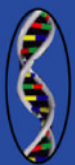


Gregory V. LaMann
Editor



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Veterinary Parasitology



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VETERINARY PARASITOLOGY

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VETERINARY PARASITOLOGY

GREGORY V. LAMANN
EDITOR

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CONTENTS

Preface		vii
Chapter I	Histomonas Meleagridis: A New Focus on a Re-emerging Protozoan Parasite <i>Nele Bleyen, Jan Mast, Koen De Gussem, Jeroen De Gussem, Maarten De Gussem and Bruno M. Goddeeris</i>	1
Chapter II	Parasite Control and Livestock Production in Nigeria <i>Nicodemus M. Useh</i>	49
Chapter III	Common Avian Parasites and Emerging Diseases <i>Margit Gabriele Muller</i>	87
Chapter IV	Primary Immune Response and Parasite Dissemination in Canine Visceral Leishmaniasis <i>P.S.T. Veras, W.L.C. dos Santos, T.F. Almeida and C.A.T. Rodrigues</i>	111
Chapter V	Epidemiologic Aspects of Bovine Neosporosis in High-Producing Dairy Herds in North-eastern Spain (Review) <i>S. Almería, I. García-Ispierto, J. L. Yániz and F. López-Gatius</i>	129
Chapter VI	Gene Expression in the Bovine Gastro-intestinal Tract During Nematode Infection <i>Robert W. Li and Louis C. Gasbarre</i>	157
Chapter VII	An Update on <i>Libyostrongylus</i> , a Gastro-intestinal Nematode of Ostriches <i>Clóvis de Paula Santos, Josiana Gomes de Andrade and Renato Augusto DaMatta</i>	179
Chapter VIII	Genital Lesions and Venereal Transmission of Canine Visceral Leishmaniasis <i>Renato L. Santos, Érica A. Costa and Kristel K. Pangrazio</i>	193

Chapter IX	The Relevance of <i>Giardia</i> Infections in Veterinary Medicine <i>Thomas Geurden and Edwin Claerebout</i>	201
Chapter X	Serological Diagnosis of <i>Parelaphostrongylus Tenuis</i> in Cervids of North America: A Review <i>Oladele Ogunremi</i>	223
Chapter XI	Tick G Protein-coupled Receptors as Targets for Development of New Acaricides <i>Felix D. Guerrero and Scot E. Dowd</i>	241
Chapter XII	Biotransformation of Xenobiotics in Lancet Fluke (<i>Dicrocoelium Dendriticum</i>) <i>L. Skálová, V. Cvilink, H. Bártíková, J. Lamka and B. Szotáková</i>	251
Chapter XIII	Cross-immunity and Interpretation of the Diagnostics of Parasitic Trematodosis in Ruminants by Means of Immunoenzymatic Probes <i>A. Paz-Silva, M. Arias, I. Francisco, F.J. Cortiñas, R. Francisco, P. Díaz, J.L. Suárez, P. Díez-Baños, P. Morrondo and R. Sánchez-Andrade</i>	271
Chapter XIV	Animal Models Suitable to Study Complex Polygenic Processes <i>Lucila Hinrichsen, Ricardo Di Masso, María Delia Vasconi and Claudio Giudici</i>	289
Index		303

PREFACE

Veterinary parasitology is the study of animal parasites, especially relationships between parasites and animal hosts, and their interactions. Parasites of domestic animals (livestock and pet animals) as well as wildlife animals are considered. Veterinary parasitology studies genesis and development of parasitoses in animal host. Veterinary parasitology also studies taxonomy and systematics of parasites, morphology, life cycles, and living needs of parasites in environment and in animal host. Diagnosis, treatment, and prevention of animal parasitoses are designed using procured observations. Data obtained from parasitological research in animals helps in veterinary practice and improve animal breeding. Major goal of veterinary parasitology is to protect animals and improve their health status. Moreover, a number of animal parasites are transmitted to humans. Therefore, veterinary parasitology is also important for public health.

Chapter I - Histomonosis, or infectious enterohepatitis, is caused by the flagellated protozoan *Histomonas meleagridis* and is one of the most important turkey diseases. This parasite produces characteristic necrotic lesions in and thickening of the caecal mucosa, a caseous core in the caecal lumen, and necrotic foci in the liver. In the field, histomonosis is often recognized by the typical sulfur-colored diarrhea and occasionally by a blue-colored head, which has led to its popular name ‘blackhead’. The presence of the parasite can be determined by microscopic analysis of the lesions or the droppings. Recently, this diagnosis can be confirmed by molecular techniques, based on the DNA-sequence of the 18S rRNA gene, which is one of the few known DNA-sequences of *H. meleagridis*.

Birds can become infected by ingesting embryonated eggs of the caecal worm *Heterakis gallinarum*, which contain *H. meleagridis*. These nematode eggs protect the parasite from the environment and from the conditions in the digestive tract. Although transmission through the intermediate host, *Heterakis*, is considered to be the natural way of infection, this route can probably not explain the rapid spread of the protozoan parasite within a flock kept in captivity, resulting in high mortality rates. In this case, “cloacal drinking” is considered as an alternative route of transmission. Recently however, cyst-like stages of *H. meleagridis* have been found, which could also suggest that cysts are responsible for the direct transmission of the parasite.

For many years, several chemical products like arsenicals and nitroheterocyclic compounds were used as chemotherapeutics and chemoprophylactics against histomonosis. However, in the last two decades, all active drugs were banned in the European Union due to

the potential carcinogenic risks they entail for the consumer or due to lack of data supporting a registration under the terms of relevant European legislation. In the USA, however, there is still one prophylactic, HistostatTM (nitarson), allowed. This ban has led to an increase in outbreaks of blackhead, causing considerable economical problems for the poultry industry.

As a lot of information, mainly on the molecular level, is still lacking, genetic approaches would improve our understanding of the parasite and its virulence factors. Recently, *H. meleagridis* has been cloned by micromanipulation, which could be an important step towards the unraveling of the molecular characteristics of the parasite. This could assist in the development of new prophylactics and chemotherapeutics.

Chapter II - Parasitic infestations constitute about 70-80% of animal diseases in Nigeria. Their control is increasingly becoming difficult because of the ever-growing parasite resistance against veterinary drugs. Moreover, the low social profile of most Nigerian farmers, who rear livestock essentially on a small scale, has made improved livestock health through parasite control almost a mirage. The overall effect of this is decreased livestock production, leading to poor animal protein supply and intake. The nomadic Fulani pastoralists of rural Nigeria, who own livestock resources in the country, prefer the use of herbal remedies to treat animal diseases. It is believed that the development of potent parasite vaccines will ameliorate the menace of veterinary drug resistance. In this chapter, the biochemical, molecular, and genetic mechanisms of parasite resistance against veterinary pharmaceuticals and the prospects of emerging biotechnologies in successful parasite control for optimal livestock production are discussed.

Chapter III - Avian parasites have been well-known for a long time and form a major part of avian diseases. This chapter includes information about susceptible species, transmission routes and distribution of the most common avian parasites. It also focuses on clinical symptoms and therapeutic approaches. Moreover, it highlights the new, emerging parasitic diseases in birds.

Parasitic diseases are among the most common infections in birds and may pave the way for other secondary infections due to immunosuppression if being unrecognized or left untreated.

Among the most common avian parasites are ectoparasites like arthropods, but also endoparasites, including protozoa, and the large groups of helminths. Helminths can be differentiated into the classes of trematodas (flukes), cestodas (tapeworms), and nematodas (roundworms). Moreover, blood parasites like *Plasmodium sp* or *Haemoproteus sp.* are present in birds and can cause considerable problems for them. However, several parasites are host-specific like *Serratospiculum seurati* or *Caryospora sp.*, which is found in falconiformes. Other parasites have very low species-susceptibility like trematodes or roundworms. Apart from common diagnostic methods like flotation or smear tests, blood smears, and staining, more sophisticated diagnostic methods like molecular analysis have found their way into modern veterinary parasitology diagnostics.

However, apart from the common avian parasites, new parasitic diseases are emerging like microsporidiosis caused by *Enterocytozoon bieneusi* or *Cryptosporidium* infections. Moreover, parasites like mosquitos may even transmit zoonotic viruses like the West-Nile Virus to birds. Evolutionary changes and mutations in parasites like *Plasmodium sp* pose new challenges for veterinarians. Due to their possible implications on human health and a possible transmission of avians to humans, these new, emerging parasitic diseases may pose a considerable and highly underestimated threat to both human and avian health.

Chapter IV – Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum/L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

Chapter V - Since the first identification in dogs in 1984, the intracellular protozoan parasite *Neospora caninum* has been found to infect a wide range of animals worldwide. Cattle, horses, goat, sheep, and deer have been described as intermediate hosts, and dogs and coyotes, and possibly red foxes, as definitive hosts. In cattle, *N. caninum* has emerged as an important cause of abortion. Reduction in milk yield, increased culling rate, decreased growth rate in calves, and birth of calves with congenital abnormalities have also been related to *Neospora*-infection in dairy herds. Neosporosis can persist for a long time in a herd since the parasite can be transmitted transplacentally. The efficiency of vertical transmission has been estimated to be as high as 95%. In Spain, the mean seroprevalence registered for *N. caninum* was 32.5% for individual cows and 83.2% for herds. We revise here several studies on epidemiological aspects of neosporosis performed in high-producing dairy herds in North-eastern Spain. Points to be highlighted should include: that the irrespective of the herd level of *Neospora*-seroprevalence, plasma antibody titration against *N. Caninum*, is a good indicator of risk of abortion; *Neospora*-infection does not affect fertility nor compromises the subsequent maintenance of gestation during the first trimester of gestation; the use of beef semen, especially derived from the Limousin bulls, reduces dramatically the incidence of abortions; and that *Neospora*-seropositivity can be very stable throughout years so that chronically-infected cows can show a high rate of repeat abortions.

