Food consumption and disease risk

Consumer–pathogen interactions

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Part I

Factors that influence interactions between foodborne pathogenic agents and consumers

1

Introduction to foodborne illness: public health impact, pathogens, and consumers

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1.1 Introduction

Food is a key element of life. We cannot live without it, yet it also serves as a route by which we can become ill. It is a driver of much of who and what we are. It is in many ways a reflection of social status and living standards: in the most developed countries, we have supermarkets filled with foods from across the globe, brought to our doorstep regardless of season or cost. Food choices are driven by a desire to 'cut carbs' or other dietary concerns, with obesity a major societal concern. In the less-developed world, in contrast, there are real concerns about food availability/security, with children threatened by well-recognized risks associated with protein-calorie malnutrition. Interwoven among all of this is the ongoing role of food as a vehicle by which pathogenic microorganisms can be introduced into human hosts, as modified by the evolutionary potential of microorganisms, which allows them to take advantage of very different transmission pathways in developed and developing countries, and today's rapidly changing environmental and host characteristics.

The public health impact of infectious foodborne illness is substantial. In the classic paper by Mead and colleagues published in 1999, the US Centers for Disease Control and Prevention (CDC) estimated that there were 76 million illnesses, 325 000 hospitalizations, and 5000 deaths caused by foodborne disease in the United States each year (Mead *et al*., 1999). While these estimates may be high, similar ranges have been reported from other major developed countries. For 2002, Australia estimated that there were between 4.0 and 6.9 million cases of foodborne illness per year (OzFoodNet, 2003). England and Wales, for 2000, came up with a much lower estimate of 1.3 million foodborne cases; however, estimated rates of bacterial foodborne illnesses (for which the best data are

available) were comparable to those from the United States (Adak *et al*., 2002). Rates of illness are much more difficult to estimate for the developing world. WHO estimates that in 2000 alone 2.1 million people died from diarrheal diseases (WHO, 2002). While it is not possible to say what percentage of these deaths was due to foodborne illness or how many people were ill for each person who died, in the mélange of transmission routes by which these illnesses and their causative pathogens were acquired, food and water undoubtedly played a major role.

These estimates, despite their uncertainties, are sufficient to place foodborne illnesses among the major global public health problems. They also underscore the ongoing difficulties that arise in both developed and developing countries in trying to 'count cases'. This is due in part to deficiencies in public health infrastructure, which make case identification problematic, at best. In the United States, the foodborne disease surveillance system known as FoodNet was established, in part, to address these concerns, providing intensive, active (and expensive) surveillance for foodborne pathogens in a limited number of defined geographic areas. However, even with this intense surveillance, there is clear undercounting of cases, as reflected in the continued use by CDC of multipliers to come up with estimates of actual disease rates. An even more basic problem arises in identifying the food(s) responsible for transmission of a foodborne pathogen (or even saying that transmission was due to a food). Interventions tend to be product- or food-specific, so development of rational control strategies requires that we be able to attribute cases, and the pathogens that cause them, to specific products or product lines (such as poultry, or produce). While we have a basic idea of where pathogens originate, we are finding that there are limits on the availability of quantitative data on 'food attribution', of the type necessary to develop up-to-date risk rankings and risk assessment and management models (Batz *et al*., 2005).

The recognition of the need for interventions to try to prevent foodborne diseases is long-standing, and has led to ongoing efforts to establish regulatory frameworks for disease prevention. Dietary laws, dictated, at least in part, by empiric observations regarding 'high-risk' foods, date back to early civilizations, and are preserved today in a number of religious systems, including Jewish Kosher and Moslem Halal law. In the more recent past, concerns about foodborne illness paralleled the migration of increasing numbers of people from rural to urban areas. In the United States, prior to the 1870s, almost all of the food consumed was either made in the home or purchased from neighbors, with the exception of a few staples such as flour. Gradually, however, more and more food came from factories or was shipped long distances to market, so that consumers were unaware of the source of the food, the ways in which it had been processed and handled, or even what it contained (Alsberg, 1970; Roe, 1956). At the same time, 'competition in sales and in the development of products created incentives for illegal profits through the debasement of manufactured foods and the mislabeling of those products' (Roe, 1956). Concerns that life expectancy was decreasing in the rapidly growing nineteenth-

century cities led to demands for government intervention to control epidemics of disease and assure safe food and water for a population that was increasingly dependent on other people for their provision (Hutt and Merrill, 1991). These demands, in turn, played an important role in the development of the modern public health system, and the associated system of public health laws (including food safety laws) that were put in place in the United States in the early part of the twentieth century.

These approaches were guided by the best available science at that time, and were effective in controlling what were perceived in the early 1900s as major problems, including exclusion of dead, diseased, disabled, or dying animals from the food supply; accurate labeling of food products; and monitoring of shellfish growing areas for fecal contamination (NRC, 2003). However, in the past 50 years there have been substantive increases in our understanding of foodborne pathogens and their modes of transmission, which, when combined with the emergence of new problems, have underscored the need for new approaches to the control of foodborne illness. At a regulatory level, these factors have led to the implementation of new, science-based regulatory systems, including the US Department of Agriculture's Pathogen Reduction: Hazard Analysis and Critical Control Points (HACCP) System (in 1995) and the US Food and Drug Administration's Seafood HACCP system. While these systems appear to have been effective in decreasing rates of some of the major foodborne diseases, we are far from eliminating the problem and, based on past history, we can fully expect that new pathogens will continue to emerge, or 'old' pathogens will re-emerge, moving through new transmission pathways that are opened up by ongoing changes in food production practices.

As a classic example of this process, *Escherichia coli* O157:H7 impinged on the awareness of the scientific and public health community for the first time in 1982, and, in a series of outbreaks in fast food restaurants (and school lunchrooms) became an internationally feared cause of foodborne illness (Riley *et al*., 1983; Bell *et al*., 1994; Watanabe *et al*., 1996). The reasons for its rapid emergence as a major 'developed world' pathogen are still not completely clear: contributing factors include its extremely low infectious dose (<10 micro-organisms can cause illness), combined with evolutionary changes in the food industry (Armstrong *et al*., 1996). The latter includes increasing reliance on high-volume production of ground beef, providing greater opportunities for spread of the microorganism from a single contaminated carcass to a large lot of ground beef; changes in farm and feeding practices, with a greater concentration of cattle in smaller numbers of feedlots; and, for the school outbreaks in Japan, widespread dissemination of what appear to have been contaminated seed lots used in the production of sprouts.

This same pattern of emergence of new or newly identified infectious agents, facilitated by changing food production practices, has been seen multiple times. In the early twentieth century, shellfish sanitation regulations in the USA were effective in controlling typhoid fever in shellfish, the purpose for which they were designed. However, the major shellfish-associated pathogens in the past

several decades have been *Vibrio* species, including *Vibrio vulnificus* (a species identified for the first time in the late 1970s), *V. parahaemolyticus* (which is currently in the midst of a global pandemic associated with strains carrying a unique set of genetic markers), and non-O1 *V. cholerae* (Morris, 2003). These species are naturally occurring in shellfish harvest waters, particularly during warm summer months, and their presence does not correlate with fecal coliforms (i.e., current regulatory approaches are ineffective in their control). Economic pressures that led to summer harvesting of oysters have resulted in major increases in the incidence of *Vibrio* disease; there have also been recent suggestions that increasing incidence is related to global warming and increases in water temperatures in shellfish harvest areas (McLaughlin *et al.*, 2005). As another example: while scrapie in sheep has been known for more than 200 years, the presumed jump of the causative prion agent into cattle (resulting in bovine spongiform encephalitis (BSE)), and from cattle into humans, was not anticipated; indeed, a number of scientists expressed doubts initially about whether prions even caused TSEs. Again, however, the emergence of the agent was linked with changes in agricultural production practices, including changes and consolidations in the rendering industry, combined with economically driven procedures intended to maximize the recycling of animal protein, which together permitted widespread dissemination of the infectious agent, prion or not, to cattle in cattle feeds.

Paralleling the increasing commercialization of food production has been the increasing globalization of our food supply, which has resulted in even further separation of the consumer from food sources. This has resulted in raspberries from Guatemala (Ho *et al*., 2002) and green onions from Mexico (Wheeler *et al*., 2005) causing outbreaks thousands of miles from where they originated. The economic drivers and public health consequences of globalization are discussed in more detail in Chapter 3. In addition, food preparation patterns are changing. Our great-grandmother is likely to have raised the chicken that was eaten for Sunday dinner. Our mother and grandmother may have bought the raw chicken from a supermarket, but made certain it was thoroughly cooked and was handled properly. Families now are more likely to eat at restaurants, or mom will bring a pre-cooked chicken (with raspberry glaze) home from the supermarket or local take-out counter.

As is discussed in detail in Chapters 9 and 10, the demographics of our population are changing. Increasing numbers of people are living longer, with the elderly having greater susceptibility to infection with many foodborne pathogens. Immunity to these pathogens may also be affected by cancer chemotherapy, immunosuppression associated with organ transplants, or HIV. The developing world is also changing, with increasing commercialization of food production, and the development of new food production processes designed to feed burgeoning (and, in some areas, increasingly affluent) populations, trends that open the door to the emergence of a host of new problems.

Taken together, these observations underscore the highly dynamic nature of foodborne illness as we enter into the twenty-first century. It is unquestionably a

major public health problem, in both developed and developing nations. If we are to optimally protect the health of the public, we need a continually updated understanding of potential foodborne pathogens, the foods by which they are transmitted, how these transmission routes are affected by economically driven production practices, and host susceptibility to infection. This book provides such an updated knowledge base. It is hoped that such knowledge can lead to the development of new interventions, including new regulatory approaches, to further reduce the age-old link between food and disease.

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2

Populations at elevated risk of foodborne disease

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2.1 Introduction

Of the hundreds of pathogens transmitted to humans through foods, only a few cause most of the foodborne illnesses when a pathogen is identified (Hillers *et al*., 2003). The FoodNet system in the United States tracks the incidence of nine pathogen species (Centers for Disease Control and Prevention (CDC), 2005) and there are others that have public health significance to general or selected population groups (Hillers *et al*., 2003, Kendall *et al*., 2003) (see Table 2.1). There were almost 16 000 cases of laboratory-confirmed cases of foodborne illness reported to FoodNet in 2004. Incidence per 100 000 persons was highest for *Salmonella* (14.7), *Campylobacter* (12.9), and STEC O157 (0.9) (Anon, 2005a). Encouragingly, since the inception of FoodNet in 1996, there has been a decline in the incidence of reported cases of *Campylobacter*, *Cryptosporidium*, *Escherichia coli* O157, *Listeria*, and *Yersinia*. On the other hand, the incidence of *Shigella* did not change, and the occurrence of *Vibrio* increased (Anon, 2005a).

Reported and confirmed incidence rates of foodborne illness from *Campylobacter* and *Escherichia coli* O157 are approaching public health goals for the United States. This is mainly due to changes in regulatory requirements for industry and education aimed at consumers and food handlers (Anon, 2005a). However, unreported cases may account for much more foodborne illnesses in the general and susceptible population. For example, diarrheal illnesses occur at the rate of 6% in the general population, but only 21% of these individuals sought medical care (Imhoff *et al.*, 2004). Thus, current data suggest that the actual number of individuals affected by foodborne illnesses is much greater than the number of cases reported. This discrepancy in numbers justifies

Table 2.1 Pathogens responsible for the majority of foodborne illnesses in the United States

Pathogen	Susceptible populations	Active Surveillance by FoodNet
Bacillus cereus	Elderly (Smith, 1998)	No
Campylobacter jejuni	Elderly (Gerba *et al*., 1996) HIV/AIDS (Gerba *et al*., 1996)	Yes
Clostridium perfringens	Elderly (Smith, 1998)	No
Cryptosporidium parvum	Young children (Gerba *et al*., 1996) HIV/AIDS (Gerba *et al*., 1996)	Yes
Cyclospora ssp.	Adults (FoodNet, 2005)	Yes
Escherichia coli O157	Young children (Kendall *et al*., 2003) Elderly (Gerba *et al*., 1996)	Yes
Hepatitis A	General population (Gerba *et al*., 1996)	No
Listeria monocytogenes	Pregnant women (Kendall *et al*., 2003) Neonates (Kendall *et al*., 2003) Elderly (Kendall *et al*., 2003)	Yes
Nontyphoidal *Salmonella* ssp.	Young children (Gerba *et al*., 1996) HIV/AIDS (Gerba *et al*., 1996) Elderly (Gerba *et al*., 1996)	Yes
Norovirus	General population (Gerba *et al*., 1996)	No
Shigella ssp.	Young children (Gerba *et al*., 1996) HIV/AIDS (Gerba *et al*., 1996) Elderly (Gerba *et al*., 1996)	Yes
Staphylococcus aureus	Elderly (Smith, 1998)	No
Toxoplasma gondii	Pregnant women (Kendall *et al*., 2003) HIV/AIDS (Kendall *et al*., 2003)	No
Vibrio ssp.	Elderly (Kendall *et al*., 2003) Chronic disease (Kendall *et al*., 2003)	Yes
Yersinia ssp.	Young children (Kendall *et al*., 2003)	Yes

continued efforts to improve the safety of foods commonly consumed by susceptible populations, and education to increase the awareness of risk by all consumers.

2.2 Consumer groups at risk of foodborne illness

2.2.1 Infants and children

Certain consumer groups are at high risk for acquiring foodborne illnesses. For example, infants and children are more highly susceptible to infections because of their immunological naivety. Repeated exposure to pathogens or immunizations creates antigenic memory as adaptive immunity matures. Therefore, the

Table 2.2 Incidence of foodborne pathogen infection in children 2003[1,2]

Pathogen	Less than 1 year of age	1–9 years of age
Campylobacter ssp.	27.2	15.5
Escherichia coli O157	1.6	3.2
Salmonella ssp.	131.9	33.5
Shigella ssp.	9.8	33.2

[1] Cases per 100 000 population.
[2] FoodNet (2005).

younger the child, the less able it is to mount a productive immune response to prevent illness from occurring. Even small doses of pathogen may be sufficient to infect infants and young children. And, as expected, the propensity to be exposed is greater for infants and young children who are cared for by providers with poor hygiene habits or from cross-exposure to other children in environments such as daycare centers. This is confirmed by FoodNet data which show that incidence of foodborne illness from the fecal pathogens *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia* were common in children aged 0 to 4 years (FoodNet, 2005) (see Table 2.2).

Neonates who are breastfed receive antibodies from the mother that provide the baby with initial immunity (Strober and Fuss, 2001). Breast milk contains IgA produced by B cells in the mammary gland and other immunological compounds (e.g. lysozyme, lactoferrin, and cytokines) that provide some but not complete protection in the first weeks of life. For example, protection against sporadic salmonellosis was attributed to breast-feeding in a case-control study of infants reported to have nontyphoidal *Salmonella* infections (Rowe *et al*., 2004). Formula feeding does not provide the immunological protection of breast milk. Infection from an emerging foodborne pathogen, *Enterobacter sakazakii*, has been reported in very low birth-weight infants supplemented with powdered formula in a hospital nursery (Anon, 2002b).

In 2002, bacterial sepsis was the eighth leading cause of infant mortality in the United States (Kochanek and Martin, 2005). Pathogen infections in newborns may occur by transplacental infection during pregnancy, by vaginal exposure during the birthing process, or through environmental cross-contamination. The influence of progesterone during pregnancy causes a switch in the type of T cell in and around the placenta to prevent transplacental transfer of maternal cytotoxic effectors and to prevent the rejection of the fetus as a foreign antigen (Piccinni *et al*., 2000). This immunological state favors a humoral or antibody-mediated immune response to maternal infection as opposed to a cell-mediated response. Primary infection from *Listeria monocytogenes* (Genovese *et al*., 1999) or *Toxoplasma gondii* (Dubey, 1986) is more likely to occur in this environment. Either pathogen can cause the infant to be stillborn or congenitally infected with poor developmental outcomes for the child (McLauchlin, 1996; Smith, 1997). For *Listeria monocytogenes*, environmental contamination can occur and care

providers who practice poor hygiene may cross-infect other healthy infants in their care (Colodner *et al*., 2003). McLaughlin reported numerous cases of hospital cross-infection of *Listeria* between the environment or care providers and other infants (McLaughlin, 1996). Environmental contamination was also the source of *Yersinia enterocolitica* in newborns in homes where chitterlings were prepared (Anon, 2003a). Infants and children aged 0 to 4 years had the most confirmed cases of yersiniosis in the 2003 FoodNet report (FoodNet, 2005).

In 2003, infection with *Escherichia coli* O157 was common in children up to young adulthood and most common in young children 0 to 4 years of age (FoodNet, 2005). Risk factors for infection are consumption of undercooked hamburgers, foods likely contaminated with animal feces, and recent exposure to farm animals (Kassenborg *et al*., 2004a). Michel *et al*. (1999) found that frequency of verocytotoxigenic *E. coli* (VTEC) was clustered in rural areas of Ontario, Canada, which also correlated with the density of cattle in the same geographic areas. This raised the question of whether rural or urban residency was a risk factor for infection. To investigate this, antibodies that indicated prior exposure to VTEC have been found at higher concentrations in rural residents than urban residents (Reymond *et al*., 1996). However, farm-residency was found in another study to be associated with higher presence of antibodies to *E. coli* O157:H7 and also *Campylobacter jejuni* and lower incidence of disease in rural children (Belongia *et al*., 2003). Non-farm rural children showed less evidence of exposure or the ability to mount an immunological response to an infection. This may indicate that rural residency is a risk factor for exposure to at least some zoonotic pathogens, and that frequent versus periodic exposure to farm animals seems to provoke more protection against active infections.

Hemolytic uremic syndrome (HUS), one of the most common causes of sudden, short-term kidney failure, may occur after infection by Shiga-toxin producing *Escherichia coli* (STEC) (Bell *et al*., 1997). Risk for HUS seems to be associated with: (1) young age (Noris and Remuzzi, 2005), (2) use of anti-motility drugs and severity of disease (Bell *et al*., 1997), and (3) administration of antibiotics (Wong *et al*., 2000). No treatment was associated with more positive outcomes in both of these studies when the case was confirmed as caused by *Escherichia coli* O157 or other STEC. This study recommended prevention of pathogen infection as the most prudent means to prevent HUS in young children (Bell *et al*., 1997).

2.2.2 The elderly

At the opposite age extreme of infants and children, the elderly are also highly susceptible to foodborne illnesses. Hypochlorhydria from use of H_2-receptor antagonists, proton pump blockers or frequent use of antacids reduces the ability to resist infection (Feldman *et al*., 1996; Klontz *et al*., 1997; Donskey, 2004). Interestingly, histamine H_2 blocker therapy and antacid use, which is sometimes advocated as a calcium source for women, were risk factors in a reported outbreak of listeriosis in the elderly (Cobb *et al*., 1996).

There is a decrease in intestinal motility (Bitar and Patil, 2004) and mucosal immune function (Fujihashi and McGhee, 2004) with aging. Fecal impaction may result from loss of smooth muscle contractility, but it is not clear if prolonged exposure of pathogens to intestinal epithelium is a risk factor for pathogen infection. However, studies have found that the elderly are at greater risk of infection from the senescence of gut-related immune tissues (Fujihashi and McGhee, 2004).

Systemic infections are more likely because of disruption in cell-mediated immunity in the aging body (Cakman *et al*., 1996; Pahlavani and Richardson, 1996). Undernutrition, which is common in the elderly, may exacerbate immune dysfunction because of nutrient deficiencies (High, 1999). Risk for undernutrition has been associated with eating alone, depression and dementia, dental pain and poverty (American Dietetic Association, 2000). Deficiency of protein, zinc, selenium, iron, copper, vitamins A, C, E, B-6 or folic acid in the elderly have been associated with decreased immune function (Lesourd, 1997).

2.2.3 Individuals with chronic disease

Chronic disease may increase risk of foodborne illnesses because of disease-associated immune suppression or pharmacological immune suppression. Advanced age and male gender were common descriptors of non-pregnancy associated cases of listeriosis in France (Goulet and Marchetti, 1996). Of the identified cases, the greatest risk of illness was found in patients who also had an immunosuppressing condition, such as diabetes mellitus, malignancy, organ transplantation, or AIDS.

Diabetes leads to susceptibility to infection in later stages of the disease because of persistent hyperglycemia (Umpierrez and Kitabchi, 2003; Maldonado *et al*., 2004) or loss of microcirculation efficiency (Dinh and Veves, 2005). *Salmonella* (Acheson and Hohmann, 2001), *Listeria monocytogenes* (Nolla-Salas *et al*., 2002; Chougle and Narayanaswamy, 2004) and *Toxoplasma gondii* (Yamamoto *et al*., 2003) have been isolated from infected tissues of patients with advanced diabetes. Elderly who are diabetic are especially prone to infections in general (Rajagopalan, 2005), which may include foodborne infections.

Blood-borne cancers or those that are treated with chemotherapy or radiation are immuno-suppressive conditions that lead to susceptibility to opportunistic infections (Bow, 1998; Zinner, 2000). Neutropenia is the marker for infection risk and may be constant or periodic, depending on the duration and intensity of the cancer treatment regimen (Safdar and Armstrong, 2001). Again, the elderly are particularly vulnerable to the toxic effects of cancer treatment and the risk for infection is more pronounced (Repetto, 2003).

Transplant surgery, like all major surgeries, leads to short periods of immune suppression during which the patient may be at increased risk for infection (Cryer, 2000). Graft survival rate has greatly improved since the introduction of cyclosporine. However, pharmacological suppression of the immune system can lead to infection, a leading cause of mortality in kidney transplant patients

(Tanphaichitr and Brennan, 2000). The elderly are especially vulnerable and are thus poor candidates for transplant surgery (Meier-Kriesche *et al*., 2000). After about 6 to 18 months post-transplant, immune suppression ameliorates and infection risk becomes more similar to that of the general population (Tanphaichitr and Brennan, 2000).

HIV infection is characterized by gradual loss of immune function because of destruction of CD4 T lymphocytes. The onset of symptomatic AIDS results in increased risk of opportunistic infections that may be life-threatening (Sneller and Lane, 2001). Patients who are successfully receiving highly active antiretroviral therapy (HAART) may not be at increased risk for foodborne illnesses since severe immune suppression is not typical until the symptomatic phase of HIV infection is marked by low T cell counts (Crowe and Mills, 2001).

2.3 Racial/ethnic or socioeconomic status and foodborne illnesses

Because data on foodborne incidence in racial/ethnic groups are sparse and in socioeconomic groups are unavailable, incidence of diarrhea is useful as a surrogate for cases of foodborne illnesses. Health insurance coverage can indicate which demographic groups are likely to seek medical care for gastroenteritis. The percentage of persons in the United States without health care insurance in 2002 was 16.1% and 15.4% had public health plan coverage; most of those without health care insurance were categorized in the poor or near poor socioeconomic strata (Cohen *et al*., 2005). Herikstad *et al*. (2002) reported incidence of diarrhea by demographic descriptors and included data on medical care, which provides some insight into which members of various racial/ethnic groups and socioeconomic strata are seeking medical care for foodborne illnesses.

2.3.1 Differences in foodborne illness by demographic group

Hispanics reported more cases of diarrhea, but Asians were significantly more likely to seek medical advice for their illness (Herikstad *et al*., 2002). African Americans were hospitalized for diarrhea more often than other racial/ethnic groups. Females have been reported to have more cases of diarrhea and were significantly more likely to be hospitalized for their illness. Foodborne illnesses reported by FoodNet in 2003 were generally more common in Caucasians; notable differences from this generalization were the incidences for *Shigella* and *Yersinia*, where high incidence was reported for both groups for *Shigella* and for *Yersinia* and African Americans (FoodNet, 2005).

Food handling knowledge and practices of racial/ethnic or socioeconomic groups are rarely reported in the literature. Hand washing after using the rest room or changing diapers was less frequently acknowledged as an appropriate

behavior by low-income participants of all racial/ethnic or socioeconomic groups (Meer and Misner, 2000; Wenrich *et al*., 2003). This may provide some explanation for incidence rates for *Shigella*. Risk for yersiniosis has been associated with consumption of pork products. In one study, incidence of *Yersinia enterocolitica* illnesses occurred mostly in African American infants ($n = 8$) and one Hispanic infant who were exposed in households where chitterlings were being prepared (Anon, 2003a). Incidence of listeriosis is more common in Caucasians than other racial/ethnic groups, but a recent increase in incidence in Hispanic populations has become a matter of public health concern because of consumption of a specific high-risk food. An outbreak of listeriosis in Hispanic women was associated with homemade fresh cheese, called queso fresco, which is common to Latin American cultures and is frequently made from raw milk (Anon, 2001a; Van Hekken and Farkye, 2003). Hispanics have been reported to be more likely than other racial or ethnic groups to consume unpasteurized milk (3% versus 1%) and soft cheese made from raw milk (28% versus 10%) (Banerjee *et al*., 2002).

2.3.2 Medical insurance and likelihood of seeking medical care

Hispanics were more likely than other racial/ethnic groups to have no health-care insurance coverage (24.2%) (Ni and Cohen, 2005). African Americans are more likely to have public health plan coverage than other minority groups (17.0%). Weighted prevalence of diarrheal illnesses and hospitalization for that illness was higher for respondents without medical insurance. However, those with insurance were significantly more likely to seek medical advice and to have their illnesses diagnosed with stool sample analysis. In fact, none of the respondents without medical insurance reported having laboratory confirmation of their diagnosis. However, to say that insurance coverage is the determinant of access to health care is an oversimplification. Individuals may refuse the health care that is available if they do not perceive the need for the care, physical barriers make access difficult, cultural beliefs cause doubt in the efficacy of care, or refusal to cope with inefficient service (Gulliford *et al*., 2002). While lack of health insurance has been found to be associated with poor health (Baker *et al*., 2001), both public or private health insurance have been shown to have a positive impact on health care outcomes (Quesnel-Vallée, 2004). Poor health outcomes were more closely associated with poor health status and low socioeconomic status in childhood.

2.4 Consumer awareness and knowledge about food safety

Consumers are the gatekeepers of food safety in their homes, since previously safe food can be contaminated while being handled, prepared, or stored. To explain the epidemiologic incidence of foodborne illnesses data gathered through FoodNet and other means of passive surveillance, numerous studies of

Table 2.3 Pathogens, control factors and foods frequently related to incidence of foodborne illnesses[1]

Pathogens of concern	Primary control factor	Foods of concern
Norovirus *Shigella* spp. Hepatitis A	Personal hygiene	Food contaminated by infected food handler Food exposed to contaminated water Raw/undercooked vegetables, fruits, eggs, meat, poultry or seafood
E. coli O157:H7 *Salmonella* spp. *Campylobacter jejuni* *Yersinia enterocolitica* *Toxoplasma gondii*	Adequate cooking and/or avoiding cross-contamination	Unpasteurized milk Raw/undercooked eggs, meat, or poultry
Vibrio spp. *Listeria monocytogenes* *Cryptosporidium parvum*	Avoid unsafe foods and water	Unpasteurized milk Raw/undercooked meat, poultry or seafood Contaminated water
Bacillus cereus *Clostridium perfringens* *Staphylococcus aureus*	Keep foods at safe temperatures	Cooked foods

[1] Adapted from Medeiros *et al.* (2001b) and Hillers *et al*. (2003).

consumer food handling practices have been conducted. The result is a confusing array of information that may or may not have an impact on public health. Redmond and Griffith (2003) published a meta-analysis of 87 studies that used a variety of research methodologies to gather information on consumer food handling. These authors noted considerable differences in study outcomes based on whether or not consumers were asked to respond to queries about food safety or if their actual food handling behaviors were observed. To this end, self-reported information tends to under-report risky behavior and generally presents a more positive picture of consumer awareness, knowledge or behavior as related to food safety.

Practice of safe food handling is related to the prevention of foodborne illnesses and can be more directly linked to epidemiologic data on disease if the relationship between the practice and likelihood of specific pathogen contamination is validated. Medeiros *et al*. (2001b) suggested that pathogens are more effectively controlled when groups of related food handling behaviors are practiced. These authors proposed five control factors that, when practiced, are the primary means by which illness from specific pathogens can likely be prevented (see Table 2.3). The same research group used an expert panel to validate individual behaviors to the primary control factors (Medeiros *et al.,* 2001a), pathogens (Hillers *et al*., 2003), and population groups particularly susceptible to foodborne illnesses (Kendall *et al*., 2003).

2.4.1 Personal hygiene practices of consumers

Personal hygiene includes cleanliness of the hands, hair, clothing, and body in general. Hand washing is most frequently the sentinel behavior for assessment of personal hygiene in consumer food safety studies. From a Hazard Analysis and Critical Control Point (HACCP) perspective, the critical control point for ensuring the safety of foods that are prepared to be served without heating is personal hygiene. Controlling the transfer of pathogens from the hands to food is important for almost all foodborne illnesses, but especially: (1) raw vegetables and fruits; (2) some types of desserts; (3) raw or undercooked foods exposed to polluted water; and (4) previously cooked foods handled by consumers and served without additional heating. It is estimated that 5% of Hepatitis A cases are foodborne, 20% of *Shigella* cases, and 40% of Norovirus cases are estimated as being foodborne (Mead *et al*., 1999). Thus, hands contaminated with fecal pathogens can be the source of pathogens in foods (Feachem, 1984).

Foods at risk

Numerous outbreaks of Norovirus infections were reported in 2000 by the CDC that were associated with foods such as raw oysters, fruit or vegetable dip, submarine sandwiches, butter cream frosting, pasta salad or chicken nuggets (Anon, 2002a). Outbreaks of Hepatitis A were reported that were associated with green onions, sushi or guacamole (Anon, 2005b). A *Shigella* outbreak occurred due to contaminated parsley (Naimi *et al*., 2003). Surveys of food safety practices have probed consumers for their habits regarding consumption of high-risk foods, or foods likely to cause foodborne illnesses (Klontz *et al*., 1995; Yang *et al*., 1998; Wenrich *et al*., 2003; Cody and Hogue, 2003). Raw oysters, sushi or cerviche are not commonly consumed by consumers in the United States (85–95% claming no consumption, depending on the study) and may be more localized to specific populations or regions. For example, a study completed in Arizona that reported higher consumption of raw fish or seafood than other studies (30% of respondents) included 32% Hispanic respondents (Meer and Misner, 2000). It is possible that cerviche consumption, which is common to Central American cuisines, could account for this observation. However, this was not discussed by the authors. Shiferaw *et al*. (2000) reported in their survey that Hispanics were more likely than other racial or ethnic groups to consume raw shellfish, as were respondents residing in coastal areas.

Consumer knowledge and practices of safe food handling

Consumer knowledge about the use of personal hygiene in food handling to decrease the risk of illness does not always lead to good practice by the consumer. For example, Altekruse *et al*. (1996) found that 86% of the respondents to their survey believed that washing hands before preparing food decreased risk for foodborne illnesses. However, Cody and Hogue (2003) found that almost half of their respondents were likely to forget to wash their hands before cooking food. Participants in a community education program for low-income families knew they should wash their hands before preparing or handling

food (over 90% correct), but fewer associated the need to wash after sneezing, using the restroom, or touching pets (Wenrich *et al*., 2003). Thus, these studies suggest that a gap exists between consumers' knowledge about the impact of personal hygiene on decreased risk for foodborne illnesses and their practices during food preparation and handling.

Worsfold and Griffith (1997) conducted simulated food preparation studies with consumers to actually observe their food handling behaviors. They found that the participants washed their hands prior to handling food only 66% of the time. Videotaping of consumers preparing foods verified that hand washing before touching food is either sporadic or is performed in a manner that could have little impact on the sanitation of the hands (Jay *et al*., 1999; Anderson *et al*., 2004; Kendall *et al*., 2004). Health care providers were observed to have poor hand washing compliance in patient care situations where they are likely to have contact with fecal pathogens and responded only moderately to educational efforts to improve hand washing rates (Pittet *et al*., 1999; Bischoff *et al*., 2000). Placement of alcohol-based sanitizer dispensers improved hand washing rates, suggesting that in the health care setting convenience is primary to compliance (Bischoff *et al*., 2000). The difficulty in convincing health care providers to properly clean and sanitize their hands when they are very aware of the consequence of not doing so could be predictive of poor educational outcomes when the same skills are taught to consumers who do not have the same awareness, motivation, or skill.

2.4.2 Cross-contamination and adequate cooking

In addition to transfer of pathogens by failure to wash hands prior to handling foods, hands contaminated during food preparation also can transfer pathogens to previously safe foods. This primary control factor differs from personal hygiene because the immediate source of the pathogen is the food rather than hands contaminated by non-food related routes. The critical control point for foods at risk for causing foodborne illnesses is either cross-contamination of foods eaten without additional heating (i.e. raw vegetables or salads), or food that has been contaminated after cooking. The pathogens that can be controlled by preventing cross-contamination also can be controlled by adequate heating, provided re-contamination does not occur after pasteurization (see Table 2.3). Any surface that is touched by contaminated hands or foods can transfer microorganisms to other foods.

Foods at risk

According to a survey conducted by FoodNet, examples of risky foods that are sources of cross-contamination and that can be made safer by cooking are undercooked eggs, undercooked hamburgers, and unpasteurized milk (Shiferaw *et al*., 2000). Older adults (age 40 and up), Hispanics, and rural residents not living on a farm report the greatest frequency of undercooked egg consumption. Undercooked hamburger consumption is highest among males, college

graduates, Hispanics, those with incomes above US$100 000, and suburban residents. Unpasteurized fluid milk consumption was found in the FoodNet study to be more common among Hispanics, those with education levels less than high-school graduation, and farm residents. Shiferaw *et al*. (2000) report in their sample population that young adults (age 18–25) consumed unpasteurized milk more frequently than other age groups, but this was not supported in another study. The elderly are more likely to consume raw milk than younger populations according to data used in the *Listeria monocytogenes* Risk Assessment, which is based on nationally representative food consumption surveys (US Department of Health and Human Services, US Department of Agriculture, 2003).

Although typically controlled by heat pasteurization, foodborne illnesses from *E. coli* O157:H7 have been associated with unpasteurized apple juices made from fruit contaminated with fecal matter (Cody *et al*., 1999). Chicken, typically a risky food for *Campylobacter* contamination (Friedman *et al*., 2004), has also been identified as a risk factor for *Salmonella* Enteritidis (Kimura *et al*., 2004). Eggs, a risky food for *Salmonella* Enteritidis, are also a risk factor for *Salmonella* Heidelberg (Hennessy *et al*., 2004).

Other sources of infectious organisms

Touching farm animals has been identified as the source of STEC transferred either directly to humans or food cross-contaminated without washing hands (Belongia *et al*., 2003). Cats are the primary source of *Toxoplasma gondii* and may contaminate humans directly or through human consumption of infected food animals (Dubey, 1986).

Methods for controlling cross-contamination

Cross-contamination can be controlled by proper cleaning and sanitizing of food preparation surfaces, hand washing after touching contaminated food or surfaces, and by cooking foods to temperatures sufficient to destroy heat-labile pathogens. Washing and/or sanitizing cutting boards after preparation of meat, poultry, or seafood, or washing hands after touching raw meat or poultry are common food handling behaviors included in food safety surveys. Anywhere from 7% to 40% of the consumers self-reported they did not follow these practices (Altekruse *et al*., 1996; Shiferaw *et al*., 2000; Yang *et al*., 1998; Cody and Hogue, 2003). Klontz *et al.* (1995) reported that respondents to their survey used contaminated cutting boards with little or no cleaning (26%), washed it with soap or bleach or used another board (58%), or did not respond to the question (16%).

Consumers have knowledge, but lack skill in prevention of cross-contamination

Worsfold and Griffith (1997) observed behaviors of consumers during food preparation and found numerous factors that could lead to cross-contamination: use of single cutting board for all tasks (60% of occurrences), use of unwashed/sanitized cutting board (25% of occurrences), handler washes raw meat/poultry

(33% of occurrences), not washing hands after handling raw meat/poultry (58% of occurrences) or contaminated packaging in work area (18% of occurrences). Kendall *et al.* (2004) verified self-reported behavior with direct observation of consumers who were preparing food. Respondents first completed the food preparation activity and then completed a self-reported interview of their perceived food safety behavior. Eighty-four percent stated they wash food contact surfaces during food preparation, and 88% were observed doing so. However, only 63% followed a cleaning procedure that would adequately prevent cross-contamination (e.g. hot, soapy water). Ninety-seven percent stated they washed their hands after touching raw meat, chicken, or seafood, and 88% were observed completing this behavior. Correct hand washing (e.g. soap and warm running water) was not frequently observed (20% of occurrences). These researchers concluded that behaviors that prevent cross-contamination are typically practiced by consumers, but the skill of completing the task adequately to prevent foodborne illnesses is lacking.

Reducing the risk of contamination and illness by adequate cooking
Contamination of food by heat-labile pathogens can be eliminated by adequate cooking. The United States Department of Agriculture consumer education materials advocate the use of a food thermometer to ensure that cooking is complete and that food is safe to consume (Anon, 2001b). Color of food has not been found to be a reliable indicator of adequate cooking of ground meat, a known source of *Escherichia coli* O157:H7 (Anon, 2003b). About 37% of respondents to a food safety survey used temperature as their indicator of doneness of foods, but they also used touch, taste, sizzling, or appearance to cue them to doneness (Wenrich *et al*., 2003). Kendall *et al.* (2004) observed that consumers do not rely on food thermometers to verify temperatures, but cooked chicken and hamburger to at least 71.1 ºC (160 ºF) (89% and 93% of occurrences, respectively). Worsfold and Griffith (1997) observed a slightly higher percentage of failure to reach adequate cooking temperatures (15% of occurrences).

2.4.3 Keep foods at safe temperatures

Mead *et al.* (1999) reported that 100% of the cases of *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus* illness are foodborne, but FoodNet does not conduct active surveillance of foodborne pathogens that are primarily controlled by storage temperature. Reported incidence of illnesses associated with improperly stored foods currently is low, perhaps because illnesses caused by these pathogens are largely self-limiting and medical treatment is infrequent (Mead *et al*., 1999), or because of the emphasis consumer education programs have traditionally placed on food handling behaviors that control these pathogens (Medeiros *et al*., 2001b). However, consumers frequently practice behaviors that could lead to illnesses from these pathogens. For example, a food safety survey of consumers found that almost half of the respondents were likely

to consume food that had been left at room temperature for more than two hours (Cody and Hogue, 2003). Only 48% of respondents to another survey knew that leftovers should be stored in shallow containers (Wenrich *et al.,* 2003). Another example comes from Worsfold and Griffith (1997), who observed that cooked foods were not properly cooled in 35% of the occurrences in their observational study. In addition, 69% of participants in a community education program did not know their refrigerator's temperature (Meer and Misner, 2000). Clearly, continued diligence in educating the public about properly stored foods is justified.

2.4.4 Avoid unsafe foods and water

For certain population groups, consumption of foods and water that may be sources of *Vibrio* species, *Listeria monocytogenes,* or *Cryptosporidium parvum* should be avoided. These groups are immune compromised or suppressed because of age, pregnancy or chronic disease, and/or medical treatments. Raw or undercooked seafood, raw milk or milk products, improperly handled packaged foods or polluted water can be contaminated with these pathogens. Even though foodborne illnesses can be avoided by practicing behaviors to prevent cross-contamination and ensure adequate cooking, it is prudent for individuals who are susceptible to infection to avoid consumption of such risky foods (Kendall *et al*., 2003).

2.5 Travel as a risk factor for foodborne illness

Gastroenteritis is a worldwide phenomenon shared by developing and developed countries. Estimating the contribution of foodborne illnesses to the total diarrheal burden is greatly complicated by the lack of medical or surveillance systems to track illnesses (Flint *et al*., 2005). Incidence rates by specific pathogens vary by country and could be influenced by diet and the possibility of immunity among indigenous populations. A comparison of incidence of foodborne illness in England and the United States shows higher population rates in the United States for illnesses in general (Adak *et al*., 2002). However, both countries have high incidence of *Escherichia coli*, *Salmonella*, *Yersinia*, and Norovirus infections, and England and Wales reported higher incidence of *Aeromonas* and Rotavirus infections. Denmark reports increased mortality from *Salmonella*, *Campylobacter*, *Yersinia*, and *Shigella* (Helms *et al*., 2003). There are also literature reports on incidence of infection from *Salmonella* in France (Gallay *et al*., 2000), *Listeria monocytogenes* in Israel (Siegman-Igra *et al*., 2002), and *Campylobacter* in England (Rodrigues *et al*., 2001).

Non-native visitors to a country are prone to bouts of traveler's diarrhea when they are unaccustomed to water and food handling or preparation practices in that country. Recent international travel by Americans has been identified as a risk factor for illnesses from *Campylobacter* (Kassenborg *et al*., 2004b) and

Salmonella Enteritidis (Kimura *et al*., 2004). Information about gastroenteritis for the benefit of international travelers can be found on the Centers for Disease Control and Prevention website (Anon, 2005c). *Escherichia coli*, *Shigella*, *Giardia*, *Cryptosporidium*, and Norovirus are common causes of food-related diarrhea. Travelers are at the greatest risk for developing illnesses if they fail to take precautions to protect their health in international countries, such as prudent selection of foods, prophylactic immunizations, or prompt and proper treatment when diarrhea does occur, as suggested by health organizations. While travel-associated enteric disease can occur anywhere, travel to developing countries of Latin American, Africa, the Middle East and Asia has been identified as a specific risk factor.

2.6 Future consumer trends

In the United States, there has been a trend away from at-home meal preparation and consumption toward meals eaten out of the home, or more recently, meals prepared away from home and consumed in home (Collins, 1997). The USDA Economic Research Service predicts that consumer spending in full-service restaurants will increase by 18% by 2020 and by 6% in the fast-food market (Stewart *et al*., 2004). Changes in demographics will drive the increase to full-service vendors because of the trend toward households consisting of a single person or multiple adults without children, and a better educated population. Elderly adults will continue to prefer full-service restaurants and the increase in the older population is reflected in the slower growth of the fast-food industry. Hispanic and African American households are less likely than Caucasian and Asian households to spend money in full-service restaurants; however, spending at fast-food restaurants will moderately increase for all racial/ethnic groups by 2020. Saturation in the food-eaten-away-from-home market in the United States is causing the restaurant and food industry to look for growth in international markets (Seid and Ainsley, 2005). Factors driving this trend are stronger economies of some developing countries, changes in international lifestyles, lower labor costs and greater disposable incomes internationally.

Concern for food safety has apparently given way to a greater desire for convenience and time savings. The FoodNet Working Group surveyed consumers about food handling practices and found that 85% of the interviewees had eaten food outside the home in the previous week (Shiferaw *et al.,* 2004). Of those, 3.3% reported a gastrointestinal illness during the same week as compared with only 2.4% in the group of respondents who did not eat a meal away from home. Consuming chicken eaten at a restaurant has been identified as a risk factor for sporadic cases of *Campylobacter* infection (Friedman *et al*., 2004). A preference for undercooked hamburgers associated with high risk for *Escherichia coli* O157:H7 was reported by 33% of consumers who eat in fast-food or sit-down restaurants more than six times per week (Garman *et al*., 2002).

Consumers must rely on the training and diligence of commercial food workers who have assumed the role of assuring the safety of foods during preparation. Safe storage of foods prepared away from home then transported to the point of consumption raises concerns that temperature abuse could lead to re-emergence of some types of low-incidence foodborne illnesses, such as *Staphylococcus aureus* or *Bacillus cereus* (Little *et al*., 2002). These researchers concluded that establishments where the manager had participated in food safety training have less food contamination than those without trained managers. Cohen *et al*. (2001) evaluated the efficacy of an in-house food safety training program and learned that success was dependent on the motivation of the workers to practice safe food handling behaviors. Workers in food establishments in England, most of whom (95%) had received some type of food safety training, were surveyed regarding their food handling practices (Clayton *et al*., 2002). Surprisingly, 63% admitted they did not practice what they knew when lack of time or insufficient staff interfered. In the United States, food workers admitted they touched ready-to-eat foods without gloves (60% of occurrences), handled food with potentially contaminated gloves (33% of occurrences), failed to use thermometers to check adequate cooking of food (53% of occurrences), or worked with active cases of vomiting or diarrhea (5% of occurrences) (Green *et al*., 2005).

Since food handler behavior is dependent on motivation or skill, alternative methods to ensure food safety may become necessary to have continuous improvement in the incidence of foodborne illnesses. Irradiation will sterilize foods and will present a safe product to the food preparer, but it is poorly accepted by consumers (Sapp, 2003). Communication processes are key to acceptance of new technologies. Consumer groups have called for food safety labeling to warn of potential harm from consumption of foods considered to be risky for some immune compromised or otherwise susceptible groups (Anon, 1999). A study was conducted to determine consumer opinions about food safety labels in response to a required rule of the United States Department of Agriculture to labels foods about processes used to prevent risk of infection from *Listeria monocytogenes* (Anon, 2004). Respondents highly endorsed packages (82%) as the place they desired to find food safety information. Shiferaw *et al*. (2000) reported high consumer awareness of food safety information on package labels, but poor compliance with basic practices such as hand washing. This indicates that safety labels do not replace either motivation or education.

2.7 Sources of further information and advice

Information about incidence and protection from foodborne illnesses is available from numerous public health sources. Information is readily available to consumers through the World Health Organization. In the United States, the Centers for Disease Control and Prevention and the FoodNet system public websites, the Food and Drug Administration, and the Food Safety and Inspection

Service of the Department of Agriculture also have consumer education resources available through their websites. Educational materials for educators and fact sheets for consumers or individuals with high risk for foodborne illnesses are available through these sources. Links to other sites are an integral part of most, if not all, of these sources of information. The World Health Organization is recommended as a source of information for international information.

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3

Globalization of the food supply and the influence of economic factors on the contamination of food with pathogens

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3.1 Introduction

Evidence is lacking on whether the globalization of the food supply increases or decreases foodborne disease risks. As a first effort in understanding potential linkages, this chapter takes a broad view of the economic factors that influence human exposure to and infection with foodborne pathogens. As the foundation, this chapter looks at some of the demand-side and supply-side factors that have driven the substantial increase in international trade in agricultural products and commodities. This increase in trade and changes in the composition of trade have implications for human exposure to different pathogens.

Demand-side factors that support the increased globalization of the world's food supply include increases in income levels and urbanization. These factors are important in that they affect the mix of food purchased and consumed domestically and the composition of global food trade. Different foods have a different mix of potential microbial foodborne illness hazards to consumers and also vary in the risk of contamination. In turn, hazards in the different foods vary in the likelihood and severity of acute illness and chronic complications (e.g. mild illness from *Salmonella* in cantaloupe, kidney failure from *E. coli* O157:H7 in ground beef). Additionally, wealthier nations tend to demand safer food.

Supply-side factors that support the increased globalization of the food supply include changes in food production, processing, storage, transportation, and distribution due to technological advances and other factors as well as structural changes measured by the number, size, and organization of firms in a particular food industry. Many of these supply-side factors have led to the mass

production and distribution of food, which multiply potential opportunities to disseminate foodborne pathogens more widely and to a greater number of people. On the other hand, some of these advances and structural changes make food safer.

Many of the economic factors affecting food safety risks are interrelated. The food system is a complex network of food production, consumption, and trade that is also heavily influenced by overarching factors such as countries' and multinational agencies' food regulation and oversight (e.g. regulatory standards, tariff and nontariff barriers to trade) as well as global macroeconomic conditions, such as relative economic growth rates and currency exchange rates. For example, the general income level of a country is a factor that can be viewed from different perspectives, such as which foods the average citizen can afford, which food safety technologies food companies can afford to implement, and to what extent governments are willing and able to pay for food safety regulation.

To date, no one has systematically looked at all of the economic factors that affect the global risks from microbial pathogens and no one has developed a comprehensive framework. Therefore, this chapter aims to provide a starting discussion of some of the key economic factors that need further research. The chapter provides examples of untested and arguable hypotheses of how changes in these economic factors might affect foodborne disease risks. The chapter also provides examples of the magnitude of the problem in terms of adverse economic consequences. Attention will be paid to differences between countries with different levels of development, with a focus on high-income countries such as the United States.

The key conclusion of this analysis is that although the globalization of the food supply can amplify the consequences of foodborne pathogens by expanding the reach of contaminated food to greater and more distant populations, some of these economic factors, particularly the supply-side factors in the food industry, can make food safer. In short, there is no clear-cut answer about whether globalization of the food supply increases or decreases overall foodborne illness risks because there are so many confounding effects and so little supporting data.

3.2 Demand-side factors

In general, consumers benefit from increased trade through lower prices, year-round supplies, and greater variety in the type and quality of food (e.g. off-season produce, seafood from distant waters). International food trade also can provide foreign exchange to food-exporting countries, which is important to spur on economic development in many low-income level countries (WHO, 1998). However, the globalization of the food supply means that new food safety risks can be introduced into countries (e.g. emerging bacteria), previously controlled risks can be reintroduced into countries (e.g. cholera), and contaminated food can be spread across greater geographical areas and cause illness worldwide. For

example, during 2000–02, outbreaks of *Salmonella* Serotype Poona infections occurred in 12 states and Canada due to contaminated cantaloupe from Mexico (Anderson *et al*., 2002).

International agricultural trade has increased from US$339 billion in 1993 to US$522 billion in 2003 (FAO, 2005), though the rate of growth has slowed in recent years. There are many reasons why agricultural trade has increased, but one important factor is certainly the need for greater quantities of nutritious and safe food to feed the ever-expanding world population, which is currently growing by over 73 million people a year (US Census Bureau, 2005). Given finite amounts of land, other limited resources, and climate and seasonal differences among countries, international food trade is one avenue to match world food demand with world food supply. Overall, human health risks from most internationally traded food appear to be low, based on reported foodborne outbreaks in the United States. Food safety incidents also are rare considering the total volume of trade.

When talking about global food safety risks from internationally traded foods, it is too simplistic to focus solely on the total quantity of food trade. It is much more informative to focus on the mix of foods consumers demand in countries with different levels of development and how the mix or composition of international trade for the different commodity groups (e.g. meats, produce, and grains) may change in response to changes in consumer demand. The premise here is that the bundle of global foodborne disease risks is affected by the types of foods traded internationally, which are in part determined by consumer demand for the different foods. The premise is based on the view that different food products are prone to different food safety hazards and that these hazards vary in the likelihood and severity of acute and chronic health complications. For example, mycotoxin risks are primarily associated with consumption of grains, histamine risks are associated with consumption of seafood, and risks from *Trichinella* are primarily associated with consumption of raw or undercooked pork and meat from wild carnivores, such as bear or crocodile.[1] This premise is by no means perfect as many pathogens, such as *Salmonella*, can be found in a wide array of food vehicles.

To better understand how demand-side factors affect global foodborne disease risks, the remainder of the section follows a two-step analysis. First, dietary differences among countries with different levels of income and urbanization are explored. This analysis alone is incomplete because it does not take into consideration what foods are domestically produced or acquired through international trade. Therefore, to put these countrywide differences in consumption into perspective, the second step layers the analysis of what food

1. Mycotoxins are toxic byproducts of fungal infestations affecting as much as one-quarter of the global food and feed crop output (Dohlman, 2003). If mycotoxin-contaminated feed is fed to food animals, it can also contaminate animal products. Food contaminated with mycotoxins, particularly the subcategory of aflatoxins, can cause fatal acute illness and is associated with increased cancer risk (Dohlman, 2003).

groups are consumed in countries with different levels of development with a look at the composition of world agricultural trade.

3.2.1 Income and urbanization levels affect the mix of foods consumed in different countries

According to a study on global consumers by Regmi and Pompelli of USDA's Economic Research Service (2002), income is the factor that has the greatest influence on dietary changes. The analysis below updates the framework used by Regmi and Pompelli (2002) with more recent and expanded data on the food available for consumption in different countries from the United Nations Food and Agriculture Organization. The World Bank's country classifications (2005) are used to help illustrate that the demand for particular food groups depends on a country's income level. The analysis below also extends Regmi and Pompelli's framework by discussing how global differences in consumption may be associated with global differences in microbial foodborne disease risks.

Regmi and Pompelli found that in low-income level countries, such as Cambodia, Haiti, and Nicaragua, consumer food demand tends to be focused on low-value staple food products to meet basic calorie requirements. Updated data in Table 3.1 support this finding and show that low-income level countries have a higher per capita consumption of cereal products and roots and tubers (e.g. sweet potatoes, cassava) than countries with higher income levels. In general, consumers in lower-income countries tend to spend a higher proportion of their budget on food than other countries (Regmi *et al*., 2001, Seale *et al*., 2003). They are also more responsive to changes in general food prices and income and therefore, make larger adjustments to their diets when food prices and incomes change (Seale *et al*., 2003). This is particularly true for higher-value food items, such as meat and dairy, while household budget allocations for staple foods tend to undergo smaller changes (Regmi, 2001). Populations that predominantly rely on cereal crops for nutrition may be more at risk from foodborne illness from mycotoxins compared with populations that rely relatively more on other commodity groups, such as meats, if they live in areas where grain production and/or storage conditions are conducive to mycotoxins. For example, in the United States, grain-consuming vegetarians are probably not at greater risk from mycotoxins than non-vegetarians because mycotoxins are not particularly a problem here compared with countries with warm and humid climates.

In middle-income countries, most consumers can easily meet their basic caloric needs so food demand is often shaped by cultural trends, taste, and other factors (Regmi and Pompelli, 2002). (To simplify the discussion, lower middle-income level countries, such as Peru and South Africa, are combined here with upper middle-income countries, such as Mexico and Saudi Arabia.) Consumers in middle-income countries are the most responsive to price changes of staple foods and often substitute staple foods with other more expensive sources of nutrition, such as fresh food products like fresh fruits and vegetables, seafood,

Table 3.1 Average supply of major food commodities by country level of development and percentage change between 1992 and 2002

	1992 (lbs per capita)	2002 (lbs per capita)	Change 1992–2002 (%)
Cereals (excluding beer)			
Low-income countries	295.3	298.9	1.2
Lower middle-income countries	336.6	327.8	−2.6
Higher middle-income countries	274.6	276.2	0.6
High-income countries	247.2	261.3	5.4
Roots and tubers (dry equivalent)			
Low-income countries	63.1	68.5	8.6
Lower middle-income countries	27.8	31.1	11.9
Higher middle-income countries	26.8	29.5	10.1
High-income countries	28.2	27.2	−3.5
Meat (livestock and fish primary equivalent)			
Low-income countries	34.8	34.3	−1.4
Lower middle-income countries	72.1	78.6	9.0
Higher middle-income countries	120.1	128.6	7.1
High-income countries	171.6	190.1	10.8
Fish, seafood (livestock and fish primary equivalent)			
Low-income countries	21.4	20.3	−5.1
Lower middle-income countries	32.3	39.2	21.4
Higher middle-income countries	50.0	50.7	1.4
High-income countries	64.9	70.9	9.2
Fruits (excluding wine)			
Low-income countries	104.7	102.2	−2.4
Lower middle-income countries	143.0	160.0	11.9
Higher middle-income countries	225.5	243.5	8.0
High-income countries	232.4	244.3	5.1
Vegetables			
Low-income countries	82.9	90.2	8.8
Lower middle-income countries	158.1	204.0	29.0
Higher middle-income countries	145.0	164.3	13.3
High-income countries	247.4	258.9	4.6
Oils			
Low-income countries	30.5	32.7	7.2
Lower middle-income countries	37.0	44.7	20.8
Higher middle-income countries	46.0	50.2	9.1
High-income countries	65.4	69.5	6.3

Source: Data calculated from the United Nations Food and Agriculture Organization Food Supply Data, Aug. 27, 2004, http://faostat.fao.org/. Oils data include coconut, cottonseed, groundnut, maize germ, oilcrops, palmkernal, palm, rape, mustard, ricebran, sesameseed, soyabean, sunflower, and vegetable oils. This table uses the World Bank's classification of member economies, and all other economies with populations of more than 30 000. Economies are divided among income groups according to 2003 gross national income (GNI) per capita, calculated using the World Bank Atlas method. The groups are: low income, $765 or less; lower middle income, $766–3035; upper middle income, $3036–9385; and high income, $9386 or more.
http://www.worldbank.org/data/countryclass/countryclass.html For a similar table with 1961–1998 data and world estimates, see Regmi and Pompelli (2002).

and meat (Regmi, 2001). Therefore, broadly speaking, the mix of food safety hazards is different for consumers in low- and middle-income countries. For example, consumers in middle-income countries are more likely to eat greater quantities of relatively high-valued meat and poultry than consumers in low-income countries, and these products are more likely to be contaminated with certain foodborne pathogens, such as *Salmonella* and *E. coli* O157:H7, than are cereal products.

In high-income level countries, such as Canada, Japan, and the United States, consumers, in general, are even more willing to pay extra income for a wider variety of foods, particularly foods that are labor-saving or of higher quality. Some consumers in developed countries also are willing to pay a premium for foods they perceive to be in line with positive environmental effects and ethical issues (e.g. farm worker safety, animal welfare).

In general, the demand for foods with higher food-safety levels (e.g. pasteurized milk) has tended to increase with growing consumer affluence and awareness of food safety issues. Accordingly, wealthier countries with more information about food safety risks tend to demand more stringent food safety standards on both domestically produced and imported food and generally also are willing to pay more for higher levels of food safety. For example, wealthier countries tend to buy higher-value, safer seafood products while less wealthy countries tend to buy lower-value products with fewer food safety assurances (Wessells, 2002). However, some technologies that reduce food safety risks may not be universally accepted by consumers because consumers consider many attributes in their decision making, not just food safety risks, and because consumers vary in the bundle of attributes that they consider in their decision making. For example, irradiated meat and poultry are not widely accepted in the United States.

Consumers in many high-income countries have an increased demand for uncooked foods with perceived enhanced nutritional value, such as fresh fruits and vegetables, even though cooked or processed versions may pose lower food safety risks. In the United States, per capita consumption of fresh fruits and vegetables increased almost 26 per cent between 1980 and 2003 (256.5 pounds to 322.3 pounds) (ERS, 2005). Meanwhile, over the past two decades, fruit and vegetable imports have more than doubled by weight (Jerardo, 2004). Perhaps partly as a result of dietary changes and increased imports, produce has taken on a more prominent role in reported foodborne illness outbreaks in the United States. An increasing proportion of all reported foodborne outbreaks with a known food item have been linked to produce – increasing from 0.7% in the 1970s to 6% in the 1990s (Sivapalasingam *et al*., 2004).

Additionally, in higher-income countries and in more urbanized areas, consumers tend to have more household amenities, such as microwave ovens and refrigerators, that enable them to purchase, store, and safely prepare perishable food products (Regmi, 2005). If properly used, these amenities should decrease food safety risks. Consumers in high-income counties also have greater access to processed ready-to-eat (RTE) products, such as factory-packaged lunch meat.

RTE products are products that consumers should be able to safely consume without further cooking or other treatment step that would kill any remaining pathogens on the product. Therefore, if an RTE product is contaminated with pathogens, illness may result. Risks of infectious foodborne diseases are likely to be different for processed and unprocessed RTE foods. For example, RTE processed foods likely pose lower food safety risks than unprocessed, street-vended food in less-developed countries with little routine oversight by governments. One example of microbial contamination in RTE foods is that 27 out of 100 samples of RTE roasted chicken in Mexico City tested positive for contamination with *Campylobacter* (Quiñones-Ramírez *et al*., 2000).

In addition to income, changes in food consumption patterns are also driven by other demographic factors (e.g. increased education levels) and lifestyle changes brought about by urbanization, increased levels of information, and the away-from-home employment of women (Regmi, 2005). These factors and others are intertwined but here we will combine them as 'urbanization' for simplicity. If current trends in population growth and migration continue, it is estimated that two-thirds of the world's people will reside in urban areas by 2030 (Saker *et al*., 2004). Urbanization is important to global foodborne illness risks because it affects the composition of foods consumed. Urban areas tend to have access to a wider array of fruits and vegetables, meats, and processed food products.

Because of increased access, one could argue that urban consumers are more likely to consume food away from home in restaurants, fast-food outlets, and other outlets. In the United States in 1997, outbreaks outside the home accounted for over 76% of outbreaks where the place of consumption could be identified (Olsen *et al*., 2000). Although this proportion may be inflated because these outbreaks are more likely to be recognized by health officials during outbreak investigations, away-from-home venues could potentially contribute to food-borne disease through practices such as the pooling of eggs, incomplete cooking of certain foods (e.g. hamburgers), and the holding of hazardous foods at temperatures that allow growth of certain pathogens (Altekruse *et al*., 1997). In general, public health agencies in many developed countries appear to be getting better at spotting foodborne outbreaks and quickly mitigating their impact. These countries have the resources to take advantage of advances in molecular biology and electronic communications. Data are unavailable on the proportion of foodborne illnesses attributed to away-from-home food in developing countries and these data are limited even in developed countries.

The greatest impact of away-from-home foods on foodborne illness is the multiplier effect, whereby one cook or one restaurant chain with poor food preparation practices can make dozens or thousands of people sick. For example, over 550 people became ill with hepatitis A after working or dining at a restaurant in Pennsylvania in 2003 (Dato *et al*., 2003). Preliminary analysis implicated green onions as the likely food vehicle. When foods such as green onions are contaminated during growing, harvesting, or packaging and distribution (e.g. by unsafe irrigation or processing water), the contamination entering home or commercial kitchens may be the same. However, even then, illnesses

generated by food service, where a few contaminated onions will be spread throughout a large batch of chopped onion, will potentially exceed the number of illnesses generated by home preparation where illness from one contaminated onion will be limited to members of that household. Although food from away-from-home sources are not necessarily riskier in terms of foodborne disease than food prepared at home, the possibility exists, particularly in outlets which are not regulated or inspected. In many countries, effective food safety education and control is not keeping pace with the increase in the number of food service establishments (WHO, 2005).

Other changes in dietary habits and food demand, particularly in high-income countries and urban areas, include the increased demand for so-called 'ethnic' foods. Ethnic foods are a niche market, bringing foods or cooking practices from low- or middle-income countries to high-income countries. For example, a cholera outbreak in 1992 in Thai immigrants living in the United States was linked to consuming commercial fresh frozen coconut milk imported from Thailand at a picnic (Taylor *et al*., 1993). This coconut milk was produced in a plant in Thailand that was later determined to have violated several sanitary standards. Additionally, as countries acquire a taste for new foods (e.g. sushi), they may be less familiar with how to reduce any inherent foodborne illness risks while preparing those foods.

3.2.2 Composition of world agricultural trade

The second step in understanding the impact of demand-side factors (income and urbanization) on global foodborne disease risks requires a closer look at actual changes in global food trade. In particular, how does global food trade change as a result of changes in consumption, which are partly influenced by global income levels and urbanization? If global incomes among developing countries continue to rise, one would expect to see consumption trends that more closely follow developed countries. If this happens, what are the implications for foodborne disease risks? A simple schematic on the demand-side factors is:

Income (Increases in global income levels and urbanization)	→	**Consumption** (Dietary upgrades or changes)	→	**Trade** (Changes in food trade to support new consumer demand)	→	**Food safety risks** (Changes in the bundle of global foodborne disease hazards)

Consumption changes driven by changes in countries' per capita income levels are the most important factors explaining historical changes in the composition of global food trade. Evidence of this was verified by simulating the individual contribution of household income, changes in factors of production, policy, and transportation costs on the changing composition of food trade from 1980 to 1995 (Coyle *et al*., 2001; Gehlhar and Coyle, 2001).

Over the past 20 years, the composition of world agriculture trade (food and nonfood, such as livestock feed) has changed. The share attributed to bulk goods (e.g. wheat, corn, and coffee) has been declining while the share of processed consumer goods (particularly chocolate products, pastry, and prepared foods) has been increasing (Fig. 3.1). Although shares of intermediate processed goods (e.g. soybean oil) and fresh horticultural goods have remained fairly steady, their total quantities traded have kept pace with the increasing volume of total agricultural trade (Gehlhar and Coyle, 2001).[2]

Processed foods accounted for around three-quarters of all global food sales (Regmi and Gehlhar, 2005). Processed foods are often differentiated by unique labels and brands and help satisfy consumer demand for variety and labor-saving products (Gehlhar and Coyle, 2001; Regmi and Pompelli, 2002). In wealthy, developed countries, the growth in imports of processed consumer goods is particularly notable. Meanwhile in developing countries, imports remain dominated by bulk and intermediate products though processed consumer goods are also making inroads (Coyle *et al*., 1998). International food trade is highly asymmetric with respect to exports as well-developed countries are mostly net exporters of more processed foods while developing countries mainly export unprocessed products or products with a low level of processing (Mathews, 1994).

If urbanization and income levels among developing countries continue to rise, it is likely that consumption trends in these countries will more closely follow the trends in developed countries. In particular, if developing countries change their diets to incorporate more processed consumer goods and fresh-food products, such as fresh seafood, fruits, vegetables, and meats, and international food trade increases to reflect these dietary upgrades, then we might see a greater share of global foodborne disease risks associated with these food products. However, the analysis is by no means straightforward as consumption trends may or may not be reflected in increased imports into a particular country. The demand for imports depends on each country's food production capacity. For example, increased livestock demand may not be satisfied with greater imports of livestock products when it is more economical to import feed and expand domestic production (Gehlhar and Coyle, 2001).

Data from the US Food and Drug Administration (FDA) on import detentions (i.e. imports detained for food safety or other technical violations) provide one view of which food safety issues tend to arise for the different product categories. In an analysis of January–May 1999 detention data, pesticide residues and microbial contamination were the most common reasons for food safety detention for fruits and vegetables, while microbial contamination, drug

2. Intermediate processed products are essentially processed bulk commodities (e.g. vegetable oil made from oilseeds) so part of the import demand for bulk commodities has been satisfied with increased growth in intermediate processed products (Gehlhar and Coyle, 2001).

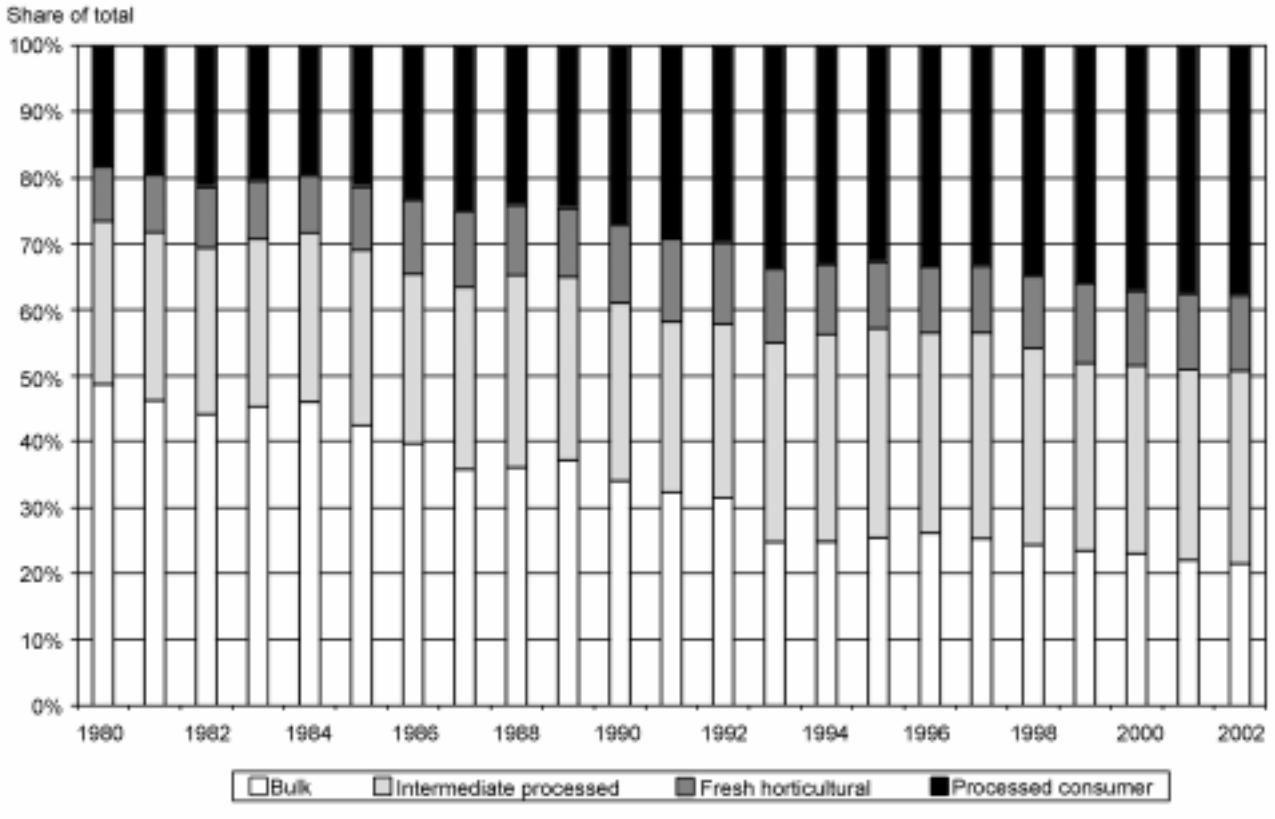

Fig. 3.1 Composition of world agricultural trade.

residues, parasites, and zoonotic diseases were issues for meat, poultry, and fishery products (Unnevehr, 2000; Unnevehr and Hirschhorn, 2000). Mycotoxin hazards were present on certain plant products. A shift in the composition of imported foods into the United States will result in changes in the relative proportions of the different food safety risks. Additionally, the detention data show that the top three categories where sanitary issues occurred were vegetables, fishery products, and fruits. This finding is particularly meaningful if consumers in developing countries adopt diets similar to those of consumers in developed countries and if international trade of these products increases to meet consumer demand. Meats had few detentions, presumably because of USDA's pre-certification of inspection systems in exporting countries (Unnevehr, 2000; Unnevehr and Hirschhorn, 2000).

In an examination of food safety issues and fresh food exports from less-developed countries, Unnevehr (2000) speculates that fresh food products have relatively higher food safety risks than other traded foods, such as bulk grains. The foundation for this theory is that fresh food products have more opportunities for food contamination because they are handled at all stages of the food production and marketing chain and are then consumed in their fresh form (Zepp *et al*., 1998; Unnevehr, 2000; Unnevehr and Hirschhorn, 2000). Meanwhile, processed or manufactured foods are less likely to deteriorate during shipping and handling and have more widely established and recognized standards (Unnevehr, 2000). However, the consistent use of such standards is less common in countries with lower levels of development. Better data and a more comprehensive analysis are needed to provide supporting evidence that fresh food products pose greater global food safety risks than bulk grains and processed or manufactured foods.

3.3 Supply-side factors

The food production and marketing chain has changed notably over the past few decades. Supply-side factors that support the increased globalization of the world's food supply and affect the mix of international agricultural trade include: (1) changes in the food production, processing, storage, transportation, and distribution resulting from technological advances and other factors, as well as (2) changes in industry structure. These two changes contribute to a general trend towards mass production and distribution of food. The impact on food-borne disease risks as a result of these supply-side factors is unclear. Although there is some anecdotal evidence or speculation that some technological advances or increased consolidation or concentration of firms may increase overall quality of a food product, there is no empirical evidence in the literature about the impact on specific food safety risks. After a brief discussion of some of these advances and changes, two sub-sections focus on how mass production and mass distribution can affect global foodborne illness risks.

3.3.1 Changes in the food production chain and food industry structure

There have been many changes in the food production and marketing chain (i.e. food production, processing, storage, transportation, and distribution) over the past century owing to technological advances and other factors that have ultimately affected foodborne disease risks. Early improvements in food hygiene and sanitation included pasteurization of milk, refrigeration, shellfish monitoring, and chlorination of water (Altekruse *et al*., 1997). More recently, other technologies have been implemented or are under consideration, such as packing innovations, controlled atmosphere technologies, and fruit and vegetable coatings. Technologies that reduce foodborne disease risks extend beyond product, process, and transportation technologies to include advanced information and communication technologies (e.g. Internet for E-commerce) and quality management systems and standards that help ensure that quality levels are reached and maintained.

Not all changes in the food production and marketing chain have resulted from technological advances – some changes are due to other factors such as a balancing of global supply and demand for certain food products. Additionally, some changes in the food production chain may increase some food safety risks or alter the mix of risks. For example, the use of aquaculture is becoming more common as wild fisheries become increasingly over-harvested and less cost-effective for some species and areas. US aquaculture production increased by over 50% between 1990 and 2000 (NMFS, 2002) and the aquaculture share of the world production also has increased (FAO, 2000). Farm-raised fish pose a different set of food safety challenges from those of wild-caught fishery products. Farm-raised fish are subject to contamination from residues by production inputs (e.g. vaccines, feed additives, and antibiotics), whereas wild-caught seafood may be more subject to histamine risks from poor temperature control.

In addition to changes in the food production chain, there have been several important changes in the structure of the food industry, particularly in developed countries that have supported the globalization of the food supply. In certain food sectors, there is greater *concentration* of firms (i.e. fewer and larger firms) resulting from industry *consolidation* (e.g. through mergers and acquisitions). For example, the four largest beef processors in the United States supplied about 70% of the beef market on a value basis in 2002, up from 26% in 1967 (Stewart and Martinez, 2002).

In addition to industry concentration and consolidation, there is also greater *vertical coordination* in some industries whereby successive stages of the production and marketing of a food product are synchronized with respect to quality, quantity, and timing of product flows (Martinez, 2002a). One type of vertical coordination that is becoming increasingly popular in the United States and other developed countries is *vertical integration* in which a single firm controls the flow of the commodity across two or more stages of production (Martinez and Reed, 1996). It can better guarantee the safety and quality of a firm's inputs and enhance the ability to trace product ingredients or processes back through the food production and marketing.

Contracts are another type of vertical coordination. Food producers in developed countries are increasingly engaging in long-term contracting with their suppliers, and are carefully vetting those suppliers for compliance with food safety principles or standards (Mitchell, 2003). In the United States, vertical integration and production contracts now account for over 90% of production in the poultry, egg, and pork industries (Martinez, 2002a). Food safety specifications can be an important component of these contracts. For example, after the large 1993 outbreak from hamburgers contaminated with *E. coli* O157:H7, Jack in the Box, Inc. revamped its quality control programs, suspended all existing contracts with hamburger patty suppliers, and designed new and more stringent contract specifications (Salay *et al*., 2004).

To a lesser extent, developing countries also implement the same kinds of technological advances in the food production and marketing chain and also see the same kinds of changes in the food industry structure for certain industries. For example, supermarkets in Thailand and Brazil have initiated total quality management programs for perishable foods such as seafood, meat, and vegetables (Trienekens *et al*., 2003). And just as in developed countries, the supermarket sector has undergone rapid consolidation in Latin America and East/Southeast Asia (Reardon *et al*., 2003). For example, the top five supermarket chains in each country in Latin America make up 65% of the total supermarket sector (Reardon *et al*., 2003). In general, improvements in food industry infrastructure are often necessary to accompany demand-induced changes in the food supply.

3.3.2 Mass production of food

But does mass production of food increase or decrease the risk of infectious foodborne disease? The answer is not clear cut and data are insufficient to reach an answer to which all can agree. Some claim that modern production methods that result in mass production of foods can lead to reduced costs and production efficiency but also can increase the chance of accidental contamination of foods and amplify their consequences (Morse, 1995). Claiming that the chance of accidental contamination is greater in mass-produced foods is certainly an arguable point that would require more research to substantiate. But perhaps one thing on which we all might agree is that mass production means larger batches of food that may be fed to larger numbers of people, so simple mathematics shows that if a batch is contaminated, the consequences could be amplified. However, what also must be considered is that the food industry has developed and implemented many critical control steps, processes, or checks to reduce food safety risks in mass-produced foods. Therefore, one could also argue that mass-produced food is actually safer (e.g. the probability of contamination may be lower).

In the literature, there have been several well-cited examples of large foodborne disease outbreaks linked to industry consolidation and mass distribution of contaminated foods (Altekruse *et al*., 1997). For example, an outbreak of

salmonellosis affecting roughly 200 000 people was linked to pasteurized milk from a large dairy plant in 1985 (Ryan *et al*., 1987). In 1994, another large outbreak of salmonellosis occurred when pasteurized ice cream base (premix) was transported in tanker trailers that had previously carried liquid raw egg (Hennessy *et al*., 1996). In this outbreak, around 224 000 people developed *S. enteritidis* gastroenteritis (Hennessy *et al*., 1996). In short, when products from large centralized food processors are distributed to wide geographic areas, there is the risk for dispersed outbreaks (Altekruse *et al*., 1997). Of course, outbreaks that are prevented by industry action to minimize food safety risks are not represented in the literature.

Those who support the theory that mass-food production increases food safety risks often point to the increase in concentration of animal production (particularly in developed countries) and claim that large-scale confinement operations and higher densities of food animals increase risks of disease. For example, in large-scale production units, infection of one animal can lead to wider contamination through exposure of other animals (Unnevehr and Roberts, 2002). And in many developed countries, large poultry flocks are reared in communal housing – a practice that leads to large numbers of birds having common risk profiles (Saker *et al*., 2004). The implication is that flocks with less genetic diversity may be more uniformly susceptible to certain diseases. Higher densities of farm animals also may pose steeper challenges for disposal of manure and other wastes to minimize the contamination of water, farm animals, and agricultural land. Chapter 4 more fully discusses the trends in agricultural management and land use that influence contamination of food with pathogens.

On the other hand, looking more closely at hog production as an example, there are many schemes that can limit the transfer of pathogens between groups of pigs in large-scale operations and some of these schemes may also, in turn, reduce food safety risks in pork (e.g. *Salmonella*). In particular, the risk of some disease outbreaks may be reduced by the specialization in phases of production (Martinez, 2002b), such as between breeding, nursery, and finisher production stages. Also, separating baby pigs from the sow before her maternal antibodies are depleted decreases the likelihood that they will contract any diseases that she might carry (Bell, 1998). Other risk-reducing schemes include using multiple-production sites with at least 200 yards between sites as well as biosecurity measures, such as rodent control, foot baths for workers entering nursery rooms, and limiting visitor and vehicular traffic between sites (Bell, 1998). The use of antimicrobial agents may be easier to administer in large confinement operations and may provide satisfactory protection from many diseases.

Additionally, in large-scale processing facilities, contaminants from a few carcasses or produce items can contaminate larger amounts of food. Current hamburger production methods make it possible for a single hamburger patty to come from the meat of dozens or even hundreds of cattle (Armstrong *et al*., 1996). Furthermore, all lots (e.g. one lot may be one hour's worth of production) of hamburger produced in a single day are often produced sequentially in a

continuous throughput process in the same meat grinder, making it possible for one lot to contaminate subsequent lots produced that day (Armstrong *et al*., 1996). For example, an analysis of the largest *E. coli* O157:H7 outbreak to date found that all the implicated hamburger patties sold to a chain in Washington State were produced at a single patty-making plant in California (Bell *et al*., 1994). This outbreak also caused illnesses in California, Idaho, and Nevada for a four-state total of more than 700 illnesses and four deaths (AGA, 1995).

In general, arguments against the theory that mass food production increases food safety risks point to the many critical control steps, processes, or checks that the larger food firms have developed and implemented to reduce food safety risks. Some contend that mass production of foods actually has made food safer. There is no doubt that private system approaches to reduce food safety risks are becoming more widespread and stringent (Caswell and Henson, 1997), particularly in developed countries. In addition to vertical integration, private approaches fostering food safety include self-regulation, third-party certification, and common approaches to risk identification, assessment, and management such as Hazard Analysis and Critical Control Point (HACCP) systems and voluntary guidelines or good agricultural practices (GAPs).[3]

Slaughter plants tend to use multiple interventions, which might include washing hides, chemically removing hair, sanitizing carcasses through steaming or washing them, and using electronic imaging to detect residual traces of surface contamination (Maday, 2004). Smaller plants and plants in developing countries may use a smaller set of these and other risk-reducing techniques. According to USDA records, the percentage of ground beef samples testing positive for *E. coli* O157:H7 has declined dramatically since its peak in 2001, largely because of post-harvest interventions (Maday, 2004). Many producer groups have instituted quality assurance programs, and firms often use a mix of approaches.

Large food companies have a lot at stake so one might argue that they would be even more diligent about minimizing the frequency and extent of pathogen contamination in their food products. Firms that face a large public recall of product or that are implicated in a foodborne illness outbreak may suffer business losses, such as from lost reputation, reduced stock prices, plants closed for cleanup or permanently shut down, food poisoning lawsuits, premiums raised for product liability insurance, and demand for product reduced enough to threaten entire markets or industries (Buzby *et al*., 2001). One example of a whole industry being affected by a foodborne illness outbreak is the Guatemalan raspberry industry. After repeated *Cyclospora* outbreaks in the United States and Canada from contaminated raspberries, only 3 Guatemalan raspberry producers

3. Third-party certification provides assurances to consumers that the information supplied by firms is correct (Golan *et al.*, 2000) (e.g. the International Organization for Standardization has its ISO 9000 series or 'EN 29000' in Europe). HACCP essentially identifies, monitors, and controls hazards at critical points in food production and processing.

remained in business of the 85 prior to the outbreaks (Calvin, 2003). In short, the private sector, both here and abroad, has incentives to prevent food safety crises and to mitigate their impact if they arise.

3.3.3 Mass distribution of food

This century has seen new technologies in transportation and distribution, particularly new developments in ocean shipping, which facilitate worldwide distribution of food (i.e. mass distribution). For example, although containerization has reduced world transportation costs since the 1950s, it was not until the 1960s, when refrigerated containers called 'reefers' were developed, that perishables could be integrated into the flow of general cargo (Coyle *et al*., 2001). Reefer containers have their own refrigeration units and use ship-generated power for climate control (Coyle *et al*., 2001). These refrigerated containers allow high-value, perishable products to reach distant markets while maintaining product quality and reducing delivery times and costs (Coyle *et al*., 2001). In particular, these and other advances, such as packaging techniques, have helped high-volume processed products and fresh horticultural products meet rising global demand in developed countries.

Combined, all the technological advances and changes along the food production and marketing chain have increased the potential for distributing pathogen-contaminated foods more widely. The geographical distribution and incidence of an increasing number of foodborne illnesses has expanded (Beuchat, 1998), alongside increased technical advances in food production and distribution. For example, iceberg lettuce imports, possibly from Spain, were implicated in *Shigella sonnei* infections in several North West European countries in 1994 (Frost *et al*., 1995). Although dioxin is not a foodborne pathogen, one glaring example of the potential reach of contaminated food and feed through international trade occurred in 1999 when animal feed in Belgium was contaminated with cancer-causing dioxin and polychlorinated biphenyls (PCBs). This feed was subsequently fed to chicken, swine, and other food animals. This incident affected a large array of agricultural industries and temporarily interrupted trade with more than 30 countries for dozens of products, such as the many different types of meat and milk-containing products (Buzby and Chandran, 2003).

Some might speculate that the best evidence of an impact from changing industry structure on foodborne disease it that there is increasing awareness that a new kind of outbreak scenario is developing. Traditionally, most foodborne illness outbreaks in the United States were acute and highly local with a high-attack rate and a high-inoculum dose (Tauxe, 1997). Now, however, widespread and diffuse outbreaks have been identified that may involve many states and countries (Tauxe and Hughes, 1996). It is believed that widespread distribution of commercial food products that are contaminated at low levels is responsible for these outbreaks (Tauxe, 1997). However, it is also true that their detection now is the result of advances in microbial detection methods (e.g. 'genetic

fingerprinting') that have made it possible to recognize and link together dispersed, seemingly unrelated sporadic cases. More research is needed to determine the proportion of these outbreaks that represent the new, added burden of foodborne disease rather than enhanced ascertainment.

As international food trade is increasing, more producers in more countries (some of which have relatively low food safety standards and controls) are exporting food. For example, an increasing number of countries are exporting seafood and some of these countries have poor internal control systems and/or are in tropical areas where some marine biotoxin and bacterial hazards are intrinsically higher (Ahmed, 1991). The US Food and Drug Administration detains and inspects samples of imported seafood at the port of entry and refuses adulterated shipments. The FDA import detention data for seafood products indicate that out of 130 countries represented, 86 had one or more shipments detained in 2001 and 80 had violations for adulteration (safety, packaging integrity, or sanitation problems) (Allshouse *et al*., 2003). In total, there were 4912 detentions for seafood products for 6405 violations (detentions can be for multiple violations). *Salmonella* accounted for 34% of the 5356 adulteration violations (other violations were for misbranding, etc.). Shrimp, by far the largest volume seafood item imported into the United States, accounted for one-quarter of all detentions. The three countries with the greatest number of detentions for adulteration were Vietnam, Thailand, and Indonesia.

Additionally, to meet export demand, low-income countries may cultivate non-indigenous crops that may be more susceptible to indigenous pathogens (Saker *et al*., 2004). A prime example of this is when Guatemalan producers grew non-native raspberries for commercial export (Saker *et al*., 2004). In 1996 and 1997, *Cyclospora* outbreaks in Canada and the United States were attributed to Guatemalan raspberries (Calvin, 2003). In short, the globalization of the food supply has the potential to create new agricultural and ecological challenges.

3.4 Discussion

Beyond the complexity of the world food supply, another reason for scarce empirical evidence on whether increased international trade (and the demand-side and supply-side factors behind this trade) increases risks from foodborne pathogens is the issue of attribution. A main reason why so little is known about the economic factors that affect foodborne illness is that little is known about which foods cause foodborne illness. Only a few research groups are attempting to attribute national pathogen incidence across all food categories and to evaluate such incidence in terms of illness severity and economic burden. One of the first groups to systematically study infectious diseases to determine which foods have the greatest risk is the Health Protection Agency's PHLS Communicable Disease Surveillance Centre in the United Kingdom (Adak *et al.*, 2005). Additionally, as part of the Foodborne Illness Risk Ranking Model (FIRRM), the Food Safety Research Consortium in the United States is

developing a computer model of the attribution of pathogen incidence and valuation (FSRC, 2005). Earlier studies estimated the extent of foodborne infections from particular pathogens at the national level but did not attribute these infections to particular foods (i.e. Mead *et al*., 1999; Adak *et al*., 2002). The lack of reliable epidemiological data in many countries has hindered the public health community in recognizing the importance of food safety and has limited the emphasis on food safety programs (Käferstein *et al*., 1997).

Foodborne pathogens can impose a substantial financial burden upon individuals, families and nations (WHO, 2005). Overall, costs of foodborne illnesses are likely to be relatively higher in developing countries with larger at-risk populations, while at the same time these countries lack the financial and technical resources and the institutional framework to address these risks and contend with their results. The lack of food safety systems in these countries not only results in greater domestic foodborne illness, which in turn lowers productivity, but it also results in the loss of export markets, which in turn decreases access to foreign currency and investments, inhibiting economic development. Additionally, the lack of public health surveillance means that foodborne illnesses and their economic impact are not documented – information important to support the need for foreign aid. Therefore, it is essential for developing countries to build capacity to identify and document domestic foodborne illness and to respond to existing and emerging food safety problems.

Although, for the United States at least, there is no evidence that imported food, as a whole, poses higher food safety risks than domestically produced food, globalization of the food supply may be introducing new food safety risks, reintroducing previously controlled risks, and spreading contaminated food more widely. Risks from imported food sources are similar to the kind and extent of risks from domestic sources, but the United States has limited food safety oversight in countries from which we import, which are increasingly countries with lower levels of economic development. The potential for increased food-related illnesses from continued increases in internationally traded food will challenge government food safety systems and private firms to develop and implement improvements in prevention, inspection, and control systems.

3.5 Future trends

Global food trade will continue to increase owing to expected increases in global income levels, improved transportation networks, and growing populations requiring greater quantities of nutritious and safe food. Similarly, the demand for a wide array of labor-saving processed food products and foods consumed away from home is expected to increase alongside increasing incomes and urbanization. As income levels of developing countries increase, we may well see food consumption trends more closely match those in developed countries. In particular, consumption as well as imports of fresh horticulture products, processed goods, and foods of animal origin likely will increase.

With heightened awareness of food safety concerns and a rapidly changing food system, food safety standards worldwide are becoming more stringent and responsive to new hazards (Roberts and Unnevehr, 2003). Countries that trade internationally may have different domestic food safety goals and food safety regimes as well as different costs of complying with regulations. These differences may lead to trade conflicts or reductions in trade (Mitchell, 2003). On the other hand, these differences may lead to increased dialogue between countries that could lead to elevated and harmonized food safety systems and goals.

Food safety, both domestically and internationally, is managed and assured by both private and public sector efforts (Caswell and Henson, 1997). Some of the private sector efforts have been discussed previously. New approaches to food safety regulation emerged in industrialized countries during the 1990s following advances in science, changes in markets, and increased awareness of food safety risks. Roberts and Unnevehr (2003) identify seven main trends for food safety regulation in industrialized nations. They find that regulatory agencies are increasingly:

(1) organized into one agency that can focus on food safety;
(2) using risk analysis to design regulation;
(3) stressing a farm-to-table approach in addressing food safety hazards;
(4) adopting the HACCP system as a basis for new regulation of microbial pathogens in food;
(5) adopting more stringent standards for many food safety hazards;
(6) adding new and more extensive regulation to handle newly identified hazards; and
(7) improving market performance in food safety through provision of information to consumers (e.g. safe food handling labels).

As countries become more industrialized, it is likely that their regulatory agencies will follow the same trends. More international cooperation in identifying, controlling, and preventing foodborne disease is needed both because of the globalization of the food supply and because of greater movements of potentially contaminated or infected food animals, animal feed, and people (e.g. international travel, refugees, and migration). International cooperation is particularly important because human responses to diseases are conditioned by jurisdictional boundaries whereas microbes are unhindered by international borders but can rather move freely around the world (Fidler, 1998).

Additionally, intersectoral cooperation is also important, such as between national public health and veterinary services in the case of the zoonotic agents that cause foodborne diseases in humans (Abdou, 1998). One recent example of a disease linking animals, food, and human health is bovine spongiform encephalopathy (BSE) in cattle, which has caused regulatory changes affecting production, imports, and market access worldwide (Mathews *et al*., 2003). In short, the global food system is both complex and dynamic at all levels – consumption, production, and trade – and therefore solutions to identify, control, and prevent foodborne illness are also complex.

3.6 Sources of further information and advice

An abundance of information on international food consumption and trade can be obtained through USDA's Economic Research Service. In particular, the previously cited articles by Regmi, Gehlhar, Coyle, and other ERS economists as well as many other related publications can be obtained at www.ers.usda.gov. The Food and Agriculture Organization of the United Nations (www.fao.org) and the World Health Organization (www.who.int/en/) are two other good sources of information.

Further information on international trade and food safety for several commodity sectors can be found in a US Department of Agriculture report (Buzby, 2003, see http://www.ers.usda.gov/publications/aer828/). In general, literature on the economic factors that influence human exposure to and colonization with foodborne pathogens is limited. Therefore, the field is wide open for further research. This research is needed because quantifying the economic and health consequences caused by foodborne pathogens can help policymakers better understand the costs and benefits of implementing new or expanded food safety programs to protect public health.

Epidemiology, risk assessment, and economic analysis are tools that help us better understand the hazards and impact of foodborne disease. These tools can be used to estimate baseline incidence of disease risks, distribution of disease severity, and economic impact, which can then be used to monitor progress of risk reduction efforts. These tools also can help policymakers in priority setting when allocating scarce resources among different intervention efforts. However, better epidemiological data are needed to improve the accuracy of economic and risk analyses that estimate the costs and benefits of different intervention strategies.

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4

Trends in agricultural management and land use and the risk of foodborne disease

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4.1 Introduction

In this chapter we discuss trends in agricultural management practices and land use that may influence the contamination of food with pathogens. The identification of the trends and their relationship to food markets today, or in the future, is not intended to prove causal relationships between production practices and food risks. Rather, it serves to point out changes in agricultural production that can be scientifically argued either to possess elevated risk factors or else have the potential to influence the transmission of foodborne disease.

We focus on the agricultural system in the United States to illustrate systems elsewhere in the developed world and indicate the direction of future development in the developing world. The relative extent of change may differ in other developed countries, but the linkages between the trends in modern agricultural management and land use and the risk of foodborne disease will not, except where actions have been undertaken to reduce this risk.

The way that our foods are produced has undergone huge changes over the past few decades. Government and private investment in the science of food and food production practices has led to new technologies that alter not just how food is produced, but also the nature of the food that is consumed. This technology, and the management system it has spawned, has in turn enabled greater economies of scales in production, greater consistency and uniformity in consumer product quality, and greater top-down control over the attributes of food (McDonald *et al*., 2004). On the other hand, the system has led to less efficient use of the by-products of animal agriculture, such as litter, dead

animals and animal waste. It has led to greater reliance on regulation and technology screening to reduce the risk of low-probability but high catastrophic failure of the system with concomitant consequences for pathogen transmission to food and ultimately to humans.

Key trends in agricultural management and land use are the following:

- Specialization of farms in the production of animals and animal products – that is, the growing separation of crop from livestock agriculture across farms and specialization in phases of animal production.
- Greater concentrations of animals of similar or identical genetic stock in confined or open animal feeding operations (concentrated animal feed operations, CAFO, and animal feeding operations, AFOs, respectively).
- Regional concentration of production of certain livestock, particularly of CAFOs, often in association with granaries, feedgrain depots that supply the feed or processing plants or slaughterhouses or meat processing plants to convert the livestock into food products.
- Concentration of livestock production in the vicinity of population centers.
- Relocation of confined operations in areas where fresh water is increasingly scarce.
- The economic transformation of manure and by-products of animal production into waste products with little or no local value.

4.1.1 Separation and specialization of crop and livestock agricultures and concentration of animal production on less land

As discussed in Chapter 3, the trend in the past 40 years has been for crop and livestock agricultures to separate. Farmers previously grew crops that were fed to their own or their neighbors' livestock. The livestock, in turn, produced manure and waste products that fertilized subsequent crop production on the same farm or neighboring cropland. The trend has been away from dual production farms and towards specialization in one type of agriculture. Farmers who specialize in crop production generally produce only crops. Livestock producers focus on the production of livestock and generally only one type of livestock, such as dairy, poultry, or swine. In the latter case, the waste is spread on the land and crops are grown primarily as the cheapest means of waste disposal.

Even the geographic locations of these disparate activities have separated. Large regional feed and grain dealers substitute for the traditional link between crop and animal production. These dealers, in many developed nations, such as the United States and member nations of the European Union, often buy government-subsidized feedstock that is transported to locations advantageous to the marketing or production of the livestock commodity. There is little direct, short-term economic advantage to produce livestock and crops in the same geographic vicinity. The caloric or nutritional value of grass or feedstocks grown locally no longer constrains the production of livestock within an area. Where primarily feed crops are grown, chemical fertilizers have largely

Table 4.1 Live chicken exports and imports (2004)

Country	Exports	Imports
Romania	260 000	16 178 000
Russia	1 351 000	11 724 000
Turkey	4 155 000	1 821 000
Ukraine	1 802 000	11 827 000

Source: *FAO Stat*, FAO, Rome 2005.

displaced manure as the source of nitrogen and phosphorus for the growing of crops. This de-linking of crop and livestock agricultural production has allowed them individually to move to where they have comparative economic advantage.

This geographic shift applies not only within a country, such as the United States, but also across national boundaries. In a globalized economy, feed grain is shipped halfway around the world to feed animals. The meat, the value added product from this grain, can even be shipped back to the region or nation from which the grain originated. But it is not just the process animal being shipped. Large numbers of live animals grown in certain regions or countries are also transported around the world, as evidenced by the export of live poultry (Table 4.1).

In the United States, there are fewer livestock operations where the feed is grown and more in areas where crop production is marginal (NRCS, 2000). A greater share of total livestock production occurs in operations where animals are confined (CAFOs) and the availability of cropland is scarce. The size of these operations has increased as well (see Table 4.2). Moreover, since the 1970s, the combined forces of population growth, i.e. new migration towards urban centers and expansion of these centers into traditionally rural areas, and re-location of operations closer to consumer markets and processing sectors have resulted in more animal operations located near densely populated areas (USGAO, 1995; McBride, 1997; Kohls and Uhl, 1998). This increases the

Table 4.2 Change in livestock farm size from 1982 to 1997.

Farm size category	Percentage change 1982 to 1997
Farms with fewer than 25 animal units	−64
Farms with 25 to fewer than 50 animal units	−53
Farms with 50 to fewer than 150 animal units	−43
Farms with 150 to fewer than 300 animal units	−12
Farms with 300 to fewer than 1000 animal units	36
Farms with 1000 or more animal units	88

Source: NRCS (2000), Manure nutrients relative to the assimilative capacity of cropland and pastureland to assimilate nutrients: spatial and temporal trends. December.

possibility of broad human contact with animal operations either directly or through contamination of water or air with animal waste.

Worldwide, such industrial livestock farming systems scarcely existed some 30 years ago (Holmes, 2001). They are now growing at twice the rate of traditional mixed farming systems and six times as fast as grazing-based production. Industrial systems account for 74% of the world's poultry production, 40% of pork, and 68% of the egg supply (Delgado *et al*., 2003). More than half of all poultry and pork occurring in confined feeding or 'landless' operations occurs in the developed world. Intensification and concentration of livestock production is particularly acute in East Asia (Wagner, 2002). The trend for beef production is toward industrial systems, too, with these large-scale feedlots common in the United States.

The situation in the United States regarding the amount of land available for manure spreading illustrates the problem of excess manure. Land that is controlled by CAFOs relative to the number of animals in CAFOs has increased by 60% between 1982 and 1997, the latest date for which the US Department of Agriculture provides complete data. The result is the generation of excess manure and animal residuals (manure and other by-products, such as dead animals or body parts) that cannot, with reasonable certainty, be accommodated by crops through nutrient uptake.

