treatment, less ability to afford medical treatment, and the health systems in their communities are under-funded and often perform poorly.

Although poor people are at greater risk from zoonoses as a result of their lifestyles, animals and animal source food also bring many benefits. Perry and Grace (2009) identify three poverty reduction pathways mediated by livestock. The first pathway is reducing vulnerability by their important role in the household's asset base and in generating flows of benefits through draft power, food and, manure production and sales. The second pathway is specialization and intensification to increase the productivity of livestock, increasing household incomes and promoting accumulation of other assets. The third pathway is improving access to market opportunities (opening new markets, reducing transaction costs) to increase the profitability of livestock activities and create incentives to increase production and sales.

46.2 Zoonoses of Poverty

46.2.1 Most Important Zoonoses in Poor Countries

In order to understand zoonoses and poverty, information is needed on which zoonoses pose risk to the poor. One of the earliest attempts to prioritise zoonoses was conducted by the International Livestock Research Institute (ILRI) (Perry et al. 2002). In this, zoonoses were first listed and then ranked by veterinary practitioners and experts in sub-Saharan Africa, South Asia, and Southeast Asia. The most important zoonoses were considered to be brucellosis, cysticercosis, tuberculosis and sleeping sickness (in decreasing order of importance).

Recent years have seen several other prioritisation exercises for zoonoses and animal health, although most did not focus on poor countries. These were recently reviewed by the ENHanCE group (http://www.liv.ac.uk/enhance/). Most prioritizations use experts, criteria setting, and weighting to come up with lists. Zoonoses appearing in multiple listings according to the ENhanCE review, in declining order of number of appearances, were:

- First place: Salmonellosis
- Joint second place: Leptospirosis = rabies

- Joint fourth place: Campylobacteriosis = tuberculosis = West Nile virus infection = toxoplasmosis
- Joint eighth place: Listeriosis = anthrax = echinococcosis = *E. coli* infection = BSE = botulism
- Joint fourteenth place: Cryptosporidiosis = Japanese encephalitis = Q fever = Rift Valley fever = tetanus

Another prioritisation was conducted by ILRI in 2012 (Grace et al. 2012b). This first identified 56 priority zoonoses from five different credible sources. These 56 zoonoses were then ranked by five criteria: human mortality; human morbidity; livestock sector impacts; amenable to intervention; risk of emergence. This produced a short-listing of 12 zoonoses, which were responsible for an estimated 2.2 million human deaths and 2.4 billion cases of illness each year. Nine of the 13 top-ranked zoonoses were considered to have high impact on livestock, all have a wildlife interface, and all were amenable to agriculture-based interventions.

Disease	Wildlife interface	Deaths human annual	Affected humans annual
Gastrointestinal (zoonotic)	Important	1,500,000	2,333,000,000
Leptospirosis	Very important	123,000	1,700,000
Cysticercosis	Some importance	50,000	50,000,000
Tuberculosis (zoonotic)	Some importance	100,000	554,500
Rabies	Important	70,000	70,000
Leishmaniasis	Important	47,000	2,000,000
Brucellosis	Some importance	25,000	500,000
Echinococcosis	Important	18,000	300,000
Toxoplasmosis	Important	10,000	2,000,000
Q fever	Important	3,000	3,500,000
Trypanosomiasis (zoonotic)	Important	2,500	15,000
Anthrax	Some importance	1,250	11,000

(Grace et al. 2012)

46.2.2 Top 12 Zoonoses in Poor Countries

In the last section of the chapter, we consider some of the priority zoonoses in greater depth.

46.2.2.1 Gastrointestinal Zoonoses

In developing countries, typically 50–60% of diarrhoea cases are bacterial in origin. The most important of these pathogens are zoonotic, including *Salmonella*, toxigenic *Escherichia coli, Listeria,* and *Campylobacter* (Thorns 2000; Schlundt

et al. 2004). Other zoonoses of somewhat lesser importance include: *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium spp*. The bacterial pathogens responsible for acute gastrointestinal disease can also cause chronic effects (which include abortion, arthritis, developmental defects, paralysis, septiciaemia, and seizures) are of similar impact to acute diarrhoea (Lindsay 1997). In developing countries, less is known about the pathogens that cause diarrhoea, the prevalence of zoonotic diarrhoea, high-risk foods, or the cost of illness. However, it is likely that gastrointestinal disease is the single most important zoonotic disease in terms of impact on human health and economic loss. Many gastro-intestinal bacterial pathogens do not cause serious disease in the animal reservoir.

46.2.2.2 Leptospirosis

Leptospirosis is an infectious disease caused by pathogenic organisms belonging to the genus *Leptospira*. There are many serovars (>250) but typically only around 10–20 are found in a given region. Serovars can be grouped into 25 serogroups. Infected animals often become carriers. Wild animals are affected and can be important reservoir hosts. Leptospirosis may be an important cause of disease in animals in developing countries. Evidence is especially strong for swine production in Asia.

46.2.2.3 Cysticercosis

Cysticercosis is a systemic parasitic infestation caused by the pork tapeworm (*Taenia solium*). Humans are at risk not from consumption of pork with cysts but from consumption of tapeworm eggs shed by themselves or another human carrier. The disease persists in poor, pig-keeping communities where pigs have access to human faeces. Cysticercosis is believed to be the most common cause of adult onset epilepsy in poor, pig-keeping communities. The disease is not an important cause of illness in livestock but reduces the value of meat produced.