Chapter VI - Gastro-intestinal nematodes have been ranked among the top three diseases that may have a significant economic impact on the American cattle industry with an estimated annual cost in excess of \$2 billion. At least 41 different species of parasitic nematodes have been described in the bovine gastrointestinal tract with predilection sites in the abomasum, small intestine and large intestine. Understanding molecular mechanisms that contribute to acquired immunity, immunosuppression and innate resistance is an important prerequisite for developing sound alternative nematode control strategies, such as vaccination and breeding for resistant populations based on genetic variations in the host genome.

Evidence suggests that parasitic nematode infection elicits drastic changes in gene expression patterns in host cells. Gene expression profiling represents the first step in understanding of the mechanism underlying protective immunity and host resistance. In this chapter, we organize our discussion into four topics: 1) A summary of recent advances on alterations in cytokine expression profiles during nematode infection; 2) A discussion of gene expression patterns of cell adhesion molecules, such as collectins, galectins, and cadherins, and their possible roles in host immune responses; 3) The effect of mucins and mucin biosynthesis during nematode infection and cell-specific patterns of expression of select genes; and 4) Perturbations in pathways and regulatory networks during nematode infection. The gene expression data accumulated over years will provide insight into cattle-parasite interactions and protective immunity against gastro-intestinal nematodes.

Chapter VII - Ostriches are commercially-reared mainly in South Africa and are spreading rapidly all over the world. Among the factors that lower the productivity of this poultry are endo- and ectoparasites. In this aspect, the nematode of the *Libyostrongylus* genus deserves attention. Although this parasite has been described for a long time (since 1882), only recently attention has been given to this genus, and a new species was identified in 1995. The occurrence of this new species has been neglected in the word probably due to its small dimension, less known diagnostic methods, and lack of knowledge on how to collect and differentiate the species. This article reviews the *Libyostrongylus* genus as a gastro-intestinal pathogen, principally its characteristics, distribution worldwide, control, biology, diagnosis, and future perspectives.

Chapter VIII - Visceral leishmaniasis (VL) is a major zoonotic disease endemic in several regions in the world, particularly tropical and Mediterranean countries. The dog is the most important reservoir for human VL. The disease is caused by *Leishmania chagasi* in the Americas, *L. infantum* in Europe, and *L. donovani* in Southern Asia. Transmission of VL is heavily dependent on the biologic vector, which are sand flies belonging to the genus *Lutzomyia* (in the Americas) or *Phlebotomus* (in the Old World). However, several cases of VL transmission in the absence of the invertebrate vector have been documented. Although VL in the dog is characterized by a chronic debilitating disease, with splenomegaly, hepatomegaly, lymphadenopathy, and emaciation among other classical signs, a diverse range of atypical clinical manifestations has also been reported. This prompted us to study genital lesions associated with VL in the dog. Interestingly, *L. chagasi* has a tropism to the male genital system, where it is located preferentially in the epididymis and external genitalia, and is associated with epididymitis, balanitis, and posthitis. Conversely, bitches naturally infected with *L. chagasi* do not develop significant genital lesions. In addition, the organism is shed in the semen, and the natural mating of dogs shedding *Leishmania* in the semen with *Leishmania*-free susceptible bitches may result in the venereal transmission of the disease.

Chapter IX - Worldwide prevalence studies indicate a high prevalence of *Giardia* in domestic animals, mainly in livestock and in companion animals. In mammals, *Giardia duodenalis* is the most prevalent species. Phylogenetic analysis revealed that *G. duodenalis* is in fact a species complex, comprising 7 so-called assemblages (assemblage A to G). Some of these assemblages are host-specific and others are zoonotic. In domestic animals, infections with only one assemblage are most often reported, and to a lesser extent mixed infections. Until recently, most research on *Giardia* in domestic animals has been inspired by the concern for zoonotic transmission, and not from a veterinary perspective. Despite the high prevalence and the alleged pathogenicity, *Giardia* is often neglected as a pathogen in

veterinary medicine. This is mainly due to the vagueness of the clinical symptoms, ranging from growth retardation and ill thrift to more overt symptoms, such as acute or chronic diarrhea. Although several clinical studies do indicate an association between *Giardia* infection and clinical signs, other studies report the presence of *Giardia* trophozoites or cysts in fecal samples from apparently healthy animals. Whether there is a difference in clinical outcome when animals are infected with one particular assemblage or another or with multiple assemblages has not yet been determined, and certainly warrants further studies. At present, only fenbendazole/febantel is registered in dogs for the treatment of *Giardia*. Several other chemotherapeutics are efficacious, but none are registered. Whether chemotherapeutic treatment is useful in the prevention of infection is uncertain, and additional hygienic or management measures seem to be needed.

Several questions still surround the relevance of *Giardia* infections in domestic animals. Is treatment and control of giardiasis in livestock economically justified? From a public health point of view, should chemotherapeutic treatment be advocated to prevent environmental contamination or direct zoonotic transmission? Is vaccination a valid alternative for chemotherapeutic treatment?

Chapter X - The meningeal worm (*Parelaphostrongylus tenuis*; Family: *Protostrongylidae*) of white-tailed deer (*Odocoileus virginianus*) in eastern North America is a cause of neurologic disease and mortality in co-habiting, native cervid species and certain livestock. The traditional method of identifying animals exposed to the parasite relies on detecting excreted larvae in feces and has been found inadequate. New enzyme-linked immunosorbent assays (ELISAs) using excretory-secretory antigens of the infective larvae of *Parelaphostrongylus tenuis* to detect antibodies in infected white-tailed deer, elk (*Cervus elaphus*), and moose (*Alces alces*) have been developed. Subsequently, the tests were rigorously evaluated to assess their fitness-for-use as tools for preventing the spread of the parasite through animal translocation and for clinical diagnosis. For the purpose of developing reliable performance parameters for the ELISAs, serum samples obtained from the three cervids species, either naturally- or experimentally-infected, were tested. All three tests showed high sensitivity ($\geq 97\%$) and specificity ($\sim 100\%$), good repeatability, and reproducibility, and are deemed useful for detecting cervids with either early exposure to *P. tenuis* or with long-standing infections. The tests are now commercially available for diagnostic use. The application of the ELISA test in a free-ranging elk showing characteristic clinical signs was instrumental in achieving the first antemortem laboratory diagnosis of *P. tenuis* infection.

Chapter XI - The GPCR class of receptors is a source of many pharmacologicals in human medicine and is still being pursued in research programs as promising targets for drug development. GPCRs have been identified in arthropods, and with the advent of expressed sequence tag (EST) and genome projects in the ticks *Ixodes scapularis*, *Rhipicephalus (Boophilus) microplus*, and *Rhipicephalus appendiculatus*, there is an opportunity for mining these sequence databases to identify tick GPCRs. These tick GPCRs can be identified using GPCR-targeted bioinformatic approaches and investigated for their potential in the development of novel tick control chemical technologies. There is precedence for developing acaricides targeting membrane-associated proteins like the GPCR family, as the pyrethroid, formamidine, and macrocyclic lactone families of acaricides target membrane-associated proteins. Current acaricides are becoming less efficacious due to problems with resistance and new chemistries which attack new targets are desperately needed by the cattle industry. We

have used bioinformatics approaches to identify and classify putative tick-specific GPCRs in our *R. microplus* gene index of assembled EST sequences. Opportunities for using high throughput screening approaches are identified and discussed which can facilitate discovery of chemical compounds which inactivate the GPCR and can serve as an acaricide.

Chapter XII - Biotransformation of xenobiotics serves as an efficient defense against potential negative action of foreign compounds in an organism. The activities of biotransformation enzymes determine desired, as well as undesired, effects of drugs and knowledge of drug metabolism is necessary for safe and effective pharmacotherapy. While human and mammalian biotransformation enzymes have been intensively studied for many years, the ability of helminth parasites to metabolize xenobiotics has been relatively little investigated so far. The aim of the present study was to test the activities of biotransformation enzymes towards model substrates and to study the biotransformation of albendazole (ABZ) in lancet fluke (*Dicrocoelium dendriticum*). Dicrocoeliosis, lancet fluke infection, is a frequent parasitosis of small ruminants and ABZ belongs among anthelmintics effective in control of this parasitosis. *In vitro* experiments (subcellular fractions of fluke homogenate), as well as *ex vivo* experiments (adult flukes cultivated in medium), were performed in the study. Biotransformation of many model xenobiotics was tested and activities of peroxidases, catalases, aldo/keto reductases, glutathion S-transferases, and UDP-glucuronosyl transferases were found. On the other hand, no activities towards model substrates of cytochromes P450 and flavine monooxygenases were detected. LC-MS analyses of ABZ metabolites revealed that lancet fluke enzymes metabolized ABZ via sulfoxidation, but they were not able to form any conjugate of ABZ.

Chapter XIII - Trematodosis caused by *Fasciola hepatica* or *Paramphistomum* spp are responsible for severe lesions in ruminants which affect production and cause important economical losses. These are parasitic infections with an analogous external phase in their life cycle and periods of high risk also. The occurrence of fasciolosis and/or paramphistomosis needs for the presence of an aquatic snail acting as the intermediate host, so the ruminants grazing in humid areas are at a highest risk for developing these diseases.

The routine procedure for the detection of infections by parasitic trematoda is based on the coprological sedimentation, and by the detection of the presence of eggs in the feces the existence of mature adult flukes in the definitive host is concluded. The main disadvantages are that a period longer than 10 weeks after infection is required, and that during many chronic infections fluke eggs are erratically excreted.

Immunoenzymatic techniques focused on the detection of antibodies against several antigens have been frequently applied in the last decade. Although good results can be achieved, the presence of cross-reaction might difficult the exact diagnosis of these diseases.