4.1.2 Vertical integration of agricultural production

Production in livestock agriculture occurs more and more within the framework of vertically integrated companies. These vertically integrated companies shift the decisions of what, where, and how to produce away from farmers and landowners and give the power to marketing entities. In vertical integration, a single firm controls administrative operation of two or more successive stages of production. In vertically integrated firms, management directives dictate the transfer of resources across stages of production and marketing. Vertical coordination, which refers to the synchronization of the successive stages of a production and marketing system (Martinez and Davis, 2002), is achieved through contracting.

Vertical integrators tend to be large grain brokers with established marketing links. They contract with independent growers or producers to farm the livestock. Contracts generally spell out the technology to be used by contract growers, the responsibilities of the growers in the use of that technology, and, often by default, the ownership of animal waste. The vertical integrators provide the feed, medicines, and livestock, the ownership of which they retain. The contractees provide the housing in which the animals are grown, water, and the production services.

Contract production is becoming more common as food processors and distributors seek to gain greater control over their products and ensure market outlets (Martinez, 2002). Contracts allow more information and control over factors related to quality attributes, such as the genetics of the livestock and the feed given to the animals.

4.2 Trends within specific livestock categories

Dairy farms are becoming larger with continuing consolidation and concentration in specific locations (Lakshminarayan *et al*., 1994). Nearly 50% of dairy production occurred under contract in 1998. From 1950 to 1987, the number of farms reporting milk cows declined by roughly 94%, with the average number of cows per farm increasing from fewer than 6 to 50. Regional trends suggest a shift of dairy production from the Midwest and Northeast to the West and the southern regions of the United States to take advantage of more favorable climate that contributes to lower financial outlays. The high cost of transporting dairy waste to where it can be used in crop production partly explains the transformation of manure from a valued commodity as fertilizer to a waste with little or negative value (Manale and Narrod, 1994).

The swine industry has rapidly restructured over the past 15 years. From 1993 to 2001, contracts for total hogs sold increased from 10% to 72% (Martinez, 2001). Despite the roughly constant inventory of hogs, the number of farms or operations producing swine decreased from some 200 000 in 1994 to roughly 80 000 in 2001. Hogs on farms with more than 1000 head represented 71% of the swine population in 1997, 47% in 1992, and 37% in 1987. Farms with more than 2000 head accounted for 29% of hogs in 1992 compared with 55% in 1997. Operations with 5000 or more head accounted for half of all hogs in 2001 with an average of 16.7 hogs per 0.4 hectare (1 acre) of land (Martinez, 2001).

Operations have concentrated within certain regions, generally clustering around feed granaries or slaughterhouses to reduce transportation costs. In contrast to the traditional locus of swine production in the Midwest, where most crops for feed originate, newer operations and expansion of existing operations occur in the Southeast and Southwest. In these areas, less arable land is available for the spreading of manure on crop and pasture land, the cheapest option for the disposal of animal waste. In the Midwest, producers are more likely to use pit storage for manure and slurry spreaders to deliver the fertilizer to fields and inject it into soils; in the Southeast, producers generally use lagoons for manure storage and sprinkler irrigation linked to the lagoons for delivery to fields (Ribaudo, 2003). The success of lagoon technology in preventing environmental problems depends upon proper siting and adequate storage capacity, particularly in the event of intensive storms. Sprinkler irrigation leaves the manure on the surface of fields where, unlike being injected in the soil, it can more easily wash off in intensive rain.

The poultry industry led the trend towards industrialization of livestock production. Technology developed since the 1950s enabled the automation of chicken and turkey production. Nearly all broilers and egg layers and more than half of all turkeys are produced under contracts to large integrators, with most poultry operations located within 32.19 km (20 miles) of the integrator (Ollinger *et al*., 2000). This limits the amount of land available for spreading and hence the ease and cost of disposal of the waste.

Even cattle production has undergone major changes. In 1998, some 25% of cattle were produced under contract and, in 1999, 32% were bought under

contracts or fed and owned by the beef packers. As the Congressional Research Service points out, the largest 1% of the beef feedlots produces 71% of the fed beef, yet control only 2% of the cropland on fed beef farms. The smallest 92% of feedlots produce 10% of the total but control 75% of the cropland (CRS, 1998).

A great deal of research over the past 40 years has documented the potential of large concentrations of farm animals adversely to affect water quality. The pollution containing both pathogens and nutrients originate, not just from where the animals are raised and the waste products stored, but also from the fields to which the manure and by-products have been applied. Animal waste generated in CAFOs is generally stored in storage pits or anaerobic lagoons until it can be spread on fields (Sweeten, 1992). The lagoons have been known to fail, in part because design specifications to accommodate 25-year storm events have faced more frequent storms (Pagano and Abdalla, 1994). Excessive application, inappropriate methods and timing of application, or poor selection of locations to spread the manure can exceed the assimilative capacity of plants and contaminate ground and surface waters used for drinking or washing of foods (Vanderholm, 1994; USEPA, 2004a). The United States Environmental Protection Agency (USEPA, 2004b) has identified some of the pathogens contained in manure and animal carcasses that can adversely affect human health, other livestock, aquatic life, and wildlife when introduced into the environment. Several pathogenic organisms found in manure can infect humans. Runoff from feedlots, the generally open air space within which animals such as dairy and beef cows are confined, also contains much higher levels of pollutants, including pathogens (Novotny, 1999).

Growing livestock in close quarters, as occurs in CAFOs, increases the stress level of animals and their susceptibility to disease. The possibility of disease transmission within a facility is further enhanced with the low amount of genetic diversity of the animals. Although this use of standardized animal attributes in confined settings allows for efficient production of the animals, it also allows for fast infection rates since the pathogen faces no or few genetic barriers.

On the plus side of the ledger, production of animals in confinement can reduce the interaction and contact between humans and livestock. This decreased interaction reduces the likelihood of transmission of pathogens from animals to humans and vice versa. The retention of the ownership of the living stock by the integrators creates the strong financial incentive to provide whatever assistance, both diagnostic and therapeutic, necessary to ensure the health of the animals until they are harvested.

In the developing world, the transmission of pathogens from animal to animal and animal to human is a recurrent problem (Delgado *et al*., 2003). Stock animals are raised in close contact with humans. Flocks and herds are mixed in market settings and small landowner herds can come into contact with animals in large concentrated operations. Infectious agents can become endemic in an area through the reservoir of animals raised in small operations, and backyard farms becoming re-infected through inadequate diagnostic and therapeutic services since these producers typically lack access to diagnosis and control

programs. Witness the recent concern regarding avian flu virus evolution and progression in the developing regions of East and Southeast Asia.

To reduce the incidence of microbial infection, therapeutic or prophylactic antibiotic and pesticide use in feed is commonplace and often standard practice in large confined operations. Antibiotics and pesticides in feeds are also commonly used as growth enhancers. An estimated 70–80% of all antibiotics are used globally for non-therapeutic uses in livestock (Schreier, 2002). By reducing the severity and occurrence of low-level infections, more of the animal's energy can be applied to growth rather than defending against disease. According to Schreier, most of the excess winds up in water supplies and drainage systems, the fate of which is largely unknown.

The inadvertent selecting of microbial strains for antibiotic or antiviral resistance through prophylactic use can accelerate the natural evolution of newer strains. Over-applying and spreading animal waste on crop or pasture land can contaminate agricultural land with these newer strains. From there, these new strains can be transported into surface or ground waters and introduced into human settings (JETACAR, 1999). Pathogens originating within a confined operation or inadvertently introduced into a confined operation (through human contact, feed, contaminated implements, or other means) can be retransmitted through the spreading or disposal of the waste on land.

Antibiotics are used in most phases of swine production, with their use increasing between 1990 and 1995, the only years for which data are available. For preventive purposes in feed, 39.1% of operations used antibiotics in 1990, compared with 45.5% in 1995. The 1995 survey by the US Department of Animal and Plant Health Inspection Service (APHIS) found that 92.7% of all swine at the grower/finisher stage received antibiotics in their diet at some time during this growth phase (APHIS, 1996). Virginia Cooperative Extension found that 80–90% of all starter pig feeds, 70–80% of all grower pig feeds, 50–60% of all finisher pig feeds, and 40–50% of all sow feeds are fortified with antimicrobial feed additives (Harper, 2004). For disease prevention and the promotion of growth, 91% of all operations used antibiotics in feed. In farrow-to-finish phase, 89.5% received antibiotics. In cattle production, roughly 25% of small feedlot operations and 57% of large operations used antibiotics. In dairy operations, there are regional differences in antibiotic use. The Midwest has a 95.1% antibiotic use rate and the Southeast has an 80% use rate (APHIS, 2005).

Bacteria can become resistant to the antibiotics used in feed. The US Food and Drug Administration (FDA) concluded in October 2000 that two antibiotics used in poultry had spawned drug resistance (Consumer Reports, 2005b). Soil and waterborne bacteria seem to be acquiring tetracycline resistance genes from bacteria originating in pigs' guts (Ananthaswamy, 2001). Prophylactic feeding of antibiotics to animals can lead to the emergence of resistant strains of gut bacteria, such as *Salmonella* and hence enhance pathogenic risks.

The developed world is not without its incubators of animal diseases that can and do infect animal vectors that can serve as transmission vehicles. In the United States, a trend is to growing of wild animals, such as elk, bison, and deer,

in confined settings. These confined animals can serve as a reservoir and amplifier of pathogenic diseases that can then be passed to domesticated animals or other wild animals (Bulmer, 1989; Meagher and Meyer, 1994). The organisms can thence be transmitted to humans through direct contact (CDC, 2005) or through exposure to a contaminated wild animal.

4.3 Policies and decisions affecting location of livestock production

The shift to vertical coordination of livestock production in poultry and swine has led to changes in decisions that growers and landowners make affecting the location of livestock production. To reduce transportation costs, integrators generally contract within 30–50 kilometers (20–30 miles) of the plant, granary, or processing facility. The volume of production within a watershed will depend primarily upon the decisions of the integrator or integrators regarding animal slaughter. This in turn determines how many animals are fed by growers within the contracting area – the area within a certain number of kilometers of the processing facility or feed grain elevator. Hence, the availability of non-grower land upon which to spread excess manure can fall outside the control of the landowner or grower if the landowner or grower must compete for available land upon which to dispose or spread manure. The decisions of the vertical integrator thereby also affect the 'value' of the manure (specifically by decreasing the value) as an input in crop production. Hence the responsibility for the consequences of the decision most critical for the environment has shifted away from the person or entity with greatest control or influence over the decisions – the integrator or processor – to the grower or producer with the least leverage or set of options for managing the environmental risk other than land application of the waste.

There is some evidence suggesting that large-scale operations with large amounts of liquid animal waste, such as pigs and dairies, locate in certain areas, such as the more arid areas of the southwest, to take advantage of lower precipitation. Doing so enables large-scale operation, reduces unit costs, and thereby achieves some cost savings for waste treatment (ERS, 2000).

With the globalization of food production, regulatory and policy decisions affecting how food is produced by some trading partners become important for all trading partners in the market. Locational decisions are but one example. The European Union (EU) regulations for nitrate (nutrient) emissions from livestock operations (the Nitrate Directive) influenced producers to curtail production in the EU and locate facilities in the United States (ERS, 2000).

Another is the safety of the imported products since the share of imported livestock products in the diet of the United States is increasing (Putnam and Allshouse, 2001). The history of the transmission of mad cow disease illustrates this point. Decisions made decades ago in Britain to use dead livestock as a protein supplement for beef production led to the transmission of the disease to

other livestock not just within Britain, but also in its trading partners' herds (Kirby, 1999).

Agricultural policies in the developed world related to the production of grain and oilseeds have also affected the location of livestock production. Support to farmers and agricultural operators has often been in the form of direct and indirect subsidies for the production of grain and other crops grown as feedstock. The subsidies have lowered the effective cost of feedstock and hence helped shift the competitive balance away from extensive, grass-fed or grazed livestock production to confined production (Delgado *et al*., 2003).

4.4 Change in availability of land for waste disposal in the vicinity of concentrated livestock operations

Excess manure nutrients, such as nitrogen and phosphorus, in quantities that are not assimilated by crops and hence are available for transport from fields to water bodies, have largely followed the shift in production location. More and more counties in the US have faced the problem of excess nutrients since 1982 (Fig. 4.1). The occurrence of excess nitrogen and phosphorus is important in the issue of pathogen transmission for the following reasons: not only do they provide the medium in which pathogens may flourish when they contaminate water supplies since both serve as 'feed' for microbial populations, but they also indicate where excess manure may have been applied to the land in general, and hence possible sources of greater pathogen loads.

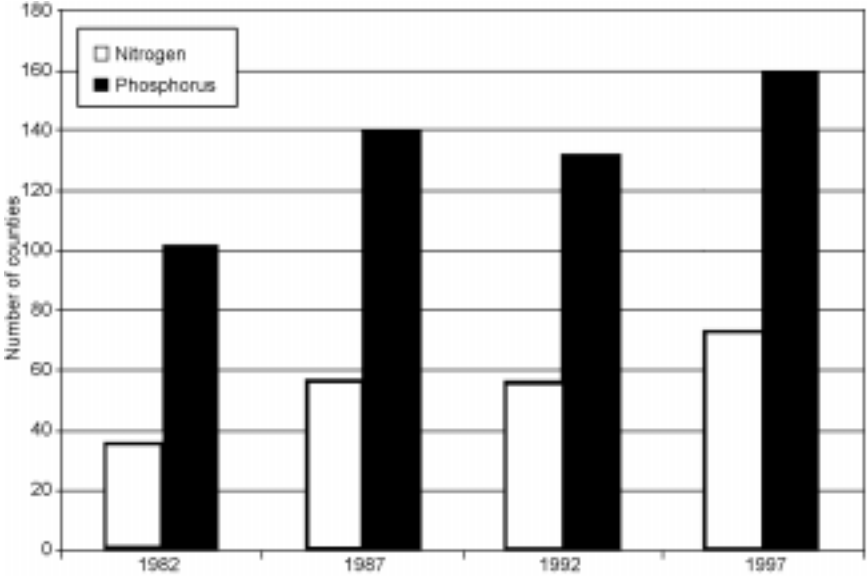

Fig. 4.1 Number of counties with county-level excess nutrients. Source: NRCS (2000).

The situation in other parts of the world with large concentrations of animals is not significantly different. World Resources Institute reports that 'manure quantities in East Asia are so great that capacity of plants to use the nutrients provided is sometimes exceeded by 1000 kg of nitrogen per hectare' (Fritschel and Mohan, 1999). Taking into account cattle, sheep and goats, horses, water buffalo, and camels, livestock densities globally range from less than 50 head to over 50 000 head of livestock per square kilometer. Some of the highest densities in the world are in the Middle East, Asia, and Australia (Holmes, 2001).

4.5 Implications of land management on contamination of animal- and plant-based foods

Where pathogen and nutrient contaminants run off from the land and into water supplies, the nutrients in the water bodies can create the medium for microbial growth. They can also contaminate shellfish beds and cause fish kills. The nitrogen and phosphorus from runoff into water bodies can contribute (through nutrient loading) to periodic algae blooms, especially of *Microcystis aeruginosa* and *Pfiesteria piscicida*, which can adversely affect human health through direct contact. The algae blooms of Chesapeake Bay and other estuaries of the United States serve as examples (Magnien, 2001; Burkholder, 2001). It is harmful to humans because contact with, inhalation, or consumption of water with *Microcystis* and related blue-green algae blooms can cause illness in humans and death in livestock or pets. Pathogen-contaminated water used in the irrigation of crops can transfer the pathogens to plant-based products destined for raw consumption.

A growing concern in the United States is the potential transfer of pathogens from animal agriculture to crops through the use of untreated animal waste or inadequately treated composted waste. In recent years, there have been a number of reports on USDA recalls of fresh vegetables owing to their contamination with pathogens from manure used as fertilizer (FDA, 1998; Cornell University, 2005).

Nevertheless, there are scant data on how manure is used on crops. A survey published by USDA's National Agricultural Statistics Service (NASS, 2001) suggests that manure, treated (generally composted or other heat treatment) and untreated (raw), is applied to a small but growing percentage of cropland used for vegetable crops (see Fig. 4.2). In 1999, roughly 3% of cropland, upon which vegetables are grown, was fertilized with manure, the latest year for which data are available. However, some 6% of head lettuce, 2% of other types of lettuce, and 12% of celery are grown with manure. The only national data that can provide insight into trends are the data collected in 1998 and 1999 regarding the percentages of farms applying manure in the growing of vegetable crops. As Fig. 4.3 indicates, there was a slight – 1% – increase in manure use on farms growing vegetables from 1998 to 1999, which is consistent with anecdotal information.

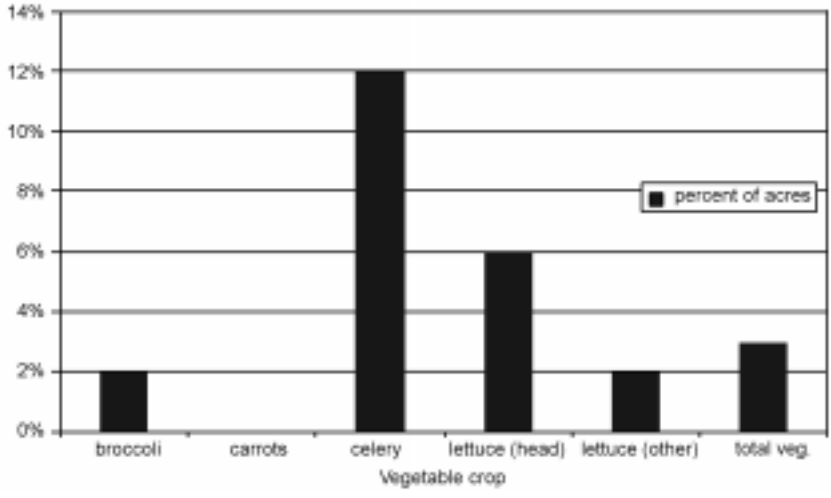

Fig. 4.2 Manure applied to cropland in 1999. Source: USDA NASS.

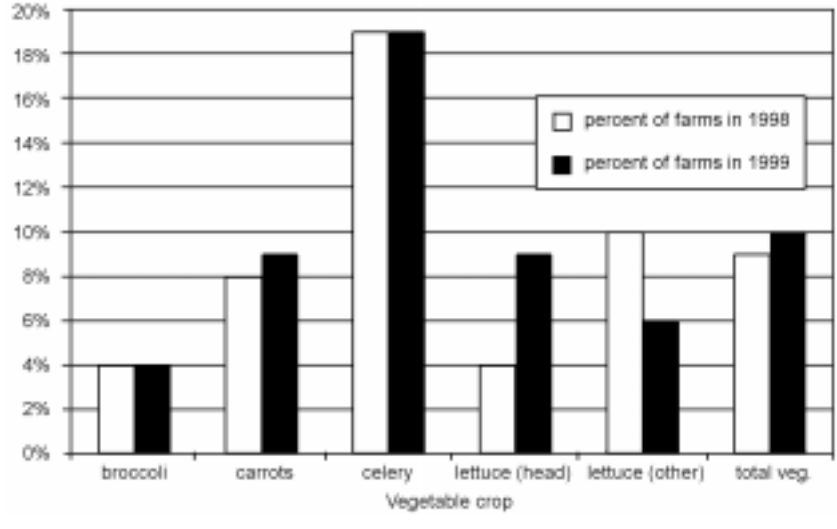

Fig. 4.3 Manure applied to cropland, 1998 versus 1999. Source: USDA NASS.

4.5.1 Recycling of animal products

The surplus of animal waste and by-products has led to innovative ways to dispose of the excess. Poultry litter, floor wastes from coops, including feces and plastic pellets, are permitted as roughage in feed (CFR Title 21, vol 6, revised as of April 1, 2005. 21CFR589.2000). Meat and bone meal are also permitted. In fact, 10–30% of feed for cattle and chickens can contain constituents other than plant-based materials (Consumer Reports, 2005a).

In Britain, poultry waste has been a legally permitted feed ingredient and was extensively used until about ten years ago. About 25 years ago, both human and animal waste – both liquid and solid – were used in feed in the United Kingdom (Kirby, 1999).

Though proper treatment of the waste eliminates the possibility of transmission of most pathogens, it does not suffice for prions, infectious protein agents responsible for such diseases as BSE (mad cow disease). Composting, for example, which destroys most pathogens in manure through the heat generated in the composting process, does not destroy the heat-resistant prion. Moreover, proper treatment of waste requires oversight to ensure compliance with standards for treatment. Hence, the possibility remains of the transmission of pathogens through waste from one species to another and from animals in one location to many animals throughout a region or even the world.

4.5.2 Implications for clean water supplies

Agriculture, with few exceptions, converts large amounts of clean water into reduced quantities of lower-quality water. Contamination with pathogens is just one by-product of agriculture, particularly in the conduct of intensively managed animal agriculture. According to Schreier (2002), if livestock numbers globally were translated into human waste equivalents, the total waste would be equivalent to that produced by 18 billion people. With current trends in management of animal production, the more intensive and concentrated the operation within a watershed, the greater the local impact.

Yet the supply of fresh water worldwide for consumptive use is finite. The more high-quality water that is used in livestock agriculture worldwide, the less there is for humans, except at considerable expense of purification and decontamination. As Fig. 4.4 shows, increasing global population is accompanied by a commensurate increase in demand for fresh water.

As more people demand more meat and livestock-related products, the transformation of fresh water to lower quantities of poorer-quality water can be expected to grow at least linearly with this trend, if not exponentially. Wastewater, particularly sewage waters, may already be used on a tenth of the world's irrigated crops (Pearce, 2004). Humans have already appropriated approximately 50% of accessible global freshwater runoff and this could increase to 70% by 2025 (Postel *et al*., 1996) with adverse environmental consequences (Rosenberg *et al*., 2000). The US Geological Survey estimates that during 1995, the last year for which data are available nationally, 20.75 billion litres (5490 million gallons) per day were withdrawn for total livestock purposes in the United States (USGS, 1995). This is 22% more than in 1990. The consumptive use in 1995 was 58% of withdrawals.

The global supply of renewable fresh water per person has followed a decreasing trend since the 1950s, with an expected overall decrease of 60% by 2030 (Postel *et al*., 1996). For every pound (0.45 kg) of milk produced per day, 24 to 52 gallons (91 to 197 litres) of water are consumed by the dairy cow, not

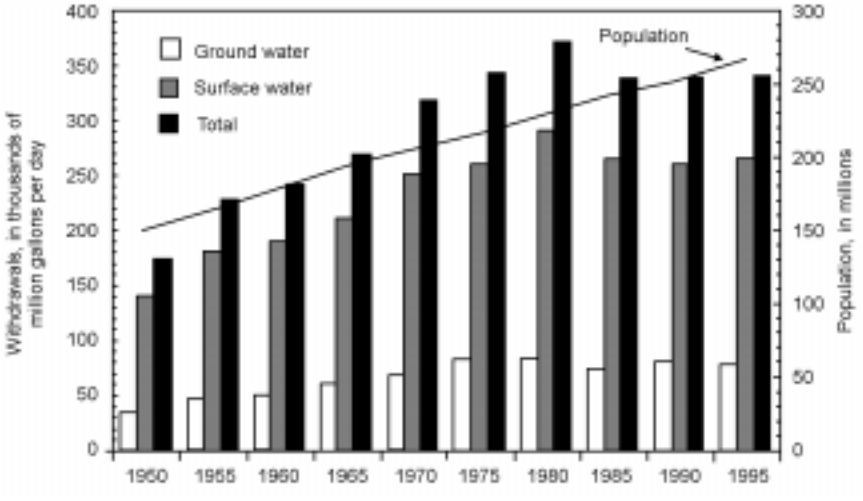

Fig. 4.4 Trend in fresh ground and surface water withdrawals and population, 1950–1995. Source: USGS.

including water needs for cleaning and other purposes (Univ. of Nebraska, 1993). The Agri-Food Research & Development Initiative in Canada has estimated the total daily water usage in the production of a sow from farrow to finish, as 89.5 l with a range of 71.3 to 110 l (ARDI, 2001). Of this, some 7.6–30.1 l of wastewater is generated per sow per day through gestation and farrowing. Water needs for beef range from 15 000 to 70 000 kg of water per kg of food (Schreier, 2002). Compare these numbers to the US Environmental Protection Agency estimated requirement of 2 l of water per day for a person in the United States. Moreover, because of the volumes of water needed by livestock operations, especially fresh water, these operations will continue to be located in areas where competition with human consumption is likely to occur.

4.6 Future trends

There has always been a tendency in the United States to look for technological fixes for all social or economic problems. Often the political path of least resistance from consumers who want cheap food to powerful agribusiness interests who want cheap raw inputs (i.e. animals and crops) is to call for more research and hope for the discovery of a new technology that solves the political problem, at least for today. The future can find its own solutions.

In the United States and in most of the developed world, the human waste generated in cities is treated. Animal waste, on the other hand, is believed to merit different treatment and is allowed to be dumped on the land where it is thought to be managed in a non-harmful manner. This assumption that animal waste is a valuable commodity that will be handled in an environmentally sound

manner – without government or other external pressure – because of its importance as a crop fertilizer appears no longer to apply to animal waste from industrialized agriculture.

New technologies are being developed to treat human-generated waste more cheaply and efficiently to render it less harmful to humans and the environment (USEPA, 1999). Despite the applicability of these technologies to animal-generated wastes, the economics of doing so have been prohibitive until now.

Excess animal waste applied to not enough land as a source and pathway for pathogens into the environment and ultimately to humans begets its own technological grail. The major hurdles to resolve the problem are posed by, among others, large initial investment costs, the high shipping cost of transporting high moisture-laden wastes, and the potential health and environmental hazards posed by the movement of large quantities of wastes from where they are generated to where they can be safely and properly disposed of. Options currently being actively explored, though none appears to be the 'silver bullet,' include the following:

- composting;
- burning;
- biotechnological changes to the feed that alters the characteristics of the waste (CRS, 1998).

Composting, to eliminate the weight and volume of the waste, presumes a market for the compost that facilitates transfer from the facilities where manure is generated to the agricultural or forest enterprise that can utilize it. This 'commoditization of compost' requires a system of oversight to assure attributes of consistent quality, just like every other commodity where buyers and sellers are geographically disconnected. Such a system exists now almost exclusively for organic fertilizers used in organic agriculture.

Burning to generate energy solves the pathogenic issue but does not solve the problems of eliminating all the constituents of waste that do not combust, such as phosphorus or nitrogen. Moreover, the economics of competing with conventional fuels are unfavorable except at relative high fossil fuel costs.

Biotechnological fixes include enzymes in feed that reduce the phosphorus in animal excrement. They do nothing for the pathogenic potential of the excrement, however, nor do they do much to reduce the volume of waste that is spread on land. On the other hand, they resolve one of the drawbacks of burning, which is the volume of residual material after combustion. Other new biotechnologies, such as anammox bacteria (Pilcher, 2005) may help in the future to fix some remaining problems with residuals, assuming that there is regulatory or market pressure to adopt such technologies by the livestock industry. The issue is not the existence of the technology for treating the waste and rendering it into a less harmful form, but rather the unfavorable economics of applying off-the-shelf technology. Land application of untreated or at best combusted waste remains, without strong regulatory pressure, the most effective option for disposal.

Precision agriculture, whereby new technologies such as global positioning systems and geographic information systems aid decision-making promises,

more accurately delivers manure and nutrients to plants on cropland. New devices can keep precise records of where manure has been applied to prevent over-application (Ess *et al*., 2005). These promising technologies work only if the overall nutrient value of the manure applied does not exceed the potential for uptake by the crops and incorporation into new soils.

Aseptic production of animals in a closed or largely closed system can serve to minimize the likelihood of transmission between groups of animals, and to control outbreaks of disease within production groups. The technological and capital requirements would likely lead to considerable increases in the cost of production, with implications for further consolidation of production and concentration of production in certain regions, exacerbating the problem of what to do with the waste. Moreover, the system is not closed until waste can also be treated before it is returned to the environment. Combustion of animal waste may reduce the likelihood of the release of pathogens into the environment, but it carries its own set of environmental problems. These include contributing to greenhouse gas emissions and, perhaps more importantly, reducing the supply of carbon residuals that are important for rebuilding and replenishing cropland soils. Composting of the residual waste can serve to control pathogens in the residual and ensure the availability of organic matter for restoring and enhancing cropland soils. However, strict regulation or other oversight may be required to ensure control over the process of waste management and compliance with guidelines.

Better siting of confined animal feeding operations is another option that can be practiced without undue expense (Manitoba Agriculture, Rural and Food Initiatives, 2005). New livestock operations are restricted in areas where there is an abundance of animals and encouraged to locate where the waste generated can be better assimilated by crops and other living matter and the risk of water contamination by nutrients and pathogens is reduced. Siting requires planning and assessment of the assimilative capacity of the landscape to absorb the manure and an institutional structure to conduct these activities. This is certainly possible in the developed world, but less so in developing countries where the necessary institutional structures may be lacking.

Considering the vertical organization of much livestock production and who makes key production decisions, the question of siting should not be limited to individual livestock operations themselves, but also the grain depot and the animal processing facility. As discussed earlier, the extent of production within a watershed or region is determined to a large extent by the decisions of the integrator or processor with regard to processing throughput. Engaging the processors along with farmers and livestock managers and owners in the question of proper management of livestock waste would simply reflect the current reality of how key decisions are made related to livestock production practices and location and who makes them.

Less intensive or extensive agriculture with more genetically diverse animals can reverse the trends and the associated risks described in this chapter. There is some indication that growing consumer concerns about the problems of

industrial animal and crop production has led to niche markets in the agricultural and livestock products of alternative agricultural or sustainable techniques. One example is 'organic foods' in the United States which are grown under less intensive production practices, without prophylactic use of pesticides and antibiotics and with a more genetically diverse living stock. Though representing only a small portion of total food production in the United States, about 1–2% (Dimitri and Greene, 2002), the share of consumer expenditure on 'organic' food continues to grow. Disease risks from pathogen transmission through raw crops remain, however, through the inadvertent treatment of crops with poorly composted manure or other inappropriately generated organic fertilizers. Hence, even in the case of organic production of foods, prevention of pathogenic transmission to humans requires guidance and oversight.

In 2003, the US Environmental Protection Agency (USEPA, 2003) issued a final rule, effective April 14, 2003, for regulating discharges from confined animal feeding operations to protect water quality. The rule strengthened existing regulations on CAFOs by requiring all CAFOs to apply discharge permits and implement a nutrient management plan. This regulatory action is expected to lead to greater adoption of practices that better control emissions by the estimated 15 500 CAFOs and roughly 270 billion kg (300 million tons) of manure EPA estimates they produce each year. How it will affect locational decisions of livestock operations remains to be seen.

4.7 Sources of further information and advice

World Resources 'Global Livestock Densities' (World Resources Institute, 2005) presents a global perspective on the problem of livestock densities. For a closer look at structural changes in a particular animal sector in the United States, I recommend Economic Research Service's production, Economics and Structural Relationships in U.S. Hog Production, AER-818 (Ribaudo, 2003). For a glimpse into the future of consumer demand for meat globally, read Delgado *et al.*'s '2020 Vision for Food, Agriculture, and the Environment' (Delgado *et al.*, 1999). For a general overview of how agriculture and the nature of food production has changed in the United States since the beginning of the twentieth century, I suggest National Agriculture Statistics Service's, 'Trends in U.S. Agriculture' (NASS, 2005). United States Environmental Protection Agency's NPDES (National Pollutant Discharge Elimination System) website http://cfpub.epa.gov/npdes/pubs provides a list of documents on the environmental impacts of confined or concentrated livestock production from a US Government perspective. There are also numerous documents presenting EPA's arguments for regulating discharges from confined animal feeding operations. I also recommend EPA's Proposed Rule Economic Documents for Concentrated Animal Feeding Operations (CAFOs) (USEPA, 2001) for a presentation of arguments for why CAFOs need to be regulated to protect public health and the environment along with the evidence for pathogen loadings from animal feeding

operations. Finally, I suggest USDA's report, 'Waterborne Pathogens in Agricultural Watersheds' by Barry H. Rosen, for a discussion of how agricultural livestock practices can cause pathogen contamination of water bodies, the pathogens of concern, and control methods.

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5

Influence of food processing practices and technologies on consumer–pathogen interactions

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5.1 Introduction

All raw foods contain microorganisms that will eventually cause spoilage unless they are controlled or destroyed. Many of the thousands of microorganisms that have been discovered and identified perform some useful function, such as the production of fermented foods (breads, cheese, wine, beer, sauerkraut, sausages, olives, tea and chocolate, to name just a few). However, it is also true that many raw foods contain pathogens that, if not controlled, can result in human illness. Thus, there are two major roles of food processing – to preserve food against spoilage and to render a food safe for consumption by eliminating or controlling pathogens. Pathogen control in foods results from preventing contamination, applying an inactivation treatment, preventing pathogen growth, or a combination of these practices.

This chapter will cover the role of food processing in increasing or decreasing contamination, growth and survival of pathogens in food. There is a long history of preservation of foods; however, the science behind the safety of food preservation is relatively new, as is noted in Section 5.2. Section 5.3 addresses how science and technology have influenced production and manufacturing processes, including the impact of the adoption of the Hazard Analysis and Critical Control Point (HACCP) system in the food manufacturing sector. Section 5.3 also describes how foodborne illness outbreaks and new surveillance strategies have resulted in the development of new food safety control measures. Section 5.4 describes how consumer preferences can sometimes have a negative impact on public health, when preservation systems are reduced to meet

consumers' desires for fresher, more natural products. Future trends (Section 5.6) include better identification of risk mitigation strategies and use of on-farm controls.

5.2 Historical perspective on food processing – Roman sausage to canning to space food

Early humans were hunters and gatherers. Getting food was a daily process, and food spoilage and foodborne illnesses must have been common. Agricultural production of grains and animal husbandry followed the hunting/gathering stage, although hunting and gathering remained common means of obtaining food. Early forms of preservation such as salting, drying, smoking and fermenting were practiced long before people understood why they worked, and were likely discovered by accident. Although food safety was probably not at the forefront of early man's concern when they were just trying to get enough food to survive, these food preservation techniques that inhibited food spoilage microorganisms had the added benefit of inhibiting many pathogenic organisms. Early attempts at fermentation were probably especially fraught with dangers. *Clostridium botulinum* is derived from the Latin term *botulus*, meaning sausage. The 'controlled spoilage' under the specific conditions of fermentation allows yeast and lactic acid bacteria to grow and prevents the growth of putrefactive bacteria. Even today, there are outbreaks of botulism associated with Inuit meat fermentation practices, probably because of variation from the traditional practices. One can imagine the cases of foodborne disease that may have occurred during the evolution of current technologies appropriate for safety.

In nontropical climates, where plant crops were seasonal, in order to have some of the food last through the lean times of the winter, humans would apply the techniques of drying, salting, and fermenting. Since there was a limited amount of plant food and animal feed during the winter, it was necessary to slaughter most of the livestock and apply these same preservation techniques to their meat (Thorne, 1986). Cultures in warmer climates did not have to worry about having enough food to last through the winter, but they had to deal with rapid spoilage of fresh food year round. These cultures developed methods to ferment milk and air dry other foods. Mesopotamians (3000 BC) used salt to preserve meat and fish (Bottero, in CAST, 1997) and Cato (234–149 BC) described salting meats and vegetables to preserve them (CAST, 1997). Asian cultures developed methods for fermenting soy products to preserve foods.

While early humans were probably more preoccupied with identifying the source of their next meal than they were with food safety, it is easy to assume that through observation they were able to figure out what foods or practices led to acute illness or death. Illnesses due to the consumption of certain plants or rotten flesh or observing foodborne outbreaks among groups of people that ate certain foods such as shellfish or pork may have taught early humans to avoid these foods. It is easy to go from these types of assumptions about early man's

food safety education to the conclusion that religious dietary laws were the first food safety regulations. In a review of Jewish kosher and Muslim halal food laws, Regenstein *et al*. (2003) state that this is not the case. Both Jewish kosher and Muslim halal laws address specific animals that are prohibited from being consumed, prohibition of blood and methods of slaughter. Halal further prohibits consumption of carrion and intoxicants. While both sets of dietary laws may seem to have their origins in food safety, Regenstein *et al*. (2003) state that the laws are divinely inspired and are not to be questioned as to their origins. The authors argue that while it may be commonly assumed that prohibition of pork evolved from attempts to prevent trichinosis, this is not the case. While other types of meat are sources of pathogens that can be controlled by fully cooking the meat, there are no religious guidelines to do such. *Trichinella* has not been isolated from samples of ancient mummified pork and the 10–14 day incubation period was probably too long for early humans to associate consumption of pork with onset of illness.

While vegetarianism is commonly practiced among Buddhists, Hindus and Seventh Day Adventists, this is more of a cultural discipline than religious doctrine. The exception is the prohibition of beef consumption by the Hindus. Vegetarianism seems to have its roots more in the health of the practitioner and in other ethical and spiritual ideals than in food safety.

5.2.1 Canning

The history of canning began with Nicholas Appert, a French confectioner, who heated foods in wide-mouthed, corked glass bottles in boiling water to preserve them for use by the French military. At the time, preservation was primarily through drying or the addition of 'a foreign substance for the purpose of impeding fermentation or putrefaction' (sugar, salt, vinegar), each process having specific drawbacks (Appert, 1812). The French government, at war with several countries, offered rewards for the development of means to use 'indigenous substances' and 'diminish the consumption of foreign commodities' (specifically, sugar). The French navy began using Appert's 'canned' (bottled) meats, vegetables, fruits and milk around 1806, which led to his reward of 12 000 francs in 1809 for his process for preserving foods (Drummond and Lewis, 1939). Although Leeuwenhoek had described bacteria viewed through his microscope in 1683, their role in food was unknown at the time. In 1810 Appert published the first book on canning (translated into English in 1812), in which he provided detailed descriptions of his methods for preserving a wide variety of products, including meats, gravy, vegetables, fruits, eggs, milk, and how to prepare the preserved products (Appert, 1812). He believed that the combination of heat and the exclusion of air prevented decomposition, a view that was widely held for most of the nineteenth century. Some 50 years later, Louis Pasteur showed that certain microorganisms are responsible for fermentation and decay. He conducted experiments on food preservation, and the term 'pasteurization,' first used to destroy undesirable microorganisms in wine, bears his name.

August de Heine and Peter Durand patented the use of iron and tin containers for preserving foods in 1810, and John Hall and Bryan Donkin applied Appert's process to food in metal containers beginning in 1811 (Drummond and Lewis, 1939). 'Tinned foods' became used frequently by the British navy and army and on explorations for the Northwest Passage and on Arctic explorations in the first half of the nineteenth century (Drummond and Lewis, 1939). Processes achieving higher temperatures by addition of calcium chloride to the water were introduced in the mid-nineteenth century (Downing, 1996; Drummond and Lewis, 1939; Jackson, 1979). However, the introduction of large containers (9–32 lbs) led to significant spoilage (Drummond and Lewis, 1939). Canning in the United States was first used by William Underwood in 1819 and tin cans became widely used in the United States in the mid-1800s; the containers were originally known as 'canisters,' which was shortened to 'can' (Jackson, 1979). The firm Chevallier-Appert introduced the concept of pressure processing of canned foods (Downing, 1996) and Shriver introduced the closed retort in the United States in 1874 (Jackson, 1979). This, along with the mechanization of food preparation and can manufacture led to mass production of canned foods, and the number of canning plants and canned products increased during the American Civil War and the years following it (Jackson, 1979). The development of the open top, 'sanitary' double-seamed, three-piece can at the beginning of the twentieth century permitted additional mechanization and the development of the canning industry (Downing, 1996; Jackson, 1979).

Early canning processes were arbitrary – canning was more of an art than a science – and there were frequent losses due to spoilage. Food safety scares were abundant in the United States at the beginning of the twentieth century, including numerous cases of botulism from canned foods. This resulted in passage of the Pure Food and Drugs Act (and the Meat Inspection Act) and led to the establishment of the Food and Drug Administration from the Bureau of Chemistry of the US Department of Agriculture. It was the formation of the National Canners Association in 1907 and the establishment of its first laboratory in 1913 that led to science-based thermal processes for canned foods. NCA scientists developed the use of thermocouples for heat penetration studies to set processes for canned foods (NCA, 1920) and the thermal death time (TDT) techniques for determining the heat resistance of spores (Esty and Williams, 1924). Using this technique, Esty and Meyers (1922) established the 'classic' heat resistance of *Clostridium botulinum*, which served as the basis for the '12D' concept still used today. C.O. Ball (1923) pioneered the combination of TDT and heat penetration data with mathematical calculations to establish thermal processes, and the science was fully established for production of safe canned foods.

5.2.2 Refrigeration and freezing

Canning was not the only method being developed for preservation of food. The use of cold temperatures as a means of preserving foods had long been

recognized. As long ago as 1000 BC, the Chinese used ice cellars to preserve foods (Archer, 2004). In Egypt, Greece, Rome, and India evaporative cooling was used to cool water in clay vessels at night (Tressler and Evers, 1943; Woolrich, 1968). Packing foods in snow or in ice cut from lakes or ponds in the winter was common in many countries until the development of mechanical refrigeration in the 1800s. An entire industry developed around cutting large blocks of ice from Northern lakes in the winter, transporting them to southern parts of the United States, and using the blocks of ice as a means of holding perishable products for extended periods of time. Natural methods of cooling foods were supplemented with chemical solutions such as saltpeter in water or calcium chloride in snow to reduce the temperature. Some of these solutions were used commercially in machines to freeze foods, but were soon rendered obsolete by mechanical refrigeration and freezing (Woolrich, 1968). Compression systems were developed in the early 1800s, leading to the development of refrigeration machines in Texas and Louisiana in the United States and in Sydney, Australia, by the middle of the century (Woolrich, 1968). Carl von Linde, in Germany, patented an ammonia compressor in 1876 that demonstrated the possibilities for ammonia refrigeration and led to systems with improved condensers and evaporators and a variety of refrigerants (Tressler and Evers, 1943; Woolrich, 1968). These systems were used for both refrigeration and freezing. Early uses included freezing of fish and meat and opened up distant markets. In the 1870s frozen beef and mutton were successfully transported from Australia to England (Lund, 2000).

Modern freezing technology began with Clarence Birdseye, who, based on his experience with frozen seafood, developed rapid freezing technology that better preserved the quality of foods. A significant percentage of foods sold today are frozen (Lund, 2000). Although it was long held that refrigeration prevented growth of pathogens, we now recognize there are a number of pathogens capable of growth at refrigeration temperatures, including *Listeria monocytogenes* and some strains of *Clostridium botulinum*. Nevertheless, refrigeration significantly slows the growth even of those pathogens that can grow at low temperatures. Freezing, on the other hand, prevents growth of bacterial pathogens and sometimes kills them (Archer, 2004). Refrigeration and freezing are of key importance in the safety of foods, and are thus commonly used by the processing industry.

5.2.3 Milk pasteurization

One of the most significant advances in food processing impacting the safety of the food supply was the development of processes to pasteurize milk. Although the heating of milk was recommended to extend shelf-life as early as 1824 by William Dewees, its role in microbial destruction was not recognized until the work of Pasteur (Westhoff, 1978). Early proponents of heating milk supported its use for infant feeding, but its popularity grew because of the increase in shelf-life. Commercial pasteurization was a common practice in Denmark and

Sweden in the mid-1880s to prevent spread of tuberculosis (Westhoff, 1978). There was much debate over the benefits and disadvantages of milk pasteurization. Even medical authorities, who recognized the treatment as a means of preventing milkborne diseases, were opposed to milk dealers heating milk owing to the potential for recontamination. Many were skeptical about the methods, particularly high temperature, short-time (HTST) treatments, because of the great variability of flow rate, temperature, and holding time (Westhoff, 1978). In the 1920s a major research effort established not only the appropriate time and temperature that would inactivate microorganisms of concern in milk (human and bovine tubercle bacilli, typhoid and paratyphoid bacilli, diphtheria bacilli and hemolytic streptococci) but also uncovered the defects in the then available pasteurization equipment. These studies led to improvements in engineering, construction, and controls of milk pasteurizers to ensure proper safeguards for public health (Westhoff, 1978). Based on these studies, the first federal standard for milk pasteurization (61.7 ºC (143 ºF) for 30 minutes), was established in 1924 based on destruction of *Mycobacterium tuberculosis* (Meanwell, 1927; Westhoff, 1978). In the 1950s pasteurization processes were increased to 63 ºC (145 ºF) for 30 minutes when it was recognized that *Coxiella burnetii* (the agent of Q fever) was more resistant than *M. tuberculosis* (Westhoff, 1978). An HTST pasteurization (72 ºC (161 ºF) for 15 seconds) process also was established.

5.2.4 Irradiation

One of the newer methods of food processing is irradiation – the exposure of foods to ionizing radiation such as gamma rays, X-rays, or electron beams. Radiation extends shelf-life by delaying ripening of fruits and vegetables or destroying spoilage microorganisms; it inhibits sprouting of vegetables such as potatoes and onions; and, most importantly for public health, it inactivates many pathogens. Although the benefits of irradiation in making food safer and more plentiful have been studied extensively and the safety of the treated foods has been well established, this process has generated significant negative consumer reaction (WHO, 1988), as will be noted later. The lack of public acceptance has limited the use of an effective food safety tool.

5.2.5 Foods for special uses

Food processors are aware of the special needs of consumers such as infants, the elderly (http://nutritionandaging.fiu.edu/downloads/Med_Fds_Background_final.doc), and immunocompromised patients. In addition to providing foods that are highly nutritious and meet physical requirements for chewing and swallowing, the foods may be produced with an extra degree of safety for these especially sensitive groups. Recently, *Enterobacter sakazakii* was recognized as a potential pathogen in powdered infant formula for infants under 2 months old (WHO, 2004). Processors of powder infant formula use special precautions in monitoring of ingredients, the processing environment, and finished product for

this organism, as well as applying a lethal step of pasteurization to the wet formula prior to drying. Nevertheless, since absence of *E. sakazakii* cannot be guaranteed, proper use by the consumer is important to prevent illness.

Military food and foods for space travel represent areas with specialized needs. They share requirements of high levels of nutrition, portability, stability without refrigeration, possible compaction and ease of rehydration. For the military, the food has to be organoleptically desirable to ensure that soldiers will consume enough to replace calories used under extreme physical demands such as battle. The history and current state of development of military food can be found at http://www.militaryfood.org/ and http://www.usariem.army.mil/nutri/milrat.htm. Space food has additional challenges, such as minimizing crumbs or enhancing aromas in a zero gravity environment, being lightweight, and having packaging that is easily disposed of. There will be additional requirements on extended lunar and Mars missions in the future. More can be found on the development of space food at http://spaceflight.nasa.gov/living/spacefood/, http://advlifesupport.jsc.nasa.gov/Food/index.html and http://www.ag.iastate.edu/centers/ftcsc/pages/insig.htm. Obviously, since outbreaks of foodborne disease on the battlefield or in the confines of outer space would be highly undesirable, processing and packaging of these foods to destroy pathogens and prevent recontamination are always in the forefront of their development.

A recent trend is toward the consumption of 'functional foods,' which are foods or dietary components designed to support health and reduce the risk of chronic, diet-related illnesses and conditions, including cardiac disease, osteoporosis, and cancer (Hasler, 1998). Most examples of functional foods are plant based, such as oats, soy, flaxseed, garlic, tomatoes, broccoli and other cruciferous vegetables, citrus fruits, grapes, olive oil, and cranberries. Fatty fish and eggs from chickens fed flaxseed are good sources of omega-3-fatty acids. Fernandez-Gines *et al*. (2005) reviewed meat products that are formulated with additional plant products and have reduced or modified lipid content as functional foods.

Dairy products have long been known to be a functional food, as a good source of calcium. Additionally, fermented dairy products such as yogurt and kefir are a source of probiotics, live microbial food ingredients that are beneficial to human health (Roberfroid, 2000). Some probiotics are not part of a fermented milk product but are added to the milk as freeze-dried cultures. Intestinal microflora play an important role in the maintenance of health. Consumption of these live bacterial cultures are thought to affect the microbial ecology of the intestinal tract by colonization and replacement of non-beneficial bacteria (see also Chapter 8). Proposed benefits of the consumption of high levels of certain exogenous bacteria such as strains of *Lactobacillus* and *Bifidobacterium* include resistance to enteric pathogens, anti-colon cancer effect, strengthening of the immune response and alleviation of lactose intolerance (Sanders, 1999). Prebiotics are inulin-type fructans, i.e. carbohydrates that are indigestible by humans but are available in the lumen of the gut to stimulate the growth, activity, and colonization of probiotic and resident organisms in the intestinal tract.

5.3 Influence of science and technology on production and manufacturing processes

Science and technology have always influenced production and manufacturing processes, resulting in higher-quality and safer food products, as well as the development of new types of products. In the development of new products and processes, the effect of the new technology on pathogens has been investigated to ensure that technologies are effective in producing safe products and do not create new safety issues. Many of the recent scientific and technological developments that have been investigated with respect to reduction of foodborne illness from pathogens during production and manufacturing are the result of implementation of HACCP by the food industry.

5.3.1 The role of HACCP in the production of safe food

HACCP is a systematic framework for identifying hazards of concern in a product, determining the points critical to control the hazard during production, applying appropriate controls, and documenting delivery of the control parameters. The successful application of HACCP depends on understanding the hazards (e.g. a pathogen), the sources of contamination, and the effects of processing steps on the hazard.

HACCP was developed by the Pillsbury Company, the US Army Natick Laboratories and the National Aeronautics and Space Administration (NASA) in response to the food safety requirements imposed by NASA for 'space foods' produced for manned space flights. It was recognized that existing methods for quality control, including microbiological testing of raw materials and finished products, did not provide sufficient assurance of safety. A concept known as 'modes of failure' was adapted to foods – by determining what could go wrong (hazard analysis) and selecting specific points in the process to assess process control (critical control points), manufacturers would be able to prevent hazards from occurring (Stevenson and Bernard, 1999). The HACCP concept was presented at the 1971 National Conference on Food Protection and gained considerable interest. However, the time and expertise to set up a HACCP program limited its use to a few large companies until interest was rekindled in 1985 by a report from a Subcommittee of the Food Protection Committee of the National Academy of Sciences (NAS, 1985). As a result of this expert group's strong endorsement of HACCP as the most effective and efficient means of assuring the safety of the food supply, the National Advisory Committee on Microbiological Criteria for Foods was established as an expert scientific advisory panel to the Secretaries of Agriculture, Commerce, Defense and Health and Human Services. One of their first charges was to develop the HACCP concept and provide guidelines for its use. They developed a document in 1989, revised in 1992 and 1997, which serves as the basis for the application of HACCP in industry throughout the United States (NACMCF, 1997). The document served as the basis for US regulations mandating the application of HACCP to the production of seafood, meat and poultry, and juices. A similar

document was prepared by the Codex Alimentarius Committee on Food Hygiene that serves as an international basis for application of HACCP.

The meat and poultry and juice HACCP regulations contained performance standards related to pathogen reduction that have had a significant impact on the use of science and technology in the production and manufacturing of safe food. Requirements for reduction of pathogens on raw meat and poultry have been somewhat controversial, especially when the presence of pathogens has served as the basis for regulatory enforcement. Nevertheless, it is recognized that such requirements have spurred the development of interventions to reduce pathogens on animal carcasses.

Interventions at meat and poultry slaughter

The USDA Pathogen Reduction/HACCP regulation requires slaughter (and grinding) establishments to meet performance standards for *Salmonella* based on prevalence of the organism as determined in nationwide baseline studies. Each establishment is required to at least meet these percentages consistently over time. The performance standards are expressed in terms of the maximum number of *Salmonella*-positive samples that are allowed per specified number of samples (sample set). The number of samples in a sample set varies by product, and the maximum number of positive samples allowed in a set provides an 80% probability of an establishment passing when it is operating at the standard. The assumption was made that reducing the prevalence of *Salmonella* on carcasses will reduce the risk of foodborne illnesses and that a clear standard, coupled with implementation of HACCP, will lead to significant reductions in contamination rates (USDA FSIS, 1996).

Even prior to implementation of the regulation, establishments were conducting studies on technologies to determine their ability to reduce pathogens on the surface of carcasses. A variety of methods were developed long before the implementation of HACCP to decontaminate bacteria, including pathogens, on carcasses (Dickson and Anderson, 1992). These included rinsing with water containing chlorine, organic acid rinses (e.g. acetic and lactic acids) and hot water rinses. With the renewed emphasis placed on reducing pathogens on raw meat and poultry, industry continued the search for new and better means of removing pathogens from carcasses at slaughter. Castillo *et al*. (2002), Dorsa (1997), and Huffman (2002) have reviewed some of the more widely researched post-slaughter intervention strategies that are in use today, including the studies demonstrating the effect on microbial populations. One of the most common interventions used by industry is a hot water rinse (70–96 ºC), which has been shown to be superior to ambient water sprays used in the past. Steam vacuuming, in which a spray nozzle delivers hot water (88–94 ºC) to a targeted area of the carcass while simultaneously vacuuming the area, is used for removal of fecal and ingesta contamination from small areas. Whole carcass steam pasteurization systems have been demonstrated to result in lower microbiological counts and have been successfully employed in many large beef slaughter facilities in the United States. Chemical treatments, generally solutions

of organic acids such as lactic and acetic, are commonly used on beef carcasses. Trisodium phosphate (TSP) sprays and chlorine in the chill water are used for poultry decontamination. New proprietary chemical solutions such as Mionix's $Safe_2O^{®}$ containing acidified calcium sulfate and Sanova (acidified sodium chlorite) from Alcide (now part of Ecolab) show promise for carcass decontamination (among other applications) and are being used by industry. The limitations of individual interventions, as indicated by in-plant data, have demonstrated the importance of using a multiple hurdle approach. Thus, many of these interventions are being used in combinations to effect control of pathogens and achieve the highest possible reduction. However, the use of these products may be limited by cost, the extensive testing needed to demonstrate efficacy, and the occasional lack of consistent results, probably owing to the variability in microbial populations associated with the live animal and the lack of understanding of all the factors that impact efficacy of the treatments.

Although there is much debate about the validity of the performance standards (CAST, 2004), the result of industry's implementation of HACCP and other controls to comply with the performance standards and requirements from customers for better control of pathogens is a reduction in the prevalence of pathogens in raw meat and poultry. FSIS verifies that establishments are meeting the *Salmonella* performance standards by having federal inspection personnel collect randomly selected product samples for analysis at FSIS laboratories. FSIS provides periodic progress reports on its testing results (see http://www.fsis.usda.gov/Science/Progress_Report_Salmonella_Testing/index.asp). In 2005, FSIS tested 40 714 non-targeted samples for *Salmonella* (over 47% were ground beef). Table 5.1 shows the data from samples collected across all sizes of plants during 2005 for 'A' sample sets (an initial sample set or one that follows a passed set) compared with the baseline prevalence. Although the data are not strictly comparable (FSIS verification sampling was designed to track establishment performance rather than to estimate nationwide prevalence of *Salmonella* in products), in all product categories, *Salmonella* prevalence was

Table 5.1 Prevalence of *Salmonella* in FSIS verification samples from 2004 compared with baseline (pre-HACCP) levels

	Prevalence of *Salmonella* in raw meat and poultry	
Product	Baseline prevalence (%)	2005 FSIS verification percent positive
Broilers	20.0	16.3
Market hogs	8.7	3.7
Cows/bulls	2.7	1.3
Steers/heifers	1.0	0.6
Ground beef	7.5	1.1
Ground chicken	44.6	32.4
Ground turkey	49.9	23.2

lower than in the baseline studies conducted before the implementation of the Pathogen Reduction/HACCP regulation. New baseline studies are needed to demonstrate reduction in prevalence compared to the original baseline studies.

The presence of *Salmonella* does not render raw meat and poultry adulterated, as does the presence of *Escherichia coli* O157:H7 in raw ground beef (and trim for grinding) or other non-intact beef products (e.g. injected beef products and mechanically tenderized steaks). FSIS requirements to address the risk from *E. coli* O157:H7 in HACCP plans for such products has resulted in the search for interventions that can reduce this pathogen on raw materials. This resulted in major changes in operations, including installation and validation of new technologies at slaughter to specifically control this organism. The multiple hurdle approach (Huffman, 2002) is being employed for beef carcasses, and chemical treatments (e.g. organic acid washes) are being employed for beef trim to be used for grinding. Many plants have also increased their testing for *E. coli* O157 to verify their food safety systems, diverting products that test positive for *E. coli* O157:H7 to further processing (cooking or canning).

These efforts seem to be paying off. Positive *E. coli* O157 regulatory (FSIS) ground beef samples declined more than 80% from 2000 to 2004 (Fig. 5.1). Between 1996 and 2002 illnesses from *E. coli* O157 varied but showed only a limited decline. However, in April 2004, the Centers for Disease Control and Prevention (CDC), in its annual report on foodborne illness in selected sites in the US (CDC, 2004a), reported a 36% reduction in illnesses from *E. coli* O157:H7 in 2003 compared with 2002. There was a further decline in 2004 to a level that exceeded the public health goals for 2010 (CDC, 2005b). The number of FSIS recall actions related to *E. coli* O157:H7 also continued to drop. There were five recalls related to *E. coli* O157:H7 in each of 2005 and 2004, compared with 12 in 2003 and 21 in 2002.

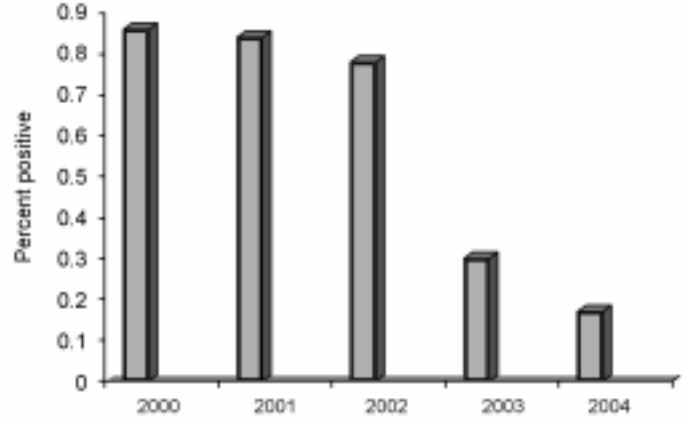

Fig. 5.1 Positive *E. coli* O157 regulatory ground beef samples 2000–2004. Source: FSIS HACCP Verification Program http://www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPHS/ecoltest/tables1.htm.

We are unaware that the treatments applied to reduce pathogens on raw meat and poultry have selected or created microorganisms resistant to the treatment or that have enhanced virulence. Nevertheless, this is an area that warrants monitoring.

Pre-harvest interventions for animal production

It has become increasingly clear to industry that interventions at slaughter can reduce microbial pathogens but not eliminate them. To further reduce the risk from pathogens in raw meat and poultry, strategies to control pathogens at the farm level will be needed. Research has been conducted on a number of intervention strategies to reduce pathogens in animal production (CAST, 2004; Huffman, 2002; IFT, 2002). These include animal husbandry practices, competitive exclusion, and vaccines.

Feed withdrawal and diet alteration may affect the presence of pathogens in the feces of food animals (CAST, 2004). Significant research has been conducted on the effects of dietary modification on the shedding of pathogens by animals; however, there have been no proven husbandry methods that effectively reduce pathogenic bacteria in animals destined for slaughter (Huffman, 2002). Other animal production practices that have been investigated relate to feed additives (e.g. sodium chlorate) and treatments (e.g. pelletizing, heating); water additives (e.g. organic acids, sodium chlorate) and treatments (e.g. cleaning and disinfecting water troughs, chlorination); and poultry litter treatment (addition of antimicrobials) (CAST, 2004).

Competitive exclusion (CE), sometimes called the 'Nurmi concept,' is the introduction of microbial cultures that outcompete pathogens. The concept was introduced by Nurmi and co-workers, who determined that exposing newly hatched chicks to cecal or fecal bacterial flora from adult chickens could prevent colonization with *Salmonella* (Nurmi and Rantala, 1973; Nurmi *et al*., 1992). Commercial competitive exclusion products have been developed (e.g. Preempt^{TM}, Broilact®, Aviguard®, Mucosal Starter Culture (MSC^{TM})) using defined and partially characterized, or undefined, cultures and are usually administered by spraying the chicks during hatching or in the first drinking water. Although CE does not provide complete protection against *Salmonella*, it has been shown to significantly reduce the number of *Salmonella*-positive birds (Bailey *et al*., 2000; Ferreira *et al*., 2003). This reduction in *Salmonella* on the birds was carried through processing to the final processed carcass, thus potentially reducing consumer exposure to *Salmonella* (Bailey *et al*., 2000). Undefined cultures have been reported to be most effective in preventing *Salmonella* colonization (O'Keefe, 2004). MSC^{TM}, developed by the US Department of Agriculture's Agriculture Research Service (USDA ARS), is currently being used in Japan and Brazil, and other undefined CE cultures are being used successfully in Europe; however, no undefined CE cultures are licensed for use in the United States at this time (O'Keefe, 2004). Competitive exclusion products that make pathogen-reduction claims are classified as drugs under the Federal Food, Drug, and Cosmetic Act and thus subject to approval by FDA, a process that requires the products to be characterized and can take many years (CAST, 2004).

In addition to their use in humans, probiotics have been used in food-producing animals to maintain a healthy gut flora and enhance health (IFT, 2002). CE cultures are probiotics that specifically claim to reduce pathogen colonization. While CE is associated most often with poultry, the use of probiotics has expanded to many food-producing animals (IFT, 2002). Zhao *et al*. (1998) found that selected *E. coli* isolates reduced carriage of *E. coli* O157:H7 in cattle. Lactic acid bacteria (LAB) have been fed as probiotics to cattle to improve animal performance. Recently Brashears *et al*. (2003) isolated a strain of LAB considered appropriate for treating cattle to reduce *E. coli* O157:H7. According to the National Cattlemen's Beef Association, a probiotic called Bovamine, which was shown by researchers at Colorado State University to be effective (http://www.fass.org/2004/abstracts/125.PDF), is being used commercially to reduce *E. coli* O157:H7 in cattle.

It has been hypothesized that vaccination may reduce colonization of animals with pathogens such as *E. coli* O157:H7 and *Salmonella*. One approach has been to develop vaccines against the proteins involved in attachment to the intestinal mucosa (CAST, 2004; Judge *et al*., 2004; Huffman, 2002). Studies have demonstrated that vaccination of broiler breeder flocks (Feberwee *et al*., 2000) and laying chickens (Woodward *et al*., 2002) reduces infection with *Salmonella* Enteritidis, resulting in decreased excretion of the pathogen and reduced horizontal spread (Holt *et al*., 2003). Vaccination of breeder flocks of chickens using either live, genetically altered or killed cells (or both) is being used by industry to reduce the percentage of birds that are positive for *Salmonella* (O'Keefe, 2004). Vaccination has been successfully used in the United Kingdom to reduce *Salmonella* Enteritidis in layer chickens (CAST, 2004), and has been correlated with a substantial decrease in cases of human salmonellosis (Van den Bosch *et al*., 2003).

5.3.2 The role of foodborne disease outbreaks in changing production and manufacturing processes

Unfortunately, in many instances it is an outbreak of foodborne illness that first alerts us to production and manufacturing processes that may be inadequate to control a foodborne pathogen. This can result in changes to the production or manufacturing process for specific products.

Illnesses from E. coli *O157:H7 in sausages*

In 1994 an outbreak of illnesses due to *E. coli* O157:H7 was attributed to dry-cured salami, a product produced by fermentation and drying (CDC, 1995a). USDA Food Safety and Inspection Service (FSIS) and industry representatives met to discuss ways to ensure that the production process adequately controlled the pathogen. Scientific studies to validate the efficacy of salami processes led to the inclusion of heating steps for many fermented sausages (Calicioglu *et al*., 1997; Hinkens *et al*., 1996; Luchansky *et al*., 1996).

Outbreaks from pathogens in juice

Foodborne outbreaks from juice led to changes in the production of juices that emphasized controls for enteric pathogens such as *Salmonella* and *E. coli* O157:H7. In 1995 there were 62 cases of salmonellosis in 21 states associated with unpasteurized orange juice sold at a Florida theme park (CDC, 1995b). This was the first orange juice outbreak associated with a commercial processing facility (others had been associated with food service). Outbreaks from *Salmonella* Typhimurium in Australia in 1999 (Anonymous, 1999) and from *Salmonella* Muenchen in 1999 (CDC, 1999b) and *Salmonella* Enteritidis in 2000 (Anonymous, 2000) in the United States further alerted us to the risk of illness from unpasteurized orange juice. In 1996 in the United States, 66 cases of illness and one death from *E. coli* O157:H7 were associated with unpasteurized apple juice (CDC, 1996). That same year, outbreaks from apple cider due to *E. coli* O157:H7 and to *Cryptosporidium* also occurred (CDC, 1997). Outbreaks such as these ultimately led to US regulations mandating that juice be produced under HACCP (FDA, 2001). The regulation included a performance standard (a 5-log reduction of the most resistant pathogen of public health significance in the juice) to assure juice safety. Because it had previously been thought that pathogens were not a concern in products such as fruit juices with low pH (they do not grow, and it was expected that the acid pH would result in microbial death) there were no data on inactivation of pathogens in these products. Research was conducted to determine the thermal resistance of bacterial pathogens in juice and establish criteria for 5-log reduction processes (Mazzotta, 2001). When it was determined that *Cryptosporidium* was slightly more heat resistant than bacterial pathogens (Harp *et al*., 1996; Deng and Cliver, 2001), the recommended process time was increased for apple juice by the FDA (FDA, 2004).

The regulation also led to investigation into alternative technologies that produced juice with characteristics similar to fresh, unprocessed juices but at the same level of safety as that achieved by thermal processes. The use of ultraviolet (UV) irradiation to inactivate *E. coli* O157:H7 and *Cryptosporidium* in apple cider has been demonstrated and the process has been used commercially to pasteurize apple cider (Basaran *et al*., 2004; Hanes *et al*., 2002; Quintero-Ramos *et al*., 2004). High-pressure processing of juices also has been commercialized. Other novel processes with potential application include pulsed electric fields and pulsed light (IFT, 2000).

In spite of all these efforts to control pathogens in juices, in May and June 2005 an outbreak of illnesses due to *Salmonella* Typhimurium was epidemiologically linked to one brand of unpasteurized orange juice. An estimated 126 cases in 22 states occurred (Jack Guzewich, FDA, personal communication). The US regulations that require juices to be produced under HACCP and to be subject to procedures that provide a 5-log reduction of the pathogen of concern in the juice have a provision that citrus juices can receive a cumulative 5-log reduction all or in part through treatments of the surface of the fruit prior to extraction. Such processes may be more difficult to control and do not provide a

large safety margin. They are highly dependent on stringent adherence to good manufacturing practices (GMPs) and have little margin for error.

Outbreaks from pathogens in produce

In recent years a number of foodborne illness outbreaks have been associated with fresh produce such as tomatoes (CDC, 2005a), melons (Tamplin, 1997), lettuce (Ackers *et al*., 1998), and green onions (CDC, 2003). Outbreaks have also been associated with sprouted seeds (NACMCF, 1999) and raw almonds (CDC, 2004b; Isaacs *et al*., 2005). Products that do not receive a kill step for pathogens present significant challenges for the food industry. One approach has been the development of 'best practice' documents such as the FDA's 'Guide to minimize microbial food safety hazards for fresh fruits and vegetables' (FDA, 1998) and Cornell University's Good Agricultural Practices document (Rangarajan *et al*., 2000). United Fresh Fruit and Vegetable Association, along with the National Food Processors Association (now the Food Products Association) and the International Fresh-cut Produce Association, developed 'Field cored lettuce. Best practices' (UFFVA, 2001). Commodity-specific best practices for produce such as lettuce, tomatoes, and cantaloupes have been developed. Best practice guidelines generally follow a HACCP-like approach but do not provide the same assurance of safety, in part because they are often applied to products for which there is no definitive control step that can assure the removal or inactivation of pathogens.

Decontamination procedures for produce have been investigated and, in some instances, applied commercially. Chlorinated water is routinely used when washing produce. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) reviewed the safety of sprouted seeds and made recommendations for interventions, including the pre-soaking of seeds for sprouting in 20 000 ppm calcium hypochlorite. FDA published guidance for production of sprouts recommending the use of such treatment (FDA, 1999). However, the treatment does not guarantee total elimination of pathogens (FDA, 1999) and outbreaks implicating sprouts from treated seeds have occurred (Brooks *et al*., 2001). Because of a lack of antimicrobials that can eliminate pathogens from seeds for sprouting, the FDA also recommended testing spent irrigation water from each production lot for *Salmonella* and *E. coli* O157:H7 (FDA, 1999).

As a result of two outbreaks of salmonellosis from consumption of contaminated raw almonds (in 2001 and 2004), the Almond Board of California developed an Action Plan to ensure that all almonds entering the marketplace have undergone a treatment to reduce the potential for pathogen contamination (5-log reduction of *Salmonella* recently changed to 4-log) without compromising quality and flavor (http://www.almondboard.com/). The Almond Board is researching technologies to achieve this goal, including working with private companies who are developing appropriate technologies. Blanching, oil roasting, and treating with propylene oxide have been validated with respect to achieving a 5-log reduction, but an acceptable technology that achieves the

reduction while providing all the organoleptic characteristics of a raw almond requires additional research.

Outbreaks of listeriosis from dairy, meat, and poultry products

Probably no other pathogen has had a bigger impact on processing practices in the food industry than *Listeria monocytogenes.* The first major outbreak associated with food occurred in Nova Scotia in 1981; coleslaw was identified as the vehicle (Schlech *et al*., 1983). However, it was a large outbreak in 1985 associated with Hispanic-style cheese (Linnan *et al*., 1988), along with several other outbreaks associated with milk and cheese and numerous recalls of dairy products (e.g. ice cream) contaminated with *L. monocytogenes*, that focused the industry's attention on this organism and resulted in major efforts to determine how to control it. Case control studies associating listeriosis with meat products (Schwartz *et al.*, 1988) and a single case of listeriosis associated with a turkey frank (CDC, 1989) alerted the meat industry that this organism was likely a concern in meat products as well as dairy products. This concern was justified by a major outbreak of listeriosis associated with hotdogs and luncheon meats in 1998–1999 (CDC, 1998, 1999a). Extensive research was conducted by industry, academia, and government research laboratories to determine sources of contamination; incidence, growth, and inactivation of *L. monocytogenes* in food products; effect of antimicrobials and sanitizers; virulence; and detection and isolation methodology. Guidelines for controlling environmental contamination were developed (FDA *et al.*, 1988; Tompkin *et al*., 1999) that recognized the limitations of trying to eradicate *L. monocytogenes* from the processing environment to totally eliminate the potential for contamination of finished products.

Through attempts to control *L. monocytogenes* contamination, industry developed an awareness that product contamination with *L. monocytogenes* occurs at some very low frequency in a haphazard manner without apparent illness among consumers. However, a substantial risk of foodborne illness occurs when the organism has become established in a niche or harborage site in the production environment where it can persist and grow (Tompkin *et al*., 1999; Tompkin, 2002). When this happens, routine cleaning and sanitizing become ineffective, and during operation the organisms can work their way out of the niche and contaminate food contact surfaces and product (Tompkin *et al*., 1999).

With the emphasis on control of *L. monocytogenes*, the production of ready-to-eat (RTE) foods has changed considerably. Control of *L. monocytogenes* has been addressed through properly designed lethality treatments (as appropriate to the product), attention to design of equipment to prevent harborage sites, stringent GMPs (including control of product flow and traffic patterns in the plant and sanitation practices focused on *L. monocytogenes*) and environmental monitoring for *Listeria* spp. or *Listeria*-like organisms to identify problem areas, locate contamination sources in the plant, and to confirm that problem solving procedures have been effective (Scott *et al*., 2005; Tompkin *et al*., 1999).

Also, the meat industry has determined that it cannot completely prevent contamination with *L. monocytogenes*, even in the best managed plants

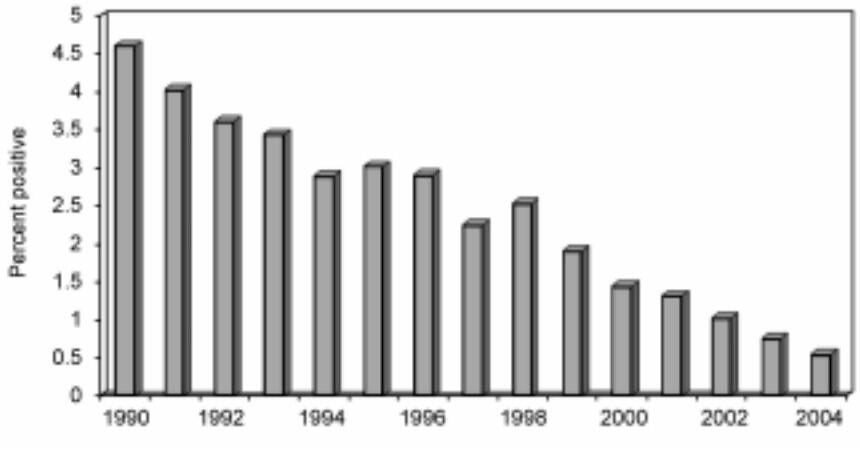

Fig. 5.2 Percentage FSIS samples of ready-to-eat meat and poultry products positive for *Listeria monocytogenes*.

(Tompkin, 2002). Therefore, many establishments such as those producing hotdogs and luncheon meats have made changes to formulations (e.g. addition of lactate and diacetate; Legan *et al*., 2004; Seman *et al*., 2002) to minimize growth of *L. monocytogenes* and are using new technologies such as high-pressure processing of packaged product (Hayman *et al*., 2004) and other post-packaging lethality treatments such as heat (Muriana *et al*., 2002, 2004; Murphy *et al*., 2005) to enhance safety of these products. As a result of these efforts, the percent of meat and poultry regulatory samples positive for *L. monocytogenes* has dropped from 1.91% in 1999 to 0.55% in 2004 (Fig. 5.2). Much of the impetus to implement such controls is a result of an FSIS interim final rule (FSIS, 2003) on control of *L. monocytogenes* that imposes the most stringent requirements on manufacturers of RTE products that rely solely on sanitation practices to minimize the risk from recontamination with *L. monocytogenes* and lesser requirements on manufacturers that use post-lethality treatments and/or antimicrobial agents or processes, as well as sanitation, to reduce the risk.

5.3.3 The impact of new foodborne illness surveillance strategies in changing food safety control measures

We noted above the role of foodborne disease outbreaks in changing production and manufacturing processes. In 1996 the CDC established the Foodborne Diseases Active Surveillance Network (FoodNet) as the foodborne disease component of its Emerging Infections Program (EIP). FoodNet is a collaborative project of the CDC, ten EIP sites, USDA, and the FDA. Its active surveillance for foodborne illness, combined with complementary epidemiologic studies, has resulted in more precise estimates of the burden of foodborne illness associated with specific pathogens in the United States, as well as data on trends. Another objective is to determine the proportion of foodborne diseases attributable to

specific foods and settings in the United States. These data have been used to assess where additional food control measures may be needed. For example, the lack of a decline in illness from *E. coli* O157:H7 prior to 2003 suggested the need for implementation of more stringent control measures, as noted previously under interventions for meat and poultry slaughter.

The combination of enhanced quantitative data on the incidence of foodborne disease from active surveillance and improved outbreak detection and food attribution that results from the molecular subtyping of clinical and food isolates has led to a better understanding of the role of certain foods in foodborne illness. In investigating an outbreak of illnesses due to *Escherichia coli* O157:H7 in hamburgers from a fast food chain in 1993, the utility of applying pulsed field gel electrophoresis (PFGE) to characterize food and clinical isolates and the potential for its use in other outbreak investigations became apparent to CDC. As a result, CDC developed standardized PFGE typing and pattern analysis technology for specific pathogens, standardized pattern nomenclature, and a means of electronic transfer of patterns to a national database at CDC. Designated PulseNet, this national molecular subtyping network for foodborne disease surveillance, uses pulsed-field gel electrophoresis (PFGE) to characterize foodborne pathogens (e.g. *E. coli* O157:H7, nontyphoidal *Salmonella* serotypes, *L. monocytogenes, Campylobacter*, and *Shigella*) and to detect clusters of foodborne illness (Swaminathan *et al*., 2001). The value of this network has clearly been demonstrated through the early recognition of foodborne illness outbreaks and the rapid identification of their sources. Outbreaks that would have gone unrecognized in the past because cases were not clustered in space and time have been detected by PulseNet. For example, PulseNet detected the 1998 outbreak of listeriosis from meat products. This led in part to the many changes noted previously in how industry controls *L. monocytogenes*, including the use of environmental monitoring to detect *Listeria* in the plant environment, formulation of products to prevent growth of the organism and the use of in-package lethality treatments.

PulseNet also was responsible for determining that an outbreak of 75 cases of *Salmonella* Newport in 13 states was from a common (and unusual) source – mangos (Sivapalasingam *et al*. 2003). It has been responsible for the detection of numerous outbreaks of illness from contaminated produce such as illnesses from *Salmonella* in tomatoes. PulseNet is now widely recognized for its ability to assist in the early detection, rapid investigation, and effective intervention in the control of local, state, national, and even international outbreaks of foodborne disease as it has expanded to cover not only all the United States but also has gone international – PulseNet Canada, PulseNet Europe, PulseNet Asia Pacific, and PulseNet Latin America are all in various stages of development.

The identification of outbreaks from specific pathogens associated with specific food types through PulseNet has resulted in a focus on the need for new control measures for pathogen/food combinations. The identification of fresh produce outbreaks has resulted in controls targeted at the farm level, through use of good agricultural practices. It has resulted in the investigation of numerous disinfectants for washing fresh produce, including chlorine, chlorine dioxide,

acidified sodium chlorite and hydrogen peroxide, as well as the use of hot water. It has been determined that immersion of warm produce in cool washing solutions can result in infiltration of the wash solution and any contaminating microorganisms into the product through cuts, stomata, stem-end tissue, etc. A negative temperature differential of 15 ºC allowed *Salmonella* Montevideo to infiltrate the core of tomatoes (Zhuang *et al*., 1995). An understanding that washing and sanitizing steps are key mitigation strategies to be controlled has gained widespread recognition in the fresh produce industry in recent years.

In an earlier section, we discussed outbreaks of illness attributed to juices largely through PFGE-related improvements in investigations, and the resulting development of alternative technologies such as UV irradiation and high-pressure processing for ensuring the safety of juice products. Juices treated with these technologies are commercially available.

The control measures for *L. monocytogenes* noted above have been driven in large part by new foodborne illness surveillance strategies. PulseNet has identified outbreaks and led to their sources, as noted above. FoodNet has provided estimates of the incidence of listeriosis and trends over time that have prompted actions to effect greater control. For example, the rate of listeriosis in the United States declined from around 0.5 per 100 000 population to 0.3 per 100 000 between 1998 and 2000. The rate then did not decline further for several years, and even appeared to increase slightly in 2003 (Fig. 5.3). The lack of progress in achieving the Healthy People 2010 public health goal of reducing listeriosis from 0.5 in 2001 to 0.25 in 2010 was one of the factors motivating FSIS to take a more risk-based approach to control of *L. monocytogenes* in its interim final rule (FSIS, 2003). The availability of FoodNet data on incidence and PulseNet for identifying outbreaks, coupled with regulatory pressure, prompted industry efforts to develop control strategies, including the use of antimicrobials such as lactate and diacetate, treatments such as high-pressure processing, and environmental monitoring, that have been described in various *L. monocytogenes* control guidelines.

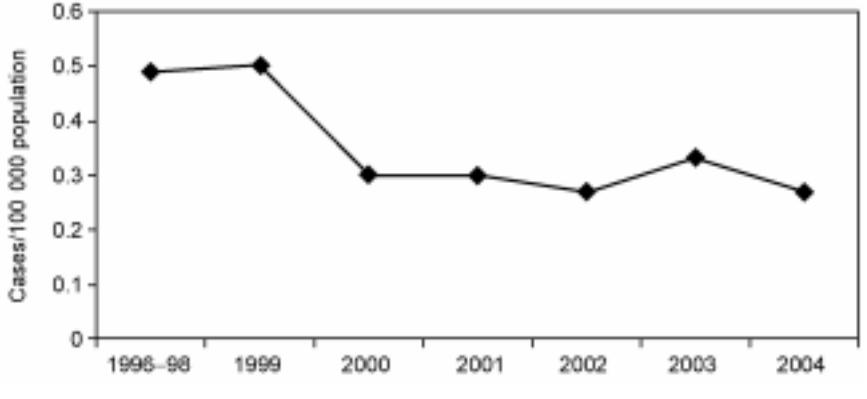

Fig. 5.3 Rate of listeriosis in the United States (from FoodNet).

5.4 Consumer preferences and public health

The twentieth century saw dramatic changes in the way food was consumed. In the early years of the twentieth century, meals were primarily prepared at home from locally produced raw ingredients. Many items were seasonal. The processed food industry changed all that – consumers could enjoy seasonal fruits and vegetables year round as a result of the widespread availability of canned and frozen items and the development of improved transportation and refrigeration. Food markets changed also, from commodity-specific butcher shops, fruit and vegetable stands, and dry-goods stores to the modern supermarket. Continuing cultural evolution is replacing supermarkets with warehouse stores and mega-marts. Lifestyles have changed as well. The stereotypic middle-class US family of the mid-twentieth century – a working father, stay-at-home mother and two children – exists at present in only a small percentage of US households (Senauer *et al*., 1991). According to the US Census Bureau, the average household in 2000 was about 2.59 people; this can be compared with 4.8 persons in 1900 and 3.8 in 1940 (Senauer *et al*., 1991). Marriages are delayed until later in life, and there are more divorces than previous times, resulting in more single person households. Single parent households also are expected to rise another 7% between 2005 and 2010 (Sloan, 2005). One of the most significant changes with respect to food buying habits is the result of the increased participation of women in the US labor force. The number of working women is projected to rise from 65 to 77 million by 2010 (Sloan, 2005). Families also are busier than ever with work, school, sports, and other activities. Singles often do not want to prepare extensive meals for one, and working couples often are faced with time constraints. As a result, convenience is now one of the most important attributes of food products (Senauer, *et al*., 1991; Sloan, 2005). Convenience is reflected in more meals consumed outside the home and in the purchase of prepared foods (cooked or ready-to-eat fresh food). Processors are developing foods for these markets – the store applies a minimal heat treatment and sells the item hot – and stores have become food processors, manufacturing specialty items such as sausage, sushi, and smoked seafood. Many consumers want meals prepared for them rather than having to cook a meal. For the years 1999 through 2002, consumers spent an average of over 40% of their food expenditures on food consumed away from home (US Department of Labor, 2004). The percentage was even higher for single consumers and households with two earners. These consumer preferences pose food safety challenges for the food industry.

Consumers are more concerned about nutrition and health implications of food than ever before. In recent decades consumers have changed their eating patterns because of health concerns (Senauer *et al*. 1991; Zink, 1997). This has resulted in increased consumption of produce, from 287 lb per capita in 1990 to an estimated 332 lb per capita in 2004 (Produce Marketing Association, http://www.pma.com/Content/ContentGroups/Fact_Sheets/Produce_Statistics/FS-Consumption2005.pdf). Consumers in general are looking for natural, fresher preservative-free foods with reduced salt, fat, and sugar (Zink, 1997). 'Gen

Y-ers' (persons born between 1977 and 1994) in particular are looking for healthier, fresher, more natural foods (Sloan, 2005). Although they are unwilling to give up convenience, consumers prefer food that has been subjected to minimal chemical treatment, both preharvest and postharvest (Senauer *et al*., 1991), and they understand less than people once did about potential naturally occurring hazards and proper preparation methods. Reformulation of foods to meet consumer demands may result in foods that are more likely to support growth of pathogens. Food additives are used to enhance the safety and shelf-life of foods, which should be viewed as desirable from the perspective of the consumer. Yet these additives are frequently perceived as unnatural and unsafe, in part because of controversies that have arisen over the years about potential harmful effects of additives such as cyclamates, saccharin, food dyes (e.g. red no. 2), and nitrite, and an apparent distaste for and distrust in science and technology.

5.4.1 Reduction of preservatives

As a result of concerns about the potential for formation of carcinogenic nitrosamines in products containing nitrite, there have been numerous studies, reports, and debates about safe levels. However, nitrite serves as a means of preventing growth of *Clostridium botulinum* and is thus an important safety component of these products; restricting its use presents an increased risk of botulism from cured products (Marriott *et al*., 1981; Tompkin, 1980). The residual level of nitrite in today's cured meats is five times lower than in the 1970s (CAST, 1997), as a result in part of introducing other compounds such as ascorbates in the curing system to allow reduction of nitrites while maintaining the ability to inhibit *C. botulinum* (Marriott *et al*., 1981). Similarly, salt plays a key role in the safety of many products. Salt levels decreased considerably during the twentieth century, from levels greater than 6% in the first half of the century to around 2% today (CAST, 1997). Much emphasis has been placed on reducing levels of sodium in foods to decrease hypertension. Reduction of sodium in meat products has been investigated, including the substitution of potassium and magnesium chloride for sodium chloride. However, one study determined that sodium chloride was better than potassium chloride or magnesium chloride for inhibiting botulinum toxin production in turkey frankfurters (Barbut *et al*., 1986). Thus, the food industry recognizes the need to take caution in changing the formulation of products to meet consumer desires, as the reformulated product may not have the same microbial stability.

Consumers have also become more conscious about calories, and as a result, manufacturers have responded with products containing low-calorie sweeteners. Manufacturers must evaluate the impact of such changes on the safety of the products being produced. In at least one instance such a change resulted in an outbreak of botulism. Canned hazelnut purée sweetened with aspartame instead of sugar was used in yogurt produced in the UK (O'Mahony *et al*., 1990). The process given the hazelnut purée (pH 5.0–5.5) was inadequate to destroy spores

of *C. botulinum*. Apparently, the manufacturer of the hazelnut purée neglected to consider the effect that changing sugar to aspartame would have on factors such as water activity, and the process delivered was thus inadequate.

5.4.2 Concerns about refrigerated foods

While consumers focus on the convenience of prepared foods, they still want 'fresh' foods. And while they want 'fresh' foods, they want to keep the foods longer. The consumer demand for high-quality convenient meals that require minimal preparation has resulted in an increase in refrigerated foods that are lightly processed to preserve flavor, texture, nutrients, and other quality factors. This has often been combined with packaging in a vacuum or modified atmosphere to help extend shelf-life. One such process is known as 'sous vide:' a food is vacuum packaged, given a minimal heat treatment, quickly chilled and then reheated just before serving. The process retains many of the flavor, nutritional and texture aspects of fresh product. Concerns have been raised about the potential for growth of psychrotrophic strains of *C. botulinum*, since the packaging provides an anaerobic environment, competing microflora have been destroyed by the heat treatment, and the shelf-life might provide the time needed for growth and toxin production (Conner *et al*., 1989; Juneja, 2003). Moreover, surveys of commercial storage and distribution systems and home refrigerators indicate that, at some point during its shelf-life, a refrigerated product is likely to be exposed to temperatures in excess of the manufacturer's recommendations. Historically, mild or transient temperature abuse of refrigerated foods has not led to a loss of food safety, and concerns about botulism from these products have been unrealized, likely owing to control measures over the quality of raw materials, refrigeration temperatures, and processes that have been implemented. More recently, the focus has been on the risk from *L. monocytogenes* in refrigerated products that have been exposed to the environment after a heat treatment or in ready-to-eat refrigerated foods that are not given a heat treatment.

To respond to these microbiological concerns, industry developed guidance and has implemented a variety of controls (ECFF, 1996; FSA, 2004; NFPA, 1989). These controls have included heating product to inactivate spores of the non-proteolytic strains of *C. botulinum*, using time/temperature integrators to indicate when product should no longer be consumed, and the addition of secondary barriers to growth (sometimes called hurdles) in the event of exposure to abusive storage temperatures.

5.4.3 The hurdle concept

Reducing the negative impact on the quality of food by a single physical (e.g. thermal processing) or chemical (e.g. salt) preservation method can be achieved by combining two or more methods to provide an equivalent level of safety. This approach is not a new concept and has been used empirically for years to

preserve foods; for example, in smoking of salted meat, some microbial cells are destroyed by the heat while survivors are inhibited by chemicals present in the smoke that are deposited on the meat and by reduced water activity due to both heat and the presence of salt. Thus, using multiple hurdles can inhibit microbial growth or, in some cases, even enhance inactivation of microbes associated with food (Leistner and Gorris, 1995; Leistner and Gould, 2004).

An advantage of applying hurdle technology is that foods can be produced that meet the consumer requirement for being less heavily processed, fresher, and more natural. While some factors may not be effective or practical when used independently, two or more factors may interact in an additive or synergistic manner to enhance the safety of the product. Hurdles include, but are not restricted to: (a) pH; (b) type of acidulent; (c) controlled moisture or water activity; (d) competitive microbial flora; and (e) preservatives. To fully take advantage of hurdle technology, the inhibitory mechanism of each preservation method and how microorganisms react to this stress must be understood. Combining methods that disturb multiple homeostasis mechanisms simultaneously are the most effective and can possibly prevent adaptive responses by the target microorganism(s) to a single method.

In using predictive models to describe the effect of combinations of temperature and water activity, McMeekin *et al*. (2000) found that these factors acted independently (additively) on growth rate but at the juncture of growth/no growth, they acted synergistically. This implies that slight changes to combinations of hurdles can be more effective at the growth/no growth interface than under conditions where the combined hurdles are merely slowing microbial growth. The combination of known inhibitory factors is not always more effective than a single factor. Casey and Condon (2002) demonstrated that sodium chloride in combination with acidic pH was less effective in destroying *E. coli* O157:H45 than acidic pH alone. These studies point to the importance of understanding the underlying antimicrobial mechanisms that the various inhibitory methods employ to take full advantage of their combinations.

5.4.4 Increasing demand for fresh-cut produce

Consumer demand for healthy foods such as fresh produce, combined with their desire to minimize food preparation time, has resulted in a segment of the food processing industry that barely existed 20 years ago – the fresh-cut produce industry. These products are trimmed, peeled, cut up and packaged to provide convenience and freshness for consumers. Chemical dips, modified atmosphere packaging and high-pressure processing have been used to extend shelf-life. However, research has shown, that without proper controls, the potential exists for pathogens to survive and, in some cases, to grow. Thus, much research is being conducted to find washing or other processes to reduce or inactivate pathogens while retaining a fresh product. As noted in an earlier section, the produce industry has focused on the implementation of 'good agricultural practices' and the development of commodity-specific guidance to minimize the

potential for pathogens to be present on raw produce. Nevertheless, with limited controls available and increasing consumption, produce is likely to continue to be identified as a vehicle for foodborne illness.

5.5 Influence of emerging technologies and potential negative impacts

We have over 80 years of experience in the development of safe processes for thermally processed canned foods. The increasing desire by the consumer for fresh-like, convenient foods has spurred research in the development of non-thermal, or 'cold pasteurization,' processes to minimize organoleptic changes but inactivate pathogens of concern. High pressure processing, ultraviolet radiation, pulsed electric fields, and chemical treatments (e.g. ozone, chlorine dioxide) have been shown to effectively reduce the most resistant micro-organism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage (pasteurization) (NACMCF, 2006). In general, these new technologies do not appear to result in unique microbiological hazards. For new technologies we will need to determine the most resistant pathogen of public health concern that is likely to be present in the product, determine the level of inactivation needed, and define the critical operating parameters to ensure the process is adequately delivered to the food.

In addition to new physical processing technologies, manufacturers are exploring novel food preservation systems, including the use of natural preservatives such as bacteriocins, competitive microflora, lysozyme, chitinases, lactoferrin, and lactoperoxidase, to name a few. By combining physical processes with these novel food processing systems, it may be possible to design processes for the precision destruction (or inhibition) of pathogenic and/or spoilage organisms, yet allow the desired fresh-like characteristics of the food to remain.

As with any new food processing technology, these emerging technologies will need to be evaluated to determine their impact with respect to eliminating competitors such that surviving or recontaminating pathogens become a concern; selecting for more resistant microorganisms such as pathogenic spore-formers; or sublethal injury resulting in pathogens that can repair themselves and cause foodborne illness (NACMCF, 2006). We must consider and conduct surveillance to determine whether a new technology may have unintended consequences on any surviving microorganism, such as potentiating adaptive responses and cross-protection against food-associated stresses, or impacting the expression of virulence genes.

5.5.1 Impact of injury and stress

Exposure of microorganisms to sublethal stresses has long been known to injure them. This could have the effect of allowing an overestimation of the lethality of

the process when viable, injured cells are not recovered and are presumed dead. Microorganisms are also known to adapt to stressful environments and even become more resistant to the stress. Exposure to physical and chemical stresses encountered during food processing, such as heat, pressure, increased osmolarity, or weak organic acids can cause an adaptive response. Exposure to one stress (e.g. low pH) may induce cross-protection to another stress (such as heat). This is particularly true for microorganisms stressed due to starvation in the stationary phase of growth. This phenomenon is a concern because parameters for minimal processes that are based on the response of non-resistant microorganisms may be inadequate for these microorganisms once their stress response mechanisms have become activated by the process. Davidson and Harrison (2002) reviewed the potential for antimicrobials and sanitizers used in food processing to impart resistance to microorganisms. There has not been much evidence generated to demonstrate acquired resistance to food antimicrobials or sanitizers. However, since the stress response is known to occur in the laboratory, more research is needed into the frequency and mechanism of resistance, mechanisms of action of antimicrobials and development of strategies to prevent the acquisition of resistance to stresses in the microbial ecology of food.

Archer (1996) stated that traditional food preservation systems work well to inhibit the growth of toxin-producing bacteria such as *S. aureus* or *C. botulinum* that require relatively high numbers for the toxin to cause disease. However, he expressed concern that infectious bacteria such as *E. coli* O157:H7 and strains of *Salmonella* may increase in virulence during stressful conditions of food preservation. Stresses such as starvation and extremes of temperature, pH, and osmolarity cause adaptive responses, one of which may be to potentiate expression of virulence genes or, even worse, create unpredictable mutations in the virulence genes. To date there is little evidence that this occurs in food production, but it warrants vigilance.

5.5.2 Impact of technologies that reduce competitive microflora

One potential disadvantage of new technologies may be the impact they have on reducing microorganisms that would compete with pathogens. Jay (1997) has proposed the idea that new technologies for extending the shelf-life of foods not only reduce the targeted pathogens but also that the numbers of spoilage microorganisms may be creating foods with a greater risk of causing foodborne illness because of the removal of the normally harmless bacteria that are antagonistic to pathogens. He suggested that since there are now fewer food producers and that foods may require longer shelf-lives for distribution, the appearance of large foodborne disease outbreaks may be due to the destruction of the natural microbial interference that once held the growth of pathogens in check over a relatively short shelf-life of the product. Processes that reduce all microorganisms in a product can significantly increase product shelf-life. However, if pathogens remain in the product or recontaminate the product and the product is exposed to temperatures that allow the pathogen to grow, the absence of competitors can provide an

environment with no means of blocking growth (similar to when the player carrying the football gets beyond the final defensive player of the other team, and nothing stands between him and the goal).

Jay (1996, 1997) uses beef to illustrate his argument, but the more recent outbreaks in fresh-cut produce may also support this hypothesis. Manufacturers of fresh-cut fruits and vegetables employ good agricultural practices to reduce contamination of the produce by pathogens. Since fresh-cut produce has a short shelf-life and will be consumed without any other intervention step by the consumer, the processors thoroughly wash the produce and possibly apply sanitizing agents or use surface pasteurization to eliminate the offending spoilage bacteria and pathogens. In some cases they may use clean room techniques to prevent recontamination of the clean produce. While cleaner is definitely better in the world of food processing, products such as fresh-cut fruits and vegetables that are ultra-clean may be providing an environment that is conducive to the growth of pathogens, particularly if the produce is temperature abused during distribution. Koseki and Isobe (2006) found that the bacterial growth on lettuce treated with sanitizers (ozonated water or sodium hypochlorite) grew at a faster rate than on the unwashed lettuce, possibly due to the reduction of competing microorganisms. Delaquis (2005) stated that destruction by heat of a substantial portion of the native microflora on fresh-cut produce could provide a competitive advantage to surviving microorganisms. One possible lesson that may be learnt from this is that the closer one gets to complete sterilization of the food, without actually reaching it, the greater the risk that a surviving or recontaminating pathogen would have free rein to grow and produce foodborne disease. This is not meant to suggest that we should not clean our food, but rather to point out there may be an advantage of treatments targeted at a specific pathogen rather than a 'broad spectrum' approach.

An example of a new food preservation technology that presents a potential concern is the use of vacuum packaging and modified atmosphere packaging of foods to extend shelf-life. This would seem to be a very useful technology for packaging fresh fish, which is highly perishable. Greater shelf-life could increase consumption of what has become recognized as a very healthy food. However, a natural concern would be to question whether this technology would create conditions that would allow neurotoxin to be produced by non-proteolytic *C. botulinum* sooner than in fish stored in non-oxygen-reduced environments. In one study, not only was neurotoxin produced faster in modified atmospheres, it was produced before the fish was considered spoiled (Post *et al*., 1985). Therefore, a technology that provides a longer shelf-life for this very perishable food may suppress the natural warning system for consumers by suppressing the growth of the natural spoilage microorganisms.

5.5.3 Active packaging

Packaging of food has traditionally been used to contain the product, to protect it from contamination by microorganisms and insects, and to maintain quality by

reducing the rate of oxidation and moisture loss or pickup. In addition to this passive role of packaging in food preservation, a new breed of packaging being developed is known as active or smart packaging. Active packaging interacts with the food and the atmosphere surrounding the food to create an environment that extends shelf-life by inhibiting the growth of spoilage and pathogenic microorganisms, maintaining desirable quality attributes, and indicating migration of contaminants (Ozdemir and Floros, 2004; Tewari, 2002). Examples of some of the types of active packaging include material that absorbs oxygen, carbon dioxide, ethanol, moisture, ethylene, and flavors from the atmosphere within the package. Some systems may release carbon dioxide, ethanol or flavors, depending on needs of the product. Gas-permeable films used to maintain the quality of fresh-cut produce, incorporation of time–temperature integrators, and susceptors (metalized package materials used to intensify microwaves to provide browning and crisping of foods) for microwave heating are also examples of active packaging.