46.2.2.4 Tuberculosis

Worldwide and historically, most human tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (which probably gave rise to cattle TB). *M. bovis* is responsible for cattle tuberculosis. It affects a wide range of animals and is responsible for zoonotic TB in humans. In West Africa, *M. africanum* causes up to half of human tuberculosis. Atypical mycobacteria are found in the soil and environment and can infect both people and animals. Tuberculosis is an important cause of reduced productivity in livestock but is believed to make only a small contribution to the global TB burden (Müller et al. 2013). However, it can be a more serious problem in some high risk communities (especially cattle-keepers in Africa)

46.2.2.5 Rabies

Rabies is one of the most feared of all zoonoses. It is a disease of poor and vulnerable communities where deaths are often unreported: more than 95% of human deaths occur in Africa and Asia. Dogs are the most important reservoir, but many domestic and wild animals can be affected. Where rabies is common it can be of minor importance as a cause of mortality in livestock.

46.2.2.6 Leishmaniasis

Leishmaniasis is a protozoal infection transmitted by the bite of the sand fly. The most common forms are cutaneous leishmaniasis, which causes skin sores, and visceral leishmaniasis, which affects several internal organs (usually spleen, liver, and bone marrow). In some areas, humans are the main reservoirs, while in others animals have an important role in transmission. The disease affects mainly dogs and cats but livestock may have a role as reservoirs.

46.2.2.7 Brucellosis

The most important species of *Brucella* are zoonotic: *B. abortus*, responsible for bovine brucellosis; *B. melitensis*, the main etiologic agent of ovine and caprine brucellosis and an increasing cause of cattle brucellosis; and *B. suis*, causing pig brucellosis.

The main risks for people are occupational (contact with livestock) and consumption of dairy products. In some areas, brucellosis may be maintained in reservoir wild animal hosts (African buffaloes and North American bison); in other cases disease spills-over to wildlife and, if eliminated in cattle, brucellosis will die out in wildlife. Brucellosis is an important disease of livestock in developing countries.

46.2.2.8 Echinococcosis

Cystic echinococcosis (CE) is a condition of livestock and humans that arises from eating infective eggs of the cestode *Echinococcus granulosus*. Dogs are the primary definitive hosts for this parasite, with livestock acting as intermediate hosts and humans as aberrant intermediate hosts. A small number of countries bear the great majority of the burden of CE, In descending order: China (Tibetan plateau), Turkey, India, Iraq, Iran, and Afghanistan. A preliminary estimate of the annual global burden of CE has suggested approximately 1 million DALYs are lost due to this disease (Budke et al. 2006). In addition the losses to the global livestock industry is around $ 2 billion lost annually and cost of illness is around the same. As for cysticercosis, the main livestock impacts are through lost meat production.

46.2.2.9 Toxoplasmosis

Toxoplasomosis is one of the most common parasitic infection of humans and animals and is found worldwide. In most healthy adults it does not cause serious illness, but it can cause blindness and mental retardation in children in the womb and serious disease in immunocompromised individuals. Toxoplasmosis is probably an important cause of abortion in developing countries.

46.2.2.10 Q Fever

Q fever is an infectious disease of animals and humans caused by a species of bacteria (*Coxiella burnetii*). It is most frequently found in ruminants (cattle, sheep, and goats) but can also be detected in wildlife and companion animals. According to the literature sheep appear to be infected most frequently, followed by goats and—less frequently—cattle. Human cases are often associated with proximity to small ruminants (particularly at parturition or during abortions) and dry, windy weather. Clinical signs are rare in livestock but abortions in sheep and goats may occur.

46.2.2.11 Trypanosomiasis

Tsetse-transmitted trypanosomiasis is an infectious disease unique to Africa and caused by various species of blood parasites. The disease affects both humans (Rhodesian and Gambian sleeping sickness) and animals (nagana). *Trypanosoma brucei gambiense* (*T.b.g.*) is found in West and Central Africa; it currently accounts for over 95% of reported cases of sleeping sickness and causes a chronic infection (Gambian sleeping sickness). Most transmission is anthroponotic and can be controlled effectively through interventions targeted at human reservoirs; however, animal reservoirs have a role in the epidemiology *T. brucei rhodesiense* (*T.b.r.*) is found in eastern and southern Africa. Nowadays, this form (Rhodesian sleeping sickness) represents less than 5% of reported cases and causes an acute infection. Trypanosomiasis is the single most important disease of livestock in Africa.

46.2.2.12 Anthrax

Anthrax is endemic in sub-Saharan Africa and many parts of Asia. It is highly lethal to ruminants but in humans the most typical manifestation are skin lesions (malignant pustule). However, if spores are inhaled death may result. Anthrax is not common in most developing countries, but some are prone to outbreaks where localised losses can be high.

46.3 Conclusions

Developing countries are not all alike and there are important differences between isolated and neglected pastoralists in a declining and increasingly climate insecure economy, and the rapidly growing urban centers with their accompanying intensifying livestock systems. The most critical systems where improved understanding on the prevalence, impact and dynamics of zoonoses are needed include: rapidly intensifying agricultural systems; urban systems; and, systems where substantial land-use change is occurring especially irrigation in arid areas, and farming on forest margins.

As the importance of zoonoses in poor countries is increasingly recognized, so is the need to generate a stronger evidence-base on problems and solutions to support decision making. Information is needed on the impacts of zoonoses, the multiple costs and benefits of control, and the sustainability, feasibility and acceptability of zoonoses management.

History in the developed world shows that the burden of zoonotic diseases can be dramatically reduced. High zoonotic disease prevalence is both a cause and consequence of poverty, and investment and innovation is urgently needed to tackle zoonoses in developing countries where they currently impose massive burdens on human, animal and ecosystem health.

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