To gain more knowledge about the influence of the possible cross-immunity against *F. hepatica* and *Paramphistomum* on the interpretation of the diagnostics of fasciolosis or paramphistomosis, fecal and blood samples were individually obtained from Rubia Gallega autochthonous cattle during the 2007 year. With the purpose to select cattle with a single infection by *F. hepatica* or *C. daubneyi*, the liver, rumen, and abomasum from 492 slaughtered autochthonous cattle were examined. In addition, 1,158 fecal samples belonging to Rubia Gallega animals were analyzed by the coprological sedimentation technique. Blood samples from all of these ruminants were collected and the analyzed by means of an ELISA and different antigens of *F. hepatica* (FES) and *C. daubneyi* (CES). After collecting their excretory/secretory crude products, purification was carried out under nature conditions by

using liquid chromatography (FPLC), and under reducing conditions by electrophoresis (SDS-PAGE) and electroelution.

Five peaks were collected from the fractionation of the FES and CES under an FPLC system. The molecular weight was similar in four peaks and differed in the fourth only. After the electroelution the presence of three proteins common to both FES and CES, with a molecular mass of 106, 41, and 23 kDa was observed.

The ELISA showed that cattle with fasciolosis reacted strongly against CES-peaks P3 and P1, whereas the lowest cross-reaction against P4 (<13.7 kDa) was reached. Sera from cattle with paramphistomosis recognized FES-peaks P2 and P3, and the weakest reaction against P4 (<13.7 kDa) was obtained. By means of the electroelution procedure one protein belonging to CES (Cd2; 52 kDa) was identified, which was recognized only by the 15% of sera from cattle with fasciolosis. Furthermore, only 14% of the sera from bovine with paramphistomosis reacted with an 18-kDa protein from the FES (F8).

The elevated similarity between the excretory/secretory antigens from these parasitic trematoda under native and reducing conditions points the existence of some antigenic determinants being shared by them, hypothesis supported by the observation of an important cross-immune reaction among sera from cattle monoinfected by *Fasciola* or *Paramphistomum*. It is concluded that the application of immunoenzymatic diagnostic probes such as the ELISA requires the investigation of the possibility for a cross-immune reaction to enhance the right interpretation of the achieved results. The purification of antigens by electroelution allows the production of proteins with low cross-immunity.

Chapter XIV - Parasitic infections are chronic processes difficult to eradicate, but studies both in animals and in humans indicate that the host genetic background contributes to resistance/susceptibility and to the acquisition of the infection. Despite the high global prevalence of enteroparasitic infections in humans and the economic damage they cause in livestock, our understanding of the host-parasite relationship at the molecular level is still rudimentary, and the elucidation of the genetic basis of resistance is a major challenge in parasitology research. Laboratory animal models have the advantage of facilitating the study of the interaction among multiple genes, characteristic of complex diseases such as a parasitic infection. Furthermore, genetic heterogeneity and a range of variation of exposure to environmental agents can be controlled, allowing the identification of the different susceptibility loci. Differences in the quality and quantity of natural enteroparasitosis on the chronic phase, observed in adult mice of the CBI-IGE stock in a previous study, led us to analyze the pattern of inheritance of these variables in the reciprocal crosses of the inbred lines CBI/C and CBI-, differing in resistance. The hybrids were similar to the resistant parent, though a maternal effect was observed in the proportion of mice infected with protozoans. The helminth burden showed dominance of the resistance and a sex effect, differences among genotypes being higher in males than in females. Susceptibility to enteroparasites in the F1 differed from that of the parental lines, evincing that the host genotype is crucial in modulating the host-parasite relationship. Control of parasitic diseases based on the host genetic resistance is currently being considered as a complement or replacement of existing technologies. For this approach to be successful, a good understanding of the genes involved and the molecular pathways they control should be achieved from studies of animal models, since an organism's phenotype is the result of complex interactions between its genotype and the environment. Animal models generated through targeted gene deletion or transgenesis, though useful, involve manipulation of single major genes, making these models

inappropriate to analyze interactions among genes. Contrariwise, lines of mice generated by long-term artificial selection, a powerful technique to modify the genetic structure of a population, are valuable resources to obtain populations suitable for QTL-mapping studies to increase our understanding of the many biological processes underlying resistance to parasitic infections.

Chapter I

HISTOMONAS MELEAGRIDIS: A NEW FOCUS ON A RE-EMERGING PROTOZOAN PARASITE

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ABSTRACT

Histomonosis, or infectious enterohepatitis, is caused by the flagellated protozoan *Histomonas meleagridis* and is one of the most important turkey diseases. This parasite produces characteristic necrotic lesions in and thickening of the caecal mucosa, a caseous core in the caecal lumen, and necrotic foci in the liver. In the field, histomonosis is often recognized by the typical sulfur-colored diarrhea and occasionally by a blue-colored head, which has led to its popular name 'blackhead'. The presence of the parasite can be determined by microscopic analysis of the lesions or the droppings. Recently, this diagnosis can be confirmed by molecular techniques, based on the DNA-sequence of the 18S rRNA gene, which is one of the few known DNA-sequences of *H. meleagridis*.

Birds can become infected by ingesting embryonated eggs of the caecal worm *Heterakis gallinarum*, which contain *H. meleagridis*. These nematode eggs protect the parasite from the environment and from the conditions in the digestive tract. Although transmission through the intermediate host, *Heterakis*, is considered to be the natural way of infection, this route can probably not explain the rapid spread of the protozoan parasite within a flock kept in captivity, resulting in high mortality rates. In this case, "cloacal

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drinking” is considered as an alternative route of transmission. Recently however, cyst-like stages of *H. meleagridis* have been found, which could also suggest that cysts are responsible for the direct transmission of the parasite.

For many years, several chemical products like arsenicals and nitroheterocyclic compounds were used as chemotherapeutics and chemoprophylactics against histomonosis. However, in the last two decades, all active drugs were banned in the European Union due to the potential carcinogenic risks they entail for the consumer or due to lack of data supporting a registration under the terms of relevant European legislation. In the USA, however, there is still one prophylactic, Histostat™ (nitarson), allowed. This ban has led to an increase in outbreaks of blackhead, causing considerable economical problems for the poultry industry.

As a lot of information, mainly on the molecular level, is still lacking, genetic approaches would improve our understanding of the parasite and its virulence factors. Recently, *H. meleagridis* has been cloned by micromanipulation, which could be an important step towards the unraveling of the molecular characteristics of the parasite. This could assist in the development of new prophylactics and chemotherapeutics.

INTRODUCTION

After the discovery of blackhead or infectious enterohepatitis by Cushman in 1893 [43], the disease spread rapidly throughout the poultry industry, mainly due to the emergence of mass production. A few years later, Smith [255] described the symptoms and pathology of the disease in detail and identified a protozoan in the liver lesions of infected turkeys, which he named *Amoeba meleagridis*. This nomenclature was based on its relatively simple structure, its amoebic movements, and on symptom resemblance with amoebic dysentery in humans [255,256].

Due to its economical importance, the scientific interest in the causative agent was high. Ever since the parasite was named *Amoeba meleagridis*, conflicting views have been reported, as to its nature and taxonomy. Many researchers proposed that the disease was caused not by an amoeba, but by a trichomonad-type flagellate because of its morphology and division [103,143,277]. Subsequently, Tyzzer [279] reported that the parasites cultured *in vitro* exhibited a characteristic flagellated motility, besides the typical amoeboid activity noticed by Smith [255,256]. These discoveries led to the change of the name to *Histomonas meleagridis* [279]. Later, as the ultrastructure of *H. meleagridis* was unraveled, it became clear that this organism, which has many characteristics in common with the trichomonad line, might have arisen from an organism with a monocercomonad body plan, placing *H. meleagridis* in the Monocercomonadidae family and in the order of the Trichomonadida (figure 1) [123,250]. This was confirmed by several immunologic studies and by analysis of the 18S rRNA gene. These all revealed a close relationship between *H. meleagridis* and *Dientamoeba fragilis*, which is also a member of the Monocercomonadidae family [69,70,71,90].

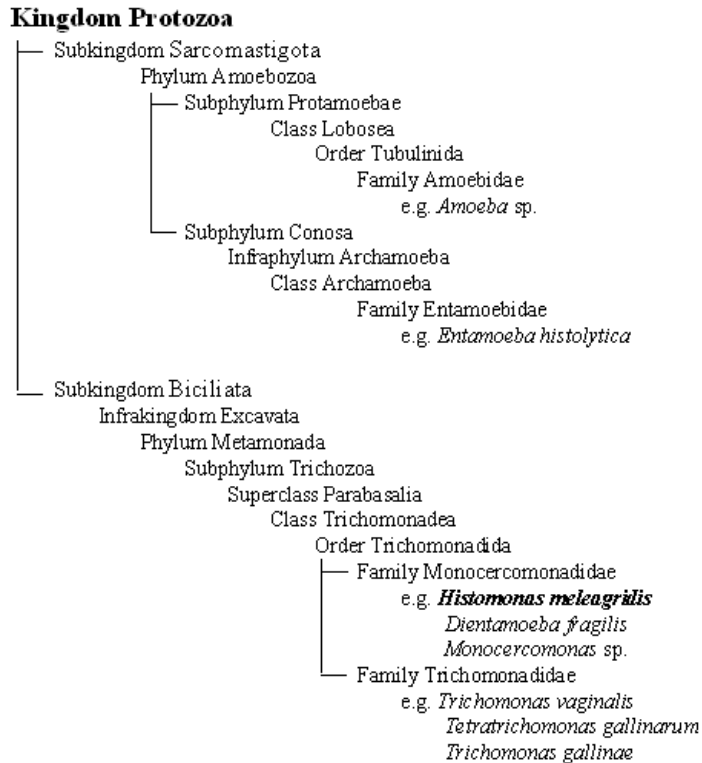


Figure 1. The taxonomic position of *Histomonas meleagridis* placed between the amoeba and the trichomonads (based on the classification system of Cavalier-Smith [21]).