Although absorbing oxygen and moisture or emitting carbon dioxide in a package can affect the growth of microorganisms, there are packaging schemes that are specifically designed to inhibit foodborne microorganisms. These fall mainly into two categories, packaging materials and edible films/coatings that contain antimicrobial agents that interfere with microbial growth on the surface of the food (Cha and Chinnan, 2004). A variety of antimicrobials can be incorporated into packaging material or applied to the interior surface of the material, ranging from weak organic acids such as acetic, benzoic, lactic, propionic, and sorbic acids; enzymes such as lysozyme; bacteriocins such as nisin or pediocin; triclosan; chitosan; and fungicides. The selection depends on the target organism and the food. Many of these same agents can be incorporated into edible coatings made from polysaccharides such as starch, cellulose, or gums. Proteins films are based on corn, soy, milk, collagen, and gluten while lipid coatings include waxes and glycerides (Cha and Chinnan, 2004). To date there are no specific active packaging regulations in the United States. Migration of the antimicrobial agents out of the package or film and into the food must be treated as food additives (Appendini and Hotchkiss, 2002).

5.5.4 Negative consequences of not using a technology

One technology that is only ‘emerging’ because of its limited application in the market is that of irradiation. Treatment of foods with ionizing radiation has been researched for decades. A report by the World Health Organization concluded that food irradiated to any dose to achieve the intended technological objective is safe to consume and nutritionally adequate (WHO, 1999). However, consumers associate the process with the negative effects of radiation on humans resulting from atomic bombs and the fear of nuclear war and accidents at nuclear power facilities such as those at Chernobyl and Three Mile Island. Activists have viewed the process as a way to mask contamination, and they claim it destroys nutrients and creates harmful chemicals. Consumer misconceptions about the

effects of irradiation on the safety and nutritional composition of foods and the belief it will be used to make unwholesome foods saleable have severely limited the use of a technology that can truly enhance the safety of foods, particularly raw foods in which contamination with microbial pathogens cannot be prevented. The use of this technology could eliminate enteric pathogens such as *Salmonella, Campylobacter*, and *E. coli* O157:H7 from raw meats, preventing not only foodborne illness from undercooked meats but also from cross-contamination in the home. Irradiation offers a practical means of inactivating parasites such as the tapeworms *Taenia saginata* and *T. solium* and the larval forms *Cysticercus bovis* and *C. cellolosae* (Monk *et al*., 1995), which are still major foodborne problems in developing countries. Thus, with this technology, the potential food safety problem results not from its use, but from not using it.

5.5.5 New technologies must be carefully evaluated

New technologies continue to be developed to meet consumers' demands for fresher, natural (no preservatives), and convenient foods and to respond to other pressures and changes in the food industry. Products derived from new technologies provide numerous challenges to food safety professionals who must assess and control potential hazards that may not have been recognized in foods derived from older technologies. Research continues at a brisk pace to describe conditions of novel processing technologies, antimicrobials, packaging, and combinations of these technologies that will provide a desired inhibition/destruction of pathogens or spoilage microorganisms in food. The potentially negative impact of the extension of refrigerated shelf-life from new processing and packaging technologies on public health demands that there should be parallel research to investigate the biological mechanisms of inhibition and the effect on microbial ecology, adaptation, and virulence.

5.6 Future trends

Just as HACCP revolutionized how the food industry focused its food safety efforts, new concepts on how to manage the risk of microbiological hazards in food are developing. Most significantly the application of risk analysis, which consists of risk assessment, risk management, and risk communication, is becoming the accepted approach in evaluating and controlling microbial hazards in food (Codex Alimentarius Commission, 2005). The process begins with the identification of a food safety issue and a microbiological risk profile. If there are sufficient data, a formal risk assessment is conducted, generally at the governmental level. This can be used in the selection of microbial risk management options, which may involve establishing a food safety objective (the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection), a performance objective (essentially a food safety objective at a point in the food

chain prior to consumption) or a performance criterion. Performance criteria may be established to provide a target for industry that is considered appropriate to control the hazard, and should be based on public health goals. Process criteria or product criteria are established by industry to achieve the performance criteria. Microbiological criteria may be established to verify adherence to the criteria or objectives. Through applying these concepts, controls used by the food industry are directly tied to public health outcomes, which can be measured through active surveillance systems such as FoodNet.

The use of these new concepts impacts industry in several ways. Risk assessments may be used to assess the relative risk of specific foods, such as was done in an FDA/FSIS risk assessment on *L. monocytogenes* (FDA/FSIS, 2003). This identified for both industry and regulatory agencies those foods that pose the greatest risk for listeriosis, and thus the foods for which stringent *Listeria* controls should be implemented. Risk assessments can be used to evaluate intervention strategies. For example, the risk assessment clearly highlighted that for foods that support growth of *L. monocytogenes*, refrigeration temperature has a major impact on risk, since growth to high numbers significantly increased risk. Risk assessments underpin many new regulations that impact the food industry, such as proposed USDA FSIS regulations for lethality and cooling of ready-to-eat meat and poultry products and those for egg products, which will establish performance standards that industry must meet.

Meeting new consumer expectations while achieving new levels of microbial control for raw, as well as processed, products assures the continued development of new technologies for processing, as well as new packaging concepts and materials. There will be more convenience foods, processed with technologies that are increasingly able to match the attributes of freshly made products. We will also see an increasing development of foods that enhance performance and prevent illness, possibly including foods that help prevent foodborne illness through the use of probiotics.

Without entirely new processing technologies, we are fast approaching the limits of what can be achieved in terms of food safety at the processing level. As a result, there will be an increasing need to develop more on-farm controls and renewed consideration of acceptable and 'fail safe' preparation technologies that can be applied easily immediately before consumption. Increasing emphasis will be placed on strategies to reduce or eliminate pathogens on incoming live animals. This will require changes in farm management practices based on scientific research (IFT, 2002). Technologies being investigated include vaccines (e.g. to immunize cattle against intestinal colonization with *E. coli* O157:H7), the administration of bacteriophage active against targeted pathogens, manipulating feed ingredients and/or practices (e.g. feed additives that prevent colonization of cattle with *E. coli* O157:H7), and control of pathogens in livestock drinking water (Huffman, 2002).

There is an increasing trend to put brand names on fresh foods, including produce, meat, and poultry. This is primarily a marketing device whereby the name 'personalizes' the product and distinguishes it from the competitors.

Branded products may be perceived as 'value added' by the consumer. Branded products are promoted for their high quality and specific characteristics (e.g. leaner meats, organic vegetables, pasteurized shell eggs, free-range chickens). In many instances such branding is viewed as providing a product with enhanced safety because of an implied commitment to taking responsibility for the safety and quality of the product. Branding also can enhance traceability, thereby providing a real food safety attribute to this marketing strategy.

Regardless of where the future leads in terms of new products and processes, food safety will be a focus of the food industry. Consumers expect their food to be safe, and to the extent that food processing practices and technologies can deliver a safe product that is acceptable to the consumer, the industry will implement such practices and technologies.

5.7 Sources of further information and advice

The most comprehensive series of books on food safety is that produced by the International Commission on Microbiological Specifications for Foods. The Commission's most recent books include:

Microorganisms in Foods 5: Microbiological Characteristics of Food Pathogens (1996)

Microorganisms in Foods 6: Microbial Ecology of Food Commodities, 2nd edition (2005)

Microorganisms in Foods 7: Microbiological Testing in Food Safety Management (2002)

A number of trade/professional bodies also provide food safety information. The foremeost of these is the International Association for Food Protection. For more information, go to http://www.foodprotection.org/main/default.asp.

The Partnership for Food Safety Education (PFSE), a nonprofit organization formed in 1997, is dedicated to educating the public about safe food handling to help reduce foodborne illness. PFSE members represent all aspects of the food and consumer industry from meat and produce to marketers, and allied trade as well as government and consumers. The PFSE developed the Fight BAC!® campaign, a public education campaign focused on safe food handling. More information can be found at http://www.fightbac.org/main.cfm.

Other useful food safety websites include:

- The gateway of US government food safety information: www.foodsafety.gov
- FDA: http://www.cfsan.fda.gov/
- USDA FSIS: http://www.fsis.usda.gov/
- World Health Organization Food Safety: http://www.who.int/foodsafety/en/
- Food and Agriculture Organization Food and Nutrition: http://www.fao.org/es/esn/index_en.stm
- US Centers for Disease Control and Prevention: http://www.cdc.gov/

- FoodNet: http://www.cdc.gov/foodnet/
- PulseNet: http://www.cdc.gov/pulsenet/

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6

Exposure assessment for foodborne pathogens

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6.1 Introduction

Foodborne illnesses have declined over much of the last century as the result of advances in processing and storage technologies such as pasteurization, canning, packaging and refrigeration, coupled with strong regulation of the food industry, increased hygiene and sanitation. The globalization of agriculture and food trade has also led to many benefits, including lower food prices for American consumers and year round access to a huge variety of fresh foods. However, the emergence of microbial pathogens such as *Campylobacter* and *Escherichia coli* O157:H7, protozoan parasites, enteric viruses, and prions such as those that cause bovine spongiform encephalopathy (BSE) have prompted new food safety concerns in recent years. Further, new food safety concerns have arisen owing to global sourcing of ingredients and distribution of foods, a larger proportion of the diet consumed outside the home, higher consumer expectations, and re-emergence of established pathogens, which exhibit enhanced persistence and virulence, and a growing sub-population of immune-deficient or immune-suppressed individuals.

To effectively manage these food safety issues, business and government leaders have been turning to the burgeoning discipline of microbial risk assessment. Risk assessment for foodborne pathogens is rooted in the chemical risk assessment methodologies. A 1983 National Academy of Sciences National Research Council (NRC) document 'Risk Assessment in the Federal Government: Managing the Process', also known as the 'Red Book', established a four-part risk assessment paradigm consisting of Hazard Identification, Dose–Response, Exposure Assessment, and Risk Characterization. Since the US

Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) publication of the first microbial risk assessment for a foodborne pathogen-product combination '*Salmonella* Enteritidis Risk Assessment: Shell Eggs and Egg Products' in 1998, several food safety risk assessments have been conducted in the United States (e.g. assessment of fluoroquinolone-resistant *Campylobacter* in chickens conducted by FDA-Center for Veterinary Medicine (CVM), assessment of *Listeria monocytogenes* among select categories of ready-to-eat foods by FDA-FSIS, and an assessment of *Vibrio parahaemolyticus* in raw molluscan shellfish by FDA-CFSAN). A microbial risk assessment framework for food safety was developed jointly by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) and has been adopted by the Codex Alimentarius.[1] The structure is similar to the NRC framework including Hazard Identification, Exposure Assessment, Hazard Characterization (Dose–Response), and Risk Characterization.

Microbiological risk assessment shares much in common with its chemical forbear. The organizational elements of the risk assessment and computer simulation models are, in both instances, designed ultimately as a decision-making tool for risk management. There are significant differences as well. Chief among these is the difficulty of determining microbial exposures, because of the sporadic and dynamic nature of bacterial contamination on foods. On the other hand, compared with chronic chemical exposure, microbiological risk assessment can link exposures to fairly robust epidemiological data on acute adverse health outcomes, including illnesses and deaths.

Microbial exposure assessments characterize the prevalence or likelihood of a pathogen's presence and the expected numbers present in food at the time it is consumed by an individual. A key difference between chemical and microbial exposure assessment is the multiple sources and pathways by which the hazard could enter the food production system in the latter case. In fact, microbial contamination can be introduced at any point, including on-farm production, during processing, distribution to retail or food service, consumer storage and handling, or final meal preparation. Identification of which sources and pathways contribute to pathogen infestation is part of an exposure assessment process. In addition, the dynamic nature of microorganism levels complicates exposure assessments. Most bacterial and fungal pathogens are capable of exponential growth when provided with proper nutrients and conditions of favorable pH, temperature, water activity, and oxygen tension. Conversely, all pathogens are susceptible to the effects of interventions that could reduce or eliminate them, including: cooking, freezing, desiccation, fermentation, antimicrobial agents, or acidification. Another confounding feature of microbial exposure assessment is the sporadic nature and uneven distribution of pathogens on foods. Exposure models are sometimes developed with the sole purpose of examining how prevalence and concentration change at points along the food chain. An exposure assessment describes the pathways through which a pathogen population is introduced, distributed, and grows, or is challenged in the production, distribution, and consumption of food.[2]

The relationship among the presence of a pathogen on food, human exposure from consuming that contaminated food, and estimation of the nature, likelihood and severity of the resulting human illness is a complex interaction of many factors. These include: pathogen factors (virulence potential and population level as influenced by growth, reduction, or elimination along the entire food chain); food factors (food choice, frequency eaten, and amount consumed); and human host factors (immune status, gastrointestinal tract status, age, disease status, medical or pharmaceutical treatments). Data for these factors, which are necessary for complete characterization of human exposure to foodborne pathogens and characterization of risk of illness, are often unavailable. Therefore, exposure assessments are usually conducted using models that represent a simplified version of this complex reality. More often, risk assessors adopt specific conventions (default values or assumptions, either implicit or explicit) to bridge missing or incomplete data/knowledge. All assessments are embedded with assumptions and this limitation should be disclosed within the risk assessment document and then recognized by risk managers when making food safety policy decisions. By describing the components, approaches, and applications of microbiological exposure assessment, this chapter provides context for exposure within a broad risk assessment and risk analysis framework. The following areas are discussed:

- Characteristics of foodborne pathogen exposure factors that need to be considered for developing exposure assessment models.
- Exposure assessment modeling approaches.
- Challenges and limitations in exposure assessment.
- Policy implications and future considerations.

6.2 Foodborne pathogen exposure factors

6.2.1 Pathogen characteristics and microbial ecology

The biological and pathogenesis characteristics and microbial ecology of a specific pathogenic microorganism must be considered in the risk assessment design. Foodborne pathogens are classified as infectious or toxigenic and these distinctions influence how modeling is approached. The former group (including *Salmonella*, norovirus (formerly Norwalk-like virus), and protozoan parasites) must first adhere to the gastrointestinal epithelium after oral ingestion and then replicate to form colonized foci. In turn, these infections stimulate localized disturbances, typically causing inflammation, upsetting electrolyte balances, and damaging the muscosal lining. This results in vomiting, watery or bloody diarrhea or other symptoms. Many infectious agents (e.g. *Listeria monocytogenes*, Hepatitis A virus) can penetrate the intestinal epithelium and invade the physiology, resulting in systemic or selective organ disease.

Toxigenic microorganisms, such as *Staphylococcus aureus*, produce protein toxins, pre-formed in foods, or formed post-ingestion *in vivo*. For risk assessment purposes both infectious and toxigenic (chemical-like) characteristics must

be evaluated. Likewise, some pathogens have inactive and environmental stress-resistant life-cycle stages, such as bacterial spores (e.g. *Clostridium botulinum*), which render many processing technologies ineffective in reducing numbers or eliminating them from foods.

Considerable genetic distance exists within most microbial species, which influences the potential for a pathogen to survive in a food environment and to cause disease. These sub-type differences result from the presence or absence of virulence genes and polymorphism within a single gene. Likewise, phenotypic differences resulting in the switching on or off of virulence genes influence each strain's response to specific environmental stimuli. Sub-typing is performed using genotypic and phenotypic tools, including: serotyping, phagetyping, ribotyping, and pulsed field gel electrophoresis (PFGE). These techniques classify the relatedness of isolates beyond the species level. In recent years sub-typing has been used to link geographically disparate foodborne illness outbreaks and to associate bacterial, viral, and parasitic isolates from clinical cases with those from foods, contributing to the establishment of cause and effect. For risk assessment purposes, however, there is a pressing need to relate those molecular epidemiological classifications to the environmental persistence and virulence potential of those microorganisms.

Microbial levels vary significantly through growth or inhibition as food transitions through the segments of the production, processing, distribution, and meal preparation chain. This characteristic necessitates consideration during exposure modeling. Although viral and parasitic pathogens cannot grow in foods, bacteria and fungi are capable of exponential growth in the environment if favorable conditions exist. Similarly, most foods undergo some level of cleaning or processing that can reduce or eliminate pathogen loads. Extrinsic factors, such as nutrient density and balance, pH, temperature, water activity, and oxygen tension contribute to growth or inhibition.

Exposure assessment is dependent upon an estimation of levels of pathogens on specific foods, yet this information is frequently lacking. The customary approach to the microbiological analysis of foods has been the determination of presence or absence typically at a level of sensitivity at 0.04 colony-forming units per gram of food. Exposure assessment is limited by time, cost, and lack of validated methods for conducting enumeration analysis.

Microbial ecology needs be considered for exposure assessments. Microbial contamination on foods that results from human hand contact, animal waste, or contaminated tools or equipment, is frequently unevenly distributed on products. Similarly, natural pathogen reservoirs are sometimes unknown. For example, the recent emergence of *Enterobacter sakazakii* in infant formula and resulting fatalities has sparked a search to identify its environmental reservoir. Furthermore, multiple possible routes of contamination, such as food, water, or air may limit or make more complex product pathway analysis. Finally, microbial contamination may occur at any point of food production or handling and contamination patterns may be temporally, seasonally, or geographically uneven.

6.2.2 Food handling

Despite increasing automation of food production, some forms of human food handling occur at all stages of food production (e.g. hand-harvesting perishable produce, cooking meals). Improper food-handling practices have been associated with outbreaks of *Salmonella* sp., *Campylobacter jejuni*, norovirus, and *Escherichia coli* O157:H7.[3–6]

Food preparation hygiene and cooking practices may have the greatest impact on the risk of foodborne illness. Of the 2751 foodborne disease outbreaks reported between 1993 and 1997, the Centers for Disease Control and Prevention (CDC) identified the following six categories of contributing factors to foodborne outbreaks (listed in the order of highest and lowest number of outbreaks (in parentheses)):[7]

1 improper holding temperatures (938);
2 poor personal hygiene (623);
3 contaminated equipment (400);
4 inadequate cooking (274);
5 other causes (222); and
6 food from unsafe sources (133).

Failures in the refrigeration process provide an opportunity for the growth of pathogens in contaminated food products. While cooking at high temperatures eliminates most live bacterial pathogens, cooking at low temperatures may allow pathogens to multiply and increase. Cooking behaviors, time, temperature, method and quantity of food cooked are variables of interest when assessing pathogen exposures. The frequencies with which these behaviors occur, and the effect of each behavior on live microbial pathogen levels are also important factors in exposure assessment. Cross-contamination from contaminated surfaces (i.e. other carcasses, equipment, tabletops, utensils, containers, and hands) during processing or in the consumer kitchen may be an important potential source of exposure. Data on cross-contamination are limited or non-existent and thus this important variable is rarely addressed in exposure and risk assessments.

6.3 Environmental antecedents

The CDC National Center for Environmental Health (CDC-NCEH) has defined environmental antecedents as 'variables in the environment that, in the absence of control, may create contributing factors that lead to adverse health outcomes.' There are five categories of environmental antecedents.[8]

6.3.1 Category 1: Food and its inherent properties

Inherent properties of foods influence the extent to which pathogens can survive and grow. They include pH, water activity, specific heat, and density. Con-

sideration of these factors can help to focus on foods that are more likely to be impacted by pathogens given their intrinsic properties.

Food sourcing is another important variable. Globalization reduces traditional geographic barriers to emerging and traditional pathogens.[9] The international food trade, ease of worldwide shipment of foods, development of new food industries, altered production methods, environmental and demographic shifts in developing countries, degradation of sanitation and the immediate human environment, and mass tourism present opportunities for pathogen introduction into foods.[10,11]

6.3.2 Category 2: The people factor

Knowledge, beliefs, attitudes, skills/education, background (culture), supervision, and management structure are all factors that influence how people handle food at work and at home. This human variable may be the most important environmental antecedent factor that contributes to foodborne illnesses. Studies that examined the beliefs of food handlers toward food safety have identified a number of barriers to implementing safe food-handling practices, including lack of time, staff, and resources.[12,13] It has also been noted that self-reported practices did not correspond to observed behaviors with regard to consumer food-handling in the home.[14,15] Studies have also identified a number of high-risk food-handling behaviors, including eating raw or undercooked food products, and not washing hands or food preparation implements with soap and water after handling raw meat or chicken.[2,16,17]

6.3.3 Category 3: Equipment

Equipment type, materials, capacity, maintenance, and location are environmental variables that can create factors contributing to foodborne pathogens. The equipment used during food production or processing is susceptible to contamination. For example, machines used to defeather chickens may incorporate present contaminants into the carcasses of the chickens, which are then put into a 'fecal bath of chilled water' where *Aeromonas hydrophila*, an antibiotic-resistant bacterium, may be present.[18] Food processing equipment is vulnerable to 'biofouling' or the accumulation of biofilms on the surfaces of the equipment. Biofilms occur when microorganisms 'form a slime layer upon a surface and provide an environment for pathogens to proliferate.'[19] Some common microorganisms that form biofilms on various food processing equipment are *Staphylococcus aureus* (with poultry), *Listeria monocytogenes* (with meat and dairy), and *Bacillus cereus* (dairy).[18] However, biofilms and other sources of cross-contamination can be avoided with the use of cleaning agents and disinfectants in addition to using machines with a sanitary equipment design (i.e. antimicrobial coatings) and performing routine/preventative maintenance on the food processing equipment.[19]

6.3.4 Category 4: Process

Various stages of the food production continuum from farm to table, including growing, harvesting, slaughtering, processing, packaging, transport, and storage are variables belonging to this category of environmental antecedents. Food types, such as ready-to-eat, cook/serve, and their preparation steps, are also included here.

6.3.5 Category 5: Economic factors

Economic changes in the food industry, particularly economies of scale, and changing incentives have resulted in a rise in foodborne illnesses. As the food distribution has become more centralized, contamination becomes a bigger danger in large distribution centers where one contaminated food item can come into contact with edibles destined for geographically disparate locations, causing a widespread epidemic.[20] Economic incentives in the food industry have changed recently encouraging companies to engage in dangerous behaviors that previously would not have been economically profitable. For example, infections with *Vibrio vulnificus*, an oyster pathogen, have increased as oystermen increasingly harvest from the Gulf of Mexico during the summer when *V. vulnificus* is active. This change in source occurred because the Chesapeake Bay oyster population has plummeted in recent years making the slightly riskier Gulf of Mexico population an attractive summer option.[20] The final economic influence on the food industry is the Government. Through uneven appropriations to regulatory agencies at the federal level and inconsistent funding to food illness monitoring at the local level, the Government allows certain pathogens to persist in the food supply far longer than others.[21]

6.4 Farm-to-table continuum

During the food production process, there are many opportunities for pathogens to enter the food supply chain, such as improper food handling, that can lead to food contamination. Once food contamination occurs, when control measures are not applied to mitigate and/or to prevent further food contamination, the pathogens remain in the food supplies and potentially grow until time of consumption. Depending on pathogen virulence and host immunity status, consumption of such foods may lead to foodborne illnesses. Clearly, there are many factors involved along the continuum, from growing, producing, processing and packaging, transportation and storage, food preparation and consumption, that influence exposure and must be considered (Table 6.1). Unless actual measurements are taken at each of these stages, they must be modeled based on the knowledge that already exists.

6.4.1 Pre-harvest

Food production from plants and animals begins on a farm, orchard, ranch, or other facility. This earliest stage in the food production process is generally referred to as ‘pre-harvest.’ A complete exposure assessment starts at this stage

Table 6.1 Farm-to-table continuum – foodborne pathogen exposure factors

Food production stages	Exposure factors	
	Pathogen sources	Control failures
Pre-harvest	Ill workers Contaminated water supply for plants and animals Unsanitary handling of animal wastes Poor animal health Unsanitary or careless farming practices Human or other wastes in fields	Failures to isolate, treat, or remove infected animals Failure to exclude workers exhibiting vomiting, diarrhea, or jaundice Contaminated irrigation water Failure to use latrines Failure to exclude children from fields
Post-harvest (production and processing)	Ill workers Crowding Slaughter practices HACCP practices Improper processing Cross-contamination and introduction of extraneous matter to foods	Use of contaminated water or inadequate rinsing Inadequate processing or heating Packaging failures Failure to exclude ill workers Poor GMPs
Transportation and storage	Improperly cleaned trucks can introduce pathogens from previous shipments to subsequent shipments (*Salmonella* and ice cream) Storage: temperature/conditions may favor contaminants and/or growth of pathogens	Poor temperature control Poor transporter sanitation Unsecured transporters
Preparation and food handling	Health of food preparer: sick individuals can introduce viral, bacterial and other pathogens to foods Cleanliness of plant, kitchen: leaking vent and air conditioning systems can contaminate food products Heating, refrigeration capabilities Improper handling can introduce pathogens from foods that will be cooked to foods that will not be cooked	Inadequate heating and/or cooking can fail to kill pathogens Inadequate heating or refrigeration can allow pathogens to multiply Failure to exclude ill food handlers Inadequate storage Poor sanitation

so that the effect of the environment can be included. For example, green vegetables or berry crops may be affected by contamination from soil, manure, irrigation, or silage. Insects may also play a role in the spread of pathogens to crops. Pathogens may survive in manure, soil,[22–24] or inside protozoa[25,26] for long periods of time. Some may also penetrate the vasculature of leafy plants such as lettuce, alfalfa, and mung bean.

Exposure assessment at this stage aims to identify areas in which pathogens are introduced and the likelihood for such introduction. There are a number of factors that could lead to the introduction of pathogens during this stage:

- *Indigenous factors*: during production, animals, fruits, and vegetables are exposed to natural hazards such as viruses, bacteria, molds, and parasites as part of the environment. The natural prevalence of any given pathogen will vary with geography, climate conditions, and season. Although *L. monocytogenes* does not occur naturally in oceans, some aquatic environments may become contaminated with *L. monocytogenes* from human or animal sewage or from soil, such as from cultivated and uncultivated fields carried in rainwater runoff. In such cases, *L. monocytogenes* may contaminate fish, shellfish, and vegetables.
- *Food handling*: the process of pre-harvest food production requires handling of the potential food product as well as the environment around it. The food-handling practices used during production can play a major role in the type and amount of hazards that follow the food into processing, that is, post-harvest. Some sources of 'microbial contamination in foods include: improperly cleaned hands, lack of hygiene, dirty clothes, hair, as well as the presence of minor cuts and infection in hands and face and mild generalized diseases (e.g. flu, strep throat, and hepatitis A) may amplify the situation.'[27]
- *Equipment cleanliness/maintenance*: equipment used in the 'harvesting, transportation, processing, and storage of foods' is another source of microbial contamination in foods.[27] These microorganisms enter the equipment 'from the air, raw foods, water and personnel'.[27] Also, continuous use of equipment without cleaning and maintenance increases the risk for microbial contamination of food. The rate at which microbial contamination occurs in foods depends on the environment and time.
- *Hygiene and sanitation practices*: practices on the farm can be a determinant for pathogen introduction. Poor hygiene and sanitation at production facilities, animal housing, and production equipment may foster growth of microbial agents. Poor rearing practices such as inadequate cleaning of facilities between flocks or herds of new animals may lead to the spread of pathogenic microorganisms. Spread of pathogens among livestock can occur from direct contact between animals, drinking from shared water sources, or eating contaminated feces (chickens are notorious for doing this).
- *Farming inputs*: farming inputs such as sources and quality of irrigation water supplies, sources and types of feed, and quality and sources of seeds may provide opportunities for introduction of pathogens into this stage of food production.

- *Wildlife*: proximity to wildlife harboring pathogens can infect domestic herds and flocks.

After harvest, preliminary washing or cleaning of the product will remove some of the initial contamination. Transport may introduce additional or new pathogens. At each of the succeeding stages of production, changes in prevalence and concentration are likely to occur. However, unless actual measurements are taken at each of these stages, they must be modeled based on the knowledge that already exists.

6.4.2 Post-harvest (food processing and packaging)

Food processing occurs after food products are harvested from a farm or other production facility and transported to another facility for additional steps in preparing the food item for consumption. For example, processing includes turning apples into cider, milk into cheese, corn into a microwaveable dinner, or cattle into steaks. Food processing ranges from only a few steps in a small building, such as making apple cider, to complex processes conducted in several separate buildings and requiring many steps, such as producing steaks from a live steer. Different types of food products have different packaging and processing challenges.

The likelihood for contamination to occur during food processing is also related to economic factors. This includes processing/producing a product in large quantities 'at a faster rate in a centralized plant'.[27] Other sources of microbial contamination are packaging materials (wrapping materials and containers) used in ready-to-eat (RTE) foods, as well as flies and rodents that may enter food processing facilities.[27]

Meat processing

Cross-contamination is a key process enabling exposure during this stage of food production. This is in addition to the contamination that may have been introduced during pre-harvest. In poultry processing where hundreds of birds are simultaneously processed in de-feathering machines and the same water-filled tanks for scalding and chilling, cross-contamination is a major consideration. Although large livestock such as cattle and swine are usually processed on a singular basis, cross-contamination could also occur from using shared equipment for processing. The most likely vehicles for spreading pathogens among carcasses during processing are the surfaces in the slaughter or processing plant, machinery, tools such as scalding and chilling tanks and the accompanying solutions, packaging machinery, or carcass-to-carcass contact.

Holding, mixing and aggregation, fermentation, heating, pasteurization, brining, smoking, and pickling are the processing steps to decrease the prevalence and concentration of pathogens in RTE meats. Environmental contamination such as aerosols from cleaning water and dirty equipment may be the sources of *L. monocytogenes* contamination in processing plants. Cooked products such as

processed RTE meats may become recontaminated during handling and contact with equipment before final packaging. Slicing operations are common sources of re-contamination of cooked products.[28,29]

Fruit and vegetables

Fruits and vegetables can arrive at the dinner table in two forms: processed or fresh. The most likely source of contamination for both types is on the farm. Contaminated fertilizer (untreated manure), contaminated irrigation water, and field workers with poor personal hygiene are the most likely sources of contamination. Contamination can also occur through water used to wash the produce after leaving the field if the pH is too high or it is not properly chlorinated. *Listeria monocytogenes* is the biggest concern in fruit and vegetable processing facilities owing to the cold wet conditions under which it thrives.[30]

Fresh fruits and vegetables are far more likely to carry pathogens than their processed counterparts because fresh produce does not undergo a kill step. Therefore, if contamination has occurred at the field, it is best controlled through keeping temperatures within safe ranges, typically below 4 ºC (40 ºF). Processed fruits and vegetables tend to undergo kill steps including acidifying, brining, freezing, cooking, blanching, and irradiation. Although these steps are likely to kill many pathogens, unclean machinery, contaminated employees, or contact with other contaminated produce can still infect processed fruits and vegetables after undergoing a kill step.

Transportation and storage of food

The process of shipping animals to slaughter facilities stresses the animals and can increase the shedding of pathogenic organisms into feces. Transfer of pathogens from the digestive tract of some livestock, usually via feces, to the hide or feathers of non-colonized livestock and processing equipment presents the greatest risk to uncontaminated carcasses and the final meat product. Hazards can spread from animal to animal during transportation because of cramped shipping conditions or via pathogen-contaminated feces that can smear onto the feathers or hides of other livestock. Smaller livestock, especially poultry, are often shipped in stacked cages with wire flooring that allow feces from upper cages to contaminate the feathers of animals in lower cages – a process that can spread pathogens to uninfected animals. Although the uninfected animal has little time to acquire infection, the feathers or hide can contaminate slaughterhouse equipment and carcasses during processing. Similar factors apply to transportation and storage of processed food as to post-harvest production food destined for processing.

6.4.3 Process control systems

Process control is part of a systems approach to assuring the quality of products or services. Through process controls fewer pathogens would be introduced during production, hence, fewer of these hazards would be transported with the

food to processing, storage, preparation, and consumption. In process control systems (PCS) actions are taken on a number of steps in the process (i.e. critical control points) to ensure quality of the product. Hazard Analysis and Critical Control Points (HACCP) is an example of a PCS.

HACCP includes seven principles:[27]

1 Conduct a hazard analysis to determine risks associated at all stages, from growing raw materials and ingredient to final product ready for consumption.
2 Identify critical control points (CCPs) to control this hazard. Examples of CCPs in the farm-to-table process are cooking ready-to-eat foods, refrigerating processed foods, or irradiating fresh produce.
3 Implement conditions to control hazards at each CCP.
4 Implement effective procedures to monitor the control conditions for each point.
5 Implement corrective measures to be taken if a deviation occurs at a point.
6 Implement effective record-keeping systems for HACCP activities.
7 Implement procedures to verify that the plan is working effectively.

However, poor design and/or improper implementation of the HACCP system can lead to unsafe food products. Exposure assessment of foodborne pathogens must take into consideration whether CCPs are properly identified and controls are properly used. Guidelines are available in the published literature and from governmental and non-governmental food safety organizations, but no blanket approach is possible because every operation is different.[31] Exposure assessment can be employed as a tool in the development of HACCP plans to help assess hazards, potential consumer exposure levels for specific formulations or processes, and to determine the potential to reduce or eliminate the exposure.

6.4.4 Food preparation and consumption

Whether inside or outside the home, food preparation is the combining of foods to prepare meals. Combinations of menus, culinary approaches, and ethnic and cultural preferences determine the likelihood of introduction and/or growth of pathogens.

Outside the home

There is no clear definition of 'retail food services.' In general, these are places where food is prepared for immediate consumption by consumers. For the purpose of this chapter, these establishments include grocery stores (including bakery, deli, butcher shops), convenience stores, restaurants (quick service, full menu, off-site catering, etc.), and institutional food (hospitals, nursing homes, schools, etc.)

There are a number of food preparation scenarios outside the home that can lead to the presence of pathogens in foods. If pathogens are already present in foods at low levels when they are delivered to retail establishments, levels of microorganisms can increase during storage and display. Pathogenic levels may

also increase through recontamination or cross-contamination from portioning opened packaged products through slicing or chopping and then repackaging. Ambient temperatures can permit the growth of pathogens on contaminated slicing equipment, cutting boards, etc., increasing the pathogenic population in food.

At home

An understanding of consumer variables concerning food selection, handling, preparation and consumption habits are critical in an exposure assessment. Culture, ethnicity, lifestyle, age, and gender have significant influence on consumers' food behaviors.

Cultural identity and ethnicity

Food preparation can be influenced by cultural practices. For example, native subsistence communities in Alaska have had outbreaks of botulism owing to their practice of fermenting and putrefying food rather than thermally processing it. Indigenous communities in northern and southeastern Alaska have the highest rates of botulism, reflecting their dietary dependence on marine mammals and salmon, respectively.[32]

Ethnicity has been shown to be the most important predictor of food selection and frequency of food consumption.[33–36] For example, an outbreak of *Salmonella* in several western states in late 1999 associated with Brazilian mangos most affected people of Asian or Latino backgrounds.[37]

Lifestyle

Studies have found that changing consumer lifestyles can affect food behavior. For example, the increasing number of women in the workforce, limited commitment to food preparation, and a greater number of single heads of households have been identified as contributing factors.[38]

Geography

Geographic differences have been observed in the patterns of exposure and foodborne illness. As part of the Foodborne Diseases Active Surveillance Network (FoodNet), CDC and its federal and state partners have conducted a population survey and produced an Atlas of Exposures. The observed geographic differences in consumption of high-risk foods from the Atlas are shown in Table 6.2.

Food consumption patterns will likely differ based on population demographics (age, gender, ethnicity, socioeconomic group) and seasonal and regional (both national and international) differences in food availability. For exposure assessment, considerations of food consumption patterns for sensitive subpopulations (e.g. young children, pregnant women, the elderly, and the immunodeficient) and of high-risk consumer behavior (e.g. consuming unpasteurized dairy products or undercooked meat products) are also particularly important.

Table 6.2 Percentage reporting consumption of high-risk foods in the past seven days by state[39]

Foods	California	Colorado	Connecticut	Georgia	Maryland	Minnesota	New York	Oregon	Tennessee
Lettuce in the home	76.2	74.8	75.8	68.5	71.7	70.6	75.8	74.9	65.1
Raw uncooked sprouts	13.1	9.2	7.5	5.3	7.7	6.9	6.8	10.7	5.6
Uncooked mangoes	14.8	8.6	7.5	6.3	7.9	4.4	3.8	7.0	4.7
Raw fresh fish	9.8	4.1	3.9	2.5	3.4	1.9	2.9	3.0	1.9
Unpasteurized milk	2.8	4.4	3.6	3.9	3.0	3.4	3.4	3.2	3.6
Unpasteurized juice	13.8	11.1	10.8	10.9	11.6	7.2	10.3	10.0	7.7

Source: http://www.cdc.gov/foodnet/surveys/pop/2002/2002Atlas

6.5 Exposure assessment models

As previously discussed, microbial hazards in foods can arise at any stage in the food chain, and can be affected by subsequent processing and handling steps. Therefore, a continuum of the entirety of the food production system, from the point of production (farm) to the point of consumption, must be considered. The exposures and risks posed by the pathogens at one point in the food chain are not treated in isolation from the system as a whole. Hence, creating a flow chart to show the origin of pathogens and the relationships and operations that can change the level and prevalence of the pathogen in the food along the continuum from farm to table is the simplest way to begin an exposure assessment. An example of a generic flow chart for microbial food safety exposure assessment is shown in Fig. 6.1.

Although much attention has been paid to modeling risk from farm to fork, it is not always necessary to include the entire food chain. To date, as part of most food microbiological risk assessments, the exposure assessments have been conducted beginning from either production stages or retail stages. Some assessments have modeled prevalence and concentration at the time of consumption by allowing for the effects of time and temperature on growth and survival of a pathogen from an earlier point in the chain (such as the FDA-FSIS *Listeria monocytogenes* in RTE foods). A few have started the exposure assessments as far back in the food chain as the farm (e.g. USDA-FSIS *E. coli* O157:H7 in ground beef). The scope of the risk assessment will typically influence the breadth of the exposure assessment.

In general, an assessment of exposure to foodborne pathogens requires two types of information:

1 the amount of food consumed and by whom, and
2 where in the food chain the microbiological hazards arise, and what factors affect the prevalence and concentration of the pathogen in the food at the time of consumption.

The key desired outputs of an exposure assessment for foodborne pathogens are prevalence, concentration, and, if possible, the physiological state of pathogens in foods at the point of consumption. However, since not all pathogens and food contamination carry the same severity of public health implications, a gradation of exposure assessment approaches, from qualitative to quantitative assessments, can be applied, and the resulting exposure metrics can include:

- a qualitative expression, e.g. high, medium, low exposure;
- an estimate relative to some known or existing level of exposure, e.g. risk to consumers of a particular food;
- a single numerical estimate based upon a series of point estimates, e.g. average or the worst case;
- a set of estimates that describes the range of possible outcomes, e.g. average, minimum, maximum, and most likely;
- an estimate derived by combining the frequency distributions of individual

Point in food continuum	Variables affecting dose		
	Consumption	*Concentration in contaminated units*	*Prevalence of contaminated units*
Raw ingredients		Environmental sources affecting concentration in ingredients	Season, harvest area, fodder/feeding regimes, irrigation water
↓			
Processing		*Volumetric changes*: dilution/concentration (e.g. evaporation, whey removal)	Cross-contamination, mixing with other ingredients
↓		*Growth/inactivation changes*: binding, heating, cooling, holding times	Splitting into smaller units for retail/food service
Transport and storage		Time, temperature, product composition	
↓			
Retail sale		Time, temperature, product composition, splitting into smaller units	Packaging, cross-contamination, portioning, splitting into smaller units
↓			
Home/food		Time, temperature, product composition	Cross-contamination, mix with other foods
↓	Frequency and amount consumed affected by: season, wealth, age, sex, culture/region, etc.		
↓		Heating, mixing with other ingredients (e.g. salad dressing), splitting into smaller units	Splitting into smaller units/serving portions
Consumption			

Fig. 6.1 A generic exposure assessment model for pathogens in foods.[40]

variables in the assessment, characterized by a frequency distribution of possible outcomes. This approach provides the range of possible outcomes and the probability of each outcome. It requires the greatest amount of information and mathematical modeling techniques.

6.5.1 Conceptual and mathematical models

Conceptual exposure models

A conceptual exposure model describes the variables and their interactions that result in an exposure to foodborne pathogens. For example, the numbers of ingested *L. monocytogenes* by consumers are usually unknown. However, an

estimate can be derived based on models of the effect of physical processes and conditions that the food undergoes through the farm-to-table chain. Empirical data from studies conducted on products or their ingredients at different stages throughout the chain and/or modeled data are used to quantify and combine these variables.

Typically in an exposure model, the various steps in the food production continuum are broken down to several components or steps (e.g. pre-harvest, post-harvest, transportation and storage, and preparation and food handling) and the behavior of the pathogen is either measured or modeled (increase, reduction, or no change) throughout each of the steps. The concentration at the conclusion of one step is the initial concentration for the next step. For example, a slaughter model for a risk assessment of beef might include modeling pathogen prevalence and concentration at the end of the following steps: stunning, sticking, head removal, dehiding, evisceration, splitting, chilling, washing, fabrication, and packaging of meat cuts for further processing or retail establishments.[41,42]

Mathematical approaches such as predictive microbiology models, which predict the growth of pathogens in foods, can also help provide necessary information and fill some of the data gaps. Predictive models for microbial growth, survival or inactivation could be developed for each step in an exposure model, from production up to preparation and prior to consumption. Predictive microbiologists have determined that temperature, pH, and water activity can account for almost all fluctuations in bacterial growth and death rates in food. Temperature is the most powerful determinant of the three and the only one that the bacteria themselves cannot influence. Predictive microbiology begins by creating a primary model, an empirical model of bacterial growth with respect to time. This growth curve is then modified into a secondary model by introducing a key variable such as temperature. Large databases of empirical data and the resulting models are maintained by joint effort between the USDA and UK Food Standards Agency.[43]

Mathematical models, variability, uncertainty, and sensitivity analysis

A mathematical model explicitly defines the mathematical relationship of the variables described in the conceptual exposure model. By assigning values (i.e. data) to the variables in the model (i.e. model inputs), the equations describing the origin and amount of a pathogen in the food and its activity level can be solved to yield a numerical estimate of exposure.[40] In a probabilistic exposure assessment (PRA), the equations are solved stochastically and model inputs (assumptions) and outputs (results) are specified as distributions. PRA models can be built for the whole farm-to-table food production and processing chain. Alternatively, several models could be built for segments of the food chain and then linked together.

A distribution that describes an input or output of a model is composed of two components: uncertainty and variability. Variability refers to temporal, spatial, or inter-individual differences (heterogeneity) in the value of an input.[44] Variability is an inherent property of all physical, chemical, and biological systems.

Uncertainty refers to the incompleteness of one's knowledge or information. Random and systematic measurement errors, as well as reliance on models or surrogate indicators, are all sources of uncertainty.[44] Often uncertainty exists when assumptions have to be made about the ranges of an unknown quantity and their probabilities of occurrence. The degree of uncertainty can be reduced by the acquisition of new data or knowledge, whereas additional data will not decrease variability.

Sensitivity analysis is used to measure the potential importance of model inputs as contributors to variation in model outputs. Such an analysis can provide insight into how a real world system is sensitive to perturbation of some of its components or processes, assuming that such relationships are adequately represented in the model.[44]

In recent years, with increasing computational capabilities and modeling software, the development of computer models capable of complex calculations and simulations using sophisticated mathematical techniques, such as Monte Carlo, has been expanding. Computer simulation modeling software such as @Risk, Crystal Ball, and Analytica can provide a means to calculate the results for complex food systems. These software programs calculate all of the possible combinations of factors by calculating exposure many times (i.e. iterations). For each iteration, these programs select a random value from each variable range according to the probability distribution describing that variable. The outcome is then calculated for that specific iteration. Values from all iterations are collated to generate a probability distribution of possible outcomes.

Model validation

Validation is a process by which a computer simulation model is evaluated for its accuracy in representing a system. The type of validation that is needed depends on the purpose of the model. For models that are developed for the sole purpose of defining the functional relationships between input quantities and overall behavior of a system, evaluating relative changes in model predictions as a function of changes in input is more appropriate than attempting to validate whether the model prediction is true. For example, for an exposure model constructed to examine the impact of various intervention strategies on the prevalence and levels of *Listeria monocytogenes* in deli meat, evaluating the impact of model prediction by changing model inputs would be an appropriate validation approach. It is only when a model will be used for making predictions that it should be subject to a full validation process.[44]

One of the ways microbiological risk assessments have been validated is to compare model prediction of illness with epidemiological data. For example, in the USDA-FSIS risk assessment for *Escherichia coli* O157:H7 in ground beef, the public health risk estimates derived from the risk assessment model were compared with estimates of foodborne illness derived from the CDC epidemiology data, and adjustment to the dose–response/hazard characterization was made until the public health risk estimates were similar to the epidemiology data.[42] An underlying assumption with this validation approach is that the

exposure model (and its inputs) component of the risk assessment is correct and that only the dose–response/hazard characterization aspect of the risk assessment was anchored to the epidemiology data. While this approach is not ideal, a full validation of a risk assessment of this scope and scale would be difficult, if not impossible.

As a practical matter, for large and complex models, validation for the entire model may be impossible, but evaluation of some components of the model might be feasible. This is referred to as partial validation.[44] For instance, predictive microbiology models that are used to fill in data gaps in the full exposure model could be validated using products of similar microbial ecology to the product of interest. An example would be models that have been developed for growth, survival, and inactivation of *L. monocytogenes* in laboratory broth media and some foods. The most reliable of these models are the ones developed from systematic studies under carefully controlled conditions, which include temperature, water activity (A_w), sodium chloride (NaCl) concentration, pH, and levels of preservatives (i.e. organic acids and nitrite). These conditions are known to exert a major influence on *L. monocytogenes* growth. Nevertheless, these models may have to be modified for specific foods and their full complement of ingredients.[40]

6.6 Challenges in exposure assessment

Significant challenges exist in exposure assessment for foodborne illness. Often the lack of data prevent adequate quantification of the long list of input variables in an exposure model, such as the prevalence and concentration of a pathogen in foods, the composition of the food, serving sizes and frequency of consumption. Knowledge about the food system, from farm to fork, is also often incomplete and poses great challenges in modeling such a system. Further, assumptions that may be made in the incorporation of data and information about the food system into an exposure model may lead to significant sources of model uncertainty.

6.6.1 Model uncertainty

Simple reflection of reality

Models are often a simple reflection of the real world. Thus, the structure of mathematical models employed to represent scenarios and phenomena of interest is often a key source of uncertainty.[44] There are four major sources of model uncertainty in microbial risk assessment. The first is the simplification of complex processes into mathematical models for physical processes. Second, the exact point at which any given pathogen becomes inactive or begins to grow is very difficult to predict. Third, the process of extrapolating from small sets of scenarios to all scenarios of importance necessarily introduces some uncertainty. Finally, assumptions are often made dealing with the operation of complex

processes based on readily observable components of those processes that encounter the same extrapolation difficulties as the scenario sets.

The results of an exposure assessment that employs computer simulation modeling techniques will depend on the model, the data ranges and distributions that are used, and on the assumptions made in setting up the model. While it is easy to develop spreadsheet models, it is also easy to make mathematical and logical errors in the construction of the model. There is a need to verify both the accuracy of the mathematical model as a description of the system being assessed (model validity) and its mathematical reliability (the ability to produce consistent results upon repetition).[45–47] The many potential pitfalls in simulation modeling have been well-documented in general references and guidelines for their use in risk assessment.[46–49]

6.6.2 Data limitations

An assessment of exposure to a foodborne pathogen typically requires data that:

- describe the prevalence of the pathogen in food ingredients, or specific finished products of interest, or both;
- describe the concentration of the pathogen in ingredients, specific finished products of interest, or both;
- describe the amount of the product eaten at each meal or serving, the frequency of consumption, and if possible, the consumption characteristics of subpopulations that are particularly susceptible to the pathogen;
- allow the determination of the prevalence and concentration of the pathogen at one or more points in the food chain, e.g. data about storage times and temperatures, pathogen growth potential in the food;
- help to simplify and prioritize the assumptions and process model to be included in the exposure assessment, since it is impossible to include in a model all of the situations that a food may experience.

The information on which exposure estimates rely is often limited. A number of different types of data are used, including microbiological sampling data, food consumption data, epidemiological surveillance data, outbreak data, and survey data. Often, the only data available are from studies intended for other purposes (e.g. regulatory compliance) and are not ideally suited for the objectives of exposure assessment. Population characteristics are usually inferred from observations made on a sample drawn from the population at a specific point in time, and observed phenomena are extrapolated to the situation under study.

The source, quality, and amount of data can vary considerably. Sources include books, published studies, government reports, and unpublished data. While generally accepted data quality guidelines have not been developed for microbial risk assessment, data sources may be excluded for many reasons, including poor quality, small sample size, non-English language, unpublished sources, and publication prior to a given date, etc. In some cases where there is

no current information, data that were collected and analyzed decades ago may be used. Any change in practices, such as the implementation of HAACP or technological advances, would not be reflected in exposure assessments using these old data. Arguments have been made in favor of unpublished data, including the fact that published data are likely to be biased away from the null. The following sections discuss several key data limitations.

Microbiological sampling

Information on the extent of contamination (prevalence) and level of a pathogen contamination in the food product or material (i.e. concentration or enumeration) in the production steps are necessary to conduct an exposure assessment. Flock or herd prevalence data can be obtained from on-farm samples such as fecal samples or internal swabs (e.g. rectal swabs). The USDA-FSIS periodic baseline sampling of carcasses from various livestock, which tests for various pathogens and surrogates of public health concern such as *E. coli, Salmonella, Listeria*, *Campylobacter*, is a major source of microbiological sampling data (see Table 6.3).[50]

Ideally, the prevalence and enumeration studies used in exposure assessment should be comprehensive national surveys of the specific foods in question. However, these are rarely available. Furthermore, in a farm-to-table analysis microbiological enumeration data covering several points in the production continuum are needed to verify that modeled estimates are in realistic value ranges. However, these data are often lacking. For example, based on a review by the WHO, it was noted that smaller surveys within several countries often have to be used to estimate the contamination of RTE foods by *L. monocytogenes* and enumeration data are often not available from these studies.[40] The zero-tolerance regulatory approach adopted by many authorities towards *L. monocytogenes* in RTE foods and other foodborne pathogens and the time and cost associated with collecting enumeration data have both contributed to this lack of data.[51] Zero tolerance implies regulations that require that the hazard not be detectable in a test sample of specified size. Many countries specify the absence of *L. monocytogenes* in a 25 gram test sample in RTE foods as the tolerable limit.[52]

Table 6.3 Prevalence of microorganisms on cattle, chicken, and swine carcasses as measured by USDA-FSIS

Microorganisms	Cattle, n = 1881		Chicken, n = 1225		Swine, n = 2127	
	n	%	n	%	n	%
E. coli	312	16.6	1167	95.3	937	44.1
Salmonella	23	1.2	107	8.7	147	6.9

Sources: http://www.fsis.usda.gov/PDF/Baseline_Data_ Cattle.pdf, accessed 21 June 2005; http://www.fsis.usda.gov/PDF/Baseline_Data_Young_Chicken.pdf, accessed 21 June 2005; http://www.fsis.usda.gov/PDF/Baseline_Data_Swine.pdf, accessed 21 June 2005.

Cross-contamination

Data detailing pathogen transfer and cross-contamination are limited. Models usually rely on assumptions about cross-contamination based on expert opinions. This adds to the uncertainty of subsequent exposure estimates.

Food preparation and handling

Food preparation involves the combination of different food products to make a dish or a complete meal. Combinations of menus, culinary approaches, ethnic and cultural preferences determine the likelihood for introduction and/or growth of pathogens. Time and temperature are the two most important factors in food preparation, followed by hand washing, and cleaning utensils and cooking surfaces properly. Since food preparation and handling information are not always available, assumptions about food preparation and handling practices are assumed for various scenarios in an exposure assessment, contributing to additional uncertainty.

Food consumption[53]

Food production statistics, food consumption surveys, retail food purchase data and household surveys are useful in providing data regarding food production and food consumption. Data from the US Department of Agriculture (USDA) and the National Center of Health Statistics (NCHS) nationwide food consumption surveys, including the USDA Continuing Survey of Food Intakes by Individuals (CSFII) 1985–86, Nationwide Food Consumption Survey (NFCS) 1987–88, CSFII 1989–90, 1990–91, 1994–96 and 1998, the NCHS 3rd National Health and Nutrition Examination Survey (NHANES III), and now NHANES 1999+ have been and continue to be relied upon for exposure assessment in risk assessment.

Food consumption surveys for individuals are frequently conducted to assess the nutritional status of a population rather than to characterize consumption of specific foods. Although these surveys may provide information about consumption by specific age and gender groups, they may not describe foods in sufficient detail, include enough participants from sensitive subpopulations or even collect the information necessary to identify these subpopulations. The food description may not indicate whether foods such as milk or juices are pasteurized or the degree to which foods such as eggs or ground beef are cooked. Raw data from the surveys are not always available, requiring the exposure assessor to rely on aggregated data, which may not be sufficiently detailed or targeted to the foods of concern.

Information on consumer behavior that may increase or decrease the risk of foodborne illness is absent from most food consumption surveys. The ability to link food consumption data to information about an individual's propensity for consuming high-risk foods (e.g. eating undercooked hamburgers, raw shellfish, or temperature-abused foods) would be extremely useful in estimating exposure to microbiological hazards in foods.

6.7 Prioritizing data needs

The lack of adequate data to fill in key variables will lead to uncertainty in the exposure estimates. Reducing this source of uncertainty will increase confidence in the exposure estimates. One type of data that is much needed for quantitative exposure and risk assessment is enumeration data. Often these data are limited or unavailable. Depending on the organism and analytical laboratory, the enumeration cost per sample could range from $30 to $175 (see Table 6.4). Coupling the typically high per sample cost with large sample sizes that may be necessary given the typical lack of homogeneity of organisms in the food matrix, the cost of enumeration could run very high. Clearly, as a practical matter, data needs must be prioritized based on potential for reducing uncertainty and balanced against the costs and difficulties of developing more and better data.

Table 6.4 Samples of microbiological enumeration costs

Microorganisms	Bio Research Laboratories lab/cost (US$)	Biosan Laboratories lab/cost (US$)	Colorado Dept. of Public Health & Env. lab/cost (US$)
Campylobacter confirmation			90
Campylobacter culture			35
Campylobacter, *Shigella* *Salmonella* culture			100
E. coli		40	
E. coli (food)			85
E. coli (petrifilm)	30		
E. coli O157:H7	50	65	
Listeria monocytogenes	60	75	
Listeria (food)			75
Salmonella sp.		65	
Salmonella (TECRA)	45		
Salmonella (FDA BAM)	40		
Yersinia enterocolitica culture			35
Yersinia pestis confirmation			90
Yersinia pestis culture			35
General food pathogen screen (*S. aureus*, *Salmonella*, *E. coli*, total bacteria, yeast and mold count)		175	
Food poisoning/standard food pathogen includes: *E. coli*, *Salmonella*, aerobic plate counts (APC), total coliform, yeast/mold, *S. aureus*)	125		

6.8 Policy implications and future direction

The ultimate target audience for a pathogen exposure assessment is the policy maker whose concern is safeguarding public health. The primary purpose of a pathogen risk assessment is to inform decisions by government regulators, policy makers or food producers about whether and how exposures should be reduced (i.e. risk management). Several issues arise concerning exposure assessment in this context:

- The assessment must address a set of focused risk management questions, e.g. the selection of scenarios to be modeled must parallel the risk management tools available.
- The exposure estimate should not be taken literally. Sophisticated models produce a false sense of confidence in the accuracy of the predicted outcome and can give the impression that the modeled exposures and illness outcomes are real.
- The propensity of the public sphere to prefer certainty and simplicity should not be allowed to obscure the uncertainties and limitations of the model used and the resulting estimates.
- If clearly articulated and explained, the sensitivity and uncertainty analyses that are included in a scientifically sound exposure assessment are as informative to the risk manager as the exposure estimate itself. These analyses tell the decision maker when risk management intervention may be premature and more study of a specific variable is a better option.

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Using surveillance data to characterize and analyze risk factors for foodborne illness

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7.1 Introduction

Over the past decade, intensified public health efforts have reduced the incidence of foodborne illness in the United States. Nevertheless, with ever-changing food industry practices, consumer eating habits, and sociodemographic patterns, new pathogenic agents emerge and known agents take on new importance. In this chapter, we will discuss the role that epidemiological surveillance takes in helping to measure and control the burden of foodborne illness within the United States.

In the first half of the chapter, we introduce the basic concepts of surveillance for foodborne illness. Surveillance is critical to identifying disease trends and is also an important element in evaluating the effectiveness of new food safety interventions. However, its accuracy and usefulness are often limited by case reporting failures, biased reporting, and the inability to link cases to specific foods and/or agents. Current, 'active' approaches to surveillance, coupled with web-based reporting and improved laboratory methods have enhanced the reliability and usefulness of surveillance data.

Unfortunately, surveillance data are commonly thought of as simply reporting what has been observed. In the latter half of the chapter, we demonstrate how active surveillance, because of its increased accuracy, holds the possibility of generating new information beyond traditional trend data. Finally, we extend our discussion to global foodborne disease surveillance efforts. It is clear that

monitoring the burden of foodborne disease across the world's nations and food chains will allow us not only to prioritize public health efforts, but also to identify new foodborne disease challenges.

7.2 Surveillance data

7.2.1 Foodborne illness surveillance

Systems for monitoring foodborne illness

Foodborne diseases are commonly regarded as one of the most widespread health problems (Motarjemi and Kaferstein, 1997). An estimated 76 million illnesses, 325 000 hospitalizations, and 5000 deaths annually are associated with foodborne illness in the United States alone (Mead *et al*., 1999). Within the framework of the US National Food Safety Initiative, it has been recognized that the availability of reliable epidemiologic data on foodborne disease is an important prerequisite to assessing food hazards and evaluating the cost-effectiveness of prevention programs (Binder *et al*., 1998). As such, surveillance data ultimately provide the grounds for purposeful changes to food safety regulations.

Historically, evaluating the magnitude of foodborne disease in developed countries relied on statistics on foodborne illness from 'passive' surveillance. Passive surveillance methods require clinical microbiology laboratories and physician's offices to report cases of foodborne disease to state, provincial, and/or regional health departments which in turn communicate to a national entity such as the US Centers for Disease Control and Prevention (CDC). Several systems based on this principle exist at the national level in the United States (CDC, 1997; Mead *et al*., 1999): the National Notifiable Disease Surveillance System (NNDSS), the Public Health Laboratory Information System (*Salmonella* and *Shigella*), and the Foodborne Disease Outbreak Surveillance System. The Foodborne Disease Outbreak Surveillance System contains data on more than 20 000 US foodborne disease outbreaks reported to the CDC since 1973 (Batz *et al*., 2005). Perhaps most importantly, the Foodborne Diseases Active Surveillance Network (FoodNet) maintains an 'active' surveillance of illnesses caused by nine pathogens that frequently are foodborne agents. National surveys of health care agencies provide information on patient symptoms, diagnoses, and length of hospital stays (Mead *et al*., 1999). For instance, the National *Salmonella* Surveillance System (NSSS) received 5000 reports of *Salmonella* isolations from state health departments (CDC, 2003). More than 6000 cases of salmonellosis were reported to FoodNet in 2002 (CDC, FoodNet, 2004). The Foodborne Outbreak Response and Surveillance Unit indicates that 29 outbreaks of *Salmonella enterica* serovar Enteritidis were reported to the CDC in 2002. These outbreaks resulted in 840 reported illnesses, 52 hospitalizations, and no deaths among residents of 23 states (CDC, 2002).

In most US counties, doctors and clinical laboratories notify a local health department when a nationally notifiable disease is diagnosed. Botulism, cryptosporidiosis, cyclosporiasis, listeriosis, shigellosis, salmonellosis, and infections

caused by *E. coli* O157:H7 are several foodborne diseases that are among the nationally notifiable diseases. At the local level, staff members implement appropriate control measures and forward the information to the state level. Next, state health departments forward the information to the CDC. Core surveillance data include date, county, age, sex, race/ethnicity, and disease-specific information. Surveillance data may be reported either as individual cases or as aggregated data for a group of cases.

Surveillance has been defined as an ongoing and systematic collection, analysis, interpretation, and dissemination of descriptive information on health events (Declich and Carter, 1994). Essentially, surveillance systems focus on describing when and where health problems are occurring and who is affected, i.e. the quintessential epidemiologic triad information of time, place, and person (Buehler, 1998). In this process, a key constraint is the balance between information needs and the feasibility of data collection. As a continuous process, the long-term sustainability of a surveillance system relies on the participation of the involved health professionals upon whom only a proportionate burden can be put. Surveillance data are thus generally less specific or precise than those from research studies, and their analysis and interpretation impose caution.

Limitations to surveillance data

Surveillance data are useful for analyzing disease trends and determining relative disease burdens. However, it is commonly accepted that their absolute figures result in large underestimates of the actual incidence of foodborne illnesses. Surveillance of foodborne illnesses is complicated by several factors (Mead *et al*., 1999). While diarrheal diseases can be severe or even fatal, milder cases usually do not require medical care and thus go unreported. Second, the role of foodborne transmission is obscured by the fact that many pathogens transmitted through food also are spread through water or from person to person. Food attribution, or the capacity to attribute cases of foodborne disease to a food vehicle, may be determined either epidemiologically or microbiologically (Batz *et al*., 2005). However, in the period from 1993 to 1997, a specific food was not identified in 20–40% of reported cases associated with outbreaks (Olsen *et al*., 2000). Finally, the infectious etiology of foodborne illness often remains undefined. In 1993–97, approximately 40% of reported outbreak cases were associated with an unknown agent (Olsen *et al*., 2000). In fact, some proportion of foodborne illness is likely to be caused by microorganisms whose role as foodborne pathogens has yet to be recognized – the role of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Cyclospora cayetanensis* as causes of foodborne illness was unrecognized just two decades ago.

Additionally, surveillance data are susceptible to external influences. Reports may be influenced by varying diagnostic and reporting procedures, as well as varying resources and priorities of state and local officials. New methods for public health surveillance and new detection methods may also cause changes in disease reporting that are independent of the true incidence of disease.

FoodNet active surveillance

Statistics on foodborne illnesses have historically relied on 'passive' surveillance. The Foodborne Diseases Active Surveillance Network (FoodNet) was established in 1996 as a collaborative effort by the CDC, the US Department of Agriculture, the US Food and Drug Administration, and selected state health departments. FoodNet records laboratory-confirmed cases associated with seven bacteria (*Campylobacter* spp., *Escherichia coli* O157, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Yersinia enterocolitica*) and two parasitic protozoa (*Cryptosporidium* spp., *Cyclospora* spp.). In 2006, the system covered regions in ten US states (California, Connecticut, Georgia, Minnesota, Oregon, Maryland, Tennessee, Colorado, New Mexico, and New York) for a population of 44.1 million inhabitants (equivalent to 15.3% of the national population).

FoodNet has four main objectives: (1) to measure the frequency and severity of foodborne diseases; (2) to establish the relative importance of specific food items; (3) to describe the epidemiology of new and emerging foodborne pathogens; and (4) to monitor temporal trends (CDC, 2000d). The core component of FoodNet is a population-based active surveillance with participation of over 300 clinical microbiology laboratories distributed in several states. The word 'active' essentially means that FoodNet investigators contact these laboratories either weekly or monthly to collect information on all laboratory-confirmed cases of diarrheal and listeriosis infections. Specific information such as possible vehicle and demographic characteristics also are collected for each case. As the type of reporting implies, most specimens are obtained for diagnostic purpose from ill persons.

The results of the FoodNet active surveillance are viewed as a comprehensive and timely database of foodborne illness in a well-defined population (CDC, 1997). They are presented in yearly reports (CDC, 2000a,b,c,d; 2001a). Table 7.1 summarizes the infection rates per 100 000 individuals at the five original FoodNet sites (i.e. California, Connecticut, Georgia, Minnesota, and Oregon) for

Table 7.1 Incidence per 100 000 inhabitants of selected pathogens detected by FoodNet, 1996–1999

Pathogen	1996	1997	1998	1999
Campylobacter	23.5	25.2	21.4	17.3
Salmonella	14.5	13.6	12.3	14.8
Shigella	8.9	7.5	8.5	5.0
Cryptosporidium	n/a	3.0	3.4	2.9
Escherichia coli O157	2.7	2.3	2.8	2.1
Yersinia	1.0	0.9	1.0	0.8
Listeria	0.5	0.5	0.6	0.5
Vibrio	0.2	0.3	0.3	0.2
Cyclospora	n/a	0.3	<0.1	<0.1
Total	51.3	53.6	50.3	43.6

the period from 1996 to 2000 (CDC, 2001a). These data show that the relative importance of the individual pathogens has remained relatively constant over the 5-year period. In particular, *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. have been – in decreasing order – the three most frequently reported agents. Important year-to-year and regional fluctuations complicate the identification of secular incidence trends (CDC, 2001a). For instance, an enlargement of the area under surveillance can explain annual variations for some sites. Further, the occurrence of large outbreaks also has an important impact. For instance, the spike in shigellosis cases registered in 2000 was attributed to outbreaks in California and Minnesota. When the 1996 and 2000 data are compared, the incidence of both *Campylobacter* and *Salmonella* infections declined. While *Salmonella* rates were fewer in all five original sites, *Campylobacter* declined in only four sites. Nevertheless, fluctuations in the years between 1996 and 2000 could also suggest that the decline is merely part of a random process. Especially in the case of *Shigella* infections, the incidence varies substantially from year to year and from site to site. Overall, these few points clearly show that, based solely on a descriptive analysis, the identification of temporal trends is challenging and cannot be established with reasonable certainty.

Besides contrasting the results of different surveillance years, the annual FoodNet reports describe the influence that select demographic characteristics have on the number of reported cases. The reports for the years 1997, 1998, and 1999 discuss age and gender effects, and conclude that, especially for *Campylobacter* and *Salmonella* infections, the annual incidence of foodborne illness varies by age and gender. However, from the text of the FoodNet reports, it is not clear whether the highlighted differences are the result of statistical analysis. The reports also present data on ethnicity and race, i.e. number of cases and percentage of population distribution for each pathogen/site. Unfortunately, absence of information on these traits' distribution in the surveyed population makes a more detailed analysis impossible.

In addition to measuring the incidence of disease associated with these foodborne pathogens, surveys on the frequency of diarrhea in the general population, the proportion of ill persons seeking care, and the frequency of stool culturing by physicians and laboratories for selected foodborne pathogens are investigated within the framework provided by FoodNet. In the context of food safety initiatives, FoodNet can be used to help evaluate the efficacy of enacted regulatory measures, such as the USDA Food and Safety Inspection Service's 1996 Pathogen Reduction and Hazard Analysis and Critical Control Points (HACCP) Rule, in decreasing the number of cases of foodborne diseases in the United States. To illustrate this point, the incidence rates of foodborne disease per 100 000 inhabitants estimated through FoodNet are presented in Table 7.1. While *Campylobacter* spp. and *Salmonella* spp. remain the leading causes of foodborne diarrheal illnesses, these figures indicate that the total burden of disease and particularly the incidence of campylobacteriosis have diminished between 1996 and 1999. Regulatory and policy officials note that this coincides

with the staggered implementation of HACCP in poultry and beef slaughter plants, which occurred over the same period. Among its key findings, the 1999 FoodNet report mentions that *Campylobacter* incidence decreased by 18% between 1998 and 1999, and by 26% between 1996 and 1999 (CDC, 2000d). As poultry is the most common source of *Campylobacter*, the decline is linked to changes in poultry processing plants required by the implementation of the HACCP Rule. This example shows that inferences drawn from FoodNet surveillance data have implications that reach into the core of the food safety policy-making process. Understanding the limitations of the surveillance tool has to be viewed as an important element in safeguarding the scientific soundness of that process.

The main limitations of the FoodNet surveillance have been discussed (CDC, 2001a). Currently the system encompasses nearly one-fifth of the US population, although the data may not be entirely representative of the national situation. Secondly, FoodNet does not completely eliminate underreporting. Since most foodborne illnesses will not require in-depth medical investigation, data still merely reflect the fraction of cases that are laboratory-confirmed. Even if an illness case becomes the object of a laboratory work-up, procedural and performance differences among laboratories may influence the isolation outcome. For instance, while stool specimens are routinely tested for *Salmonella* and *Shigella* and often for *Campylobacter*, testing for *E. coli* O157 is only carried out half the time, and other pathogens even less frequently. Importantly, some reported cases may be the result of an exposure not linked to food, such as drinking water or person-to-person contact, and the source of infection on a population basis generally only is established during sporadic case-control studies. Finally, cases are recorded by site of occurrence, but exposure may well have occurred at another location.

Estimating the burden of foodborne illnesses in the United States

Several attempts have been made over the years to estimate the total burden by foodborne diseases in the United States based on surveillance data (see Table 7.2). Although inevitably flawed by a paucity of data and thus requiring a large number of assumptions, such estimates have had a critical role in setting public health priorities. A closer look at the processes used to generate such estimates is thus warranted.

By coupling information on the underreporting of salmonellosis with data on other foodborne pathogens, Archer and Kvenberg estimated in 1985 that 24 to 81 million foodborne illnesses, inclusive of all pathogens, occurred in the United States each year (Archer and Kvenberg, 1985). Illnesses due to known pathogens were estimated at 8.9 million. In 1987, Bennett *et al*. computed incidence figures for all known infectious diseases and for different transmission modes. It was concluded that foodborne transmission of known pathogens caused 6.5 million illnesses and up to 9000 deaths. Todd (1989) employed a combination of methods, including extrapolation from Canadian surveillance data, to derive an estimate of 5.5 million foodborne illnesses and

Table 7.2 Estimated number of illnesses and deaths due to foodborne hazards in the United States

Source	Number of illnesses (in millions)	Number of deaths
Archer and Kvenberg (1985)	24 to 81	–
Bennett *et al*. (1987)	6.5	8980
Todd (1989)	12.5	520
CAST (1994)	6.5 to 33	9000
Mead *et al*. (1999)	76	5200

170 related deaths in the United States. A group of experts convened by the Council for Agricultural Science and Technology concluded in 1994 that illness cases likely ranged between 6.5 and 33 million and that deaths might be as high as 9000 (CAST, 1994).

Most recently, Mead *et al*. (1999) compiled and analyzed information from multiple surveillance systems, including FoodNet. The analysis entailed three basic assumptions that concerned the degree of underreporting, the proportion of foodborne transmission for the individual pathogens, and the frequency of acute gastroenteritis in the general population. These investigators concluded that foodborne diseases cause approximately 76 million illnesses and 5200 deaths annually. Known pathogens were estimated to account for 14 million illnesses and 1800 deaths.

The same authors recognized two limitations in these estimates (Mead *et al*., 1999). First, separate calculation methods were necessary for estimates specific to bacterial, parasitic, and viral pathogens because of different surveillance information. Second, some rare infectious agents, such as *Plesiomonas*, *Aeromonas*, and *Edwardsiella*, as well as noninfectious agents, such as mushroom or marine biotoxins, metals, and other inorganic toxins, were not considered because of a lack of surveillance data. Mead *et al*. (1999) also discussed possible explanations for the discordance among estimates obtained by different authors. First, it is noted that the various figures often refer to different groupings of pathogens, i.e. either to known pathogens or to all causes of foodborne illnesses (known and unknown, infectious and noninfectious). Second, the single analyses used data from different sources. Finally, different rates of foodborne transmission were assumed in all cases.

7.2.2 Analytical methods for surveillance data: a *Salmonella* case study

Outbreak versus sporadic cases

While the cause of the majority of foodborne outbreaks is unknown, the number of outbreaks with definitive etiology remains relatively constant (Fig. 7.1). Although outbreaks are often the newsworthy effect of foodborne diseases, the number of cases that they cause is merely a fraction of all foodborne illnesses that occur each year. Hence, 'sporadic' cases – that is, cases of foodborne

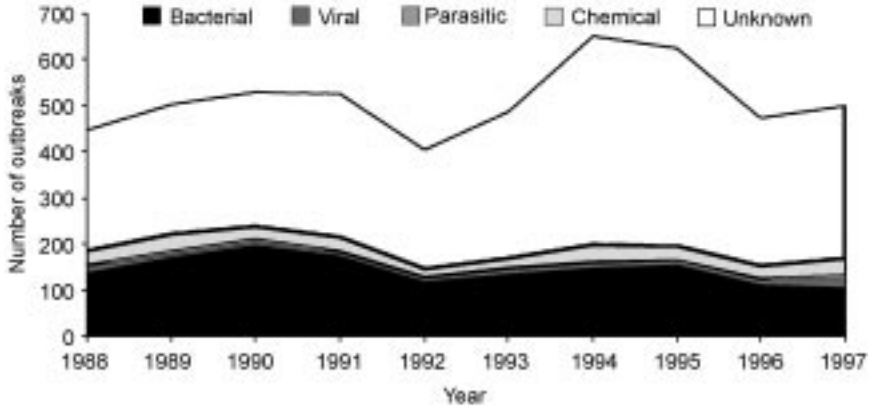

Fig. 7.1 Number of foodborne outbreaks in the United States, 1988–1997.

illnesses that are not or cannot be linked to an outbreak – make up the majority of foodborne illnesses. This point is illustrated in the case of *Salmonella* isolates (Fig. 7.2). In the period from 1988 to 1997, more than two-thirds of the *Salmonella* isolates that were reported to CDC were from sporadic cases (range: 68% in 1996 to 93% in 1988). The proportion of sporadic cases for other foodborne pathogens is even higher than the one observed with *Salmonella*. Although the distinction between outbreak and sporadic cases is somewhat artificial, given the working definition of an outbreak used by CDC (i.e. two or more linked cases), the observation is meaningful in the context of microbial dose–response assessment. In fact, a high proportion of sporadic cases may be suggestive of a small attack-rate and thus of low-dose exposure. This conclusion is consistent with the postulate that the actual dose ingested in sporadic cases of

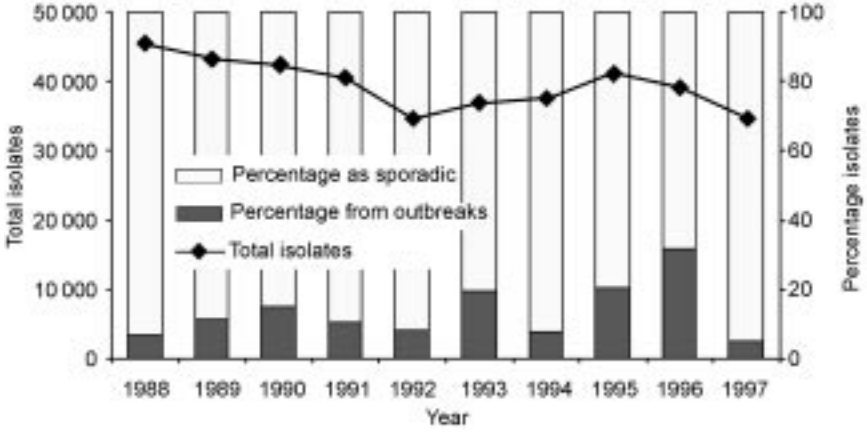

Fig. 7.2 Total *Salmonella* isolates and percent distribution between outbreak and sporadic cases in the United States, 1988–1997.

human salmonellosis frequently may be the 1% infective dose (ID_1) (Blaser and Newman, 1982).

Host factors

The findings of epidemiologic studies probably offer the best opportunity to identify host factors that influence the risk of foodborne disease. A review of two dozen analytical studies, i.e. case-control and cohort studies, related to *Salmonella* infection is presented below (Table 7.3).

Age

A common observation is that age of patients with *Salmonella* infections is distributed according to a bimodal distribution with peaks in children and the elderly. In a Belgian hospital-based study covering isolates for a 20-year period (1973–1992), *S.* Typhimurium and *S.* Enteritidis were mainly isolated in children of less than 5 years of age (Le Bacq *et al*., 1994). The age distribution was, however, less accentuated for *S.* Enteritidis than for *S.* Typhimurium. Both serovars were more likely to lead to bacteremia in middle and older age groups than in those younger than 5 years of age (Le Bacq *et al*., 1994), confirming a previous observation made in the United States (Blaser and Feldman, 1981). Another study reported on *Salmonella* isolates obtained by a Hong Kong hospital for the period 1982–1993 (Wong *et al*., 1994). Among both intestinal and extraintestinal isolates, *S.* Typhimurium, *S.* Derby and *S.* Saintpaul predominated in infants. In patients older than 1 year of age, *S.* Derby and *S.* Typhimurium remained the most common intestinal isolates, while *S. typhi, S.* Typhimurium, and *S.* Enteritidis were the most common extraintestinal isolates. In a British population-based study, the highest age-specific isolation rates for *S.* Enteritidis were observed in children aged less than 2 years, and for *S.* Typhimurium, in those under 1 year (Banatvala *et al*., 1999).

Table 7.3 Risk factors for foodborne non-typhoidal salmonellosis reported in case-control and cohort studies

Factor category	Reported factors
Demographic and socioeconomic factors	Age Gender Race and ethnicity Nutritional status Social/economic/environmental factors Travel abroad
Genetic factors	*HLA-B27* gene
Health factors	Immune status Previous exposure Concurrent infections Underlying diseases Concurrent medications

In children younger than one 1 year of age, the peak incidence is generally observed in the second and third months (Ryder *et al*., 1976; Davis, 1981; CDC, 1983). The study from Hong Kong showed, however, a peak at 12 months of age (Wong *et al*., 1994). In a study on Peruvian children, the IgG and IgM titers against *Salmonellae* serogroups AO, BO, and DO were higher at 12 months of age than at 2 or 3 months of age, which was interpreted as an indication of acquired immunity (Minh *et al*., 1998).

It should be pointed out that association with age might be spurious. It is likely that children and the elderly with diarrhea are more frequently cultured than other age groups (Banatvala *et al*., 1999). Further, age influences the relative exposure to specific serovars. This may explain an increased risk of infection with resistant *Salmonella* serovars, which has been observed in infants (Lee *et al*., 1994). Moreover, age association may reflect behavioral characteristics. For instance, eating snow, sand, or soil – a behavior more likely in children – was found to be associated with *S.* Typhimurium O:4-12 infection (Kapperud *et al*., 1998b).

Gender

In terms of number of isolates, men seem to be more likely to become infected with *Salmonella* than women. A male-to-female ratio of 1.1 has been reported on various occasions (Blaser and Feldman, 1981; Le Bacq *et al*., 1994; Wong *et al*., 1994). The significance of such a finding does not appear to have been addressed. Several factors, such as proportion of the two genders as well as different age distributions for males and females within a country or hospital catchment area may play an important role. In the evaluation of single studies, it should be pointed out that the occurrence of other factors, e.g. use of antacids or pregnancy, tend to be gender specific, and gender may thus have the effect of a confounder.

Race and ethnicity

The potential role of race and ethnicity has seldom been considered. An association with black race and Hispanic origin was reported for resistant *Salmonella* infections (Riley *et al*., 1984; Lee *et al*., 1994). In the former case, the association was explained by differences in the distribution of infecting serovars among ethnic groups, which in turn depended on varying food preferences or methods of food preparation.

Nutritional status

An association between altered nutritional status and acute gastroenteritis has been shown in AIDS patients (Tacconelli *et al*., 1998). Apart from this report, no direct reference to the role of nutritional status was found in the recent literature.

Social/economic/environmental factors

Isolation rates of several *Salmonella* serovars have been compared among groups of different socioeconomic strata on the basis of the Townsend score, an

index for deprivation (Banatvala *et al*., 1999). While isolation rates for *S.* Typhimurium were not related to the Townsend score, highest isolation rates of *S.* Enteritidis were observed in less-deprived areas. It was hypothesized that populations living in the less-deprived areas more frequently ingested vehicles, such as raw eggs, harboring *S.* Enteritidis.

Sanitation deficiencies have been associated with high rates of enteric disease, but direct reference to the potential role of *Salmonella* spp. is scarce. In the 1950s, lack of sanitation, poor housing, limited water supply, and poor personal hygiene were associated with high *Shigella* rates in Guatemala (Beck *et al*., 1957). A similar observation was made in the United States where, in areas of inadequate sanitary facilities, poor housing, and low income, *Shigella* infections were the major causes of diarrheal diseases. In particular, there were nearly twice as many cases of diarrhea among persons living in dwellings having outhouses than among those whose houses had indoor toilet facilities (Schliessmann *et al*., 1958). In certain Guatemalan villages, the habits of the people and the density of the population were found to be more important determinants of diarrheal disease than the type of housing (Bruch *et al*., 1963). In a study conducted in Panama, six representative types of dwellings were considered as an index of social and economic influences on the prevalence of specific enteric pathogens among infants with diarrheal disease (Kourany and Vasquez, 1969). Each dwelling type differed characteristically from one another, but five of the six types were considered substandard and their occupants were of low socioeconomic status. Infection rates for enteropathogenic *Escherichia coli*, *Shigella*, and *Salmonella* among infants from the various groups of substandard dwellings ranged from 6.0 to 10.2%, in contrast to the zero infection rate observed in infants from the better housing type. It is worth noting that the literature on sanitation and housing was mainly published in the 1950s and 1960s. It is possible that improved waste-water management and drinking water quality consequent to economic development has diminished the importance of those factors in some countries.

A French study on sporadic *S.* Enteritidis infections in children investigated the influence of diarrhea in another household member in the 3–10 days before a child showed clinical symptoms. The strength of the association with such a factor appeared stronger for cases in infants (1 year of age or less) as compared with cases in children between 1 and 5 years of age (Delarocque-Astagneau *et al*., 1998). On the basis of this observation as well as other results of the study, it was postulated that *S.* Enteritidis infection in children of less than 1 year of age is mainly related to exposure to a household contact, while children between 1 and 5 years of age are more likely to contract infection by consuming raw or undercooked egg products or chicken.

A seasonal pattern in isolations, which generally shows increased rates during warmer months, has been documented. For instance, in a British study, increased isolation rates for *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, and *S.* Newport were observed in summer (Banatvala *et al*., 1999). The French study mentioned above noted that the association between *S.* Enteritidis infection and prolonged

storage of eggs was stronger during the summer (Delarocque-Astagneau *et al*., 1998).

Travel abroad

Travel abroad is a risk factor for *Salmonella* gastroenteritis that has been consistently demonstrated in both North America and northern Europe. For California residents, Kass *et al*. (1992) demonstrated an association between sporadic salmonellosis and travel outside the United States within 3 weeks prior to the onset of illness. Possible variations in sporadic salmonellosis were cited in a Swiss study (Schmid *et al*., 1996) where travel abroad within 3 days prior to disease onset was found to be associated with both *S.* Enteritidis and serovars other than Enteritidis, although to a greater extent for the latter case. While most patients with *S.* Enteritidis infection were more likely to have traveled within Europe, the majority of non-Enteritidis infections might have originated outside Europe. Individuals of a British region with *Salmonella* infection were more likely to have reported travel abroad in the week before the onset of illness (Banatvala *et al*., 1999). Frequency of overseas travel between patients with *S.* Enteritidis or *S.* Typhimurium infection was no different, but it was among patients with other serovars. Indication of how travel abroad may lead to increased risk of salmonellosis was reported in a study of Norway residents (Kapperud *et al*., 1998a). This study suggested that about 90% of the cases from whom a travel history was available had acquired their infection abroad. The study failed to show an association with either foreign travel among household members or consumption of poultry. However, consumption of poultry purchased abroad during holiday visits to neighboring countries was the only risk factor considered in the study that remained independently associated with disease. Only cases of *S.* Typhimurium allowed for a separate analysis, which showed an association with both poultry purchased abroad and foreign travel among household members.

Genetic factors

As far as acute gastroenteritis caused by *Salmonella* is concerned, no genetic factors related to the host have been reported. Reports concerning race and ethnicity probably should be considered in light of eating habits. There are, however, genetic determinants for chronic disease sequelae associated with *Salmonella* exposure. For example, a putative association of the gene Human Leukocyte Antigen B27 (*HLA-B27*) for patients with spondyloarthropathies, in particular reactive arthritis and Reiter's syndrome, has been described. The *HLA-B27* gene has a very high prevalence among the native peoples of the circumpolar arctic and sub-arctic regions of Eurasia and North America, and in some regions of Melanesia. In contrast, it is virtually absent among the genetically unmixed native populations of South America, Australia, and among equatorial and southern African Bantus and Sans (Bushmen) (Khan, 1996). Fifty percent of Haida Indians living on Queen Charlotte Islands of the Canadian province of British Columbia have the *HLA-B27* gene, which is the highest prevalence ever observed in a population. The prevalence among Americans of

African descent varies between 2 and 3%, while 8% of the Americans of European descent possess the gene (Khan, 1995).

Immune status

The host immune status is, as in any other infectious disease, a very important factor in determining both infection and clinical illness. In general terms, its importance does not seem to have been the direct subject of any formal work, and has thus to be indirectly assessed though other factors, e.g. age and underlying conditions.

Concurrent infections and underlying conditions

Persons infected with Human Immunodeficiency Virus (HIV) tend to have recurrent enteric bacterial infections. Such infections are often severe and associated with extraintestinal disease (Smith *et al*., 1988; Angulo and Swerdlow, 1995). The following six risk factors for enteric salmonellosis have been identified in HIV-infected patients: increasing value on the prognostic scoring system Acute Physiology and Chronic Health Evaluation (APACHE II); altered nutritional status; previous antibiotic therapy; ingestion of undercooked poultry/eggs or contaminated cooked food; previous opportunistic infections; and stage C HIV infection (Tacconelli *et al*., 1998).

The risk represented by other underlying conditions was evaluated in a large nosocomial foodborne outbreak of *S.* Enteritidis that occurred in 1987 in New York (Telzak *et al*., 1991). Gastrointestinal and cardiovascular diseases, cancer, diabetes mellitus, and alcoholism, as well as use of antacids and antibiotics, were the factors considered. Of these, diabetes was the only condition that was independently associated with infection after exposure to the contaminated meal. Although people with diabetes were more likely to develop symptomatic illness than those without, the difference was not statistically significant. Decreased gastric acidity and autonomic neuropathy of the small bowel (which leads to reduced intestinal motility and prolonged gastrointestinal transit time) are the two biologically plausible mechanisms for the increased risk of *S.* Enteritidis infection among diabetics. Among patients with sporadic salmonellosis in Northern California, diabetes mellitus and cardiac disease were the only two health conditions (out of a total of 14) that were associated with clinical illness (Kass *et al*., 1992). Nongastrointestinal medical conditions and, to a larger extent, a recent history of gastrointestinal disorder, were associated with sporadic *S.* Typhimurium O:4-12 infection in Norway (Kapperud *et al*., 1998b). It was, however, noted that physicians are more likely to recommend a stool culture for patients with preceding illness. In a British epidemiologic study, cases of *Salmonella* infection were more likely to report a long-term illness (including gastroduodenal conditions) than controls (Banatvala *et al*., 1999).

Concurrent medications

Although the use of gastric acidity reducers and antimicrobial medication are often considered risk factors for enteric diseases, the evidence found in the

literature concerning their association with human salmonellosis is inconsistent. While some studies have shown an association with antacid use (Banatvala *et al*., 1999), others have failed to do so (Telzak *et al*., 1991; Kapperud *et al*., 1998a,b). A similar situation is found for the use of antibiotics in the weeks/days preceding the infection or disease onset: some studies have demonstrated an association (Pavia *et al*., 1990; Kass *et al*., 1992; Bellido Blasco *et al*., 1998) but other have not (Telzak *et al*., 1991; Kapperud *et al*., 1998a,b; Banatvala *et al*., 1999). Having a resistant *Salmonella* infection has been associated with previous antibiotic use (Lee *et al*., 1994). An association between serovars other than *S.* Enteritidis and intake of medications other than antacids was shown in Switzerland (Schmid *et al*., 1996). Regular use of medications was a risk factor for *S.* Typhimurium O:4-12 infection in Norway (Kapperud *et al*., 1998b). In the same study, use of antacids and antibiotics were not risk factors.

Application of analytical methods to FoodNet surveillance data to explore typical risk factors for salmonellosis

Surveillance data have essentially been the object of descriptive rather than analytical investigations. For instance, annual FoodNet reports describe the temporal trend of foodborne diseases by contrasting yearly rates and interpreting the potential effect of demographic covariates through frequency tables (CDC, 2000a,b,c,d). A more analytical approach would harness the multivariate and longitudinal characteristics of the FoodNet data and could provide additional epidemiologic insights.

Common goals of surveillance data are to estimate incidences (rates of illness or infection per population at risk) and to establish the potential relationship between incidence and a set of available explanatory variables (e.g. site, age). In applying higher-order statistical methods to these goals, specific challenges are likely to emerge. First, surveillance data are less specific or precise than those from controlled research studies (Buehler, 1998), and may not be amenable to the assumptions constraining statistical analyses. For example, a quantitative approach would have to respect two constraints specific to surveillance data: (1) the discrete (rather than continuous) count characteristic of the dependent variable; and (2) the likely correlation among measurements repeated annually (i.e. autocorrelation). Additionally, exposure may not be well characterized by the available explanatory variables (place, time, covariate). Finally, the interrelationship among these effects may be complex.

Surveillance data often come in the form of discrete counts of events, in this case, the number of cases of infections that often are foodborne. When frequencies are counted, an adequate assumption is that the counts follow a Poisson distribution (Stokes *et al*., 2000). Such an approach has previously been applied in epidemiology. For instance, Shahpar and Li (1999) performed an age–period–cohort analysis to characterize the temporal trends and birth cohort patterns of death rates from homicide in the United States. Other recent examples are the analyses of (1) mortality trends for multiple sclerosis in Italy (Tassinari *et al*., 2001); (2) age-incidence relationships in cervical cancer in

Sweden (Hemminki *et al*., 2001); and (3) age, sex, geographic and socio-economic effects in hospital admissions for anaphylaxis in the United Kingdom (Sheikh and Alves, 2001). A useful characteristic of the Poisson log-linear model is that, similar to logistic regression, the exponentiation of the parameter coefficients leads to measures of relative risk, i.e. the incidence rate ratio (IRR).

In capturing the temporal trend of health events, most surveillance systems collect data over consecutive time periods. Similar to time-series data, observations within a specific site (one cluster) are likely autocorrelated. Therefore, drawing valid statistical inferences requires respecting the longitudinal structure of the data in the analysis (Diggle *et al*., 1994). If ignored, inefficient estimates of the regression coefficients (i.e. imprecise estimates) and incorrect inferences about those coefficients would result. The Generalized Estimating Equations (GEE) method is an extension of the Generalized Linear Model that provides a semi-parametric approach to longitudinal analysis (Liang and Zeger, 1986).

In this study, we seek to extend the usefulness of surveillance data by applying an investigative analytical method to FoodNet data or other active surveillance data sets. We use Poisson regression analysis as an analytical tool to model rates of foodborne illnesses as a function of age, gender, site, and year. Parameters are estimated through the GEE method. Specific outcomes are incidence rate ratios of salmonellosis for the different levels of two covariates (age and gender). Such relative risks can be employed to characterize inter-individual variability in susceptibility which is useful within the framework of microbial risk assessment.

Methods

Counts of *Salmonella* infections corresponding to the years 1996, 1997, 1998, and 1999 were extracted from the relative annual FoodNet reports (CDC, 2000a,b,c,d). Specifically, the frequency tables describing the distribution of age and gender stratified by site were consulted.

FoodNet surveillance does not necessarily cover an entire state and not all states were covered for the entire 4-year period. Data for the whole 4-year period were available for five sites: California, Connecticut, Georgia, Minnesota, and Oregon. Data for the years 1998 and 1999 were available for Maryland and New York. The level of data aggregation was not only different for the seven sites, but it also changed over the 4-year period. The 1996 data cover the entire states of Minnesota and Oregon, and selected counties in California, Connecticut, and Georgia (CDC, 2001a). Twelve Georgia counties and one county in Connecticut were added in 1997. In 1998, the surveillance became statewide for Connecticut, and selected counties in Maryland and New York were added. Finally, the remaining counties in Georgia and eight counties in New York were added in 1998. From 1996 to 1999, the total population in catchment areas went from 14.3 to 25.9 million.

State- and year-specific censuses stratified by age and gender were obtained from the US Census Bureau (US Census Bureau, 2002). As counties under surveillance are not specified in the FoodNet reports, state censuses were

reduced proportionally to the site-specific populations listed in those reports. It was thus assumed that the age and gender distribution in each site was equal to that at the state level. By combining infection counts and census figures, two data sets – one with counts stratified by eight age categories, the other with counts stratified by gender – were obtained.

After calculation of the annual incidence rates, data were explored qualitatively using graphs in which two of the three explanatory variables (age, gender, and site) were contrasted. Poisson regression was implemented in SAS/STAT version 8.01 (SAS Institute, Cary, NC) with the PROC GENMOD software procedure. The GEE method was used to estimate model parameters. Specification of the REPEATED statement in which the variable identifying clusters was the crossing of age group and site resulted in the implementation of the GEE method (independent covariance structure). Since there are no readily specified procedures to assess goodness-of-fit within the GEE framework, goodness-of-fit of the final model was investigated through analysis of Anscombe residuals (Cameron and Trivedi, 1998). Plots of the Anscombe residuals against the observed number of cases and levels of the explanatory variables were used to assess the goodness-of-fit of the final models. The normality of the residual was checked through the Kolmogorov–Smirnov test and normal probability plots.

Univariate analyses were used to screen explanatory variables to be included in the multivariate models. Specifically, only those variables with a significance level smaller than 0.25 were considered further. This arbitrary threshold was chosen in accordance with standard epidemiological practices (Hosmer and Lemeshow, 1989).

Multivariate analyses started with the model specifying, in addition to the three main effects, all first-order interactions of the retained variables. Through backward selection, the interaction or main term that was the least significant (at a >0.05 level) was subsequently eliminated. The procedure stopped when no term or effect in the model exceeded the 0.05 significance level. Incidence rate ratios were calculated through exponentiation of the parameter estimates. Additional, more technical discussion of the analytical framework used in this study is provided in the Appendix.

Results

Graphical results: Graphical representation of the incidence rates offers insight into potential interactions among covariate (age/gender), place (state), and time (year). Figures 7.3–7.7 systematically contrast two variables by stratifying for the third one. In each figure, the upper and lower series of graphs essentially show the same information, where the levels of the *x*-axis variable in the upper series become the lines in the lower set of graphs, and vice versa. Horizontal lines imply that the *x*-axis variable has no influence on the infection rates; vertical distance among the lines reflects the effect of the other variable. Lines that are parallel between two subsequent levels of the *x*-axis variable suggest a lack of interaction between the two variables. Such parallelism should be evident in both series of graphs. If a similar pattern of lines emerges within each

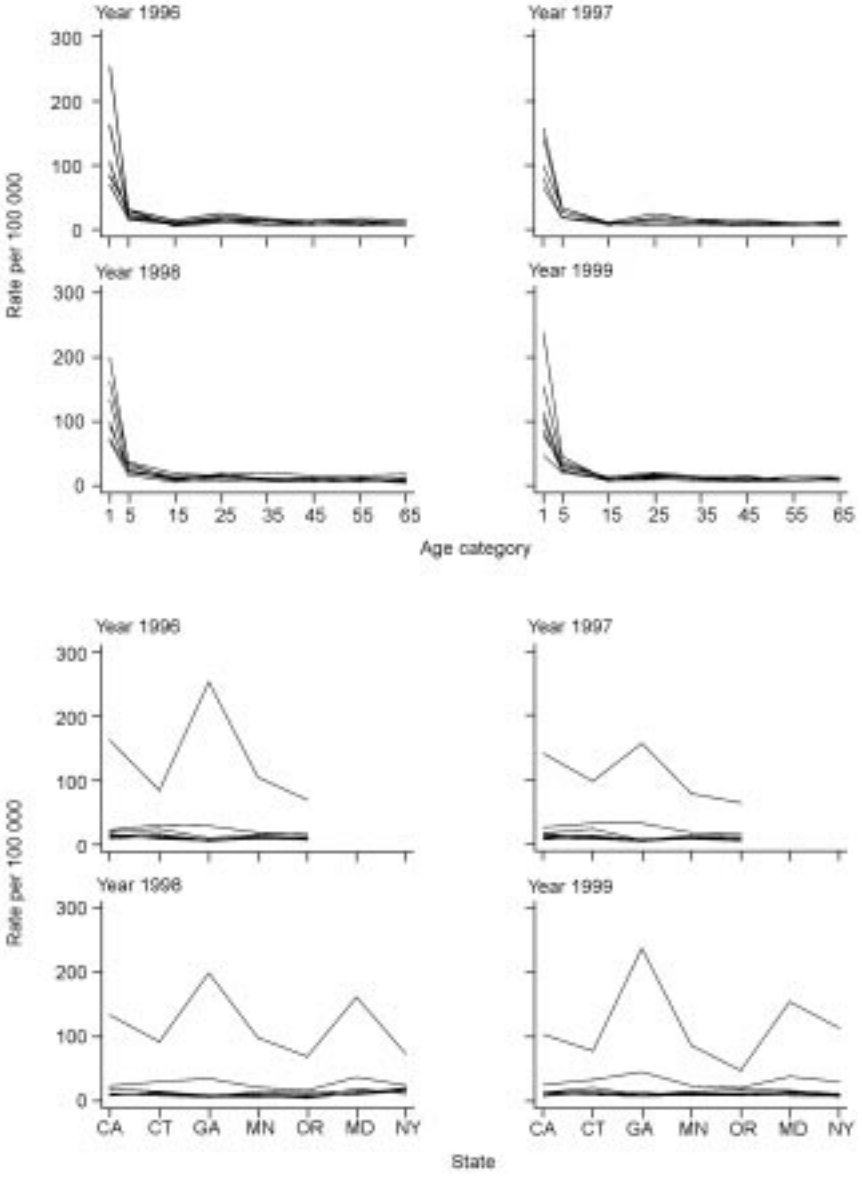

Fig. 7.3 Age and state effects on *Salmonella* isolation rates. Each line connects the rates of age category (upper set of graphs) or a specific state (lower set of graphs).

series of graphs, one would infer that infection rates do not change at different levels of the stratifying variable. The effect of age is presented first.