In the 20th century, treatment and prevention of blackhead has been an important research topic in veterinary medicine. Many compounds have been tested, all *in vitro* and *in vivo*, resulting in a number of commercially used drugs. As this has led to the decrease of outbreaks of histomonosis, little research on the parasite has been performed. However, in the 1990s and the beginning of the 21st century, the European Union banned all chemotherapeutics and prophylactics from use in food-producing animals and only one chemoprophylactic is still available in the USA. This has led to a severe increase of outbreaks and a re-found interest in *H. meleagridis* [19,74,81,106,115,154]. Therefore, at the moment, blackhead is considered to be one of the most important turkey diseases.

LIFE CYCLE

Natural Infections

As *H. meleagridis* is very sensitive to temperatures lower than the body temperature of birds, the protozoa use an intermediate host, *Heterakis gallinarum*, and occasionally other vectors to survive in the environment outside the host's body [9,284,289]. As the parasite can also spread rapidly through a poultry-growing facility, direct transmission is suggested (figure 2).

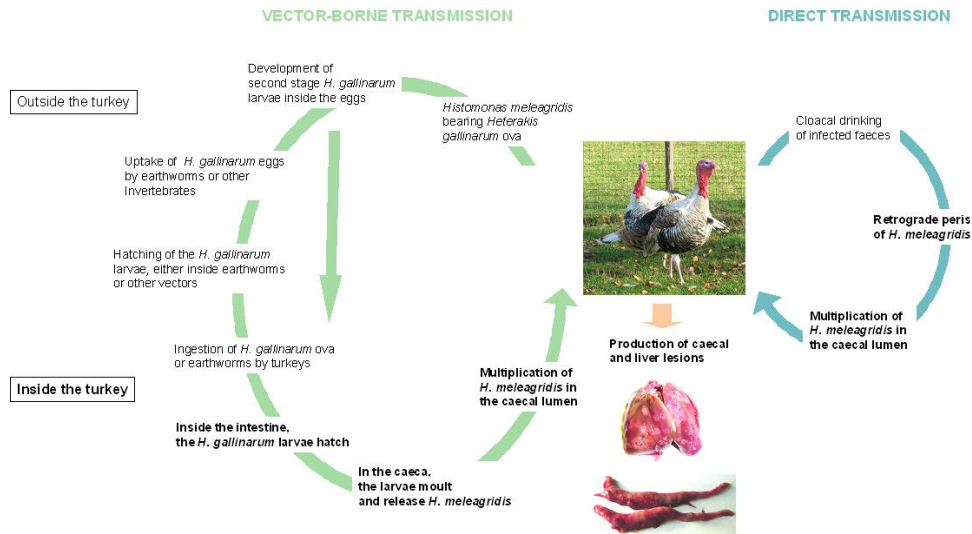


Figure 2. Life cycle of *Histomonas meleagridis* in turkeys. Green arrows: Transmission of *H. meleagridis* via embryonated eggs of *Heterakis gallinarum*. When earthworms (or occasionally other invertebrates) take up these eggs, the larvae of *Heterakis* hatch in the intestine of the earthworms. Therefore, ingestion of earthworms carrying these larvae or of the eggs themselves will lead to the transmission of *H. meleagridis*. Mortality usually surfaces 14 to 20 days post infection. Blue arrows: Direct transmission postulated through cloacal drinking. *H. meleagridis* could be taken up by cloacal drinking from infected droppings followed by retrograde peristalsis. This transmission route anticipates that mortality usually surfaces 8 to 14 day post infection. Both infection rates depend on the virulence and the dose of the strain, as well as the breed and the age of the turkeys.

Transmission by an Intermediate Host

Heterakis gallinarum (further stated as *Heterakis*) is a nematode which is commonly present in the caeca of birds. It is relatively non-pathogenic, as the only damage it causes is a mechanical disturbance of the cells of the caecal wall [295]. This worm is most likely to be found in outdoor pens or organic farms but is represented less in well-managed intensive animal production facilities [23], indicating that transmission through this nematode usually occurs in the former type of housing system.

Birds can become infected with *H. meleagridis* through ingestion of embryonated eggs of *Heterakis*, which contain the protozoan organism, or through the consumption of female nematodes carrying these embryonated ova [98,288]. The larvae hatch in the intestinal tract and migrate to the caeca, thereby carrying the protozoa to their primary site of invasion [33,179,270]. The protozoa are released during one of the moulting phases of the larvae, either in the caecal mucosa or in the lumen [85,179]. Histomonosis is therefore not induced upon ingestion of unembryonated eggs [270]. In the caecal lumen, the protozoan parasite will start to multiply and later on will invade the caecal tissue [179].

In the caecal lumen of the bird, the worm can take up the protozoa, resulting in the retention of *H. meleagridis* by *Heterakis* and the transmission of the protozoa [150,178,284]. This acquisition is usually accomplished by nematode larvae older than ten days because they tend to remain in the lumen [180]. After ingestion, *H. meleagridis* can migrate to the intestinal lumen of the worm, through the wall, and into the pseudocoel. In a female *Heterakis* worm, the protozoa will invade the germinal zone of the ovary and finally go through the

multiplicative and developmental stages before entering the egg [157]. On the other hand, *H. meleagridis* can also invade the reproductive organs of the male worm and nest itself amongst the developing spermatids [91,158]. The female worm can become infected with the protozoa during copulation. After this, *H. meleagridis* has to penetrate the wall of the uterus, enter the pseudocoel, invade the ovaries, and subsequently will embed the eggs in the same manner as described after ingestion [157,158]. It is believed that although this latter way of contaminating the eggs occurs, far more female worms become infected by taking up *H. meleagridis* themselves [158].

When the egg shells are formed upon the fertilized ova, they enclose the protozoa [157,174,258,284,288]. Moreover, it is suggested that *H. meleagridis* is present within the membrane surrounding the *Heterakis* embryo [288]. When voided into the environment within the droppings of the bird, the eggs remain infected with *H. meleagridis* for 3 to 4 years [84,175]. They will protect *H. meleagridis* in the environment from extreme conditions e.g. temperatures and drought, but also in the upper intestinal tract (i.e. the acidity of crop and gizzard) when healthy birds ingest the nematode eggs [97,179] and a new infection cycle starts. Field outbreaks of blackhead seem to occur more frequently in the hottest months of the year, although occasionally outbreaks are reported during the winter [19,97,259,289].

Young and wild turkeys, in contrast to chickens, respond severely to an infection with *H. meleagridis*, rendering the caeca untenable habitats for *Heterakis*. Therefore, in young and especially wild turkeys, the worms fail to mature or are lost completely, which means that these birds do not assist in the perpetuation of the nematode, or of *H. meleagridis* [184,187,189,193,259,284]. In contrast, adult turkeys and chickens can be infected with *H. meleagridis* without showing severe pathologic signs due to recovery. Therefore, *Heterakis* has a greater chance to mature and thus transmit *H. meleagridis*-bearing eggs in adult turkeys and chickens [184,187,257,259,289]. This shows that it is important to regard not only chickens but also adult turkeys as potential sources of *H. meleagridis*-bearing *Heterakis* ova [184].

Earthworms are reported to serve as carriers for *Heterakis* and thus ultimately for *H. meleagridis* [40,147,194,196]. When ingested, the nematode eggs hatch in the intestinal tract of the earthworm. Then, *Heterakis* larvae invade the body cavity and remain embedded in the tissues of the earthworms, although not in the digestive tract as the chance of expulsion would be too high. The larvae are retained by the earthworm until the worm is consumed by the avian host [147,194]. Earthworms protect *Heterakis* larvae from extreme environmental conditions with regards to dehydration, freezing, fungal activity, and predators. They will also cause a rapid spread and a concentration of the parasites [179,194]. As turkeys are not very keen on earthworms, this vector will not play a large role in the transmission of *H. meleagridis* in this avian host [observed by professor B. M. Goddeeris].

Several other invertebrates, such as grasshoppers, flies, *Alphitobius diaperinus*, and sowbugs, are capable of serving as vectors for *H. meleagridis* [87,137,261,278], although they appear much less important as compared to earthworms. In addition, the caecal worms can be carried mechanically by vertebrate animals or by humans, working or present in poultry facilities [40].

Direct Transmission

Although transmission via *Heterakis* eggs is still considered to be the natural way of infection, this probably cannot explain the rapid spread of *H. meleagridis* within a flock

resulting in high mortality rates within a few weeks. In addition, direct transmission is observed when droppings of sentinel (in-contact) birds became positive for *H. meleagridis* within 2 days post intracloacal inoculation of seeder (infected) birds [95,114,117,206], which is too fast to be transmitted via *Heterakis*. Moreover, transmission of the protozoa is occasionally reported in the absence of any demonstrable vectors [35,131,286].

At first, direct transmission of *H. meleagridis* was postulated to occur by oral uptake of the protozoa from the caecal discharges [255,284,286]. As the caeca are the primary site of infection, it is logical to assume that the disease is transmitted through ingestion of *H. meleagridis* containing droppings, which was been proven in 1898 [225]. However, oral inoculation with homogenized infected tissues, cultured protozoa, or faeces fails to induce histomonosis in a consistent manner [4,9,40,41,125,127,133,173,235,272,278,284,286,288] probably because the protozoa do not always survive the acidity of the crop and gizzard in sufficient amounts [127,173,272]. Only when the pH in the gizzard and crop is raised by starving the birds and/or feeding them an alkali mixture do they have a higher chance of becoming infected through oral administration of *H. meleagridis*. Therefore, in birds that are well fed and taken care of, oral transmission of the parasites by pecking at faeces is presumed to be unimportant [127,173].