Age: Rates of *Salmonella* infection are the highest for children less than 1 year of age (Fig. 7.3). Frequencies for the age groups 1–9 and 20–29 appear to be

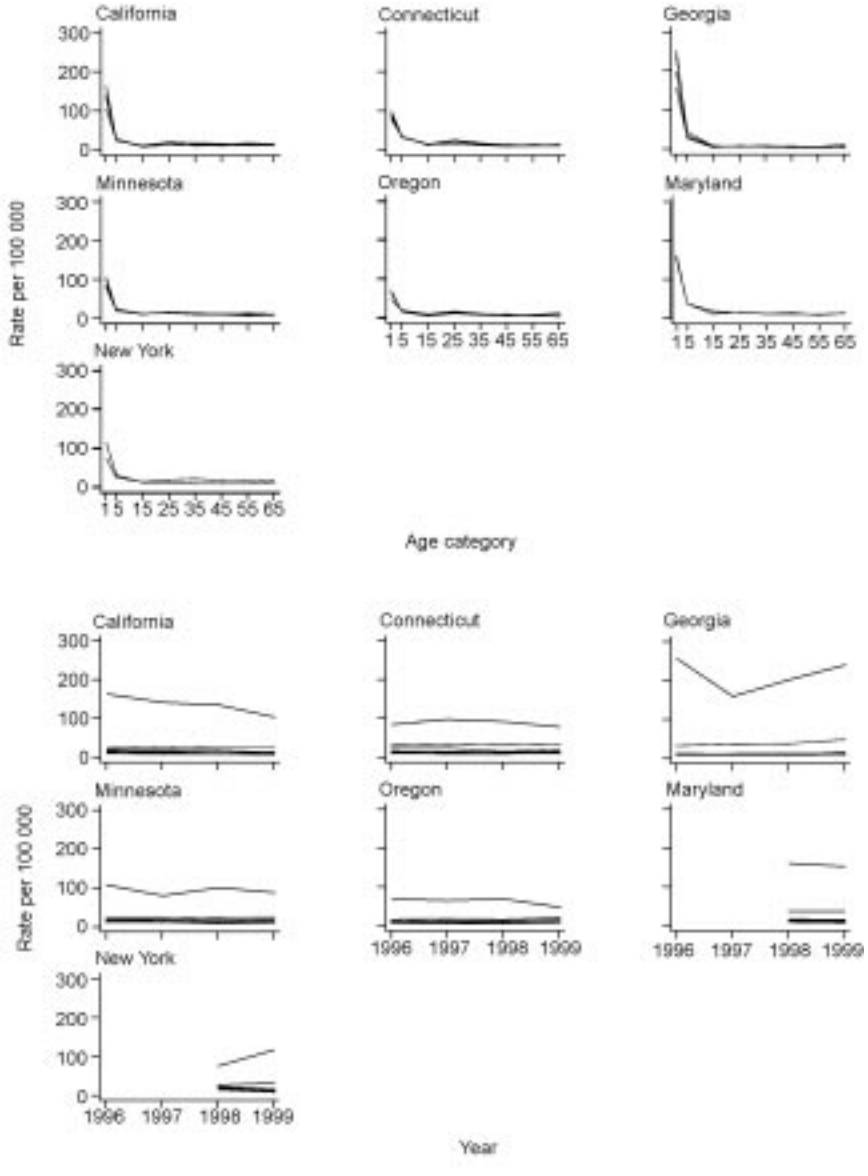

Fig. 7.4 Age and year effects on *Salmonella* isolation rates. Each line connects the rates of age category (upper set of graphs) or a specific year (lower set of graphs).

higher than those of the remaining age categories. The lower series of graphs shows that infection rates vary among states only for infants. For each of the four surveillance years, Georgia has the highest rates among infants, followed by Maryland and California. For the other age groups, the infection rates are fairly constant across sites. Figure 7.4 confirms the highest risk is for infants, but also

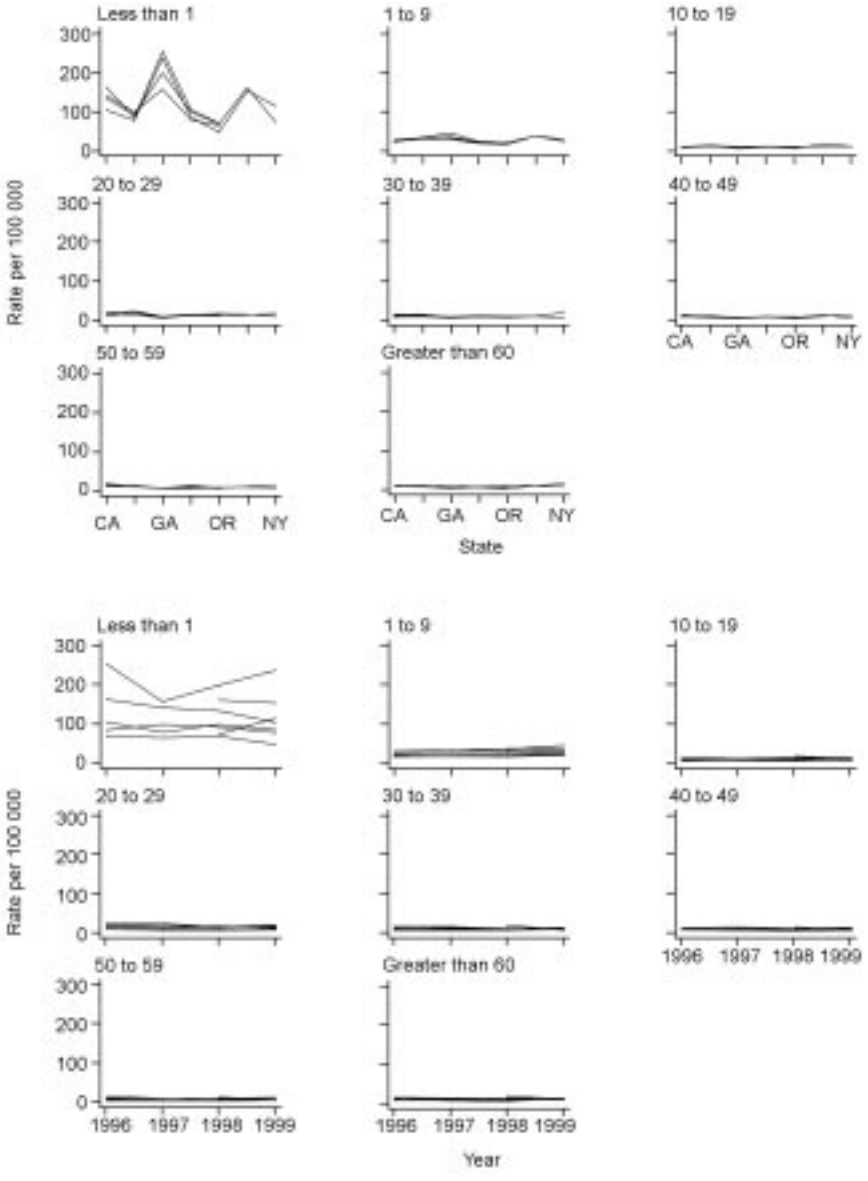

Fig. 7.5 State and year effects on *Salmonella* isolation rates. Each line connects the rates of a state (upper set of graphs) or a specific year (lower set of graphs).

suggests that such risk can vary across surveillance years in an unpredictable manner (lower set of graphs, e.g. constant decline for California, decline and surge for Georgia, increase for New York). The dependence of *Salmonella* infection rates in infants on the variable state and – to a lesser extent – year is again evident in Fig. 7.5. However, this figure clearly shows that, for all other

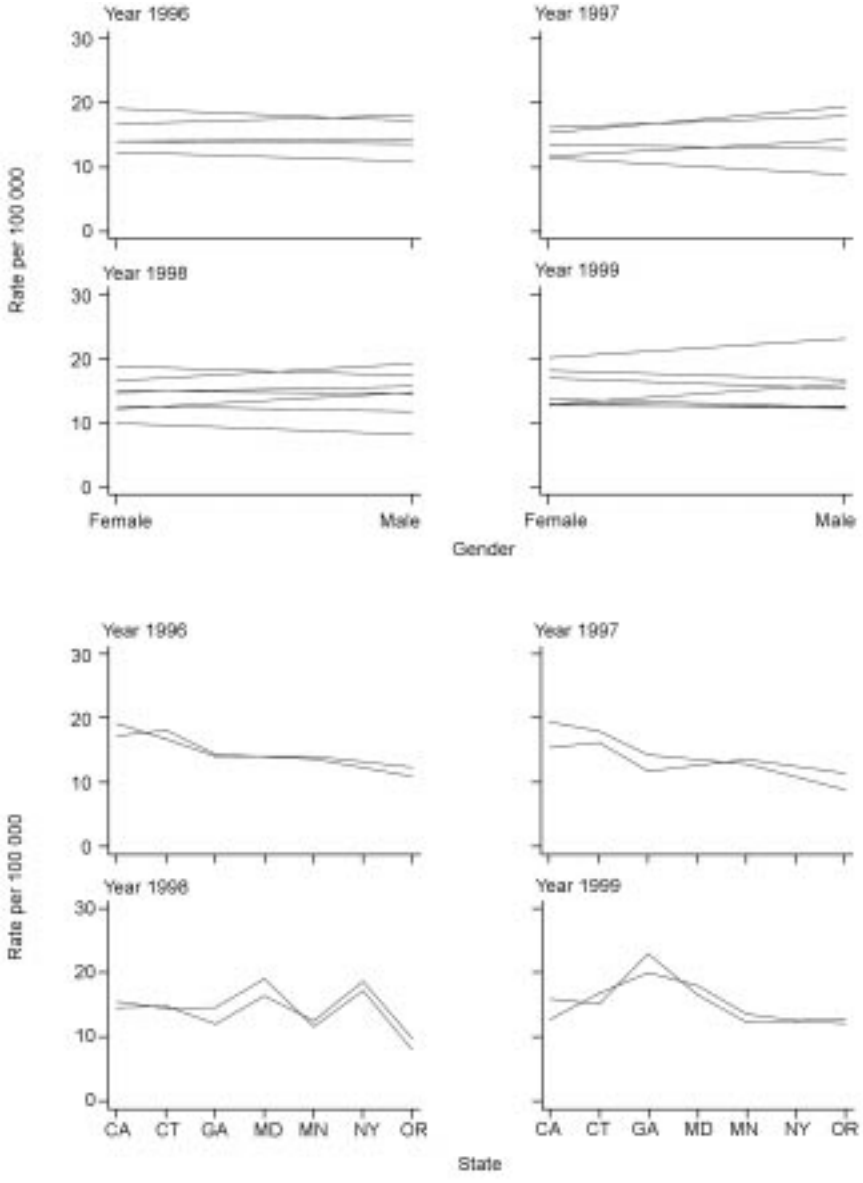

Fig. 7.6 Gender and state effects on *Salmonella* isolation rates. Each line connects the rates of gender (upper set of graphs) or a specific state (lower set of graphs).

age groups, the frequencies are largely unaffected by those two variables. In summary, Figs 7.3 to 7.4 show that *Salmonella* infection rates are higher in infants than in other age groups. However, location (state) and time (year of surveillance) influence the specific risk in infants, which would suggest interaction between the considered variables.

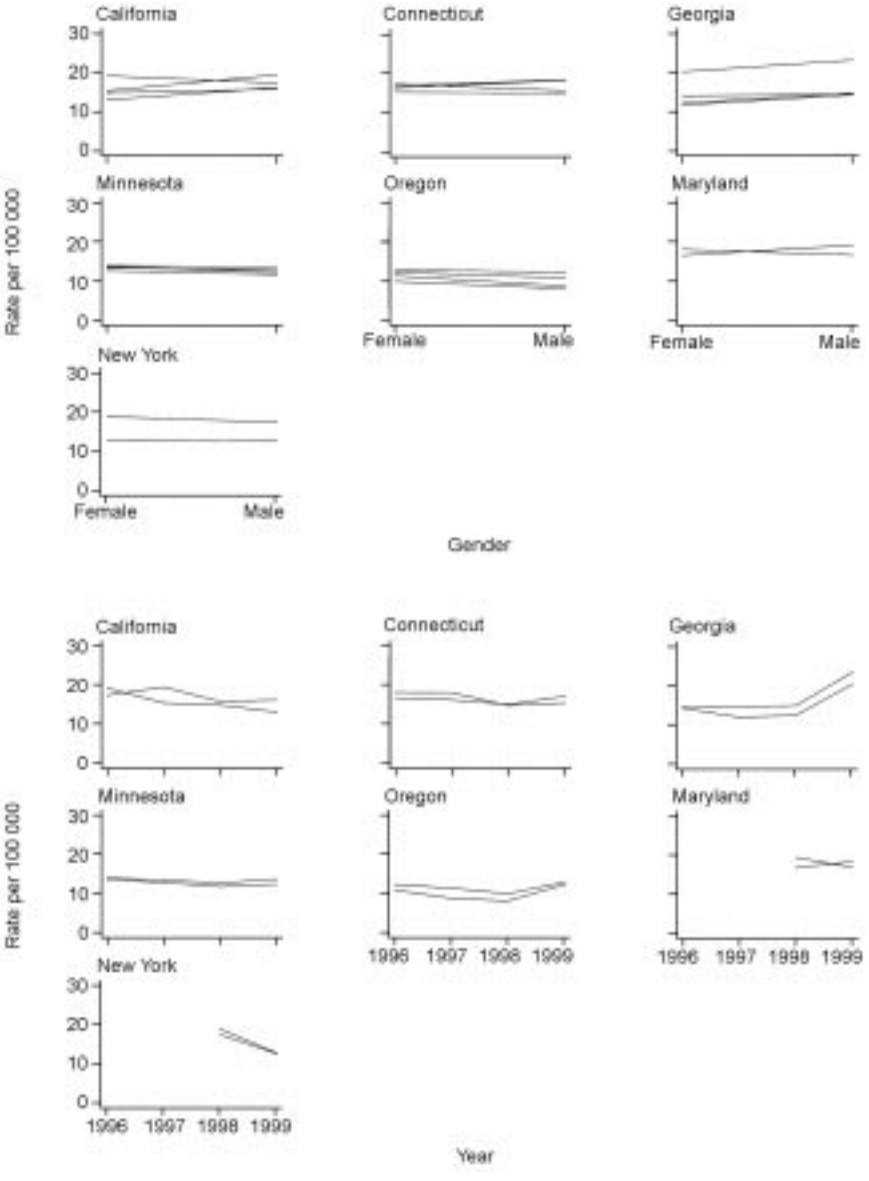

Fig. 7.7 Gender and year effects on *Salmonella* isolation rates. Each line connects the rates of gender (upper set of graphs) or a year (lower set of graphs).

Gender: Figure 7.6 displays the influence of gender on *Salmonella* infection rates. The influence of gender on *Salmonella* rates does not follow a clear pattern. Depending on the state, the frequencies for females can either be higher than, equal to or lower than the frequency in males. The same consideration is true when the combined effects of age and surveillance year are combined (Fig.

Table 7.4 Annual incidence per 100 000 for reported cases of infections with *Salmonella*

	Crude rate (model with year effect)				Age-state adjusted rate (model with all main effects)		
	Observed	Estimate	LCL	UCL	Estimate	LCL	UCL
1996	14.5	14.5	12.1	17.3	11.3	8.4	15.2
1997	13.7	13.7	11.4	16.5	10.6	7.8	14.3
1998	13.7	13.7	11.4	16.4	10.2	7.6	13.6
1999	15.2	15.2	11.9	19.4	11.3	8.4	15.1

Observed, calculated from FoodNet Reports 1996 to 1999 based on number of cases with known age; LCL, lower 95% confidence limit; UCL, upper 95% confidence limit.

7.7). Of the four variables considered in this study (age, gender, state, year), the influence of gender appears to be the least important.

Analytical results: The first analytical results are incidence rates for each surveillance year estimated from the age-stratified data (Table 7.4). The second column of Table 7.4 presents 'observed' crude rates, i.e. rates that were calculated based on the number of reported cases of known age and the population in catchment areas as reported in each FoodNet report for the years 1996 through 1999. (These figures differ from those reported in Table 7.1, where rates for only the five original sites are reported and cases with unknown age also are included.) 'Estimated' crude rates were determined using a model specifying only year as the explanatory variable, and can be directly compared to the observed rates. As one would expect, the point estimates are equal. However, the analytical approach has advantages in that it delivers confidence limits (columns 4 and 5 of Table 7.4), and allows one to formally compare the single point estimates. Statistical comparison of *Salmonella* incidence by year indicated no significant difference ($p \geq 0.119$).

By fitting a model with all three main effects (age, state, and year), 'age-state adjusted rates' were obtained (columns 6–8 of Table 7.4). While these rates are abstract (they can no longer be compared with the observed crude rates), the adjusted rates reflect the specific impact of time since age and state effects are controlled. The adjustment leads to minor rectification of the previous conclusions – the *Salmonella* rates for 1998 become lower than those of 1996 and 1999 ($p = 0.012$ and $p = 0.019$, respectively), but the other contrasts are statistically indistinguishable ($p \geq 0.064$).

Age: The incidence rate ratios for the univariate and multivariate analyses of the age-stratified data set are reported in Table 7.5. In these tables, the reference levels of each variable are an age between 20 and 29 years, site Oregon, and surveillance year 1999. (Note that the incidence rate ratio for the year effect from the univariate analyses reported in Table 7.5 is equivalent to the ratio of the rates listed in Table 7.4.) Where the confidence interval does not include the

Table 7.5 Incidence rate ratio for *Salmonella* (Poisson model with generalized estimating equations, independent correlation structure)

Parameter	Univariate analysis			Multivariate analysis		
	IRR	LCL	UCL	IRR	LCL	UCL
Age						
<1	9.16	5.71	14.70	9.15	5.83	14.34
1–9	1.98	1.40	2.80	1.97	1.43	2.72
10–19	0.71	0.53	0.94	0.71	0.53	0.96
20–29	1.00	–	–	1.00	–	–
30–39	0.78	0.59	1.04	0.78	0.58	1.06
40–49	0.66	0.50	0.89	0.67	0.50	0.90
50–59	0.63	0.47	0.84	0.63	0.47	0.86
≥60	0.70	0.54	0.91	0.70	0.54	0.92
State						
CA	1.50	0.99	2.29	1.42	1.17	1.73
CT	1.48	0.97	2.27	1.49	1.23	1.79
GA	1.38	0.68	2.80	1.32	0.97	1.80
MD	1.62	1.00	2.65	1.62	1.37	1.93
MN	1.20	0.82	1.75	1.19	0.98	1.45
NY	1.34	0.88	2.03	1.32	1.10	1.57
OR	1.00	–	–	1.00	–	–
Year						
1996	0.95	0.82	1.10	1.00	0.91	1.09
1997	0.90	0.79	1.02	0.94	0.86	1.02
1998	0.90	0.79	1.02	0.90	0.84	0.97
1999	1.00	–	–	1.00	–	–

value 1.00, there is a suggestion that the given level differs statistically from the reference level. In the univariate analysis, an age of 9 years or younger is linked to a statistically greater infection risk when compared with the reference age group 20–29. With the exception of the age group 30–39, the remaining age groups are at lower risk than the 20–29 age group. In contrast, all levels of the variables state and year appear to represent indistinguishable risk of *Salmonella* infection.

The overall age and year effects are statistically significant at a level <0.25 in the univariate analysis ($p = 0.038$, $p = 0.202$, respectively) and so are included in the multivariate analysis. The state effect largely exceeds that threshold ($p = 0.567$), and was excluded from the multivariate analysis. Completion of the backward selection procedure led to a final model that contains age as the only explanatory variable. This is equivalent to a univariate analysis with that variable.

The graphical representations of the Anscombe residuals (data not shown) suggest that, although the fit is not particularly good for the variables excluded from the model (state and year), a pattern is absent for age, i.e. the main variable

of interest. Specifically, the residuals seem to be normally distributed with mean −0.047 and standard deviation 2.367 (Kolmogorov–Smirnov statistics $p > 0.15$). Refitting the model after deletions of all observations that generate Anscombe residuals greater than 2.6 does not notably change the values of the parameters (data not shown). It is thus concluded that outliers did not have particular leverage on parameter estimates.

Gender: In the univariate analysis of the gender-stratified data, the gender effect resulted in significance levels greater than 0.25 ($p = 0.868$) and were not considered further in a multivariate analysis. The relative incidence rate ratio was 0.98 (0.81–1.20).

Discussion

Children, the elderly, pregnant women, and immunocompromised persons are commonly thought to be at the greatest risk of illness and mortality from food- and waterborne enteric microorganisms (Gerba *et al*., 1996; Smith, 1998, 1999). Analogously, a main interest of this study was to investigate the potential influence of the covariates age and gender on the incidence rates of *Salmonella* infection recorded through the FoodNet surveillance system for its first 5 years. The justification for carrying out an analysis that also considers the effects of location and time is that the obtained estimates are covariate-specific. By controlling for state and surveillance year, one more efficiently assesses the underlying effect of age or gender.

Generally speaking, indications obtained from the graphical representation of the rates (Figs 7.3–7.7) find confirmation in the statistical analysis (Table 7.5). Specifically, analysis of *Salmonella* rates show that infants (<1 year) have a 9-fold greater risk of infection than young adults. Rates for the age group 1 to 9 are twice those of the reference age groups. In contrast, individuals between 10 and 19 years of age and those older than 40 years display comparable, lower rates. These results are consistent with available literature. For instance, in a Belgian hospital-based study covering isolates for a 20-year period (1973–92), *S.* Typhimurium and *S*. Enteritidis were mainly isolated from children of less than 5 years of age (Le Bacq *et al*., 1994). The highest age-specific isolation rates for *S.* Enteritidis were observed in children under 2 years of age and for *S.* Typhimurium in those under 1 year in a British population-based study (Banatvala *et al*., 1999). The association of age with increased salmonellosis rates might have several explanations including more frequent culturing than other age groups and behavioral characteristics as mentioned above (Banatvala *et al*., 1999; Kapperud *et al*., 1998a,b).

Graphical analysis suggests a slightly lower infection risk for females than for males; however, the differences were not statistically significant. When numbers of *Salmonella* isolates are compared, a male-to-female ratio of 1:1 has been reported on various occasions (Blaser and Feldman, 1981; Le Bacq *et al*., 1994; Wong *et al*., 1994). Seemingly, the significance of such a finding has not previously been addressed. Several factors, such as proportion of the two

genders as well as different age distributions for males and females within a country or hospital catchment area, may play an important role. Unfortunately using these methods, we were unable to test for the compounded effect of gender and age.

The results of this study are subject to limitations in both data and methods. In the food safety arena, a commonly held perception is that, among others, children and the elderly are more susceptible to foodborne pathogens (Gerba *et al*., 1996; Smith, 1998). It is not always made clear whether that statement implies greater susceptibility to illness, infection, or both, or increased risk of exposure. Within the context of FoodNet surveillance, a similar ambiguity exists. A case is defined as the first isolation of an enteric pathogen from a given person (CDC, 2001a). While this definition would only reflect infection, it is also thought that, since data are collected through clinical laboratories, most cases also represent a clinical illness. There is ambiguity as to what extent calculated rates represent risk of illness over and above risk of infection. While our results (and, in general, the literature) point to a higher risk in children, no specific increased risk for the elderly was evident from our analysis of FoodNet data. Mims *et al*. (1995) point out that the evidence for a general reduction in resistance to infectious disease in elderly people is weak. Children could be more susceptible to both infection and illness (because of a naïve immunity), while the elderly could merely be more likely to incur a serious illness (for physical and physiological reasons). As the considered data do not differentiate among degrees of illness severity, our analysis could not have identified the kind of risk typical for the elderly. Furthermore, it has been advanced that case ascertainment in children and, possibly, the elderly is relatively more efficient than that of other age groups (Tauxe, 1992; Banatvala *et al*., 1999). If this is the case, the result could be an overestimate of infection rates for children and the elderly.

A further limitation of our analysis arises from the age classification used in the FoodNet reports. In particular, the age groups comprising children of 1 to 9 years of age and adults older than 60 years may reflect greater heterogeneity in susceptibility to enteric infection/illness than the remaining age groups. Under these conditions, grouping into a unique age category essentially implies an averaging of the risk over the respective age span. Within the age group $\geq$60, this issue is potentially greater because increasing age is associated with a diminishing statistical weight of the relative age stratum. That is, even if older people (e.g. older than 80 years of age) had a much increased risk, their numerical proportion would be too limited for the effect to become apparent. This phenomenon alone could explain why, contrary to expectations, no increased rates for the elderly were found in our analysis.

Another limitation of this analysis is that, since cross-tabulated data for age and gender are unavailable, the joint effect of age and gender cannot be tested. As the influence of age seems to be relatively large, this shortcoming could possibly affect the interpretation of the gender effect. Our results suggest that males are overall at a higher risk than females, but that the increased risk is not

statistically significant; however, the possibility that a decreased risk for males in specific age categories is balanced by an increased risk in other age groups cannot be excluded.

The GEE method has theoretical appeal when it comes to the analysis of surveillance data. Nonetheless, some practical aspects of its implementation are less well established when compared with the MLE framework. Our analysis required making choices regarding the inference and goodness-of-fit tests to be employed. Specifically, the score statistic was used to assess variable significance in Type III contrasts. This statistic tends to be more conservative than the Wald statistic (Stokes *et al*., 2000). Had the latter statistic been chosen, more complex models, i.e. models that would contain more interaction terms, would have resulted (data not shown). The graphical representation of the infection rates seem to indicate that such interactions are present. Rather than being predictive, the intent of our study was explanatory, i.e. the emphasis was on revealing underlying influences on the modeled variables. This justifies having opted for the less sensitive, yet more specific test. Nonetheless, the model building strategy used in this study did not always lead to well-fitted models.

In conclusion, the FoodNet surveillance system is expected to provide epidemiological information important to the food safety policy-making process (Binder *et al*., 1998). While FoodNet undoubtedly represents a qualitative improvement upon previous passive surveillance systems, it would seem most of the emphasis has been on data generation rather than data analysis. For instance, the 1999 FoodNet report concluded that a decline in *Campylobacter* infection rates observed for the period from 1996 to 1999 was likely related to changes in poultry processing plants because of implementation of the HACCP Rule (CDC, 2000d). Nonetheless, in 2000, *Campylobacter* rates surged from the low of 1999 (CDC, 2001a). The extent to which core facts – whether temporal trends or host characteristics – are teased out from a noisy, multifaceted background largely depends on the availability of an adequate analytical framework. This study proposed and investigated the application of the Poisson regression model estimated by means of GEE. From a theoretical perspective, such a statistical approach permits a multivariate analysis while respecting the intrinsic characteristics of the FoodNet data. In practical terms, however, its implementation, such as establishing a model's goodness-of-fit, is not yet well established and still requires judgment from the analyst. The question is not so much whether the identified, underlying findings are in accordance with the expectations; our results are generally consistent with available literature. Rather, the main issues are whether the quantitative estimates are sufficiently reliable and specifying the bounds of confidence we have in our findings.

7.3 Future trends

In this chapter we have demonstrated how epidemiologic surveillance methods have been used to estimate and monitor the burden of foodborne disease in the

United States. Additionally, classic epidemiologic study designs such as case-control studies can be combined with surveillance data to identify risk factors for various diseases. However, little effort has been applied toward validating the apparent trends or developing analytical rather than descriptive methods to identify risk factors.

We expect that with increasing emphasis on risk-based decision making, there will be a concomitant increased need for rigorously established epidemiologic data sets. We will need to identify subpopulations at greater risk, whether because of exposure or susceptibility, and we will need to establish vehicles of illness, especially in newly emerging illnesses. Some of these data needs can be answered by using advanced statistical methods to evaluate the surveillance data that have already been collected, as demonstrated in this chapter. Additionally, once the power of such analytical methods is recognized, surveillance approaches may be refined to facilitate greater and more detailed analyses. Perhaps the greatest benefit of using advanced statistical techniques in the analysis of observational data is for more rigorous characterization of trends. This, in turn, can be used to develop hypotheses that can be tested using carefully designed analytical epidemiological approaches. This last point cannot be overemphasized. While descriptive epidemiological studies on foodborne disease have been an important development over the last decade, targeted analytical, field-based epidemiological studies will remain a critical component in the identification of disease risk factors.

7.4 Sources of further information and advice

The focus of our discussion has been surveillance of foodborne disease within the United States; however, one of the many implications of the globalization of the food supply is the requirement for better surveillance information on a global scale. Currently, the WHO is mobilizing a global network of surveillance networks. With full participation from member nations, this global network will be able to monitor the endemic incidence of foodborne disease across the world, as well as identify spikes and emerging diseases. This global approach offers three key advantages. First, with knowledge of the worldwide burden of foodborne illness, relief agencies will be able to prioritize efforts to areas that most need aid. Second, ongoing observation of endemic levels of illness provides a baseline understanding of the nature and level of foodborne disease in an area. If a new agent emerges or an outbreak threatens, the global surveillance network can quickly identify the new hazard so that control efforts may be rapidly implemented. Finally, a global network promotes the concept of harmonization among nations that should facilitate and stabilize international trade efforts. We encourage interested readers to visit the WHO and European websites listed below for further information.

- **WHO Network-of-Networks on Foodborne Diseases:** a global network of existing networks involved in the surveillance of foodborne diseases.

- **WHO Global Burden of Disease Project:** ongoing global incidence and mortality data for over 130 specified causes.
- **Enter-net and Salm-gene:** surveillance of foodborne pathogens internationally, predominately within Europe, coupled with the Salm-gene molecular typing network.

7.5 References and further reading

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7.6 Appendix: Analytical framework for surveillance data

Surveillance data often come in the form of discrete counts of events. When frequencies are contained, an adequate assumption is that the counts follow a Poisson distribution (Stokes *et al*., 2000). Computational implementation of the Poisson regression is carried out within the framework of Generalized Linear Models (GLM) (Stokes *et al*., 2000). One assumes that the dependent variable Y is Poisson-distributed with mean and variance μ. If only a single explanatory model is considered, the base GLM regression model for μ is written as

$$g(\mu) = \alpha + x\beta$$

where g is the link function. The Poisson regression model applies the log function as g, and results in the loglinear model:

$$\log(\mu) = \alpha + x\beta$$

When the interest lies in modeling rates, one needs to define an exposure variable N (e.g. population at risk, time at risk). The rate is then Y/N. The expected value becomes μ/N, which is modeled as

$$\log\left(\frac{\mu}{N}\right) = \alpha + x\beta$$

This model can be rearranged as follows

$$\log(\mu) = \alpha + x\beta + \log(N)$$

where the term $\log(N)$ is called an offset and needs to be considered in the estimation process. For multiple explanatory variables, the model is written as

$$\log(\mu_i) = \log(N_i) + x_i'\beta$$

With real-life data, the observed variance of counts usually exceeds the nominal variance (i.e. the mean) of a Poisson distribution (McCullagh and Nelder, 1989). This situation is called overdispersion. When Poisson regression is estimated by maximum likelihood (MLE), overdispersion has an important impact on hypothesis testing. Specifically, while the parameter estimates are still consistent (provided that the conditional mean is correctly specified), their standard errors will be underestimated (Cameron and Trivedi, 1998). This eventually leads to an overly optimistic conclusion on the statistical significance of the considered parameter. As long as outliers and a misspecified regression model can be excluded, overdispersion in MLE can be accommodated by adjusting the variance of the Poisson distribution with a scaling factor or by

applying a more flexible model, such as the negative binomial model (Stokes *et al*., 2000). Alternatively, other estimation approaches could be applied.

To capture the temporal trend of health events, most surveillance systems collect data over consecutive time periods. Similar to time-series data, observations within a specific site (one cluster) are likely autocorrelated. In drawing valid statistical inferences, the longitudinal structure of the data needs to be respected in the analysis (Diggle *et al*., 1994). If ignored, imprecise estimates of the regression coefficients and incorrect inferences about those coefficients would result. The generalized estimating equations (GEE) method is an extension of GLM that provides a semi-parametric approach to longitudinal analysis (Liang and Zeger, 1986). In contrast to GLM, the GEE approach accounts for the structure of the response covariances through its specification in the estimating process. By defining a common link and variance function, the analyst describes the random component of the model for each marginal response. The method then manages the covariance structure as a nuisance parameter, and models the function of the marginal expectation of the response variable as a linear function of explanatory variables. Although the specification of a working correlation matrix is required, the approach is robust to misspecification of this matrix. Even when the assumed correlation structure is incorrect, the GEE method relies on the independence across clusters to consistently estimate the parameter variances.

GEE are ideal for repeated, discrete response data such as binary outcomes and Poisson count (Stokes *et al*., 2000). In addition to modeling the correlation structure, they are inherently resilient to overdispersion. Further, because of the method's flexibility, it can handle continuous explanatory variables, a moderate number of explanatory categorical variables, time-dependent explanatory variables, and randomly missing data. Similar to MLE, GEE rely on asymptotic theory, and a sufficiently large number of clusters is needed to produce consistent estimates. With a limited number of explanatory variables, 25 clusters may be enough. With 5–12 explanatory variables, more than 100 clusters (possibly 200) are preferred. This issue also has implications when it comes to assessing the significance of an explanatory variable through Type 3 contrasts. The Z statistics and the Wald statistics require about 200 clusters to be reliable at the 0.05 confidence level. The score statistics are often more conservative in the presence of small numbers of clusters. Finally, unlike MLE, there are no readily specified procedures to assess goodness-of-fit within the GEE framework.

Part II

Human host factors that influence foodborne disease

8

Nonspecific host defenses against foodborne pathogens

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8.1 Introduction

Foodborne pathogens face a wide array of host defenses that they must survive and overcome to cause infection. These defenses range from biochemical and physical host factors including saliva, gastric acid, peristalsis, intestinal mucous and the barrier formed by enterocytes or intestinal epithelial cells (IEC), to the different levels of immune responses mounted by the host at the level of gut-associated lymphoid tissue (GALT). Successful foodborne pathogens have strategies to defy and evade many of these defenses, allowing them to establish infection in the host and inflict damage on host tissues. The focus of this chapter is on the nonspecific host defenses used against foodborne pathogens and how these defenses are evaded by foodborne pathogens. The potential for bolstering host defenses through the use of probiotics to improve the ability of the host to deal with foodborne pathogens is also considered.

8.2 A look at nonspecific host defenses

8.2.1 Physical barriers

Initial access of foodborne pathogens into the body is barred by physical barriers established by enterocytes, by the mucous layer coating the intestinal mucosal surface, and by the continual motion of the gastrointestinal tract provided by peristalsis. While these physical barriers provide a highly effective defense against foodborne pathogens, microbial mechanisms of evasion have evolved.

Enterocytes or *intestinal epithelial cells (IEC)* form a cohesive barrier preventing ready access of pathogens and many other agents to the internal tissues beyond. These multifunctional cells are active in nutrient transport, exchange of water and electrolytes, hormone production, and also form a protective barrier at the gastrointestinal mucosal interface. In addition to all these roles, IEC are now acknowledged as interactive participants in the mucosal immune response, mediating crosstalk between bacteria in the gut and cells of the gut-associated lymphoid tissue (reviewed in Lu and Walker, 2001; Nagler-Anderson, 2001; Sansonetti, 2004). IEC are polarized cells, with an apical surface covered in microvilli, providing an absorptive surface adapted for nutrient uptake and digestion, yet resisting ready entry by bacteria. *Tight junctions* composed of specialized junctional transmembrane proteins (claudins and occludins) seal each IEC to its neighbor, rendering the epithelial barrier 'leak proof' by inhibiting diffusion across the epithelium, and impeding the ability of foodborne pathogens to invade between IECs. Tight junctions also act to restrict movement of membrane proteins within the IEC membrane, creating a clear separation between apical and basal surfaces, and so maintaining IEC polarity. Microvilli also play a role in further reinforcing and regulating the physical barrier formed by IEC, with their dense actin filament network forming links between IECs through adherens and tight junctions (Sansonetti, 2004).

Turnover of IEC is continual and frequent, making the epithelial lining a dynamic barrier that is readily repaired, justifying the energy cost to the body of such rapid turnover. Crypt cells, located at the base of intestinal crypts, divide at a high rate, and migrate upwards to renew the IEC layer. Since microbes attached to the surface of epithelial cells are also shed along with the IEC, a strategy of many successful foodborne pathogens is to have mechanisms allowing IEC entry and invasion, usually involving dramatic rearrangements of the host cell cytoskeleton (Goosney *et al.*, 1999a). For example, *Salmonella* Typhimurium produces a specialized structure, the type III secretory pathway apparatus, which extends from the cytosol of the bacteria into the IEC membrane. This hollow tube allows *S.* Typhimurium to deliver virulence proteins directly into the IEC, which then trigger cytoskeletal rearrangements by promoting actin polymerization and depolymerization. These rearrangements manifest as a 'ruffling' effect on the IEC surface, resulting in uptake of the *S.* Typhimurium and their successful invasion into the cell (Galan, 1996).

Shigella flexneri uses a similar tactic, triggering membrane ruffling and secreting proteins that induce colonic microfold cells (M cells) to endocytose this pathogen (Raupach *et al.*, 1999). Both enteropathogenic and enterohemorrhagic *E. coli* use the Tir protein to insert into the host cell membrane and bind to the host cell intimin protein. This process recruits host cell signaling proteins and drives host cell cytoskeletal rearrangements, resulting in the formation of actin-based 'pedestal' structures that form attaching and effacing lesions (Goosney *et al.*, 2001). *Bacillus cereus* has also recently been reported to induce F actin disruption and necrosis of intestinal epithelial cells (Minnaard *et al.*, 2004). *Yersinia enterocolitica* uses a different approach, binding to B1

integrin proteins on the IEC surface using invasin, an outer membrane protein. This interaction between *Y. enterocolitica* and IECs stimulates morphological changes in the enterocyte cell membrane which then surrounds the bacteria, facilitating their internalization (Finlay and Cossar, 1997).

How do foodborne pathogens cross the IEC barrier?

The key route for foodborne pathogens and antigens into and through the IEC barrier has long been considered to include binding to M (microfold) cells, specialized epithelial cells located in the follicle-associated epithelium, resulting in transport into the subepithelial dome of the Peyer's patches. The Peyer's patches (PP) are lymphoid follicles located mainly in the small intestinal ileum. PP provide a microenvironment ideally suited for generating an immune response, allowing for close contact of antigens with cells of the immune system. Certain foodborne pathogens, including *Yersinia enterocolitica, Salmonella* Typhimurium*, Shigella flexneri*, and enteropathogenic *E. coli* readily bind to M cells, and enteropathogenic *E. coli* can bind to IEC (Donnenberg *et al.*, 1997) leading to their uptake across the epithelial layer through a transepithelial vesicular transport pathway (Kraehenbul and Neutra, 2000). *Listeria monocytogenes* has also been reported to be rapidly translocated across the epithelial barrier after invading IEC, rather than showing a requirement for uptake via M cells (Daniels *et al.*, 2000).

Recently, however, additional routes of entry across the IEC barrier have been detected. In addition to M cells in PP, uptake of gut bacteria by distinct M cells located in the epithelial layer of intestinal villi has recently been reported (Jang *et al.*, 2004). Dendritic cells (DC) may also participate in uptake of antigen and pathogens through a 'direct sampling' process. DCs extend their dendritic processes through epithelial tight junctions, and 'sample' bacteria from the GI tract by bringing them across the IEC barrier (Rescigno *et al.*, 2001). Antibody produced by the specific immune response may participate in assisting antigens – and potentially foodborne pathogens – in crossing the epithelial barrier by binding to bacteria and facilitating transport via M cells (Rey *et al.*., 2004).

The mucous layer covering the intestinal surface contains a complex polymeric mix of polysaccharides and glycoproteins whose role is to trap microbes and block attachment of bacteria to the intestinal epithelial cells. This continuous mucous gel varies in thickness from the stomach to the colon, and is at its thinnest in the small intestine, with minimal or no covering of the Peyer's patches, allowing them to effectively sample the luminal contents (Forstner *et al.*, 1995). Mucins secreted by goblet cells (specialized columnar epithelial cells) effectively reduce the numbers of microbes (and of toxin molecules) which come into direct contact with the intestinal epithelium. Lactoferrin, lysozyme, and defensins are also present in mucin, providing an extra arsenal of defenses. Since mucin is continually expelled and the mucin layer renewed, foodborne pathogens trapped in the mucous layer are shed from the body, owing to the action of peristalsis (Hecht, 1999). Intestinal mucus is now known to act as a 'dynamic defensive barrier', rather than simply a static physical defense,

potentially due to interactions of intestinal microbes with goblet cells (Deplancke and Gaskins, 2001). Mucin granules are constitutively released from goblet cells through the process of baseline secretion, while the response to various mucin secretagogues (such as inflammatory mediators, hormones and neuropeptides) stimulate the release of centrally stored mucin granules through the process of compound exocytosis (Forstner *et al.*, 1995).

Motility and mucin-degrading ability may be assets for the successful foodborne pathogen at this point, allowing movement through the mucous layer to make contact with the underlying epithelium. *V. cholerae* is able to readily penetrate through the mucous layer using mucinases (Schneider and Parker, 1982). For certain other bacteria, such as *Yersinia enterocolitica*, the presence of flagella appears to be essential for infectivity (Young *et al.*, 2000). In contrast, nonmotile foodborne pathogens such as *Shigella* spp. still manage to reach and invade the epithelium without expressing flagella or exhibiting motility (Butler and Camilli, 2005). Foodborne pathogens that retaliate against the mucous barrier by producing mucinases are able to degrade mucin in a multistep process that usually involves several bacterial enzymes, clearing a pathway to the intestinal epithelium (Corfield *et al.*, 1992). Correlations between the type of mucin glycoconjugate bound by certain pathogens and the type of mucin-cleaving enzymes they produce have been noted. For example, sialidase-producing *S.* Typhimurium binds well to mucin glycoproteins that contain sialic acid, suggesting that when these pathogens adhere to mucus, they are stimulated to produce sialidase in order to escape being trapped in the mucous layer (Deplanke and Gaskins, 2001).

The mucin layer varies in thickness, with low-viscosity layers interspersed between thicker layers recently secreted from goblet cells, and it has been suggested that foodborne pathogenic bacteria may take advantage of this lack of homogeneity, moving through low-viscosity layers to gain access to the epithelial surface. While mucus has protective properties, it should be noted that it also serves as a nutrient source for bacteria. The foodborne pathogens *Vibrio cholerae* and *Listeria monocytogenes* can trigger mucin exocytosis at the intestinal epithelium, presumably benefiting from the release of this rich source of carbohydrates, peptides, vitamins and minerals (Coconnier *et al.*, 1998; Lencer *et al.*, 1990). Notably, the normal enteric microflora also breaks down mucin to utilize as a nutrient source (Simon and Gorbach, 1986), providing mucin with yet another potential role in host defense, through the ability to promote maintenance of and colonization by non-pathogenic commensal bacterial (reviewed in Hecht, 1999).

Attachment to the intestinal epithelium is important from the perspective of the foodborne pathogen, not only to initiate the infection process, but also to evade the outward flow of *peristalsis*. Peristalsis is a process of hydrodynamic flow driven by intestinal muscle contractions designed to keep intestinal contents moving along the gastrointestinal tract and to spread chyme along the surface of the intestinal mucosa. Ingestion of food stimulates a gastroenteric reflex leading to increased rates of peristalsis, especially in the small intestine.

Food moves from duodenum to jejunum to ileum, allowing for nutrient absorption through active and passive transport processes. Bacteria colonizing the gastrointestinal tract must attach to the intestinal epithelium to avoid being swept away through peristalsis, whether they are foodborne pathogens or are part of the normal gut microflora. Mucins also aid in the process of removal of pathogens by trapping bacteria for removal by peristalsis.

8.2.2 Digestive tract secretions

Saliva is the initial host defense encountered by an ingested foodborne pathogen. Salivary flow itself has a flushing effect, contributing to the removal of bacteria from oral mucosal surfaces. Saliva is relatively neutral (pH 6.0 to 7), but it contains several antimicrobial components (reviewed in Walker, 2004). While salivary IgA is involved in preventing microbial adherence, other nonspecific factors in saliva are also active. Saliva contains complement component C3, which is required for initiating the alternative complement cascade, a process leading to bacterial destruction through formation of channels in the cell wall. Saliva also contains lysozyme, lactoferrin and lactoperoxidase, all of which have antimicrobial activity.

Lysozyme enzymatically degrades the bacterial cell wall component peptidoglycan by cleaving linkages between *n*-acetylglucosamine and *n*-acetyl-muramic acid. Lactoferrin, a high-affinity iron-binding protein, acts to bind up available iron, keeping it sequestered from bacteria, and so inhibits bacterial growth. Most pathogenic bacteria require free iron concentrations that are 10^{11}–10^{12} fold higher than physiologically available iron concentrations (Andrews *et al.*, 2003). This situation drives many pathogens to have *siderophores*, specialized systems for acquiring iron (Shaible and Kaufmann, 2004). The foodborne pathogen *Listeria monocytogenes* produces a transferrin-like siderophore, and *E. coli* has three iron uptake systems (reviewed in Andrews *et al.*, 2003; Shaible and Kaufmann, 2004). Both *E. coli* and *Vibro cholerae* express cytolysins that release iron from host cell intracellular iron complexes, allowing them to scavenge host iron (Andrews *et al.*, 2003). Lactoperoxidase is a bactericidal peroxidase utilizing thiocyanate/halide-H_2O_2, and generating toxic superoxide radicals, which then damage bacterial membranes. Lactoperoxidase is most effective against Gram-negative bacteria. In an interesting new application, the lactoperoxidase system is currently being assessed for its activity as a biopreservative effective against foodborne pathogens when it is applied directly to a food surface (Elliot *et al.*, 2004).

Once they are swallowed, foodborne pathogens encounter the hostile environment of the stomach. Here, the acidic pH (typically about pH 2) provided by hydrochloric acid present in gastric juice, and the activity of digestive enzymes, such as pepsin, act to further attack invaders. Food composition can influence survival of bacteria during passage through the stomach; for example high fat foods tend to protect microorganisms from gastric acid. Rapid passage through the stomach when it is full can also act to protect foodborne pathogens from

prolonged exposure to the stomach defenses. Evidence of the role played by gastric acid in dealing with foodborne pathogens is seen in situations where individuals with impaired gastric acid secretion (achlorhydria) show increased susceptibility to foodborne infections (Cook, 1985; Holt, 1985) and in reports of associations between excessive antacid intake and increased vulnerability to foodborne pathogens (Banatvala *et al.*, 1999; Noriega *et al.*, 1994).

Bacterial pathogens best able to cause foodborne infections also tend to be more acid tolerant than their more 'innocent' counterparts. For example, the low infective dose of *Shigella* spp. relative to Enteroinvasive *E. coli* (10^3 cells *vs* 10^9 cells) is believed to reflect the higher acid resistance of *Shigella*, allowing it to more effectively survive passage through the stomach. *E. coli* O157:H7 (which also has a very low infective dose) and *Shigella* spp. both use specialized acid-resistance systems that can protect them from pH values as low as 2.5 (Foster, 2004). Gastric digestive enzymes include proteases such as pepsin and lipases, and these may contribute to damage to foodborne pathogens.

Bile is released from the gall bladder and reaches high concentrations in the small intestine and colon. While the primary role of bile is in fat digestion, bile also provides an additional defense against bacteria owing to its detergent-like ability to disrupt bacterial cell membranes and to break down endotoxin from Gram-negative bacteria into non-toxic components (Bertok, 2004). Bile deficiency may thus be linked to endotoxic shock, a potential pathogenic outcome of infection with Gram-negative foodborne pathogens.

8.2.3 Resident gut microflora: a dynamic role in host defense

The resident gut microflora contains an impressively diverse array of bacteria, many of which have not yet been fully characterized. Commonly detected bacteria in the human colon include *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Lactobacillus* and *Peptostreptococcus*. Anaerobic niches in the gastrointestinal tract provide unique opportunities for obligate anaerobes to thrive, and aerobic and facultative bacteria are present in high numbers. Total microscopic counts exceed counts obtained as CFU/gram, suggesting that many of the 6×10^{13} bacteria present in the average human colon are not culturable by traditional methods, and so remain unidentified (Tannock, 2000). Colonization of the gastrointestinal tract increases in density proceeding down the tract. Duodenal counts are typically in the range of 10^2 to 10^3/mL, 10^3 to 10^4/mL in the jejunum, increasing to 10^5/mL in the upper ileum and 10^6 to 10^7/mL in the lower ileum, and 10^{10}/mL in the colon (Adams and Moss, 2000). Location also influences the composition of the microbial population present at different intestinal locations. Lactobacilli and Streptococci are present in high numbers in the small intestine, while the large intestine harbours high numbers of *Bacteroides* and *Bifidobacterium*, obligate anaerobes that may account for as much as 99% of the large intestinal flora. Other residents of the small intestine include *E. coli* and other Enterobacteriaceae, enterococci, *Clostridium* and *Fusobacterium* species.

Several benefits arise from our intensive colonization with the resident gut microflora. One beneficial outcome is *competitive exclusion*, a process whereby the normal gut microflora acts to prevent the adherence of pathogens to the epithelial surface, and to compete with them for available nutrients. In addition, certain members of the Gram-positive microflora, such as the Lactobacilli, produce *bacteriocins*, peptides that can kill susceptible target bacteria (reviewed in Cotter *et al.*, 2005). Bacteriocin production by Lactobacilli is thought to be a means of decreasing competition by killing susceptible bacteria and thus preventing colonization of the intestinal surface by competitors. An alternative role for bacteriocins has recently been revealed. Lactacin F production has been shown to promote intestinal colonization by its producer *L. johnsonii* VPI 11088, suggesting that it may be acting as a communication signal between bacteria to promote colonization of the gastrointestinal epithelium (Tannock, 2000).

The protective role of the resident gut microflora is illustrated by cases of outgrowth of *Clostridium difficile* leading to pseudomembranous colitis following antibiotic therapy. *Clostridium difficile* can be present in the gut microflora, but is normally held in check by its competition. Disturbances of the microflora due to antibiotic treatment give *C. difficile* a competitive advantage, to the detriment of the host. Prolonged antibiotic treatment has also been reported to be associated with increased susceptibility to foodborne infections, further illustrating the beneficial impact of the resident gut microflora (Vanden Eng *et al.*, 2003; Salminen *et al.*, 1995).

GALT faces challenges unique to its location in the body. Owing to the continual barrage of stimuli in the form of food components, normal gut microflora and pathogens, the GALT must distinguish between signals it should mount a response against and those to which it should remain nonresponsive (Smith and Nagler-Anderson, 2005). Immunity and tolerance are two very different outcomes of the initial contact of GALT with antigens. *Oral tolerance* is a state of selective nonresponsiveness of the immune system that is seen for certain stimuli contacted at the gastrointestinal interface. Gut microflora tend to induce oral tolerance, and this is also often the response to antigens from foods, an outcome that prevents unnecessary and detrimental immune reactivity in the gastrointestinal tract. Several mechanisms have been proposed as participants in establishing oral tolerance (recently reviewed in Mowat, 2003; Nagler-Anderson, 2001). Normal gut microflora are essential for development of the mucosal immune system, and the intimate contact between gut microflora and cells of the intestinal epithelium and GALT also plays a role in the development of oral tolerance (Sudo *et al.*, 1997). In recent studies examining the effects of human normal microflora in a murine model, development of oral tolerance was shown to require colonization with more than one strain, and it has been suggested that sequential colonization of the gastrointestinal tract may be required (Gaboriau-Routhiau *et al.*, 2003). Interactions between specialized DCs in the GALT with resident gut microflora are currently believed to be the driving force in establishing oral tolerance (Stagg *et al.*, 2004).

Recent evidence also shows that IEC respond differentially to nonpathogenic and pathogenic enteric bacteria, and this has been linked to different patterns of intracellular signaling, providing a potential mechanism for some of the beneficial effects of the normal gut microflora on the gut epithelium (Neish *et al.*, 2000). Commensal enteric microbes, including certain *Salmonella* strains (*S.* Typhimurium $PhoP^c$ and *S.* Pulloram), are able to attenuate inflammatory responses through their ability to inhibit, rather than activate, the DNA-binding protein NFkB (Neish *et al.*, 2000). An outcome of this is that these commensal *Salmonella* strains are able to block epithelial IL-8 secretion in response to various pro-inflammatory stimuli, reflecting their ability to interfere with the intracellular activities that normally lead to NFkB activation, and so inhibiting acute inflammatory responses. This anti-inflammatory effect of the resident gut microflora is believed to be crucial in maintaining the state of 'tolerance' or hyporesponsiveness of the gastrointestinal epithelium to the continual barrage of stimuli the microflora provide.

8.3 Mucosal immune responses

Foodborne pathogens encounter the gastrointestinal mucosal surface, and must face the mucosal immune system as well as the physical barriers the location presents. The mucosa-associated lymphoid tissues (MALT) includes the GALT, a location reported to contain more lymphocytes than are found in the total content of all other secondary lymphoid organs (Nagler-Anderson, 2001). The GALT is composed of lymphoid aggregates, including the Peyer's patches (located mainly in the small intestinal distal ileum), where induction of immune responses occurs (Fig. 8.1). The lamina propria serves as a homing location for mature effector B and T cells; these cell types are essential for the specific adaptive immune responses of the GALT. Several cell types involved in the innate immune response are present in the PP microenvironment, including macrophages and dendritic cells.

Intraepithelial lymphocytes (IELs) are a T-cell subpopulation located above the basement membrane and in between intestinal epithelial cells, below the intercellular tight junctions connecting the IEC. IEL are cytolytic, and also have immunoregulatory activity. IEL are activated in response to infection by pathogens, but use a limited T-cell receptor (TCR) repertoire to recognize invaders (Hayday *et al.*, 2001). IELs have been reported to originate from cryptopatches, cell clusters located in the crypt lamina propria (Suzuki *et al.*, 2000), and their development is assisted by secretion of the cytokine IL-7 by IEC (Laky *et al.*, 2000), further illustrating the extent of cell–cell interaction essential for efficient GALT activity. IELs express $\gamma\delta$ TCR rather than $\alpha\beta$ TCRs. The $\gamma\delta$ TCR repertoire is oriented toward more conserved antigens than is the $\alpha\beta$ TCR repertoire, recognizing such stimuli as heat shock proteins and phospholipids. A key difference between the $\gamma\delta$-TCR-expressing IEL and more 'conventional' T cells with $\alpha\beta$ receptors is that IELs do not require that antigen

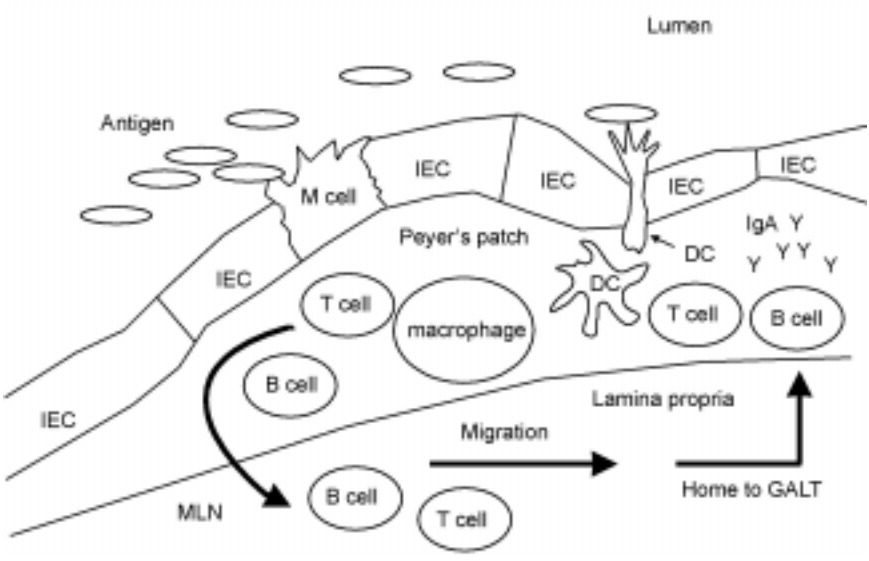

Fig. 8.1 GALT: Gut-associated lymphoid tissue. Intestinal epithelial cell (IEC), Dendritic cell (DC).

be associated with MHC molecules. Owing to their unique properties, IEL are often viewed as a cell type that is midway between the innate and adaptive immune systems. The extent and nature of their role in defense against foodborne pathogens still remains to be clearly elucidated.

In contrast, the role of IEC has been the focus of much recent attention and their importance in defense is becoming increasingly clear. IEC are multi-talented cells with several roles necessitated by their location at the mucosal interface. In addition to carrying out the uptake of dietary nutrients, IEC face a continual barrage of dietary components, gut microflora, and potential pathogens. In their role as 'sentinels of the intestinal mucosa' (Jobin and Sartor, 2000), IEC are uniquely placed to sense and respond to foodborne pathogens, and to interact and communicate with the GALT (Philpott *et al.*, 2001). Interaction with pathogens stimulates IEC to produce several defensive mediators. In addition to producing and releasing pro-inflammatory cytokines, IEC also express inducible nitric oxide synthase (iNOS), leading to nitric oxide release. IEC can also respond to the presence of pathogenic stimuli by COX activation and subsequent prostaglandin production (Philpott *et al.*, 2001). The unique location of IEL gives them the ability to interact directly with cells of the innate immune system, including macrophages and DCs. DCs can be found in the intestinal epithelium, as well as in the underlying lamina propria (reviewed in Nagler-Anderson, 2001). Cytokine production by IEC is a key means of communication with cells of the GALT, and provides IEC with a means to choreograph the resulting immune response (Pitman and Blumberg, 2000). Cytokines are proteins that carry out communication between cells of the

immune system, and are essential in stimulation and regulation of the immune response. When IEC respond to certain microorganisms in the gastrointestinal tract by producing cytokines, they become active participants in the mucosal immune response and part of a mucosal cytokine network that regulates responses to pathogens (Hedges *et al.*, 1995).

Which cytokines are produced by IEC in response to challenge by foodborne pathogens? Pathogenic bacteria have been shown to induce the production of several different cytokines by IEC (Chae-Jung *et al.*, 1995; Hedges *et al.*, 1995; Stadnyk, 1994). Certain of the *chemokines* are IEC products. Chemokines are a subfamily of chemoattractant cytokines that act as key mediators in controlling the attraction and migration of different cells of the immune system (Baggiolini, 2001). IL-8 is a chemokine secreted by IEC that is integrally involved in the inflammatory response (Baggiolini *et al*., 1995). Another chemokine produced by IEC is RANTES. IL-8 is a C-X-C chemokine that acts as a potent chemoattractant for neutrophils (PMNs), and frequently is secreted by IEC following entry of various bacterial pathogens, where it promotes transendothelial migration of neutrophils to sites of infection (Eckmann *et al.*, 1993; Hersh *et al.*, 1998). RANTES, a C-C chemokine, chemoattracts monocytes and T cells to sites of infection, and may be involved in IEC activities in later stages of mucosal inflammation (Yang *et al.*, 1997). Other cytokines produced by IEC include tumor necrosis factor α (TNFα), interleukin-1 β (IL-1β), and the immunoregulatory cytokine transforming growth factor β (TGFβ) (Jiang and McGee, 1998). TGFβ can down-regulate production of several cytokines, control DC, monocyte and macrophage activity, inhibit IEC division, and promote IEC differentiation (Stadnyk, 1994). TGFβ also participates in tissue repair through control of collagen, fibronectin, and connective tissue growth factor production (Letterio and Roberts, 1998). TNFα production is usually associated with pro-inflammatory responses (Barbara *et al.*, 1996), while TGFβ plays a varied role, reflecting its activity as an immunoregulatory cytokine (Letterio and Roberts, 1998).

Enteric pathogens such as *E. coli* stimulate IEC to produce pro-inflammatory cytokines, including IL-8 and TNFα, a talent associated with the ability of these pathogens to cause host damage. For example, TNFα release by IEC can increase IL-8 secretion by endothelial cells, in turn leading to increased neutrophil chemoattraction and activation at the site of infection (Baggiolini *et al*., 1995). Pro-inflammatory cytokine production stimulates the innate immune system to respond to the presence of invaders such as foodborne pathogens. However, excessive production of such pro-inflammatory cytokines as IL-8 and TNFα can also play a role in the damage these pathogens wreak on host tissues. Elevated IL-8 levels promote bacterial attachment to epithelial cells by up-regulating expression of adhesion proteins on the IEC surface (Baggiolini *et al*., 1995). *Helicobacter pylori* can induce IL-8 production at the gastric epithelium, an attribute reported to be linked to its ability to produce peptic ulcer disease (Hersh *et al.*, 1998). Induction of macrophage apoptosis by *Shigella flexneri* leads to extensive release of pro-inflammatory cytokines (including IL-6 and

TNFα) as the macrophages die. These cytokines trigger an inflammatory response that in turn causes destruction of gastrointestinal mucosal tissue (Hersh *et al.*, 1998; de Silva *et al.*, 1993). Cytokine production by IEC is thus both a boon and a bane – it is necessary for effective communication of IEC with the rest of the immune system, and allows for stimulation of responses. If, however, production of pro-inflammatory cytokines is excessive, it plays a part in the overall pathogenesis of lesions inflicted by foodborne pathogens on the host.

8.4 Innate cellular responses: front-line defenders

The immune system includes a complex array of cells and biomolecules, which interact to provide protection from challenge by pathogenic microbes, and impaired immune function leads to increased risk of infection by foodborne pathogens. *Antigens* – substances that induce an immune response – are often components of invading microbes. The immune system can be divided into two branches – the *innate* (or nonspecific) and the *adaptive* (or specific) immune response. The innate response (the focus of this chapter) includes certain cell types and molecules able to react to the presence of invading microorganisms and their components, but without a high degree of specificity. Innate immunity is characterized by the speed of its response, and by a lack of memory. Rapid response speed is essential for initial host protection, and reflects the ability of cells in the innate immune system to react rapidly to contact with pathogens, a property enhanced by their pre-positioning at the luminal interface. Lack of memory of such contact, however, means that the efficiency and effectiveness of the innate immune response does not improve with repeated exposure, unlike the adaptive immune response.

Cells of the innate immune system include *neutrophils*, *monocytes*, and *macrophages*, all of which are phagocytes. Phagocytic cells ingest invading pathogens, with the goal of digesting and destroying the invader, using an array of enzymes, reactive oxygen intermediates, and nitric oxide. Macrophages are the key phagocytic cell type in tissues. Monocytes, their precursors, are present in the blood, as are neutrophils. Macrophages recognize invading microorganisms through receptors that detect non-self components, including carbohydrates such as mannose (Delves and Roitt, 2000). Macrophages and neutrophils both interact with complement (a protein component of the innate immune response) and antibodies (components of the adaptive immune response) to improve their rates of phagocytosis of invading microbes through the process of opsonization. Once foodborne pathogens are phagocytosed, they are exposed to an impressive intracellular array of defenses, including lysozyme, antimicrobial peptides, and nitric oxide, and the respiratory burst-derived mediators superoxide anion, hypochlorous acid and hydroxyl radicals. Neutrophil killing of pathogens is now believed to be mainly due to the activity of destructive enzymes within intracellular vacuoles, rather than to the direct actions of reactive oxygen species (Segal, 2005).

Neutrophils play an essential role in defense at the intestinal epithelial layer. IEC respond to foodborne pathogens, such as *Salmonella* Typhimurium, by producing IL-8, a chemoattractant cytokine, which is then secreted from the basolateral side of the epithelial layer, stimulating neutrophil migration to sites of infection (Gewirtz *et al.*, 1999). *S.* Typhimurium challenge leads to release of a second chemoattractant (pathogen-elicited epithelial chemoattractant, or PEEC) from IECs in the apical direction. PEEC release stimulates neutrophil translocation across the epithelial lining to the lumen. From this vantage point, neutrophils can actively phagocytose and destroy bacteria (reviewed in Sansonetti, 2004).

Some foodborne pathogens succeed in invading the host and causing disease owing to their ability to counteract macrophage activity. *Shigella flexneri* initially infect IEC and multiply inside them, inducing actin nucleation into comet-like tails that propel the bacteria through the cytoplasm. The resulting cellular extensions can penetrate into neighbouring IEC and other cell types, and allow *Shigella* to infect adjacent cells without being seen by cells of the immune system. *Shigella flexneri* is then able to defeat macrophages by binding to caspase 1 and inducing programmed cell death (apoptosis) following macrophage infection (Zychlinsky *et al.*, 1992). It has been suggested that enteroinvasive *E. coli* (EIEC) use this host evasion strategy as well (Kaper *et al.*, 2004), allowing both *Shigella* and EIEC to evade macrophages and cause serious illness. *Listeria monocytogenes* also induces actin polymerization and 'comet tail' formation, causing it to be projected through the IEC and directly into the membranes of adjacent cells, thus escaping detection by phagocytes (Daniels *et al*., 2000). Enteropathogenic *E. coli* use their ability to signal host cell cytoskeletal rearrangements to inhibit phagocytosis through a process involving tyrosine dephosphorylation of infected macrophage proteins (Goosney *et al.*, 1999b). Yersinia *enterocolitica* is also able to inhibit phagocytosis, using certain of its *Yersinia* outer-protein (Yop) components (such as YopJ), to effectively paralyze macrophages. *Y. enterocolitica* inhibits host inflammatory responses by both macrophages and IEC through inhibition of MAP kinases and NFkB signaling (reviewed in Boyer and Lemichez, 2004).

Natural killer (NK) cells are also participants in the innate immune response. NK cells are closely related to T cells, and use a range of cell surface receptors to recognize their targets and regulate their cytolytic activity (reviewed in Lanier, 2005). Their key role is to respond to virus-infected cells in the early stages of infection by killing infected target cells. NK cells are capable of detecting and killing certain types of malignant cells. They can recognize targets through antibody-dependent cellular cytotoxicity, or through unique NK receptors that respond to certain molecules present on all cell types. The killing ability of NK cells is kept in check by killer-inhibitory receptors (KIRs), which recognize (major histocompatibility) MHC class I molecules. Down-regulation of Class I MHC molecules following certain types of virus infection or on malignant cells releases NK cells from KIR-mediated inhibition, allowing them to kill infected or transformed target cells (reviewed in Hamerman *et al.*, 2005).

Target cell killing involves the insertion of perforin (a pore-forming protein) into the target cell membrane, and injection of granzymes, cytotoxic molecules that trigger target cell apoptosis. Owing to their mode of action and target recognition, NK cells are involved in innate defense against intracellular bacteria such as *Listeria monocytogenes* and *Salmonella* (Chin *et al.*, 2002; Wick, 2004). NK cells also provide a source of interferon γ, which then acts to activate the adaptive immune response and promote the activation of T helper 1 cells (Chin *et al.*, 2002).

NKT cells are a recently identified cell type expressing a semi-invariant TCR α chain, which allows them to recognize glycolipid antigens from Gram-negative bacteria (Kronenberg, 2005). This ability suggests NKT cells also play a role in responses to foodborne pathogens. For example, NKT cells have been shown to respond to *Salmonella* Typhimurium by producing interferon γ, a cytokine that promotes antimicrobial host defenses (Kinjo *et al.*, 2005). Recent studies suggest that NKT cells may be most important as a defense against bacteria that lack such TLR stimuli as lipopolysaccharide, and their precise role in defense against other foodborne pathogens remains to be determined (Mattner *et al.*, 2005).

Dendritic cells (DCs) provide a 'bridge' or interface between the innate and adaptive immune systems. Like other cells in the innate immune system, DCs react to the presence of pathogens using relatively nonspecific receptors. DCs are able to process and present antigens to the central participants in the adaptive immune response: T cells. Macrophages and monocytes can act as 'antigen-presenting cells' (APCs) and carry out a bridging role between innate and adaptive responses. Since T cells do not respond to 'free' antigen – only to antigen that is presented by APCs – this ability of DCs, monocytes and macrophages is crucial in bridging the transition from innate to adaptive immunity. DCs, macrophages and monocytes also exert their influence over the adaptive immune response through cytokine production, discussed in the preceding section. Interdigitating dendritic cells continually endocytose antigen, becoming activated when certain of their cell surface receptors recognize pathogen-associated molecular patterns (PAMPs). PAMPs include such microbial components as lipopolysaccharide, mannans, teichoic acids, and CpG motifs in DNA (reviewed in Akira and Takeda, 2004). Receptors for PAMPs, often referred to as pathogen recognition receptors (PRRs), include the toll-like receptors (TLRs), the LPS receptor CD14, and the Nod receptors.

TLRs are an evolutionarily conserved family of cell surface receptors that play a central and essential role in innate immunity through recognition of certain key microbial determinants (reviewed in Vasselon and Detmers, 2002). TLR-2 is involved in recognition of components of Gram-positive bacteria, including lipoteichoic acid (LTA), peptidoglycan and lipoproteins. TLR-4 recognizes LPS from Gram-negative bacteria, TLR-5 recognizes bacterial flagellin, TLR-9 recognizes unmethylated CpG dimers from bacterial DNA and TLR-3 recognizes viral double-stranded RNA (Akira and Takeda, 2004). Nod receptors are nucleotide-binding oligomerization proteins located in the cell

cytoplasm rather than on the cell surface, and are also able to recognize peptidoglycan (Mumy and McCormick, 2005). Nod 1 recognizes D-Glu-*meso*-DAP, a degradation product of peptidoglycan that is naturally released by Gram-negative bacteria. Nod 2 recognizes muramyl dipeptide, which is essentially the minimal peptidoglycan unit, giving Nod 2 the ability to detect both Gram-negative and Gram-positive bacteria (Carneiro *et al.*, 2004). Their intracellular location allows the Nod proteins to play a key role in detection of invasive enteric pathogens, such as *Salmonella* and *Shigella.* Overall, the recognition array composed of PRRs allows cells of the innate immune system to detect a broad range of invaders such as foodborne pathogens without requiring a high degree of specificity.

8.4.1 How do cells participating in the innate immune response recognize pathogens?

IECs and other cell types involved in the innate response react to foodborne and other pathogens detected through the TLR and Nod systems by activating the pro-inflammatory pathway controlled by nuclear factor kB (NFkB) (Mumy and McCormick, 2005). NFkB is a DNA-binding protein that acts as a central control point for expression of several genes encoding pro-inflammatory cytokine production following TLR activation (Medzhitov *et al.*, 1997). NFkB is present in the IEC cytoplasm in an inactive form, in a complex with a member of the inhibitory IkB family, IkBα. Pro-inflammatory signals, including those sent by many PAMPs through TLR binding, lead to phosphorylation, ubiquitination and subsequent proteolysis of IkBα, releasing NFkB. In this free state, NFkB moves into the nucleus, and binds to regulatory control sequences in DNA, activating transcription of genes encoding pro-inflammatory cytokines (Thanos and Maniatis, 1995). Several enteric pathogens such as enteroinvasive *E. coli*, enteropathogenic *E. coli*, *Salmonella* Dublin and *Yersinia enterocolitica* stimulate the production of pro-inflammatory cytokines by IEC, including IL-8 and TNFα, through NFkB activation (Elewaut *et al.*, 1999; Savkovic *et al.*, 1997). In this way, the IEC response to infection with enteroinvasive foodborne pathogens is coordinated through activation of a common signaling pathway, leading to a pro-inflammatory response.

8.5 Other nonspecific host defenses

Defensins are antimicrobial peptides of 3–4 kDa in size that provide one of the key defenses at mucosal surfaces such as the intestinal epithelium (Sansonetti, 2004). Owing to their amphipathic structure, defensins insert into cell membranes of bacteria causing membrane lysis and cell death (White *et al.*, 1995). Two classes of defensins, α defensins and β defensins, are active in defense at the gastrointestinal epithelium. Alpha defensins or cryptidins are produced by Paneth cells, present in the crypts of the small intestine, and are released into the

intestinal crypts when pathogens are encountered. The two main α defensins are human defensin 5 (HD5) and HD6 (Ouellette and Bevins, 2001). HD5 has been reported to provide protection from *Salmonella* Typhimurium in mice transgenic for this human α defensin (Ouellette, 2005).

Beta defensins are expressed by several cell types, including IEC and phagocytic cells (macrophages and neutrophils). High defensin concentrations accumulate within phagosomes inside phagocytes, and neutrophils contain high defensin levels, even though they cannot produce defensins after maturing past the promyelocyte stage (Mumy and McCormick, 2005). In IEC, β-defensin production is constitutive, and is also upregulated in response to pathogens (Ganz, 2003). More specifically, human β defensin 1 (hBD1) is expressed constitutively, while hBD2 shows low level expression, and is upregulated following infection (O'Neil *et al.*, 1999). Both LPS and peptidoglycan have been reported to act through TLRs to stimulate β-defensin-2 expression in intestinal epithelial cells (Vora *et al.*, 2004). Upregulation of human β defensin-2 expression and release by epithelial cells in response to Nod-2 ligand-recognition has recently been reported (Voss *et al.*, 2005). Both plants and animals produce defensins in response to microbial challenge, and these highly conserved peptides are believed to be one of the most primitive innate host defences (reviewed in Lehrer and Ganz, 2002a; Selsted and Ouellette, 2005). Defensins are differentially expressed throughout the gastrointestinal epithelium, and it has been suggested that they may have distinct roles in innate defense against enteric bacteria at the gastrointestinal mucosa (Eckmann, 2004).

Another antimicrobial peptide constitutively expressed at the intestinal epithelium is CAMP or LL37, a cathelicidin (Lehrer and Ganz, 2002b). The cathelicidins are believed to play additional roles in coordinating the innate immune response, as they are involved in communication between cells and can stimulate neutrophil chemotaxis and recruit mast cells (Di Nardo *et al.*, 2003). It has also been reported that certain of the defensins have chemotactic activity, acting on macrophages, T cells and dendritic cells (Ganz, 2003). Defensins may also play a signaling role, stimulating a pro-inflammatory response and inducing IL-8 production – a role that may thus have both protective and potentially damaging outcomes for the host (Lin *et al.*, 2004).

8.6 Modulating host nonspecific defenses to foodborne pathogens

8.6.1 Can innate host defenses against foodborne pathogens be enhanced?

Strategies for bolstering defenses at the gut mucosal level that are currently receiving attention are the inclusion of probiotics and prebiotics in foods, including both traditional applications and nontraditional innovations. Yoghurt is perhaps the best-known traditional approach to the use of probiotic bacteria in foods, and much research has focused on the immunomodulatory actions of lactic acid bacteria as a result of interest in the potential health benefits of

yogurt. Development of new food products containing prebiotics and probiotics and using the synbiotic approach are intriguing new avenues in food science. The term *functional food science* has been used to describe the branch of food science that focuses on claims dealing with proposed health benefits of functional foods, and the development of new food products incorporating functional ingredients (Roberfroid, 2002). Future directions will potentially include development of new functional food products targeted at specific health issues, such as improvement of gastrointestinal health (Johnson, 2001; Salminen *et al.*, 1996), or countering the effects of atopy and allergy (Laiho *et al.*, 2002).

Prebiotics are defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of indigenous bacteria' (Gibson and Roberfroid, 1995). Prebiotics act by selectively stimulating growth of certain intestinal microbes with potential health-promoting effects, and they are used with the aim of controlling the gut microflora in a beneficial manner (reviewed in Roy, 2004). Prebiotics in the form of nondigestible oligosaccharides, which are resistant to digestion in the upper gastrointestinal tract but are broken down and fermented in the large intestine, are used to promote the growth of Bifidobacteria and Lactobacilli. This increases the production of short-chain fatty acids, which can then provide energy for the host and alter fecal pH (reviewed in Farnworth, 2001). An additional mechanism through which prebiotics may act to reduce host susceptibility to infection is to act as 'decoys' for pathogen binding, by mimicking the host receptors, and so preventing pathogen binding to the host (Gibson *et al.*, 2005).

Dietary substrates used as prebiotics must meet three key criteria: they must not be broken down in the stomach or small intestine, they must selectively enhance the growth of beneficial colonic bacteria such as the Bifidobacteria, and their fermentation should have beneficial effects for the host (Roy, 2004). Oligosaccharides with potential prebiotic activity include the inulin-like fructans, soy oligosaccharides, *trans*-galactooligosaccharides, lactulose, raffinose, stachyose, and the sugar alcohols sorbitol and xylitol (Farnworth, 2001; Roberfroid, 1999). Naturally occurring sources of some of these oligosaccharides are fruits, vegetables, and beans; however, some prebiotics, such as fructo-oligosaccharides, are also produced commercially by polysaccharide hydrolysis or enzymatic synthesis from simpler sugars. The efficacy of prebiotics in improving innate immune defences against pathogens is currently an area of investigation; however, studies that produce clear and reproducible results have been difficult to design and conduct. Recent studies indicate that prebiotics may be effective in managing certain gastrointestinal conditions and in modulating the immune response (Manning and Gibson, 2004; Saavedra and Tschernia, 2002; Schley and Field, 2002). Use of prebiotics in the elderly has been suggested as a strategy to improve not only immune responses, but also nutritional and gastrointestinal issues, such as calcium absorption and constipation (Hamilton-Miller, 2004). Lack of an immune-enhancing effect of specific prebiotic treatments in the elderly has also been reported, with raftilose treatment showing no improvement of the response to

influenza or pneumococcal vaccines (Bunout *et al.*, 2002). Such variations in reports of the effectiveness of prebiotics underscore the need for further study in this area. Many factors come into play in incorporating prebiotics into the diet, and it is not surprising that not all approaches will be equally effective in every subpopulation.

Probiotics are defined by the FAO/WHO as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Recent research has examined the potential ability of probiotics to reduce the impact of infection by pathogens, and to improve host defenses. Several mechanisms have been suggested for protective effects of probiotics at the mucosal interface. Competitive exclusion, through mechanisms including inhibition of adhesion, competition for nutrients, and release of bacteriocins and other antimicrobial factors could provide a strategy for interfering with the infection process of foodborne pathogens (Coconnier *et al.*, 1998; Lu and Walker, 2001). Strengthening of the epithelial barrier through effects of probiotics on tight junctions has also been reported, an effect that would prevent the translocation of foodborne pathogens across the intestinal epithelium (Blomberg *et al.*, 1993; Salminen *et al.*, 1996). Certain probiotic strains have been shown to ameliorate pathogen-induced decrease in transepithelial resistance of human intestinal epithelial cells, suggesting a mechanism through which probiotics can counter pathogen-induced damage at the intestinal epithelial cell level (Sherman *et al.*, 2005). It has been suggested that probiotics may be involved in protection of the intestinal epithelium from damage through the prevention of cytokine-induced apoptosis of IEC (Yan and Polk, 2002). Enhanced intestinal mucin release is triggered by *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG through increased gene expression of certain mucins (MUC2 and MUC3) (Mack *et al.*, 1999). Strains of Lactobacilli able to adhere well to IEC also show the ability to induce MUC3 expression, suggesting mucin-inducing activity underlies the ability of these probiotic bacteria to prevent attachment of enteropathogenic *E. coli* to IEC (Mack *et al.*, 2003).

Probiotic bacteria can occupy the gastrointestinal microenvironment, and rapidly accumulating evidence supports their ability to influence the mucosal immune response and to interact with IEC, thus influencing innate host response at the 'mucosal frontier'. A wide array of effects on the immune system have been reported for the lactobacilli, and effects on all of the innate immune defenses have been observed, with considerable variation between strains (reviewed in Clancy, 2003; Cross, 2002; Gill, 2003; Green-Johnson, 2004; Isolauri *et al.*, 2001; Vaarala, 2003). Probiotic bacteria have been reported to induce production of the defensin hBD-2 by IEC (Wehkamp *et al.*, 2004), to enhance the activity of human NK cells (Chiang *et al.*, 2000; Sheih *et al.*, 2001), and phagocytic cells (Chiang *et al.*, 2000; Donnet-Hughes, 1999), and to induce macrophage nitric oxide production (Korhonen *et al.*, 2001). Most recently, probiotic bacteria have been shown to interact with dendritic cells (Christensen *et al.*, 2002; Stagg *et al.*, 2004). Certain probiotic bacterial preparations (VSL#3) have been reported to upregulate the expression of cell surface markers asso-

ciated with DC activation, and also to promote release of the immunoregulatory cytokine IL-10 (Drakes *et al.*, 2004). Certain species of Lactobacilli have recently been shown to prime monocyte-derived DCs to promote the development of regulatory T cells, leading to increased IL-10 production (Smits *et al.*, 2005). The surface molecule through which these probiotics interact with DC have also been identified as the DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and it has been suggested that probiotic bacteria able to bind to DC-SIGN may be those best able to exert anti-inflammatory effects. Interactions of probiotic bacteria through DC-SIGN leading to DC activation may be responsible for anti-atopic activity and anti-inflammatory effects of probiotics in inflammatory bowel disease. Interactions between resident gut bacteria and specialized DCs in the GALT are essential in establishing oral tolerance, and it is possible that probiotics could also act through this route (Stagg *et al*., 2004).

Probiotic lactobacilli have also been shown to influence cytokine production profiles by IEC, providing another potential route for probiotics to improve innate immune defenses. Induction of COX-2 expression by IEC, an action that would influence prostaglandin production, has recently been reported (Korhonen *et al.*, 2004). In their interactions with IEC, probiotics tend to counter the strong pro-inflammatory effects of gastrointestinal pathogens, and several strains of lactobacilli have been shown to downregulate both constitutive and pathogen or LPS-induced IL-8 production (Bai *et al.*, 2004; McCracken *et al.*, 2002; Wallace *et al*., 2002). This downregulatory effect has also been reported with isolated lipoteichoic acid (LTA), suggesting LTA in lactobacilli may be able to interfere with the effects of LPS on IEC (Korhonen *et al.*, 2004). Probiotic lactobacilli have been reported to act through TLR-2 on macrophages and through TLR-2 and TLR-4 on human cord blood mononuclear cells (Karlsson *et al.*, 2002; Matsuguchi *et al.*, 2003). It has also been suggested that IEC and other cells of the innate immune system could recognize probiotics by using TLR-9 to detect bacterial CpG DNA motifs (Jijon *et al.*, 2004; Lammers *et al.*, 2003).

While lactobacilli have been shown to induce NFkB activation in both human and mouse macrophages (Miettinen *et al.*, 2000; Korhonen *et al.*, 2002), their interactions with IEC may have a different effect, leading to the downregulation of the pro-inflammatory response normally seen with NFkB activation in response to foodborne pathogens. It has been suggested that the ability to block NFkB activation, observed with commensal enteric bacteria, may also be a mode of action used by probiotic bacteria to inhibit acute inflammatory responses (Neish *et al.*, 2000), a hypothesis that is in keeping with the anti-inflammatory effects reported in several studies. A recent study by Bai *et al.* (2004) found that both *B. longum* and *L. bulgaricus* inhibited NFkB activation in a human IEC line, further supporting this route for anti-inflammatory activity by probiotic bacteria. This observation also suggests that the impact of probiotics on cells of the innate immune system may vary with the responding cell type.

8.7 Using probiotics and prebiotics in functional foods: issues to consider

Several issues must be addressed in the process of developing probiotics and prebiotics for use in improving host defenses, and these are outlined in Fig. 8.2. Strain variation, host condition and genetic makeup, and the pre-existing resident gut microfloral populations all have the potential to influence the actions of probiotics and prebiotics. Different species and strains of probiotic bacteria show varied effects on the immune system, probably reflecting variation in the efficiency of their interactions with specific cell types, such as macrophages versus intestinal epithelial cells. Certain strains have been reported to induce very different profiles of cytokine production (Perdigon *et al.*, 2002; Cross *et al.*, 2002). For this reason it is unlikely that one strain of probiotic bacteria will be suited for all immunomodulatory purposes – different strains are best suited to different activities. Differences in host condition can also contribute to variation in the effect of probiotics between individuals, and effects of probiotics on the immune system will probably be most evident in recipients with underlying health problems.

Strain variation is a consistent finding with regard to the effects of probiotic bacteria on the immune response, so strains must be chosen carefully for particular applications. Mixing combinations of probiotics is another strategy that may allow optimal growth and activity, for example in fermented food products. Recent studies have shown that certain interactions between strains occur, and can influence their immunomodulatory activity, a factor that will need to be considered in designing approaches for use of mixtures of probiotics (Christensen *et al.*, 2002). This capacity for strain interactions could also occur when probiotics interact with resident gut microflora, potentially contributing to

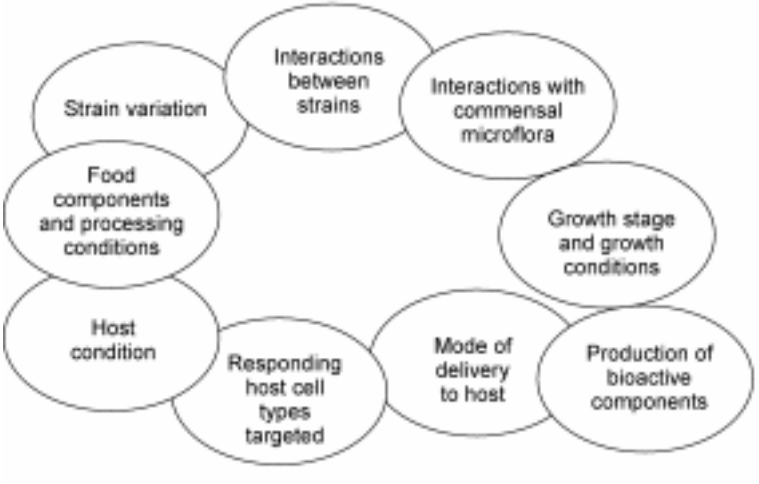

Fig. 8.2 Overview of factors influencing activity of probiotics.

individual variation in the host response to probiotics. Combining prebiotics and probiotics as *synbiotics* is another approach that aims to enhance the growth and colonization potential of the probiotics, and maximize their potential benefits to the host. Synbiotics are combinations of prebiotics and probiotics (reviewed in Holzapfel and Schillinger, 2002). This combined approach aims to both stimulate endogenous bacteria and to promote the growth of the co-delivered probiotics, which ideally will be able to use the prebiotics as substrates, resulting in selective growth promotion of the probiotic. This approach has not yet been as widely used, and relatively little information about the impact on human immunity is available to date, although studies such as the SYNCAN project have recently examined the effects of synbiotics on human volunteers in terms of modification of intestinal flora and probiotic survival of gastrointestinal transit (Van Loo *et al.*, 2005).

A key issue to consider in the functional food context is the implication of including probiotics and prebiotics from the perspective of food processing. Processors will need to consider the impact of conditions within foods, such as pH, substrate utilization ability, and storage temperatures, all of which could influence the growth and activity of added probiotics. Stabilizing prebiotics and probiotics in complex food mixtures has been identified as an issue that food science needs to address in the development of functional foods (German *et al.*, 1999). Strain selection based on the growth, survival and stability of probiotics in specific types of food products is also essential (Champagne, 2004). Food processing steps such as freezing and spray-drying can also have an impact on probiotic viability (Knorr, 1998). The ability of lactobacilli to influence IEC has been reported to vary with the growth stage, with bacteria collected in exponential phase showing different properties from those collected in stationary phase (Haller *et al.*, 1999). This will be an added issue for food processors to consider in determining optimal growth conditions for probiotics added to foods.

The efficacy of probiotics may also vary with the mode of delivery. For example, certain foods may provide added protection from the gastric environment, and allow for more effective delivery of probiotics to the intestine. Encapsulation of probiotics is one strategy that has been investigated to give additional protection during transit into the intestine. A related issue is the efficacy of digestion of the components used for encapsulation and the extent of the subsequent release of probiotics into the large intestine (Champagne, 2004). An added layer of complexity in the incorporation and utilization of probiotics in foods arises from studies showing that some probiotics produce or release bioactive components (Coconnier *et al.*, 2000; Fiander *et al.*, 2005; Leblanc *et al.*, 2002; Menard *et al.*, 2004; Prioult *et al.*, 2004) and the impact of food processing on such bioactive components is another consideration for the food processor (Korhonen *et al.*, 1998).

Probiotics hold promise as a strategy for modulating the innate immune response and bolstering host defenses against foodborne pathogens. Additional factors to consider are ensuring safety and standardizing efficacy. Lactobacilli have a long tradition of safe use in the context of fermented foods, and have

rarely shown the ability to cause disease. Recent studies examining the effects of live probiotics ingested in infant formula (Saavedra *et al.*, 2004), or delivered orally to HIV patients (Salminen *et al.*, 2004) support this view. However, the targeted use, in immunocompromised persons for example, of probiotic strains that have been associated with sepsis requires that approaches for screening and verifying safety for intended use be developed and consistently applied (Cannon *et al.*, 2005; Land *et al.*, 2005; Salminen *et al.*, 2006). Several factors to consider in safety assessment have been identified, including binding ability, metabolic activity, translocation ability, mucosal effects, dose–response effects, and immunomodulatory ability – an issue that reflects the overall balance that must be maintained by the immune system in dealing with challenges (reviewed in Ishibashi and Yamazaki, 2001; Salminen *et al.*, 1996). Mouse model systems have been used to evaluate translocation ability and persistence of certain strains such as *L. reuteri* (Wagner *et al.*, 1997) and *L. plantarum* (Pavan *et al.*, 2003), and a rabbit endocarditis model has been used to assess several strains of Lactobacilli for safety and pathogenicity (Asahara *et al.*, 2003). *In vitro* testing approaches including adhesion characterization have also been proposed as a means of 'quality assurance' for probiotics, a step that will be important especially for probiotics delivered in different formulations and products (Tuomola *et al.*, 2001). Adding to our knowledge about the mechanisms these bacteria use to interact with host cells will increase our ability to tailor their use and optimize their delivery in food products and other forms in a safe manner.

8.8 Sources of further information and advice

Further information about the innate immune system and intestinal epithelial cells:

- Man the barrier: Strategic defences in the intestinal mucosa. Cathryn Nagler-Anderson. 2001. *Nature Reviews Immunology* **1**: 59–67.
- The Immune System: Part 1. P.J. Delves and I.M. Roitt. 2000. *N. Engl. J. Med.*, **343**: 37–49. Part 1 of this set of two comprehensive reviews dealing with the actions and participants in the immune system covers the different levels of defense and the cell types involved in the immune response.
- *Instant Notes in Immunology*, 2nd edition, by P. Lydyard, A. Whelan, and M.W. Fanger. 2004. BIOS Scientific Publishers, Taylor and Francis Group. This concise text covers key cell types, interactions and organization of the immune system.

Further information about foodborne pathogens and their interactions with the infected host:

- *Food Microbiology* by M.R. Adams and M.O. Moss. The Royal Society of Chemistry. 1995. This textbook provides an excellent overview of commonly encountered foodborne pathogens, their physiological characteristics, and a concise description of basic host defenses in the gastrointestinal tract.

- *Bacterial Pathogenesis: A molecular approach*, by A.A. Salyers and D.D. Whitt. 1994. ASM Press, Washington, DC. This textbook gives additional details about the interactions of several foodborne pathogens with the host and the immune system.

Further information about probiotics and prebiotics:

- *Trends in Food Science and Technology*, 1998. Vol. 9. Special issue: Functional food science in Europe. This special issue covers several topics related to food processing and functional foods, including probiotics.
- *Functional Dairy Products*, 2003, edited by T. Mattila-Sandholm and M. Saarela. CRC/Woodhead Publishing Ltd.
- *Handbook of Nutrition and Immunity*, 2004, edited by M.E. Gershwin, P. Nestel and C.L. Keen. Humana Press.
- *Handbook of Nutraceuticals and Functional Foods*, 2000, edited by R.E.C. Wildman. CRC Series in Modern Nutrition.

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9

Specific immune mechanisms of defence against foodborne pathogens

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9.1 Introduction

The human gastrointestinal tract represents a complex anatomical site with an enormous surface area (400 m^2) in which the external environment comes into contact with the host. This region is highly specialised in order to permit adsorption of nutrients from food whilst preventing penetration by pathogens and toxic agents. The lumen of the GI tract also represents an extremely complex microbial ecosystem in which 300–500 different bacterial species compete to reach levels of approximately 10^{11} CFU/g faeces (Simon and Gorbach, 1984). This ecological diversity is further highlighted by recent molecular studies that demonstrate a vast array of unculturable microorganisms in the human GI tract (Eckburg *et al*., 2005). In order to monitor the GI tract for pathogens and to prevent inappropriate responses to the commensal flora and potential allergens, higher mammals have evolved a complex local immune network that covers the entire GI tract and interacts with the systemic immune and neuronal systems. In humans, this gastrointestinal immune system is thought to make up approximately 70% of the total immune system.

In the response to pathogens, it is clear that the cellular localisation and nature of the pathogenic agent are important factors that influence the ensuing immune response. Epithelial cells, dendritic cells and macrophages coordinate the early immune response through recognition of microbe-associated molecular patterns (MAMPs) (Niedergang *et al*., 2004) such as lipopolysaccharide (LPS), flagellin and CpG DNA. This priming of the immune response induces chemokines such as IL-8 and CCL20/MIP-3α that serve to recruit inflammatory phagocytes and dendritic cells to the site of infection. Dendritic cells and other professional antigen presenting cells then migrate to local immune centres to

present antigen to T and B lymphocytes. Production of specific mucosal antibody (sIgA or sIgM) by plasma cells is key to the removal of extracellular pathogens such as *Vibrio cholerae* and *Giardia lamblia* from the lumen of the intestine. Intracellular pathogens including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and *Shigella* spp. are eliminated following induction of a T_{H1} cell-mediated immune response in which cytokines such as interferon gamma (IFN-γ) prime phagocytes to kill internalised pathogens and cytolytic $CD8^+$ T cells specifically target infected host cells. In contrast, the response to parasitic nematodes including *Trichinella* and *Nippostrongylus* spp. requires the production of IL-4 and IL-13, cytokines that define a T_{H2} response. These diverse responses are in turn influenced by the presence of the gastrointestinal flora and are modulated by virulence factors produced by microbial pathogens during pathogenesis.

9.2 Interactions between pathogens and the gastrointestinal mucosa

9.2.1 Intestinal M cells in foodborne infection

Membranous epithelial (M) cells are specialised epithelial cells that reside in the follicle-associated epithelium (FAE) and are associated with underlying lymphoid tissue. These cells are characterised by reduced surface microvilli, a thin glycocalyx and an increased capacity to transcytose particles across the intestinal mucosa (Clark and Jepson, 2003) (Fig. 9.1). This mechanism of antigenic sampling is essential for the stimulation of underlying lymphocytes and plays a role in intestinal homeostasis by providing a constant source of information to the underlying immune system (Didierlaurent *et al*., 2002). The M cell is derived through interactions between lymphocytes and specialised dome epithelial cells within the FAE (Gebert *et al*., 1999). This interaction can be recreated *in vitro* where the combination of cultured epithelial cells and lymphocytes in transwell culture provides for a useful *in vitro* model of M cell development and function (Kerneis *et al*., 1997). *In vivo*, this process is also influenced by bacterial interactions with the mucosa and both *S.* Typhimurium and *Streptococcus pneumoniae* are capable of inducing rapid formation of M cells within the Peyer's patches in animal models (Savidge *et al*., 1991; Borghesi *et al*., 1999).

Many foodborne pathogens have exploited the antigen sampling potential of M cells in order to transit the gastrointestinal mucosa. Both *S.* Typhimurium and *S.* Typhi specifically target and invade M cells, an interaction that induces a process of significant membrane rearrangement (ruffling) at the M cell surface (Clark *et al*., 1994; Jones *et al*., 1994; Pascopella *et al*., 1995). Invasion is followed by destruction of M cells and significant damage to the FAE (Jones *et al*., 1994; Pascopella *et al*., 1995), a scenario that may account for the frequent occurrence of intestinal ulceration in patients with typhoid (Clark and Jepson, 2003). Efficient targeting of M cells by *Salmonella* requires expression of long

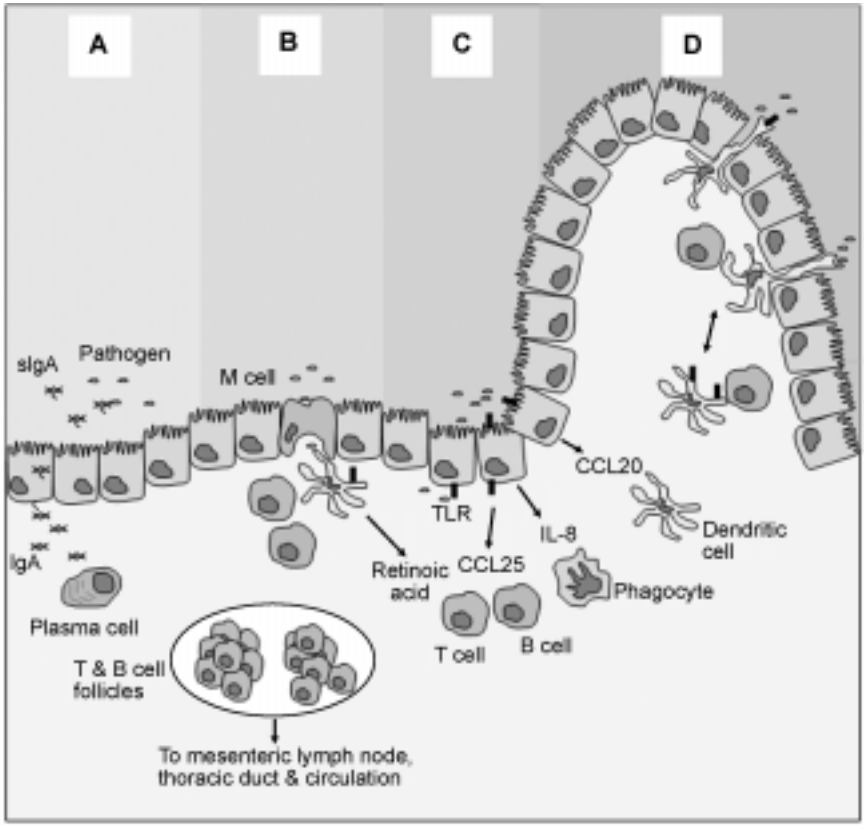

Fig. 9.1 Local immune responses to pathogens within the GI tract. **A.** Preferential homing of IgA^+ B cells or local class-switching of B cells to the IgA isotype results in an abundance of plasma cells that secrete IgA antibody. Dimeric IgA interacts with secretory component on the basal surface of enterocytes and is transcytosed through the epithelial layer to the apical surface of the epithelial cell where it enters the lumen of the gut. **B.** Membranous (M) cells are specialised epithelial cells characterised by reduced surface microvilli, expression of IgA receptors, a thin glycocalyx and an increased capacity to transcytose particles across the intestinal mucosa. These cells function to sample antigens from the lumen of the gut for presentation to immune cells that reside in close proximity to the M cell. **C.** Interaction between bacteria and toll-like receptors (TLRs) stimulates an inflammatory immune response. TLRs associated with epithelial cells may be stimulated by microorganisms in the lumen of the GI tract. Also, invasive microorganisms react with TLRs on the basal surface of epithelial cells or on local dendritic cells. Subsequent production of retinoic acid by dendritic cells or CCL25 by epithelial cells results in migration of lymphocytes to the site of invasion. Epithelial cell production of IL-8 or CCL20 results in migration of inflammatory phagocytes or dendritic cells respectively. **D.** Dendritic cells are capable of sampling the gut lumen directly by extending cellular processes across the epithelial membrane. Dendritic cells may then present antigen directly to B cells and can induce class-switching to the IgA isotype. Alternatively dendritic cells migrate to mesenteric lymph nodes where further priming of the immune response can occur.

polar fimbria (LPF) (Baumler *et al*., 1996), although other microbial factors are also thought to be involved (Clark and Jepson, 2003). Interestingly, proteins encoded within *Salmonella* pathogenicity island 1 (SPI1) are absolutely required for entry into cultured epithelial cells but are less important for entry into M cells (Murray and Lee, 2000).

Yersinia enterocolitica and *Y. pseudotuberculosis* clearly target M cells during intestinal infection (Autenrieth and Firsching, 1996; Neutra *et al.*, 1999; Clark *et al*., 1998). Although the bacterial protein Invasin is clearly essential for pathogenesis of *Yersinia* spp. via the oral route, the precise means of adhesion to the M cell surface remains to be established (Pepe and Miller, 1993; Clark *et al*., 1998; Hamzaoui *et al*., 2004). Recent studies indicate that this bacterial factor is expressed at ambient temperature and not at 37 ºC (Hamzaoui *et al*., 2004). Similarly *Shigella* invasion of the gastrointestinal mucosa is mediated through initial targeting of M cells and subsequent basolateral bacterial spread (Sansonetti and Phalipon, 1999). The massive inflammatory response occurring post-infection results in destruction of the FAE and initial symptoms of the disease (Sansonetti *et al*., 1996).

9.2.2 Sampling of luminal pathogens by dendritic cells

In addition to their essential role in bridging the innate and acquired immune responses to gut pathogens, dendritic cells also serve as a means of antigenic sampling of the gastrointestinal lumen. Recent work has shown that dendritic cells are recruited to sites of inflammation and can sample pathogenic *Salmonella* or non-pathogenic *E. coli* by extending cellular processes (dendrites) across the epithelial membrane (Rescigno *et al*., 2001). Local signals induce dendritic cells to express tight-junction proteins that allow them to extend through the mucosal layer without disrupting the barrier function of the GI tract (Rescigno *et al*., 2001). This method of translocation may account for the ability of SPI1 mutant *Salmonella* strains to penetrate the epithelial barrier.

9.2.3 Epithelial cells and the sentinel response

Epithelial cells and dendritic cells comprise mucosal sentinel cells that monitor the contents of the gastrointestinal lumen and respond appropriately to distinct microbial signals. They form key components of the proposed danger response in which antigen presenting cells (APCs) respond to alarm signals generated through inflammation and cell damage (Matzinger, 2002). The principal signal from invading pathogens is mediated through interaction between toll-like receptors (TLRs) and individual ligands associated with microorganisms. Since it is now clear that commensal organisms as well as pathogens can induce TLR responses (Bambou *et al*., 2004; Rachmilewitz *et al*., 2004), it has been proposed that the term microbe-associated molecular patterns (MAMPs) (rather than pathogen-associated molecular patterns) should be used to describe these bacterial danger signals (Niedergang *et al*., 2004). Dendritic cells are replete

with a variety of TLRs, and the manner in which they first encounter microbial antigen has a major influence on the subsequent development of acquired immune responses (see below). However, epithelial cells also express TLRs on their apical and basolateral surfaces and therefore represent a first point of contact where the host immune system can respond to the luminal contents of the GI tract.

Epithelial cells located in the crypts of the GI tract are most likely to express TLRs as this region is less prone to encounter constant stimulation from commensal organisms (Mavris and Sansonetti, 2004). The activation of a danger response in these cells is therefore likely to occur through infection of the mucosa or toxin production by foodborne pathogens or through a breach of the barrier function of the mucosa. The close association between adherent or invasive pathogens and the epithelial cell brings MAMPs into direct contact with the appropriate TLR, thereby triggering a proinflammatory (danger) response. In contrast, endoscopic studies in humans have shown that the normal commensal flora does not appear to closely interact with the surface of healthy epithelial cells in the GI tract (Mahida, 2004). This lack of close contact is likely to prevent constant triggering of the inflammatory response.

The key TLRs expressed by epithelial cells include TLR4 which, in complex with MD2 and CD14, senses lipopolysaccharide; TLR5, which is expressed on the basolateral surface of epithelial cells and senses bacterial flagellin; and TLR9, which senses unmethylated bacterial DNA. Triggering of epithelial cells initiates the expression of specific chemokines that result in the recruitment and differentiation of polymorphonuclear neutrophils (stimulated by IL-8) and dendritic cells (stimulated by IL-8, CCL20/MIP-3α and/or thymic stromal lymphpoietin (TSLP)) (Soumelis *et al*., 2002; Neidergang *et al*., 2004).

Normal epithelial cells express relatively low levels of TLR4 and demonstrate low responsiveness to LPS (Jump and Levine, 2004). In contrast, epithelial biopsies from patients with inflammatory bowel disease are seen to express high levels of TLR4 consistent with a hyperinflammatory condition (Cario and Podolsky, 2000). Recent work suggests that cytokines such as gamma interferon have the potential to increase the expression of TLR4 in healthy epithelial cells particularly within the crypts, suggesting that potential for TLR4 signalling may be further upregulated during the infectious process (Alvarado *et al*., 2005).

Signalling through interaction between TLR5 and bacterial flagellin is thought to be a major means of triggering the inflammatory response (Tallant *et al*., 2004). Gastrointestinal epithelial cells express TLR5 on their basolateral, but not their apical surfaces (Gewirtz *et al*., 2001). Therefore, pathogens such as *S.* Typhimurium that can invade the epithelial mucosa directly or transit via M cells, gain access to the basolateral compartment where they trigger a pro-inflammatory response mediated by the TLR5–flagellin interaction (Gewirtz *et al*., 2001). During pathogenesis of *S.* Typhimurium, this leads to IL-8-mediated neutrophil infiltration and recruitment of immature dendritic cells through production of CCL20 (Sierro *et al*., 2001). Interestingly, *S.* Typhi infection fails to stimulate a TLR5-mediated neutrophil inflammatory response and patients

with typhoid fever rarely exhibit neutrophil invasion in gastrointestinal foci of infection (Raffatellu *et al*., 2005). This appears to be due to reduction of TLR-dependent IL-8 production mediated by the presence of the *S.* Typhi capsule (Raffatellu *et al*., 2005). Finally, flagellin from another invasive pathogen, *L. monocytogenes* has been shown to stimulate TLR5; however, non-flagellated *Listeria* mutants are not affected in virulence potential or ability to stimulate adaptive immune responses (Way *et al*., 2004). Further work is therefore necessary to determine the role of the flagellin–TLR5 interaction in the early local immune response to *L. monocytogenes*.

TLR9 is expressed within the FAE of the Peyer's patches (Shimosato *et al*., 2005) and expression is seen to localise to the basolateral membrane in gastric epithelia undergoing *Helicobacter pylori* gastritis (Schmausser *et al*., 2004). The ligands for TLR9 are unmethylated CpG motifs associated with bacterial DNA. In particular DNA from *E. coli* has been shown to activate epithelial IL-8 production through interaction with TLR9 and it is likely that this plays a role in stimulating the innate immune response during infection (Akhtar *et al*., 2003). Interestingly, the DNA from probiotic commensal bacteria has been shown to stimulate TLR9 and to induce an anti-inflammatory response in a mouse model of colitis (Rachmilewitz *et al*., 2004). Clearly much more research is required to determine how bacterial DNA is processed and presented to TLR9 receptors both during bacterial infection and in the normal healthy GI tract.

Recently it has emerged that the intracellular foodborne pathogen *Shigella flexneri* stimulates epithelial IL-8 production through interaction with an intracellular TLR, Nod1 (Mavris and Sansonetti, 2004). Nod1 was initially thought to recognise LPS, but is now known to interact specifically with a GM-tripeptide associated with Gram-negative peptidoglycan (Girardin *et al*., 2003). Similarly, the intracellular foodborne pathogen, *L. monocytogenes* stimulates a host response gene expression profile that is specific for Gram-positive cytoplasmic infection (McCaffrey *et al*., 2004). Further work is necessary to define the receptor–ligand interaction that results in this distinct host response.

9.3 Dendritic cells as a bridge between innate and acquired immunity

Following penetration of the epithelial layer, foodborne pathogens are exposed to a complex network of immune cells that comprise the mucosa-associated lymphoid tissue (MALT). Uptake of antigen by dendritic cells leads to co-ordination of subsequent immune responses through dendritic cell differentiation, cytokine production and antigen processing and presentation to lymphoid cells. Dendritic cell activity may occur locally with the presentation of antigen to B cells to drive production of secretory IgA (sIgA). Alternatively, dendritic cells can migrate to the mesenteric lymph nodes (MLN) where they further drive adaptive immunity by inducing proliferation of antigen-specific T and B cells. The specific features of the ensuing response are influenced by the cellular

pathogenesis of the pathogen and the means of penetrating the epithelial barrier, which in turn influences the local cytokine profile.

Immature dendritic cells are attracted to foci of infection through production of CCL20 by epithelial cells (Niedergang *et al*., 2004). Furthermore, dendritic cells are found in high numbers in the FAE and in close contact with M cells (Iwasaki and Kelsall, 2001). Immature dendritic cells have a high capacity to internalise and process antigens but express low levels of co-stimulatory molecules such as CD80 and CD86 and therefore have a poor capacity to present antigen to T cells (Banchereau *et al*., 2000; Sundquist *et al*., 2004). Engaging dendritic cell TLRs with LPS or CpG DNA or local cytokine production (TNF-α, IL-1) induces dendritic cell maturation (Sundquist *et al*., 2004). Maturation of dendritic cells increases expression of co-stimulatory molecules and decreases ability to capture antigen. Importantly, mature dendritic cells display increased responsiveness to chemokines, a property that promotes migration to sites of antigen presentation (Sundquist *et al*., 2004). In addition, recent evidence suggests that LPS directly increases migration of dendritic cells from the lamina propria to mesenteric lymph nodes and the spleen (Turnbull *et al*., 2005).

9.4 Acquired immunity to foodborne pathogens

9.4.1 Antigen presentation and development of cytolytic $CD8^+$ memory T cells in the GI tract

It is clear that dendritic cells migrate from the intestine to the mesenteric lymph nodes in response to chemotactic signals (Kobayashi *et al*., 2004). In turn, signals produced by dendritic cells directly influence the migration of naïve T cells to the GI tract by inducing T cell expression of the mucosal integrin $\alpha_4\beta_7$ (Mora *et al*., 2003). In addition, T cells expressing the chemokine receptor CCR9 home to the gut in response to local epithelial cell production of CCL25 (Kunkel *et al*., 2000). Recent work has also shown that mucosal dendritic cells produce retinoic acid, a metabolite of vitamin A (retinol), which enhances T cell expression of both CCR9 and $\alpha_4\beta_7$. Indeed, vitamin A deficiency is seen to significantly reduce the numbers of mucosal T cells (Iwata *et al*., 2004). Efficient homing of T cells to the GI tract has been shown to be essential for resistance to Rotavirus infection in mice (Rose *et al*., 1998; Kuklin *et al*, 2000).

Much of the work examining subsequent antigen presentation of orally acquired antigen has focused upon the *Salmonella* model of infection (reviewed in Sundquist *et al*., 2004). It is evident that dendritic cells from a number of anatomical sites (including the mesenteric lymph nodes) are capable of presenting *Salmonella* antigens in association with MHC-I and MHC-II to $CD8^+$ and $CD4^+$ T cells respectively (Sundquist *et al*., 2004). The fact that *Salmonella* antigens are presented in association with MHC-I is interesting since such antigens are normally synthesised *de novo* in the cytoplasm of antigen-presenting cells, while *Salmonella* reside in the endosomal compartment. However, it has been demonstrated that components of the MHC-I processing

pathway, including the transporter associated with antigen processing (TAP), are invoked during antigen processing of *Salmonella* by dendritic cells (Yrlid *et al*., 2001). In addition, studies utilising adoptive transfer of immune T cells have demonstrated that $CD8^+$ T cells as well as $CD4^+$ T cells are required for maximal protection against *Salmonella* in the murine oral challenge model (Mastroeni *et al*., 1992, 1993). This work is supported by more recent studies in Class I-deficient β2-microglobulin$^{-/-}$ mice (Lo *et al*., 1999) and by experiments that show direct killing of *Salmonella*-infected cells by primed $CD8^+$ T cells (Pope *et al*., 1994). In addition to resistance to *Salmonella* infection, CD8+ T cells have been shown to be necessary for resistance to other orally administered foodborne pathogens including Rotavirus (Rose *et al*., 1998), *L. monocytogenes* (Pope *et al*., 2001) and *Toxoplasma gondii* (Lepage *et al*., 1998).

Indeed, gut-associated $CD8^+$ T cells provide a long-term memory function in the gastrointestinal mucosa (Cheroutre and Kronenberg, 2005). Following primary challenge, these effector cells reside as intraepithelial lymphocytes that can specifically target pathogen-infected host cells. Although the initial functional differentiation of the gut $CD8^+$ T cell response is $CD4^+$ T cell-independent, the development of a lasting $CD8^+$ T cell memory response requires interaction with specific $CD4^+$ helper T cells (Masopust *et al*., 2001). These gut-associated $CD8^+$ T cells differ significantly from their counterparts in the spleen and exhibit longer survival and enhanced cytolytic activity. This is thought to provide an immediate and highly efficient localised response to invasion of foodborne pathogens (Cheroutre and Kronenberg, 2005).

9.4.2 Induction of T_{H1} or T_{H2} T cell responses in the GI tract

Protective immunity against invasive foodborne pathogens such as *Salmonella* (Mastroeni and Menager, 2003), *L. monocytogenes* (Hsieh *et al*., 1993) and *Shigella* (Sinha and Bagchi, 2004) is dependent upon a robust T_{H1} response. Development of such a response depends upon production of the cytokine IL-12 by dendritic cells, which directly induces secretion of the pro-inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) by T cells (Cheroutre and Kronenberg, 2005). IL-12 is essential for resistance to oral *Salmonella* infection (Kincy-Cain *et al*., 1996) and is essential during the systemic phase of *L. monocytogenes* infection (Tripp *et al*., 1994).

Inappropriate and unregulated T_{H1} cytokine production is associated with the chronic inflammatory condition, Crohn's disease (Russell *et al*., 2004). This condition is linked to mutations in the gene encoding *NOD2*, an intracellular pattern recognition receptor that plays a role in sensing a component of peptidoglycan, muramyl dipeptide (Cario, 2005). A regulated inflammatory response mediated through *NOD2* is essential for resistance to both *Salmonella* and *L. monocytogenes* (Cario, 2005). However, mutation of *NOD2* in mice leads to an inappropriate reaction to commensal bacteria and an increase in T_{H1} cytokines (TNF-α and IFN-γ) that result in the symptoms of chronic inflammation (Netea *et al*., 2004; Cheroute and Kronenberg, 2005). Cells from mice with *NOD2*

mutations fail to produce appropriate levels of the immunoregulatory cytokine IL-10 when stimulated *in vitro* (Netea *et al*., 2004), and both IL-10 and the cytokine transforming growth factor-β (TGF-β) are required for downregulating T_{H1}-mediated inflammatory conditions in mice (Fuss *et al*., 2002). The induction of regulatory T cells that produce IL-10 and TGF-β in the GI tract is, at least in part, mediated by a distinct subset of dendritic cells known as plasmacytoid dendritic cells (Bilsborough *et al*., 2003). These dendritic cells are capable of inducing the regulatory T cell phenotype *in vitro* and may be central to the down-regulation of immune responses *in vivo* and the maintenance of immune homeostasis in the GI tract (Cheroute and Kronenberg, 2005).

T cell priming in the presence of IL-4 and absence of IL-12 results in development of a T_{H2} T cell response in which T cells are induced to secrete IL-4, IL-5 and IL-13 (Mosmann, 1986; Murphy and Reiner, 2002; Finkelman *et al*., 2004). Such a reaction is essential for host resistance to intestinal nematode parasites such as *Trichinella spiralis* through induction of local mast cell mastocytosis, eosinophilia and IgE responses that lead to worm expulsion in the mouse model (Finkelman *et al*., 2004). In addition, direct effects of IL-4 and IL-13 on non-bone marrow-derived cells in the intestine give rise to increased mucus production and increased smooth muscle contractility that also contribute to elimination of the parasite (Finkelman *et al*., 2004). Interestingly, the ability of intestinal helminth infection to induce a robust T_{H2} response has suggested that such infections may actually protect against T_{H1}-mediated colitis (including Crohn's disease) by skewing the cytokine profile away from the destructive proinflammatory T_{H1} response (Hunter and McKay, 2004).

9.4.3 Role of secretory antibodies in defence against foodborne pathogens

Resistance against foodborne pathogens that reside within the lumen of the gut is primarily mediated through mucosal secretion of secretory IgA (sIgA) or sIgM, a phenomenon known as immune exclusion (Brandtzaeg, 2003). Since secretory antibodies are not produced in neonates, acquisition of such antibodies from breast milk is essential for initial resistance to mucosal infection in the newborn (Dickinson *et al*., 1998; Brandtzaeg, 2003). However, adults secrete between 3 and 5 grams of IgA into the gut lumen per day (Brandtzaeg, 2003) and IgA is the most abundantly produced Ig isotype in the body (van der Heijden *et al*., 1987).

The majority of IgA-producing plasma cells in the intestine reside in the Peyer's patches. These accumulate through preferential homing of IgA^+ B cells, but not IgM^+ or IgG^+ cells, in response to production of the chemokine CCL25 by epithelial cells in the villous crypts (Bowman *et al*., 2002). In addition, the germinal centres of the Peyer's patches contain large numbers of T cells and dendritic cells that are capable of inducing class-switching to IgA antibody production (Fagarasan and Honjo, 2004). T cell production of IL-10 and TGF-β within MALT induces antigen-specific B cells to differentiate into IgA^+ plasma cells that become terminally differentiated as they home to the epithelial surface (Brandtzaeg, 2003). Locally produced antigen-specific dimeric IgA interacts

with secretory component on the basal surface of the epithelial cell and is transcytosed through the epithelial layer to the apical surface of the epithelial cell where it enters the lumen of the gut (Brandtzaeg, 2003). This transport process is generally constitutive but can be positively influenced by local cytokine production. Interestingly, sIgA has the capacity to neutralise viral antigens during intracellular transport of antibody through the epithelial cell (Brandtzaeg, 2003). This may play a role in neutralisation of virus during rotavirus infection of the intestinal epithelia (Burns *et al*., 1996; Brandtzaeg, 2003).

There is a clear and established role for secretory antibody in resistance to foodborne pathogens and in the maintenance of gut homeostasis. Recent studies have shown that mice deficient in sIgA develop a compensatory sIgM response. However, these mice also demonstrate a significantly altered gut flora in which unculturable filamented bacteria which are highly immunostimulatory predominate (Suzuki *et al*., 2004). IgA is also essential for local responses to the commensal flora that may occur when commensals breach the intestinal barrier. This process is mediated by dendritic cells that accumulate and retain small numbers of live commensals before migrating to mesenteric lymph nodes, where they induce commensal-specific IgA responses (Macpherson and Uhr, 2004). Furthermore, luminal sIgA may also be important for regular development of mucosal immunity through antigen sampling, as M cells within the Peyer's patches express receptors for IgA (Mantis *et al*., 2002).

Antigen-specific sIgA directly impedes microbial pathogenesis in the gut through direct blocking of pathogen–host interactions, neutralisation of toxins such as cholera toxin or by enhancing the binding of pathogens to secretory mucous (Phalipon *et al*., 2002; Brandtzaeg, 2003). Foodborne pathogens such as rotavirus (Ward *et al*., 1989; McNeal *et al*., 1994), *V. cholerae* (Quiding *et al*., 1991), *G. lamblia* (Eckmann, 2003), *Cryptosporidium parvum* (Riggs, 2002), enterohaemorrhagic *E. coli* (Noguera-Obenza *et al*., 2003), *Clostridium difficile* (Johnson *et al*., 1992; Dallas and Rolfe, 1998) and *L. monocytogenes* (Manohar *et al*., 2001) are capable of eliciting significant IgA responses. In particular, studies have demonstrated that IgA antibodies are actively protective against *G. lamblia* (Eckmann, 2003), *V. cholerae* (Freter, 1969), *C. difficile* toxin A (Johnson *et al*., 1995), *C. jejuni* (McSweegan *et al*., 1987) and rotavirus (Ward *et al*., 1989; McNeal *et al*., 1994), while for other pathogens including *Cryptosporidium* spp. (Riggs, 2002) and *Citrobacter rhodentium* (MacDonald *et al*., 2003) sIgA appears to play a lesser role in exclusion of the pathogen. Interestingly the secretory component of mucosal antibody can directly inhibit epithelial cell adhesion of *E. coli* (Giugliano *et al*., 1995), indicating that this component, even in the absence of specific antibody, may be directly inhibitory to certain pathogens.

9.5 Vaccine delivery and future research

One of the major goals of future research into mucosal immune responses is the development of oral vaccines that are capable of stimulating lasting systemic

immunity as well as local mucosal immune responses. Such vaccines must stimulate active immunity rather than immunological tolerance and the immune responses induced must be appropriate for control of the target pathogen. Currently a limited number of oral vaccines are approved for human use; these include the oral polio vaccine, a live-attenuated typhoid vaccine and an oral adenovirus vaccine (Brandtzaeg, 2003). A live-attenuated rotavirus vaccine was recently withdrawn, owing to serious adverse reactions to the vaccine. However, new rotavirus vaccines are currently in the later stages of development (Glass *et al*., 2005).

An objective of research into mucosal vaccines is the creation of a multivalent vaccine that can offer protection against a number of food- and waterborne pathogens (Walker, 2005). Organisms such as *V. cholerae, Shigella* and *S.* Typhi have been suggested as possible platforms for the creation of multivalent vaccines (Walker, 2005). However, current research has examined the ability of inactivated and live attenuated vaccine candidates for ability to protect against individual agents (reviewed in Walker, 2005). An advantage of live attenuated whole pathogen vaccines is that they provide significant immunity following a single administration. However, there are concerns about the ability to store viable live vaccines and about the safety of such vaccines (Walker, 2005). Alternatively, multiple doses of inactivated whole cells can provide protection against pathogens such as *C. jejuni* (Rollwagen *et al*., 1993), *S. flexneri* (Chakrabarti *et al*., 1999) and *V. cholerae* (Clemens *et al*., 1990). Adjuvants, such as *E. coli* heat labile toxin (Rollwagen *et al*., 1993), cholera toxin (Hartman *et al*., 1999) and unmethylated CpG DNA (Jiang *et al*., 2003), have demonstrated promise in improving responses to inactivated whole cell vaccines.

It is evident that the commensal flora greatly influences the development and maturation of the local mucosal immune system and also has profound effects upon systemic immunity (Macpherson and Harris, 2004). Indeed, the presence of commensal bacteria greatly alters the pattern of gene expression in intestinal epithelial cells (Macpherson and Harris, 2004). This raises the intriguing possibility that live beneficial commensal organisms (probiotics) or defined pharmaceutical extracts from such organisms may have far-reaching effects, including influencing the outcome of vaccine administration or controlling local inflammation. Recent studies indicate a clear role for probiotic commensals in controlling local inflammatory responses in animal models of colitis (Madsen *et al*., 1999; McCarthy *et al*., 2003). These findings have also been extended to human populations where feeding of *Bifidobacterium infantis* has been shown to significantly alleviate symptoms in patients with irritable bowel syndrome (O'Mahony *et al*., 2005).

Specific genetic manipulation of probiotic commensals and related GRAS (generally regarded as safe) microorganisms has the potential for further manipulation of local immune responses. Recombinant *Lactobacillus plantarum* has been shown to induce tolerance to the house dust mite allergen in a murine model (Kruisselbrink *et al*., 2001). In addition, genetically engineered

commensals, including *Lactobacillus* spp., have been proposed as vectors for vaccine delivery (Seegers, 2002). Such strains are capable of efficiently delivering model antigen to the mucosal immune system (Seegers, 2002). However, much further work is required to test the safety of such systems before engineered vaccines can be used routinely in disease prevention.

Finally, probiotic commensals and related bacteria can also be used to deliver immunomodulatory compounds to the mucosal immune system. Recombinant vaccine strains of *Lactococcus lactis* expressing foreign antigen and murine IL-2 and IL-6 are more efficient at inducing immune responses than strains expressing antigen alone (Steidler *et al*., 1998). Furthermore, *L. lactis* strains expressing the anti-inflammatory cytokine, IL-10, have been shown to significantly reduce colitis in a mouse model of inflammatory bowel disease (Steidler *et al*., 2000). The development of safe and effective methods to modulate the GI immune system remains an exciting and achievable goal for future research.

9.6 References

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10

Enhanced susceptibility to foodborne infections and disease due to underlying illnesses and pregnancy

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10.1 Introduction

While new infectious diseases can emerge because of changing features of pathogens, they can also arise through alterations in host susceptibility to infection. These can occur through impaired immunity caused by other infections (such as the Human Immunodeficiency Virus), treatments (such as cancer or transplant chemotherapy), or changes in the biological status of the host (such as aging or pregnancy) (Morris and Potter, 1997).

Although foodborne diseases are generally worse in the very young, the old, or those who are already ill, certain foodborne agents and human conditions are particularly associated with more severe disease. This chapter will review the following examples:

- Medications that reduce gastric acid and increase susceptibility to enteric infections.
- *Vibrio vulnificus* infections in those with underlying liver disease.
- Salmonellosis in people with AIDS.
- Listeriosis in pregnancy – a risk to the woman and her newborn child.
- Toxoplasmosis – a foodborne disease without outbreaks.

While associations of these diseases, treatments, or conditions with foodborne disease have been well documented, the relationships are dynamic, owing to changes in the pathogens, treatments of humans, or food production methods. These changes can alter the burden of foodborne disease, requiring continuing

surveillance in those with underlying illnesses. However, collecting data about underlying illnesses and foodborne disease through routine reporting systems is difficult because of the necessary time, medical review required, and confidentiality concerns. Surveillance strategies that can cope with these difficulties will be discussed.

10.2 General features and trends of compromising illnesses

Over the past two or three decades, the incidence of diagnosed cancer has increased in the United States. For white males, cancers at all sites increased from about 364/100 000 in 1973 (NCHS, 1997) to 542 cases/100 000 in 2001. Cancer in white females increased from 295 cases/100 000 in 1973 to 418 cases/100 000 in 2001. The increased incidence has been accompanied by increased cancer survival rates, leading to an even greater number of people living with cancer. Comparing the years 1974–79 and 1992–2000, the 5-year survival rates for all anatomical sites have improved from 50.9 to 60.3% in whites and 39.3 to 54.0% in blacks (NCHS, 2004). These individuals receive chemotherapy, which can lead to greater susceptibility to infection. Furthermore, they receive antibiotics, altering their intestinal tract flora, allowing easier infection with other organisms. Organ transplants have also steadily increased over the years in the United States from 12 619 in 1988 to 27 035 in 2003 (Organ Procurement and Transplantation Network, 2005). The treatments that transplant recipients receive to prevent organ rejection can produce immunocompromised individuals at greater risk of infections, including those from foodborne sources.

Since the early 1980s, the Acquired Immunodeficiency Syndrome (AIDS) has progressed from an almost unrecognized syndrome to the leading cause of death in persons 25 to 44 years old in 1995 (CDC, 1997). Since 1996, the use of highly active antiretroviral therapy (HAART) has been widespread in the United States, markedly improving survival of people with AIDS. The incidence of AIDS in the United States has remained relatively constant in recent years, 41–43 000 new cases per year from 1999 to 2003. On the other hand, the number of persons living with AIDS in the United States has increased, from about 311 000 in 1999 to 406 000 in 2003 (CDC, 2004). The risk of foodborne diseases in AIDS, and the subsequent effects of treatment with HAART, and antibiotic prophylaxis will be discussed in further detail below.

One of the challenges of pregnancy is that half the child's genetic traits are from the mother and half are from the father, generating a fetus that is antigenically different from the mother. In order to prevent fetal rejection by the mother, the cell-mediated immune system response is decreased through progesterone production. Unfortunately, the decreased cell-mediated immunity leads to increased vulnerability to a variety of infectious pathogens, such as *Brucella*, *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Yersinia*, Hepatitis A and E, *Coxiella burnetti*, and *Toxoplasma gondiae* (Smith, 1999). Later in this chapter, *Listeria monocytogenes* infections will be discussed in more detail.

While the focus of this chapter is on issues related to the United States and developed countries, a major cause of increased susceptibility to infection worldwide is malnutrition. Increased host susceptibility can occur in a variety of ways, such as through weakened epithelial integrity, decreased gastric acid production, decreased cell-mediated immunity, decreased immunoglobulin production, and phagocytosis defects. A 'vicious cycle' can occur, where food shortages lead to malnutrition, which can lead to increased susceptibility to infection (Morris and Potter, 1997). This can lead to an increased risk of foodborne disease, when food is available.

10.3 Increased susceptibility to enteric infections in those receiving gastric acid inhibitors

Hydrochloric acid is secreted in gastric juice by parietal cells in the stomach lining. This plays an important role in protecting the body from pathogens in food or water. *In vivo* and *in vitro* data documenting the bactericidal effects of gastric juice have been reviewed extensively (Smith, 2003; Martinsen *et al*., 2005). Decreased gastric acidity can occur through a variety of mechanisms. These include acquired disease states (atrophic gastritis) and surgery (vagotomy, gastric resection). However, the most common mechanism for gastric acid reduction is through medications. These include antacids (such as calcium carbonate), histamine H_2 antagonists (such as cimetidine), and proton pump inhibitors (such as omeprazole). These are used for a variety of disorders, including peptic ulcer disease and gastroesophageal reflux disease (GERD) (Del Valle, 2005). These medications are very frequently used. A survey of prescription drug use data in the United States from 2000 to 2001 found that 'antiulcerants,' including Prilosec (omeprazole), Prevacid (lansoprazole), Aciphex (rabeprazole), and Zantac (ranitidine) were the second largest source of prescription drug expenditures after antidepressants (National Institute for Health Care Management, 2002). Furthermore, the total use of gastric acid inhibitors is even greater than these data suggest, since some histamine H_2 blockers have become available without a prescription.

Several epidemiologic studies of gastric acid inhibitor use and foodborne disease have been performed. In a foodborne outbreak of listeriosis in a Boston hospital, patients receiving antacids or histamine H_2 antagonists were more likely to become infected than controls (Ho *et al*., 1986). In the United Kingdom, a case-control study of sporadic salmonellosis cases in North East Thames in 1993 revealed an association between illness and gastric acid-lowering medications (antacids, H_2 antagonists, or proton pump inhibitors) (Banatvala *et al*., 1999). A case control study of campylobacter gastroenteritis from January 1992 to August 1993 in Nottingham, UK, showed a 10-fold increase of infection with omeprazole use (Neal *et al*., 1996). A third UK study using the General Practice Research Database from January 1991 to December 1994 (Rodriguez and Ruigomez, 1997) found a weak association of bacterial

gastroenteritis with having ever used omeprazole (relative risk 1.1; 95% CI = 1.0–2.4). An association was not found in those who used omeprazole in the year before illness onset. While this study was the largest of the three in the United Kingdom, the study population consisted of 170 000 people who had used gastric acid suppressing drugs during the study period. Unfortunately, this biases the study toward not finding an association between illness and gastric acid suppressing drugs, since it excludes people who had never used these medications during the study period.

Although the widespread use of gastric acid inhibitors will continue to play an important role in resistance and susceptibility to foodborne infections, other factors may have significant effects as well. In recent years, *Helicobacter pylori* has been found to be a major cause of peptic ulcer disease. While fewer than 10% of infected people develop an illness, more than 80% of duodenal ulcers and 60% of gastric ulcers are related to *H. pylori* colonization (Atherton and Blaser, 2005). Antibiotic treatments are effective in eradicating *H. pylori*, and thus treating peptic ulcer disease caused by this pathogen. In turn, this may reduce the need for chronic gastric acid inhibitor use.

10.4 Increased severity of *Vibrio vulnificus* infections in those with underlying liver disease

Vibrio vulnificus is a Gram-negative bacterium that lives in salt and brackish water, which can cause gastroenteritis, wound infections, and primary septicemia (Shapiro *et al*., 1998). Wound infections are acquired through contact with contaminated seawater or marine animals. Gastroenteritis and primary bacteremia are caused by consumption of raw or inadequately cooked seafood. While the pathogen exists in warm marine environments worldwide, the implicated food or exposure in a particular area depends on dietary or food production procedures. For example, in the United States, most primary septicemia and gastroenteritis cases are associated with consumption of raw oysters from the Gulf Coast in warm months (Shapiro *et al*., 1998). In Japan, an outbreak was associated with 'ana-jako' consumption (Matsui *et al*., 2004), or *Upogebia major*, a shellfish related to the hermit crab. While this shellfish is normally eaten boiled, in the area with the outbreak, it is sometimes eaten raw. On the other hand, a *V. vulnificus* outbreak was reported in Israel, where the exposure was handling, rather than consuming a farmed fish, *Tilapia* spp. (Bisharat *et al*., 1999). Prior to the outbreak, fish-pond managers began selling live fish to consumers. Cases were injured at the time of purchase, or while cleaning the fish at home.

Vibrio vulnificus infections are frequently associated with underlying illnesses. The first published case series on this pathogen (Blake *et al*., 1979) reported that 23 of the 24 foodborne cases submitted to the US Centers for Disease Control and Prevention between 1964 and 1977 had pre-existing liver disease, hemochromatosis, a history of alcohol abuse, thalassemia major, or diabetes. In a more recent US study, 68% of wound infections, 97% of primary

septicemia cases, and 35% of gastroenteritis cases had significant pre-existing medical conditions. In particular, *V. vulnificus* infections were associated with underlying liver disease for both wound infections (OR, 4.9; 95% CI, 0.95–24.81), and primary septicemia (OR, 2.8; 95% CI, 1.6–4.7). Liver disease was a strong predictor of a fatal outcome (OR, 7.4; 95% CI, 5.2–10.6) (Shapiro *et al*., 1998). Elevated iron due to primary hemochromatosis, frequent transfusions, and other causes is recognized as a risk factor for many pathogens (Wright *et al*., 1981). The availability of iron has long been recognized as an important determinant in bacterial survival and growth in serum, and later investigations demonstrated that excess serum iron compromised host iron-sequestering capability and immune response (Hor *et al*., 2000). One of the mechanisms by which liver disease directly increases the risk of *V. vulnificus* septicemia is the impaired production of acute phase proteins (like C-reactive protein), which allows early bacterial growth. The increased susceptibility of persons with end-stage renal disease may be due to their treatment with intravenous iron to combat anemia, in addition to their chronic hypogammaglobulinemia and dialysis-associated neutrophil dysfunction (Barton *et al*., 2003).

In response to the *V. vulnificus* reports, a variety of warnings, regulations, and control measures have been implemented. Following the outbreak in Israel, the sale of live Tilapia or carp was prohibited; scales, fins and intestines were removed before marketing, and refrigeration was required throughout processing (Bisharat *et al*., 1999). A primary reference for *V. vulnificus* prevention in the US is the document, *Guide for Control of Molluscan Shellfish, 2003*, produced by the FDA (2003). This includes a 'Model Ordinance' for 'Post Harvest Processing,' or treatment of the finished product to reduce pathogens. In 2003, the State of California prohibited the sale of raw Gulf Coast oysters from April 1 through October 31 unless the oysters were treated by an approved method to reduce *V. vulnificus* to 'non-detectable' levels (CCR, 2003). Collection and analysis of *V. vulnificus* surveillance data in California to determine the effect of this control measure are pending.

10.5 Salmonellosis and AIDS

A hallmark of AIDS is the occurrence of opportunistic infections and the increased risk of common infections that also occur in the general population. This same pattern exists for foodborne diseases. Compared with the general population, HIV-infected persons have been reported to have a 20–100 times greater risk of salmonellosis, a 39 times greater risk of *Campylobacter* infections, and a 150–280 fold greater risk of listeriosis (Angulo and Swerdlow, 1995). While considerable information is available about all of these pathogens and HIV/AIDS, the remainder of this section will focus on salmonellosis, because its trends over time have been best documented with HIV/AIDS.

Not only is the risk of *Salmonella* infection much greater in people with HIV/AIDS than the general population, but the risk of severe disease is much greater

as well. Early in the AIDS epidemic, a study in San Francisco found that 45% of blood cultures of AIDS patients were positive for *Salmonella*, compared with 9% of blood cultures from people without AIDS (Celum *et al*., 1987). Furthermore, despite antibiotic treatment, when *Salmonella* bacteremia occurs, it is recurrent in about 45% of cases, probably because of incomplete clearance of the bacteria from impaired cell-mediated immunity (Sperber and Schleupner, 1987). On the other hand, non-typhoidal recurrent *Salmonella* septicemia (RSS) in the general population is extremely rare. For this reason, RSS has been included as an AIDS-defining opportunistic infection.

However, since the advent of improved treatments for AIDS, such as HAART, the incidence of nearly all AIDS-defining opportunistic infections decreased significantly in the US (Kaplan *et al*., 2000). Along with these other infections, the proportion with RSS as the first diagnosis of AIDS has gradually decreased, from 0.44% in 1985–1990, to 0.28% in 1991–1992, to 0.1 from 1992 to 1997 (Angulo and Swerdlow, 1995; Jones *et al*., 1999). Regional differences have been identified in proportions of AIDS cases with RSS. The northeast region of the United States had the highest proportion (0.53%), compared with 0.28% in the south, 0.37% north-central states, and 0.28% in the west. The distribution of AIDS-associated RSS was likely due to the geographic distribution of different serotypes of *Salmonella*, which have different tendencies to produce RSS. In particular, *Salmonella* Enteriditis is more associated with RSS, and was more prevalent in the Northeastern United States when the investigation was performed (Angulo and Swerdlow, 1995).

While salmonellosis has been a significant problem in HIV/AIDS patients in the developed world, the problem has been worse in the developing world because of limited resources for antiretroviral therapy, and a much higher risk of exposure to *Salmonella* and other enteric pathogens. Non-typhoid Salmonellae, as well as *S*. Typhi are increased. In one Kenyan study, *Salmonella* septicemia was the leading preventable cause of death in people with AIDS (Gilks, 1993). In the Ivory Coast, *Salmonella* was the most frequent cause of sepsis in people hospitalized with HIV (Vugia *et al*., 1993).

10.6 Listeriosis in pregnancy – a risk to the woman and her newborn child

Listeria monocytogenes is a small Gram-positive bacillus that can be found in soil, decaying vegetation, and stool of mammals (Lorber, 2000). It is an infrequent cause of illness, but when it occurs, it can cause severe disease, including bacteremia and meningoencephalitis. Listeriosis tends to occur in pregnant women, their developing fetus or newborn child, and people with underlying illnesses. Active surveillance for listeriosis was performed for about 2 years from 1989 to 1990 in several locations in the United States, with a total study population of almost 19 million (Schuchat *et al*., 1992). During this interval, 301 cases were identified, with an annual incidence ranging from 4.8 to

9.3 per 100 000, depending on the surveillance site. About one-third of these cases were pregnant women. Of the cases that were not pregnant, 98% had at least one underlying illness. Malignancy, corticosteroid use, and HIV/AIDS were the most common conditions.

Pregnant women are more vulnerable to listeriosis, probably because of decreased cellular immunity, and because the organism can proliferate in the placenta in areas not reached by defense mechanisms (Lorber, 2000). When pregnant women are infected, central nervous system involvement is very uncommon. Instead, patients tend to develop a bacteremia with self-limited fever, myalgias, arthralgias, headache, and backache. Therefore, it is easy for listeriosis in pregnancy to escape detection, unless a blood culture is obtained during the mild febrile illnesses. Perinatal infections are much more severe, with stillbirth, neonatal death, or premature labor as frequent outcomes. There are two types of neonatal infections: an early onset sepsis syndrome, related to an *in-utero* infection, and late onset meningitis, caused by infection at the time of birth (Lorber, 2000; Mylonakis *et al*., 2002).

During the 1980s, *Listeria monocytogenes* was identified as a cause of foodborne outbreaks (Schlech *et al.*, 1983). In a 2-year case-control study in the 1990s, a substantial amount of sporadic listeriosis was found related to food-borne transmission (Schuchat *et al*., 1992). Illness was associate with soft cheeses (OR, 2.6; 95% CI, 1.4–4.8), and food from delicatessen counters (OR, 1.6; 95% CI, 1.0–2.5). However, documenting foodborne transmission in outbreaks was difficult because of a variable and prolonged incubation period, ranging from 3 to 70 days, with a median of 3 weeks (Heymann, 2004, p. 311), and a lack of useful epidemiologic markers to distinguish outbreak- from non-outbreak-related strains of the pathogen.

Recent developments have markedly improved listeriosis surveillance and investigation. A standardized protocol for pulsed field gel electrophoresis (PFGE) has produced a subtyping system for *Listeria monocytogenes* (Graves and Swaminathan, 2001). The data are routinely collected and analyzed in a national network in the US, PulseNet (Swaminathan *et al*., 2001). Also*, Listeria monocytogenes* has been declared an adulterant by the USDA and FDA, and routine monitoring for the pathogen is being performed on ready-to-eat foods (FSIS, 2003), in particular ready-to-eat foods of animal origin. When foods are epidemiologically implicated, it is possible to link food isolates with outbreaks through PFGE. This has led to increased detection of geographically dispersed outbreaks of foodborne listeriosis, even though the overall incidence of reported *Listeria monocytogenes* infections has decreased in the United States (Olsen *et al*., 2005).

10.7 Toxoplasmosis – a foodborne disease without outbreaks

In 1998, the CDC convened a national workshop on toxoplasmosis (Lopez *et al*., 2000), which included many of the world's experts on the pathogen. In their

findings, they reported an estimated 750 people died in the United States per year from toxoplasmosis, and about 50% of these were caused by eating contaminated meat. This made toxoplasmosis the third leading cause of foodborne deaths in the United States (after listeriosis and salmonellosis). The deaths have probably decreased since that time, since many were AIDS patients with toxoplasmosis of the brain. In recent years, this disease, along with other opportunistic infections, decreased with improved AIDS treatments (Kaplan *et al*., 2000). However, the public health impact of toxoplasmosis is still large. From 400 to 4000 cases a year of congenital toxoplasmosis occur in the United States. These have the potential for severe outcomes, such as mental retardation, blindness, and epilepsy. Serologic surveys of US women of childbearing age have found 14% positive, indicating a past history of infection for *Toxoplasma* (Lopez *et al*., 2000). In contrast to other foodborne pathogens, outbreaks of toxoplasmosis are uncommon. With 100 cases, the largest outbreak of toxoplasmosis ever reported was in 1995 in British Columbia, Canada, from a contaminated water supply (Bowie *et al*., 1997). The lack of outbreaks is due to the natural history of the disease. As described below, most initial infections are asymptomatic or with relatively mild symptoms. The severe effects are frequently delayed, after the birth of an infected child, or in a reactivated infection in an immunologically compromised person.

Toxoplasmosis is caused by the protozoan parasite, *Toxoplasma gondii*. It has a complex life cycle, with members of the cat family (Felidae) as definitive hosts. After acute infections, they shed oocysts in the feline host's stool for about two weeks. Oocysts are hardy and can survive for about a year in warm, moist soil. After 1–5 days, they sporulate and can infect a variety of mammals, including humans and livestock. In immunocompetent people, 90% of infections are asymptomatic, and the majority of symptomatic people have mononucleosis-like illnesses with low-grade fever, malaise, headache, and lymphadenopathy. Acute infections can be severe in people with decreased immunity resulting from chemotherapy, HIV infections, or other causes. Furthermore, tissue cysts (bradyzoites) from previous infections can reactivate if immunity is compromised at a later time (Kravetz and Federman, 2005).

In women who have normal immune function, congenital toxoplasmosis occurs only when they are infected for the first time during their pregnancy. It is very rare for congenital toxoplasmosis to be caused by infections that occur before conception, although it is possible for reactivation of bradyzoites in pregnant women to cause congenital toxoplasmosis. About half of infections occurring during pregnancy are transmitted to the fetus. Infections earlier in pregnancy are less likely to be transmitted to the fetus than those that occur later in pregnancy (0–9% in the first trimester *vs* 35–59% in the third trimester). However, fetal infections that occur earlier in pregnancy are more likely to be severe (Kravetz and Federman, 2005). Epidemiologic studies of congenital toxoplasmosis have found rates ranging from 1 to 10 cases per 10 000 births (Lopez *et al*., 2000).

Other than maternal transmission, the primary external routes of infection are 1) exposure to oocysts from cat feces, or other items that cat feces might

contaminate, such as garden soil or unwashed fruits and vegetables; and 2) ingestion of raw or inadequately cooked infected meat. About one-half of toxoplasmosis cases may be acquired through food. A case-control study involving pregnant women from six large European studies found that 30–63% of toxoplasmosis infections in pregnant women could be attributed to undercooked or cured meat products (Cook *et al.*, 2000). A case-control study comparing seropositivity among vegetarians to rates among omnivores revealed that 24% of vegetarians had *Toxoplasma* titers compared with 50% of controls (Roghmann *et al.*, 1999). Measures to prevent toxoplasmosis include cooking meat thoroughly, peeling or washing fruits and vegetables, hand washing, and cleaning cutting boards, surfaces, and utensils after they have been in contact with raw meat. Other interventions include prenatal and newborn screening for toxoplasmosis, with treatment of infected individuals. Screening and treatment programs have been performed in high-risk countries, such as France and Austria, and in some regions of the United States, such as the New England Regional Screening Program (Lopez *et al*., 2000; Boyer *et al*., 2005).

10.8 Need for continuing surveillance

Underlying diseases have been investigated extensively as risk factors for foodborne illnesses on many occasions. However, the interaction between the humans and pathogens is dynamic and not static, so ongoing surveillance is necessary. For example, although gastric acid inhibitors may increase the number of people susceptible to enteric infections, antimicrobial treatment of peptic ulcer disease caused by *H. pylori* may decrease gastric acid inhibitor use. On the other hand, gastric acid inhibitor use may become more widespread, because histamine H_2 antagonists are available without a prescription. The number of people with HIV infections or AIDS has increased, but HAART administration and antibiotic prophylaxis have decreased the number of HIV-infected people with opportunistic infections, including foodborne illnesses; the impact of resistance to antiretroviral therapies on foodborne infections among AIDS patients is not yet understood (Vella and Palmisano, 2005). Exposure to pathogens may change because of changes in food manufacturing, distribution, or marketing practices. Because the risk of foodborne disease for people with underlying illnesses may vary over time, it is important to monitor this risk factor with ongoing disease surveillance. This information can be used to identify groups where additional food safety precautions are necessary, and to guide food safety policies for the population as a whole.

Unfortunately, collecting information about underlying illnesses through routine communicable disease reports can be difficult. Interviews for routine reports are usually done by local health department staff with little time, resources, or understanding of pathophysiologic relationships between foodborne pathogens and their human hosts. Even when previously collected information, such as hospital discharge data or death certificates are available, the

information may not always be accurate or easily linked to surveillance data. In an investigation of hospitalizations and deaths due to infections with *Salmonella* by the US FoodNet system, it was found that although salmonellosis was seldom listed as a cause of death on the hospital chart or death certificate, medical record review showed that *Salmonella* infection was a significant contributing factor for the deaths (Kennedy *et al*., 2004).

Potential strategies to obtain ongoing data about underlying illnesses and foodborne disease include the following. FoodNet, a network of 10 surveillance sites in the US (MMWR, 2005) exists to perform active surveillance and research on foodborne diseases beyond that which is possible through routine state and local health department activities. Ongoing surveillance for underlying illnesses in people with foodborne diseases can be done at FoodNet sites, especially in diseases where the incidence is relatively low, but the frequency of ill people with underlying diseases is high, such as *Listeria monocytogenes*, and *Vibrio vulnificus* infections. Furthermore, information about underlying illness can be obtained on all foodborne illnesses where hospitalization or death occurs. Where unusual patterns occur, case-control studies can be carried out to verify the association and quantify the risk. Finally, registries exist for various underlying illnesses and conditions, such as cancers (CDC, 2005) and organ transplants (Organ Procurement and Transplantation Network, 2005). Record linkage between these registries and the FoodNet sites could monitor foodborne disease in people with underlying illnesses, while reducing the amount of medical record review required. Similar work has been published using AIDS and *Salmonella* registries in New York City (Gruenwald *et al*., 1994).

Surveillance of toxoplasmosis poses different problems from other foodborne pathogens, which are acute illnesses and generally are required to be reported to public health departments. In contrast, toxoplasmosis is not usually a reportable disease, and acute infections are usually asymptomatic. More frequently, the severe consequences of toxoplasmosis occur long after the acute infection, with the birth of an infected child or reactivation of a previous infection in an immunocompromised individual. Furthermore, toxoplasmosis laboratory tests do not provide the same epidemiologic tools that exist for other foodborne pathogens. Laboratory diagnosis is performed with serologic tests. In most situations, these can only show that the infection occurred at some time in the past. Even a specimen with a high IgM titer can only show the infection occurred within the previous 3 months (Lopez *et al*., 2000). Determining the time of infection is particularly important when evaluating suspected toxoplasmosis in pregnancy. Infections which lead to congenital transmission almost always occur after conception. Finally, current laboratory tests lack epidemiologic markers to identify sources of infection. Consequently, interviews of mothers with affected and unaffected children must be performed to determine the proportions of toxoplasmosis attributable to food or other sources. Since these exposures occurred many months before, recall bias is a significant problem.

10.9 Future trends

It is likely that populations with impaired resistance to infection will continue to grow. As described at the beginning of this chapter, the incidences of cancers and organ transplants have increased. HIV was unrecognized 30 years ago and has become a major source of immunologically impaired persons. Furthermore, even if the incidences of cancer, HIV-positive individuals, and organ transplants do not increase, the numbers in the population will probably increase because of increased survival through improved treatment.

At the same time, surveillance for foodborne diseases and identification of infection sources has improved through systems such as FoodNet and PulseNet. While the importance of PFGE and PulseNet in listeriosis investigations has been discussed in this chapter, these surveillance tools have had a similar impact on investigations of *E. coli* O157:H7, salmonellosis, and shigellosis outbreaks as well. Furthermore, systems similar to PulseNet USA are being established throughout the Western Hemisphere, Europe and Asia (Swaminathan *et al*., 2001).

The improvements in surveillance and investigations in people with underlying illnesses are particularly prominent. Since foodborne illnesses in these people are more severe, they are more likely to be diagnosed by physicians and laboratories and reported to public health authorities. When national food safety policies are made, they should consider all people, not only those who are healthy. It is very likely that severe foodborne illness will continue to occur in people with underlying illnesses. When they occur, with current laboratory methods and surveillance systems, it is very likely that these people and a suspect food will be identified.

10.10 Sources of further information and advice

10.10.1 Fact sheets

A variety of fact sheets exist on websites of the US Department of Agriculture (USDA, www.fsis.usda.gov), the US Food and Drug Administration (FDA, www.cfsan.fda.gov), and the US Centers for Disease Control and Prevention (CDC, www.cdc.gov). The following are examples:

- USDA, Food Safety and Inspection Service Fact Sheet: Food Safety for Persons with AIDS (http://www.fsis.usda.gov/Fact_Sheets/Food_Safety_for_Persons_with_AIDS/index.asp)
- Eating Defensively: Food Safety Advice for Persons with AIDS (http://www.cfsan.fda.gov/~dms/aidseat.html)
- Listeriosis and Pregnancy, What is Your Risk? (http://www.fsis.usda.gov/oa/pubs/lm_tearsheet.htm)

10.10.2 Food safety training modules

The following modules are for college nutrition and food services courses regarding 1) foodborne disease in vulnerable populations, 2) foodborne disease caused by *E. coli* O157:H7, and 3) pregnancy and listeriosis.

- Cody M (2003) Food Safety Modules, Georgia State University (http://www2.gsu.edu/~wwwfsm/)

10.10.3 Food safety for moms-to-be education campaign

- http://www.cfsan.fda.gov/pregnancy.html

This is an FDA public health educational program in Spanish and English about foodborne illnesses of particular concern for pregnant women. The focus is on *Listeria*, methylmercury, and toxoplasma. The materials include a video, DVD, posters, and fliers. On the website, there are a downloadable Educator's Resource Guide, PowerPoint presentations, handouts, posters, and flyers.

10.10.4 Regulatory documents

- California Department of Health Services, Prevention Services, Division of Food, Drug and Radiation Safety, Food and Drug Branch, Food Safety Program (http://www.dhs.ca.gov/ps/fdb/HTML/Food/indexfoo.htm) – under Shellfish and seafood safety
- US FDA, Center for Food Safety and Applied Nutrition, National Shellfish Sanitation Program, Guide for Control of Molluscan Shellfish, 2003 (http://www.cfsan.fda.gov/~ear/nss2-toc.html).

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Part III

Agent factors of pathogenicity and virulence that influence foodborne disease

11

Evolutionary parasitology: the development of invasion, evasion, and survival mechanisms used by bacterial, viral, protozoan, and metazoan parasites

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11.1 Introduction: evolutionary parasitology

Evolutionary parasitology studies evolutionary processes at intraspecific and interspecific levels in both parasite and host species. At the intraspecific level, it focuses in particular on changes in virulence, resistance against the host's defense mechanisms and against human therapeutic interventions, changes in life-history parameters arising from coevolutionary struggle with the host species, development of host specificity and development of resistance, tolerance and life-history parameters in host species. At the interspecific level, it focuses on the origins of parasitism, cladogenesis of parasitic taxons and co-speciation of parasite and host species. The main practical objectives of evolutionary parasitology include the development of therapeutic and preventive measures to prevent increase in virulence and resistance in parasites or even help reduce them.

Parasitism is a form of symbiosis, i.e. a form of long-term coexistence of two organisms. A parasite is an organism that lives in a long-term close contact with another organism (the host), benefiting from this contact to the detriment of the other organism. If one organism benefits from the coexistence while the other organism suffers no harm, the relationship involved is not parasitism but commensalism. If coexistence is beneficial for both symbionts, it is mutualism. If the host suffers from the coexistence but the other organism neither benefits nor suffers from it, we speak of ammensalism. Both parasites and ammensals,

which have a negative effect on the viability and thus also the biological fitness of their hosts, are considered pathogens. The rate at which the parasite or ammensal (hereinafter referred to as the parasite) reduces the biological fitness of its host is called virulence (Combes, 2001). In medical terms, a parasite's degree of virulence largely coincides with the usual extent of pathogenic manifestations of the relevant infection. In epidemiological and evolutionary terms, however, a parasite that, for example, redirects all or part of the host's energy allocated for reproduction to the host's growth and protection against external influences (so-called castrator), basically enhances the host's viability, but shows higher evolutionary virulence than a parasite that causes infections associated with severe pathogenic symptoms, but does not limit its host's fertility in any major way.

The host's ability to resist the negative impact of a parasite and prevent it from entering the organism or multiplying afterwards is called resistance. Tolerance, on the other hand, means the ability of the host to resist a parasite's negative (pathogenic) activity without being able to prevent the infection or multiplication. Higher resistance or tolerance usually requires partial reallocation of resources from growth or reproduction to protection against the particular type of parasite. Therefore, if the given parasite disappears from the environment or if its abundance drops significantly, less resistant and less tolerant host lines will gradually prevail in the host's population. This creates favourable conditions for the parasite's return into the population and subsequently for a new rise in the frequency of genes for resistance or tolerance. As a result, resistance and tolerance of the members of a population may change in cycles. Genes for tolerance have more chance of becoming fixed in a population than genes for resistance (Roy and Kirchner, 2000). If the genes for resistance start spreading in the population, the size of the parasite population begins to diminish. As the size of the population diminishes, selection pressure of the parasite on the host population decreases proportionally, slowing in turn the spread of resistance genes in the genetic pool of the host population. A spread of the genes for tolerance, though, is not accompanied by a decline in parasite population. Thus, in this case, the intensity of the relevant selection pressure does not decrease and tolerance genes can become fixed in the population.

11.2 Microevolution in population of parasitic species

Microevolution is a set of evolutionary processes unfolding inside the populations of a particular species. Macroevolutionary novelties arise from mutations. At the level of the population, however, recombinants created in sexual reproduction as a consequence of genetic recombination and migrants introducing new alleles into the population from other populations represent a much more significant source of evolutionary novelties. While new mutations are mostly harmful, or neutral, for their bearer in terms of impact on biological fitness, migrants are much more likely to introduce potentially beneficial variants of

genes into the population. That is, only alleles that are useful to their bearers at least under certain conditions can be present in the population with a frequency so high that a randomly selected migrant is likely to carry their copies in its genome.

Most parasite species build highly structured populations (Poulin, 1998). In this respect, parasite species do not in fact differ too much from the host species. The sum of all continually appearing and disappearing populations created by the members of a particular species is called the metapopulation. In addition, and in contrast to host species populations, the individual populations show distinct internal structure. This is because they consist of separate infrapopulations, i.e. groups of individuals parasitizing one member of the host species. While populations often persist at one place for a very long time, infrapopulations are by definition ephemeral and disappear with the death of the host organism. In a non-structured population, individual selection is the main driver of adaptive evolution. Such selection favours the fixation of 'selfish genes', i.e. alleles enhancing the biological fitness of their bearer, often to the detriment of the biological fitness of other members of the population. If members of a species can, within the population, distinguish individuals genealogically related and unrelated to them, kin selection can naturally also play a role (Hamilton, 1964). Kin selection produces the fixation of alleles whose manifestations enhance biological fitness of either their bearer or his or her relatives (who are highly likely to carry copies of the same alleles). In a structured population, moreover, group selection can also be at play (Alexander and Borgia, 1978). Group selection can, under certain circumstances, produce the fixation of 'altruistic genes', i.e. alleles whose manifestations enhance average fitness of the members of a population, often even to the detriment of biological fitness of the allele bearer.

Group selection and kin selection are often at work in parasitic organisms in particular. As we shall illustrate later, the basic growth constant R_0, or the average number of new hosts infected by each previously infected host in a naïve host population, is the fundamental parameter as regards biological fitness of members of a certain line of parasite species organisms. Consequently, alleles whose manifestations enhance biological fitness of all members of an infrapopulation have a high chance of becoming fixed, even if they essentially harm their bearer. While in a nonstructured population the chances of fixing an allele that reduces the speed of its bearer's multiplication are low, in the infrapopulation of a parasite species an allele that limits the pathogenic manifestations of parasitosis can significantly increase the number of the parasite's invasive stages that the whole infrapopulation will release into the environment during the host's lifetime. This means that in a parasite species kin selection can fix alleles that are detrimental to their bearer but enhance biological fitness of genetically related members of the infrapopulation. Group selection between infrapopulations can also fix alleles that are detrimental to their bearer but enhance biological fitness of genetically unrelated members of the infrapopulation.

11.2.1 Host–parasite coevolution: an evolutionary arms race

There is a constant coevolutionary struggle between the parasite and the host. The parasite develops evolutionary adaptations that help it overcome its host's defence mechanisms, infect the host successfully and produce new invasive stages. The host species, on the other hand, develops evolutionary adaptations that increase the organism's resistance to the attacks of the parasite. The parasite is usually a step ahead of the host in this never-ending struggle. This is because its evolutionary strategy gives it a number of advantages. During its lifetime, a parasite typically produces more offspring than a free-living species. For example, a tapeworm *Taenia saginata* can produce about 720 000 eggs per day. Only a fraction of the offspring survives until reproductive age. As a result, the processes of selection in a parasite species are much more effective than in a free-living species. Another factor facilitating a parasite's lead in the coevolutionary struggle with its host is aptly described by the so-called life–dinner principle (Dawkins and Krebs, 1979; Dawkins, 1982). A rabbit runs faster than the fox because in the race the rabbit is fighting for its life while the fox is only fighting for dinner. Similarly, a parasite defeated in the competition with its host loses the possibility to reproduce, while for the host the defeat usually means just a certain decrease in biological fitness. The last and probably the most important factor facilitating the parasite's lead in the coevolutionary struggle with its host lies in greater consistency of the relevant selection pressures. While all ancestors of any parasite have in the past met with the host, the same is not true for the host species, since in each generation only some individuals in a population are exposed to attacks by the parasite (Dawkins, 1982).

11.2.2 Maximization of basic reproduction rate (R_0) by optimization of virulence

The basic growth constant R_0, or the average number of hosts infected by one infected host in a naïve host population, is a critical parameter that determines biological fitness of members of a parasite species. This also largely determines the direction in which the microevolution of a parasite species will progress. In the course of microevolutionary processes, phenotypic properties increasing the R_0 under given circumstances become fixed, while properties decreasing the R_0 disappear. The idea that a parasite's virulence gradually decreases during the evolution of the parasite species has become an integral part of the scientific folklore. The parasite, which at the beginning caused severe harm to its host or even killed it, gradually adapts to the host, so that in the end it not only does not kill it but even minimizes the negative impact of its activities on the host's biological fitness. This notion is largely incorrect (Ewald, 1994). If it is useful for the parasite to reduce its virulence in order to increase the R_0, then it will indeed reduce it over time. However, if in a different situation it proves useful to increase the virulence in order to maximize the R_0, then an originally harmless commensal can gradually develop into a dangerous pathogen.

Observation of the natural environment and experience from the preparation

of attenuated vaccines by passaging human parasites in an animal host show that after so-called parasite capture (the transmission of a parasite to a new host species) the parasite is usually not able to multiply in the new host quite as efficiently, as a result of which it tends to show lower virulence. The parasite's virulence in the new host grows as it gradually adapts to it, until the pathological manifestations of parasitosis become so severe that they considerably shorten the life of the infected host and thus also the average duration of an infrapopulation. At that point the force of individual selection for the parasite's faster reproduction and hence for increased virulence becomes balanced by the force of kin and group selection operating in the opposite direction. A parasite's virulence is, of course, determined first and foremost by evolutionary constraints, for example, by which of the host's organs it parasitizes and which resources it takes from the host. Yet the resulting virulence of a parasite species or even a local parasitic population is determined by a whole range of external factors, some of which we are even able to affect by targeted (and naturally also non-targeted) interventions.

One important factor capable of affecting a parasite's virulence involves the genetic heterogeneity of the infrapopulation. An increase in the infrapopulation's genetic heterogeneity usually leads to an increase in virulence. It is known that parasites with a high mutation rate (e.g. the RNA viruses) or parasites with high-intensity recombination tend to be more virulent than parasites with a lower mutation rate (DNA viruses) and with a low or zero recombination rate (e.g. clonal organisms) (Bonhoeffer and Nowak, 1994; Ewald, 1997). Superinfections, i.e. infections of an already infected host by a genetically unrelated line of the parasite, represent an extremely significant source of genetic heterogeneity in a population. If superinfections are very frequent, or even regular, it is not worthwhile for the parasite to make its host last, but it is much more useful to multiply as fast as possible and thus produce the highest number of invasive stages before the host is killed by another strain of the parasite, faster in multiplying and hence also more virulent. A high likelihood of superinfection is one of the main causes of increased virulence of many different parasitoses during wartime, and it also seems to be the cause of the unusually high virulence of parasites causing nosocomial infections (infections in hospitals) (Bonhoeffer and Nowak, 1994; Ewald, 1994).

The way in which a parasite is transmitted from host to host represents another factor significantly affecting its virulence. Parasites borne by a vector (be it insects or running water) tend to be more virulent than parasites transmitted by direct contact of the infected hosts (Ewald, 1983, 1991). This is because it is in the interest of a parasite transferred in direct contact (e.g. the influenza virus) not to harm its host too much, allowing the host to contact and thus also infect as many members of the host species as possible. High virulence is also typical for so-called sit-and-wait parasites, i.e. parasites producing persistent spores which remain in infectious stage on the site of the host's death for a long time and wait for the arrival of a new uninfected host (e.g. anthrax) (Ewald, 1995). Foodborne parasitoses tend to show high virulence. Parasites

whose life cycle involves transmission from prey to predator, in particular, often cause significant harm to their host, thus increasing the chances of the infected host becoming prey of the predator (e.g. tapeworm echinococcus) (Ewald, 1995). The lowest virulence is shown in parasitoses of mostly vertical transmission within the population from parents to offspring, since in this case it is in the interest of the parasite that the host should have as many offspring as possible (Bouma and Lenski, 1988; Bull *et al*., 1991; Clayton and Tompkins, 1994). If the parasite's transmission is exclusively vertical through the host's gametes, biological interests of the parasite and the host become so closely interlinked that the parasite apparently dissolves in its host (Law and Dieckmann, 1998). This, for example, has been the fate of the predecessors of today's mitochondria and plastids. Low virulence also tends to be typical of sexually transmitted parasitoses, where the parasite benefits from the host having as many sexual partners as possible.

Host population characteristics also have a big impact on the virulence of parasites. Short-lived hosts (e.g. rodents) tend to have more virulent parasites, which have to use their host's resources before he dies a natural death (Ebert and Herre, 1996; Restif *et al*., 2002). Similarly, if mortality in the population of any species increases for any reason (for example, because of the appearance of a new parasite or in a human population during war), this mostly benefits the fast-reproducing lines of parasites whose infrapopulations can produce a maximum number of infectious stages before their host dies for other reasons. Parasites in a growing host population show higher virulence than parasites in a population stagnating in numbers, since in a growing population the number of immunologically naïve hosts increases, offering the parasites a big chance to establish new infrapopulations (Knolle, 1989; Ebert, 2000). For similar reasons, higher virulence is also characteristic of parasites in dense populations, populations with frequent contacts of a high number of individuals, and populations with individuals migrating over long distances (Haraguchi and Sasaki, 2000).

11.2.3 Evolution of host specificity

Evolution of a parasite species often leads to the narrowing of the host spectrum, i.e. to specialization in one or a few, mostly phylogenetically related, host species. A considerable similarity between the phylogenetic trees of the parasite and the host taxon suggests that parasites remain fairly loyal to their hosts even on large time scales and that parasite capture by an unrelated host remains more of an exception (Brooks, 1993). Similarity between the trees also suggests that cospeciation, i.e. speciation of the host species (split into two daughter species) accompanied by speciation of its parasites, is a very frequent event in the course of evolution.

Specialization in a particular host species or a particular small circle of host species develops with different intensity in different groups of parasites, depending on the form of the parasite's coexistence with the host (endoparasite, ectoparasite, social parasite), as well as on other ecological and physiological

characteristics of the parasite and host species. Generally speaking, the parasite's host-specificity increases over time as the consequence of evolutionary trade-offs, evolutionary fixation of a mutation enhancing the parasite's ability to infect members of one host species, which at the same time reduces its ability to infect (survive, reproduce in) members of another species (Poulin, 1998). The constraints of successful infection of other species can be divided into two groups. On the one hand, there are ecological constraints, consisting in the parasite's inability to meet representatives of the relevant species with sufficient frequency. On the other hand, there are physiological constraints, consisting in the parasite's inability to multiply in the members of the new species quite as efficiently (Combes, 2001). Experimental studies suggest that ecological constraints seem to be more important and that if, in particular, there is repeated contact between the parasite and a new, initially unsuitable species, the parasite will mostly manage to adapt to the new species successfully. Characteristically, humans acquired a large part of their parasites, around 260 species are usually mentioned, not from their phylogenetically related ancestors but from the phylogenetically unrelated farm animals with which they have lived in long-term close physical contact for centuries (Ashford, 2000). In some cases, parasite capture simply broadens the parasite's host spectrum, while in other cases the subpopulation of parasites that adapts to the new species loses ability to parasitize the original species efficiently, thus giving rise to a new species of parasite.

11.3 Mechanisms of invasion, evasion, and survival

Biological interests of the parasite and its host are in many respects antagonistic. Anything that enhances the biological fitness of the parasite usually reduces the biological fitness of its host. Most parasite adaptations take the form of arms that allow the parasite to overcome its host's defence mechanisms, while many adaptations of the host take the form of counter-arms designed to defend against specific and non-specific parasites. A parasite's most important defences include various mechanisms allowing the propagules to seek members of suitable host species and penetrate their bodies (on their bodies in the case of ectoparasites), to remain, mechanically, inside or on their bodies, to draw the necessary amount of suitable resources from their bodies in an efficient way, to resist the host's defence (e.g. immune) systems, put them out of operation or re-direct their activity in a way that renders them harmless to the parasite. The most important defences of the host include the individual components of the immune system, designed to kill parasitic organisms or reduce their vitality and fertility, together with various patterns of behaviour that reduce the risk of contact with the parasite's infectious stages (Moore, 2002). These include, for example, avoiding places contaminated with feces or avoiding cannibalism. Some defence mechanisms operate at the population level and have probably developed in the course of evolution through some form of group selection. These are, for

example, the removal of feces (many species of birds), avoiding contact with other members of the population that are in bad health, and migration of sick individuals (birds, rodents, and possibly even humans) over larger distances (thus protecting the related individuals from infection) (Poulin, 1994; Rozsa, 2000). In some cases infected individuals even seem to commit active or passive suicide. The parasitized individuals are in such cases sometimes killed by an apparently non-adaptive overreaction of their own immune system, at other times, in the case of butterfly caterpillars for example, they move to places where they are easily spotted by insectivorous birds (Trail, 1980).

Characteristics preserved within a population by group selection from the part of parasites are often considered to include sexuality. Many believe that the reason why asexual individuals (who can reproduce twice as fast because they do not have to produce males) do not prevail in the population of a sexually reproducing species is that sexual reproduction offers a more effective defence against parasites (Bell, 1982). The Red Queen model assumes that the disadvantage of asexual reproduction lies in the fact that biological fitness often shows negative heritability owing to the existence of parasites. Parasites are indeed fast in adapting to individuals with the most abundant genotype, that is, individuals that are currently most biologically fit. In the next stage, their offspring are exposed to the hardest attack by the parasites and thus, in turn, become the least biologically fit. In sexually reproducing organisms, the genotype is not inherited from generation to generation but is created *de novo* every time by a random mixing of genes coming from two parent individuals. The parasites therefore cannot adapt to the most successful individuals and thanks to that the sexual line of the host will in the medium-term defeat the asexual lines.

Obviously, group selection is also at play in the evolution of parasites. As has been mentioned earlier, competition among infrapopulations leads to optimization of the growth rate and thus also to optimization of the parasite's virulence. However, group selection or species selection can also be responsible for investment of a large part of the reproductive potential in the production of long-distance migrants (who will not compete with their relatives in the local host population) and possibly also for introducing vectors in the life cycle of the given parasite species.

11.3.1 Parasite invasion strategies

In the course of their evolution, parasite species have developed a whole series of extremely varied invasion mechanisms that allow them to invade host organisms successfully and overcome their defence systems. The best-known invasion mechanisms include the development of indirect life cycles, avoidance of immune system surveillance, molecular mimicry, immunosuppression, immunomodulation, and manipulation with the host's endocrine and neural systems.

One of the most successful and widespread ways of invading host organisms consists of the introduction of intermediary hosts into the parasite's life cycle (Poulin, 1998; Combes, 2001). Life cycles of parasites are divided into direct

and indirect. Parasites with direct life cycles will make do with one host species and can only switch between two stages during their life cycle – the parasite stage and the free-living stage, designed solely to facilitate transfer from one individual of the host species to another by getting across open environment. Depending on parasite species, the two stages can, but do not have to, differ morphologically and physiologically. It may not be easy for the free-living parasite stages to find a new host and a large part of them may die in the open environment. That is why in the course of their evolution a considerable number of parasite species developed indirect life cycles of varying complexity, involving a higher number of successive host species in different roles. The parasites often use an ecological, mostly trophic, relationship between different species for effective transfer from host to host. The intermediary host would in this case be a species eaten by the final host species. The final host becomes infected by hunting down and eating an infected intermediary host. Or, the intermediary host can be a micropredator, such as a mosquito or flea, which repeatedly suck on a large number of members of the host population and can thus transfer parasites from host to host.

Very often the parasite does not just passively wait in its host for the trophic event to occur but actively promotes it, often in a very sophisticated way (Fig. 11.1). A number of trophically transmitted parasites can influence the behaviour of their host in ways that increase the likelihood of the host being hunted down by the corresponding predator. For example, the *Toxoplasma gondii* protozoan, whose final hosts are felines, reduces the fear of cats in parasitized rats (Berdoy *et al*., 2000), while in parasitized mice and humans (the incidence of *Toxoplasma* in humans reaches a global average of around 30%) it decreases psychomotor performance of hosts and lengthens the reaction time of infected mice and humans (Webster, 2001; Havlíček *et al.*, 2001). In humans (as opposed to other primates or mice), a longer reaction time (observed in computerized simple reaction time tests) does not increase the chances of *Toxoplasma*'s transfer into the intestine of a feline (i.e. infection does not increase the likelihood that people with toxoplasmosis will be eaten by cats) but it can, for example, increase the risk of car accidents, and the higher prevalence of toxoplasmosis in victims of car accidents suggests that it could be increasing that risk up to 2.6-fold (Box 11.1). The *Leishmania* protozoan, for its part, can damage the suction system of its vector (the sandfly) so the micropredator cannot suck enough blood, is always hungry, and moves from member to member of the host population in attempts to satisfy its appetite (Schlein, 1993).

Another strategy that helps the parasites to colonize host organisms successfully consists of avoiding the immune system surveillance. Bodies of organisms contain certain so-called immunoprivileged organs and tissues in which the common immune mechanisms do not operate or operate to only a limited degree. These organs include, for example, the nervous system, in which a strong immune reaction would result in death or cause severe damage to the individual, and the eye lens, in which a similar reaction could easily lead to blindness. To a certain degree, it also is true in mammalian red blood cells

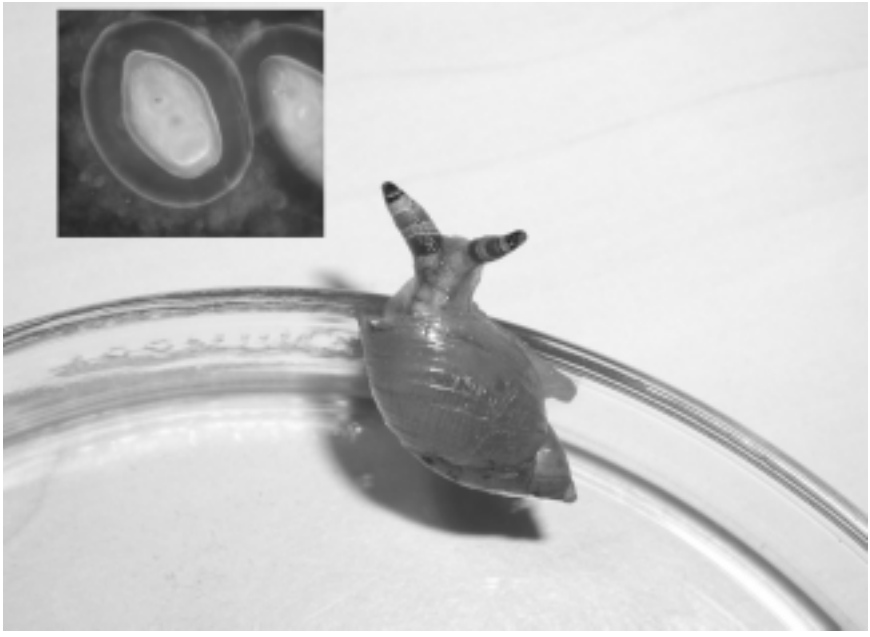

Fig. 11.1 Increased probability of the parasite's transmission by manipulation of the intermediate host morphology and behavior. Trematodes of the genus *Leucochloridium* must get from their intermediate host (land snail, here the amber snail *Succinea putris*) to the definitive host, an insectivorous bird. Their large elongated sporocysts with brightly pigmented brown, green, and white bands move into the snails's tentacles and transform the tentacles into large pulsating caterpillar-like organs. Inside the sporocysts, hundreds of infective metacercariae (inset) wait for their opportunity to infect the bird definitive host. (Photo author).

which, owing to the absence of nucleus and proteosynthesis, do not express class I MHC antigens on their surface so they cannot signal the presence of intracellular parasites to T lymphocytes. Many parasite species, including viruses (human cytomegalovirus – brain), bacteria (meningococcus – brain), protozoa (*Toxoplasma* and *Frenkelia* – brain, *Thaileria* and plasmodium – erythrocytes), and helminthes (schistosomes, cysticerci of some tapeworms – brain), specialize in the use of these immunoprivileged tissues and organs.

Another strategy used by a whole range of parasite species involves molecular mimicry (Damian, 1964; Moloo *et al*., 1980). The parasite tries to mimic the molecular set-up of the host species with its own molecular set-up as much as possible. In some cases it achieves this by synthesizing the relevant proteins directly from its own genes, which it had either directly stolen from its host in the past, e.g. the tapeworm *Spirometra mansonoides* (Phares and Cox, 1987), or created from its own genes through a series of mutations, e.g. schistosomes (Dissous and Capron, 1995). In other cases it simply steals molecules from the cells of the host organism and attaches them to its own surface.

Box 11.1

About 30–60% of the population in both developed and developing countries is infected with the parasitic protozoan *Toxoplasma gondii*. Definitive hosts of *Toxoplasma* are various species of felids and the intermediate host can be virtually any mammal or bird species, including humans. The human hosts usually acquire infection by consumption of raw or undercooked meat containing tissue cysts of *Toxoplasma* or by ingestion of food contaminated by cat feces containing *Toxoplasma* oocysts. In an immunocompetent human the acquired toxoplasmosis, characterized by rapid reproduction of tachyzoites in cells of different tissues, is a relatively mild disease. Usually, it is unrecorded or is misdiagnosed as a common viral or bacterial disease. Within weeks or months the tachyzoites disappear and tissue cysts form in various tissues, mainly in the brain and muscles. The latent toxoplasmosis, i.e. life-long presence of these cysts and presence of anamnestic concentrations of anti-*Toxoplasma* antibodies in immunocompetent subjects, is considered asymptomatic and harmless. However, a recent study of blood donors showed that subjects with latent toxoplasmosis have significantly impaired psychomotor performance (prolonged simple reaction times) in comparison with *Toxoplasma*-negative subjects (Havlíček *et al*., 2001). It is also known that subjects with latent toxoplasmosis express specific changes in some personality factors measured with 16PF questionnaire (Flegr and Hrdý, 1994; Flegr *et al*., 1996, 2000). The differences in psychomotor performance and personality factors increase with duration of infection (Havlíček *et al*., 2001; Flegr *et al*., 2002). A retrospective case-control study showed that people infected with parasitic protozoan *Toxoplasma gondii* have about 2.6 higher risk of traffic accidents than uninfected subjects. The underlying basis of the increased risk cannot be determined on the basis of these results; however, both the decrease in psychomotor performance and the personality changes observed in the infected persons could play an important role. Infected men have decreased superego strength, i.e. they have tendency to disregard rules, and infected women have higher affectothymia, i.e. they are more outgoing and easygoing. Because of extremely high prevalence of life-long latent toxoplasmosis (about 30% of people worldwide are probably infected with the *Toxoplasma*) and high incidence of traffic accidents (according to WHO data about 3.5 million people are killed in traffic accidents/year), latent toxoplasmosis, which is mostly considered to be more or less harmless, could be indirectly responsible for more than 150 000 fatalities/year. If this is correct, then latent toxoplasmosis, a common foodborne disease, could be the second most important protozoan killer (after malaria).

Probably the most common strategies of parasite's protection against the host's immune system consist in immunosuppression and immunomodulation (Chandra, 1982; Binaghi, 1993). In the former the parasite directly switches off immune mechanisms of the host's immune system, for example by killing a specific subpopulation of immunocytes (e.g. HIV and CD4 T lymphocytes) or by damaging an immune organ. Given that an immunosuppressed host can easily die of infection by another parasite species, this parasitic strategy is not particularly convenient for the infrapopulation. That is why many parasite species have developed much more sophisticated mechanisms that allow the parasite to re-direct the activity of the immune system to areas where it does not pose any danger to the particular parasite, without limiting the host's overall capacity of defence in any major way.

Adaptive immunity consists of two basic defence components – humoral immunity and cellular immunity. Humoral immunity provides defence mainly against parasites that live freely in the host's tissues and against their toxins, using antibodies as its principal effector. Cellular immunity, on the other hand, is mainly aimed against intracellular parasites and also against multicellular parasites. In some situations, both components of the immune system cooperate closely. It is also true, however, that activation of the humoral component usually leads to a suppression in the cellular component activity and vice versa. A number of parasites, including an intestinal nematode *Trichuris* (Grencis and Entwistle, 1997) can produce molecules of specific immunomodulators to switch the functions of the immune system in a way that suits them suppressing the component that is dangerous for them and enhancing the component that does them no harm (Wilson, 1993). Until historically recent times, people lived in a certain form of permanent symbiosis with intestinal helminths. Only since around the middle of the last century have people living in developed countries become free of these parasites as a result of better hygiene and public health infrastructure. It has been postulated that the absence of helminth intervention in the operation of our immune system has produced shifts in the activity of the immune system components and that this shift is responsible for the surge in the incidence of allergies seen in the developed countries in recent decades (Bell, 1996).

Another weapon used by parasites in the fight with their host consists of purposeful interventions in their endocrine system. A number of parasites can produce substances with hormonal activity through which they are able to alter the physiology, immunity and behaviour of the host organism for their own benefit. A partial or complete parasitic castration represents a frequent type of parasite's hormonal intervention into its host's physiological condition. By hormonally castrating the host organism the parasite achieves a reallocation of resources from reproduction to growth and maintenance, which are, from the point of view of horizontally transmitted parasites, functions substantially more usefully than the host's reproduction (Wilson and Denison, 1980). Through these interventions, the parasite (for example, larvae of some species of flukes) significantly enhances its host's vitality to the detriment of its fertility (hence to the detriment of its biological fitness) (Box 11.2). A parasite's hormonal interventions in the

Box 11.2

The phenomenon of parasitic castration was studied on various parasite–host systems. For example, the ant *Allomerus demerarae* living in symbiosis with the tree *Cordia nodosa* castrates its host plant by biting off all the flowers. *Allomerus* workers protect new leaves and their associated domatia (specialized plant organs inhabited by an ant colony) from herbivory, but destroy flowers, reducing fruit production to zero in most host plants. Castrated plants occupied by *Allomerus* provide more domatia for their associated ants than plants occupied by three species of Azteca ants that do not castrate their hosts. *Allomerus* colonies in larger plants have higher fecundity. As a consequence, *Allomerus* appears to benefit from its castration behaviour, to the detriment of *C. nodosa* (Yu and Pierce, 1998). Parasitic castration and gigantism is extensively studied on trematode parasites of snails. It was observed in *Schistosoma–Biomphilaria*, *Trichobilharzia–Lymnea* and *Diplostomum–Lymnea* systems. It is widely believed that the host-derived factor called schistosomin, which appears in the snail hemolymph at the time trematode cercaria develop, influences the parts of the neuroendocrine system that regulate reproduction and growth (de Jong-Brink, 1999). However, it must be admitted that some parasitologists consider the gigantism to be a host strategy that was more consistent with the snail making the best of a bad situation, and some suggest that certain cases of gigantism were artifacts of abundant nutrients provided during the laboratory studies.

physiology of its host can also aim to change the function of the immune system indirectly through sex change. It is, for example, known that female mice are much more susceptible to infection by *Taenia crassiceps* tapeworm than males. However, when a male becomes infected, he experiences a 200-fold increase in estrogens and a 10-fold decrease in testosterone, which brings the defence capacity of this feminized male close to the defence capacity of a female (Larralde *et al.*, 1995). Bacteria of the genus *Wolbachia*, which can only be transmitted to the next generation in eggs, not sperm, can even change the sex of their host, a land crustacean for example, from male to female. The crustacean's population then consists almost exclusively of females, but a more detailed analysis will show that a large part of the females are, in fact, genetic males (Knight, 2001).

11.3.2 Parasite evasion strategies

Several basic evasion strategies can in general be distinguished, used by different parasite groups to a varying extent and in different specific forms.

Hit and run strategy

The principle of this strategy consists of the parasite's multiplying as fast as possible after having penetrated the host organism, to produce the maximum

number of propagules and infect the maximum number of other host population members before the host builds a sufficiently effective immune response to eliminate the parasite. If the immunity against a particular parasite species lasts for a longer time or even for the lifetime of a previously infected individual, the disease tends to behave in an epidemic fashion, returning to the population in waves at regular or irregular intervals. This strategy (rare in macroparasites) is used by a large group of viruses and bacteria.

Good guy strategy

Parasites using this strategy try to behave as inconspicuously as possible in the host organism. The host's immune system detects the presence of a dangerous foreign agent through, *inter alia*, the presence of molecules from damaged tissues and also through the dynamics of a growing concentration of foreign molecules in the population. If the parasite does not damage host tissues and cells, and does not multiply within the host but only produces propagules that are released into the outer environment, the host's immune system may not detect the parasite's presence at all. A number of parasites behave in the host organism in such a restrained way that from the ecological point of view they can rather be considered neutralists or even mutualists. This strategy is used by a number of macroparasites and other parasites, causing chronic or latent (more-or-less symptom-free) diseases. However, many of these parasites have a back-up strategy should the condition, and thus also the life expectancy, of their host deteriorate. A good guy can then very often turn into a bad boy and by using the hit and run strategy quickly use up all the host's remaining resources.

Moving target strategy

This is a relatively sophisticated and effective strategy used by a number of bacterial and single-celled eukaryotic microparasites (Seifert and So, 1988; Michael and Turner, 1999). In a more primitive form, though, the strategy is even used by some viruses. It is in principle a variation on the hit and run strategy. The parasite multiplies in the host organism and gradually induces the host's immune response. After some time this response is so strong that the rate at which parasites are killed exceeds the rate of their multiplication, making the numbers in parasite populations drop fast. At that time, however, the parasite population already contains a limited number of immunologically distinct variants that are not affected by the host's immune response. Their populations start to multiply and in time again provoke an immune response against themselves. Thus, waves of different peaking antigenic variants of the parasite replace one another until the parasite uses up its repertory of antigenic variants or until the host succumbs to total exhaustion. In eukaryotic parasites, such as trypanosomes causing sleeping sickness, the parasite's genome contains a ready-made reserve of as many as hundreds of genes for the immunodominant surface coat antigen, while in viruses antigenic variants are usually generated through mutations.

Smokescreen strategy

This strategy is based on the fact that the host's immune system usually cannot chase a large number of 'rabbits' (i.e. antigens) at the same time. If the parasite can induce an immune reaction against other antigens present at that moment in the host's body, it can itself escape the surveillance of the immune system. A number of parasites, including a number of viruses with a relatively small genome, include genes for the so-called superantigens such as, for example, molecules that bind to the constant regions of antigen receptors on T lymphocytes, thus activating the T lymphocytes (Llewelyn and Cohen, 2002). As a result, a high percentage of T lymphocytes are activated simultaneously in the body of the infected organism and the immune system may start to build an immune response against antigens inherent to the body. Such a situation can of course be deadly to the host, but from the parasite's perspective it is beneficial because behind the smokescreen of general activation the parasite can easily escape the surveillance of the immune system. The parasites sometimes bring live helpers to the host to create a smokescreen, such as viruses or bacteria of other species. For example, helminths of the genera *Steinernema* and *Heterorhabditis* inject the body of the parasitized host with bacteria of the genus *Xenorhabdus* which quickly multiply and put the defence system of the invertebrate host perfectly out of action (Forst *et al.*, 1997). Given that the life span of activated immunocytes (in both invertebrates and vertebrates) is limited, it is sometimes difficult to decide whether the objective of the strategy is to create a smokescreen or to eventually suppress the activity of the immune system.

Mafia strategy

In most cases this strategy seems to have developed from a strategy of starting as a good guy and switching to hit and run if things go wrong. Under normal circumstances, the parasite tries not to damage the host organism too much and uses the resources in a moderate way. However, as soon as the organism starts attacking it through the immune system or in any other way, its behaviour changes and it starts to harm the host actively (Zahavi, 1979). This strategy was first described in a nest parasite, the great spotted cuckoo. The cuckoo lays an egg in another bird's nest and regularly checks to see how the involuntary foster parents treat it. If the involuntary hosts throw the egg out of the nest, the cuckoo will destroy their whole clutch during the next check.

The same strategy seems to be used by a whole range of microparasites (Soler *et al.*, 1998). For example, the *Corynebacterium diphtheriae* bacterium, which causes diphtheria, behaves in a rather orderly way in the host until the host withholds all reserve iron, which most parasite species need for growth, in response to their multiplication. At that point the bacteria start to produce their diphtheria toxin that kills the surrounding cells, making them release, among other substances, the iron. The original objective of this strategy is, of course, to obtain the otherwise unavailable iron. A long-term use of the strategy produces the side effect of selection for hosts that will not withhold iron if infected by this species of bacteria.

Drug dealer strategy

A whole number of symbionts, formally classified as mutualists, are in fact parasites using the drug dealer strategy. The strategy consists in making the host addicted in some way to the parasite's presence. The addiction sometimes emerges spontaneously when the host starts using a resource produced by the parasite and, as a result of the parasite's long-term presence in the host's populations, eventually loses the ability to obtain the resource in any other way. For example, beetles of the genus *Sitobius* lose their ability to fly if purged of their bacterial symbionts with the help of antibiotics. In other cases, however, the parasite actively helps to induce the addiction by putting an organ of the host organism out of operation and then replacing its function. A parasitic crustacean, *Cymothoa exigua*, can be used as an illustrative although not typical example (Brusca and Gilligan, 1983). It bites out the tongue of its fish host and then clings to the stub permanently with its hind claws, using the front claws when the fish 'sticks it out' from the mouth to collect pieces of food for the fish (and for itself).

11.4 Future trends

For the food processors, evolutionary parasitology has two pieces of news. As such things go, one is good and the other is bad. The good news is, without question, that as a result of the rising sanitary and veterinary standards applied to both the agricultural and industrial parts of food production, not only is the risk of foodborne infections becoming lower but, in addition, a gradual micro-evolutionary decrease in virulence of the parasitic infections is under way. This is due to the fact that if the average success rate of parasite transmission from host to host decreases, the situation by definition favours those parasite lines within the species that are able to treat their host with consideration if possible and make him last longer. The good guy strategy, for example, gains significant competitive edge over the hit and run strategy. Obviously, in developed countries the same phenomenon is also effectively enhanced by the overall increase in life expectancy owing to better nutrition, better health care and a lower risk of war conflicts. Here again, it is convenient for the parasites to make their long-living hosts last and avoid damaging the host organism unnecessarily so as not to shorten the time during which they enjoy the host's resources. It must also be recalled, however, that microevolution of the parasite species populations is not irreversible and that any deterioration in the living conditions of human populations (even if only occurring at a local scale) can turn this trend around.

And this brings us to the bad news. Many processes taking place ever faster in the human population lead, in contrast, to an increase in the virulence of parasitoses and to a general increase in parasitic risks. In the first place I would like to mention the growing connectivity of human populations in general, as well as the increasing mobility of individuals. An outbreak of the most virulent

Ebola-type parasitoses would be practically impossible without modern means of transport. Before the infected individual could reach the next village, he or she most likely succumbed to a disease of such virulence, which prevented the infection from spreading to a larger number of people. Today, however, even a highly virulent infection can quickly reach any international airport and a sufficiently infectious disease can easily persist in the dense populations of the present-day urban agglomerations despite its high virulence. Another risk of emergence of new pathogens and increase in the virulence of known pathogens is linked with the globalization of world trade. This makes it possible for people in any part of the world to come in contact with parasites and pathogens to which their populations are not adapted at all and which they cannot fight. Globalization comes hand in hand with the trend of decreasing natural global genetic diversity in farm animals and cultural plants. Reduced genetic diversity renders any species much more vulnerable to parasites, which can quickly adapt to the most abundant genotypes thanks to their greater speed of microevolution. Globalization and increased mobility of individuals, in contrast, result in higher local genetic diversity of parasite species. As has been mentioned above, increased genetic diversity in parasitic populations spurs their evolution towards higher virulence. More virulent lines begin to prevail at the population level and more virulent species at the level of community.

Globalization, together with other trends currently under way in our environment, also significantly increases the risk of new parasite capture, i.e. the risk that a parasite until now adapted to another species would jump to humans. This risk is enhanced by the global trade in exotic pets, as well as exotic foods. Last but not least, new parasite capture can be facilitated by the growing proportion of old or immunosuppressed individuals in the general population. The latter include both the 'naturally' immunosuppressed persons, such as those with HIV who live ever longer thanks to advances in medicine, and persons whose immune system has been switched off artificially, for the purpose or in consequence of medical interventions (transplants, radio- or chemotherapy, treatment of allergies).

The last risk that must not be omitted, especially in connection with food processing, consists of the emergence of parasite lines resistant to chemotherapeutics as a result of the frequent and extremely intensive use of these substances in farming. Resistance to chemotherapeutics develops very easily and very fast in the parasites, often with a cross-over effect, meaning that resistance to antibiotics used to boost meat production in animal farming can also equip the parasites with partial or total resistance to antibiotics used in human medicine. It may be useful to recall that different species of parasites are quick and willing to exchange the genes for resistance, making it possible for resistance developed in one species to spread very easily to many other (even unrelated) parasite species.

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12

Foodborne microbe mechanisms of colonization, attachment, and invasion

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12.1 Foreword

Salmonella enterica serovars are important foodborne pathogens worldwide. One study estimated that 1.4 million cases of nontyphoidal salmonellosis occur each year in the United States (Mead *et al.*, 1999). Consumption of poultry or poultry products is associated with approximately 40% of these cases of salmonellosis (Olsen *et al.*, 2001). A decade ago, in response to public and governmental concerns about food safety, the US Food Safety and Inspection Service oversaw implementation of a program in poultry processing plants to limit *Salmonella* contamination of finished poultry products. The efforts of that program were found to reduce the frequency of poultry carcass contamination from ~20% to 10% (Schlosser *et al.*, 2000). Despite these programmatic efforts, studies in the United States have demonstrated that commercially available beef, pork, and poultry products are still contaminated with levels of *Salmonella* sufficiently high to pose a threat to consumers (White *et al.*, 2001). Similarly, despite implementation of monitoring and control measures in most European countries, *Salmonella* contamination of poultry products, and the threat of human infection, remains high (Heyndrickx *et al.*, 2002; van Duijkeren *et al.*, 2002; Wilson, 2002).

While substantial progress has occurred in reducing the contamination of poultry and meat with *Salmonella*, detection of this pathogenic organism in food is still a critical mandate for public health and food safety laboratories. In addition to detection, efforts are underway to quantify the levels of organisms in food so that contamination information acquired by food safety laboratories can be correlated with the risk of acquiring salmonellosis after consumption of meat or poultry (see Chapter 17). Thus, reliable estimations of how food contaminated

with known numbers of organisms is likely to impact human public health can be developed (Rocourt *et al.*, 2003). Quantitative approaches to food safety use both traditional and newer microbiological methods for isolating and identifying *Salmonella* in food. These include selective enrichment in media that reduces the growth of other bacteria, serological tests specific for *Salmonella* serotypes as well as new media formulations, automated instrumentation, DNA/RNA probes, antibody-dependent assays, and polymerase chain reaction. Together, these microbiology methods are overcoming some of the limitations of detecting *Salmonella* in food and are increasing timeliness, limits of detection, and differentiation of virulent and nonvirulent isolates.