A more plausible route of direct transmission is through a physiological process called 'cloacal drinking'. When any liquid material is applied to the cloacal lips, especially when followed by tactile stimulation of the dorsal lip, the material is rapidly transported by the vigorous sucking movements of the vent into the cloaca and from there to the bursa of Fabricius and via retrograde peristalsis into the caeca [34,133,260]. As this technique evokes histomonosis when applying *H. meleagridis* to the cloacal lips, it is possible that contact between the cloaca of the healthy bird and *H. meleagridis*-containing droppings is sufficient to ensure infection. However, *H. meleagridis* is very sensitive to temperatures lower than the body temperature of birds as they die out completely within a few minutes or a few hours, depending on the outside temperature [9,181,284,289]. Therefore, cloacal drinking from the watery infected diarrhea should always occur quite quickly after discharge in order to obtain a sufficient amount of viable infectious protozoa in the caeca to cause blackhead. On the other hand, direct transmission of *H. meleagridis* through cloacal drinking could not be observed in chickens [135], although cloacal drinking of other material can be observed in this bird species [34,260]. These results suggest that the dynamics of direct transmission through cloacal drinking could differ between host species.

Recently, a cyst-like form of the parasite has been detected in the faeces of an infected chicken. This cyst-like stage probably is more resistant to environmental factors compared to the flagellated form and consequently remains infective outside the bird's body for a longer period of time [222,228,229]. Therefore, this could also help to explain the often very rapid spread of the disease through a flock.

Experimentally-Induced Infections

Over the years, several methods for the artificial infection of birds with *H. meleagridis* have been employed.

To obtain blackhead infections in a more natural manner, some researchers infected birds by placing them on soil contaminated with *Heterakis* [40,53,65,66,100,113,257,288]. Rearing

birds with carrier birds can also be employed in order to simulate natural conditions [144,257,289]. However, both infection methods are susceptible to highly varying parameters, leading to inconsistent results.

Oral inoculation with infected tissues, faeces, or cultures can induce blackhead [4,9,40,41,98,125,127,133,173,225,235,272,278,284,286,288], although this method is not consistently successful. It only seems remotely reliable when very high concentrations of the protozoa are inoculated most likely because a large amount of protozoa does not survive the passage through the crop and gizzard [286]. Thus, to increase the success rate of this oral infection method, the pH of the crop and (especially of) the gizzard should be increased [125].

Blackhead can also be produced subcutaneously [54,278,287,289], intracaecally via laparotomy [3,92,243], or intravenously [92,220], usually with good results. However, these methods in no way resemble the natural infection route and usually produce atypical lesions.

Experimental infection of birds with *H. meleagridis* bearing eggs of *Heterakis* has been a generally used method to induce blackhead. The more ova fed to the birds, the higher the chance for infection and the more severe the infection will be [97,221,258,270,287,289]. Apparently, it is important that the birds are fed before the *Heterakis* eggs are administered, as these eggs have to be retained in the digestive tract long enough to permit the hatching of the larvae [179,303].

Intracloacal inoculation of birds with a mixture of infected tissues, a culture of *H. meleagridis* or fresh caecal discharges, is a reliable and convenient method in inducing blackhead [9, 45, 284, 286]. Material administered in this way is taken rapidly into the caeca by retrograde peristalsis, especially when combined with holding the birds in an inverted position for a certain amount of time [16,114,133,172]. Although both the homogenized caeca and liver can be used as inoculum, infections with homogenized caecal material are far more effective [110,183]. However, maintaining *H. meleagridis* by continuous *in vivo* intracloacal passages revealed that *H. meleagridis* loses (some of) its pathogenicity [3, observed by J. De Gussem].

Compared to *in vivo* infections with *H. meleagridis* bearing *Heterakis* eggs, this experimental infection route causes disease more rapidly. In addition, this method has the great advantage that the exact concentration of *H. meleagridis* in the infection mixture can be determined, in contrast to the infection dose inside the *Heterakis* eggs [182]. Moreover, the individual inocula usually differ somewhat in with regards to egg counts and the hatchability of the eggs, indicating that this method could lead to variations in the infected groups [174]. However, intracloacal infection usually causes severe lesions in both caeca, whereas in natural conditions, the lesions frequently only occur in one caecum [286]. Moreover, in contrast to infected *Heterakis* ova, cultured protozoa are not always readily available and cannot be stored easily without losing pathogenicity [221]. Nevertheless, over the years, the intracloacal inoculation method with either cultured *H. meleagridis* or homogenized infected tissues became the common method for experimental infections.

HOST SPECTRUM

The susceptibility to *H. meleagridis* varies greatly between gallinaceous birds. Turkeys, both domestic and wild, and chukar partridges have been shown to be the most susceptible, although turkeys are economically the most important [140,166,179,188,190,282]. The pathology of blackhead in young peafowl is very similar to that of turkeys, although the disease develops more slowly in the former [186,190,282].

Chickens, both broiler breeders and laying hens, can become infected with *H. meleagridis*, but the disease frequently does not cause systemic lesions, and thus does not become fatal [284]. Histomonosis in chickens usually results only in a decrease in zootechnical parameters and mortality is rarely noticed [65]. Frequently, these birds recover spontaneously, with the parasites persisting in the caeca, leaving the host as a carrier animal [9,40,41,174,177,183, 190,258,278,281,282,284,289]. Although blackhead is detected more often in turkeys than in chickens, probably due to its fatal outcome, an increase in outbreaks in chickens could be recorded over the recent years [35,75,81,89,120].

In guinea fowl, blackhead usually causes low morbidity and mortality rates, indicating that this disease is of little importance in this bird species. Moreover, although *Heterakis* thrives well in the caeca of these birds, the nematodes gathered from this species do not carry a large amount of *H. meleagridis* in their embryonated eggs. Therefore, they are considered to be less important carrier animals than chickens [26,27,42,187,188]. The pheasant, on the other hand, though still less susceptible to *H. meleagridis* infections, is considered the best host for *Heterakis* and the highest contaminator of soil with *H. meleagridis*-containing nematode eggs [186,188,190,236].

In summary, *H. meleagridis* has also been reported to infect rhea [59], ostriches [12,238], a number of game birds such as quails [60,61,151,155,185,240,276,277,308], ruffed grouse [277,282], and sometimes even zoo birds [205]. Non-galliform birds, such as ducks, are poor hosts for *Heterakis*, but sometimes they can serve as a carrier for *H. meleagridis*. This usually occurs without the development of any clinical signs of blackhead [1,18,195].

PATHOGENESIS

General External Symptoms

Some of the earliest symptoms of blackhead – although nonspecific – are listlessness, weakness, a hunched posture, unsteadiness, and indifference to food. Since these diseased birds are often not noticed in the flock, the disease can progress until it is too late to contain it. In a later stage of the infection, the birds begin to walk more slowly, they often stand up with ruffled feathers, have drooping wings, with their heads drawn to their bodies and their eyes closed [179,218,255,278,301]. Chickens usually display only growth retardation, while turkeys show this in the beginning of the infection, and effective weight loss when blackhead progresses [177,218]. In some cases, the skin of the face and comb turns into a dark blue color, to which the name ‘blackhead’ refers [44]. One of the most apparent symptoms of histomonosis is the shedding of sulphur-colored watery droppings, probably coming from the coagulated necrotic material in the caecal lumen [45,119]. It is also suggested, however, that

the yellowish faeces are a result of a disturbance of the bile secretion and a general dysfunction of the liver and kidneys [7].

Histomonosis in Turkeys

Domestic and wild turkeys are very susceptible for *H. meleagridis* and both develop severe lesions when infected with this parasite [193]. The severity of histomonosis in turkeys is correlated with many factors. First of all, breed differences can result in varying susceptibilities to *H. meleagridis* [3]. Generally, turkeys of 3 to 12 weeks appear to be the most susceptible since the disease tends to be fatal at this age [19,40,179,204,256], while mature or nearly mature turkeys appear to recover from the disease after showing subclinical signs of infection [40,179,256,284]. Furthermore, the severity of the disease is also related to the infection dose [172] and the virulence of the strain [179,285]. When a strain is highly virulent, the infection dose does not have a severe impact on the outcome of the disease [167]. In conclusion, the mortality rate is thus case-dependent, but can reach 100% in uncontrolled turkey outbreaks and *in vivo* experiments.

As the caecum is the primary site of a natural blackhead infection, this is the first organ showing lesions. Upon entry, the parasites start to multiply in the caecal lumen, from where they invade the lamina propria. A few days later, they will infiltrate the submucosa, the muscle layers of the caeca, and even the caecal tonsils [230,256,306]. Initially, the longitudinal ridges of the distal end of the caecum start to flatten and the mucosal surface appears to undulate. The submucosa will distend; oedema arises frequently and the color and shape of the caeca alter when they become hyperemic [83,197,306]. In a second stage, the caecal lumen begins to fill with exudates from the mucosal glands, which could possibly be due to the disturbance of the blood and lymph flow [256]. To rehydrate the tissues, water is absorbed from these gelatinous and fibrinous exudates and a typical caseous core is formed. When this occurs, part of the epithelium is lost in the core. The rate of the deposition and the consolidation of the exudates is cyclic, resulting in a core consisting of concentric cylinders and often centrally hollow [42,256]. In addition, due to the rapid multiplication and migration of the parasites in the caecal wall, the inflammatory reaction extends into the muscular layers. Consequently, yellowish ulcers, necrotic patches, and occasional hemorrhagic areas can begin to develop on the serosa of the caeca. All gradations of these severe lesions can be observed—even down to a few small necrotic patches (figure 3) [42,83,197,230,256,306].

In young turkeys, the fibrinous material of the core completely blocks the lumen, and the mucosa usually is destroyed beyond repair [256]. In contrast, in the infected caeca of mature turkeys, often only patches of necrosis and exudation of fibrinous material occur. Therefore, frequently, young turkeys do not reach the recovery stage, which is in contrast to the older turkeys. When these birds recover, the small core fragments in the caecal lumen break up and are discharged. The thickened wall of the caeca will slowly shrink as it repairs itself [179].



Figure 3. Typical hepatic (A) and caecal (B) lesions seen in a turkey infected with *Histomonas meleagridis*. The liver is covered with large lesions, showing concentric circles which indicate the periodical growth. Lesions of different sizes are observed, as the protozoa migrate over a period of time to the liver. The serosa of the caecal wall is severely swollen, ulcerated, and shows necrotic foci. The caecal lumen is filled with fibrinous, in some places even hemorrhagic exudates, covering the mucosa. Necrotic lesions of the mucosa can be detected.

Through the capillaries in the submucosa and the portal vein, *H. meleagridis* can migrate from the caeca to the liver [220]. In the beginning of the infection, the liver lesions tend to be small, white, and spherical or lenticular. As the lesions grow larger, the periphery (where *H. meleagridis* multiplies rapidly) usually is elevated as opposed to the inside of the lesion tissue, which is destroyed and therefore subsided [256]. Large and old liver lesions often show concentric circles, indicating periodical growth (figure 3). Lesions of different sizes and different stages can occur in one infected turkey, as *H. meleagridis* can reach the liver in a period of several days [179,220].

Ultramicroscopical observations suggest that *H. meleagridis* releases proteolytic enzymes and possibly also nitric oxide to cause the cellular damage, in a similar manner to *Entamoeba histolytica* trophozoites in hepatic granulomas in humans [156,253,298]. In *E. histolytica* infections, neutrophils might also play an important role in the augmentation of the parenchymal liver damage by causing aspecific damage to cells surrounding the protozoa [298]. This could also be the case for heterophils in histomonosis, but should be investigated further.

H. meleagridis can be transported through the blood vessels to other organs such as the kidneys, the spleen, the pancreas, the thymus, the heart, the brain, the bone marrow, and the lungs, where they occasionally cause mild to moderate microscopic and/or macroscopic pathological changes [96,112,138,166,197,253,287]. Without entering the bloodstream, they can also reach the bursa of Fabricius where they often cause severe inflammation with

lymphoid necrosis [96,112,114,200]. However, as the parasite is sometimes detected in the blood vessels of the bursa of Fabricius, hematogenous transmission is not excluded [253]. Furthermore, the parasite can also cause pathological changes, though rarely, in the proventriculus, the duodenum, and the jejunum. It could be explained by retrograde progression of the parasite or by migration through the bloodstream [96,112,138,254,256].

Although not frequently observed in birds unless the protozoa caused excessive damage to the caecal wall, bacteria and other micro-organisms like *Tetratrichomonas gallinarum* (figure 4) may also enter the portal vein to the liver [9,35,50,54,92,93,110]. In some severe cases, this can lead to peritonitis and secondary infections.

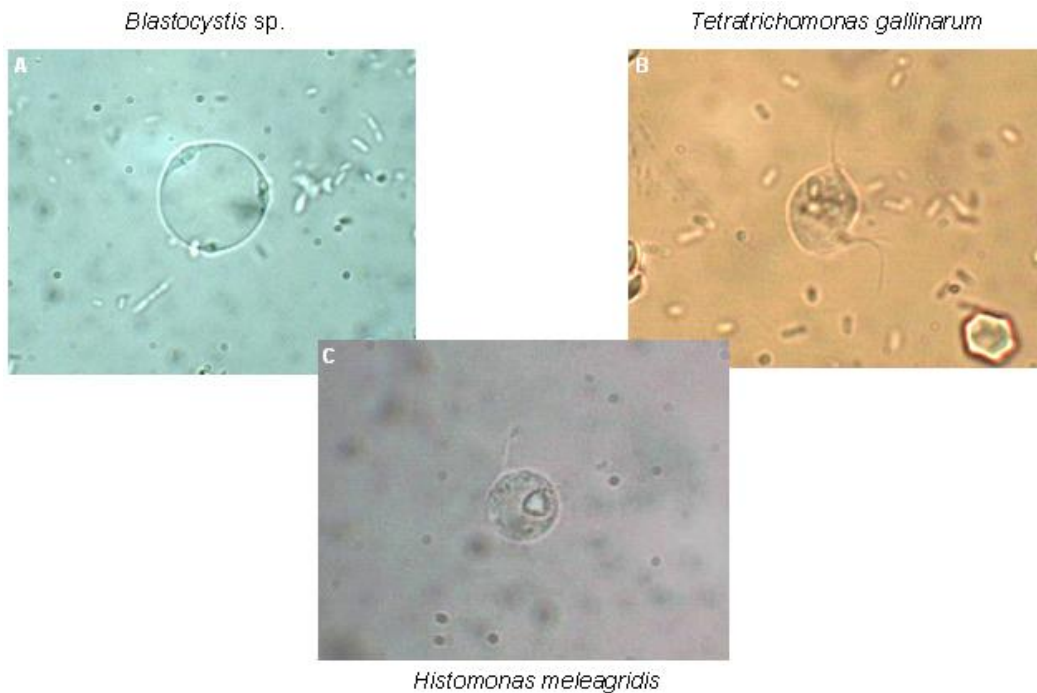


Figure 4. Light microscopical observations (1250x) of *Histomonas meleagridis* and two other protozoan organisms frequently occurring in the caecal content of turkeys, i.e. *Blastocystis* sp. and *Tetratrichomonas gallinarum*. A. Unstained wet mount of *Blastocystis* sp. The cytoplasm is a thin rim surrounding a central vacuole in which organelles are barely distinguishable. B. Unstained wet mount of *Tetratrichomonas gallinarum* (fixated in 4% paraformaldehyde), exhibiting four anterior flagella and an undulating membrane extending over the entire length of the body, finishing in a long posterior flagellum. C. Unstained wet mount of a cultured form of *Histomonas meleagridis* (fixated in 4% paraformaldehyde). This spherical organism displays only 1 flagellum and a food vacuole clearly with a rice starch particle.

Histomonosis in Chickens

Although histomonosis tends to be less severe in chickens than in turkeys, blackhead often results in a decreased production and therefore leads to an economic problem in the chicken industry [81, 177]. However, blackhead can also cause high mortality rates in chickens in field outbreaks [3,7,9,35,89,120,227,275]. The severity of blackhead in chickens

depends on the virulence of the *H. meleagridis* strain, on the breed and age of the chickens (susceptibility increases at 4-6 weeks of age), and on stress [3,9,27,120,177,179,186,231]. An increased susceptibility to *H. meleagridis* has been reported in the presence of sub-lethal dosages of p,p'DDT, an insecticide frequently used in the past [241].

The pathogenesis of severe blackhead infections in chickens resembles those of turkeys [9,89,179,227]. In addition to their common lesions in the liver and caeca, *H. meleagridis* can also cause necrosis in the bursa of Fabricius [35].

When the infection is mild, only the caeca are involved and the liver shows no or small lesions [81,127,258]. First, the caecal contents become fluid and a few days later the bird might start defecating blood. A few pin-point hemorrhages are apparent in the caecal mucosa and the protozoa are found in the mucosa and submucosa. The caecal wall becomes inflamed and a caecal core may form in the same way as in turkeys, although it becomes less rigid. Moreover, the cores often contain a large amount of erythrocytes originating from the hemorrhages [127,258]. In contrast to turkeys, chickens start to void the caseous core in the subsequent days. Lymphocytes migrate to the injured submucosa and mucosa and the chickens start to repair their caecal wall. Two weeks later, the thickened caecal wall is shrunken to its original proportions and the destroyed mucosa is covered again by epithelium or replaced by scar tissue. All protozoa which have entered the body are phagocytized and the liver shows either no or very small scar tissue [127,179,258]. Nevertheless, the parasite can be detected by molecular diagnostics in various organs of the recovered chickens, namely the caeca, the bursa of Fabricius, the kidneys, the heart, and the brain [96], indicating that the parasite is suppressed but still present.

Due to this recovery, the caeca of infected chickens are a much more sustainable environment for *Heterakis* than the caeca of turkeys. Therefore, chickens are good hosts for the nematode and will contribute to the infection with *H. meleagridis* via the intermediate host [174,177,183,190].

Effect on Serum and Tissue Molecules

Plasma enzyme levels usually alter proportionally with tissue damage at the different stages of histomonosis. In turkeys, the concentrations of glutamic oxalacetic transaminase (GOT), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and glutamate dehydrogenase (GLDH) are significantly elevated after infection with *H. meleagridis* starting from the development of liver lesions. This is related to the severity of the infection and indicates the disturbance of the biological functions caused by the infiltration of the parasite [2,3, 19,209,249].

Severe infections with *H. meleagridis* cause blue coloring of the head, which is caused by a severe increase in the blood methemoglobin level. This elevated level lowers the oxygen-carrying capacity of the blood and thus may contribute to the clinical process of the infection [201]. Furthermore, the plasma-bilirubin concentration, especially the protein-bound, is increased after infection with *H. meleagridis*, suggesting a destruction of erythrocytes (i.e. hemolytic jaundice) during the course of histomonosis [119].

Due to an increase in the permeability of the capillaries of the inflamed caecal mucosa, plasma will leak into the caecal lumen and the peritoneum, and a sharp decline of the albumin concentration in the blood is noticeable. When the disease progresses and the liver cells are

destroyed, the albumin production is reduced, resulting in a further decrease in the albumin concentration. However, in chickens and adult turkeys, due to their recovery of the disease, the albumin concentration will rise again to a normal level [31,208]. In turkeys, the plasma level of cholinesterase, a measure for liver function, decreases after the infection reaches the liver, whereas this reduction is not noticeable in chickens where only caecal lesions are present [209]. Furthermore, during the first stage of infection in chickens, the prolactin concentration in the blood starts to rise due to dehydration or blood loss in the caeca [22]. Finally, the blood amylase levels are elevated due to the caecal disturbances caused by blackhead [209].

At the beginning of the infection, or when only the caeca are involved, the concentration of xanthophylls in the blood decreases, which results in an almost completely colorless serum or plasma [208]. Prior to and after invasion of the caecal wall by the parasites, a decrease in activity and distribution of acid phosphatase and non-specific esterases can be observed, correlated with the progression of the disease. In the liver, a reduction in the amount of glycogen is observed after invasion, initially only in the surroundings of the parasites but later in all liver regions [306]. Due to this depletion and the decline in food consumption, the bird develops hypoglycemia during the final days of the disease. This severe decrease in plasma glucose concentration may even be the immediate cause of death [218].