One of the goals of pathogenic microbiology is to understand the wide variety of mechanisms that contribute to the ability of these organisms to cause disease in the human host. One application of this basic research is that as pathogenic mechanisms are identified and characterized, it becomes possible to develop new diagnostic tests or assays that can distinguish one organism from another or a virulent species from a nonvirulent one. Another application is that as the virulence strategy of different bacterial pathogens are discovered and understood, at least to some degree, it becomes possible to develop intervention strategies to reduce or eliminate infection. In the case of *Salmonella*, significant efforts in basic science research, diagnostic development and food safety oversight have generated a wide range of information and tools. This review is an effort to summarize basic research data relevant to *Salmonella* adherence, colonization, and invasion in such a way that might be useful to food safety professionals. In particular, information that is being acquired about the ability of *Salmonella* to adhere and form biofilm on a variety of surfaces including glass, gallstones, tissue culture cells, and intestinal tissue is a focus of this review. If *Salmonella* that are members of microbial communities in intestinal biofilms are the important source of the organisms that are isolated from livestock animals, then efforts to understand the characteristics and behavior of *Salmonella* carriage in this metabolic state will be more important to controlling carriage and eventual contamination of food than studies on pure cultures of *Salmonella* in broth cultures. Similarly, fundamental understandings of *Salmonella* adherence factors need to be applied to the field of probiotics. A major thrust of probiotic research is to exclude colonization of the pathogen. Including the impact of probiotic strains on the ability of *Salmonella* to adhere to tissue culture cells and/or to form biofilms in various systems will help to determine more precisely how probiotics are functioning. Finally, efforts to understand, and perhaps manipulate, the ability of *Salmonella* to colonize and form biofilm on host surfaces may be a key to reducing or eradicating this pathogen from food such as poultry, pork, and beef.

12.2 Introduction

Pathogenic *Salmonella* species are an important cause of infectious diseases throughout the world. These bacterial pathogens are transmitted via the fecal–

oral route and can cause human infections ranging from self-limiting gastroenteritis to typhoid fever. The strict human pathogen, *Salmonella enterica* serovar Typhi, causes typhoid fever, a systemic disease, which results in 16 million illnesses and 600 000 deaths worldwide each year (1997). Transmission of this disease within the human population is the direct result of poor sanitation of water and food supplies in developing nations. Efforts to control disease transmission include improved sanitation practices and antibiotic treatment. However, infections with antibiotic-resistant *Salmonella* species have surfaced, posing a greater risk to human populations in endemic areas (WHO, 1997). *Salmonella enterica* serovars Enteritidis and Typhimurium cause the majority of human gastroenteritis infections: a reported 40 000 cases of laboratory-confirmed salmonellosis occur in the United States each year. These broad host-range *Salmonella* serovars are prevalent within warm-blooded animal populations that make up the human food supply, and bacterial transmission generally results from consumption of raw or undercooked food products (Darwin and Miller, 1999).

Identifying and characterizing the molecular details of *Salmonella* pathogenesis has been a focus of significant research efforts by many groups (Fierer and Guiney, 2001; Zhou and Galán, 2001; Waterman and Holden, 2003; Kuhle and Hensel, 2004; Altier, 2005; Bueno *et al.*, 2005; Guiney, 2005). Following ingestion of *Salmonella* in contaminated food or water, organisms pass through the acidic environment of the stomach and enter the small intestine. Within the small intestine a number of interactions between *Salmonella* and host cells occur as part of various microbial virulence mechanisms. The bacteria bind to the surface of intestinal cells via fimbriae and then begin to grow on the surface of the cells to form microcolonies. If the organisms are not host adapted, then growth on and invasion of intestinal epithelial cells leads to destruction of the intestinal mucosa and inflammation (gastroenteritis). Following attachment, host adapted organisms engage the invasion machinery and pass through M cells of the Peyer's patches or intestinal epithelial cells to gain access to underlying tissue, where dissemination and growth lead to more severe systemic infection.

Efforts to understand the mechanisms by which *Salmonella* causes disease have lead to the development of tissue culture cell infection systems and animal models of infection. The use of these biological tools has been instrumental in characterizing many virulence factors necessary for *Salmonella* pathogenesis. For example, infection of epithelial and macrophage tissue culture cell lines has helped to identify and characterize the genetic elements necessary for adherence, invasion, and intracellular survival of *Salmonella*. In addition, oral infection and intestinal ligated loop studies in murine models have allowed the investigation of virulence factors that mediate systemic pathogenesis reminiscent of typhoid fever, while rabbit and bovine models have provided valuable information about mechanisms of virulence that result in localized gastroenteritis (Wallis and Galyov, 2000). Thus, *in vitro* and *in vivo* studies have provided much information regarding the virulence factors required to mediate *Salmonella* disease.

12.3 Mechanisms of *Salmonella* adherence to mammalian cells

Human gastroenteritis, caused by *Salmonella* serovars, is initiated when organisms adhere to and colonize the intestinal epithelium. Following adherence, the bacteria can invade intestinal epithelial cells, disrupt the integrity of the mucosal surface and gain access to underlying tissue (Jones and Falkow, 1993; Jones *et al.*, 1994). Several studies have provided experimental evidence that fimbriae mediate *Salmonella* adherence to and growth on the intestinal mucosa in support of the idea that colonization of host intestinal tissue is an important early step of infection (Clegg and Gerlach, 1987; Lockman and Curtiss, 1992; Townsend *et al.*, 2001; Boddicker *et al.*, 2002; Humphries *et al.*, 2003).

Fimbriae are rigid, filamentous structures expressed on the surface of a bacterium that mediate attachment to a receptor. Many fimbriae are assembled using a chaperone-usher system of assembly and the filamentous structure usually comprises several subunits (Hultgren *et al.*, 1991). The shaft of the fimbriae is typically composed of a repeating major structural subunit as well as minor subunits that are integrated into the shaft of the fimbriae and have functions that are believed to be ancillary (Paranchych and Frost, 1988; Collinson *et al.*, 1996). The published *S.* Typhimurium genome sequence contains a total of 13 fimbrial gene clusters (McClelland *et al.*, 2001; Townsend *et al.*, 2001; Humphries *et al.*, 2003), including eight that have been identified by sequence homology alone (Fig. 12.1).

The best characterized of the *Salmonella* fimbriae is type 1 fimbriae. *Salmonella* type I fimbriae are morphologically similar to type I fimbriae produced by other members of the Enterobacteriaceae family of bacteria (Clegg and Swenson, 1994). Similar to *E. coli* type I fimbriae, the type I fimbriae of *Salmonella* bind to mannose residues and are characterized *in vitro* by their ability to agglutinate guinea pig erythrocytes (Clegg and Swenson, 1994; Tinker and Clegg, 2001). These fimbriae are encoded by a 9.5 kb *fim* gene cluster encoding 9 genes (Fig. 12.1) (Clegg *et al.*, 1985; Purcell *et al.*, 1987). The gene cluster includes the *fimA* gene, encoding the major structural subunit; *fimC*, a molecular chaperone that initiates fimbrial biogenesis from the bacterial cell membrane; *fimD*, an usher that facilitates ordered fimbrial biogenesis (Hultgren *et al.*, 1991); *fimH*, the adhesin that mediates mannose receptor binding specificity; and *fimI* and *fimF*, minor subunits whose functions remain unknown (Old and Duguid, 1970; Firon *et al.*, 1984; Clegg and Swenson, 1994). The *fimWYZ* genes encode regulators of type I fimbrial production in *Salmonella*. The *fimZ* gene product is a DNA binding protein, while *fimY* encodes the co-activator protein FimY (Yeh *et al.*, 2002) that functions with FimZ to induce transcription of *fimA* and the production of type I fimbriae (Yeh *et al.*, 1995). The *fimW* gene encodes the FimW protein that is a negative regulator and counteracts the activity of the FimYZ regulatory proteins (Tinker *et al.*, 2001). In addition to type I fimbrial regulation, our research group recently demonstrated that *fimYZ* negatively regulate *hilA* transcription through activation of the

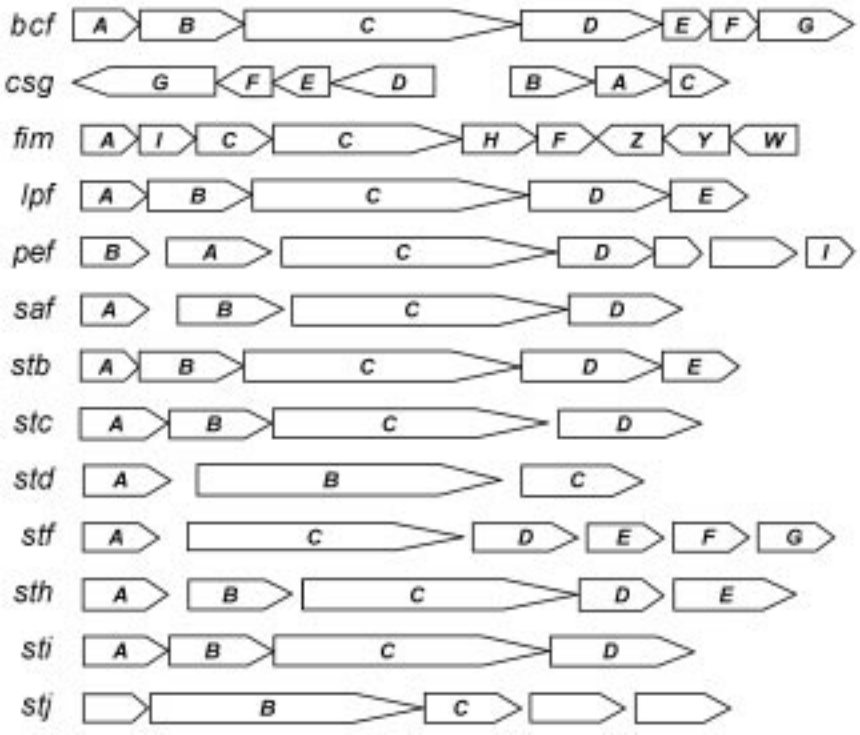

Fig. 12.1 The fimbrial genes clusters of *Salmonella enterica* serovar Typhimurium. Based on the *S.* Typhimurium genomic sequence there are 13 fimbrial operons. The name of each operon is given to the left of the gene cluster. Arrows indicate the position and direction of transcription of fimbrial genes. The length of each block indicates the approximate size of each gene.

hilE repressor gene (Baxter and Jones, 2005). Others have demonstrated that *fimYZ* negatively regulate flagellar biosynthesis and motility (Clegg and Hughes, 2002).

Figure 12.2A illustrates a *S.* Typhimurium strain expressing type I fimbriae, while Fig. 12.2B shows an isogenic strain carrying a mutation in the *fim* operon that results in a loss of type I fimbrial expression and induction of flagellar assembly. These fimbriae are expressed *in vitro* after static growth for 48 h at 37 ºC. Interestingly, several groups have published work indicating that under the growth conditions that have been tested, type I fimbriae are the predominant adherence organelles present on the surface of the bacteria (Humphries *et al.*, 2003). Our research group has recently demonstrated that these fimbriae are involved in biofilm formation on HEp-2 tissue culture cells, murine intestinal epithelium, and chicken intestinal epithelium (Boddicker *et al.*, 2002).

Several other *Salmonella* fimbriae have been identified and partially characterized. Thin aggregative fimbriae (Tafi) are atypical appendages that possess highly adhesive properties and mediate autoaggregative pellicles in broth

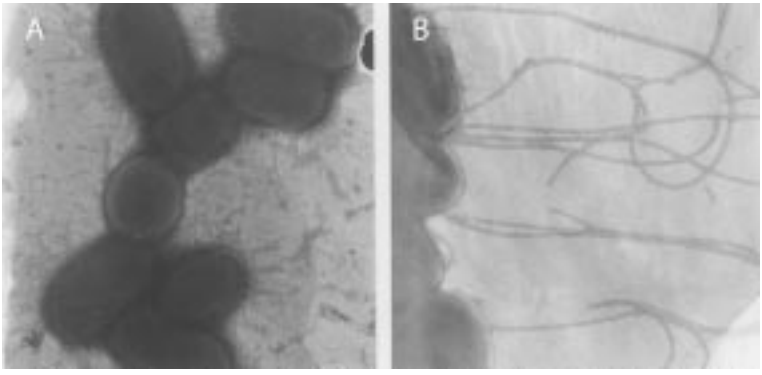

Fig. 12.2 Expression of type 1 fimbriae by *Salmonella enterica* serovar Typhimurium. Type 1 fimbriae produced by *S.* Typhimurium are shown in panel A. The organisms that are shown in panel B lack fimbriae but express flagella, which are visible.

cultures (Collinson *et al.*, 1991; Clouthier *et al.*, 1993; Austin *et al.*, 1998). Tafi are highly stable structures and are not easily solubilized under denaturing conditions (Hammar *et al.*, 1996; Wu and Fives-Taylor, 2001). Tafi mediate adherence to extracellular matrix proteins, contact phase proteins, and MHC class I molecules (Herwald *et al.*, 1998; Brown *et al.*, 2001). Tafi have also been implicated in *Salmonella* biofilm formation on glass (Romling *et al.*, 1998a) and may contribute to mouse virulence (van der Velden *et al.*, 1998). The components of Tafi are encoded by two divergently transcribed operons: *csgBAC* and *csgDEFG* (Fig. 12.1) (McClelland *et al.*, 2001). CsgA makes up the majority of the Tafi shaft with the CsgB subunit also present in the fimbrial shaft as a minor component. Tafi uses a unique mechanism of assembly known as 'nucleation precipitation'. In this system, CsgA and B are secreted into the extracellular environment by the bacteria where a soluble CsgA subunit binds to an anchored CsgB subunit to undergo a conformational change. CsgB appears to have nucleator functions and is believed to act as an anchor and a polymerization starting point for CsgA aggregation in the bacterial cell wall (Bian and Normark, 1997). The conformational change, catalyzed by CsgA binding to CsgB, leads to rapid CsgA self-polymerization and Tafi assembly on the surface of the bacterium (Bian and Normark, 1997).

The roles of CsgC, CsgE, and CsgF are not well understood although there is evidence to suggest that CsgE is involved in fibronectin binding and CsgF participates in nucleation (assembly). CsgG is a lipoprotein that stabilizes the CsgAB protein complex in a manner analogous to a molecular chaperone (Loferer *et al.*, 1997; Wu and Fives-Taylor, 2001). Environmental signals regulating Tafi biosynthesis in rich media proceed through the LuxR-type transcriptional regulator, CsgD, which directly regulates expression of the major subunit, CsgA (Romling *et al.*, 1998b; Brown *et al.*, 2001; Gerstel *et al.*, 2003; Gerstel and Romling, 2003). In addition to regulating expression of Tafi, CsgD also regulates cellulose biosynthesis in rich media, through the *adrA* gene (Brown *et al.*, 2001; Gerstel *et al.*, 2003; Gerstel and Romling, 2003; Garcia *et*

al., 2004). Cellulose has been found to be a component of exopolysaccharide matrices in *Salmonella* biofilms that form on surfaces such as precipitated bile salts and the surfaces of tissue culture cells (Prouty *et al.*, 2002; Solano *et al.*, 2002; Prouty and Gunn, 2003; Garcia *et al.*, 2004; Ledeboer and Jones, 2005). Expression of Tafi is tightly regulated by such environmental conditions as osmolarity, temperature, and growth conditions (Olsen *et al.*, 1993). *In vitro* expression of Tafi is optimal at low temperatures (>25 ºC) and during stationary phase growth (Romling *et al.*, 1998a; Wu and Fives-Taylor, 2001).

The long polar fimbriae (Lpf) are encoded by the *lpfABCDE* genes (Fig. 12.1) and have been implicated in colonization of the murine intestinal mucosa (Baumler and Heffron, 1995; Baumler *et al.*, 1996c; Kingsley *et al.*, 2002). The Lpf fimbriae are believed to use a chaperone-usher system of assembly with *lpfA* encoding the major subunit, *lpfB* and *lpfC* encoding a chaperone and usher, respectively; and *lpfD* and *lpfE* are believed to encode minor subunits (Baumler and Heffron, 1995; Baumler *et al.*, 1996c; McClelland *et al.*, 2001). *Salmonella* strains have been found to phase vary from Lpf 'on' to Lpf 'off' states (Norris T *et al.*, 1998; Norris and Baumler, 1999; Kingsley *et al.*, 2002). Regulation of Lpf expression by phase variation suggests that the fimbriae are immunogenic and that the ability of the organisms to modulate expression of the fimbriae, and their antigenic properties, is an advantage for *Salmonella* when interacting with the immune system of a host.

S. Typhimurium carries a large plasmid (90 kb) that carries virulence genes required for colonization of the lymph nodes, spleen, and liver (Friedrich *et al.*, 1993). In addition to intracellular survival genes, the plasmid possesses conjugation genes (*tra* genes) and a fimbrial operon known as the plasmid encoded fimbriae (*pef*) (Friedrich *et al.*, 1993). The presence of these fimbriae does not appear to be required for *in vitro* adherence to various tissue culture cell lines nor for invasion of the intestinal epithelium (Friedrich *et al.*, 1993). The *pef* genes are encoded by a 13.9 kb cluster of DNA consisting of genes *pefBACD* and *pefI* (Fig. 12.1) (Friedrich *et al.*, 1993; Nicholson and Low, 2000). The *pefB* gene of this operon encodes a putative regulatory gene; *pefA* encodes the major subunits of the fimbrial shaft; *pefC* is a putative outer membrance protein; *pefD* is likely to be a chaperone as it displays considerable homology to molecular chaperones; and *pefI* is a negative regulator of *pef* gene transcription (Friedrich *et al.*, 1993; Nicholson and Low, 2000). It has also been demonstrated that Pef fimbriae are expressed in acidic growth medium (LB broth at pH 5.1). The role of the plasmid-encoded fimbriae in *Salmonella* pathogenesis has been partially defined by Baumler *et al.* (1996a) who demonstrated that *Salmonella* strains expressing the Pef fimbriae can adhere to murine intestinal epithelium and cause fluid accumulation in the intestinal lumen. Adherence and fluid accumulation decrease in strains lacking expression of the Pef fimbriae. In addition, a *pefC* mutant had an LD_{50} for mice twofold higher than the parent strain (Baumler *et al.*, 1996a). Similar to the long polar fimbriae, the plasmid-encoded fimbriae use phase variation to alter their expression. The mechanism of phase variation is analogous to the *E. coli* Pap system in which Dam-dependent methylation of the

pefI regulator controls expression of the fimbrial gene cluster (Nicholson and Low, 2000).

The remaining fimbrial operons *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj* have all been putatively identified by sequence analysis (see Fig. 12.1 for structure of operons). Each of these operons, with the exception of *saf* and *stj* are believed to contain a major subunit located near the 5′ end of the gene (Humphries *et al.*, 2003). Optimal conditions for expression of these fimbriae have not been identified and their expression on the bacterial surface has not been demonstrated (Humphries *et al.*, 2003).

Models of *Salmonella* pathogenesis assume that colonization of the intestinal mucosa precedes invasion and gastroenteritis. As described above, Baumler *et al.* (1996a) demonstrated that the *pef* operon is necessary for fluid accumulation in mice and a *pef* mutant exhibits a twofold increase in dose required to kill 50% of mice compared with wild type. In addition, Baumler *et al.* (1996c) have determined that the long polar fimbriae are necessary for adherence to the murine intestinal mucosa. Our research group has demonstrated that type I fimbriae are necessary for bacterial adhesion to tissue culture cells, murine intestinal tissue, and chicken intestinal tissue (Boddicker *et al.*, 2002; Ledeboer and Jones, 2005). However, the role in pathogenesis of the remainder of the fimbriae that *S.* Typhimurium possesses remains unclear. There appear to be two primary reasons for this lack of understanding. First, the large number of adherence factors, which may have overlapping functions, complicates the analysis of the role of a single adherence factor. Second, the expression of the majority of the fimbriae appears to be very low during *in vitro* growth. It is possible that the role of each of the various fimbriae will only be unraveled when the conditions required for the *in vivo* expression of each is identified. Another confounding issue is that several non-fimbrial adherence factors are present in *Salmonella* strain that include flagella, lipopolysaccharide (LPS), and extracellular matrix. The role of these factors will not be discussed in this review.

12.4 The biology of biofilm formation

A significant advance in microbiology has been the realization that bacteria form complex communities in which microbial growth and development are based on the survival of the entire community rather than a single organism. These complex communities often occur as biofilms, defined as a population of microorganisms that is concentrated at an interface and is surrounded by an extracellular polymeric substance (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004). Since the first description of biofilm formation (Allison *et al.*, 1990) it has become clear that biofilms constitute a unique growth phase in which bacteria express a significantly different repertoire of genes than planktonically grown (liquid grown) bacteria.

A multi-stage model of biofilm development has been proposed (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). In the model, biofilm formation begins with a loose

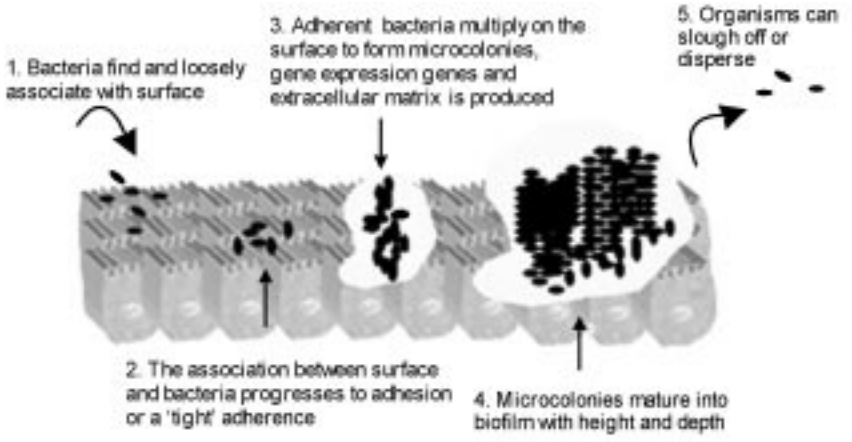

Fig. 12.3 Model of biofilm formation on eukaryotic cells. Biofilm formation on cells is a sequential process. First, adherent organisms encounter and loosely associate with the surface of the cells. Next, this association becomes more permanent. Third, the organisms begin to multiply and form microcolonies that express extracellular polysaccharide. Finally, mature biofilm is formed which is characterized by almost complete coverage of the epithelial surface and significant height of bacterial growth on top of the mucosa. A final step is that some organisms slough off or disperse from the biofilm to return to a planktonic form of growth.

association between the bacteria and a solid surface (Fig. 12.3, step 1). This association advances to 'adhesion' to the substratum (Fig. 12.3, step 2) (Costerton *et al.*, 1987, 1995; Hall-Stoodley *et al.*, 2004) and at this step the bacterial cells alter their phenotypes in response to their proximity to the surface (Costerton *et al.*, 1995). Once attached, sessile or planktonic cells surrounding the newly attached bacteria aggregate with the attached bacteria to form microcolonies (Fig. 12.3, step 3) (Hall-Stoodley *et al.*, 2004). Microcolonies are differentiated from adherent bacteria by production of an extracellular matrix (Costerton *et al.*, 1987, 1995, 1999). From the microcolony stage the biofilm develops into a mature biofilm (Fig 12.3, step 4). Mature biofilms can adopt different structures such as 'mushroom' or 'flat' phenotypes that are dependent upon the nutrient source of the bacteria in the biofilm (Hall-Stoodley *et al.*, 2004). As the biofilm matures, large sections of biomass can slough off and bacteria in the detached material can separate and return to a planktonic state (Fig. 12.3, step 5) (Hall-Stoodley *et al.*, 2004).

In natural settings, biofilms are often composed of multiple bacterial species and are believed to be a prevalent mode of growth for many bacterial populations (Costerton *et al.*, 1999). Biofilms can also be a persistent source of infections. For instance, *Pseudomonas aeruginosa* biofilms contribute significantly to persistence of the bacteria in cystic fibrosis lung infections as the organisms in the biofilm are more resistant to antibiotic treatment and the presence of the biofilm provides a constant source of infecting bacteria (Whiteley *et al.*, 2001). Recent evidence indicates that following adherence to a solid surface

(i.e. glass, plastic or eukaryotic cells) serotypes of *S. enterica* are able to form complex, three dimensional biofilms (Collinson *et al.*, 1996; Boddicker *et al.*, 2002; Garcia *et al.*, 2004; Ledeboer and Jones, 2005). The ability of *Salmonella* to form biofilm is likely to be important for intestinal carriage in food-producing animals (Althouse *et al.*, 2003; Ricke, 2003; Morgan *et al.*, 2004). *Salmonella* biofilm formation on non-biological surfaces in food processing facilities and equipment also may be an important consideration for the food processing industry (Joseph *et al.*, 2001; Prouty and Gunn, 2003).

12.4.1 Mechanisms of biofilm formation by *Salmonella enterica* species

While *S. enterica* serovar Typhi causes systemic infections in humans by traversing the intestinal epithelium and invading and growing within macrophages, it is well known that bacteria can also be carried to the gallbladder where a carrier state can develop (Dutta *et al.*, 2000; Prouty *et al.*, 2002). Patients in a carrier state can asymptomatically shed bacteria for years, even when administered antibiotics. To determine if biofilm formation might contribute to the carrier state of *S.* Typhi, Prouty *et al.* (2002) grew *Salmonella* spp. in media containing human gallstones, in the presence or absence of bile. Interestingly, both *S.* Typhi and *S.* Typhimurium formed biofilms on gallstones within 14 days of inoculation and biofilm formation was dependent on the presence of bile in the growth medium. To determine what bacterial products were necessary for biofilm formation, Prouty *et al.* created knockouts in biosynthetic genes for capsule, flagella, fimbriae (Pef, Csg, Lpf, Fim), LPS, and the AI-2 quorum-sensing signaling molecule (Prouty *et al.*, 2002). The flagellar mutant was able to form a weak biofilm after 14 days that was significantly different from the wild-type strain. The biofilm from the flagellar mutant contained fewer bacteria and significantly less exopolysaccharide (EPS) as detected by scanning electron microscopy (SEM). In examining the roles of the Pef, Lpf, Csg, and Fim fimbriae in gallstone biofilm formation, the authors detected no differences in the ability to form biofilm between the wild type and any of the single fimbrial mutants. It was concluded that these different fimbrial species are not required for biofilm formation on gallstones (Prouty *et al.*, 2002).

To analyze the role of LPS in biofilm formation Prouty *et al.* (2002) examined the ability of a *galE* mutant to form biofilm. *GalE* mutants are unable to add sugar side chains to the O-antigen of the LPS molecule. In addition, a *galE* mutation disrupts the production of colanic acid, which is a component of EPS. The authors observed that a *galE* mutant had a reduced biofilm formation phenotype on gallstones, although they were unable to attribute the observed defect to an incomplete LPS molecule or a defect in EPS production using this method (Prouty *et al.*, 2002). Later work showed that an *rfaD* mutant that is disrupted for O-antigen biosynthesis showed no difference in biofilm formation from the wild type (Prouty and Gunn, 2003), suggesting that the biofilm defect observed for a *galE* mutant is due to a defect in EPS synthesis. The authors concluded that the LPS molecule is not directly involved in biofilm formation (Prouty and Gunn, 2003).

Prouty and Gunn (2003) have also compared biofilm formation on glass to biofilm that forms on gallstones. This work demonstrated that colanic acid is not a major component of biofilm EPS on a glass surface while a cellulose biosynthetic mutant produced reduced biofilm on glass (Solano *et al.*, 2002; Prouty and Gunn, 2003). Neither sugar appeared to be required for biofilm formation on gallstones (Prouty and Gunn, 2003). Additional work to identify polysaccharides essential for biofilm formation on gallstones was unsuccessful (Prouty and Gunn, 2003). The data led to the suggestion that *Salmonella* has evolved mechanisms to form biofilms in a diversity of environments. It is likely that some bacterial factors participate in biofilm formation in all environments while signals unique to each environment may induce a customized EPS that is appropriate for individual surfaces on which the bacteria adhere and grow (Prouty *et al.*, 2002; Prouty and Gunn, 2003).

Other studies of *Salmonella* biofilm formation have focused on EPS components in biofilms formed as pellicles at an air/liquid interface. In pellicles, cellulose is a primary component of EPS for both *S.* Typhimurium and *S.* Enteritidis. Accordingly, Solano *et al.* (2002) searched for cellulose biosynthetic mutants by screening for isolates that had lost the ability to bind to calcofluor. Calcofluor is a fluorescent compound that binds to cellulose. Mutants in cellulose biosynthesis were identified by this technique and found to be defective for biofilm formation on glass.

A relatively unexplored research area is the study of the interactions between *Salmonella* and the intestinal epithelium of poultry. Typically, *Salmonella* species will colonize chickens and turkeys within 2 days of hatching owing to ingestion of fecal material by the chicks. *Salmonella* colonization of the intestine will last throughout the life cycle of the bird and the organisms can also be introduced to the surface of eggs through contact with feces. Poultry meat is also usually contaminated by the contents of the intestine while processing the carcasses. The microbiology lab at the University of Iowa has recently examined the ability of *Salmonella* to form biofilms on host cells, with the goal of gaining insights into the interactions of *Salmonella* with host intestinal surfaces. It was initially demonstrated that two different *S.* Typhimurium strains, LT2 and SL1344, had marked differences in their ability to adhere to tissue culture cells *in vivo* (Boddicker *et al.*, 2002). Subsequent work revealed that both strains expressed type 1 fimbriae at high levels but that the SL1344 strain *fimH* gene sequence, which encodes the type 1 fimbrial adhesin, varied from the LT2 *fimH* gene at two positions that encoded amino acids 61 and 118. These changes did not affect hemagglutination of guinea pig red blood cells but they rendered the type 1 fimbriae unable to bind to HEp-2 tissue culture cells. Transfer of the *fimH* gene, by P22 transduction, from the highly adherent strain to the nonadherent strain conferred a highly adherent phenotype to the transduced SL1344 strain. Further work revealed that highly adherent *Salmonella* strains can colonize and form biofilm on monolayers of HEp-2 cells but nonadherent strains are unable to form biofilms on tissue culture cells. These findings were extended to show

that the highly adhesive type 1 fimbrial allele mediates biofilm formation on both murine intestinal tissue and chicken intestinal tissue.

Subsequent work in the lab has focused on identifying additional factors that contribute to the ability of adherent *Salmonella* to form biofilm on tissue culture cells and poultry intestinal epithelium. Microarrays were used to probe gene expression of *Salmonella* grown as biofilm organisms compared with gene expression when the bacteria were grown planktonically. Two classes of genes have been identified and characterized for their roles in *Salmonella* biofilm formation on host cells. The first class of genes is involved in the production of sugars, specifically colanic acid and cellulose, as components of biofilm EPS (Ledeboer and Jones, 2005). Disruption of the *wcaM* gene, which is involved in colanic acid biosynthesis, altered biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium but not on tissue culture-treated plastic. Disruption of the *yhjN* gene, which is involved in cellulose biosynthesis, caused alterations in biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium and on a plastic surface. The second class of genes identified by the microarray analysis is a subset of the fimbrial gene clusters encoded by *S.* Typhimurium that was discussed above in Section 12.3. Genes encoding plasmid-encoded fimbriae, long polar fimbriae, thin aggregative fimbriae, and the Sth fimbriae were found to be upregulated at least fivefold by biofilm conditions. *S.* Typhimurium mutants were constructed in a required gene for each of these fimbriae and the ability of each of the mutants to form biofilm was compared to the parent strain. The *pef* and TAFI mutants were defective for biofilm formation on plastic, tissue culture cells and chicken intestinal epithelium while the *lpf* mutant exhibited a complete loss of ability to form biofilm on chicken intestinal epithelium but only an intermediate loss on tissue culture cells and plastic. A mutation in a *sth* gene had no effect on the ability of the strain to form biofilm on any of the three surfaces.

Collectively, these results establish that several fimbrial species (type 1, Pef, TAFI, and Lpf) are involved in forming biofilm communities on host cells. Others, such as Sth, appear to have no role in colonization and biofilm formation, at least under the conditions examined. In addition, colanic acid and cellulose are important sugars in the extracellular matrix that is also important in establishing and maintaining these communities.

The role and importance of biofilm in the *Salmonella* lifestyle is still being determined. Biofilm formation on inanimate surfaces such as plastic, glass, or metal is important in various food processing environments and is relevant to food safety. Understanding the role of biofilms in establishing a carrier state in food-producing animals remains an active area of research. Work examining the ability of *Salmonella* to colonize and form biofilms on gallstones appears to provide a starting point for understanding carrier states in more detail. The ability of *Salmonella* to create biofilms on host cells may be an important contributing factor to various host interactions. Invasive organisms penetrate the intestinal epithelium by interacting with the apical surface of cells. *In vitro* studies with tissue culture cells have provided some conflicting evidence that

suggests that *Salmonella* adherence factors are not particularly important to entry of the bacteria (Jones and Richardson, 1981; Ernst *et al.*, 1990; Baumler *et al.*, 1996b, 1997; Dibb-Fuller *et al.*, 1999). However, in the context of a host intestinal environment with a highly polarized epithelial surface that is colonized by large numbers of bacteria and is protected by mucociliary movement, the potential of adherence (and colonization) to potentiate invasion seems logical. In addition, the contribution of adherence to *Salmonella*–host interactions may be more important in gastroenteritis models of infection than in systemic infection models. Thus, a variety of experiments still need to be performed in relevant animal models to determine the contribution of adherence and biofilm to the *Salmonella* virulence strategy.

12.5 Mechanisms of intestinal invasion by *Salmonella*

Work performed more than 30 years ago revealed that *S.* Typhimurium, introduced orally into mice, became primarily associated with the lymphoid follicles or Peyer's patches rather than with the mucosal epithelium (Carter and Collins, 1974; Carter, 1975). More recent work demonstrated that invasive *Salmonella* organisms invade and induce their own uptake into the specialized M cells that are found within the lymphoid follicles (Clark *et al.*, 1994; Jones *et al.*, 1994). Using a variety of experimental systems and mutagenic approaches, a large complement of genes that actively mediate uptake of *Salmonella* into mammalian cells has been identified at centisome 63 of the *Salmonella* chromosome (Galán and Curtiss, 1989; Lee *et al.*, 1992; Behlau and Miller, 1993; Groisman and Ochman, 1993; Jones and Falkow, 1994; Bajaj *et al.*, 1995, 1996; Mills *et al.*, 1995; Galán, 1996; Hong and Miller, 1998; Bakshi *et al.*, 2000). This genetic region has been named *Salmonella* pathogenicity island 1 (SPI-1) and encodes a type III secretion system (TTSS) and its translocation machine, transcriptional regulators, chaperone proteins, and some of the effector molecules that mediate the interactions between *Salmonella* and the cell that has been targeted for uptake (Mills *et al.*, 1995; Galán, 1996).

Early work characterizing *Salmonella* invasion established that entry disrupts the actin cytoskeleton of the host cell (Finlay *et al.*, 1989). Subsequent work has established that the TTSS system injects various effector proteins into the host cell that collectively disrupt the normal regulation of the cytoskeleton of the cell and subvert it so that ultimately the bacteria gain entry to the intracellular environment of the cell. These proteins have specific functions that have been characterized. SipA and SipC bind to actin directly and alter actin dynamics to presumably enhance the efficiency of bacterial invasion (Hayward and Koronakis, 1999; Zhou *et al.*, 1999; McGhie *et al.*, 2001). SopE facilitates exchange of guanine on GTPases and activates RhoA, RhoG, Rac1, and CDC42 (Hardt *et al.*, 1998). SptP has the activity of a GTPase-activating protein (GAP) and is believed to act in concert with SopE to alternately activate and then deactivate GTPases to facilitate actin remodeling to allow bacterial uptake and

to shut down cytoskeletal activation to allow the cell to return to normal (Fu and Galán, 1999; Galán and Zhou, 2000). SopB is an inositol phosphate phosphatase that indirectly activates GTPases (Norris F *et al.*, 1998).

Experiments examining the interactions of SPI-1 gene mutants with lymphoid follicle tissue by electron microscopy revealed that noninvasive mutants were unable to invade into M cells or into intestinal enterocytes at detectable numbers (Penheiter *et al.*, 1997). Furthermore, the virulence of noninvasive *S.* Typhimurium mutants for mice following oral infection was attenuated approximately 60-fold while virulence via the intraperitoneal route was unaffected. Those results indicate that invasion is an important virulence phenotype for penetration of the intestinal epithelium at M cells and enterocytes in mice.

An alternative mechanism for transport of *Salmonella* through the intestinal epithelium has been described. CD-18 positive phagocytes and dendritic cells have been found to have the capacity to transport *Salmonella* across the epithelial barrier, presumably by passage between epithelial cells (Rescigno *et al.*, 2001; Hurley and McCormick, 2003). It is not known to what extent this pathway of bacterial transport across the mucosal epithelium contributes to *Salmonella* dissemination and disease although it seems likely to contribute to the ability of *Salmonella* to penetrate the intestinal epithelium when the invasion genes may not be expressed or as a complementary uptake pathway.

12.6 Current research frontiers

The majority of the research efforts that have been discussed in this review are still ongoing. Efforts continue to define the roles of various *Salmonella* adherence factors in mediating attachment to nonbiological surfaces, as well as to biological (epithelial) surfaces. The properties of biofilms on nonbiological and biological surfaces continue to be explored as part of the effort to control and eradicate the bacteria. One particular area of interest is how pathogens, such as *Salmonella*, overcome the barrier of normal flora organisms to interact with host tissue in a manner that favors the pathogen. Our lab has recently examined the ability of *E. coli* and *Salmonella* to form biofilm on biological surfaces in the presence of a pre-existing biofilm (Esteves *et al.*, 2005). Interestingly, *Salmonella* could insert itself into biofilms formed by *E. coli* but *E. coli* was unable to establish itself in the *Salmonella* biofilm. These findings suggest that evolved pathogens may have highly competitive adherence mechanisms to facilitate their ability to bind to host cells, and suggest that experiments designed to mimic complex polymicrobial environments may be important to increase our understanding of pathogen adherence and biofilm mechanisms. Finally, the information gained from studying the pathogenic mechanisms of adherence, biofilm formation, and invasion into epithelial cells can be used as the basis to search for new antibacterial therapeutics. Novel approaches to disrupt adherence, colonization, and invasion could provide new treatment alternatives to pathogens that are rapidly acquiring resistance mechanisms to traditional antibiotics.

12.7 Sources of further information and advice

A large number of reviews has been published on *Salmonella* invasion and intracellular survival and adherence mechanisms that are available for additional information through PubMed.

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13

Hijacking the host cell: foodborne pathogen strategies for reproduction and defense evasion

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13.1 Introduction

In infectious disease, events that occur between host cell and pathogen are critical in determining the outcome of infection, whether that outcome be asymptomatic infection, clearance of the pathogen, or an infection that overwhelms the body's defenses and results in disease or death. The study of these events at the host–pathogen interface has spawned the emerging field of cellular microbiology – a field that depends on the integration of knowledge from bacteriology, virology, cell biology, and immunology. Central to cellular microbiology is the concept of host–pathogen cross-talk. When a pathogen enters an immunocompetent host, both pathogen and host sense each other's presence. If the invader is recognized as dangerous, the host deploys specialized immune cells and a plethora of chemical signals intended to eradicate the pathogen. The pathogen, on the other hand, often deploys chemical signals to thwart the host's immune response or to otherwise alter the host in such a way that the pathogen has increased chances for survival and reproduction. Thus, the pathogen and the host are engaged in battle with chemical signals being chief weapons of both.

This chapter will focus on the strategies utilized by foodborne pathogens to gain the upper hand at the host–pathogen interface. While great strides have been made in recent years to understand the epidemiology and pathogenesis of foodborne disease-causing viruses and parasites (Koopmans and Duizer, 2004; Slifko *et al.*, 2000), much more is known about the bacteria that cause foodborne disease. Furthermore, even though viral pathogens cause higher numbers of known human foodborne infections (approximately nine million per year in the

United States as compared with approximately four million caused by bacteria), bacterial foodborne pathogens give rise to the majority of fatal infections (72% of fatal foodborne infections are caused by bacteria as compared to 7% caused by viruses) (Mead *et al.*, 1999). Bacterial pathogens will thus be used as examples to demonstrate the various concepts discussed herein. This chapter will begin with an introduction to the human immune system and the general strategies employed by foodborne bacterial pathogens to escape immune destruction. Next, Section 13.3 will explore in greater detail how bacterial pathogens avoid phagocytosis and intracellular killing, while Section 13.4 will examine the roles of eukaryotic cellular receptors in pathogenesis. Section 13.5 will discuss the general mechanisms bacterial pathogens use to secrete protein toxins, while Section 13.6 will explore ways that pathogens hijack cellular processes by interfering with host NF-$\kappa\beta$. The chapter will end with a discussion of bacterial-induced apoptosis or cytolysis in Section 13.7 and a look at current research frontiers in Section 13.8.

13.2 Evasion of immunity

The human immune response consists of both innate and adaptive defenses. The first line of protection against infectious agents is provided by the defenses of the innate immune response, which are activated in response to multiple types of infectious organisms. In contrast, the adaptive immune response, which targets highly specific antigens and is marked by the clonal expansion of B and T lymphocytes, develops only after 3–5 days following initial exposure to an infectious agent (Medzhitov and Janeway, 2000). Consequently, innate immune defenses are the first obstacles that pathogens must overcome to establish a successful infection. Components of the innate defense system include anatomical barriers such as intact skin, physiologic barriers such as stomach acidity and antimicrobial peptides such as lysozyme and defensins, as well as the general inflammatory response and the action of professional phagocytic cells such as macrophages and neutrophils (Medzhitov and Janeway, 2000). Several excellent reviews have been written on the subjects of innate immune responses (see Chapter 8) and strategies employed by pathogens to overcome innate immunity (Medzhitov and Janeway, 2000; Kahn *et al.*, 2002; Alonso and Garcia-del Portillo, 2004; Moine and Abraham, 2004); however, this section will focus specifically on strategies bacteria use to avoid destruction by the inflammatory response as well as the advantages to the pathogen of accessing the interior of a host eukaryotic cell.

13.2.1 Immunosuppression

Many pathogens utilize strategies to suppress the immune responses of their host, thus increasing their own chances of establishing a successful infection. A well-studied example of host immune suppression occurs following infection

with pathogenic *Yersinia* species, which include the causative agent of bubonic and pneumonic plague, *Yersinia pestis*, as well as the foodborne pathogens *Yersinia entercolitica* and *Yersinia pseudotuberculosis*, which can cause gastroenteritis and/or pseudoappendicitis (Naktin and Beavis, 1999; Putzker *et al.*, 2001). All three pathogenic *Yersinia* species carry a 70 kB plasmid (pCD1), which encodes important virulence proteins, some of which facilitate suppression of the host's immune response. One of the immunosuppressive virulence proteins encoded by pCD1 is the *Yersinia* V antigen, also known as the LcrV (low calcium response V) protein. During infection with pathogenic *Yersinia*, LcrV is excreted by the bacterium into the extracellular space where it binds to host membrane receptors CD14 and toll-like receptor 2 (TLR-2) on the surface of macrophages and neutrophils (Pujol and Bliska, 2005). Binding of LcrV triggers an increased production of the immunosuppressive cytokine IL-10, which consequently decreases secretion of TNF-α in host cells and results in an overall less effective immune response to the bacterial infection (Sing *et al.*, 2002a,b; Underhill, 2004).

Other *Yersinia* pCD1-encoded proteins including YopB and YopD (*Yersinia* outer membrane protein B/D) have been shown to decrease the amount of the chemotactic cytokine IL-8 secreted by the host cell, thus effectively decreasing the number of polymorphonuclear leukocytes attracted to the site of infection (Schulte *et al.*, 1996). YopB has also been shown to inhibit the production of TNF-α in stimulated murine macrophages (Beuscher *et al.*, 1995). Thus, pathogenic *Yersinia* species have evolved multiple mechanisms to interfere with the host's ability to mount a successful defensive inflammatory response, thereby increasing their own chances for survival and replication. For a review of the strategies other bacteria use to interfere with host cytokine networks, see Wilson *et al.* (1998).

13.2.2 Intracellular parasitism

Another general pathogen strategy for evading the immune system is intracellular parasitism. From the perspective of a bacterial pathogen, an important advantage of accessing the inside of a host eukaryotic cell for all or part of the bacterial life cycle is avoidance of humoral and several innate immune responses. Specifically, pathogens residing in an intracellular niche are protected from circulating antibodies and complement (parts of the humoral branch of the immune system), as well as from phagocytic destruction by monocytes, macrophages, and neutrophils involved in innate immunity. Thus, the host organism becomes largely dependent upon a single branch of the immune system, cell-mediated immunity, to clear the intracellular pathogen.

Another potential advantage of intracellular parasitism from the perspective of the invasive bacterium is the ability to access cytosolic nutrients that enable bacterial replication. It was long thought that the cytosol represented a nutrient-rich environment permissive to the growth of many bacterial species, including those not normally invasive (O'Riordan and Portnoy, 2002). For example, the

cytosol contains a pool of free amino acids and is capable of supporting the growth of auxotrophic *Listeria monocytogenes* mutants (Piez and Eagle, 1958; Marquis *et al.*, 1993). Later, the idea that the cytosol is nutrient-rich and naturally supports bacterial growth was called into question by experiments that analyzed the growth of bacteria microinjected into the host cytosol and showed that only those bacterial species specifically adapted to cytosolic growth, such as *Listeria monocytogenes* and *Shigella* species, could grow in the host cell cytoplasm (Goetz *et al.*, 2001). These results suggested that the cytoplasm may not be an environment permissive to bacterial growth, and that growth in the cytoplasm may require either specific bacterial adaptations (such as specific nutrient adaptation systems) or bacterial-directed modifications of the host cell. For example, *Listeria monocytogenes* expresses a hexose phosphate translocase gene (*hpt*) that is expressed in the cytoplasm and it has been shown that mutant strains lacking *hpt* are less effective in their ability to proliferate in the cytoplasm of HepG2 hepatocytes, J774 macrophage-like cells, and Caco-2 epithelial cells (Goetz *et al.*, 2001; Chico-Calero *et al.*, 2002). In reality, the ability of a bacterium to replicate in the host cell cytoplasm likely depends on many factors including, but not limited to, the bacterium's route of entry into the cytoplasm, the host cell type, as well as specific genetic adaptations carried by the pathogen for intracellular parasitism (O'Riordan and Portnoy, 2002).

13.3 Avoiding phagocytosis and intracellular killing

13.3.1 Preventing uptake by inhibition of phagocytic signaling

Since phagocytosis of invading pathogens by macrophages and neutrophils represents a key component of innate immunity, an effective bacterial strategy for avoiding destruction by the host immune response is to inhibit phagocytosis. Phagocytosis by a macrophage, for example, involves first sensing the pathogen or particle to be engulfed, followed by the initiation of intracellular signaling events that result in extensive actin cytoskeleton rearrangements, and finally, the formation of pseudopod-like extensions of the cell membrane that surround the pathogen, fuse together, and engulf it. To prevent being phagocytized, a pathogen can interfere with any of these steps. Several excellent reviews have been written describing the various mechanisms pathogens employ to inhibit phagocytosis (Ernst, 2000; Celli and Finlay, 2002; Navarro *et al.*, 2005), so only a few of the mechanisms used by foodborne pathogens will be described here.

One well-studied bacterial system for avoiding phagocytosis is used by *Yersinia entercolitica* and *Yersinia pseudotuberculosis*. Both species utilize effector proteins of their type III secretion systems (discussed in further detail in Section 13.5) to disrupt several different stages of the phagocytic process. For example, the effector protein YopH is a tyrosine phosphatase that, when injected into the cytoplasm of a phagocytic cell, dephosphorylates host cellular proteins that are involved in the formation of the host cell-bacterial focal adhesion and that are responsible for initiating upstream signaling events leading to actin

rearrangement and phagocytosis. YopH is also thought to play a role in disrupting cell-to-cell signaling events responsible for macrophage recruitment to the site of infection (Yao *et al.*, 1999; Navarro *et al.*, 2005). Thus, YopH plays dual roles in preventing phagocytosis, first, by directly inhibiting phagocytosis by adjacent macrophages and, second, by inhibiting the recruitment of other macrophages as part of the inflammatory response. Furthermore, two other *Yersinia* effector proteins, YopE and YopT, have been shown to disrupt the activity of GTPase proteins such as RhoA, Rac, and Cdc42 that are necessary for the actin polymerization events required for phagocytosis (Von Pawel-Rammingen *et al.*, 2000; Navarro *et al.*, 2005). Similarly, another foodborne extracellular pathogen, enteropathogenic *E. coli* (EPEC), disrupts phagocytosis by inhibiting the activity of phosphatidylinositol-3-kinase, which is necessary for actin polymerization and cytoskeletal rearrangements (Celli *et al.*, 2001).

13.3.2 Escaping the phagosome

Despite the efforts of bacteria such as *Yersinia* species and enteropathogenic *E. coli* to avoid being phagocytozed, some bacteria, including those that lack phagocytic avoidance systems as well as those whose avoidance systems fail, will find themselves inside a host cell. Whether the pathogen was passively engulfed by a macrophage or neutrophil or actively invaded the cell, the pathogen is first enclosed in a membrane-bound vacuole known as the phagosome. In naturally phagocytic host cells, the contents of this phagosome would normally be targeted for destruction via fusion of the phagosome with a lysosome (forming a phagolysosome) and other vessicles containing bactericidal compounds such as reactive oxygen and nitrogen species (NO and NADPH oxidase), and degradative enzymes such as lysosyme (Tjelle *et al.*, 2000). Thus, a large percentage of bacteria engulfed by macrophages and neutrophils are effectively destroyed in the phagolysosome, especially in the phagolysosome of a macrophage that has been activated. For example, it has been shown that the number of *Listeria monocytogenes* found in the cytosol following vacuolar escape is reduced from 18% to approximately 4% if the macrophage is activated with interferon-γ, LPS, and IL-6 (Myers *et al.*, 2003). To avoid this fate, some foodborne bacterial pathogens have evolved the ability to escape from the phagosome prior to its fusion with a lysosome.

Two foodborne pathogens well known for this ability are *Listeria monocytogenes*, the causative agent of the foodborne human and animal disease listeriosis, and *Shigella* spp. (*flexneri* and *dysenteriae*), which cause human diarrheal disease. *L. monocytogenes* escapes the phagosome primarily through the action of the protein toxin Listeriolysin O (LLO), which creates pores in the phagosomal membrane and is synthesized in response to the acidifying pH of a maturing phagosome (Glomski *et al.*, 2002). LLO is assisted by the action of two phagosomal membrane-damaging phospholipases, including the broad spectrum phosphatidylcholine phospholipase (PC-PLC ecoded by *plcB*) and the phosphatidylinositol-specific phospholipase (PI-PLC encoded by *plcA*). All

three proteins damage the phagosomal membrane sufficiently that internalized *L. monocytogenes* are able to escape into the protected niche of the host cell cytosol. Importantly, the membrane-damaging action of LLO is confined to the phagosomal compartment; LLO contains a PEST*-like sequence on its N-terminus that targets any cytosolic LLO released during phagosomal lysis to the proteosome for degradation, thus ensuring that the integrity of the cytoplasmic membrane, and consequently, the protected intracellular niche, is maintained (Decatur and Portnoy, 2000). *Shigella* species also are able to escape from the phagosome prior to fusion with the lysosome, although the mechanism by which *Shigella* escape is not as well understood. Several proteins have been shown to be involved including IpaB (High *et al.*, 1992), IpaC (Barzu *et al.*, 1997), and IpaH (Fernandez-Prada *et al.*, 2000).

Bacteria such as *Listeria* and *Shigella* that escape the phagosome to replicate in the host cytosol still face additional host cell defense mechanisms, including escaping detection by intracytosolic recognition pathways and xenophagy (O'Riordan and Portnoy, 2002; Levine, 2005). For example, it has been demonstrated in both epithelial and bone-marrow derived macrophages, that NF-$\kappa\beta$ translocates to the nucleus and activates transcription of INF-β in response to the presence of cytosolic, but not phagosomal, Gram-positive (*L. monocytogenes*) and Gram-negative (recombinant *E. coli*) bacteria (O'Riordan *et al.*, 2002), while cytosolic *Salmonella* are destroyed by the proteosome after recognition by the ubiquitin system (Perrin *et al.*, 2004). Furthermore, use of the autophagy machinery to destroy intracellular bacterial and viral pathogens (a process termed 'xenophagy') has recently been recognized as an important host cellular defense (Kirkegaard *et al.*, 2004; Levine, 2005). Autophagy is an evolutionarily conserved pathway in eukaryotic cells by which the cell sequesters and degrades cyplasmic constituents and organelles for the purposes of nutrient recycling and cellular repair (Levine, 2005). Cellular constituents targeted for autophagy are surrounded by a double membrane-bound autophagocytic vacuole that later fuses with a lysosome. Rich *et al.* (2003) were able to demonstrate that mutant *L. monocytogenes*, defective in their ability to spread from cell to cell, can be found within autophagocytic vacuoles. Interestingly, however, intracellular *Shigella* possess a mechanism to escape autophagy that is dependent on the Type III secreted effector protein IcsB (Ogawa *et al.*, 2005). Indeed, mounting evidence suggests that many intracellular pathogens have evolved sophisticated mechanisms for escaping or even taking advantage of the autophagocytic cellular machinery (Ogawa and Sasakawa, 2006).

13.3.3 Surviving in an intracellular compartment

A second general strategy intracellular pathogens can use to avoid being killed by the phagolysosome is to interfere with host cellular trafficking events such

* P = proline; E = glutamic acid; S = serine; T = threonine.

that the lysosome is prevented from fusing with the pathogen-containing phagosome. In this way, the environment inside the phagosome remains permissive to bacterial growth. A well-studied example of an intracellular pathogen that resides inside a modified phagosome is *Salmonella enterica*. The ability to survive within macrophages is essential for *Salmonella* pathogenesis both because the macrophage provides a protected niche for bacterial replication and because macrophages facilitate extraintestinal dissemination of bacteria (Vazquez-Torres and Fang, 2001). In *Salmonella*-containing phagosomes, also known as *Salmonella*-containing vacuoles (SCVs), proteins in the phagosomal membrane involved in intracellular vesicular trafficking are remodeled such that the phagosome does not fuse with lysosomes. Similarly, *Salmonella* is able to prevent fusion of the phagosome with vesicles containing NADPH phagocyte oxidase enzyme complex (Vazquez-Torres *et al.*, 2000, 2001) and with vesicles containing inducible nitric oxide synthase (iNOS) (Chakravortty *et al.*, 2002).

The prevention of both lysosome and NADPH phagocyte oxidase-containing vesicular fusion is dependent on the gene products of *Salmonella* pathogenicity island II (SPI-2), whose expression, like the expression of LLO in *L. monocytogenes*, is activated in response to the acidification of the maturing phagosome (Rathman *et al.*, 1996). Specifically, the SPI-2 encoded gene, *spiC*, whose product is secreted through the phagosomal membrane and into the macrophage cytosol by a type III secretion system, is required (Uchiya *et al.*, 1999). Additional SPI-2 encoded effector proteins that remain to be identified target the actin cytoskeleton and promote the formation of actin focal adhesions around the *Salmonella*-containing phagosome; these SPI-2 encoded proteins are also believed to help prevent the fusion of lysosomes and NADPH phagocyte oxidase-containing vesicles with the phagosome (Guiney and Lesnick, 2005).

13.4 Cellular receptors

Pathogens infecting a host organism must contend with a group of evolutionarily conserved proteins on and within eukaryotic cells that are part of a host's innate immune response. These membrane receptors and cytosolic surveillance proteins are designed to detect the presence of the pathogen and to trigger an inflammatory response to defeat it. Collectively, these proteins have been termed pattern recognition receptors (PRRs) (also pattern recognition molecules, PRMs) because they recognize certain evolutionarily conserved motifs (termed pathogen-associated molecular patterns or PAMPs) on microorganisms. Examples of bacterial PAMPs recognized by host cell PRRs include bacterial peptidoglycan, lipoteichoic acid, lipopolysaccharide, and flagellin. Some PRRs also are able to detect bacterial and viral nucleic acid (Philpott and Girardin, 2004).

Eukaryotic PRRs can be categorized according to their cellular location. PRRs that are membrane bound belong to the toll-like receptor (TLR) family, while cytosolic PRRs belong to the nucleotide-binding oligomerization domain

(NOD) family (Philpott and Girardin, 2004). Recognition of a pathogen can occur outside or inside the host cell (Kufer *et al.*, 2005). Until recently, it has been thought that TLRs were responsible for recognizing extracellular pathogens, while NOD proteins were responsible for surveying the cytoplasm to detect intracellular bacteria and viruses. It is now becoming clear, however, that TLRs and NOD proteins monitor overlapping regions of the host cell (Philpott and Girardin, 2004; Underhill, 2004; Kufer *et al.*, 2005). The following sections will provide an overview of our current understanding of the functions of TLRs and NOD proteins, as well as explore ways in which pathogens can thwart recognition by host cell PRRs.

13.4.1 Toll-like receptors

Toll-like receptors are integral membrane glycoproteins and cell surface receptors found in eukaryotic cells. All TLRs have a similar structure and contain one transmembrane domain, an extracellular domain made of leucine-rich repeats and a cytoplasmic tail. There are 11 currently recognized toll-like receptors (Akira and Takeda, 2004; Underhill, 2004), which have been the focus of much recent research and several excellent reviews (Akira and Takeda, 2004; Netea *et al.*, 2004a,b; Underhill, 2004). Binding of a TLR to its specific PAMP ligand triggers host intracellular signals that result in the host cell turning on genes required for an innate immune response.

13.4.2 NOD proteins

NOD-LRR proteins, which also have been referred to as NACHT-LRR (leucine-rich repeats) proteins* (Kufer *et al.*, 2005), are an intracellular cytosolic family of eukaryotic cell proteins whose role is to sense intracellular PAMPs. Such intracellular PAMPs can be found on bacterial pathogens that have invaded the host cell or that result from the injection of PAMPs from extracellular bacteria into the host cell by Type III or Type IV secretion systems (Kufer *et al.*, 2005). Like TLRs, NOD proteins have an evolutionarily conserved domain structure. The N-terminus of NOD proteins contains one or more domains involved in protein–protein interactions such as the caspase-activating and recruiting domains (CARD) found in NOD1 and NOD2. The central domain of NOD proteins contains a conserved nucleotide binding region with a NACHT residue motif, while at the C-terminus is a domain containing several LRRs (Philpott and Girardin, 2004).

* A NACHT domain is a domain present in several other proteins including NAIP (neuronal apoptosis inhibitor protein), CISTA (major histocompatibility complex (MNC) class II transactivator), HET-E (plant *het* gene product involved in vegetative incompatibility) and TP1 (telomerase-associated protein 1).

13.5 Mechanisms of protein toxin delivery

Bacterial pathogenesis often is dependent upon the bacterium's ability to secrete specific toxins or virulence factors, which are usually but not exclusively proteins. By definition, toxins that are secreted from the bacterium are classified as exotoxins, in contrast to toxigenic components of the bacterium itself, such as lipopolysaccharide, which are defined as endotoxins. Although a comprehensive discussion of bacterial toxin secretion mechanisms is beyond the scope of this chapter, a brief overview is warranted here since toxin secretion mechanisms are increasingly being investigated as potential targets for antibacterial agents.

Many bacterial protein toxins in both Gram-positive and Gram-negative bacteria are secreted by the general secretory pathway (Sec), which consists of a heterotrimeric pore in the cytoplasmic membrane through which proteins containing a 15–30 amino acid N-terminal leader sequence (or signal sequence) are secreted. The signal sequence gets cleaved by a signal peptidase during translocation, and importantly, proteins secreted by the general secretory pathway are translocated as unfolded molecules. In contrast, an alternative protein secretion system that translocates folded proteins, which are often bound in the cytosol to their co-factors, has more recently been discovered. This protein secretion system depends on a leader sequence that contains a twin arginine motif and, hence, the system has been named the twin arginine translocase (Weiner *et al.*, 1998).

Gram-negative bacteria have several additional mechanisms for secreting proteins across both the inner and outer membranes. These secretion systems, known as Types I, II, III, and IV, are reviewed extensively elsewhere (Thanassi and Hultgren, 2000; Harper and Silhavy, 2001; Salyers and Whitt, 2002). Briefly, Type I systems, which also are known as ABC transporters, bypass the periplasm to translocate proteins directly from the cytoplasm to the extracellular space through a pore spanning both membranes. In contrast, proteins secreted by Type II secretion systems are first secreted by the general secretory system into the periplasm and then are transported through the periplasm and outer membrane by pilus-like structures and specialized proteins known as secretins. Type IV secretion takes advantage of the bacterial conjugation pilus and related machinery to transport proteins directly from the bacterial cytoplasm to the external environment or directly to the cytoplasm of a host cell (Salyers and Whitt, 2002).

The Type III secretion system of Gram-negative bacteria has received considerable attention in the last decade. In Type III secretion, bacterial proteins are injected from the bacterial cytoplasm directly into the cytoplasm of a eukaryotic target cell through a needle-like complex known as an injectisome (Mota *et al.*, 2005). The injected proteins, also known as effectors, target various host cellular processes and, ultimately, serve to make the host organism more permissive to infection. Effector proteins can have antiphagocytoic, cytotoxic, and/or immunomodulatory effects on the target cell. Additionally, effector proteins may promote rearrangements of the host's actin cytoskeleton to facilitate bacterial uptake or to promote strong bacterium–host cell binding.

Several Gram-negative foodborne pathogens utilize Type III secretion for pathogenesis (Mota and Cornelis, 2005; Mota *et al.*, 2005). Among them are the pathogenic *Yersinia* species, as well as enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC), *Salmonella* species, and *Shigella* species. As discussed earlier, the Type III secretion system of *Yersinia* is used to secrete effector proteins that inhibit phagocytosis (e.g. YopE and YopT) and suppress the immune response (e.g. YopH). Similarly, *Salmonella* utilizes effectors encoded by SP-2 to inhibit the lysosome from fusing to the *Salmonella*-containing vacuole. Pathogenic *Salmonella* possesses another Type III secretion system encoded by *Salmonella* pathogenicity island-I (SP-1) that is important during the invasion step of pathogenesis (Guiney and Lesnick, 2005). *Shigella* also utilize Type III secretion for both invasion and escape from the vacuole (Parsot, 2005). One of the most interesting applications of Type III secretion by a foodborne pathogen, however, is formation of the attaching and effacing (AE) lesion by enteropathogenic *E. coli* (EPEC). These bacteria utilize a Type III secretion system to facilitate their attachment to intestinal epithelial cells. Following an initial loose attachment of *E. coli* to an intestinal epithelial cell mediated by bacterial pili, the Type III secretion apparatus is assembled and injects a number of effectors involved in actin rearrangements as well as the translocated intimin receptor (Tir) into the host cell. Inside the host cell, Tir inserts itself into the host cell membrane and serves as a receptor for the *E. coli* membrane protein intimin (Nougayrede *et al.*, 2003). Binding of intimin to its injected receptor, Tir, results in intimate attachment between the bacterium and the host cell.

13.6 Pathogen control of cellular processes via NF-$\kappa\beta$

Throughout this chapter, we have encountered ways in which bacterial pathogens can interfere with eukaryotic intra- and intercellular signaling processes to achieve beneficial effects such as inhibiting inflammation (Section 13.2.1) and modulating actin polymerization either to inhibit phagocytosis by professional phagocytes or to induce uptake by nonphagocytic epithelial cells (Section 13.3.1). While the signaling pathways that bacteria are capable of targeting are diverse, many of the affected pathways converge at NF-$\kappa\beta$. A central player in a host cell's response to a pathogen, NF-$\kappa\beta$ is the eukaryotic transcription factor that, upon activation, translocates into the nucleus and activates a number of genes important in host immune response. Some of the genes activated by NF-$\kappa\beta$ include those that encode pro-inflammatory cytokines such as TNF-α, IL-1a, IL-1b, IL-6, and IL-8 (Liang *et al.*, 2004).

Interestingly, different bacterial pathogens have opposite effects on NF-$\kappa\beta$. Some, as might be expected, inhibit activation of NF-$\kappa\beta$ as part of their efforts to depress the host's immune response. For example, the Type III effector protein YopJ of *Yersinia* inhibits NF-$\kappa\beta$ by inhibiting MAP kinase pathways (Orth *et al.*, 1999; Yoon *et al.*, 2003). Similarly, a YopJ homolog and Type III effector in *Salmonella*, AvrA, also inhibits activation of NF-$\kappa\beta$ but by a

different mechanism (Collier-Hyams *et al.*, 2002). While in many cases it is beneficial for a pathogen to modulate eukaryotic cell signaling events to cause immunosuppression in its host, some pathogens may actually induce the inflammatory component of an immune response by activating NF-$\kappa\beta$. Induction of an inflammatory response may be beneficial to infectious bacteria by providing them with additional host cells (e.g. monocytes) facilitating their dissemination throughout the body. Several foodborne bacterial pathogens produce products that are capable of activating NF-$\kappa\beta$. For example, the virulence factor Listeriolysin O (LLO) produced by *Listeria monocytogenes* was shown to activate NF-$\kappa\beta$ in human umbilical vein endothelial cells (Kayal *et al.*, 1999), specifically by activating I$\kappa\beta$, which degrades the inhibitor of NF-$\kappa\beta$ (Kayal *et al.*, 2002). Another listerial virulence factor, Internalin B (InlB) has also been shown to activate NF-$\kappa\beta$ via phosphoinositide 3-kinase in macrophages (Mansell *et al.*, 2000). Gram-negative pathogens such as *Salmonella* and *Shigella* also activate NF-$\kappa\beta$, but this activation seems to be the result of a non-specific response to bacterial lipopolysaccharide rather than due to specific protein virulence factors (Philpott *et al.*, 2000; Rosenberger *et al.*, 2000).

Further support for the concept that bacterial-induced activation of NF-$\kappa\beta$ and the resulting inflammatory response provides a mechanism for bacterial dissemination comes from research indicating that individuals with leukocytosis may be more susceptible to hemolytic uremic syndrome caused by enterohemhorragic *E. coli* (EHEC), possibly because the increased number of circulating leukocytes provide additional Shiga toxin-binding sites and transport the toxin to endothelial cells (Te Loo *et al.*, 2001; Gobert *et al.*, 2005). Interestingly, the regulation of inflammation and apoptosis are closely integrated (Reed *et al.*, 2004). Besides inducing the transcription of genes involved in the inflammatory response, NF-$\kappa\beta$ also regulates the expression of several anti-apoptotic genes (Wang *et al.*, 1998; Shishodia and Aggarwal, 2002). Indeed, it has been suggested that the activation of NF-$\kappa\beta$ may serve as a bacterial self-defense mechanism to maintain the integrity of a host cell (Kitamura, 1999).

13.7 Loss of cellular integrity: cytolysis and apoptosis

Several fates can befall the eukaryotic cell that encounters a bacterial pathogen. One fate is death due to lysis of the host cell plasma membrane from damage inflicted from the inside of the cell; such loss of cellular integrity can come from bacterially produced membrane-damaging proteins (e.g. hemolysins and phospholipases) or from the physical strain of harboring growing numbers of intracellular replicating bacteria such as *L. monocytogenes* or *Shigella* species. A eukaryotic cell infected with intracellular bacteria also may be killed by cells and proteins involved in cell-mediated immunity acting external to the infected host cell. On the other hand, some intracellular bacteria have the ability to induce host cell apoptosis, which is programmed cell death without loss of membrane integrity or an inflammatory response (Knodler and Finlay, 2001).

Apoptosis results from the activation of a cascade of cellular proteases known collectively as caspases (Chang and Yang, 2000). One well-studied foodborne pathogen known to induce apoptosis in host cells is *Salmonella enterica.* For example, *Salmonella enterica* serovar Dublin was shown recently to induce apoptosis in 70% of THP-1 human macrophage-like cells by a mechanism that did not require SPI-1 or SPI-2 (Valle and Guiney, 2005). Interestingly, however, the apoptotic effects of *Salmonella* seem to be variable and dependent on both host cell type and stage of infection (Knodler and Finlay, 2001). For example, *Salmonella* has been shown to induce a rapid death in macrophages by a mechanism that activates host cellular caspase-1 and the pro-inflammatory cytokine IL-1β and that requires bacterial SipB. While this pathway has some similarity to known apoptotic pathways (e.g. the activation of caspase-1), it also differs from classical apoptosis in being pro-inflammatory and in that the killed macrophages lose membrane integrity (Jarvelainen *et al.*, 2003). Thus, it is not clear whether the *Salmonella*-induced caspase-1 dependent death of macrophages is actually apoptosis or rather a form of cell necrosis.

Salmonella also has been shown to induce delayed apoptosis of macrophages by a pathway that involves host cellular caspases 2, 3, 6, and 8 as well as bacterial SipB (Knodler and Finlay, 2001). Similarly, *Salmonella* are able to induce apoptosis in other cell types, such as epithelial cells and dendritic cells, albeit less efficiently than in macrophages (Knodler and Finlay, 2001). By controlling the temporal induction of apoptosis in multiple cell types, *Salmonella* appear to have evolved a sophisticated strategy for optimizing survival in both the gastrointestinal and systemic phases of salmonellosis; namely, rapid induction of apoptosis by the pro-inflammatory caspase-1 pathway may allow the bacteria to escape immediate immune destruction while also providing new host cells to invade during the initial gastrointestinal phase of infection, while delayed apoptosis may facilitate dissemination and enhanced survival during the systemic phase of infection (Knodler and Finlay, 2001; Jarvelainen *et al.*, 2003). Several other foodborne bacterial pathogens, including *L. monocytogenes*, *Shigella*, *E. coli*, and *Yersinia* have been shown to induce apoptosis in a variety of host cell types, and at least two excellent reviews describe the bacterial-induced apoptosis more fully (Tato and Hunter, 2002; Menaker and Jones, 2003; DeLeo, 2004).

13.8 Current research frontiers

The explosive growth of the field of cellular microbiology in recent years has yielded a wealth of information about the complex interactions that occur at the interface between pathogen and host cell. Importantly, it is now well established that a comprehensive understanding of infectious disease requires knowledge of pathogen virulence strategies, host defense strategies, and the reciprocal communication and signaling that occurs between pathogen and host. Despite all of the recent research advances in the area of host–pathogen interactions,

much more remains to be learned. One exciting research frontier is the degree to which amino acid variation in both pathogen-associated molecular pattern molecules (PAMPs) and pathogen recognition receptors (such as toll-like receptors and NOD proteins) contributes to host susceptibility to infectious agents and to the variability in outcomes of infection. Additionally, the contribution of xenophagy to innate immunity remains an area about which little is known. Finally, much work remains to be done to unravel the complex relationship between inflammation, apoptosis, and eukaryotic intracellular signaling pathways that can be modulated by pathogens. It is hoped that further knowledge and research in this area will provide novel targets and strategies for prevention of different foodborne infections.

13.9 References

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14

Role of viruses in foodborne disease

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14.1 Introduction

Enteric viruses may be the leading cause of foodborne outbreaks and diseases.[1] These outbreaks and diseases have interrelated determinants of infection including: transmission vehicles (including high-risk foods, environment, and human carriers), resistance of the virus to the environment and disinfectants, and host determinants of resistance and susceptibility. To better understand these complex determinants of infection, this review focuses on the most important features of foodborne viral disease from a public health perspective. Other perspectives have been addressed in various excellent reviews.[2–10]

Before beginning this review, it is important to understand the main difference between viruses and other infectious organisms in foodborne disease. Viruses are not free-living and are dependent on specific living cells to replicate and propagate. Since these cells are not present on our foods, viruses cannot increase in number during storage. For these reasons, the mechanism of viral contamination of food is closer to toxin contamination than to contamination of foods with bacteria or fungi[2]; at the same time, toxins, unlike viruses, rarely have secondary spread. Viruses commonly implicated in foodborne outbreaks are listed in Table 14.1. Of these viruses, only rotavirus and hepatitis A has a commercially available vaccine. The reader is encouraged to consult other reviews that have detailed the pathogenicity, epidemiology, and clinical features of the viruses listed in Table 14.1.[2,5] This review will focus on norovirus and hepatitis A because they are the most epidemiogically significant causes of viral foodborne outbreaks.

Table 14.1 Foodborne viruses

Virus	Food	Prevalence in outbreaks*	Detection in food†	Detection in clinical specimens†‡	Vaccine available
Norovirus	Yes	90–96% (38–45%)	Genome	Genome, Ag, Ab, EM	No
Hepatitis A	Yes	3–6% (1–3%)	Genome	Ab	Yes
Rotavirus	Rare	Occasionally	Genome, culture	Ag	Yes
Adenovirus	Yes (shellfish)	Rare	Genome	Genome	No
Aichivirus	Yes (shellfish)	Rare	Genome, culture	Genome, culture	No
Astrovirus	Some (shellfish)	Occasionally	Genome, culture	Genome, Ag	No
Enteroviruses	Yes (shellfish)	Rare	Genome, culture	Genome	No
Sapporovirus	Rare (shellfish)	Occasionally	Genome	Genome, EM	No
Wollan, Ditchling, Paramatta and Cockle agents, Parvovirus	Yes (shellfish)	Rare	Genome?	EM	No

* Data compiled from the CDC Foodborne Outbreak Response and Surveillance unit. % among confirmed viral foodborne outbreaks (% among all confirmed infectious foodborne outbreaks).
† Most common methods listed.
‡ Electron microscopy (EM), Antigen (Ag), Antibody (Ab). Ag and Ab are commonly detected by ELISA (enzyme-linked immunosorbent assay).

14.1.1 Clinical and epidemiologic characteristics of foodborne viruses

Noroviruses

Noroviruses (NoV), also called Norwalk-like viruses, are a large group of small, structured RNA viruses (~27–33 nm, positive-strand RNA) classified in the *Caliciviridae* family. They are the major cause of epidemic gastroenteritis in the United States (Table 14.1) and a significant cause of severe diarrhea in young children in developing countries.[11,12] NoV are also the most frequent cause of acute gastroenteritis after ingestion of raw shellfish.[13–15] NoV infection is characterized by vomiting, diarrhea, nausea, abdominal cramps, and general malaise. NoV are classified by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Disease at the National Institute of Health (NIAID-NIH) as Bioterrorism Category B Priority Pathogens based on their high transmissibility, low infectious dose, and serious public health and economic impact. Transmission of NoV occurs via ingestion of fecal-contaminated food and water, exposure to contaminated fomites or aerosolized vomitus, and direct person-to-person contact.[16–24] In rare cases, transmission can occur through organ transplantation.[23,24] A low infectious dose of less than 10 RT-PCR-detectable (reverse transcriptase polymerase chain reaction) units, or approximately two genome copies, can infect a healthy adult (Moe C. *et al*., unpublished data). In certain individuals, virus can be shed up to 3 weeks post-infection.[25–27] The incubation period is usually 24–48 hours, though in some human challenge studies the incubation period was 10–50 hours. These viruses are the second most important cause of severe gastroenteritis in young children,[28,29] and may cause about 20% of endemic gastroenteritis in families.[30] Each year in the United States, the public health impact of NoV is evidenced by the estimated 23 million infections that result in an estimated 50 000 hospitalizations and 310 fatal cases.[1] Gastroenteritis induced by NoV is self-limiting, of short duration and rarely fatal. Fatality in children and the elderly is usually caused by severe dehydration after NoV infection.[12,31,32] No vaccine is currently available.

Hepatitis A

Hepatitis A virus is a 27 nm picornavirus (positive-strand RNA virus) and is classified as a member of the family *Picornaviridae*. Hepatitis A is responsible for the bulk of infectious hepatitis worldwide and is an important cause of foodborne outbreaks (Table 14.1). The most common symptom of hepatitis A is jaundice; nausea and malaise without jaundice can also occur. Symptoms can be nonspecific and include fever, headache, fatigue, nausea, and abdominal discomfort followed by symptoms and signs of hepatitis 1–2 weeks later. The likelihood of symptoms is related to the age of the individual. Children younger than 6 years rarely develop jaundice, and infection is asymptomatic. Peak viral shedding occurs 2 weeks before onset of jaundice or liver enzyme elevation. Concentration of virus in stool decreases after jaundice appears, though prolonged shedding can occur, especially among children and infants. Hepatitis A has a median incubation period of 30 days (range 15–50 days). Infection with

hepatitis A is self-limiting, can last up to several months, and is rarely fatal (mortality ranges from 0.1 to 0.3% among infected individuals), but adults over 50 years of age or persons with chronic liver diseases have an elevated risk of death from hepatitis.[33] Hepatitis A virus is also classified by the CDC and NIAID as a Bioterrorism Category B Priority Pathogen.

Hepatitis A virus is one of two enterically transmitted viruses that cause hepatitis. Transmission of hepatitis A virus occurs person-to-person by the fecal–oral transmission route including contaminated water and foods, and contaminated fomites (reviewed in Cuthbert[34]). A number of outbreaks in the United States and Europe have also been associated with injecting and non-injecting drug users and men who have sex with men.[35] Although rare, hepatitis A can also be transmitted by transfusion of blood and clotting factor.[35] Even more rare are cases of transmission of hepatitis A by urine (reviewed in Hollinger *et al.*[36]). In one unique outbreak, hepatitis A was transmitted to navy personnel after a mentally disturbed cook urinated in the potato salad.[37] The infectious dose is unknown but presumed to be between 10 and 100 viruses. A vaccine, made from killed hepatitis A virus, and treatment with immunoglobulin, prevents infection with the virus.

14.2 Detection of noroviruses and hepatitis A virus

14.2.1 Diagnosis of human infections

Norovirus is diagnosed in individuals by detection of NoV RNA in stool samples by RT-PCR or by visualization of virus particles by electron microscopy. Several commercial enzyme immunoassays (ELISA) can also detect NoV antigen in stool samples and are a promising avenue for rapid diagnosis.[38–40] Currently, these diagnostic methods are mainly offered by reference or research labs and are not readily available. Serology is rarely used, and if used a four-fold rise in specific antibody in paired sera may be useful for diagnosis. Salivary antibodies are also being investigated as a novel diagnostic tool.[41] If laboratory tests are not available, then epidemiological evidence may provide support for the diagnosis.

Hepatitis A is diagnosed in individuals by detection of anti-hepatitis A virus IgM antibodies in the serum of recently or acutely ill patients.[33] Nucleic acid amplification is not generally used for diagnostic purposes, though hepatitis A virus RNA can be detected in stools and blood of most acutely infected persons.

14.2.2 Detection in foods

Unlike clinical samples, which usually have high titers of virus, food samples have low levels of contamination with viruses. To address this challenge, technologies to detect viruses in foods must (1) concentrate the virus and (2) amplify the virus, usually though RT-PCR amplification of the viral genetic material. These technologies must also remove inhibitors of viral amplification

from the food matrix *without* reducing the yield of RNA. Unfortunately, these technologies cannot distinguish between infectious and non-infectious virus. Infectivity assays have not yet been developed because NoV or wild-type hepatitis A virus cannot be cultured. Several excellent reviews address this topic.[7,42–46]

Bacteria, such as fecal coliforms or *E. coli*, are sometimes used as indicators of fecal contamination. Unfortunately, bacteria are not good indicators of viral contamination because (1) they have shorter persistence in the environment; (2) they are more susceptible to heat and pH; (3) their presence does not necessarily indicate viral contamination; and (4) they may multiply on foods (viruses cannot reproduce without a host cell).

Norovirus can be detected in foods by nucleic acid amplification.[47,48] The main issues in nucleic acid amplification of NoV in foods include processing of different food matrices, concentration of the virus prior to amplification, and elimination of inhibitors of nucleic acid amplification. If RT-PCR is used for amplification, an additional challenge is that, because of the vast genetic diversity of NoV strains, no one set of primers has been commonly accepted for universal NoV detection.[49] Therefore it is difficult to design a standard set of procedures for NoV detection. In addition, since RT-PCR can generate non-specific products, detection methods are usually followed by a confirmation step to increase the specificity of the assay. Once NoV are detected on foods, NoV can be genotyped, and this genotype can be used to identify potential transmission routes and clusters of related cases. These molecular methods are not yet available for routine use.

Hepatitis A virus can be detected in foods by nucleic acid amplification methods.[47,48] The challenges in detection of hepatitis A virus in food are identical to the challenges in detection of NoV in food including: (1) differences in food matrices, (2) viral concentration, and (3) amplification inhibitors. Detection of hepatitis A virus on foods must be done by specialized food microbiology laboratories and is not available for routine use.

14.3 Foodborne outbreaks associated with noroviruses and hepatitis A virus

14.3.1 Foods associated with outbreaks of viral gastroenteritis

Shellfish

Bivalve molluscan shellfish (particularly cockles, mussels, and oysters) are an important source of foodborne viral infections because these filter feeders can concentrate hepatitis A and noroviruses in their tissue and retain them for some time. These shellfish are commonly harvested from locations close to shore where water may be contaminated with viruses from sewage effluents. Because shellfish become contaminated by sewage, shellfish outbreaks often involve multiple virus strains. Shellfish also tend to cause multi-state or multi-country outbreaks. Various regulations may prohibit harvesting of shellfish from

contaminated waters, require shellfish to be heat treated, relayed to clean waters or depurated prior to harvest. Relaying of shellfish is a process by which contaminated shellfish are moved from contaminated areas to clean areas, allowing sufficient time for the shellfish to purge pathogens. Depuration is a process by which shellfish are placed in a recirculation tank with clean water. Water is circulated through the shellfish, disinfected (usually by UV treatment) and recycled back to the shellfish. Through depuration, shellfish gradually rid their bodies of fecal indicator bacteria and may be sold when fecal indicator bacteria fall below a certain level. As mentioned, bacteria are not a good indicator of the presence of foodborne viruses, and multiple outbreaks have been documented from shellfish compliant with these regulations.[50–54] For example, depuration of oysters for 48 hours may reduce *E. coli* levels by 95% but NoV levels were only reduced by 7%.[55] These data suggest that noroviruses and other enteric viruses are poorly depurated under conditions favorable for *E. coli* depuration. Long-distance transmission of foodborne viruses may occur through trade and transport of contaminated shellfish.[17,53] Lastly, shellfish frequently are consumed raw, or minimally cooked, and this increases the risk of viral infection. An example of a shellfish outbreak can be found in Case study 14.1.

Case study 14.1 Multi-state outbreaks caused by oysters from Louisiana, USA

In February 1996, 75 individuals from four states developed gastroenteritis from eating raw oysters harvested from Louisiana.[17] Though the individuals consumed oysters at different gatherings, they all had the same strain of norovirus in stool samples. The Louisiana Department of Health and Hospitals determined that the implicated oysters had been harvested in South Black Bay. A visit to the South Black Bay area revealed a functioning oil rig that had eight employees sick with diarrhea and vomiting between January and March. Four of these eight employees had elevated serum titers to norovirus, suggesting recent infection. In addition to the sick employees, the oil rig had a malfunctioning sewage facility that discharged feculent and cloudy effluent directly into the surrounding waters. Staff from the oil rig indicated that oyster harvesters routinely worked in a prohibited zone surrounding the oil rig. The investigators could not rule out the possibility that the oyster beds became contaminated from harvesters dumping their waste overboard (a practice that continued during the outbreak). This outbreak highlights the ease with which oysters can be contaminated with norovirus from inappropriately disposed sewage. A second multi-state outbreak occurred 10 months later. This outbreak was interesting because genetic sequencing of stool samples identified three distinct norovirus strains originating from three distinct geographic sequences, including South Black Bay. This finding suggested the possibility that distinct harvesters infected with different strains discharged sewage in different harvest sites.

Produce and salads

Viral foodborne illness can be linked to produce and salads. Produce and salads refer to both fruit and vegetables. Salads are more commonly implicated in outbreaks than are individual produce items. Both produce and salads are commonly handled immediately before serving, are eaten raw, and are frequently eaten without peeling. These practices increase their risk for viral contamination. Contamination may occur at any point in the food path: preharvest, postharvest, processing, food preparation, and table where produce or salad is to be eaten. Produce that are contaminated during production or harvest tend to cause multi-state or multi-country outbreaks. Unfortunately washing of produce is often not effective at eliminating viral contaminants (Table 14.2).[56] In the vast majority of cases, when this type of food is associated with an outbreak, an infected food handler is suggested as the source. Unfortunately, this causal relationship is rarely confirmed. Produce can become contaminated with enteric viruses on the farm via fecal-contaminated irrigation water or during harvest via handling by infected agricultural workers. Similar to shellfish, long-distance transmission of viruses can occur through trade and transport of contaminated produce. For example, a recent multi-state hepatitis A outbreak in the United States was linked to contamination of green onions imported from Mexico.[57] A norovirus outbreak in the United Kingdom was thought to be caused by raspberries contaminated during picking or packing.[58]

Other foods

In addition to shellfish, produce, and salads, other foods that have been implicated in viral foodborne outbreaks include: desserts, sandwiches and deli meats, foods that are consumed raw, and other minimally processed foods. In general, any food that has been handled manually is at risk for contamination. These ready-to-eat (RTE) foods are the most important cause of foodborne disease. RTEs are discussed in Section 14.4.4. Contamination may occur at any point in the food path from farm to fork.

14.4 Transmission routes of viral contamination

For foodborne viruses, all virus transmission occurs through a fecal–oral transmission route (Fig. 14.1). Environmental transit times between hosts may be prolonged or brief. Long-distance transmission may take place through water, air, and transported food. Long-distance transmission is also accompanied by exposure to the environment and dilution. Since enteric viruses cannot replicate outside their hosts, viral levels theoretically should decrease during transport and storage. Both hepatitis A virus and norovirus counteract these effects by inducing shedding high numbers of virus from the host and by being particularly resistant to environmental exposure including low pH, high temperature, drying, and various disinfectants (Table 14.2).

Table 14.2 Effect of various inactivation approaches for noroviruses and hepatitis A viruses on foods and surfaces

	Norovirus*	Hepatitis A	References
Disinfectants			
Chlorine	Effective 30 min contact time, 2.0 log reduction† pH 6, 5 ºC, 1 mg/L dose	High levels required $CT_{99.99}$ (4-log): 2-12 min mg/L pH 6–9, 0.5–25 ºC	133–142
Chlorine dioxide	High levels required 60 min contact time, 0.5 log reduction† pH 6, 5 ºC, 1 mg/L dose	High levels required $CT_{99.99}$ (4-log): 8–50 min mg/L pH 6–9, 1–25 ºC	140–144
Ethanol	Reduction (not efficient)	Reduction	82, 145, 146
Monochloramine	High levels required CT_{99} (2-log) reduction: 775 mg min/L pH 8, 5 ºC	High levels required $CT_{99.99}$ (4-log): 497–2883 min mg/L 1–25 ºC	141, 147
Organic acids	Not effective Resistant to pH 2.7 for 3 h	Not effective Resists pH 1.0 2 h	106, 146, 148–151
Ozone	Effective	Effective $CT_{99.99}$ (4-log): 0.3–1.8 min mg/L 1–25 ºC	136, 140, 141, 152–155
Sodium hypochlorite (bleach), hypochlorous acid	Effective	Effective	143, 145, 146, 156–158
UV irradiation	Effective	Effective $CT_{99.99}$ (4-log): 6–15 mW s/cm^2,† $CT_{99.9}$ (3-log): 36 mW s/cm^2, CT_{99} (2-log): 21 mW s/cm^2	141, 146, 154, 159–161

Other treatment processes/conditions			
Depuration of shellfish	Not effective	Not effective	50, 55, 162, 163
Desiccation	Still stable for >2 weeks and caused outbreak on cruise ship	Stable for >1 month	77, 80–82, 164–169
High hydrostatic pressure processing	Unknown (shellfish)	Effective (shellfish)	78, 79, 170
Irradiation	Unknown	Effective at 3 kGy (shellfish)	171
Thermal inactivation, pasteurization	Reduction 3-log reduction at 71.3 ºC (liquid) Resists 60 ºC for 30 min	Reduction 3-Log reduction 71 ºC (liquid) Resists 60 ºC for 10 min Shellfish protects from heat	36, 70, 71, 106, 146, 149, 172–174
Washing, detergents, water	Detergent may not be effective on surfaces Unknown effect on hands	Reduction Effective on hands	56, 80–82, 156

* Including only RT-PCR data on human norovirus and not feline calicivirus. Values based on reduction of NoV levels by RT-PCR.
† Unpublished data.

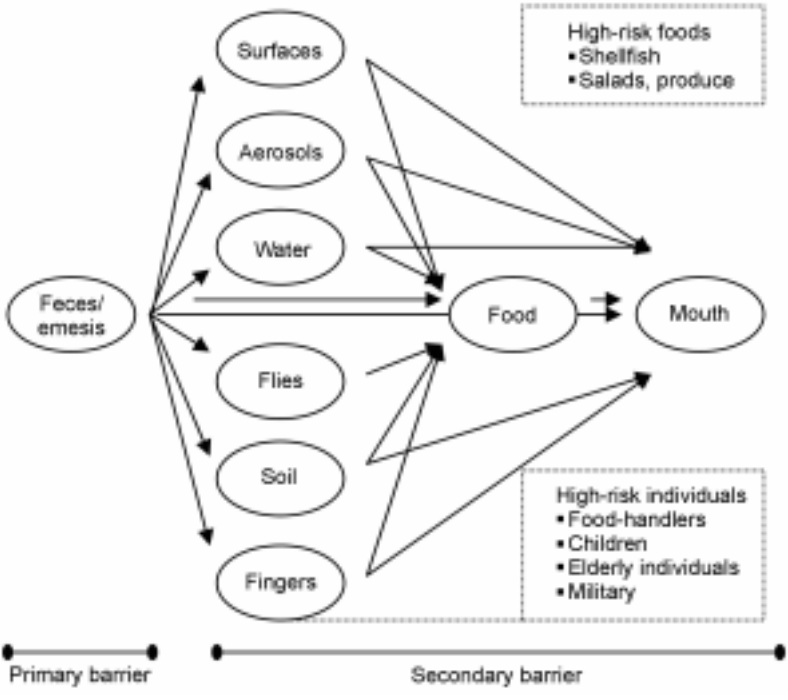

Fig. 14.1 Fecal–oral transmission route for foodborne viruses. Arrows indicate potential routes by which virus in feces or emesis from an infected individual can be transmitted to another host.

14.4.1 Fecal–oral transmission route

Both norovirus and hepatitis A virus are transmitted from person to person via fecal contamination of the environment. Therefore, foodborne transmission of these viruses is merely a subset of the fecal–oral transmission route, and food has a privileged niche because it is easily introduced into the body, has widespread distribution through trade, and is of economic importance.[2]

A simplified model of the fecal–oral transmission route is illustrated in Fig. 14.1. This figure includes documented and suspected transmission routes for norovirus and hepatitis A virus. Vomitus is grouped with feces because it may be the origin of viral contamination into the environment and foods. Each box is connected by multiple lines because contamination of food can occur through multiple routes. Each line represents a potential transmission route that could be a potential site for a public health intervention. Public health interventions can be divided into two categories: primary and secondary barriers.[59] Primary barriers are those interventions that prevent viruses from getting into the environment. Examples of primary barriers would be safe disposal and isolation of vomitus and feces (e.g. use of safe excreta disposal, appropriate cleaning and disinfection of vomitus). Viruses may enter the environment when there are no

primary barriers to keep the infectious organisms out of the environment or when the primary barrier works imperfectly. Secondary barriers are those interventions that prevent viruses from infecting another host once the viruses are in the environment. Examples of these secondary barriers may include avoiding unsafe foods (e.g. raw shellfish) or destruction of viruses (e.g. cooking foods, hand-washing). Vaccination may also be a secondary barrier. Effective interventions that inactivate viruses are listed in Table 14.2. In addition, two reviews on the effects of water and sanitation on enteric illness morbidity list several interventions (e.g. good water quality, hand-washing, safe excreta disposal) that could interrupt the fecal–oral transmission route.[60,61]

Fecal–oral transmission of hepatitis A and NoV is influenced by various risk factors already discussed above. In addition, hepatitis A and NoV transmission may persist because of asymptomatic infections. In the case of hepatitis A, asymptomatic individuals may shed large amounts of virus in feces 1–2 weeks before symptoms.[62,63] In the case of norovirus, asymptomatic individuals may shed virus in feces for as long as 3 weeks and transmit the disease unknowingly.[25–27]

14.4.2 Food contamination during growing, harvesting, and postharvest

The significance of contamination of produce during growing and harvesting is unknown because once an outbreak occurs, it is often difficult to trace the product back to a definitive source of contamination (reviewed in Seymour and Appleton[5] and Richards[6]). It is probable that virus contamination usually occurs during harvesting or during subsequent postharvest handling steps. Transmission is thought to occur mainly by surface contamination and not by uptake of viruses within damaged plant tissues during growth. Produce may become contaminated with human viral pathogens by coming into contact with inadequately treated sewage effluent, sewage sludge or human feces (as fertilizer) or otherwise contaminated irrigation water. Contamination may also occur through handling of produce by workers during harvesting.[64] There is an increased risk of contamination from workers with inadequate sanitation facilities or hygiene practices. Some outbreaks have been linked to infected field workers.[58,65] Some reports suggest that for specific produce items, handling of produce in the packing sheds increases the levels of indicators (*E. coli*) of fecal contamination.[66–68] For shellfish, contamination is rarely due to manual handling of the shellfish, but instead is due to fecal contamination of shellfish harvest waters from sewage or boat discharge.[69]

14.4.3 Food contamination during food processing

Contamination of foods can arise during processing, storage, or distribution either by contact with a contaminated environment or directly from infected people. Water must be of sanitary quality when used in food processing (such as for washing or icing) or when used as an added ingredient in food, for use in manufacturing ice, or for washing foods. As previously mentioned, food handlers must be provided with adequate sanitation facilities and training in good hygiene.

Both noroviruses and hepatitis A virus are persistent in the environment and easily transferred from surfaces to hands to other surfaces. Foods are rarely contaminated with viruses during food processing, although products served raw or only lightly cooked would be at greater risk of transmitting viruses arising from either pre- or postharvest sources. Furthermore, processing of some products, such as shellfish, may not inactivate viruses. For example, since viruses are sequestered in the gut of the mollusk, viruses may be protected due to inadequate heat.[70,71] Some NoV outbreaks have been associated with cooked oysters.[72] The effect of temperature on hepatitis A and norovirus is described in Table 14.2.

14.4.4 Food contamination during preparation

RTE foods are the main cause of foodborne illness. RTE foods are often contaminated by food handlers. Food handlers play an important role in transmission of foodborne viruses; a recent review suggested they were the most common source of viral contamination of food.[73] Food handlers generally cause single strain outbreaks. Food handlers may themselves be infected and contaminate food or may be uninfected but have contaminated hands (e.g. contact with sick relatives, working with contaminated surfaces) and go on to contaminate food. Both hepatitis A virus and norovirus may induce an asymptomatic infection with variable amounts of viral shedding. Viral shedding may occur over days and weeks for both viruses. For these reasons, it is important to prevent sick and infected food handlers from working with food for a predetermined period of time (usually 2–3 days). It is also important to institute appropriate hygiene practices and sanitary conditions when working with food (e.g. frequent hand-washing, use of gloves). Poor personal hygiene, and to a lesser degree, unsafe food source, are the most commonly identified factors associated with norovirus and hepatitis A foodborne outbreaks.[74,75] While any food handled by ill food handlers may become contaminated, certain foods have been historically associated with outbreaks including: salads, raw fruits and vegetables and bakery products (reviewed in Richards[6] and Guzewich and Ross[73]). Cross-contamination of processed products by contaminated surfaces, foods (especially raw foods), and utensils is another important risk factor for outbreaks (reviewed in Rooney *et al.*[76]). An example of an outbreak caused by food contamination during preparation can be found in Case study 14.2.

Even though special emphasis has been placed on food handlers as a risk factor for foodborne outbreaks, it is worth repeating that viral contamination of foods may occur at any point in the chain from farm to fork.

14.5 Aspects of virulence

14.5.1 Physical factors

Persistence in the environment

Norovirus and hepatitis A viruses are stable in the environment. Noroviruses have been shown to persist over 2 weeks on surfaces, and hepatitis A virus has

Case study 14.2 Cake frosting causes outbreak in Georgia, USA

In February 2000, the Georgia Department of Agriculture, a regulator of grocery stores, received complaints of gastrointestinal illness from several consumers attending events where only cake was served.[175] The Georgia Department of Public Health (GDPH) initiated an epidemiological investigation and recovered receipts of cakes purchased on the same day from the same grocery store bakery. This bakery prepared 85 cakes linked to at least 32 outbreaks with an estimated 1000 cases. The GDPH interviewed 196 persons from 29 different social gatherings that served cake or puffed rice bars with the same frosting. Of those interviewed, 123 met the case definition for primary gastroenteritis and reported vomiting (84%), nausea (90%), diarrhea (82%), and/or abdominal cramps (78%). About 24% of cases sought medical care and two were hospitalized. The overall attack rate among the population attending events serving the frosting was 85%. The median incubation period was 31 hours and average duration of symptoms was 2 days. Laboratory assays identified the same strain of norovirus in 15 cases but could not detect norovirus in the implicated frosting samples. One of the two individuals preparing frosting was ill at the time of frosting preparation. The cake decorators did not wear gloves and the ill individual had long artificial fingernails which may have harbored norovirus. This outbreak reinforces the need for good worker hygiene, furloughing of workers when ill, promoting the use of gloves and discouraging long nails when handling RTE foods.

been shown to persist over 1 month in dried feces (Table 14.2). In addition, these viruses are highly resistant to acids, probably because they need to survive in the gastrointestinal system that has regions of high acidity. Viruses may persist on various materials commonly used in institutional settings and during food preparation, including cotton, paper, aluminum, china, glazed tile, latex, and polysterene.[77] The exact mechanisms of viral persistence in the environment are unknown, but it is hypothesized that the absence of an envelope, their small size, and capsid configuration contribute to their hardiness.[5,7] Factors that can influence the stability of these viruses in the environment include humidity, degree of drying of viral medium, type of viral medium (fecal, water, etc.), virus type (perhaps strain), and the type of surface contaminated (including hands) (reviewed in D'Souza *et al.*[46]).