MORPHOLOGY

H. Meleagridis in the Host

In the caecal lumen and the droppings, the protozoa can occur as a flagellated amoeboid organism, carrying a single flagellum [156,222,281,284,285]. Here, *H. meleagridis* is structurally indistinguishable, although somewhat smaller, from its *in vitro* cultured form (figure 4) [9, 284]. In the caeca, as well as in the liver, three different amoeboid forms of the parasite are found: an invasive, a vegetative, and possibly a resistant tissue-dwelling form. Presumably they have internalized their flagella in order to adapt to living between the cells [250,277].

The flagellated stage feeds by phagocytosis upon bacteria, starch grains, and occasionally even erythrocytes [156,222,281,284,285]. Therefore, as opposed to the tissue forms, this phase regularly shows ingested bacteria and, when cultured, also starch granules (figure 4) in their food vacuoles [156,222,284,285]. At room temperature, the organisms are spherical, do not form pseudopodia, and measure approximately 10 to 20 μm in diameter, though this size can vary significantly depending on the diet. At 30°C, pseudopodia are formed randomly, helping the protozoa in their movement. When the temperature rises above 37°C, *H. meleagridis* becomes highly amoeboid, the flagellum moves more quickly, and the protozoa have a rapid directional movement using the pseudopodia. The beating of the flagellum does not aid in any way to the locomotion or orientation of the protozoa [9,121,284]. The flagellated form has a parabasal apparatus, a microtubular pelta-axostyle complex, and four kinetosomes, 3 barren and 1 giving rise to a flagellum. Together these form the mastigont system, clearly arranged according to the basic pattern of the trichomonads, thus indicating its taxonomy. The parabasal apparatus is comprised of the parabasal body (Golgi complex) and

the parabasal filament, which may serve to anchor the mastigont system to the cytoplasm [121,246,250].

In the tissue, protozoa of the invasive phase can be found. In these forms, bacteria are not found inside the food vacuoles, indicating that these forms do not need bacteria to survive as opposed to the lumen-dwelling stage [159,277]. In contrast, they seem to secrete histolytic enzymes to attack and phagocytise the host cells [156]. They can be observed as large amoeboid organisms (up to 30 μm in length), highly variable in shape and pushing their pseudopodia between the host cells. They have a small nucleus, basophilic cytoplasm containing little RNA, but many granules and food vacuoles [159,277].

The vegetative phase of *H. meleagridis* appears as a rounded cell (8-20 μm in length) with a small centrally-placed nucleus embedded in a basophilic cytoplasm (figure 5) [159,279,observed by Dr. J. Mast]. This stage is commonly found in the central and oldest part of the lesions, whereas the invasive forms spread out rapidly to the periphery of the lesions [156,159,277]. Moreover, active movements of the vegetative forms have practically ceased [277]. These forms also secrete histolytic enzymes into the environment, but in contrast to the invasive stage, the vegetative stage appears to ingest the resulting fluids and small particles by pinocytosis instead of phagocytosis. Due to pinocytosis, a clear area surrounding the cell is formed (figure 5) [159]. As this stage takes up nutrients by pinocytosis and by intake of soluble nutrients such as sugars and amino acids across the general body surface, the few large food vacuoles which can be observed in the vegetative forms are not a result of phagocytosis in this stage, but are food vacuoles left in the cytoplasm from the invasive stage [159]. In the early stages of infection, the vegetative stage can still divide; in a later phase of the disease, however, this stage is usually observed inside the macrophages or in giant cells, which are a fusion of infiltrating monocytes [159].

In contrast to the invasive stages, the cytoplasm of the vegetative forms, especially the ones recently dividing, contain more RNA and also granules [159]. A Golgi apparatus, also referred to as the parabasal body of the mastigont system, is generally found close to the nucleus; a second one is often seen in a more eccentric position. In the cytoplasm, rows of microtubules with a characteristic banding pattern can be observed. These are usually found extending from a centriole, alongside the nucleus towards the distal face of one of the Golgi complexes. The rows of microtubules and the parabasal body probably give rise to the flagella of the caecal luminal and cultured forms of the parasite (figure 5).

The resistant phase is smaller than the other tissue-dwelling forms and surrounded by a dense surface membrane. This stage could be the degenerated form of the parasite and was only observed by Tyzzer [277].

In all morphological stages, the protozoa lack mitochondria, indicating that they carry out anaerobic respiration [156,250]. Instead of these organelles, spherical, membrane-bound, electron-dense organelles are present in the cells [159,250], which are probably hydrogenomes [202,222]. These organelles are likely to convert pyruvate to acetate in an anaerobic environment, which in turn will produce energy in the form of ATP and excrete molecular hydrogen [79]. In addition, regardless of the stage, multiplication occurs by binary division. Both features confirm the position of *H. meleagridis* in the order of the Trichomonadida (figure 1) [9,121,250,277,279].

Recently, a cyst-like stage of *H. meleagridis* has been observed in the faeces of young chickens infected with blackhead [222] and after subcultivation [228, 229], suggesting the presence of a cyst stage. These 'cysts' are approximately 10 μm in size, spherical, and

surrounded by an amorphous, cyst-wall-like outer layer or two outer membranes [228]. When comparing these results with the description of the resistant phase seen by Tyzzer [277] and a few spherical forms detected by Lund [181], it could be suggested that all of these forms are cyst-like stages. The possibility that *H. meleagridis* can form cysts opens up new discussions about the direct transmission of the parasite.

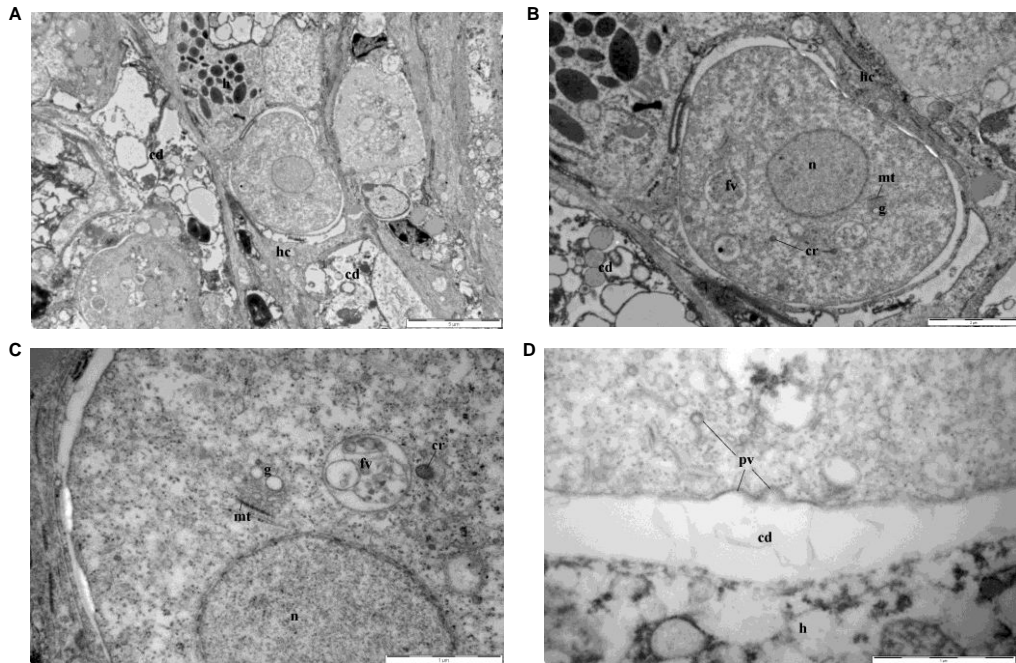


Figure 5. Transmission electron microscopic observation of the vegetative stage of *Histomonas meleagridis* observed in an infected caecum of a 3-week-old turkey (10 days post infection) (Dr. J. Mast). A. The parasites present themselves within a clear area between damaged host cells (hc), cell debris (cd) and heterophiles (h). B. Overview of the vegetative stage. Inside the cytoplasm, food vacuoles (fv), hard crystalline bodies (cr), a Golgi apparatus (g), microtubule (mt), and the nucleus (n) can be observed. C. High magnification of the perinuclear zone of *H. meleagridis*. D. The clear space between *H. meleagridis* and the host cells or heterophils contains granular material and few cell organelles in varying stages of disruption. Numerous vesicles, in various stages of pinocytosis, are present on the membrane and in the cytoplasm below it. These pinocytotic vesicles (pv) are lined with the same finely granular material covering the outer surface of the parasites.

Histomonas Meleagridis in *Heterakis*

After ingestion, *H. meleagridis* will occur in the gut epithelium of the nematodes as an intercellular organism [284], although, in one occasion, intracellular forms of the protozoa have been observed [51]. Then, they migrate through the intestinal wall and into the pseudocoel. Finally the protozoa invade the reproductive systems of both male and female worms. Electron microscopic observations have perceived that generally the stages of *H. meleagridis* in the worm appear similar to the ones described in the tissues of the host, although the protozoan stages in *Heterakis* are smaller in size (5-10 μm) [150,156,157,158]. A complex of a parabasal fibre, a Golgi complex, centrioles and microtubules, which is very

similar to the one associated with the flagellum of the lumen-dwelling form in the caeca of the avian host, has been observed in these stages. Therefore, this could give rise to the flagellum when *H. meleagridis* is released in the caecal lumen [157].

However, in contrast to their life cycle in the final avian host, *H. meleagridis* has intracellular stages in the reproductive system of both the male and female nematode, without causing any damage to the cells. In the male nematode, this stage arises when the protozoa arrive in the vas deferens. Here, *H. meleagridis* is smaller and less active than elsewhere in the nematode [91,158]. The intracellular form in the female worm occurs in the ovaries, where the protozoa nest themselves between the oogonia and gradually pass down into the growth zone as an extracellular parasite. When the oocytes begin to develop, the protozoa break through the oolemma and commence their intra-oocyte stage [157]. The intracellular form of the parasite and (especially) the effort *H. meleagridis* has to make to end up inside the oocyte prove the importance of *Heterakis* as an intermediate host and not just as a vector.