Resistance to inactivation

Both norovirus and hepatitis A viruses are resistant to multiple common disinfectants listed in Table 14.2. The methods that are most effective at reducing virus levels include UV light, ozone, thermal inactivation, chlorine, and hypochlorite (bleach). However, the efficacy of these disinfectants may be inhibited by the food matrix. For example, shellfish protect hepatitis A virus

from thermal inactivation.[70,71] Although UV is a good virucidal agent, it does not penetrate foods and can be used only for surface inactivation. Some virucidal agents may not be used on certain foods because they may affect their form or taste, or introduce toxic substances. Several groups have also determined that high hydrostatic pressure processing, a novel way to decontaminate shellfish, may be effective at reducing levels of hepatitis A virus on shellfish.[78,79] High hydrostatic pressure processing is also effective at reducing levels of feline calicivirus, a commonly used surrogate for norovirus.[78] The effect of this treatment on noroviruses is unknown. Lastly, hand-washing is effective at reducing levels of hepatitis A virus and feline calicivirus on hands.[80–83] Hand-washing is effective at reducing levels of norovirus but may not be relied upon to eliminate the virus, particularly if fecal material is trapped under fingernails.

14.5.2 Epidemiologic factors

Norovirus

Noroviruses are prevalent worldwide. The burden of disease is unknown, but it is hypothesized that over 90% of adults have been exposed to norovirus. This hypothesis is based on antibody acquisition data indicating that antibody prevalence to norovirus is lowest in children 0–5 years old and increases with age. In industrialized countries such as the United States and the United Kingdom, antibody prevalence ranged from undetectable to 20% in the 0–5 year old age group to 65–70% in the 11–15 year old age group.[84–87] In developing countries such as Bangladesh and Ecuador, antibody prevalence ranged from 75 to 100% in the first 5 years of life and remained in this high range throughout childhood.[84,85] Based on RT-PCR analyses of stool specimens, several cohort studies have identified NoV prevalence rates of 7–20% in developing countries among symptomatic and asymptomatic children.[12,88,89] It is important to note that these studies are preliminary and antibody acquisition data will differ from RT-PCR results because of the assay measurement, sensitivity, and specificity. These differences suggest higher rates of exposure in developing countries than industrialized countries perhaps due to differences in water quality, sanitation and hygiene practices. Children may be reinfected several times with different strains of virus in a short period of time.[12]

Noroviruses are divided into five different genogroups, three of which cause human disease: genogroups I, II, and IV. Genogroups I and II are most commonly associated with human disease. Genogroup IV, while associated with human disease, is rarely found.[90] Each genogroup has multiple strains categorized by phylogenetic analysis (based on either polymerase or capsid sequences) into prototype clusters. In 2004, genogroup I had over 8 prototype clusters and genogroup II had 17 prototype clusters (CDC, personal communication). New strains of NoV are routinely identified, and therefore this number is expected to increase. Among the genogroup II viruses, strains in the GII/4 cluster are most commonly associated with outbreaks in the United States and Europe. Certain NoV strains may appear in a region, predominate among outbreaks, and later be

replaced by other strains. It is hypothesized that different strains have different predominant transmission routes, infectivity, and clinical manifestations. We speculate that virus strains differ in other virulence aspects including longer shedding times, shedding higher titers, and longer persistence in the environment. In addition, host determinants of resistance and susceptibility (genetic and immunologic) are probably different for each strain (reviewed in Hutson *et al.*[90]). The genetic differences among NoV strains create challenges for the development of a pan NoV vaccine (reviewed in Hutson *et al.*,[90] Matsui and Greenberg,[91] Tacket *et al.*,[92] Estes *et al.*,[93] and Tacket[94]). It is possible that NoV vaccine development may follow the influenza vaccine model, in which yearly flu vaccines are developed for the predominant strains.

Surveillance data from Europe and the United States indicate that novel virulent NoV strains may emerge and cause pandemics. Why pandemics are caused by certain strains and not others is not understood. For example, in 1995 to 1996, a norovirus strain, belonging to genogroup II/4, was detected in the United States and spread throughout South America, Europe, Asia, and Australia.[95] In 2002, the European surveillance network detected a rise in norovirus outbreaks among ten European countries caused by the emergence of a new genogroup II/4 norovirus strain.[96] This strain had a consistent mutation in its polymerase region that set it apart from other genogroup II/4 strains detected in Europe. The factors that contribute to norovirus genetic diversity are also not understood. Several groups have suggested that within-genogroup recombination gives rise to hybrid strains capable of causing outbreaks in human populations. For example, the strain Arg320 was suggested to be a recombinant of Lordsdale (II/4) virus and Mexico virus (II/3).[97] In summary, noroviruses are highly virulent and have broad genetic diversity, and these features contribute to their epidemic and pandemic spread across the world.

Hepatitis A virus

Hepatitis A virus is present worldwide and may cause sporadic and epidemic disease. Different rates of prevalence and endemicity are observed in various world regions. In the United States, one-third of the general population has serologic evidence of prior hepatitis A infection; while in some parts of southeast Asia, over 90% of the general population has serologic evidence of infection.[33] In developing countries, hepatitis A is endemic, and most individuals are infected in early childhood without symptoms. A single hepatitis A infection renders immunity to the virus. Because of this, in developing countries, most adults are usually immune, and epidemics of hepatitis A are uncommon. As socioeconomic or sanitation conditions improve, the prevalence of hepatitis A infections decreases, and the average age of reported cases increases because people are more likely to be exposed later in life and have symptomatic infections. In industrialized countries, transmission is largely person-to-person (fecal–oral), but large common source outbreaks can occur. Hepatitis A infection often occurs in crowded institutions such as schools, prisons, and the military. Disease transmission is frequent among households, sexual contacts of acute cases,

travelers to endemic countries, injecting drug users, and men who have sex with men. Foodborne outbreaks have been reported in most parts of the world except those with the highest hepatitis A endemicity. In outbreaks, up to 20% of cases are due to secondary transmission of hepatitis A (reviewed in Koopmans *et al.*[8]).

Only one serotype of hepatitis A virus has been found of which the antigenicity is determined by an immunodominant epitope. Four distinct genotypes of hepatitis A virus have been identified in humans. These four genotypes have no important biologic differences, and all belong to the same single serotype. The hepatitis A vaccine or infection confers lifelong immunity to the virus. In the United States, increased hepatitis A vaccination has decreased the incidence rate by 76%, between the baseline period (1990–1997) and 2003.[98] In 2003, rates in the United States were 2.6 for every 100 000 individuals (61 000 new infections in 2003) compared with 1992, when rates were 9.1 for every 100 000 individuals (288 000 new infections in 1992). Hepatitis A vaccinations and immunoglobulins are also effective interventions in hepatitis A outbreak situations and may shorten outbreak duration.[62,99]

Infectious dose

An exact determination of the infectious dose of viruses in humans is difficult because conducting necessary investigations requires human challenge studies. For hepatitis A, it is believed that 10–100 virus particles are sufficient to infect an individual. For norovirus, 10 RT-PCR detectable units, or approximately two genome copies, are sufficient to infect an individual (Moe C, *et al*. unpublished). In these studies, volunteers were infected with three different strains of noroviruses. Challenge with different NoV viruses induced different dose–infectivity curves and dose–illness curves (Moe C, *et al*. unpublished). These results suggest that individual NoV strains may have different infectivity and severity.

14.5.3 Host factors

Host factors important to transmission and infection with foodborne virus include both genetic and immunologic resistance and susceptibility. For additional information on host response to viral infection, please see Chapter 15.

Norovirus

Genetic determinants of host susceptibility to NoV infection

More than 25 years ago, Parrino *et al*. conducted a Norwalk virus (NV, a specific strain among NoV) challenge study in volunteers and reported that a subset of volunteers was repeatedly susceptible to NV infection, whereas a second subset was repeatedly resistant to infection.[100] It was hypothesized that a genetic factor, possibly a receptor, could affect an individual's susceptibility to NV infection. Our group recently reported that a member of the ABO histo-blood group family, the *FUT2* allele, conferred susceptibility to NV infection.[101] Individuals who were homozygous recessive for the *FUT2* gene are considered secretor negative[102] and did not become infected regardless of NV dose. In

addition, saliva samples from these individuals did not bind to recombinant NV virus-like particles. The *FUT2* gene encodes an α-(1,2)-fucosyltransferase that produces the carbohydrate H type 1 found on epithelial cells and in mucosal secretions.[103] In addition, individuals of the B blood group are more resistant to NoV infection while those of the O blood group are more susceptible to NoV infection.[101,104] Together, these data suggest that the ABO histo-blood group components, including *FUT2*, may be necessary for NV binding and infection and are therefore important genetic determinants of susceptibility to NV infection. These genetic components apply only to specific strains of NoV, and other NoV strains may infect individuals who are 'genetically resistant' to one strain of NoV (reviewed in Hutson *et al.*[90]).

Immune determinants of host resistance to NoV

The mechanisms of immunity to NoV are not known (reviewed in Hutson *et al.*,[90] and Matsui and Greenberg[91]). Early NV challenge studies suggested that short-term immunity to homologous virus protected from subsequent infections. Volunteers that became ill after NV challenge were re-challenged with homologous virus between 6 and 14 weeks after the initial exposure and did not become ill a second time.[105–107] Interestingly, long-term immunity to homologous virus did not protect from subsequent infection or illness. Volunteers that became ill after NV challenge were re-challenged with homologous virus between 24 and 42 months, and they all became ill again.[100] Four of these volunteers were given a third inoculum between 4 and 8 weeks after the second exposure. Three volunteers did not become ill. This result confirms the previous report that short-term immunity can protect from illness. In addition, other evidence indicates that short-term immunity may sometimes protect from re-challenge with a different virus.[105] The mechanisms or components of this short- and long-term immunity are unknown.

Humoral and cellular immunity to NoV

Humoral immunity may or may not be protective against NoV infection. Various reports contradict each other in stating that pre-existing anti-NV serum antibodies are associated[108–110] or not associated[26,111–115] with protection from NV infection. This contradiction may be due to differences in host populations, infectious NoV strains or the presence of host genetic confounders. Our results indicate that a portion of genetically susceptible individuals (secretor-positive) was resistant to infection, suggesting that an anamnestic (memory) immune response or other mechanism also protected from NV infection.[101] This uninfected group exhibited a rapid rise in anti-NV salivary IgA titer *before* 5 days post-challenge. In contrast, the infected group exhibited a rise in anti-NV salivary IgA titer only *after* 5 days post-challenge. The rapid immunological response to NV challenge in secretor-positive, uninfected individuals suggests that acquired immunity may explain the difference in those secretor-positive volunteers who developed infection after challenge and those who did not. To date, there are few reports on cellular immunity induced by human norovirus

infection[116] and there is a great need to understand NoV-specific cellular and memory immune responses in order to design effective vaccines.

Hepatitis A virus

Only one serotype of hepatitis A virus has been found of which the antigenicity is determined by an immunodominant antigenic site. A single hepatitis A virus infection renders immunity against the virus for the lifetime of the individual. Once an individual is infected with hepatitis A virus, they cannot be reinfected.

The immune system, and not cytopathic damage by the virus, is thought to be responsible for disease. Large quantities of infectious virus are produced in the liver and excreted in the stool before any symptoms appear in individuals.[36,117] Hepatitis A virus is not directly cytopathic in cell culture but instead is associated with persistent infection without cell damage.[36,118] These observations suggest that the virus does not directly cause damage in the host but instead that immune-mediated injury contributes to hepatic inflammation and destruction.

Humoral immunity to hepatitis A virus

Infection with hepatitis A virus induces circulating immune complexes with associated symptoms in some patients. These complexes include IgM, IgG, and IgA antibodies directed against conformational epitopes on the virus. In addition, the level of total IgM increases in acute hepatitis A infections and is a useful marker of acute disease.[36] The majority of cases have detectable levels of IgM at 5–10 days post-exposure.[33] Levels of IgM readily decline, and after 6 months, 75% of patients have no detectable IgM antibodies left.[119] IgA is also produced for a limited period of time, but its role in immunity is uncertain. Induction of IgG antibodies is delayed compared with the appearance of IgA and IgM antibodies. However, IgG antibody levels are long-lived (over 50 years in some documented instances[120]) and are responsible for protection from infection. The antibodies are usually directed against three viral proteins though antibodies to other nonstructural proteins may also be observed. Differences in antibody recognition of these viral proteins can distinguish between active infection and vaccination.[34]

Cellular immunity to hepatitis A virus

The cellular immune response is likely involved in viral clearance. In addition, it is hypothesized that the cellular response is involved in hepatic inflammation and damage. Evidence in support of this hypothesis includes reports that lymphocytes isolated from patients with acute hepatitis A infection can lyse hepatitis A infected cells.[121–123] Other cells, such as natural killer cells and memory cells, may also be involved in host response to hepatitis A virus infection.[121,124–126]

In summary, individuals will have varying degrees of susceptibility to hepatitis A and norovirus based on genetic and immunologic predisposition and the strain (for norovirus) they are exposed to.

14.6 Implications and future studies

14.6.1 Implications for prevention

Contamination of foods by enteric viruses usually occurs through fecal–oral transmission routes. Within the fecal–oral transmission routes, the manual handling of foods is the most significant contributor to foodborne disease. Therefore, the most straightforward measures to reduce the probability of food contamination and resulting outbreaks are to implement strict sanitation and hygiene protocols (e.g. emphasize hand-washing), agree on rules for the furloughing of sick food handlers and agricultural workers, provide education on viral transmission and appropriate reporting to health facilities, vaccinate all food handlers against hepatitis A virus, and enforce appropriate barriers of protection from foods (e.g. gloves and hair nets). Nonetheless, for a variety of reasons, these measures are difficult to implement, and lack of compliance remains an important issue.

At a national and international level, foodborne disease outbreak surveillance systems have been implemented in both the United States and Europe. Continued support and expansion of surveillance activities would increase the probability that foodborne disease and outbreaks can be detected and contained more rapidly. Europe currently has an excellent foodborne outbreak surveillance system. This surveillance system includes surveillance for foodborne viruses, such as norovirus and hepatitis A. This system unites 9 countries and 11 groups to standardize laboratory detection of these pathogens, implementation of standardized reporting databases, common definitions of outbreaks, among several activities. For additional information on the Foodborne Viruses in Europe Network, please refer to the report published by the European Commission entitled 'Food-borne Viruses in Europe' describing these activities.[127] Support and implementation of these surveillance systems would help to identify the most likely avenues of contamination, responsible virus strains, and tracking of virus spread both geographically and temporally.

Contamination during growing, harvesting, and postharvest

For agriculture, research is needed to document specific practices and risk factors for viral contamination of produce. This information can then be used to conduct formal risk assessments or design of preharvest control strategies, perhaps even HACCP analyses (Hazard Analysis and Critical Control Point). The current guide, published in 1998, entitled 'Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables' identifies the recommendations by FDA and USDA on practices for the production of safe produce.[128] These guidelines are general and provide little guidance for prioritizing specific practices. Unfortunately, at this time the agricultural community does not have a firm understanding of the role of specific risk factors for produce contamination (e.g. unsanitary equipment, contaminated workers' hands, policies for sick workers, children and animals in crop fields, among others). Once these data are obtained, they can be incorporated into good agricultural practices (GAP) guidelines. Produce organizations can work with researchers and public health officials to identify high-risk practices, develop

guidelines to prevent produce contamination, and help enforce guidelines for cleaner, safer produce.

The current USDA/FDA guide proposed several recommendations on maintaining agricultural water quality and produce cleaning and disinfection. Unfortunately, washing decreases but cannot be relied upon to eliminate foodborne pathogens. Several reviews have focused on different washing protocols and reagents.[5,6,46,129] In general, of all the currently used disinfectants, chlorine, hypochlorite (bleach), and ozone are the most effective at reducing levels of bacteria and foodborne viruses on produce (Table 14.2).[46,129] Depending on the concentrations of these disinfectants, they may negatively affect the shape and taste (organoleptic properties) of produce. These disinfectants, especially hypochlorite, can also be used to decontaminate equipment surfaces. Produce contamination by workers' hands remains a concern, and hand sanitizing recommendations are described in the 'Contamination by hands' section below.

For the shellfish industry, contamination is rarely due to manual handling of the shellfish, but instead is due to contamination of shellfish harvest beds from sewage overflows, septic tank leakage, water run-off, or boat discharge. For this reason, practices to prevent shellfish contamination are different from those for other food items. Unfortunately, assays to detect viral contamination of shellfish are complex and require a research laboratory. There are also no commonly accepted technologies for viral inactivation in shellfish. Therefore, until further progress is made to develop reliable detection assays and virus inactivation practices, the shellfish industry should continue to make sure that shellfish are collected from waters with minimal fecal contamination. All individuals, but particularly those with specific health risk factors, such as the immunocompromised, elderly, or pregnant, should be made aware of the risks associated with the consumption of raw molluskan shellfish. At this time, high hydrostatic pressure processing shows promise as a method to inactivate viruses in shellfish, but further work is needed to establish its efficacy and then to transfer the technology to the shellfish industry.

Contamination during food processing

For the food processing industry, many of the recommendations outlined above would also apply to postharvest handling (good sanitation, hand-washing, communication with public health officials, etc.) and are frequently a part of the general good manufacturing practices (GMP), good hygienic practices (GHP), and HACCP plans used routinely by industry. Industry must maintain educational initiatives, and assure compliance with personal hygiene recommendations to assure that viral contamination during food processing remains a rare occurrence.

Contamination during food preparation

For the food service industry, effective and simple measures to prevent virus transmission would include defining appropriate hygiene protocols (including food barriers such as gloves) and emphasizing good hand-washing by all employees, implementing required furlough (with pay) of ill food handlers,

providing ongoing education about the causes of food contamination and pathogen transmission, and maintaining open channels of communication with public health officials to report and identify foodborne outbreaks. The food service industry may want to consult the 2005 edition (updated every four years) of the Food Code. This Code is published by the FDA, USDA, and CDC and is a reference manual for regulatory agencies that ensure food safety in food service establishments, retail food stores, other food establishments at the retail level, and institutions, such as nursing homes and child-care centers. This Code has evidence-based, practical recommendations for addressing risk factors associated with foodborne illness. In addition, mandatory vaccination (i.e. hepatitis A vaccination) of all food handlers would also reduce the risk of foodborne transmission of this virus. For example, St. Louis, Missouri, and the state of Nevada require all food handlers to receive hepatitis A vaccination.[130] Mandatory hepatitis A vaccination of food handlers is a debated topic.[35,131,132] An example of an outbreak caused by contamination during food preparation is provided in Case study 14.3.

Case study 14.3 Gastroenteritis at a university in Texas, USA

In March 1998, the Texas Department of Health received a call reporting gastrointestinal illness in a student and his roommate at a university.[176,177] Subsequent calls to local health facilities revealed 23 ill students at a local hospital and 20 ill students at the student health center in the previous day. Over several days, 125 students sought treatment at either health facility, and 65 were interviewed and reported vomiting (91%), diarrhea (85%), headache (66%), myalgias (49%), bloody diarrhea (5%), and abdominal cramping (68%). The mean duration of illness was 2 days. Illness was associated with eating at the deli bar on two days. Investigation of the deli bar revealed deficient food handling practices. One of the deli food handlers initially chose to be suspended from her job rather than to be interviewed and submit a stool sample. This food handler later reported slicing ham and serving sandwiches on the two implicated days while wearing gloves. Since she wore gloves, she felt there was no need for hand-washing. She denied having gastrointestinal illness during the outbreak period but reported that her infant had been sick with watery diarrhea. Laboratory analysis revealed an identical norovirus strain in nine ill students, one deli ham sample, and the stool of the ill infant. It is likely that the food handler was the source of the outbreak and indirectly transmitted norovirus from her child to the students. This outbreak reinforces the need for careful food hygiene practices (especially hand-washing) and care to educate food handlers on hygiene when around sick individuals. Of interest, is the finding that the source may have had an asymptomatic infection and/or was an indirect carrier of norovirus.

Contamination by hands

Because contamination of foods by hands is the common denominator for many cases of foodborne viral contamination, we describe it in its own section. Hand contamination may be addressed by regular hand-washing practices, but hand disinfectants may also help. Several studies have examined the effect of hand disinfectants on the inactivation of enteric viruses (reviewed in Richards,[6] and D'Souza *et al.*[46]). In general, alcohol and chlorhexidine gluconate-based hand disinfectants are not effective at reducing the levels of foodborne viruses.[80] Hand-washing with soap reduces levels of viruses but does not always eliminate them. A survey of several soaps found that soaps with Triclosan, a chlorophenol, were most effective at reducing hepatitis A virus levels.[80] Additional work is needed in this area to identify effective hand sanitizers against hepatitis A virus, norovirus, and other foodborne viruses. An example of an outbreak caused by contamination by hands is provided in Case study 14.3.

14.6.2 Environmental virology lessons learned

Environmental virology methods have been invaluable in the investigation of foodborne disease outbreaks and have contributed to our understanding of foodborne transmission of enteric viruses. Specifically, environmental virology methods have been helpful for:

- identifying *where* viruses occur in the food production environment (e.g. shellfishing waters and sediments);
- examining viral *persistence* in the food chain, including production, processing, and preparation;
- testing the *efficacy* of various disinfectants and treatment processes against viruses on foods and in the food chain, including production, processing, and preparation.

The overall limitation of environmental virology methods is that they are too complex and time-consuming for routine screening of viral contamination in food and other environmental matrices. In addition:

- effective virus detection methods still need to be developed for many complex food matrices;
- detection methods for both hepatitis A virus and norovirus still need to be simplified and optimized for improved sensitivity;
- molecular detection methods for hepatitis A virus and norovirus are limited because they do not necessarily indicate infectivity.

14.6.3 Future challenges and research

The two main future challenges for controlling foodborne disease are: (1) increased worldwide movement of food commodities and therefore increased opportunity for the spread of foodborne pathogens, including viruses; and (2) the emergence of new virus strains (especially norovirus).

To address the present and future challenges of foodborne viral diseases, multiple research avenues must be pursued. We list here only a few of the many research needs.

- For virus detection, we need easier and more effective assays that can detect foodborne viruses in various types of food matrices (e.g. produce, salads, and shellfish) and in the environment (irrigation waters, shellfishing waters). These assays must be sensitive enough to detect viral contamination at levels anticipated in naturally contaminated products, and hence will require improvements in upstream sample concentration and better virus extraction prior to detection. Since direct detection of foodborne viruses on foods is currently difficult and expensive, the food industry would benefit from the identification of appropriate viral indicators that can be economically measured and applied for widespread screening.
- We also need to continue to develop simple model systems to assess the infectivity of human norovirus, since we are currently limited to human challenge and epidemiology studies.
- Because NoV foodborne outbreaks continue to occur even in industrialized countries with high levels of hygiene, water, and sanitation, improved environmental conditions are probably not sufficient to eliminate foodborne transmission of NoV. An effective vaccine against noroviruses, even if used only for specific high-risk groups, could greatly reduce transmission and prevalence of epidemic gastroenteritis.
- Improved virus inactivation technologies and products would help control the transmission of enteric viruses and assure the safety of our foods. These technologies and products should be targeted towards inactivation of viruses on different food processing and preparation surfaces, in different food matrices (e.g. produce, shellfish), and on hands.
- We also need additional data on (1) the duration of viral excretion by infected individuals; (2) the persistence of viruses on surfaces, and in various foods and water; and (3) the specific food handling practices associated with viral contamination. These data can then be used for quantitative risk assessments and to provide guidance to regulatory agencies and the food industry (e.g. HACCP).

Lastly, national and international entities should continue to collaborate, develop, and maintain global surveillance systems to track and identify viral foodborne outbreaks. With increased trade and movement of food commodities across the globe, it is important to develop systems to better identify and contain foodborne outbreaks irrespective of state and national borders.

We have come a long way in our management and prevention of foodborne diseases. With continued advancements in science, and cooperation between regulatory agencies, the food industry, and academic institutions, we can continue to move forward in our protection of the safety of the global food supply.

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15

Pathogenic mechanisms of foodborne viral disease

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15.1 Introduction

Infection of a host with a virus unleashes various defense mechanisms based on the induction and secretion of cytokines, necessary to activate both innate (interferons, other cytokines) and adaptive immune responses to control and eliminate the virus. Our understanding of the dynamics of host–virus interaction has been greatly augmented by the ability to culture animal cells outside the organism,[59,269] and the ability to infect such cultured cells with viruses, first demonstrated with poliovirus.[326] The outcome of a virus infection is predicated upon the nature of these defense mechanisms and the ability of the infecting virus to block or evade such antiviral mechanisms. Depending on these factors, predicted outcomes of a virus infection can be the death of the cell or organism due to unrestricted viral replication; effective inhibition of virus replication resulting in the survival of the cell or organism; or the establishment of a persistent or latent infection with a limited amount of viral replication or viral gene expression, until some event disturbs the balance in favor of the virus.

Pathogenicity of a virus is frequently not the result of the toxic effect of a particular viral function on host cell. Programmed cell death (also know as apoptosis) is often induced by the host to eliminate the infected cells, whereas the virus may trigger apoptosis to facilitate virus spread and to circumvent the host immune response.[17,268] Certain cells of the immune system, such as cytotoxic T cells and natural killer (NK) cells, also mobilize to recognize and bind virus infected cells, and induce apoptosis. These virus and cell mediated mechanisms result in damage to infected organs, although the host organism may survive.

15.2 Factors contributing to the pathogenicity of viral foodborne diseases

The pathogenicity of foodborne viruses depend on both the stability of the virus in the environment, as well as virus–host interactions at several different levels. The route of entry into the organism, mechanism of virus spread, site of replication, effects of virus infection on cells, and the adaptive and innate antiviral responses all play roles in determining the pathogenicity of the virus. Recent studies show that the nutritional status of the host also contributes to the ultimate pathogenesis of the virus.[22,23,96] The presence of virus receptors at the cell surface determines virus susceptibility, and is genetically controlled.[181,234,323] A genetic component for susceptibility to human norovirus (NoV) infection is also suggested by the recent finding of cell surface receptors for this virus.[157,199] The role of the JAK-STAT pathway of interferon signaling during the replication of rotavirus, hepatitis A virus (HAV) and mouse norovirus (MNV) also point to the critical role played by the genetic background of the host in foodborne virus infections.[126,187,263,337]

15.2.1 Nature of the pathogens

Most foodborne viruses belong to the picornavirus, calicivirus, and reovirus families (Table 15.1). The total number of illnesses caused by these viruses has been estimated to be upwards of 30 million cases per year in the United States. However, most estimates indicate foods as a primary source of infection in only 5–6% of the incidences.[75,186,221] For reasons to be discussed in Section 15.6, direct demonstration of the presence of viruses in foods implicated in foodborne outbreaks have been achieved only in a few instances.[125,182,195] In terms of sheer numbers, Norwalk virus (NV) within the genus norovirus (NoV) is responsible for the vast majority of foodborne illnesses in the United States, followed by astro- and rotaviruses.[221] Hepatitis A virus comes in at a distant fourth, and the numbers have dropped somewhat following the development of an effective vaccine.[12] However, like many enteric viruses, the number of asymptomatic infections is high, and the reported cases may not reflect the actual number of infections. Asymptomatic individuals excrete the virus in the feces and are capable of spreading the virus via person-to-person contact, as well as through contaminated foods.[186] Poliovirus (PV) infections have been eradicated in most industrialized countries but remain endemic in some developing countries.[66] Circulating vaccine-derived poliovirus (cVDPV), however, may be of concern to non-immunized populations.[43,180] Hepatitis E virus (HEV), was once thought to be mainly a waterborne disease in the third world; however, many industrialized countries including the United States, Japan, and countries in the European Union have recently reported sporadic HEV infections from farm and game animals.[100] Particularly intriguing are reports from Japan that people who consumed undercooked meat from wild boars and deer have contracted the disease.[300]

These reports raise the question whether other viruses, such as avian influenza or hantaviruses not normally associated with foodborne outbreaks,

Table 15.1 Viruses transmitted by food or water

Virus species	Classification	Disease
Norovirus	Calicivirus	Gastoenteritis
Poliovirus	Picornavirus	Paralytic poliomyelitis
Coxsackievirus (Human enterovirus A–C)	Picornavirus	Gastroenteritis Respiratory infections Juvenile diabetes Meningitis Myocarditis, pericarditis
Echovirus (Human enterovirus B)	Picornavirus	Gastroenteritis Respiratory and skin infections
Hepatitis A virus	Picornavirus	Hepatitis
Hepatitis E virus	Unclassified	Hepatitis
Astrovirus	Astrovirus	Gastroenteritis
Rotavirus	Reovirus	Gastroenteritis
Adenovirus 40,41	Adenovirus	Gastroenteritis

Additional details regarding taxonomic classification can be obtained at www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm.

also could spread by contaminated foods. Mortality due to foodborne illnesses remains low in the healthy immunocompetent population, but increased mortality due to HAV infection is observed in immunocompromised people,[186] and for unknown reasons, among pregnant women infected with HEV.[218] Rotavirus remains a leading cause of infantile gastroenteritis and infant mortality in the third world.[57] At present, there are no effective vaccines for HEV, rotavirus, and norovirus. Foodborne infections due to coxsackievirus (CV) or echovirus (EV) are less frequent.

15.2.2 Viral genomes

The genomes of representative members of all categories of foodborne viruses have been cloned and sequenced.[13,78,164,169,184,189,232,302,311] With the exception of rotavirus (family Reoviridae), which is a double-stranded RNA (dsRNA) virus with a segmented genome, the genomes of major foodborne viruses are single-stranded RNAs of positive polarity (i.e. viral genome is mRNA). The replication of RNA viral genomes are error-prone owing to the lack of the proof-reading ability of RNA viral replicases; thus RNA viral genomes are present in infected cells as quasispecies.[72,129] A quasispecies is defined as a collection of multiple sequences of a single strain, each sequence differing from the other by a few nucleotides. As shown in Fig. 15.1, the structural organization, gene expression, and replication strategies of the two major groups of foodborne viruses, namely picorna- and caliciviruses are similar but not identical.[24,55,193,291]

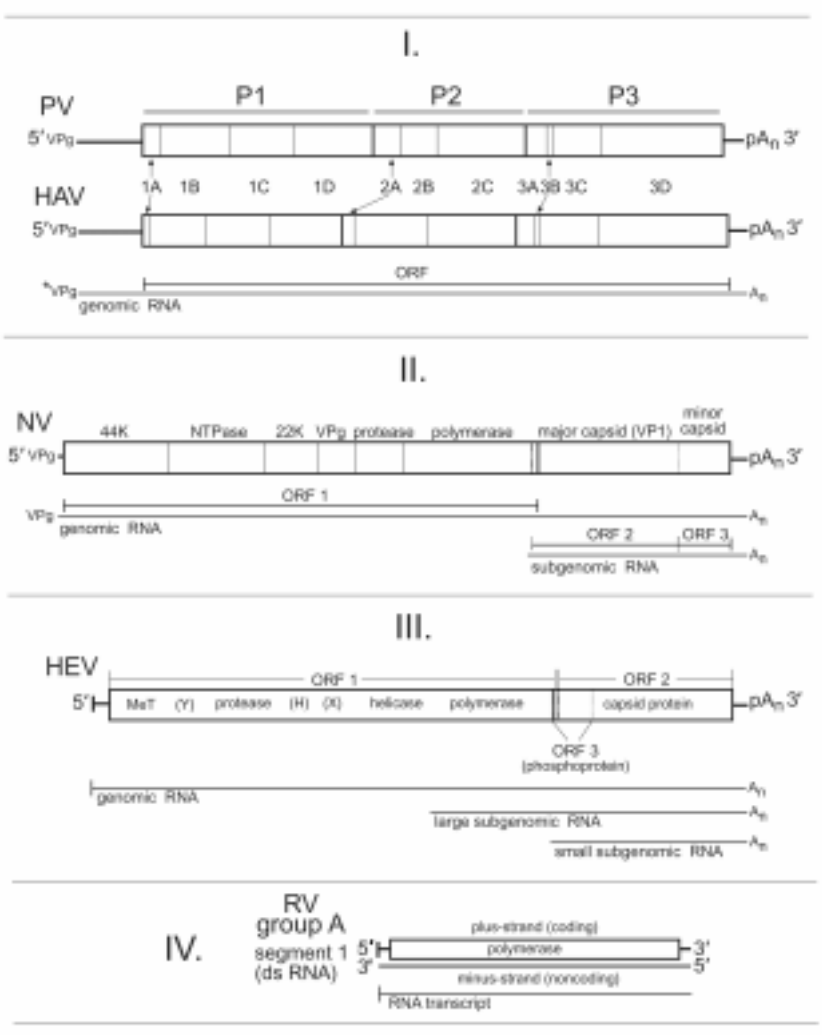

Fig. 15.1 Diagrammatic comparison of selected genomes of major foodborne viruses. Comparison of representative picornavirus species (panel I), poliovirus (PV) and hepatitis A virus (HAV); norovirus species, Norwalk virus (NV) (panel II); hepatitis E virus (HEV) (panel III) and rotavirus (RV), group A (panel IV) are illustrated.

Information derived from published materials was used to generate Fig. 15.1 and the references cited herein may be referred to as a guide for obtaining further details regarding genome organization and expression of the various encoded proteins.[9,86,104,131,150,164,184,238] The open boxed regions represent the coding regions for the respective genomes. Vertical demarcations within the boxed regions indicate the amino- and carboxy-terminal boundaries of fully processed viral proteins. The relative locations of the proline-rich hinge region (H), X and Y domains (X and Y in parentheses) encoded by HEV ORF1 are also

indicated. The significance of stippled versus solid demarcations is discussed further below. The 5′ and 3′ untranslated regions (UTRs) are shown as solid lines and the relative differences in nucleotide length of the UTRs among the various genomes is schematically illustrated by the differences in length of the line. Beneath each genome, horizontal lines define the genomic and subgenomic RNAs and delineate the respectively encoded open reading frames (ORFs), in order to highlight the differences that exist among the virus groups regarding the number, size, and genomic location of the viral genome/transcripts utilized by these viruses to produce their respective viral proteins. Panel I–III viruses have polyadenylated genomes. Their genome is the primary viral transcript used for initial viral protein translation upon host cell infection. Group I viruses encode a single polyprotein identified by one ORF, whereby capsid (i.e. structural) proteins are derived from the P1 region and nonstructural proteins (e.g. protease, RNA polymerase) are derived from the P2 and P3 regions. Group II and III viruses produced nonstructural proteins (ORF1) from their primary transcript and also produce subgenomic RNAs during infection for translation of their structural proteins, and putative accessory phosphoprotein for HEV (ORFs 2 and 3). ORF1 is differentiated from ORFs 2 and 3 by solid versus stippled vertical lines, respectively, within the open boxes. Viruses in group I and II are not capped but rather have a covalently attached VPg (viral protein g) moiety at the 5′ end of the genome. For PV, this moiety is removed (*) by a cellular enzyme following infection. Viruses in group III and IV have a 7 mG cap moiety at the 5′ end of their genomes. Unlike the single-stranded, positive polarity of the viruses in groups I–III, the rotaviruses (group IV) genomes are double-stranded and segmented. Rotaviruses contain 11 dsRNA segments which encode 12 proteins, whereby the 11th segment encodes two proteins. Shown as a representative segment in this figure, segment 1 encodes for the viral RNA polymerase. Each rotavirus genome segment is transcribed (from the negative strand) into an RNA transcript which is 7 mG capped prior to the translation of its ORF. It is important to note that actual nucleotide lengths and distances are not drawn to scale.

The common structural features include a genome linked protein VPg, a 5′ untranslated region (UTR), and a 3′ terminal poly(A) sequence. The 5′ UTR of picornaviruses is larger than the 5′ UTR in caliciviruses, and houses the internal ribosomal entry segment or IRES (Fig. 15.2). As depicted and discussed in Fig. 15.2, viral RNA translation occurs in a cap-independent manner as opposed to the translation of host cellular mRNAs.[54,78,184,194,253,311] The major difference is that picornaviral genomes are translated as a single polyprotein (Fig. 15.1), which are then cleaved by viral coded proteases 2A and 3C (only 3C in the case of HAV) to produce mature viral proteins. Calicivirus genomes, on the other hand, are organized into three different ORFs, and are translated into three different proteins, with the lone viral protease 3C performing all maturation cleavages (Fig. 15.1). Recent studies indicate that the 5′ terminal IRES also plays a significant role in pathogenesis of picornaviruses (see Section 15.2.5). For a full discussion of the various aspects of viral RNA synthesis, protein

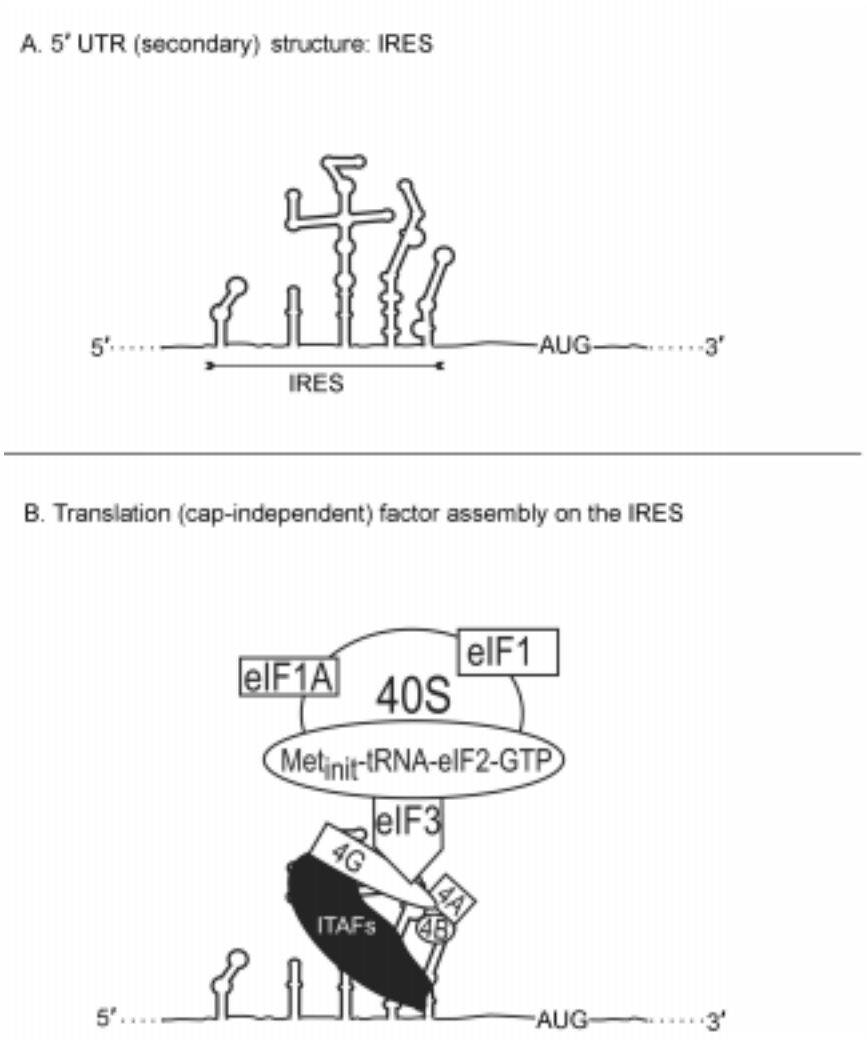

Fig. 15.2 Picornavirus internal ribosome entry sequence (IRES): structure and cap-independent translation factor assembly.

translation, and processing, the reader is referred to several recent reviews.[131,135,150,216,291] Unlike picorna- and calicivirus genomes, a 7-methyl guanosine cap structure[284] is present in the genomes of HEV,[172,208,251] and rotaviruses.[116] The double-stranded RNA genome of rotavirus is not active as mRNA.[104,116,164,240] Instead, the minus strand of each segment of the genome is first transcribed to yield the plus (coding) strand and then capped by virus coded capping enzymes, which is then translated to produce viral proteins. Unlike other foodborne viruses, neither the genomic nor the newly synthesized rotaviral RNAs are polyadenylated at the 3′ end.[229]

15.2.3 Viral capsid

The basic virion structure of all viruses, including foodborne viruses, is a nucleic acid core surrounded by a protein shell.[135,239,266,291] Rotavirus is unique in having two concentric protein shells forming the capsid, each having a different set of structural proteins. The rotavirus capsid also contains the viral RNA polymerase and capping enzymes.[229] The purpose of the protein shell or capsid is to protect the nucleic acid genome from damage by physical and chemical agents. Foodborne viruses lack a lipid envelope/membrane that is a feature of many of the larger DNA and RNA viruses which may explain their resistance to bile salts *in vivo* and detergents and organic solvents *in vitro*.[135,266] However, the enveloped avian influenza virus is stable at the low pH of the gut in birds and is excreted in bird feces at very high concentrations.[301] Thus, the absence of an envelope is not the only determinant of resistance to bile. The proteins of the capsid bind to specific receptors on cell surfaces in order to gain viral entry into the cell, and are important determinants of the host range and pathogenicity of the virus (see Section 15.3.1). The primary and higher-order structures of the capsid proteins also play critical roles in determining heat and acid stability, as well as resistance to stomach and intestinal proteases. Recent studies[230,231] showed that even digestion with proteinase K failed to expose the HAV genome to RNase activity as measured by reverse-transcription polymerase chain reaction (RT-PCR) unless the virions were also treated with heat or other chemical agents. The stability of enteric viruses to physical and chemical agents has been the subject of much experimentation and controversy owing to the lack of a standardized set of conditions to be followed for such studies.[83,84,159,207,220,236,273] Constituents of food matrices (such as sucrose in strawberry mash) also affect the heat stability of HAV.[35,87] NoV has not been grown in cell culture; thus the heat and acid stability is inferred from either experiments with a surrogate virus, such as the feline calicivirus,[97,230,288] or by the resistance of the viral genome to various treatments as detected by RT-PCR.[207,231] However, feline calicivirus infects via the respiratory route, and therefore cannot be considered truly representative of enteric human caliciviruses. The RT-PCR approach (Fig. 15.3) suffers from the small target size of the amplicon, which may not detect damage to the genome over its entire length.[34] The RT-PCR approach also suffers from the disadvantage that inactivated virus often produce positive RT-PCR signal, particularly when a small region of the viral genome is targeted for amplification.[34,230,231] The apparent lack of correlation between infectivity and nucleic acid detection by molecular methods is of great concern to food virologists and regulatory agencies concerned with food safety (see Section 15.6).

15.2.4 Routes of entry

Foodborne viruses gain entry to the host organism via the alimentary tract, and replicate initially in the small intestine before gaining access to the body via the lymph nodes.[10,53,73,104,131,225,339] Thus, the survival of the incoming infectious virus in the hostile environment of stomach acids, bile, and proteolytic enzymes

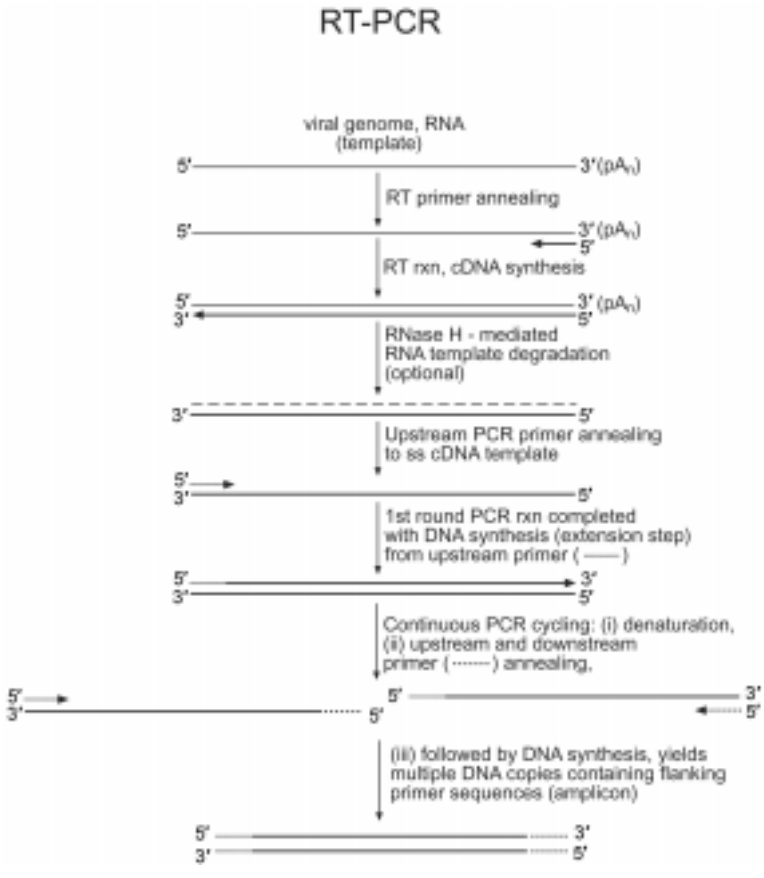

Fig. 15.3 Overview of essential steps and reactions involved in the targeted amplification of a specific RNA sequence or genomic region by the application of reverse transcription-polymerase chain reaction (RT-PCR). Viral RNA is annealed to a primer and copied into the complementary strand (cDNA) by the enzyme reverse transcriptase (RT). The primer may be complementary to sequences within the genome, the polyA region (if present) or be designed to target a broader range of sequences through the application of degenerate primer design. Degradation of the RNA strand may be accomplished through the use of an RT having endogenous RNaseH activity or by including the enzyme into the procedure (an optional step). A thermostable DNA polymerase, in combination with PCR primers which are designed as complementary sequences to the cDNA, is used to complete the PCR amplification. The typical PCR has a number of steps/cycles which usually include template denaturation, primer annealing, and primer extension. The final product of PCR is double-stranded DNA, termed an amplicon. The conditions (such as time and temperature) for each of the steps, as well as the total number of cycles, in the PCR are in part determined by the length and primary sequence of the primers, predicted length of amplicon, and target concentration. Since the target for PCR is single-stranded cDNA, the first round of PCR begins from the upstream, annealed primer. Following extension and denaturation, two complementary target DNA strands become available for both upstream and downstream primer annealing and extension. PCR cycling yields multiple copies of a sequence flanked by primer sequences. The length of the amplicon is determined by the relative position of the upstream and downstream primers on the target.

in the gastrointestinal tract all contribute to its pathogenicity. Proteases such as trypsin, elastase, pancreatin, and chymotrypsin convert non-infectious rotavirus to infectious subvirion particles (ISVPs) through the cleavage of the capsid protein VP4 to two polypeptides.[7,42,106] One of these, VP5, is responsible for the binding to the cell membrane and internalization of the virus.[42,106,226] Stomach and intestinal proteases, low pH and/or a combination of these processes result in the cleavage of capsid protein VPI and enhanced infectivity and antigenicity of HAV,[38,196] PV,[114,261] and astrovirus.[20] Virus-like particles (VLPs) assembled in insect cells infected with a recombinant baculovirus expressing the NoV capsid protein undergo proteolytic cleavage of the 58 kDa capsid protein when treated with the intestinal protease trypsin.[137] The model of infection that emerges from these studies is one in which stomach acids and intestinal proteases expose receptor binding sites in the capsid proteins enhancing infectivity and pathogenicity of the enteric viruses.

15.2.5 Viral genes and pathogenicity

Many recent insights into the pathogenic mechanisms of enteroviruses have come from the development of transgenic mouse models expressing the human poliovirus receptor.[234] Mouse is a natural host of coxsackievirus (CV) and mouse models for the investigation of CV pathology have been extensively utilized.[23,154] In addition, primate models for PV and HAV have been the source of much of the information currently available. *In vitro* studies with mammalian cells in culture, as well as in cell-free systems, have been invaluable in elucidating many of the molecular mechanisms of virus replication, and host defenses such as apoptosis.[80,121,323] These studies indicate that the viral encoded proteases 2A and 3C, as well as the 5′ UTR encompassing the IRES, play key roles in the pathogenicity of picornaviruses.

The viral IRES

As shown in Fig. 15.1, the 5′ end of all picornavirus and calicivirus genomes contain an untranslated region or UTR. The relatively long UTR of picornaviruses houses the IRES (Fig. 15.2), and is responsible for the internal initiation of protein synthesis from viral mRNA in a cap-independent manner.[24,55,193,271,291] Panel A of Fig. 15.2 shows the IRESs identified for picornaviruses are located in their 5′ UTR. Based on their conserved RNA sequences and secondary structures, most picornavirus IRESs can be divided into two groups, Type I and Type II. Type I IRESs are present in enteroviruses and human rhinovirus while Type II IRESs are present in other picornaviruses such as cardioviruses and aphthoviruses. A possible third Type III IRES has been identified in hepatitis A virus. A representative IRES structure is shown in panel A to illustrate the highly ordered secondary structures present in IRESs.[24,216,271] Panel B shows the mechanism of cap-independent translation of picornaviral genomic RNA (via the IRES structure). It is believed that 40S ribosomal subunit recognition of an IRES and subsequent translation factor assembly is facilitated in part by RNA–protein

interactions; translation efficiency is further augmented though interaction with other cellular factors termed IRES-trans-acting factors (ITAFs). The illustration depicts a general representation of translation factor assembly at an IRES (refer to Fig. 15.4 for a description of the various translation factors depicted in this figure). eIF4G (4G) has been reported to participate in cap-independent translation either as an intact protein (e.g. HAV IRES)[45] or in the truncated form (illustrated in Fig. 15.2) generated following enterovirus protease cleavage (see Fig. 15.4). ITAFs that are reported to be involved in interaction with picornavirus (e.g. enterovirus and hepatitis A virus) IRESs include pyrimidine tract binding protein, poly(rC) binding proteins and La autoantigen.[24,120,149,216]

There is general agreement that the IRES of different picornaviruses differ in their ability to initiate protein synthesis in cultured cells and cell-free systems that correlates with their ability to induce a cytopathic effect (cpe) in tissue culture cells.[44,49,52,128,241,260,276,341] The IRES may even be responsible for determining the tissue or species specificity,[49] pathogenesis and attenuation phenotype,[181,209,297,298] and efficiency of translation of the viral genome.[52,209,244] In particular, nucleotides at positions 472, 480, and 481 of the PV IRES have been reported as critical determinants of neurovirulence, although nucleotide changes elsewhere in the genome also contribute to the virulence phenotype.[177] Replacement of the PV IRES with the IRES from rhinovirus abolished the virulence phenotype.[133,134] In contrast, the IRES of cell-culture adapted HAV are more efficient in initiating protein synthesis *in vitro* and *in vivo*.[276,330,331,340]

The IRES of cytopathic strains of HAV (e.g. HM 175/18f) have a higher efficiency of translation initiation than the non-cytopathic strains, apparently because of decreased binding of the IRES to GAPDH,[275,276,340,341] a protein known to bind and destabilize RNA secondary structure. The significance of the low efficiency of translation initiation *in vitro* of HAV IRES to virulence is questionable, since chimeric HAV viruses with an EMCV IRES, although more efficient in translation, nevertheless showed no effect on the replication phenotype,[165] and the culture adapted rapidly replicating P16 strain is attenuated for virulence in animal models.[175,223,287,305] Attenuated strains of PV, on the other hand, are less efficient than their virulent parent strains in protein synthesis and host shut-off,[297,298] although such differences may be cell-line specific.[190] Clearly, IRES efficiency *in vitro* is not the sole determinant of pathogenicity in susceptible animals. The importance of other mechanisms is emphasized by the isolation of a recombinant vaccine derived poliovirus (VDPV) from a child with paralytic poliomyelitis that had the Sabin type-3 capsid antigenic site replaced by a Type II site due to recombination.[215] Remarkably, a six amino acid change in the antigenic site 1 of PV-1 (Mahoney) by the sequence of PV-2 (Lansing) rendered the non-pathogenic Mahoney strain to neurovirulence in mice.[214]

The 2A protease

The pathogenicity of foodborne viruses is dependent on their ability to kill the infected cells by either necrosis or apoptosis. Enteroviruses such as PV and CV that are highly cytolytic to cultured cells use a virally coded protease 2A-

mediated cleavage of eIF4G and other cellular proteins such as PABP (polyA binding protein) involved in translation to inhibit cellular protein synthesis (host shut-off).[4,5,107,170,178,191,255] As shown in Fig. 15.4, the host shut-off occurs because cellular mRNAs having a 5′ 7-methyl guanosine cap require functional interaction between eIF4G and eIF4E (cap binding protein) for translation, whereas the viral RNA translation initiated by an IRES remains unaffected by such cleavage events (Fig. 15.2).[24,55,247,278]

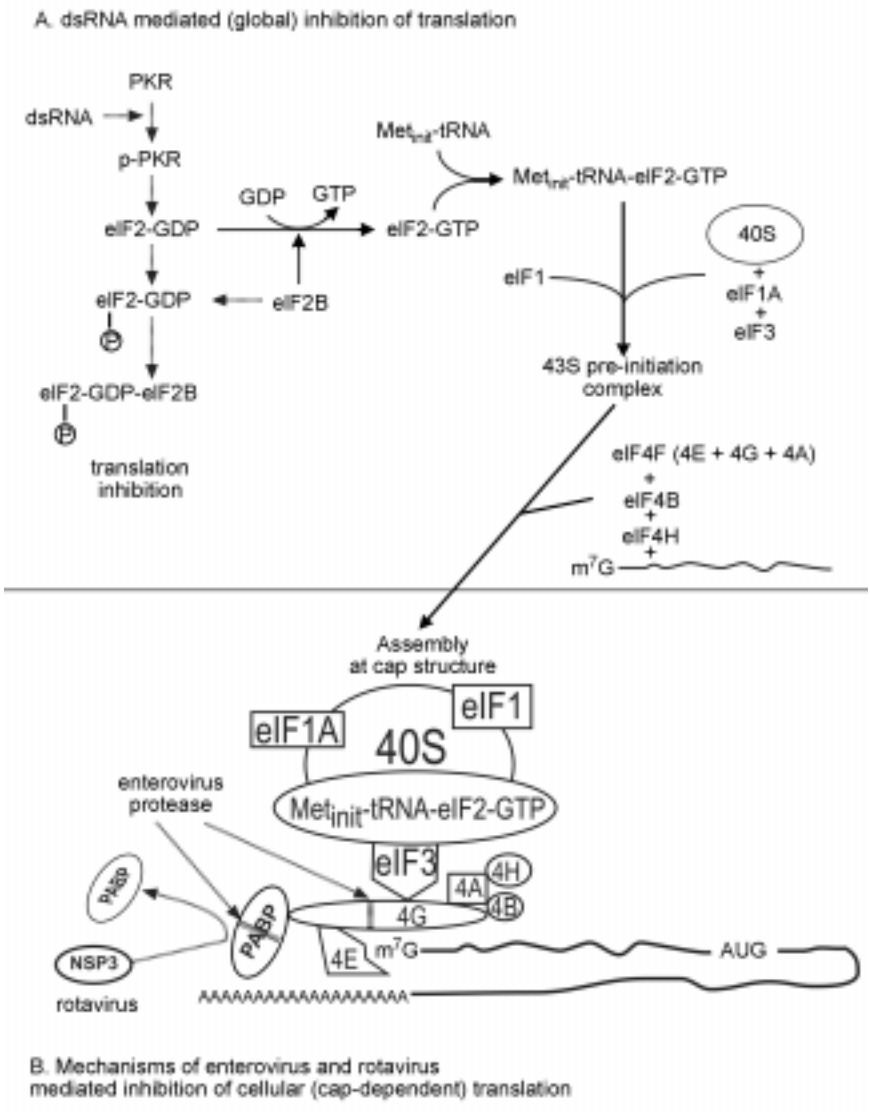

Fig. 15.4 Illustration of dsRNA and virus mediated inhibition of cellular protein synthesis.

The cap-dependent translation of cellular mRNAs shown in Fig. 15.4 is a cyclical process involving recruitment, dissociation, and recycling of various components and can be essentially divided into three phases: initiation, elongation, and termination. The study of cellular factors and mechanisms involved in this highly coordinated, regulated and complex process is the subject of numerous reviews.[24,55,74,120,149] The following discussion is limited to the events regarding translation initiation and the modes by which enteric viruses may affect this process. As shown in panel A, the recruitment of the cap binding protein complex (eIF4F) to the mRNA 5′ cap moiety m7G may be considered the beginning of translation initiation. The eIF4F complex contains the cap binding protein [eIF4E (4E)], and RNA helicase [eIF4A (4A)] and a scaffold protein [eIF4G (4G)]. The factors eIF4B (4B) and eIF4H (4H) promote RNA helicase activity through association with eIF4F. The 40S ribosome-eIF3-eIF1A complex is converted to the 43S pre-initiation complex following interaction with a ternary complex consisting of eIF2, methionyl-initiator tRNA and (eIF2-bound) GTP and the addition of eIF1.

In panel B of Fig. 15.4, the 43S pre-initiation complex subsequently associates with eIF4F through a bridging interaction between eIF3 and the eIF4G subunit to form the core of the 48S initiation complex. The tethering of eIF4F to the 43S complex is important for cap-dependent recognition and translation initiation. The scaffolding function of eIF4G is provided by its binding domains for eIF4E, eIF3, eIF4A, an eIF4A associated kinase (Mnk1), and a translation enhancing factor, poly(A) binding protein (PABP).

Not shown in Fig. 15.4 is the 5′ to 3′ mRNA scanning of the initiation complex to the start codon AUG (methionine), thus beginning the elongation phase of translation, where eIF5 facilitates the release of eIFs from the complex (factors are recycled). Following the hydrolysis of eIF2-bound GTP to GDP (panel A) which is facilitated by eIF2B, the cycle of factor assembly and translation initiation begins again. The enterovirus 2A and 3C proteases can inhibit cap-dependent translation via cleavage of factors such as eIF4G and PABP which disrupt the critical scaffolding function of these factors. The rotavirus nonstructural protein 3 (NSP3) interacts with the 3′ end of its viral mRNAs and can substitute for PABP, thereby displacing PABP from interaction with the initiation complex and negatively affecting translation. Translation initiation can be further regulated not only by the level and availability of the various factors described above, but also ‘globally’ by the modification state (e.g. phosphorylation) of particular subunits. As illustrated in panel A of Fig. 15.4, phosphorylation of the alpha subunit of eIF2 prevents eIF2B-mediated exchange of GDP for GTP and can occur (e.g. via dsRNA mediated activation of PKR, also see Fig. 17.5) following induction of an antiviral response, thus leading to a reduction in protein synthesis. It is of interest to note that at least three other kinases can also mediate this phosphorylation event, HRI (heme-regulated inhibitor), GCN2 (general control non-depressable 2) and PERK (PKR-like endiplasmic reticulum kinase). The latter is notable because of its connection to ER-stress induced apoptosis.[51,224]

HAV infection of permissive cells does not induce host shut-off. HAV lacks detectable 2A protease activity,[187,201,274] and HAV IRES driven translation requires a functional eIF4G moiety.[45] However, there may be other as yet unidentified functions of HAV 2A, since *in vitro* translation of capped mRNA was inhibited by HAV 2A,[91,210] and the 2A gene was required for maximum virulence.[21,101,139] Rotavirus uses a different mechanism. The viral NSP3 protein binds specifically to the conserved viral 3′ end sequences, and effectively out-competes PABP for interaction with eIF4G, thereby disrupting the interaction between PABP and eIF4G on cellular mRNAs.[237,245] The two consequences of NSP3 expression, therefore, are reduced efficiency of host mRNA translation and circularization-mediated translational enhancement of rotavirus mRNAs (Fig. 15.4).

The 3C protease

All picornaviruses, including HAV, as well as caliciviruses encode a second protease called 3C. The 3C protease of picorna- and caliciviruses is responsible for almost all the maturation cleavages of the viral polyprotein.[239,247,274,278] There is, however, some evidence to suggest that protein processing intermediates from entero-, hepato-, and calicivirus, such as the 3CD (protease-polymerase) are multifunctional in their precursor form and may interact with other viral proteins and viral RNA, thus affecting viral RNA replication.[27,137,138,338] In addition, the enterovirus 3C protease cleaves a variety of cellular proteins involved in transcriptional and translational regulation, including PABP, TATA binding protein, TFIIIC, CREB, and OCT-1,[247,325] and is directly involved in virus induced cell-killing by apoptosis.[18] No information is currently available on the effect of HAV or NoV 3C proteases on cellular proteins.

15.3 Mechanisms of host cell invasion

The primary mode for natural infection by enteric viruses is by ingestion, although a respiratory route of transmission may also be important for some enteroviruses[252] and norovirus.[213] In general, the age, gender, and socio-economic status of an infected individual or population can be factors that influence the probability of infection, severity of illness and sequellae, and prognosis for recovery from enteric virus infection.

Enteric virus infection may result in clinically defined illness or in asymtopmatic infection.[343] Norovirus and rotavirus infections typically give rise to localized disease due to primary replication that is generally limited to the small intestinal epithelium, although the rare development of systemic sequellae have been attributed to rotavirus infection possibly a consequence of viremia.[71,157,254] Enteroviruses initially produce a localized infection in intestinal cells or in mucosal tissue (Peyer's patches, tonsils), followed by replication in cervical and mesenteric lymphoid tissue. A systemic (viremia) phase may follow, leading to

infection/replication of other tissues/organs resulting in non-enteric illnesses such as aseptic meningitis, mild paralytic disease, acute hemorrhagic conjunctivitis or poliomyelitis (poliovirus).[238] This is in contrast to the primarily systemic infection and illness (e.g. hepatitis) observed following hepatic infection with hepatitis A virus.[150] As with non-enteric viral diseases, illnesses caused by an enteric viral infection may be due to a direct and/or immunolopathologic consequence of virus replication in the infected tissue/organ.

15.3.1 Virus binding and cell surface receptors

Once within the host, viruses gain entry into the target cell population by the interaction between the viral capsid and receptors and co-receptors on the cell surface.[90,318] Receptors and co-receptors involved in virus binding are listed for selected enteric viruses in Table 15.2. Many of these cell receptors participate in cell-specific functions such as signal transduction (e.g. DAF/CD55, integrins), cell-to-cell interactions (e.g. HBGAs, JAM), receptor-mediated uptake of nutrients/metabolic factors, cell matrix attachment (e.g. integrins) and immunologic recognition (e.g. ICAM-1, HBGAs). The cellular functions of some receptors such as the poliovirus receptor (PVR) (CD155) and HAV cellular receptor (havcr-1) remain unknown.[11,111,222,267,285] Some viruses interact with secondary receptors (or co-receptors) after the initial binding to and interaction with its primary receptor.

Table 15.2 Examples of cell receptors known or implicated in virus binding

Virus/species/serotype	Receptor/co-receptor[a]	Reference
Poliovirus	PVR(CD155)	222
Coxsackievirus B3	CAR/CD55*	31, 32, 282, 309
Coxsackievirus B1, 3, 5	CD55/αvβ6	1, 30, 282
Coxsackie A9	Integrin αvβ3, αvβ6/GRP78, MHC-1	262, 312–314, 335
Coxsackievirus A21	CD55/ICAM-1	281
Echovirus 22	Integrin αvβ3, αvβ1	250
Echovirus 11	CD55/α2β1, αvβ1	211, 312
Echovirus 1, 8	Integrin α2β1	29, 235, 314
Echovirus 3, 6, 7, 12, 13, 20, 21, 24, 29, 33	CD55	30, 185, 238, 249
Rotavirus	SA (oligosaccharides), Gangliosides, Integrin α2β1/ Integrin α2β1, α4β1, JAM	19, 113, 146, 203, 264, 296
Norovirus	HBGAs, Lewis antigen	156, 199
Hepatitis A virus	havcr-1	111

[a] PVR means poliovirus receptor. CAR means coxsackievirus-adenovirus receptor. CD means cluster of differentiation. GRP means glucose response protein. MHC-1 means major histocompatibility complex-1. ICAM means intercellular adhesion molecule. SA means sialic acid containing receptors. JAM is junction adhesion molecule. HBGAs mean histo-blood group antigens. Havcr-1 means hepatitis A virus receptor.
* CD55 is also reported as DAF (decay accelerating factor).

Picornaviruses

Picornavirus capsids are composed of four proteins (VP1–4) arranged in an icosahedral symmetry. Their surface architecture and receptor binding specificity differ due primarily to amino acid differences, and the tissue distribution/expression of receptors contributes to viral tropism and pathogenesis.[215,234,238,252] Some virus serotypes within the same species (e.g. coxsackieviruses) have been reported to use different cell receptors depending upon the target cell (Table 15.2).

Norovirus

In the absence of a suitable cell-culture model for NoV, recombinant virus-like particles (rVLP)[167,168] produced in insect cells have been used for the study of virus-cell interactions.[136,152,332] These studies have identified the human histo-blood group antigens (HBGAs), present on red blood and mucosal epithelial cells, saliva and intestinal fluid as the putative NoV receptor.[157,212,332] HBGAs were subsequently implicated in resistance or susceptibility to NoV infection, as well as the binding of the major capsid protein VP1 of NoV genogroups.[151,156,199,303,304]

Rotavirus

Human infections by rotavirus are attributed to serogroups A, B, and C. They primarily infect mature enterocytes of the small intestinal villi. However, some evidence indicates a possibly broader host tissue range based on reports of extra-intestinal spread following initial infection.[57,206,225,258] Both sialic-acid containing receptors[93,174,203] and members of the integrin family of receptors $\alpha 2\beta 1$ and $\alpha 4\beta 1$[82,146] can serve as the primary receptors for capsid proteins VP4, VP7, or VP5, the protease cleavage product of VP4.

15.3.2 Different (multiple) virus species may use the same cell receptor

As identified in Table 15.2, different virus species appear to use similar receptor types, or the same receptor, for cell binding. For example, the CAR receptor is used by both coxsackie B virus and adenoviruses.[242] PVR(CD155) is the primary receptor for poliovirus and has been reported to function as a secondary receptor for alphaherpesviruses.[118] ICAM-1 (intracellular cell adhesion molecule-1) has been identified as a primary or secondary receptor for coxsackievirus A, and also functions as the primary receptor for major group members of genus Rhinoviruses.[238,252,281]

15.3.3 Role of circulating Ab-Ag virus complexes in cell attachment

A host has many defense mechanisms which can be utilized to respond to a virus infection. These molecular and cellular defenses may be considered as part of two broad categories: innate and adaptive immunity. A detailed discussion of innate versus adaptive immunity is beyond the scope of this review and the reader is referred to examples of articles in this subject area.[3,6,25,36,50,64,310,333,343] Innate immunity includes those responses such as phagocytosis (e.g. macrophages),

cytokine release (e.g. interferon, interleukins), cell/tissue surface defenses, and cell-mediated killing of virus infected cells (e.g. NK cells) that do not require specific viral antigen recognition in order to initiate and mediate the response. The development of antiviral antibodies and cell-mediated immunity are part of the more slowly developing and longer-lived antiviral response categorized as adaptive immunity. Cellular components of an adaptive immune response (e.g. B cells, T cells) can recognize viral proteins as foreign (antigens) and develop specifically targeted responses (e.g. antiviral antibodies, antigen-dependent cell-mediated killing) against domains (epitopes) of the viral antigen. Antibodies produced (humoral immunity) in response to virus infection can include members of the immunoglobulin (Ig) classes IgM, IgG, and IgA. An initial humoral response typically involves IgM production followed by a switch to IgG production later in the infection/antibody response. The highest levels of IgG are typically observed in the serum while those of IgA are typically observed at the mucosal tissue or fluids (e.g. intestine, saliva), and in these locations is sometimes referred to as secretory IgA.

Interestingly, antiviral antibodies can provide an alternative mechanism by which a virus may gain entry into cells such as through receptor-mediated binding and entry of virus particles into a cell as antibody–virus complexes. These complexes are formed when sub- or non-neutralizing quantities of anti-virus antibodies are bound to the virus particle,[8,299,333] allowing the exposed Fc-region ('tail') of the antibody to attach to cells via Fc receptors expressed on the cell surface, thus facilitating entry into that cell. This facilitated uptake of antibody bound virus complexes has been reported for a variety of both RNA and DNA viruses in cell culture and *in vivo*.[299] The existence and utilization of this alternative pathway suggest that a mechanism exists for expanding viral tropism *in vivo* under conditions whereby a host immune response generates sub-neutralizing titers of anti-viral antibodies.[217,257,322] Similar antibody-dependent enhancement or ADE has been reported for CV[148,183] and HAV.[94,95,110] In coxsackievirus B4 infection, ADE has been implicated in increased infectivity of the virus, implicating this mechanism in the development of myocarditis, at least in a mouse model.[148,183] Dotzauer *et al.*[94] demonstrated infection of both mouse and human hepatocytes using antibody–HAV complexes, and suggested that the formation of these complexes may play a role in relapsing HAV infections through its function as a possible liver-directed carrier mechanism. It is also important to note that the expression of Fcγ receptor on phagocytic cells (viz. macrophages, neutrophils) is part of the viral clearance mechanism used by the immune system to remove virus–antibody complexes from circulation, but ironically may contribute to the risk factors associated with disease[322] as suggested by Rekand *et al.*[257] regarding allelic polymorphisms in the FcγIIIA receptor and risk factor development for acute illness (poliomyelitis).

15.4 Host cell defenses

15.4.1 Protective immunity

There are numerous physiologic hurdles that a virus must overcome to establish an infection within the host (Section 15.2), but enteric viruses are capable of surviving many of these assaults primarily because of the structure and composition of their capsids. The host, however, has mechanisms such as antiviral and immunological responses to combat a potential enteric virus infection.[333] For example, both serum and secretory antibodies play major roles in protective immunity against enterovirus infections. For HAV infection, circulating anti-HAV IgG is protective against viral disease, but it is unclear what role immunity at the intestinal surface (such as secretory Ig) may play in protection from infection.

Human volunteer feeding studies using either infectious inoculums or NoV rVLPs, and epidemiologic and serologic studies have indicated the absence of any long-term immunity to NoV infection.[105] The relatively rapid rate of recovery from NoV infection and illness suggests both an antiviral and immunological response play a role in the overall host response to infection.[39,130]

Rotavirus nonstructural protein NSP4 has been found to have a toxin-like activity that effects diarrheagenic changes without direct destruction of villus enterocytes.[254] The major outer capsid proteins VP4 and VP7 are involved in virus attachment and cell entry and induction of neutralizing and protective antibodies.[219,233,345] VP6, an intermediate layer virus structural protein, has also been implicated as part of an IgA-specific non-neutralizing, but protective immune response.[109] Primate and mouse model studies also indicate that transcytosis of serum IgG to the intestinal lumen may be important in blocking virus attachment or endocytosis.[277,329]

15.4.2 The interferon (IFN) response

To survive a virus infection the host relies on both innate and adaptive defense mechanisms which the invading virus must overcome to replicate and assure its own survival.[79,112,143,158] Since there is usually a time lag of several days before the adaptive immune response becomes functional, the cytokine response (IFN) is crucial for the survival of the organism.[3,36,173,227] There are two major IFN-controlled cellular defense mechanisms that are important in the context of foodborne viruses: the 2-5A dependent RNase L pathway and the PKR (protein kinase RNA activated) pathway (Fig. 15.5).[17,158,280,286,292]

Apoptosis is referred to as programmed cell death.[17,51,256] This process is often argued as the means by which a cell may limit virus replication and spread through initiation of this cellular 'self-destruct' mechanism. Some viruses have evolved mechanisms to either induce or suppress apoptosis. The former may function as a means to facilitate release of progeny virus from the infected host while the latter may aid in delaying cell destruction in order to provide additional time for virus replication and progeny production. The induction of apoptosis in virus infected cells may occur as a consequence of protein synthesis

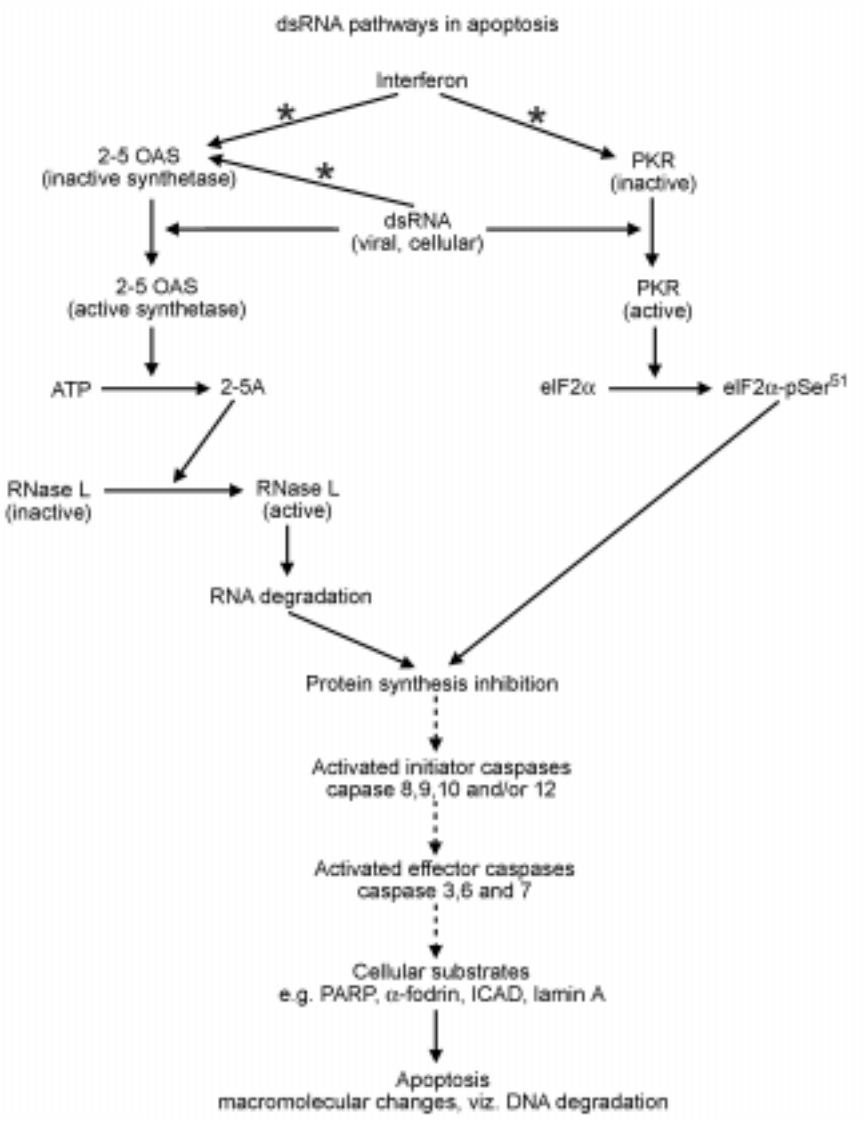

Fig. 15.5 Diagrammatic representation of the relationship between activation of the dsRNA pathways and induction of apoptosis.

inhibition resulting from, for example, modification of protein kinase signaling factors or cellular metabolic pathways leading to endoplasmic reticulum (ER) stress induction (e.g. amino acid depletion, unfolded protein response); cleavage of critical translation factors (see Fig. 15.4); and/or antiviral (dsRNA and interferon) pathway activation. As shown in Fig. 15.5, one of the cellular responses to interaction with interferon can be the induction (increased expression) (*) of 2–5 OAS (oligoadenylate synthetase) and/or PKR, whose

latent enzymatic activites are activated by viral dsRNA. dsRNA alone may function to induce expression of 2–5 OAS. Activated 2–5 OAS produces 2′–5′ adenylate oligomers which in turn activate latent, endogenous RNase L to ultimately affect protein synthesis by degrading RNA. Activated PKR phosphorylates serine 51 of eIF2 subunit α resulting in inhibition of protein synthesis (see Fig. 15.4), The link between protein synthesis inhibition and activation of initiator caspases (----›) is not fully understood and appears to be in part virus and cell/tissue dependent. Central to apoptosis is the activation of members of a family of intracellular cysteine aspartyl-specific proteases known as caspases.[77,88,103] Fourteen caspases have been identified with some reported to have a tissue-restricted specificity for activation. With few reported exceptions, capases require cleavage through an auto- or trans-catalytic process for their activation. The activation of initiator caspases (e.g. caspase 2, 8, 9, 10, 12) can occur through receptor (extrinsic) or intracellular initiated (intrinsic) mechanisms. This triggers activation of an enzymatic (caspase) cascade leading to downstream activation of effector caspases (e.g. caspase 3, 6, 7), resulting in targeted cleavage of various cellular proteins that ultimately mediate the observed physical and biochemical changes ascribed to apoptosis.

Ironically, the viral genome itself (or its replication strategy) is the Achilles heel of the foodborne viruses. This is because one or both of these two major IFN stimulated antiviral systems are triggered when the cell encounters a virus. With few exceptions, the trigger is dsRNA either in the form of viral transcript (e.g. reovirus s1) secondary structure, or in the dsRNA replicative intermediates of ssRNA viruses, although a direct role of dsRNA viral genomes has not been completely ruled out.[17,37,144,161,270] Synthesis of dsRNA is unavoidable even in dsDNA virus infected cells owing to convergent transcription from both DNA strands.[89,127] Moreover, both dsRNA triggered antiviral mechanisms are apoptotic.[17,69,280] Picornaviral 2A and 3C proteases (Section 15.2) can also induce apoptosis in infected cells by mechanisms that share some features of the apoptosis triggered by dsRNA, namely the caspase pathway.[18,56,61,123] The difference is in the timing of the onset of apoptosis. Viral proteases induce apoptosis late in infection when the viral replication cycle is complete.[4,41,47,60,61,80,121,124,255,265] With few exceptions, the dsRNA activated apoptotic pathways require prior exposure to IFN either as pre-treatment of tissue culture cells prior to virus infection, or virus-induced synthesis of IFN, which sensitizes surrounding uninfected cells to an incoming virus, and therefore induces apoptosis in these cells before virus replication can take place.[17,280,292]

15.4.3 Inhibition of virus replication by protein kinase RNA activated pathway

Activation of PKR requires dsRNA mediated autophosphorylation and dimerization (Fig. 15.5). Activated PKR phosphorylates a number of cellular proteins such as eIF2α, and I$\kappa\kappa\beta$. Phosphorylation of eIF2α renders it inactive in protein synthesis (Fig. 15.4), both cellular and viral, resulting in the inhibition of virus

replication, and in apoptotic death of virus-infected cells,[74,149] contributing to viral pathogenicity.[272] Phosphorylation of I$\kappa\kappa\beta$, on the other hand, induces the synthesis of the transcription factor NF-$\kappa\beta$, resulting in the induction of several cellular genes (including IFN) with both pro- and anti-apoptotic functions.[161]

15.4.4 Inhibition of PKR by viruses

PKR activation must be suppressed for virus specific protein synthesis and virus replication to occur.[15,37,76,293] The reovirus outer capsid protein σ3 binds to and inhibits PKR activity.[119,160,202] However, the σ3 protein is also responsible for inhibition of host cell protein synthesis.[283] A second reovirus protein μ1 may bind to σ3 and prevent its dsRNA binding function.[279,342] The NSP3 protein of reovirus, specifically an 8 kDa cleavage product, has been shown to bind and antagonize the antiviral effects of dsRNA.[192] In some cell lines of tumor origin, PKR expression is down-regulated, resulting in high-level replication of these viruses and efficient cell killing.[98,140,294] The virus induced cell-killing of certain tumor cell lines may be an interesting therapeutic application of these viruses.

Regarding other important foodborne pathogens, PV causes reduction in PKR levels by an unknown mechanism possibly involving a cellular protease and poliovirus dsRNA.[40] Evidence suggests that a reduction in cellular RNA synthesis via the degradation of a variety of RNA polymerase transcription factors[247] coupled with an inhibition of cellular protein synthesis, results in a significant reduction in PKR levels. Other enteroviruses probably employ similar mechanisms. It is not known how HAV, NoV, or HEV evade the consequences of PKR activation. Infection with apoptotic as well as non-apoptotic strains of HAV did not result in PKR activation (Fig. 15.6). Figure 15.6 shows the results of dsRNA treatment and HAV infection on 2–5 OAS levels and PKR phosphorylation in FrhK4 cells. FrhK4 cells were either mock or HAV 18f infected [48 hpi (hours post-infection)], or mock or polyI:C (i.e. dsRNA) transfected (48 h) prior to protein extraction and denaturing gel electrophoresis/immunoblot analysis (see Goswami *et al.*[126] and Kulka *et al.*[187] for experimental details) for detection of 2–5 OAS (panel A), phosphorylated PKR (panel B), or nonphosphorylated PKR (panel C). Persistently HAV clone 1 infected (>300 days pi) FrhK4 cells were mock or dsRNA treated (48 h) prior to protein extraction and immunoblot analysis. The positions of the 100 kDa and 69/71 kDa isoforms of 2–5 OAS are identified with arrows. 2–5 OAS was detected only in dsRNA-treated FrhK4 cells. Nonphosphorylated PKR (67 kDa) was present at equivalent levels in all samples, while phosphorylated PKR was not detected in any of the samples. Thus PKR phosphorylation may be defective in these cells, allowing HAV to establish a latent infection as suggested by Gale and Katze.[117] dsRNA induced synthesis of OAS, while neither the apoptotic HAV strain (18f), nor the tissue culture adapted parent strain (clone 1) caused induction of OAS. Moreover, the clone 1 virus inhibited dsRNA induction of OAS. HAV is thus equipped with an as-yet unidentified function that can

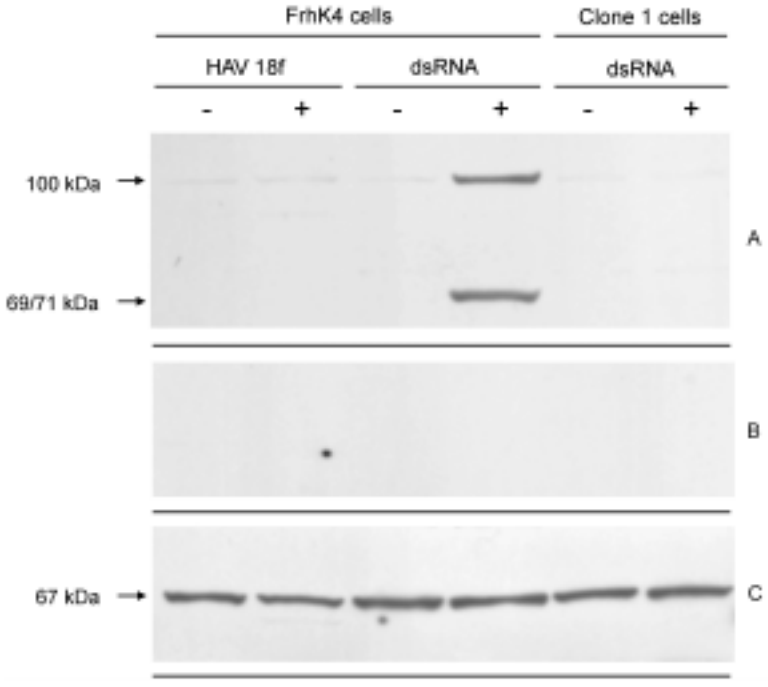

Fig. 15.6 Induction of 2-5OAS expression in response to dsRNA treatment of FrhK4 (fetal rhesus monkey kidney) cells.

interfere with dsRNA activated antiviral mechanisms. The role of cellular protein modulators of PKR activation, which work by either binding to PKR or to dsRNA,[158] are currently unknown. It has been suggested that lack of PKR activation may be the reason why some viruses are able to establish persistent or latent infection.[117]

15.4.5 Inhibition of virus replication by RNase L

A second IFN-induced antiviral pathway involved in apoptosis of virus infected cells is activation of a latent endoribonuclease RNase L (Fig. 15.5).[62,163] This ribonuclease is present in inactive form in many cell lines and is apparently involved in many physiological processes.[286] The enzyme is activated when 5′ phosphorylated trimers or higher oligomers of 2′–5′linked oligoadenylic acids (collectively referred to as 2–5A) bind to the inactive form of RNase L.[179] Synthesis of 2–5A is a function of the enzyme 2′–5′ OAS, which is induced by IFN and activated by dsRNA. Several isoforms of OAS have been described, while only a single molecular form of RNase L in a given cell type is known. The activated RNase L degrades cellular as well as viral RNAs after UpNp dinucleotides. In interferon-treated cells, degradation of both viral and ribosomal RNA (rRNA) molecules are observed following infection with several RNA and DNA viruses, or treatment with dsRNA.[62,286]

15.4.6 Viral evasion of the RNase L pathway

Little is known about how ssRNA or dsRNA viruses escape the RNase L pathway. Since cells that have not been treated with IFN contain little or no OAS, viral infection of such cells does not trigger the RNase L-mediated RNA degradation owing to the lack of the activator 2–5A. The only exceptions known to date for the requirement of IFN for RNA degradation in virus infected cells are the apoptotic strains of HAV,[125,187] and mouse coronavirus.[16] Degradation of rRNA occurred following HAV infection in the absence of prior IFN treatment of the cells, and in the absence of detectable OAS mRNA or protein (Figs 15.6 and 15.7). In contrast, treatment of the same cell line with dsRNA resulted in rRNA degradation and also induction of the 69/71 kDa form of 2–5 OAS mRNA and protein (Figs 15.6 and 15.7); degradation of viral genomic RNA in virus infected cells did not occur,[187] and virus replication was not inhibited. Along with the previous reports that HAV inhibits dsRNA-induced

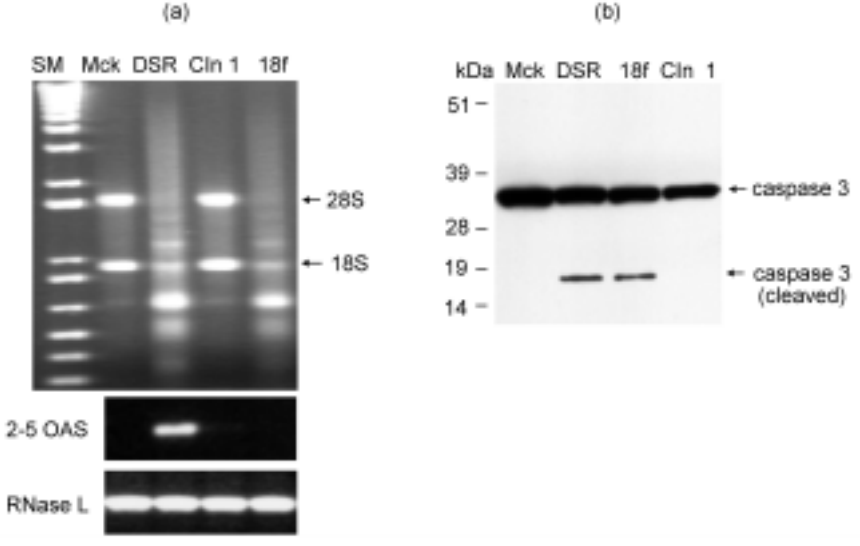

Fig. 15.7 Activation of apoptotic pathways (RNase L and caspase) following HAV infection or dsRNA treatment of FrhK4 cells. Replicate samples of FrhK4 cells either mock (Mck) treated, HAV 18f infected (18f), HAV clone 1 infected (Cln 1) or polyI:C transfected (DSR) for 48 h were subjected to either total RNA extraction/isolation for RNA analysis and RT-PCR (A, upper and lower panels, respectively), or protein extraction followed by denaturing gel electrophoresis/immunoblot analysis for caspase-3 activation (panel B) (see Goswami *et al.*[126] and Kulka *et al.*[187] for experimental details). The positions of the 28S and 18S rRNAs are indicated with arrows. Degraded rRNA appears as stained bands located both between the 28S and 18S, and below the 18S positions (A, top panel). While RT-PCR generated a 2-5OAS amplicon from dsRNA-treated FrhK4 cells, the remaining samples were negative, indicating the lack of induction of 2-5OAS gene expression in these samples. RNase L was activated in 18f infected as well as dsRNA-treated cells, suggesting 2-5A-independent activation in 18f infected cells. Activation of caspase-3 in dsRNA-treated and 18f infected cells is indicated by detection of the (cleaved) large fragment of caspase-3 (panel B).

synthesis of IFNβ[48] and the absence of activation of the JAK-STAT pathway in HAV infected cells,[125,187] it is likely that activation of RNase L in the FrhK4 cells following HAV infection is an example of the usurping of this activity by the virus to its advantage, analogous to the usurping of the PKR pathway by reovirus.[76,294]

15.4.7 Interferon, apoptosis and pathogenesis

In the context of virus infection, apoptosis is a two-edged sword. As a host defense mechanism, induction of apoptosis early in infection inhibits viral replication and formation of progeny virus. A late induction after the completion of the viral replication cycle favors the virus by circumventing the immune and inflammatory responses.[17,268] For enteroviruses, the usual outcome of a productive infection is cell lysis and release of progeny virus.[41,58,69,205] However, even with these highly cytolytic viruses, recent results indicate a significant role of apoptosis in viral-induced pathogenesis.[4,46,56,60,61,67,68,80,141,197,198,255] In general, signals for apoptotic induction converge on the enzyme cascade called caspases (Fig. 15.5), which include initiator caspases (8, 9, 10, and 12) and effector caspases (3, 6, and 7). Caspases are present in cells in inactive forms and require activation by proteolytic cleavage.[77,103,307] In this scheme, caspase 3 plays a central role, in being both activated by initiator caspases, and activating the other two effector caspases. Caspase 3 activation and caspase-mediated cleavage of cellular proteins involved in apoptosis have been observed in enteric virus infected cells.[18,26,60,67,123,126,187,265] Although it is generally thought that changes in mitochondrial membrane permeability resulting in cytochrome *c* release and activation of caspase 9 leads to activation of caspase 3 in some enterovirus infected cells, activation of upstream caspases has not been observed in HAV-induced apoptosis (Goswami and Kulka, unpublished). Similarly, the role of RNase L-mediated rRNA degradation in caspase activation remains to be established.

JAK-STAT

Central to the antiviral and proapoptotic effects of the IFN induced systems, PKR and RNase L, is the JAK-STAT pathway which controls IFN stimulated gene expression. Phosphorylation of the transcription factor STAT1 is critical for stimulation of genes containing either interferon stimulated response elements (ISRE) that are regulated by type I IFN or gamma-activated sequence (GAS) elements stimulated by type II IFN. STAT1 responsive genes include both PKR and OAS, and differential effects of the two types of IFN on the two antiviral mechanisms have been observed.[112] STAT 1−/− cells are unresponsive to IFN and sensitive to virus infection,[99,337] although the severity of the disease and vaccine derived protection may not be altered for all enteric pathogens.[321] In HAV-infected FrhK4 cells, STAT1 is not phosphorylated and IFNα/β is not induced.[48,126,187] The activation of RNase L and apoptosis in such cells is 2–5A or PKR independent (Figs 15.6 and 15.7). Clearly, the role of

STAT in the replication of enteric viruses needs to be investigated. The enhanced replication of mouse norovirus in dentritic cells and macrophages from STAT1 knockout mice also suggests a larger role of the JAK-STAT pathway in virus replication.[176,337]

15.5 Mechanisms of virus-induced damage to host cell

15.5.1 Apoptosis as a pathogenic mechanism

Considerable evidence has accumulated over the past years indicating that apoptosis plays a key role in viral pathogenicity.[5,41,61,112,204,255,265,272] Even highly cytolytic viruses such as PV and CV have been shown to induce apoptosis both in tissue culture and in animal models.[4,5,80,81,121,255,265] Because many enteric viruses are highly cytolytic in tissue culture cells, induction of apoptosis is only observed under conditions that are restrictive to virus replication.[265] In cultured cells *in vitro*, such restrictive conditions are achieved by the use of certain inhibitors of virus replication or mutant viruses. Restrictive conditions for virus replication occur *in vivo* owing to an IFNα/β response to virus infection. Moreover, poliovirus-induced apoptosis has recently been observed even under permissive conditions in a human promonocytic and enterocytic-like cell lines[204] and in the mouse central nervous system.[121,122] Apoptosis resulting from activation of the caspase pathway by the perforin/granzyme route was reported following vaccination with oral polio vaccine. IFNγ secretion by both CD4+ and CD8+ T cells was involved, again pointing to apoptosis as a mechanism of tissue damage, probably as a result of replication of the vaccine derived virus.[324] In contrast, wild-type and most cell culture-adapted HAV strains produce a noncytolytic persistent infection *in vitro*. A few cytopathic strains of HAV are capable of cell killing *in vitro* as a result of a slowly developing apoptosis.[47,124,126,187] However, unlike the enteroviruses, the relationship between apoptosis and pathogenicity in animal models has not been clearly established for HAV.

Rotavirus infection has been shown to induce apoptosis both *in vitro* and *in vivo*.[46,67,259,316,317] While induction of apoptosis *in vivo* in mice infected with a human rotavirus was correlated with the onset of diarrheal disease, the underlying mechanisms connecting these processes have not been fully elucidated.

15.5.2 Cytopathic effect (CPE)

The development of morphologic changes related to CPE in an infected cell is the subject of numerous reviews.[185,205,289] The secondary effects of viral gene expression/replication on host cell macromolecular structures and/or metabolism can ultimately lead to CPE by effecting changes in cell morphology such as cell rounding, cell lysis, syncytium formation, and inclusion body formation. The induction of CPE in cultured cells may or may not have a direct mechanistic

relationship to the pathogenesis of viral disease. The molecular events responsible for the cytopathogenesis ascribed to a particular virus infection may be delineated through investigations of the effects of viral gene expression and replication on host cell metabolism in culture. For an increasing number of viruses, the CPE observed following virus infection has subsequently been determined to be the consequence of an apoptotic response.

For example, the productive infection of non-neuronal cells (e.g. HeLa) with poliovirus gives rise to CPE but does not lead to DNA laddering that is characteristic of apoptosis. Infection of neuronal or promonocytic cells, or non-neuronal cells under conditions of restricted poliovirus replication, induces molecular events and morphologic changes consistent with cell death by apoptosis.[80,121,204] Enterovirus 71 and coxsackievirus B5 were also reported to induce apoptotic and non-apoptotic cell death depending, in part, on the cell type and multiplicity of infection.[4,60,255,327] A number of investigators have argued for the contribution of viral CPE and possibly apoptosis in the pathology of viral myocarditis and progression to cardiomyopathy.[14,153,238,328] The immunological contribution to the pathology could not be discounted. Clearly, the interplay between viral and cellular factors/mechanisms during enterovirus infections which results in either non-apoptotic CPE or apoptosis induction, is complex, and has yet to be fully elucidated with respect to natural EV infection and potential sequellae such as poliomyelitis, meningitis, and cardiac damage.

Wild-type hepatitis A virus infection in culture does not produce CPE despite replication, assembly, and spread of infectious virus. While the clinical symptoms and pathology of infectious hepatitis are attributed to a T cell mediated destruction of infected cells,[64,319,320] the contribution of virus replication *per se* to hepatic cell destruction remains unclear. There is currently no information regarding the development of either CPE or necrosis in cell culture by human NoV owing to the lack of a cell culture system for this virus. Human volunteer studies reveal upper small intestinal histopathologic lesions and mononuclear cell infiltration of the lamina propria, suggesting virus particle binding to epithelia cells (stomach) and enterocytes (small intestine).[105] While animal rotaviruses can be grown in cell culture quite easily, the culture of human rotaviruses often requires additional methods such as pretreatment and/or incorporation of trypsin in the culture medium possibly aiding in the exposure of viral antigenic epitopes for more efficient binding of the virus to its cell receptor.[174,203]

Although most culturable human (primarily group A) and non-human strains of rotavirus have been reported to develop CPE; human group B and C members have proven more difficult to culture. Histopathologic changes,[132] altered cytoskeletal organization and perturbation of calcium homeostasis,[104,254] are among the reported effects of rotavirus infection. The induction of apoptosis has been described for rotavirus infection in culture.[67,295] DNA fragmentation (not consistently reported during rotavirus infection in culture) has been correlated with the loss of mitochondrial membrane potential and release of cytochrome *c*, as well as shut-off of host macromolecule synthesis. The viral and cellular

mechanism(s) responsible for induction of apoptosis in rotavirus infected cells are not well understood and remain the subject of continuing investigation.

15.5.3 Effects on cellular metabolism: virus inhibition of protein and RNA synthesis

Owing to the limited genome capacity of most enteric viruses (Fig. 15.1), essential viral functions are co-opted to modify or inhibit cellular macromolecule synthesis and function to favor virus replication (see Section 15.2). The study of cellular factors and mechanisms involved in this highly coordinated, regulated, and complex process is the subject of numerous reviews.[120,149]

15.6 Implications for foodborne disease treatment and prevention

15.6.1 Vaccines

Norovirus

Generally, gastroenteritis caused by foodborne viruses of norovirus genus is a rather mild self-limiting disease. Thus, a nationwide program of mass vaccination is probably unwarranted, although specific segments of the population, such as military personnel destined for war zones, or food handlers and agricultural workers, might benefit from an effective vaccine. From volunteer studies it is unclear whether long-term immunity is achievable following immunization with recombinant VLP against NV,[105] or whether immunization with one genogroup confers cross-protection against a different strain of NoV.[200] Besides the lack of a cell culture or small animal host for NoV, the genetic and antigenic diversity within the genera is a great impediment towards the development of effective vaccines.

Poliovirus

The availability of both inactivated (Salk type) and live attenuated virus (Sabin type) vaccines has almost eradicated this disease in developed as well as most developing countries. The disadvantage of a live attenuated vaccine is ensuring the safety of the vaccine strain. Growth of attenuated strains of PV sometimes results in revertant strains that have regained their virulence, requiring rigorous safety testing of each new batch of virus to be used for immunization,[334] especially since VDPV with mutated phenotypes can persist in immunized populations for years.[70,147]

Rotavirus

The picture of success of vaccination programs with live attenuated poliovirus got murkier with reports of serious adverse effects during clinical trial with a live rotavirus vaccine.[33,228] Considerable resources are currently being used to develop new vaccines against rotavirus based on reassortments between human and animal rotaviruses of the capsid proteins VP4 and VP7.[155]

HAV

Two inactivated HAV vaccines are currently licensed for use.[25] They are both highly effective and serious adverse effects are not currently known.

15.6.2 DNA vaccines

DNA vaccines are based on the expression in 'vaccinated' hosts of cDNA encoding protein antigens (viz. viral coat proteins) that have been cloned into plasmid vectors carrying transcription and translation signal sequences utilized by the host's cellular/molecular machinery. Specific genetic elements can be engineered into the vector to permit replication in target cells.[145,246] Expression of the protein antigens generates both humoral (antibodies) and cell-mediated (cytotoxic T lymphocyte or CTL) responses to the antigen, conferring protection against subsequent infection by the same or related pathogen. The advantage of DNA vaccines over inactivated or live attenuated pathogens is that large quantities of highly purified pathogens are not needed, and that both CTL and antibody responses are induced, which are crucial for establishing protective (i.e. relevant) immunity. The advantage of DNA vaccines over immunization with live attenuated pathogens, which also elicit a CTL response, is the reduced risk owing to the absence of virulent pathogen production through unforeseen mutations or recombinations. The disadvantage is that the magnitude of the immune response may be low, requiring boosting of the response with purified antigen, or cytokines (see below).

Technology for the oral administration of DNA vaccines has been developed, resulting in an IgA response at the mucosal surface similar to that achieved following oral administration of live attenuated vaccines. Intramuscular or intradermal administration of DNA vaccines, or inactivated viral vaccines do not generate an IgA response,[145] although, interestingly, immunization of human volunteers with an inactivated HAV vaccine resulted in expression of IFNγ as well as interleukin (IL)-10, and a CTL response, in addition to the normal antibody response.[142] DNA vaccines against several enteric viral pathogens have been developed and tested in animal models. However, none is currently licensed for human use.[145] DNA vaccines so far have not lived up to the initial excitement, particularly when tested in large animals or humans.[102] It remains to be seen whether mucosal adjuvants such as CpG containing DNAs or cytokines will result in improved protection without the toxicity problem of older adjuvants such as cholera toxin. Targeting vaccines to mucosal cells to elicit secretory IgA response by using receptor binding proteins such as reovirus σ1 protein is a promising approach, particularly with regard to the recent development of a model for intestinal Peyer's patch M cells.[50]

15.6.3 Vaccines and cytokines

Cytokines have a profound effect on the pathogenesis of foodborne viral diseases and on innate and adaptive immunity against virus infection. The effects of

interferons, the most studied cytokines in the context of virus infections, have been described in previous sections. The effects of various cytokines may be complementary or antagonistic. The cytokine tumor necrosis factor α (TNFα) was found to down-regulate the inhibitory effect of IFNα on human rhinovirus (a picornavirus) growth, and disease potential.[28] In rotavirus infected children, the severity of the disease correlated with lower levels of IFNγ, IL-6, and IL-10, and higher levels of TNF-α.[166] However, experiments with STAT-1 knockout (Stat-1 $-/-$) mice, which are defective in IFN response showed a potent antibody and T-cell response to rotavirus challenge and were protected against rotavirus infection following immunization with capsid protein VP6.[321]

15.6.4 Inhibitors of replication

Therapeutic control of virus infections have been the subject of intense investigation ever since viruses have been recognized as disease agents, and a full discussion is beyond the scope of this review. Several therapeutic strategies are currently under investigation and include the application of interferons, capsid function inhibitors, replication inhibitors, inhibitors of receptor binding, and protease inhibitors including nitric oxide releasing compounds.[188,344] A promising new field of research is aimed at investigating the effects of virus infection on host cell gene expression with a view to identifying metabolic pathways affected by the virus.[115] Although several picornaviruses have been studied regarding their effects on host gene expression, tangible results using this approach in the form of new antivirals are lacking. The genomic approach probably is more appropriate for viruses that produce latent or chronic infections in the host. Effects on cellular gene expression following infection of FrhK4 cells with the non-apoptotic HAV strain HM175 (clone 1) was compared with the effect of infection with the apoptotic strain HM175/18f.[187] Both transcription factors c-jun (AP-1) and c-myc were significantly up-regulated in 18f infected cells compared with clone 1. However, no transcriptional induction of IFNβ, IFNα2 or IFNγ was observed with either virus. These results are similar to the results reported for echovirus and poliovirus infection.[171,243] In contrast, in a mouse model of coxsackievirus induced myocarditis, more than 150 cellular genes were shown to be affected (> two-fold increase or decrease) as a result of virus infection.[306] Surprisingly, neither IFN nor IFN-stimulated genes (ISGs) such as 2–5 OAS or PKR expression were affected. Rotavirus infection, however, caused marked increase in 2–5 OAS and PKR levels.[85] In the absence of corroborating data on protein expression or activity, the significance of the observed effects remains to be determined.

15.6.5 Other cytokines

It is recognized that cytokines other than interferons play important roles in determining the outcome of viral infections. For example PV inhibits the secretion of IL-6, IL-8 as well as IFN.[92] Tumor necrosis factor TNFα was shown

to transform a trace rhinovirus infection into full blown disease by down-regulation of the IFN response.[28] Henke *et al.*[143] reported protection of mice from a lethal injection of a CVB 3H3 strain when a recombinant CVB3 strain expressing IFNγ was administered prior to or simultaneously. In mouse models, IL-12 was protective against lethal infection by coxsackievirus.[108,248] Undoubtedly IFN response modifiers such as TNF, and interleukins will be targets for future antivirals.

15.6.6 Virus detection in foods

The ability to detect viruses in foods is a significant component of foodborne viral disease control and prevention. This has proved to be difficult for several reasons. Traditional methods for virus detection based on cytopathic effect on infected cells in culture cannot be applied to viruses of the norovirus group because a cell culture host is not available. Similarly, wild-type HAV grows very poorly in the available cell culture hosts and usually without cytopathic effect. Molecular methods based on the detection of virus genomes by RT-PCR (see Fig. 15.3) have been the basic tools for food virologists. However, attempts to detect viruses in foods by RT-PCR have met with limited success because of complicated multi-step processes required to concentrate viruses from foods. Seeding experiments have revealed severe losses of virus during such purification procedures. Most protocols also result in unacceptable levels of contaminants that severely inhibit the enzymatic reactions employed in RT-PCR. Moreover, the level of virus in foods contaminated during production or handling is expected to be low. In most cases, the distribution of virus in foods is not uniform, creating a sampling problem. Also the time lag between food consumption and onset of disease can be days to weeks, so that contaminated food is either totally consumed or discarded.

To date, only a few instances of successful detection of a suspected viral pathogen in foods are known.[35,125,182,195] The significance of these successes has been questioned since viral genomic RNA isolated from inactivated virus often produce positive signals in RT-PCR reactions.[34,230,231]

15.7 Current research frontiers

There is increasing concern that foodborne viruses, because of their resistance to inactivation by environmental factors, could be deliberately added to the food or water supply to spread diseases. It has been known for a number of years that genomes of picornaviruses can be manipulated to express foreign genes.[143,162] It is also possible to completely synthesize infectious picornaviral genomes in the absence of a viral template.[65] Thus, global control of infectious virus stocks may prove limited in effectively preventing such a malicious act, because a viral genome can be synthesized that contains mutations in critical antigenic determinants or incorporates a critical modulator of innate immunity, and therefore

will not be easily amenable to control by currently available vaccines. Major efforts will be needed to develop virus isolation and detection protocols capable of detecting such mutated or recombinant viruses in a short period of time.[63]

As discussed in Section 15.6, vaccine research, specifically DNA vaccines and vaccine cytokine combinations to augment the effectiveness of vaccinations, will remain an important area of investigation. Modulation of cytokine levels (IFN and interleukins) to control viral infections and also nonviral diseases is still in its infancy, and will undoubtedly be an active area of research.[2,108,162,248,315]

15.8 Sources of further information

15.8.1 Recommended reading

The authors suggest the following publications for further reading as sources of additional information relevant to the topics discussed in this chapter.

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16

Pathogenic mechanisms of food- and waterborne parasitic disease

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16.1 Introduction: epidemiology of parasitic diseases

Food and water are essential elements for life. Unfortunately, they can also be the transmission vehicle for most intestinal protozoan and helminth parasitic disease throughout the world. These diseases can range from intestinal disturbances to infections in one's liver, lungs, muscle tissues, and brain (see Tables 16.1 and 16.2). Accurate prevalence data for parasitic infections worldwide are unknown owing to the lack of reporting; however, some groups of organisms have estimated prevalences, which can give an idea about the scope of the disease burden. The World Health Organization (WHO) estimated in 1995 that 40 million people are infected by trematodes worldwide, primarily in southeast Asia and eastern Europe (WHO, 1995). Nematodes (*Ascaris lumbricoides, Trichuris trichiura,* and hookworms) are another huge burden, infecting an estimated 2.8 billion people with clinically evident disease in half-a-billion and 155 000 deaths annually (Montressor *et al*., 2002). Most parasitic disease occurs in developing countries because of a lack of proper sanitation facilities to prevent fecal–oral transmission of infective encysted stages of the parasites. However, raw fish and meat can also be a source of transmission for many parasites, and cultural preparation practices may allow for continual infection of certain populations (Slifko *et al*., 2000). Interestingly, sushi, a traditional Japanese raw fish dish prepared with fish such as tuna, red snapper, salmon, or flounder in restaurants, is less contaminated with parasite larvae than fish used in rural areas of Japan. Nevertheless, one should be careful when consuming raw meat or fish dishes (Nawa *et al*., 2005).

Although parasitic disease in developed countries is less frequent than developing nations, it is still present. Food- and waterborne parasite infections

Table 16.1 Helminth contaminants of food and water

Genus	Species	Common name	Disease	Infective stages for humans	Common food source	Definitive hosts	Intermediate hosts	Location in humans	Treatment	Geographic location
Trematodes										
Clonorchis	*sinensis*	Human liver fluke	Clonorchosis	Metacercariae cyst in tissue	Fish	Humans, cats, ogs, pigs, rats, badgers, ducks (rarely)	Snail, fish	Bile ducts, liver	Praziquantel	Southeastern Asia and Russia
Opisthorchis	*viverrini*	Human liver fluke	Opisthorchiosis	Metacercariae cyst in tissue	Fish	Humans	Snail, fish	Bile ducts, liver	Praziquantel	Thailand, Southeastern Asia
	felineus	Human liver fluke	Opisthorchiosis	Metacercariae cyst in tissue	Fish	Humans, cats	Snail, fish	Bile ducts, liver	Praziquantel	Poland, Germany, Russia
Echinostoma			Echinostomosis	Metacercariae cyst in tissue	Fish, aquatic vegetation	Humans	Snail, fish	Intestine	Praziquantel	Korea and Southeastern Asia
Fasciola	*hepatica*		Fascioliasis	Metacercariae cyst	Watercress other salad vegetables	Human, sheep, other herbivores	Snail, fish	Bile ducts	Triclabendazole	Worldwide
Paragonimus		Lung-dwelling fluke	Paragonimosis	Metacercariae cyst in tissue	Crabs, crayfish, shrimp	Humans	Snail, fish	Lungs and brain	Praziquantel or Triclabendazole	Asia, South America, Africa
Cestodes										
Diphyllo-bothrium	*latum*	Broad fish tapeworm	Diphyllobothriasis	Plerocercoid cyst in tissue	Fish	Humans	Snail, fish	Intestine	Praziquantel, niclosamide	Russia, Japan, Europe, North America

Taenia	*saginata*	Beef tapeworm	cysticercosis	Eggs	Meat or egg-contaminated produce	Humans	Cows	Intestine	Praziquantel	Worldwide
	solium	Pork tapeworm	cysticercosis	Eggs or cysticercus in tissue	Meat or egg-contaminated produce	Humans	Pigs	Intestine and muscle tissues	Praziquantel for intestinal stage only	Worldwide
Echinococcus	*granulosus*		Cystic echinococcosis	Eggs or encysted larvae	Meat or egg-contaminated produce	Dogs, wild carnivores	Humans, ruminents	Liver, lungs	Surgery albendazole, mebendazole, praziquantel	Worldwide
	multi-locularis		Alveolar echinococcosis	Eggs or encysted larvae	Meat or egg-contaminated produce	Red and arctic foxes, domestic dogs and cats	Humans, small mammals	Liver primarily, lungs, brain and other organs	Liver transplant albendazole, mebendazole	North America and Europe
Nematodes										
Trichinella	*spiralis*		Trichinosis	Encysted larvae	Meat, grains with animal products	Carnivores, herbivores	Carnivores and herbivores	Skeletal muscles	Albendazole for adult worms in intestine only	Worldwide
Ancylostoma	*duodenale*	Human hookworm	Ancylostomiasis	Larvae	Soil, fecal contaminated food	Humans		Intestine	Mebendazole	Middle East, Africa, Europe
Ascaris	*lumbri-coides*	The large roundworm	Ascariasis	Eggs	Egg-contaminated produce	Humans		Intestine	Mebendazole, albendazole	Worldwide, primarily tropical regions
Trichuris	*Trichuria*	Whipworm	Trichuriasis	Eggs	Egg-contaminated produce	Humans		Intestine	Mebendazole, albendazole	Worldwide, primarily tropical regions

Adapted from the following references: CDC (2004), Macpherson *et al.* (2000), Doligalska and Donskow (2003), and Muller (2002).

Table 16.2 Protozoan contaminants of food and water

Genus	Species	Disease	Potential source of infection	Infective stage for humans	Definitive host	Intermediate hosts	Location in humans	Treatment	Geographic location
Toxoplasma	*gondii*	Toxoplasmosis	Meat, contaminated produce, milk	Oocysts, encysted bradyzoites	Cats	Any warm-blooded animal	Muscle and brain tissue	Pyramethamine, sulfonamides	Worldwide
Cryptosporidium	*parvum*	Cryptosporidiosis	Contaminated water, produce	Oocysts	Humans, ruminents		Intestine	Nitazoxanide	Worldwide
Giardia	*lamblia*	Giardiasis	Contaminated water, produce	Cysts	Humans		Intestine	Nitazoxanide	Worldwide
Cyclospora	*cayetanensis*	Cyclosporiasis	Contaminated produce	Cysts			Intestine	Trimethoprim-sulfamethoxazole	Worldwide, primarily in developing countries
Sarcocystis	*hominis*	Sarcocystosis	Meat, contaminated produce, water	Oocysts, encysted bradyzoite (sarcocysts)	Humans	Cattle and swine	Intestine and muscle	None currently	Unknown
Isospora	*belli*	Isosporiasis	Contaminated food and water	Oocysts	Humans and animals		Intestine	Trimethoprim-sulfamethoxazole	Worldwide, primarily in developing countries
Entamoeba	*histolytica*	Amebiasis	Contaminated food and water	Cysts	Humans		Intestine and liver	Iodoquinol, paromomycin and metronidazole (invasive disease)	Worldwide, primarily in developing countries
Balantidium	*coli*	Balantidiosis	Contaminated food and water	Cysts	Humans, pigs, rodents		Intestine	Tetracycline	Worldwide
Microsporidia	14 species are human pathogens	Microsporidiosis	Contaminated food and water	Spore	Humans and animals		Intestine	Albendazole, fumagillin	Worldwide

Adapted from the following references: CDC (2004), Acha and Szyfres (2001), Neva and Brown (1994), and Nichols (2000).

are well documented in the United States with an estimated 2.5 million cases occurring annually. However, this may be an underestimate, since *Cryptosporidium, Cyclospora, Giardia,* and one helminth, *Trichinella*, are the only notifiable foodborne parasitic diseases. Also, diagnostic methods are challenging for these organisms since they require a skilled microscopist to examine stool samples (excluding *Trichinella*). *Giardia lamblia* leads cases with an estimated two million cases each year; however, *Toxoplasma gondii* is the third leading cause of foodborne-related deaths (21%) after the foodborne bacteria, *Salmonella* and *Listeria* (Mead *et al*., 1999). The parasites commonly found in developed countries are ones which are able to slip through the cracks of societal sanitation and food inspections. For instance, the protozoan parasites *Giardia lamblia* and *Cryptosporidium parvum* infective cysts and oocysts, respectively, are resistant to the chlorination process present at most sanitation facilities. Also, there are no regulations or inspections of meat for the presence of *Toxoplasma gondii*, a protozoan parasite that can harm the fetus of women first exposed while pregnant or cause encephalitis in immunocompromised patients. Besides the protozoan parasite examples given, helminth disease is also present in the developed world (although at a lower incidence) owing to the globalization of travel and trade (Northrop-Clewes and Shaw, 2000). Therefore, besides the importance of treating those currently afflicted with parasitic diseases, research needs to be focused on developing better detection and prevention techniques to inhibit further morbidity and mortality throughout the world. Initially, the best way to address these issues is to understand the mechanisms of how these parasites cause disease in humans.

16.2 Mechanisms of disease

A general definition of a parasite may be an organism that derives benefit from living in or on a host, ultimately leading to the detriment of that host (Northrop-Clewes and Shaw, 2000). Some organisms complete their life cycle entirely with one host (monoxenic) and others complete their cycle indirectly using intermediate hosts (heteroxenic). The advantage of having intermediate hosts is that replication of the parasite can also occur in these hosts to increase the chance of dispersal (Doligalska and Donskow, 2003). In general, the sexual stage of a eukaryotic parasite's life cycle occurs in a definitive host (usually resulting in an environmentally resistant cyst or egg). The size of parasites (i.e. single or multicellular organisms) as well as the methods parasites use to infect and survive in a host can vary. Some parasites prefer to cause minimal symptoms or destruction to a host (e.g. *Toxoplasma gondii*) while others utilize host defense mechanisms to their advantage (e.g. *Cryptosporidium parvum*) for continual transmission to occur. The following sections will give more detailed examples of how different parasites cause disease.

16.2.1 Parasites that remain in the lumen of the intestine

Ascaris lumbricoides *and* Trichuris trichuria

Ascaris lumbricoides and *T. trichuria* are two chronic intestinal soil-transmitted nematodes endemic in developing countries. Both reside within the lumen of the human intestine as adult parasites, although *A. lumbricoides* migrates through tissues in the body prior to settling again in the intestine. *A. lumbricoides* 'the large roundworm' is one of the most prevalent intestinal nematode parasites infecting almost a quarter of the world's population (1.4 billion) in Asia, Africa, and Latin America, primarily. *T. trichuria* is also highly prevalent with 1 billion infections worldwide, and commonly is present in co-infections with *A. lumbricoides* (WHO, 1995). Humans become infected by these parasites after consumption of fruits, vegetables, or anything the sticky eggs attach to after release into the environment from fecal matter. Although no major outbreaks have occurred in industrialized countries, eggs of *Ascaris* species have been detected on fresh vegetables and individual cases have occurred (Northrop-Clewes and Shaw, 2000).

Both parasites have similar life cycles with the adult stages of the worms developing in the lumen of the intestine. However, when *Ascaris* eggs hatch in the duodenum, the larvae initially migrate through the liver, lungs, and upper alimentary tract. After passage into the upper alimentary tract, the surviving larvae return to the intestine and develop into adult worms in 65 days (O'Lorcain and Holland, 2000). Adult female worms produce over 2×10^6 eggs per day and can live for 1–2 years. Eggs that are released into the environment in warm climates survive for 2–6 months but an experiment in Russia showed that the eggs can survive in soil for up to 7 years (Muller, 2002). Infections by these parasites are unique in that adult worms do not multiply inside the host, but are the result of independent infections. Severe chronic infections can result from repeated exposure in endemic areas. Clinical disease associated with *A. lumbricoides* will be described next; however, chronic infection by *T. trichuris* has similar intestinal symptoms.

Although 85% of cases can remain asymptomatic, *A. lumbricoides* can cause a range of symptoms during its life cycle. The initial larval migration clinical manifestations may include pneumonitis, fever, and conjunctivitis. During the chronic stage of infection, when adult worms are present in the intestine, abdominal pain, anorexia, and nausea may occur. Serious complications can occur when the intestine is obstructed by multiple adult worms. The death toll from this parasite ranges from 60 000 to 100 000 people annually usually due to intestine or bile duct blockage (Muller, 2002). Besides disease, it is also thought that malnutrition leading to impaired growth and cognitive development of children may be exacerbated by this parasite. However, it is hard in helminth endemic areas to attribute these effects to *A. lumbricoides* alone (O'Lorcain and Holland, 2000). Treatment is successful with anti-helminthic drugs such as albendazole and mebendazole, but there is concern that widespread blanket treatment programs of infected and noninfected individuals may result in drug resistant strains of the parasite (Bennett and Guyatt, 2000). As with most

parasites, proper sanitation and education about the disease could prevent this parasite from being so widespread.

Research has focused on understanding how nematode parasites persist chronically in the intestine evading the immune system as well as why certain individuals have higher worm burdens than others in the same population. Results from these studies may aid the development of vaccination strategies, although the worm's ability to naturally evade expulsion and reinfect most individuals makes the task challenging. In general, intestinal worm infections result in an increase in T helper 2 (T_H2) response which includes an increase in infiltrating eosinophils, mast cells, IgA, IgE and mucus secretion into the intestine. Although the mechanisms for induction and the role these cells and molecules play for the host defense to worms are not entirely known, some details are starting to be elucidated. For instance, it has been shown that mast cells are capable of killing larval cells of the nematode *Trichuris spiralis* (Bradley and Jackson, 2004; Hayes *et al*., 2004). Also, an observational study of children infected with *A. lumbricoides* and treated showed that those with high levels of *Ascaris*-specific IgE antibodies were less likely to become reinfected than those with high total IgE levels. The level of *Ascaris*-specific IgE antibodies were not found to correlate with exposure; therefore, it was believed that genetic differences in the hosts caused the variable immune response (Lobos, 1997). However, another study showed that noninfected individuals in endemic areas had elevated T helper 1 (T_H1) cytokines compared with worm-infected individuals in the same population; the authors suggested further research to determine if T_H1 cytokines are important for expulsion of worms or if they are just indicative of the absence of parasite infection (Geiger *et al*., 2002). Clearly, more research is necessary in this field to better understand the differences between persons able to clear their infections and those who remain chronically infected.

Host genetics appears to be a factor for the burden of *A. lumbricoides* and *T. trichuria* infection since certain families appear to have higher worm burdens than others. This has been studied more extensively with another nematode, *Trichuris muris*, in its mouse model of infection. Mouse models of infection are beneficial for studies such as these since researchers can use mice with either identical or genetically manipulated backgrounds. Currently, there is no animal model for *A. lumbricoides* infection, which limits direct study of this organism except for blood sampling in human populations (Bradley and Jackson, 2004). Using the mouse model for *Trichuris muris*, it was shown that small dose infections (which are more likely to occur in certain human populations) over longer periods of time promote susceptibility to infection due to an elevated T_H1 response, whereas large doses promote resistance because they generate a T_H2 response immediately. Besides the negative associations of nematode infection, it has been shown that there is a reduced risk of allergic disease and inflammatory bowel syndrome in patients chronically infected with helminths due to the shift away from the T_H1 immune response responsible for those diseases (Hayes *et al*., 2004).

The 'hygiene hypothesis' states that because of improved hygiene, vaccination and antibiotics available in developed countries, the immune systems of people in these countries is altered and it responds inappropriately to innocuous substances (e.g. pollen). A detailed review about the striking absence of allergic diseases and autoimmune disorders in developing countries is available in Yazdanbakhsh *et al*. (2002). The disorders associated with the absence of helminths calls to question the benefits of treating individuals for a disease that it usually asymptomatic. Further research on host and parasite interactions will hopefully lead to answers to that question and also may identify parasite molecules which regulate the immune system which could be used as alternative chemotherapeutic methods for allergies (Yazdanbakhsh *et al*., 2002).

Giardia lamblia

Giardia lamblia is a protozoan parasite of most mammals, including humans. It is considered the most common cause of intestinal parasite disease in the developed world and worldwide it causes an estimated 280 million cases each year (Lane and Lloyd, 2002). In the United States, 2 million cases are estimated to occur annually (Mead *et al*., 1999). In fact, from 1985 to 2000, *G. lamblia* was the causative pathogen in 39% of all reported waterborne outbreaks in the United States (Mandell *et al.*, 2005). The parasite has a two stage monoxenic life cycle consisting of an asexually replicating trophozoite and a dormant environmentally resistant encysted form. Intestinal infection occurs when a cyst, ingested from fecally contaminated water or food, passes through the acidic stomach and releases a trophozoite into the small intestine. Trophozoites primarily attach to and replicate on the lumenal surface of the small intestine. Once exposed to bile fluid, some parasites encyst and are passed out in the feces to continue the life cycle (Adam, 2001; Lane and Lloyd, 2002). Cysts are extremely stable in the environment, especially in cold water, and infection can occur with as few as 10–25 cysts (Mandell *et al*., 2005).

Giardiasis, the clinical disease caused by the parasite, causes symptoms of diarrhea and malabsorbtion of nutrients in 20–80% of patients infected. Symptoms typically occur 1–2 weeks post-ingestion of cysts, and last for 2–4 days. The infection is usually self-limiting in immunocompetent individuals, although treatment is available and can shorten the course of illness (Eckmann, 2003). The ability of *Giardia* to remain in the lumen is due to attachment of the trophozoite's ventral disk to brush border microvilli of enterocytes. Disruption of the intestinal brush border by physical attachment has been proposed as a potential mechanism for diarrheal illness and malabsorbtion of nutrients (Lane and Lloyd, 2002). However, biopsies of *Giardia*-infected intestines appear to have normal mucosa and intact epithelial cells without inflammation (Oberhuber *et al*., 1997). Regardless of the lack of macroscopic evidence for disease, subtle changes such as the reduction of microvilli height and decrease in expression and activity of digestive enzymes are apparent and may contribute to the disease symptoms. Clearance of the parasite is believed to be due primarily to secretory IgA antibodies which bind to the trophozoite and prevent attachment to the lumen

(Mandell *et al.*, 2005). The parasite has the ability to undergo antigenic variation with variant surface proteins (VSPs), and it has been shown that certain VSPs are selected against in immunocompetent hosts. However, the potential role these surface antigens play in evading the host immune response is not understood since clearance occurs within a week after infection in immunocompetent hosts (Lane and Lloyd, 2002). Other proposed roles of these molecules for *Giardia* have included protection against intestinal proteases, adaptation to a variety of hosts, and reinfection by the parasite in pre-exposed hosts (Mandell *et al*., 2005).

Animal models are available to study giardiasis; however, they are not ideal. Many studies use *G. muris* infected mice to study giardiasis since most strains of *G. lamblia* are not infective to mice. *G. muris* infection usually results in a self-limiting infection similar to humans allowing researchers to study the immunobiology of the disease. Unfortunately, the *G. muris* system does not have similar clinical manifestations, such as diarrhea, and the genetic similarities between the two parasites are largely unknown. For these reasons, the use of this model by researchers for *G. lamlia* infection is limited. The *G. lamblia* strain GS/M-H7 strain, however, is able to infect mice and is infective to humans. In spite of this, this strain belongs to a different genetic lineage (group B) from the primary laboratory strain of *G. lamblia* (group A) used by most researchers for genetic and biochemical studies making this strain unattractive for current studies (Eckmann, 2003).

Although each model system has deficiencies, they still help researchers begin to understand how *Giardia* causes disease. Using model systems, researchers have been able to verify the importance of IgA antibodies for ultimate clearance of *Giardia* as well as other immune factors which may play minor roles (e.g. NO, antimicrobial peptides) (Eckmann, 2003). Also, the interaction of *Giardia* and other organisms (pathogenic or not) in the intestine has been studied. For instance, one study showed how the presence of natural microflora in the intestine can affect the susceptibility or resistance of a host to *Giardia* infection. Mice from different vendors containing different microflora were infected with the GS/M-H7 strain and were found to be either resistant or susceptible to infection depending upon their vendor source. When the mice were given an antibiotic, both groups of mice were susceptible to disease. This study could potentially give rise to a new probiotic prevention method for giardiasis (Singer and Nash, 2000).

Besides animal models, the ability to grow *G. lamblia* axenically *in vitro* allows researchers to look at the trophozoite's interaction with cell culture intestinal epithelial cells (Eckmann, 2003). Also, *G. lamblia* can encyst *in vitro* under conditions of basic media and bile salts allowing researchers to look at this critical developmental pathway for the continuation of the parasite's life cycle. The ability to grow *G. lamblia in vitro* has led to the development of a stable transfection system allowing genetic manipulation of the organism to further aid research in understanding its biology (Adam, 2001).

A large portion of *Giardia* research has been devoted to understanding the basic biology of this ancestral eukaryote which may provide a link between early

eukaryotes and prokaryotes. In fact, some believe it is more closely related to prokaryotes than eukaryotes because of the absence of mitochondria and peroxisomes but presence of a prokaryotic-sized small subunit rRNA, and bacteria-like metabolic enzymes. Notwithstanding these prokaryotic-like metabolic differences, *G. lamblia*'s nuclei remind researchers that *Giardia* is still a complex organism. *Giardia* contains two synchronously replicating nuclei, each with five chromosomes in the trophozoite stage. Both nuclei are transcriptionally active and they cycle between a diploid and tetraploid state during the cell cycle. These peculiarities have led researchers to question how *G. lamblia* regulates its transcription and translation with two identical nuclei. The high compactness of the genome, extremely short or absent 5′ untranslated regions (5′UTRs), absense of capped mRNA, and lack of introns in most genes are peculiarities which further give evidence of *Giardia*'s closer evolutionary relationship to prokaryotes (Adam, 2000, 2001; Svard *et al*., 2003). However, despite these differences in genomic structure and RNAs, the transcription machinery still resembles that of eukaryotes. Understanding *Giardia*'s basic biology will hopefully lead to better prevention and treatment strategies which aim at targeting these genetic differences between *Giardia* and host eukaryotic organisms (i.e. humans). Also, basic research of this early eukaryote that is easily cultured and genetically manipulated *in vitro* will lead to a better understanding of more evolved and complex eukaryotic systems (Adam, 2001).

16.2.2 Parasites that invade and remain in the enterocytes of the intestine

Cryptosporidium parvum

Cryptosporidium parvum is an obligate intracellular protozoan parasite in the phylum Apicomplexa. The strains of *C. parvum* are classified into two major genotypes as either type I or type II. Type I strains usually only infect humans while type II strains infect both animals and humans (Leav *et al*., 2003). Infection occurs after ingestion of food or water contaminated with environmentally resistant oocysts shed from fecal matter. The most infamous outbreak of cryptosporidiosis occurred in Milwaukee, WI, in 1993 where over 400 000 people were infected from a contaminated water source (MacKenzie *et al*., 1995). The infectious dose of the type I strain involved in the outbreak was estimated as 1–10 oocysts (Dillingham *et al*., 2002). When oocysts are ingested, they are resistant to the acidic environment in the stomach and pass into the intestine. In the presence of bile salts and pancreatic enzymes, the sporozoites excyst and invade the epithelium surface of the intestine in both the jejunum and terminal ileum. The parasite ultimately resides in a compartment separate from the host cytoplasm, but within the host's plasma membrane. This niche is where the parasite begins its monoxenic life cycle to produce more oocysts for shedding (Tzipori and Griffiths, 1998). Approximately 10^{10} oocysts are shed during a human infection while 10^{7} oocysts/gram of feces are shed by an infected calf. This large amount of shedding allows this parasite to contaminate the environment easily and to continue its life cycle in additional hosts.

Although *C. parvum* causes many infections in developing countries where sanitation is minimal, the developed world is not entirely immune to infection by this parasite owing to its ability to evade standard water sanitation procedures such as chlorination (Dillingham *et al*., 2002).

Symptoms of gastrointestinal infection appear 5 days to 2 weeks after ingestion of an oocyst and may include severe watery diarrhea, malaise, fever, vomiting, and weight loss. Twelve liters of water can be lost per day, resulting in severe dehydration. The symptoms of the infection can last for several days to 5 weeks in immunocompetent hosts, and it can also lead to death in untreated chronically infected AIDS patients from persistent diarrhea as well as cholangitis and pancreatitis (Leav *et al*., 2003). The severity of infection in AIDS patients points to the importance of $CD4^+$ T cells for ultimate clearance of infection. When symptoms subside in immunocompetent individuals, shedding of oocysts may still occur for up to 2 weeks, indicating the importance of educating patients about good hygiene during and after infection (Dillingham *et al*., 2002). Until recently, effective drugs were unavailable for *C. parvum.* Although Nitazoxanide has recently been approved by the Food and Drug Administration (FDA) for treatment of cryptosporidiosis, it is still awaiting approval for use with immunocompromised patients, who would benefit the most from its use. This drug has been used around the world since 1996 against a broad range of protozoan and helminth parasites (Fox and Saravolatz, 2005).

A human feeding study of people aged 20–45 determined the ID_{50} (infectious dose for 50% of subjects) of a bovine type II *C. parvum* strain to be 132 oocysts in immunocompetent individuals with 30 oocysts as the lowest infectious dose. However, a third of the volunteers were asymptomatic carriers who did not experience characteristic symptoms of a *Cryptosporidium* infection (DuPont *et al*., 1995; Leav *et al*., 2003). This variance in host susceptibility is believed to be due to *C. parvum* strain differences as well as to the age and genetics of the host. One potential reason for age differences in infection is that *C. parvum* sporozoites possess surface lectins which mediate attachment to specific carbohydrates on the intestinal mucosa. The composition of the intestinal mucosal layer can vary depending on microbial flora and age of the host, and this could relate to the higher rate of infection of young and older individuals (Mosier and Oberst, 2000).

While it is known that *C. parvum* has adapted well for transmission through fecal matter in the environment (Okhuysen and Chappell, 2002), research to elucidate more details about the pathogenesis of *C. parvum* have been hindered by the lack of a long-term *in vitro* cultivation method as well as the lack of a transfection system for studying gene functions. The longest cultivation described currently is 25 days (Hijjawi *et al*., 2001). Much of the study of virulence has elucidated proteins involved in adhesion and invasion with help from homologues from other evolutionarily related parasites such as *Toxoplasma* and *Plasmodium* and monoclonal antibodies to surface proteins. Putative proteases and a haemolysin have been described but their role in infection still remains to be studied. Persistence in host tissues has been shown

to be due to activation of nuclear factor B (nFKB), which inhibits host cell apoptosis; parasite mediators of this activation remain to be elucidated (Okhuysen and Chappell, 2002). How the parasite causes the disruption of fluid and nutrient transport in the intestine also is not known. However, it has been noted that the loss of the microvilli, decreased levels of microvilli disaccharides, and presence of the organisms at the surface may interfere with nutrient absorption and fluid transport (Leav *et al*., 2003; Carey *et al*., 2004).

Although the simplest view of preventing *C. parvum* infection would be to eliminate it from drinking water, some studies elude to the potential benefit of low levels of exposure to oocysts (which do not cause clinical disease). Researchers have found that a higher percentage of seropositive individuals to *C. parvum* antigens in a town results in a lower risk for outbreaks. There is no direct evidence for protective immunity to *C. parvum* from specific serological responses; however, this research is suggestive of such immunity. Another study found 36–44% of subjects to have a strong serological response to *C. parvum* even though their public water was protected from human and animal fecal waste. This suggests that food may also be an important source of infection (Frost *et al*., 2005).

16.2.3 Parasites that are in the intestine and cross the intestinal barrier

Trichinella spiralis

Trichinella species are nematode parasites of most mammals, yet humans are more susceptible to developing clinical disease after infection. Currently, there are seven identified species of *Trichinella*; however, the species usually associated with human infection is *T. spiralis*. All life cycle stages for this parasite occur within a single host. Transmission occurs after ingesting encysted larvae present in the skeletal muscle of a host. Following release from acid pepsin digestion in the stomach, the larvae invade and remain within the small intestine epithelial cells. After maturation into sexually mature adult worms (22–24 hours post-ingestion), the worms mate and produce larvae after 4 days. These larvae travel in the circulatory system to striated muscle and the heart, brain, lungs, retina, lymph nodes, pancreas, and cerebrospinal fluid. However, only the larvae that invade the skeletal muscles are capable of long-term survival. Adult worms in the intestine only remain viable for 2–3 weeks post-infection (Bruschi and Murrell, 2002; Muller, 2002).

Transmission of this disease has been associated mainly with the consumption of contaminated undercooked pork. However, imported horse meat has been a source of infection in France. This source is puzzling since horses are considered to be herbivores. However, one study traced infection of these animals back to the feeding of contaminated raw pork by horse dealers and breeders to increase their horse's weight prior to sale. Freezing or proper cooking usually prevents infection (Murrell *et al*., 2004). Countries in the European Union inspect all pork intended for interstate and domestic consumption. Infection in the United States has largely been controlled in the pork industry

through preventative measures such as strict regulations on feed given to pigs and elimination of rodents in swine barns (Schantz and McAuley, 1991; van Knapen, 2000).

Clinical severity of trichinosis varies depending upon the species involved, amount of larvae ingested as well as host factors such as age, sex, ethnicity, and immune status. The disease typically ranges from 7 to 30 days with two phases of infection. The first phase (enteral) is characterized by an intestinal inflammatory infiltrate of eosinophils, neutrophils, and lymphocytes that occurs after the initial invasion of the parasites. Symptoms during this phase may include nausea, vomiting, pain, and watery diarrhea. The next phase, parenteral, occurs when the parasite migrates into other tissue sites causing inflammation and allergic responses. This phase can cause a range of cardiovascular, renal, and central nervous system symptoms. The combination of eosinophilia, muscle pain, fever, and gastrointestinal disturbances suggest a diagnosis of trichinosis. However, serological or PCR (polymerase chain reaction) based tests as well as muscle biopsies are used to confirm *Trichinella* infection. Albendazole is effective for removing adult worms in the intestine; however, unfortunately it is not affective against the encysted larvae in the muscle tissues. Normally patients recover but can have persistant muscle pain, fatigue, and cardiovascular pains due to chronic myositis (Bruschi and Murrell, 2002; Muller, 2002; Mandell *et al*., 2005).

Most research on *Trichinella*'s mechanism of disease has involved understanding how the host reacts to infection by the parasite. Mouse models are commonly used to study host and *Trichinella* interactions. With these models it has been shown that a T_H2 cytokine response is essential for clearance of adult worms from the intestines of mice. T_H2 ultimately results in the production of antibodies which aid in the removal of foreign antigens. However, it is unclear how this response clears adult worms from the gut since no antibodies against adult parasite antigens have been identified (Appleton and Romaris, 2001). On the other hand, there are two immunodominant groups of antigens which develop after 2 weeks ('early') or after 4–5 weeks ('late' or TSL-1 antigens) during the larval stage of trichinosis.

TSL-1 antigens are the focus of most studies because of the long-term antibody response towards them (ideal for serodiagnostic techniques) as well as the ability of antibodies against TSL-1 antigens to aid protective immunity (Denkers *et al*., 1991; Appleton and Romaris, 2001). These antigens have been found on the larval body surface and in excretory-secretory products (ESP) and are believed to be important for parasitism by this nematode. Although most of the proteins in the TSL-1 antigen group are uncharacterized, it is known that a unique carbohyrdate, tyvelose, is responsible for their immunodominance. This carbohydrate has been shown to be present only in the larval developmental stage of the parasite. In a rat model of infection, antibodies towards tyvelose are important for clearance of a challenge infection of *Trichinella* larvae in the intestine emphasizing the importance of this carbohydrate (Carlisle *et al*., 1990; Appleton and Romaris, 2001). Vaccination studies in mice using oral or nasal

routes of administration of parasite antigens have been protective (McGuire *et al*., 2002).

Besides analyzing the host immune response to *Trichinella*, researchers recently used a microarray screen to identify host genes up- or down-regulated in the muscle tissues of mice post-infection to understand how the parasite transforms what is thought to be a terminally differentiated muscle cell into a specialized (no longer differentiated) cell for survival of the parasite (Jasmer, 2005; Wu *et al*., 2005). Although the genome has not been sequenced, an expressed sequenced tag (EST) library of a *Trichinella sprialis* derived from the mRNA of adult, immature larval (prior to muscle encystation), and muscle stages was recently sequenced. The resulting data gave insight into the transcriptional differences between the three stages, and may help researchers start to understand important worm genes that induce host cell modification or aid parasite survival. In fact, only 4% of the ESTs sequenced were present in all three stages. Most work to analyze these findings will need to be done in other model systems since genetic manipulation is not possible yet for *Trichinella.* However, the identification of putative proteases, metabolic genes, glycan synthesis genes (potentially important for tyvelose synthesis), as well as secreted proteins should aid future drug and vaccine development (Wu *et al*., 2003; Mitreva *et al*., 2004).

Taenia solium

Taenia species are some of the oldest described helminths infecting humans. *T. solium,* the pork tapeworm, and *T. saginata,* the beef tapeworm, are the most-studied species today because their definitive hosts are humans. *T. solium* is the cause of both taeniasis and cystercercosis, which are infections of the intestine by the adult worm and infection of extra-intestinal tissues by the larvae, respectively (Hoberg, 2002; Levine *et al*., 2004). *T. saginata* only causes intestinal taeniasis in humans. In 1992, it was estimated that 50 million cases of human taeniasis/cysticercosis and 50 000 deaths due to neurocysticercosis (NCC) occur annually. NCC occurs when larvae infect and encyst in the neural tissues of a host. The estimates of the prevalence of infection may not be accurate since cysticercosis is not a reportable disease, and diagnosis is challenging because it is based on neuroimaging, serology, and chance of exposure (Hoberg, 2002). An estimated 1000 cases of NCC each year in the United States are due primarily to immigrants of hispanic origin or from travel to Latin American countries (Pawlowski *et al*., 2005). Prevention of foodborne transmission in the United States is most likely aided by regulations set in the US pork industry for control of other parasites such as *T. spiralis* as well as stricter sanitation measures which are lethal to the infective egg stage (Schantz and McAuley, 1991; van Knapen, 2000; Pawlowski *et al*. (2005). See Pawlowski *et al.* (2005) for further information on worldwide control of *T. solium*.

The life cycle of *T. solium* usually involves humans as definitive hosts and pigs as the intermediate hosts. The definitive cycle begins when a human ingests undercooked pork containing cysticerci (an encysted larvae). Once passed into

the intestine, the larvae can develop into adult worms which undergo sexual development to produce eggs or gravid proglottids that are passed in the feces. These eggs can remain viable for up to 8 months in warm and humid conditions. An intermediate host, pig or human, becomes infected after consuming an infective egg from fecal contamination in the environment or by autoinfection caused by reverse peristalis (only in humans). Once passed through the acidic stomach, the eggs hatch in the intestine releasing an oncosphere which can invade the epithelial layer of the gut and spread to muscle and neural tissues causing cysticercosis. After 9 to 14 weeks the parasite will develop into infective cysticerci. When this stage is eaten by a definitive host, the cycle starts again with adult worms forming 3–4 months post-infection. If left untreated, adult worms can persist in a person's intestine for up to 25 years (Hoberg, 2002; Hawk *et al*., 2005).

Clinical symptoms of taeniasis may include hunger pains, change in appetite, weight loss, diarrhea, or constipation. However, taeniasis may be asymptomatic, which poses a problem for the determination of the prevalence of this parasite in studied populations (Hoberg, 2002; Muller, 2002). Neurocysticercosis (NCC) symptoms may include epileptic seizures, sensory and movement defects, dementia, and meningitis, as well as symptoms of increased pressure in the brain, such as nausea, vomiting, and headaches. In endemic areas in Asia, Africa, and Latin America, it has been shown that 50% of late onset epilepsy is due to NCC (Hawk *et al*., 2005). Although the luminal infections by adult tapeworms can be treated readily by praziquantel, this stage of infection usually is undetected (Flisser *et al*., 2004). Also, treatment of the adult stage parasites does not eliminate the reservoir of parasites present as cysticerci in intermediate hosts. Therefore, current research has focused on understanding the host response to the parasite to develop a vaccination strategy for its eradication in animal intermediate hosts.

It has been shown that oncospheres are susceptible to complement and antibody-mediated clearance. In fact, passive immunization studies in rodents using antibodies from *T. taeniaformis* (taeniid cestode of rodents) infected animals have shown protection against oncosphere infection in naïve animals (Mitchell *et al*., 1977). On the other hand, cysticerci in tissues are not susceptible to the host immune response. This is believed to be due to several parasite-derived molecules such as a cysteine protease (which digests antibodies), taeniaestatin (a protease inhibitor that helps protect against complement-mediated clearance), and various other molecules that aid in the defense against reactive oxygen species. Regardless, vaccination development has focused on finding immunogenic oncosphere antigens to produce a recombinant protein vaccine for intermediate hosts, thereby interrupting that stage of the parasite's life cycle (White *et al*., 1997). Some researchers recently described the generation of a recombinant oncosphere antigen vaccine which induced an almost complete protection of pigs (99.5%) from challenge with *T. solium* (Flisser *et al*., 2004). This experiment gives hope for a strategy to eradicate this parasite as well as *T. saginata.*

Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite of virtually all animals. Depending on the geographic region, it is estimated that 4–85% of women of childbearing age are seropositive. Cases of toxoplasmosis are usually asymptomatic in immunocompetent individuals; therefore, the exact incidence of infection in the general population is unknown. However, pregnant women and those who are severely immunocompromised can be severely affected by the disease. Prenatal infections have an incidence of infection ranging from 1 to 120 per 10 000 births and an estimated 500–5000 newborns are diagnosed with congenital toxoplasmosis in the United States each year (Tenter *et al*., 2000; Boyer *et al*., 2005).

The definitive hosts for the parasite are in the family Felidae, which includes the domestic cat. A cat becomes infected after consuming raw meat containing encysted parasites (e.g. a mouse). The parasites are released in the cat's intestine and undergo sexual replication to form environmentally resistant oocysts. These oocysts can contaminate fresh produce and municipal water systems as evidenced by the outbreak in British Columbia in 1995 where an estimated 2800–7000 people were infected (Bowie *et al*., 1997). Once ingested by intermediate hosts, the parasite undergoes rapid asexual replication as a tachyzoite form until it converts into the slowly growing encysted bradyzoite in muscle and brain tissues. Once ingested from undercooked meat, these encysted bradyzoites are resistant to acid pepsin digestion and can continue the parasite's life cycle in either the definitive or intermediate hosts. The intermediate host cycle (virtually all warm-blooded animals) from carnivorism can exist autonomously without interaction with the cat (Dubey *et al*., 1998). Epidemiological studies suggest that the ingestion of bradyzoite cysts in raw meat are an important source of infection in the United States (McAuley *et al*., 1994; Jones *et al*., 2001).

Clinical disease depends on the immune state of an infected host. After ingestion of an oocyst or bradyzoite, the parasite invades the host's intestinal epithelium layer and begins to replicate. Immunocompetent individuals may have mild flu-like symptoms during this stage of infection, but eventually in response to the host's immune system the tachyzoites convert into encysted bradyzoites. These cysts will remain in the host as an asymptomatic chronic form in muscle and brain tissues hidden from the host's immune response. If a host becomes immunocompromised (e.g. AIDS, organ transplants), the parasite is able to reactivate into the tachyzoite form, causing encephalitis, which can potentially lead to death. In fact, 40% of AIDS patients are affected by toxoplasmic encephalitis.

Pregnant women who are infected for the first time during pregnancy can pass the parasite congenitally to their infants. Most infants will have no symptoms at birth, but may develop chorioretinitis and neurological damage by adolescence. However, 10% of infants are stillborn and another 10–23% have hydrocephalus, retinochoroiditis, or intracranial calcifications which can lead to progressive mental disabilities. Although only 15% of women of childbearing age are infected in the United States, the low seroprevalence of *T. gondii* points

to the importance of screening and educating pregnant women about the foodborne risks of *T. gondii.* This would help ensure initial infection by *T. gondii* does not occur during pregnancy. Currently, only a few women are screened for toxoplasmosis and there is a call for doctors to increase prenatal screening and education. In France, standard screening and education has helped decrease the amount of congenital infections by 50% (Boyer *et al*., 2005). Treatment is available and mostly effective for the tachyzoite stage of infection when reactivation occurs in immunocompromised patients. The drugs of choice are usually pyramethamine and sulfonamide drugs. However, many people have allergies to sulfa drugs and both of these drugs are ineffective against the chronic stage of the disease (Miller *et al*., 1999; Tenter *et al*., 2000; Bhopale, 2003a,b). Vaccine development is ongoing for animals and humans and is reviewed in Bhopale (2003a).

Toxoplasma gondii has been utilized as a model organism for studying immunity to intracellular pathogens. This is due in part to the ease of culturing *T. gondii in vitro* in virtually any cell line and the multiple mouse models available for *in vivo* studies. During the initial acute infection, tachyzoites stimulate the production of IL-12 which induces the production of IFN-gamma by natural killer and T cells. IFN-gamma in turn activates macrophages which are able to kill tachyzoites after phagocytosis. Activated macrophages also produce nitric oxide, which is capable of killing the tachyzoite form of the parasite. However, *T. gondii* tachyzoites are induced to convert into bradyzoites in the presence of IFN-gamma and nitric oxide (as well as other unknown factors) and once converted can evade killing by the immune system (Miller *et al*., 1999).

Various *in vivo* mouse models are available to study *T. gondii* infection depending on whether one is interested in studying the acute or chronic stage of infection. Acute phase refers to the initial dissemination of the parasite in the mouse, while chronic phase refers to the presence of cysts in the brain typically 3–4 weeks after infection. As with other diseases, a variety of host and parasite factors are important for the eventual pathology of infection. Type II strains (Prugniaud [Pru], ME-49) are the most prevalent cause of toxoplasmosis (Sibley *et al*., 2002). These strains are typically used to study bradyzoite development since they are easily switched to bradyzoites *in vitro* and cause high cyst levels in mouse brains *in vivo* without severe acute signs of infection ($LD_{50} > 10^3$ parasites). Similarly, certain mouse strains are either susceptible or resistant to acute infection. For instance, BALB/C mice are susceptible to acute infection and will die within a week after intraperitoneal (IP) injection of type II strains (Suzuki *et al*., 1993; Zenner *et al*., 1998; Sibley *et al*., 2002). Conversely, CBA/J and C57 mice are resistant to acute infection when injected IP with type II strains and form high numbers of cysts in their brains. The resistance of a mouse strain to acute infection has been linked to the L^d gene of MHC class I antigens of the H-2D region as well as the ability to produce IFN-γ (Gross *et al*., 1996).

Since *T. gondii* is easily cultured *in vitro* and studied *in vivo,* and genetic manipulation techniques are available for gene knockouts and complementation,

it has been named the 'model apicomplexan'. The phylum Apicomplexa includes other parasites such as *Cryptosporidium parvum* (discussed above) and *Plasmodium falciparum*, the causative agent of malaria. Apicomplexans are structurally unique from other eukaryotic organisms owing to the presence of the apical organelles named the micronemes, rhoptries, and dense granules. Neither *C. parvum* nor *P. falciparum* is easily cultured *in vitro,* and established genetic techniques are not available for *C. parvum*. Therefore, genes of those organisms have been expressed in *T. gondii* to help elucidate their functions (Kim and Weiss, 2004).

Invasion of host cells by apicomplexan organisms has been characterized in *T. gondii* and is thought to be similar in most species in the phylum. There are three basic steps after attachment led by a family of surface antigen proteins (SAG). Initially microneme proteins are released after attachment, some of which aid adhesion and motility of the parasite into the host cell (MIC2). Next, rhoptry proteins are secreted during invasion and aid the formation of the parasitophorous vacuole (PV) which encases the parasite in the host cell and remains separate from the host's endocytic pathway. Rhoptry proteins are also believed to be important for recruitment of the mitochondria (ROP2) which is believed to aid nutrient acquisition. Dense granules are the final component of invasion and are thought to further modify the PV during growth of the parasites as tachyzoites and bradyzoites. The hope is that further characterizing proteins during invasion and growth of the parasite will lead to a better understanding of apicomplexan biology in general and eventually lead to better treatments for the diseases caused by these organisms (Black and Boothroyd, 2000; Kim and Weiss, 2004).

Research on *T. gondii* biology and pathogenesis has exploded over the past 20 years owing to the AIDS epidemic and development of molecular genetic tools (Roos *et al*., 1994). The sequencing of EST libraries and the genome as well as the creation of a searchable database with bioinformatic tools will continue to expand research in the field (Ajioka *et al*., 1998; Kissinger *et al*., 2003). As with many protozoan parasites, the vast majority of genes for *T. gondii* have no known functional homologues in other organisms (about 80–85%) (Li *et al*., 2003).

One of the current research frontiers is to identify genes important for bradyzoite development and establishment of chronic infection. As mentioned earlier, there are no drugs available to treat the chronic stage of disease which may reactivate when individuals become immunocompromised. Some bradyzoite-specific proteins were identified in the past from monoclonal antibody libraries, but now genomics opens the door for a new area of research. For instance, researchers have looked at microarrays comparing the tachyzoite and bradyzoite growth stages to try to identify bradyzoite-specific genes (Cleary *et al*., 2002). In addition, insertional mutagenesis studies have been performed to identify parasites unable to undergo bradyzoite development either *in vitro* or *in vivo* (Knoll and Boothroyd, 1998; Knoll *et al*., 2001; Matrajt *et al*., 2002; Singh *et al*., 2002). Identifying genes important for *T. gondii* development not only

will help drug development, but may eventually allow for the production of a non-encysting strain for vaccine development. This vaccine could be used for agricultural animals and cats which are the primary sources of transmission for this parasite.

Entamoeba histolytica

Entamoeba histolytica is an ameboid protozoan parasite of humans causing the disease, amebiasis. The prevalence of this parasite is difficult to estimate because of poor sampling as well as the lack of a low-cost test to distinguish *E. histolytica* from *E. dispar*, a non-invasive relative believed to be the source of most asymptomatic infections. However, it is believed that up to 50 million cases of invasive *E. histolytica* occur each year, resulting in 100 000 deaths in developing nations. This makes this parasite the fourth leading cause of death due to a protozoan following the vectorborne parasites *Plasmodium falciparum*, *Trypanosoma cruzi,* and *Leishmania major* (Mandell *et al*., 2005). This parasite is also present in the United States with a 4% prevalence rate due primarily to immigrants and refugees. *E. histolytica* can live and replicate in the lumen of the intestine (trophozoite form) as well as invade the mucosal and epithelial layer. After invasion, the parasite can infect almost any other organ in the body, but most frequently it causes liver abscesses after passage into the bloodstream. The majority of people infected by *E. histolytica* have a non-invasive asymptomatic infection in which the trophozoites attach to the mucosal layer via a galactose/*N*-acetylgalactosamine (Gal-GalNac) lectin on the parasite's surface. The parasites produce cysts in the intestinal lumen which can then be ingested by other hosts after food or water is contaminated with the infectious fecal material. Extensive studies have not been conducted of people with non-invasive amebiasis; however, it is estimated that 10% of asymptomatic carriers eventually develop invasive disease and the rest clear infection within 18 months (Espinosa-Cantellano and Martinez-Palomo, 2000; Mandell *et al*., 2005).

Invasive amebiasis has a gradual onset over 1–3 weeks after the initial consumption of a cyst. The resulting symptoms can include dysentery, moderate abdominal pain, and no fever, which distinguishes it from typical bacterial gastrointestinal infections. Ulcers also form in the colon and rectum from tissue necrosis from the lytic activity of the parasite once it passes beyond the mucosal layer. After reaching this stage of infection, the parasites may migrate to the liver and cause an amebic liver abscess. A high rate of mortality is associated with ruptured abscesses. In rare cases, the parasite can also form abscesses in the brain which are associated with a high and rapid death rate without drug therapy with metronidazole (Espinosa-Cantellano and Martinez-Palomo, 2000). Antibodies (IgA type) against *E. histolytica* Gal/GalNac lectin have been found to delay the onset of reinfection in humans, and vaccination of mice with the lectin resulted in efficacies of 84–100% (Haque *et al*., 2002; Houpt *et al*., 2004). This illustrates the protective role of IgA in defense against *E. histolytica*.

Characterization of multiple parasite factors involved in *E. histolytica* pathogenesis has been aided by the ability to grow this parasite's trophozoite and

encysted stage under axenic conditions *in vitro* (Said-Fernandez *et al*., 1988, 1993). These factors have included adhesion and cytolytic molecules that aid the progression of invasive amebiasis. The Gal-GalNac lectin has been shown to be the main adhesive molecule on the parasite's surface which can bind to Gal-GalNac sugar residues on the mucosal layer and host cells (Katz *et al*., 2002). The lectin is also capable of blocking complement membrane attack complex formation, thereby preventing complement-mediated lysis. Once bound, it is thought that the parasite bombards the host cell with lytic compounds including amebapores and proteases. Amebapores are channel-forming peptides (*E. histolytica*'s is 77 amino acids) which oligomerize after binding and insertion into cell membranes (Leippe, 1997; Espinosa-Cantellano and Martinez-Palomo, 2000). These pores allow water and small molecules to pass through the cell causing lysis. However, *E. dispar*, the non-pathogenic relative of *E. histolytica*, also contains amebapores, suggesting that their activity could also be for lysing phagocytosed bacteria, the main source of food for the amoeba in the intestine (Espinosa-Cantellano and Martinez-Palomo, 2000). *E. histolytica* contains multiple cysteine proteases which appear to be secreted at 10 to 1000-fold higher levels than in the *E. dispar,* as well as a membrane bound collagenase. These proteases are believed to aid the parasite's degradation of the extracellular matrix supporting cells and tissues as well as components of complement and antibodies. The inability of *E. dispar* to cause invasive ameobiasis supports these proteases as being key factors in virulence for *E. histolytica* (Que and Reed, 2000).

Currently, the field is rapidly expanding owing to the recent sequencing of *E. histolytica*'s and *E. dispar*'s genomes (Loftus *et al*., 2005). There are also techniques available for *in vitro* culturing and genetic manipulation by gene silencing and over-expression using autonomously replicating plasmids that identify genes important for the parasite's virulence (Said-Fernandez *et al*., 1988; Bracha *et al*., 2003). Also, a microarray study is currently looking at the genetic differences between *E. dispar* and *E. histolytica* to identify genes that aid *E. histolytica* pathogenesis (personal correspondence, Upi Singh, 2005). One factor hindering *Entamoeba* research is the lack of an animal model that closely mirrors human disease. Currently, the only models of amebic liver abscesses involve direct injections of parasites into the liver of baby gerbils or hamsters (Que and Reed, 2000).

16.3 Conclusions

16.3.1 Implications for foodborne disease treatment and prevention

Currently, treatment is available for most parasitic diseases (see Tables 16.1 and 16.2). However, multiple challenges remain for the prevention and treatment of many of the most severe effects of important food- and waterborne parasitic diseases. First of all, the populations affected most by these diseases are in developing nations and live in conditions of extreme poverty without health

care. Also, basic sanitation, including proper disposal of animal and human waste as well as safe drinking water, is not available. As illustrated in this chapter, most parasitic infections are due to fecal–oral contamination. Therefore, even with free drug distribution programs promoted by multiple organizations such as the WHO to treat some of the most common infections (e.g. *A. lumbricoides* with albendazole and mebendazole), pharmacologic approaches will be only short-term solutions to a larger more challenging problem (Bennett and Guyatt, 2000; Montressor *et al*., 2002). Furthermore, there is concern that blanket treatment of an entire population with drugs regardless of the infection status of an individual will promote drug resistance. Drug-resistant helminths have already been found in the livestock industry, and there are scattered reports among human populations (Albonico *et al*., 2004; Jones and George, 2005).

Vaccination strategies are an alternative strategy to prevent parasitic infection. Vaccines for nematodes, cestodes, trematodes, and protozoans are slow in development for a variety of reasons, including the lack of appropriate animal models, lack of immune response against certain organisms, and the lack of sufficient interest from commercial industry because of their low profit potential. Also, most humans are able to be infected multiple times by some parasites; therefore, there is no lasting protective immunity in hosts, the basic foundation for vaccines (WHO, 1995). Despite these problems, some researchers have been successful in the initial development of some vaccines. For instance, there have been efforts to identify 'hidden' antigens not present on the outer surface of nematodes that produce a protective immune response (Munn, 1997). Furthermore, recombinant oncosphere antigens have been used successfully as vaccines against *T. solium* and *E. granulosus* in animal intermediate hosts. Because of the high success rate in animals, researchers believe these will be successful if used in humans as well. Unfortunately, as with other parasitic diseases, there is a lack of commercial interest in developing a vaccine for which the majority of those affected are unable to pay (Lightowlers *et al*., 2003).

Besides medical prevention options and sufficient cooking of foods, freezing of raw meats and fish prior to consumption or food irradiation are additional methods to prevent parasitic disease from raw or undercooked meat. *T. spiralis* is susceptible to freezing at −15 ºC for 20 days or −23 ºC for 10 days and *T. gondii* is generally thought to be susceptible to temperatures at or below −12 ºC (normal freezer temperatures are −20 ºC) (Tenter *et al*., 2000; Bruschi and Murrell, 2002). Also, parasites found in meat during part of their life cycle, *T. gondii, T. saginata,* and *T. spiralis* as well as parasites in fish have been shown to be susceptible to irradiation. When used at a minimum dose required for parasite inactivation, gamma irradiation does not impair the nutritional qualities or taste of foods. Although high doses are required for some parasites to be killed in certain meats, lower doses have been found to be sufficient to prevent infection by disrupting the parasite's ability to mature and replicate. Several governments, including the US, have approved the use of irradiation on certain meat products (Farkas, 1998). Further information can be found in the review (Farkas, 1998). Although freezing and irradiation may be useful techniques to

inactivate parasites in some foods, they do not solve the overall problem of poor sanitation and hygiene in developing nations which are the main factors contributing to parasitic disease.

The first step in enhancing programs to prevent food- and waterborne parasitic diseases is to identify how many people are affected and where they are located (WHO, 2002). A recent WHO report calls on all member states to strengthen surveillance of these diseases to draw attention to their public health importance. Once accurate data are available, more specific guidelines for sustainable preventive measures, including educational efforts, can be established to reduce the incidence of food- and waterborne disease (WHO, 2002). The WHO currently plans primarily to target pre-school, school-age children, and women of childbearing age for educational and soil-transmitted helminth (*A. lumbricoides, T. trichuria,* hookworms) treatment programs because these populations are most at risk for parasitic disease and their adverse affects. The adverse affects can range from intestinal disturbances to nutritional deficiencies and problems in cognitive development (Montressor *et al*., 2002).

16.3.2 Current research frontiers

Detection techniques

Determining the prevalence of certain parasitic diseases is dependent on reliable and standardized detection methods. Traditional diagnostic techniques for intestinal parasitic diseases use microscopic observation of stool and water samples. However, these rely on trained microscopists for accurate identification of organisms and can lead to incomplete and incorrect diagnoses. ELISA immunoassays of serum samples from patients or animals are also useful for detecting exposure to certain parasite antigens. Although these methods are not as dependent upon skilled labor, they too have the disadvantage of having poor sensitivity and occasional false positive results. Therefore, there is a need for high-throughput, simple, and sensitive detection techniques. Molecular biological methods currently are being looked at as the future solution. These methods are all based on parasite-specific DNA to identify the cause of infection. Methods being developed range from PCR techniques against parasite-specific genes to microarrays of multiple parasite-specific genes that can be probed with an individual sample to identify the infecting organism (see Gamble and Murrell, 1998; Morgan, 2000; Prichard and Tait, 2001 for further information). Although these techniques are not in widespread use at present, it is hoped that they will be in the near future.

Future research efforts

Parasite research has been and will be aided by the genomic era as described in the above sections. Before ESTs and/or genomes of protozoans and helminths were sequenced, much research was limited to cloning candidate vaccine antigens from parasite lysates or understanding a parasite's biology pertaining to how it metabolized a certain drug. Now with the sequencing of more genomes and EST libraries (see www.sanger.ac.uk and www.nematode.net for further

information), target genes for drug therapy or vaccine development will be easier to identify. However, initially one will need to rely upon bioinformatics to identify genes for potential drug targets or vaccines. For instance, genes that are homologous to proteases or signal transduction pathways potentially could be targets for new drug therapies that will inhibit an important parasitic function. Also, genes that are predicted to be secreted or may be present on the parasite surface are targets for vaccines since they are most likely the first parasite proteins to be interacting with the host's immune response.

Further research is especially important for developing new therapies against parasites or particular parasite developmental stages unable to be treated currently (e.g. *T. gondii* encysted bradyzoites). One of the remaining barriers to basic parasite research is the inability to culture most of these organisms in their vegetative or adult stages *in vitro*. This emphasizes the importance of model systems involving related organisms that can be grown *in vitro* and genetically manipulated, such as *Caenorhabditis elegans* (non-infectious soil dwelling) for nematode parasites or *T. gondii* for apicomplexans. Functional genomics approaches with these parasites will, it is hoped, lead to a better understanding of many parasite genes that have no homology to other organisms. Also, genes that are different between *C. elegans* and another related parasitic nematode may identify target genes to study for their importance in disease (Blaxter *et al*., 1999; Grant and Viney, 2001).

Although many parasitic diseases primarily are of importance in developing nations, the research community and general public in economically developed countries should not overlook their significance. Food- and waterborne parasitic disease is a concern not only for impoverished nations, but for the entire world owing to the expansion of global trade and travel, and the risk of exposure to some parasites in countries regardless of economic status. Also, the new threat of bioterrorism raises concerns about the potential contamination of food or water reservoirs with a variety of organisms including parasites. For example, although *C. parvum* contaminated water will not kill as many people as other bioterrorism hazards, the economic damage it would cause for a city, state, or nation due to the inability of a population to work could be devastating (Khan *et al*., 2001). Therefore, it is anticipated that funding and interest in the field of food- and waterborne parasites will continue into the future.

16.4 Acknowledgement

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17

Dose–response relationships and foodborne disease

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17.1 Introduction: microbial risk assessment and global food safety

Quantitative microbiological risk assessments (QMRAs) for foodborne disease are essential to protect public health by setting food safety objectives and to prove equivalence of level of protection in international trade. Qualitative risk assessments may be sufficient for many food safety purposes, such as detecting critical points in food chains and estimating the effects of various interventions on hazard exposure. However, the mathematical rigor inherent in QMRA is indispensable for translating public health objectives, stated in terms of frequency of disease or disease outcome, into food safety objectives at the level of consumption and performance objectives or criteria at the level of production and processing. Furthermore, when combined with economic analysis, QMRAs can be used to evaluate the cost-effectiveness of various interventions. For this quantification of risk it is necessary to relate the potential exposures during consumption (i.e. the dose) to the health impact (i.e. the response). The main problem in dose–response quantification is that one generally cannot measure by direct experimentation the effect of exposing humans at different physiologic states to pathogens of varying virulence. Thus, more indirect methods must be applied to estimate this dose–response relation, generally resulting in considerable uncertainties. These uncertainties are due to extrapolation, for example from high doses in experiments to the low doses likely to be found in contaminated food, and from the physiologically narrow range in experimental groups, be they human or

animal, to the general human population. There are also statistical uncertainties due to small dose groups in available data sets.

In addition to these uncertainties, one must also take variability into account: variability in the virulence of the organisms, in the susceptibility of the subjects, and in the specific effects of the food products consumed. Uncertainty and variability in the dose–response relationship generally determine the size of the confidence intervals around the quantification of the risk within quantitative risk assessment. Although the quantification of risk may be uncertain, the QMRA often can estimate the relative impact of various interventions with greater accuracy, and insights important to selection from among risk management options can be gained. Combined analysis of various sources of information can in time result in better understanding of dose–response relationships, although some uncertainty will always remain in the absence of data from controlled exposures of large sets of the human subpopulations of interest (e.g. pregnant women or people with HIV infections) to a wide variety of strains of the pathogens of interest; such experiments are impossible at a practical level, and are ethically unacceptable.

In characterizing the dose-response, one quantifies the relationship between the amount of pathogen consumed (exposure) and the response, i.e. the health impact. For this purpose, the whole process is usually considered as a conditional sequence of events: exposure → asymptomatic infection → symptomatic illness → total recovery or sequelae or death. The probability of consuming a certain number of pathogens is derived from exposure assessment in QMRA (see Chapter 6), and the dose–infection model relates the probability of infection to the ingested dose. Establishment of infection is a complex process involving many barriers for the pathogens (Chapters 8 and 9), each with an associated probability to survive. One example of such a barrier is the adverse conditions of the stomach. The survival of the pathogen depends on its residence time in the stomach, the protective components present in the food such as fat, and the pH of the stomach, which depends on host factors such as age and underlying health status, and the buffering effect of other constituents of the meal. The survival in the stomach also depends on the acid resistance of the organism, depending on the strain, but also on its physiological state and previous history (see Chapter 12). Takumi *et al*. (2000) modeled the survival of *Escherichia coli* in the stomach under dynamic conditions of gastric pH and food transport, and concluded that after consumption of a solid meal as much as 20–80% of the ingested bacteria may be delivered to the small intestine because shortly after the meal the pH may rise to approximately 5. Furthermore, De Jonge *et al*. (2003) demonstrated that *E. coli* O157:H7 is able to rapidly adapt itself to an acid environment, which further stimulates survival. These data suggest that the acid barrier in the stomach may be less effective than usually considered, which is an important consideration when characterizing the dose–response relationship.

After transiting the stomach, the organism has to colonize the intestines and, depending on the organism, start to produce toxins or invade intestinal cells. Depending on the immune response of the host, invasion can go further, and in

certain cases the organisms can enter the bloodstream and infect other organs. These last steps are largely influenced by the virulence of the organism and the susceptibility of the host, and to a lesser extent by the ingested dose. Also immunity (previous exposure) may influence the probability of infection and/or illness given infection. All these factors together greatly complicate dose–response assessment. On the other hand, there is a clear difference in the probability of disease after consumption of 100 cells of a virulent *Salmonella* strain and the same number of *Listeria monocytogenes* cells, justifying the effort needed to investigate these relevant factors and effects.

17.2 Biological background (single hit and independent action hypotheses)

Microbial dose–response models are based on three basic assumptions that conceptualize the biological basis of host–pathogen interaction (FAO/WHO, 2003).

17.2.1 Single hit

The single-hit assumption states that, no matter how low the dose, there is always a non-zero probability of illness and infection, however low the possibility may be. This is in contrast to the frequently used (minimal) infectious dose assumption, in which a certain threshold (MID) exists for infection. The probability of infection is 0 if the exposure is below the MID value and the probability is 1 if the dose is above the MID value. Experimental evidence supporting the single-hit hypothesis was published as early as 1957 by Meynell and Stocker (1957). These authors intraperitoneally injected mice with a mixed culture of flagellar variants of *Salmonella paratyphi* B, and demonstrated that a mixed culture could be re-isolated if the animals were exposed to high doses but a pure culture if low doses were used. Similar experiments were published by Moxon and Murphy (1978). These authors intranasally applied very low doses of streptomycin-resistant or -sensitive *Haemophilus influenzae* to rats and isolated pure cultures at doses between 3 and 9 CFU per animal and mixed cultures from doses of 26 CFU upwards. Even though a formal proof of the absence of a threshold to infection cannot be given, it is not likely that the MID concept reflects reality. Low levels of exposure have an associated probability of disease, even if very small. The MID concept might give very 'fail-dangerous' predictions and should therefore not be used in food safety.

17.2.2 Independent action

The hypothesis of independent action states that the mean probability per inoculated pathogen to cause disease or illness is independent of the number of pathogens inoculated. Experimental evidence to support this hypothesis has been

summarized by Rubin (1987). This hypothesis implies that the probability of infection by any number of organisms is a simple binomial function (see later).

17.2.3 Random distribution

In the mathematical derivation of dose–response models, it is typically assumed that microorganisms are randomly distributed in the inoculum, which can be described by a Poisson distribution. While this assumption is not necessary in QMRA, it is mathematically convenient. However, dose–response models can also be derived for other distributions, as will be briefly discussed later.

17.3 The single-hit family of dose–response models

Based on the above principles, a series of dose–response models has been constructed that is known as the single-hit family of models. They were first described in the context of infectious agents by Haas (1983) and are typically used to model the probability of infection in relation to the ingested dose. They should then be complemented with a (conditional) model of illness given infection.

17.3.1 Exponential model

In the exponential model, the probability of infection is described by:

$$P(\text{inf}) = 1 - \exp(-rD) \approx rD$$

In this model, D is the mean ingested dose and r is the dose–response parameter, indicating the probability that a single 'unit' of the organism will survive all barriers and cause infection. In practical terms, the exponential model means that the probability of infection increases linearly with the dose leveling off towards a probability of 1 at higher doses. This model requires the assumption that the bacteria have a Poisson distribution (see above), and that all cells have an equal probability of causing infection in any host. For low doses this equation can be approximated by $P \approx rD$ (as long as $rD < 0.1$ less than 5% error is made between rD and $1 - \exp(-rD)$).

17.3.2 Hypergeometric model

The assumption that every one of the bacterial cells consumed by each host has an equal probability of causing infection may not be realistic and can be replaced by the assumption that this probability follows a beta-distribution with parameters α and β. The resulting dose–response model is the so-called hypergeometric model (Teunis and Havelaar, 2000):

$$P(\text{inf}) = 1 - {}_1F_1(\alpha, \alpha + \beta, -D)$$

in which ${}_1\mathrm{F}_1(\ )$ is the Kummer confluent hypergeometric function.

This model contains one parameter more than the exponential model. In the lower dose range the model behaves the same as the exponential model (linear with the dose, $P \approx (\alpha/(\alpha+\beta))D$, but it does not have as steep a slope as it approaches a probability of 1.

17.3.3 Beta Poisson model

For cases where $\alpha \ll \beta$ and $\beta \gg 1$, the hypergeometric model can be approximated by the Beta Poisson model:

$$P = 1 - [1 + D/\beta]^{-\alpha}$$

Just as the other two models, in the lower dose range this model behaves linearly with the dose, $P \approx (\alpha/\beta)D$.

17.3.4 Models for discrete doses

The models presented thus far all relate the probability of infection to the mean ingested dose, which is the information available in an experimental data set. In risk assessment models, however, the output typically is a distribution of discrete ingested doses ($i = 1, 2, 3, \ldots, n$ cells). In that case, the dose–response model takes another form (Haas, 2002). If the dose–response parameter r is a constant, then the hypothesis of independent action predicts that the probability of infection from ingestion of i cells is simply the complement of the probability of not being infected by any of the i cells:

$$P(\text{inf}) = 1 - (1 - r)^i$$

This is the binomial dose–response model. If the dose–response parameter follows a beta distribution, then

$$P(\text{inf}) = 1 - [B(\alpha, \beta + i)/B(\alpha, \beta)]$$

where B is the beta function. This is the beta-binomial dose–response model. These models can easily be combined with any distribution of discrete doses to estimate the associated risk of infection, as illustrated, e.g. by Haas (2002).

17.3.5 Models for non-random distributed doses

In many practical situations, the distribution of pathogens in a product (food, water, etc.) does not follow a Poisson distribution but shows increased variability. There are different possibilities to describe this additional variability. Haas (2002) describes the Poisson-gamma distribution (where the parameter of the Poisson distribution is not a constant but follows a gamma distribution) and the Poisson-lognormal distribution (where the log of the concentration of microorganisms follows a normal distribution) and gives examples of how these equations can be applied in risk assessment models. An important consequence of increased variability in ingested doses is that the population risk is always

smaller than the risk associated with randomly distributed doses given the same (arithmetic) mean dose. In other words, using models based on the Poisson distribution provides an estimate of the upper bound public health risk.

17.3.6 Models for illness given infection

There has been little attention to modeling illness given infection as a function of the ingested dose. The single-hit models are frequently used to directly model the probability of illness given exposure. In that case, the implicit assumption is that there is a constant (dose-independent) probability of illness given infection. One argument in support of this approach is that the outcome of infection is largely determined by the effectiveness of the host response, which is more important than the ingested dose. Teunis *et al*. (1999) developed a conditional dose–response model for illness given infection, based on the assumption that during the infection, the host has a certain probability to become ill:

$$P(\text{ill}|\text{inf}) = 1 - (1 - \lambda)^{-\rho}$$

where ρ is the shape parameter of the underlying gamma distribution for duration of infection and λ is a function of the dose that can take various shapes, for increasing, constant or decreasing illness probability with dose: $\lambda = \eta D$; $\lambda = \eta$ and $\lambda = \eta/D$, respectively with η a constant. Fitting the model to existing data sets showed that, indeed, all shapes of the dose–response function were found.

Many other dose–response models can be found in the literature. For example, Holcomb *et al.* (1999) fitted six different dose–response models to four published data sets from volunteer experiments and demonstrated that, although most models fitted the data well in the experimental range, their low-dose extrapolations differed markedly. As long as these models accurately describe the data, they can be used for interpolation within the experimental dose range. However, for extrapolation to low doses a biological basis is necessary which is not available for other models than the single-hit family. It must be noted that some models (logistic or log-normal) would allow the probability of infection to exceed the probability of exposure, which is physically not possible. In contrast, the single-hit models have a maximum risk curve, represented by $P(\text{inf}) = 1 - \text{e}^{-D}$ (the exponential model with $r = 1$, i.e. any ingested cell is able to cause infection; Teunis and Havelaar, 2000).

17.4 Estimating dose–response relations: sources of data with their advantages and limitations

17.4.1 Human feeding studies

In the last century various human feeding experiments were carried out. These data sources can be used to determine dose–response relations for various

pathogens (Teunis *et al.*, 1996). However, in using these data one should realize the following points:

- These experiments are carried out with specific groups of people (often prisoners or students) that may not ideally reflect the response of the general population. For example, babies and the elderly, although often more vulnerable, are generally not representatively included.
- Often only a limited number of subjects have been used per dose group, with relatively high exposure doses, to increase the likelihood of measurable responses.
- These experiments primarily have been done with pathogens of importance in the last century, but current public attitudes about intentionally exposing people to potentially life-threatening pathogens make it difficult to repeat the studies with pathogens and strains that are relevant today, except for organisms where effects are very mild.
- Experiments with organisms with severe outcomes cannot be done, therefore no data are available for these organisms.
- In these experiments the doses were supplied in a specific experimental setting that was not always representative of typical foodborne exposures (for example, laboratory-adapted strains of pathogens frequently have been administered in buffer after fastening). During repeated passage on laboratory media, laboratory-adapted strains may become less virulent than naturally occurring strains.

Nonetheless, data from human feeding studies are the most direct answer to the question of the probability of disease in a human subject after a certain exposure.

17.4.2 Animal experiments

- In addition to concerns about the rights and treatment of human subjects, current public attitudes about the use of animals in research also impose limits on the use of animal experiments to predict the dose–response of humans to exposure to pathogens in food. In certain cases it has been possible to conduct animal experiments, and the data derived from these experiments are valuable in constructing dose–response models.
- Ethical and cost considerations often limit the number of subjects that are exposed per dose group, and relatively high doses are used. However, if 20 animals are exposed in one dose group and remain free of signs of illness, one can say no more than that the probability of illness is smaller than 1 in 20, a probability that is very far from the range at which one would set an acceptable risk for human foodborne disease. However, measurements are taken at a number of dose levels and a model is fitted through the data, and the combination of data from all measurements reduces the uncertainty in the value for each dose level.
- Extrapolating from animal data to the predicted human dose–response is complicated by host specific responses to various pathogens. That is, while

animal models can be very useful in studying dose–response relationships, biological differences between laboratory animals and humans constrain the inferences that can be drawn from animal data. For example, certain pathogens can be infective in a laboratory animal and not at all in a human, and the other way around, or can use different pathogenic mechanisms in laboratory animals than in humans. This problem makes it difficult to use animal data to quantitatively determine dose–response relations. In certain cases the animal experiments are more or less representative for infection but not for illness (e.g. *Salmonella* Enteritidis in rodents).

On the positive side, animal experiments have the advantage that the pathogens are exposed to a stomach, experience a real intestinal environment and enterocytes, and confront an innate and adaptive immune system. Therefore, despite their limitations, animal experiments can be very useful, especially for exploring such factors as the effect of the physiological state of the pathogen on its infectivity. Still, one needs to hypothesize that the characteristics of their infection will be similar in a human being.

17.4.3 Cell cultures

- Cell cultures share many of the deficiencies of animal experimentation without sharing the advantages of reproducing a realistic physiologic state for host–pathogen interaction. One can make use of human cell lines (for example Caco-2 cells), and even get these cells in as representative a state as possible, but these still do not reflect the exact conditions that exist within the human body at the organ or tissue level.
- In addition, the host cell–pathogen interaction is only one part of the whole infectivity pathway. The stomach passage, intestinal environment, the influence of other cells and cellular mediators, and possibly further routes after the intestinal cells cannot easily be investigated by cell culture model systems.
- It is difficult to define and set relevant states of the infecting culture (stomach and intestine residence) and culture and conditions for the cell lines.

On the other hand, without large ethical problems one can investigate the infection process, even with relatively low doses of pathogens in relation to human cells. More elaborate systems in which the stomach is simulated in fermenters and systems in which the intestines are simulated are increasingly available to study elements of dose–response modeling without employing laboratory animals or human volunteers (e.g. De Jonge *et al*., 2003; Marteau *et al.*, 1997).

17.4.4 Outbreak data

Data derived from carefully investigated outbreaks of foodborne disease are another valuable source of information on the dose–response relationship, since unintentionally, a group of people is exposed to a number of pathogens, resulting in a certain number of illnesses.

- The problem with these data is, however, that in nearly all investigations important factors are not known, or at least not accurately known. Often, for example, the exact exposure dose is uncertain, since time elapses from when the food was served to when illnesses are investigated during which the responsible food changes its character and its pathogenic load (up or down) or becomes unavailable altogether. Furthermore, concentrations of pathogens may be variable within the responsible food, and it is difficult to estimate the real exposure distribution. Also, sometimes the exact number of exposed people and the exact number of illnesses are not determined accurately.
- This method generally will only supply useful data for organisms that result in outbreaks that are sufficiently large and have a relatively short incubation period (such as *Salmonella*). For other organisms that primarily cause sporadic cases (for example, *Campylobacter*) this method can only be used for the rare outbreaks, which may have characteristics that influence the dose–response relative to that which pertains to the more usual sporadic illness (e.g. Teunis *et al*., 2005).

The great advantage of this method is that it supplies real-life data that clearly are practically relevant, being derived from exposures to actual strains of foodborne pathogens in actual food vehicles of a human population with actual variability practicing practical consumption procedures. In addition, inferences drawn from outbreak investigations may result in 'fail-safe' estimations of infectivity, since microorganisms that cause outbreaks may, in general, be more infective than the average strain of that microorganism.

17.4.5 Surveillance and annual health statistics

By making use of the total exposure of the total population for a certain pathogen and the disease burden of that pathogen, one can also estimate the dose–response factor for that population. As with the other sources of information, this method has certain drawbacks:

- Owing to underreporting the true disease incidence might be larger than estimated.
- Owing to unknown vehicles of the pathogen, certain exposures might be neglected, resulting in the fact that exposure is (largely) underestimated.
- Doses are difficult to estimate, since often the disease burden may result mainly from the very low probability of very high doses. For these extreme conditions that represent the tails of the exposure distribution, generally insufficient data are available.
- Data are available only at an aggregated level and ignore within-group variability.

But again, this method results clearly in real-life estimates from actual strains, strain conditions, population heterogeneity (of humans and pathogens), etc. Various examples of dose–response models and their parameters are given in Table 17.1.

Table 17.1 Summary of published dose–response relations

(a) Dose-infection models

Pathogen	Data source	Exposed population	Model*	DR parameters	Low dose approximation†	ID_{50}‡	Reference
Bacteria							
Campylobacter jejuni A3249	Volunteer	Young adult males	HG	$a = 0.145$, $b = 8.007$	1.78×10^{-2}		Teunis and Havelaar (2000)
Campylobacter jejuni	Outbreak	Children	HG	$a = 0.024$, $b = 0.011$	6.86×10^{-1}		Teunis *et al.* (2005)
Escherichia coli O157:H7	Outbreak	Schoolchildren	HG	$a = 0.0844$, $b = 1.442$	5.53×10^{-2}		Teunis *et al.* (2004)
		Teachers	HG	$a = 0.0496$, $b = 1.001$	4.72×10^{-2}		
Plesiomonas shigelloides	Volunteer	Adult males, buffered inoculum	BP	$a = 0.057$, $b = 1171$	4.87×10^{-5}	2.24×10^{8}	Teunis *et al.* (1996)
Salmonella anatum	Volunteer	Adult males	BP	$a = 0.451$, $b = 15177$	2.97×10^{-5}	5.54×10^{4}	Teunis *et al.* (1996)
Salmonella bareilly	Volunteer	Adult males	EX	$r = 3.19 \times 10^{-6}$	3.19×10^{-6}	2.17×10^{5}	Teunis *et al.* (1996)
Salmonella derby	Volunteer	Adult males	EX	$r = 2.19 \times 10^{-7}$	2.19×10^{-7}	3.17×10^{6}	Teunis *et al.* (1996)
Salmonella meleagridis	Volunteer	Adult males	BP	$a = 0.428$, $b = 8524$	5.02×10^{-5}	3.45×10^{4}	Teunis *et al.* (1996)
Salmonella Newport	Volunteer	Adult males	EX	$r = 3.97 \times 10^{-6}$	3.97×10^{-6}	1.75×10^{5}	Teunis *et al.* (1996)
Salmonella pullorum	Volunteer	Adult males	EX	$r = 3.48 \times 10^{-10}$	3.48×10^{-10}	1.99×10^{9}	Teunis *et al.* (1996)
Shigella paradysenteriae	Volunteer	Adult males, buffered inoculum	EX	$r = 8.26 \times 10^{-10}$	8.26×10^{-10}	8.39×10^{8}	Teunis *et al.* (1996)
Vibrio cholerae Inaba 569b	Volunteer	Healthy	EX	$r = 1.76 \times 10^{-9}$	1.76×10^{-9}	3.94×10^{8}	Teunis *et al.* (1996)
Vibrio cholerae Inaba 569b	Volunteer	Healthy, buffered inoculum	BP	$a = 0.164$, $b = 0.136$§	1.21	9.18	Teunis *et al.* (1996)

Table 17.1 Continued

Pathogen	Data source	Exposed population	Model*	DR parameters	Low dose approximation†	ID_{50}‡	Reference
Viruses							
Echovirus 12	Volunteer	Healthy adults	BP	$a = 0.401$, $b = 227.2$	1.76×10^{-3}	1.05×10^{3}	Teunis *et al.* (1996)
Poliovirus	Volunteer						Teunis *et al.* (1996)
1 sm		Adults	EX	$r = 0.491$	4.91×10^{-1}	1.411	
1 LSc2ab		Infant (newborn)	BP	$a = 0.114$, $b = 159.0$	7.2×10^{-4}	6.93×10^{4}	
1		Infant (2 months)	EX	$r = 9.1 \times 10^{-3}$	9.1×10^{-3}	76.2	
3 Fox		Infant	BP	$a = 0.533$, $b = 2.064$	0.258	5.513	
3 Fox		Premature	BP	$a = 0.299$, $b = 0.552$	0.542	5.05	
Rotavirus CJN	Volunteer	Adult males	HG	$a = 0.167$, $b = 0.191$	4.66×10^{-1}	11.9	Teunis and Havelaar (2000)
Protozoa							
Cryptosporidium parvum Ioawa	Volunteer, seronegative	Healthy adults, seronegative	EX	$r = 0.0053$	0.0053	1.31×10^{2}	Teunis *et al.* (1996)
Cryptosporidium parvum TAMU			EX	$r = 0.0573$	0.0573	1.21×10^{1}	Teunis *et al.* (2002)
Cryptosporidium parvum UCP			HG	$a = 1.15 \times 10^{-5}$, $b = 8.15 \times 10^{-6}$	0.58		Teunis *et al.* (2002)
Giardia lamblia	Volunteer	Adult males	EX	$r = 1.99 \times 10^{-2}$	1.99×10^{-2}	3.48×10^{1}	Teunis *et al.* (1996)
Entamoeba histolytica	Volunteer	Adult males	BP	$a = 0.106$, $b = 0.295$	0.359	2.04×10^{2}	Teunis *et al.* (1996)

(b) Dose–illness models

Pathogen	Data source	Exposed population	Model*	DR parameters	Low dose approximation†	ID_{50}‡	Reference
Listeria monocytogenes	Surveillance	Susceptible	EX	$r = 1–5 \times 10^{-12}$	$1–5 \times 10^{-12}$	$1–7 \times 10^{11}$	FAO/WHO (2004)
		Healthy	EX	$r = 2–5 \times 10^{-14}$	$2–5 \times 10^{-14}$	$1–4 \times 10^{13}$	
Salmonella, various serotypes	Outbreaks	Various	BP	$a = 0.1324$, $b = 51.54$	2.57×10^{-3}	9.63×10^{3}	FAO/WHO (2002)
Shigella flexneri 2a	Volunteer	Adult males	BP	$a = 0.143$, $b = 284.3$	8.26×10^{-10}	3.59×10^{4}	Teunis *et al.* (1996)
Shigella dysenteriae M131+A-1	Volunteer	Adult males	BP	$a = 0.157$, $b = 9.16$	1.71×10^{-2}	7.48×10^{2}	Teunis *et al.* (1996)

* EX: exponential model; BP: Beta-Poisson model, HG: hypergeometric model.
† EX: $= r$; BP: $= (\alpha/\beta)$, HG: $= \alpha/(\alpha + \beta)$.
‡ EX: $= \ln(2)/r$; BP: $= \beta/(2^{1/\alpha} - 1)$.
§ Violates the assumptions of the Beta-Poisson model.

In conclusion, it is clear that all data sources have major drawbacks and limitations. However, these are the data sources we have, and the combined consideration of data from all sources provides at least a rough indication of where the dose–response relationship lies, and especially which factors are most important in determining its location. Given the uncertainties, it is important to compare the results of risk assessment studies with independently obtained data from epidemiological studies and, where necessary, to adjust the risk estimates to conform to the observational data.

17.5 Implications of dose–response factors for risk-based food safety specifications

17.5.1 Effects of strain/strain variability

Figure 17.1 shows the large between-strain variability in infectivity of a single pathogen (7 orders of magnitude). It should be realized that these are experiments in mice (and thus not entirely representative of human response) and that this is the infectivity after intraperitoneal injection, not exposure *per os* that would be followed by stomach passage and contact with the luminal surface of enterocytes. Therefore, while the quantitative level of risk cannot be directly extrapolated to human beings, these results demonstrate that it is likely that a high degree of strain-related variability will exist in infectivity for humans.

Teunis *et al*. (2002a) used human volunteer studies to demonstrate differences in infectivity for three isolates of the protozoan parasite *Cryptosporidium parvum*. The low-dose approximation between the most and the least infective strain differed as much as a factor of 100 with these three strains alone.

17.5.2 Effects of host factors

The feeding status and especially the health status of the subject is also of large importance. Generally the YOPIs (Young, Old, Pregnant, and Immunocompromised) have a greater risk than the general population. In the FAO/WHO and USDA/FDA risk assessments, various factors that increase the risk from *Listeria monocytogenes* have been estimated (Table 17.2).

Teunis *et al*. (2002b) demonstrated that in subjects with high IgG levels against *C. parvum*, the infectivity of this organism was clearly less than in seronegative individuals. The data could more or less be characterized by two different dose–response models with the transition around an IgG level of 0.15 (arbitrary units). There appeared to be a 23-fold increase in ID_{50} in subjects with high antibody levels. Another feature of host effect was demonstrated in challenge studies with Norwalk virus, the prototype strain of Norovirus genogroup I (NoV GGI). Lindesmith *et al*. (2003) demonstrated that 29% of their volunteers did not express the receptor required for binding this virus, which is an ABH histo-blood group antigen. Subsequently, Rockx *et al*. (2005) showed that the absence of this antigen does not protect against infection with NoV GGII.

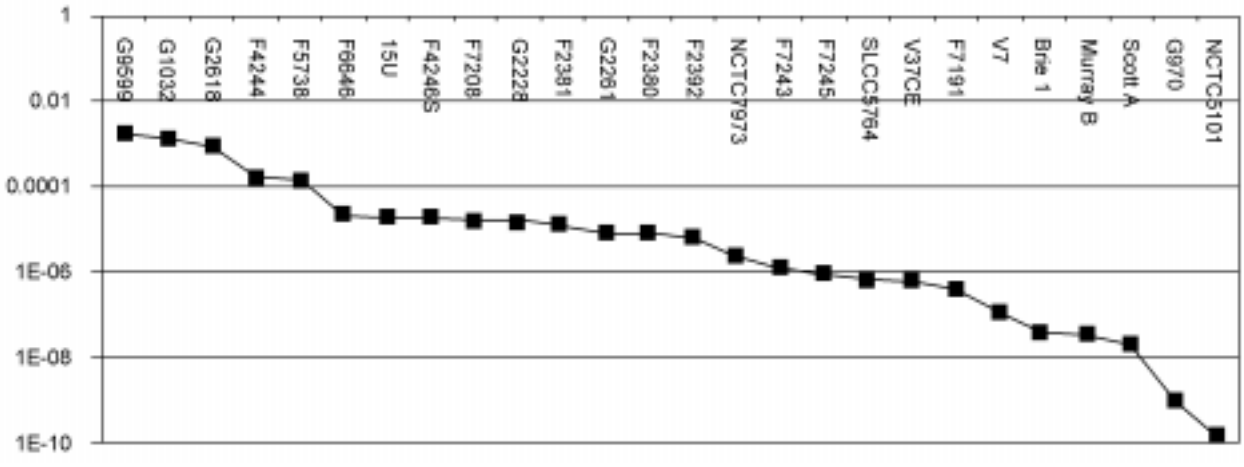

Fig. 17.1 Probability of infection of various *Listeria* strains (intraperitoneal injection in mice) from HHS/USDA (2003).

Table 17.2 Relative susceptibilities for different sub-populations based on French epidemiological data for infection with *Listeria monocytogenes* (FAO/WHO, 2004)

Transplant	2584
Cancer – blood	1364
AIDS	865
Dialysis	476
Cancer – pulmonary	229
Cancer – gastrointestinal and liver	211
Non-cancer liver disease	143
Cancer – bladder and prostate	112
Cancer – gynaecological	66
Diabetes, insulin dependent	30
Diabetes non-insulin dependent	25
Alcoholism	18
>65 years	7.5
Less than 65 years, no other condition	1

Experimental studies in rats have indicated significant difference in resistance to infection with *S.* Enteritidis that may be related to the relative response of T-helper 1 and 2 cells (Havelaar *et al*., 2004).

17.5.3 The need to extrapolation to realistic low doses from high experimental doses

At a dose of 1×10^4 cells (for example 100 g of a product with 100 cells/g), the probability of illness in Fig. 17.2 seems to be zero. Given this graph, it is tempting to define a minimal infective dose. However, this curve is calculated with the exponential dose–response model with an $r = 1 \times 10^{-10}$. For a dose of 1×10^4 this results in a probability of 1 in 1×10^6. While this seems to be a very low risk, it is important to keep in mind that it is a risk per serving. Therefore, if 50 servings of such a food product per year are consumed, the risk is 1 in 20 000 per year, so for a population of 1 million, 50 illnesses would result per year. This is clearly not zero, despite the comforting appearance of the graph.

It should be noted that by extrapolating towards these low doses, one assumes that the phenomena occurring are equal at high and low doses. That is, 10^4 cells are assumed to give 100 times lower risk than 10^6 cells. This is the best assumption as long as no better information is available, although one should realize that 10^6 cells might behave differently within the enteric microflora than 10^4 cells.

17.5.4 Impact on risk-based food safety specifications

Because of all the factors mentioned in this chapter, often the dose–response relationship can only be estimated with large uncertainty and large variability. This not only makes the accurate quantification of risk difficult, it greatly

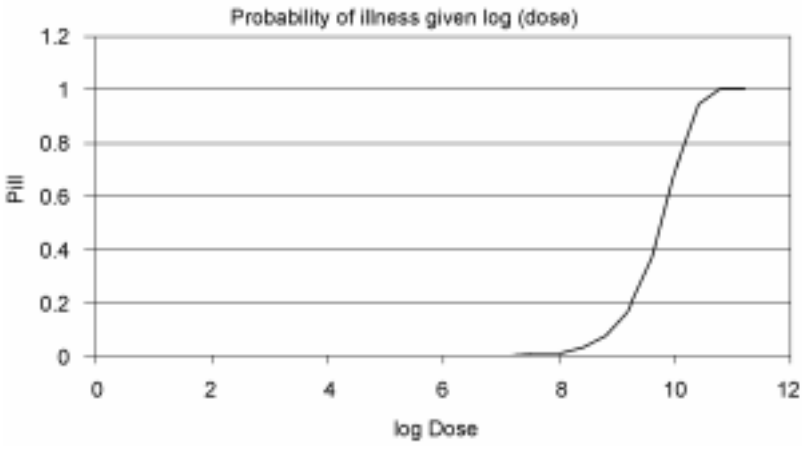

Fig. 17.2 Example dose–response relation for a relatively low virulent organism.

complicates setting the food safety objectives (FSOs, expressed in terms of numbers of pathogen per gram of food as consumed) necessary to achieve an appropriate level of protection (ALOP, expressed in numbers of illnesses within a defined population). Although this is truly a vexing problem for those who must use the FSO to establish performance criteria and performance objectives at the level of food production and processing, one should keep in mind the insights that result from making quantifications that use the best data and assumptions possible. In addition, estimations of dose–response with very wide margins of uncertainty still can provide fairly precise comparisons of the relative quantitative impact of food safety control efforts because all possible interventions will be affected equally by the dose–response relationship used by the risk model. Likewise, in determining the effect of a change in an FSO value on the level of risk, large inaccuracies in dose–response estimation generally do not translate into large inaccuracies in the determination of the effect, and the effects of variable achievement of FSOs can be estimated without very accurate knowledge of the dose–response relationship.

17.6 Future trends

Concerning dose–response relationships, one can propose many factors that should be taken into account and mechanisms to be included, but above it is made clear that the very best data that one should have are not possible to gather, so continuous improvement in the safety of the food supply will always depend on a guess based on a variety of imperfect data sources. Increasing knowledge will, however, make the guesses better and improve the bases on which quantitative risk assessments are built.

17.7 Sources of further information and advice

FAO/WHO (2003) Food and Agriculture Organization of the United Nations World Health Organization. *Hazard Characterization for Pathogens in Food and Water*. Microbiological Risk Assessment Series No. 3 ISBN 92 4 156237 4 http://www.who.int/foodsafety/publications/micro/en/pathogen.pdf

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