MOLECULAR CHARACTERIZATION

H. meleagridis has a certain amount of antigens in common with *D. fragilis* and *Trichomonas gallinae*. Some of them are at least partially structurally related, though others are identical or very closely structurally related [69,70,71]. This antigenic similarity is also reflected in their structural characteristics and their taxonomical position [62]. This close relationship between these parasites was confirmed when the 18S rRNA gene was sequenced [90].

As variations in virulence of *H. meleagridis* isolates have been reported [72] and differences of strains in the *in vitro* susceptibility to chemical or herbal prophylactics or therapeutics have been noticed [94], it becomes more and more crucial that strains of the parasites become characterized and that this is taken into account in both *in vitro* and *in vivo* trials. Therefore, the novel molecular technique of C-profiling based on the Internal Transcribed Spacer-1 sequence is designed and can be used to differentiate between *H. meleagridis* subtypes. At present, 3 different genotypes of *H. meleagridis* are identified, two of which (type I and II) are associated to strains causing clinical disease and the third one (type III), although morphologically slightly different from the other two and thus possibly not *H. meleagridis*, is related to strains causing less severe illness [293]. As the isolates for this experiment are all originating from the Netherlands, it is possible that isolates from other countries lead to other genotypes, which means that further C-profiling is necessary. Genotyping can be useful for epidemiological and pathogenesis studies, but also for future analysis of virulence factors on a molecular level.

In the hydrogenosomes, a few iron-hydrogenases, a malic enzyme, and an α -subunit of a succinyl coenzyme A synthetase (α -SCS) have been detected and (partially) sequenced. In addition, immunohistochemical staining of *H. meleagridis* suggests a supplementary role for α -SCS in the adherence to host cells [202], as previously described for *T. vaginalis* [80]. This hypothesis should be further investigated for *H. meleagridis*, but could be very interesting in regard to vaccine development.

DIAGNOSIS

To diagnose histomonosis and to study the epidemiology of the parasite and hence estimate the risk of outbreaks, it is necessary to be able to detect *H. meleagridis* or the disease in a rapid and reliable way. Clinical diagnosis of histomonosis is performed by detecting the sulphur-colored droppings and the general suspicious behavior and appearance [179]. Confirmation of blackhead in dead birds or euthanized sick birds is performed through identification of macroscopic lesions. However, diagnosis of histomonosis is complicated due to other enteric conditions causing lesions quite similar to those of blackhead, e.g. mycosis, trichomoniasis, coccidiosis, and leukosis [4,148,149,205].

Parasitological diagnosis is done by differential staining (i.e. Periodic acid-Schiff staining) of tissue sections, caecal swabs, or faecal samples [61,148,149,197,207]. Furthermore, microscopic analysis of *in vitro* cultured caecal contents or faeces could also be of help. However, microscopic diagnosis requires much experience and patience to differentiate *H. meleagridis* from artifacts, other flagellates like *T. gallinarum* pseudocysts, and other micro-organisms like *Blastocystis* sp. commonly present in the caecal content or droppings of birds. In the tissue, *H. meleagridis* is non-flagellated and similar to macrophages and yeast cells, which hinder the diagnosis significantly [9,90,284].

These days, molecular tools have been developed to detect the presence of *H. meleagridis* in tissues and droppings. Specific amplification of fragments of the 18S rRNA gene by (nested) PCR or real-time PCR is a sensitive, simple, and much-used method for the diagnosis of blackhead [10,93,105,136]. However, as PCR inhibitory components are commonly found in caecal samples and especially in droppings, PCR tests are greatly affected by certain substances in the samples [124,224,242]. Therefore, a PCR was developed with an internal control which is co-amplified with the DNA in the sample and thereby detects the inhibition of the PCR reaction and thus also inhibition of the positive samples. This gives this diagnostic PCR a great surplus value [10].

Finally, molecular tools can also be applied to localize the parasites in the tissues. *In situ* hybridization with a specific probe based on the 18S rRNA gene can be used for the accurate and distinctive detection of *H. meleagridis* in tissue samples [168]. Furthermore, the traditional routine diagnostic staining techniques have been improved by the development of an immunohistochemical staining with specific anti-*H. meleagridis* polyclonal antibodies [253].

CULTURING HISTOMONAS MELEAGRIDIS

H. meleagridis has been isolated from caecal contents [52,67,207], from caecal discharges [46,47,48,49], and from liver lesions [9,50], but it can also be isolated from embryonated eggs of *Heterakis* [245]. Storage in liquid nitrogen can successfully preserve *H. meleagridis* in the presence of 8% dimethyl sulphoxide, as a cryoprotectant, without affecting its pathogenicity [25,72,122].

Initially, a combination of a solid and liquid medium was used to culture *H. meleagridis* [64,282,284,285]. Later on, culture media were developed consisting of a basal culture medium to which growth factors and, if necessary, bacteria were added

[9,47,52,67,160,161,207,245, 291]. Different types of serum like horse, swine, or sheep serum and even chicken embryo extract or fresh cream serve well as suppliers of growth factors [67,160,161,191,207,264], although horse serum is now commonly used. Often, small grains of starch [9,46,47,48,49,52, 207,282,284,294] or charcoal [244,284] are added as the necessary source of carbohydrate. The starch granules are supposedly split by the associated bacteria or – when the particles are small enough – phagocytized completely by the protozoa themselves [282,284,294]. Increasing the amount of starch in the culture results in higher yields of *H. meleagridis* [294]. Cultures of *H. meleagridis* can be maintained without sub-culturing by regularly supplementing rice powder [284, 294]. Adding the growth factor biotin to the medium also has a positive effect, either directly on *H. meleagridis* or on the associated bacteria, resulting in faster growth [48].

The cultures should be incubated at 40-41°C to stimulate multiplication in order to imitate the body temperature of turkeys. Sometimes cultures at lower temperatures are preferred to avoid excessive sub-culturing, although incubation below 36.5°C is not recommended [9,52,54,192]. The pH of the medium appears to be important with an optimum ranging from 7.2 to 7.8 [9,47,64]. As this parasite is characterized as a facultative anaerobe organism, it is optimal to be cultured in oxygen-poor conditions [48].

In cultures originating from caecal contents or discharges, frequently other protozoa are present which are commonly found in the caeca of poultry, e.g. *T. gallinarum* or *Blastocystis* sp. (figure 4). The presence of these protozoa is known to be detrimental to the *in vitro* growth of *H. meleagridis*. This negative influence cannot yet be explained completely, but in the case of *Blastocystis* sp., this could be due to its rapid growth or to its competition with *H. meleagridis* for some essential compounds from the medium [46,284].

Furthermore, certain caecal bacterial species are also important for *in vitro* growth of these parasites, as it appears to be impossible to culture *H. meleagridis* in the absence of compatible bacteria [9,46,92,160]. However, as axenic *H. meleagridis* suspensions are needed for molecular analysis of the parasite, antibiotics can be added to suppress or even eliminate the bacterial and fungal growth [46,160,264]. In addition, cultures can also be cleared from bacteria by washing the protozoa after exposure to 15-30% horse serum [134]. Nevertheless, to ensure protozoan growth, bacteria or bacterial growth factors, which are proven to be heat-labile, have to be added regularly [92,160,264]. When the bacterial growth factors are replaced by fresh hamster liver and metallic ions, *H. meleagridis* is presumed to grow axenically [162], although this method still demands further confirmation.

Recently, Hess et al. [116] established a mono-eukaryotic culture of *H. meleagridis* using a micromanipulation approach. This cloned culture is infectious and highly pathogenic in turkeys, but less in chickens [114,167], indicating that the technique does not affect the strain's pathogenicity. The cloned protozoa are maintained in the presence of living bacteria [116]. Keeping in mind that Lesser [160] demonstrated that only bacterial growth factors are necessary for culturing *H. meleagridis*, in the future, possibly lysed bacteria might probably support the mono-eukaryotic cultures.

As bacteria appear to be an important growth factor in the culture medium of *H. meleagridis*, it is necessary to know which bacterial species are preferred. In *in vitro* cultures, the protozoa grow favorably in the presence of certain members of the *Enterobacteriaceae* family [92,164,264], suggesting that these bacterial species carry the heat-labile factor [160]. Moreover, *in vitro* growth of *H. meleagridis* in the presence of turkey caecal flora is superior to the growth of the protozoa with chicken caecal flora [163], which could contribute to the

difference in susceptibility and pathogenesis between both avian hosts. Furthermore, the presence of one or more of certain bacterial species, like *Clostridium perfringens*, *Escherichia* sp., *Lactobacillus fermenti*, *Streptococcus faecalis*, and *Bacillus subtilis* is required to induce blackhead *in vivo*, although not all of these species generate equally good results [13,14,63,88,146,262]. Moreover, they are not all commonly found in the caecal contents of turkeys [109].

Maintaining the cultures for a prolonged period can affect their pathogenicity, although no uniformity could be established between various strains of *H. meleagridis* regarding the rate of decrease in pathogenicity [117,127,191,192,282,283,284,285]. While the structure and basic activity of these attenuated strains remain virtually unchanged, nutritional and both quantitative and qualitative antigenic changes do occur, which can all be related to the loss in pathogenicity [68,73,191,192]. A gradual attenuation of virulence is observed which is fully reversible in the beginning by consecutive passages in avian hosts. However, after further attenuation, the loss of virulence is no longer reversible [72,284]. Attenuated strains which have lost their ability to cause blackhead in chickens are sometimes still pathogenic for turkeys. Therefore, it is necessary to perform pathogenicity tests whenever these attenuated strains would be applied for *in vivo* studies [284,285].

Table 1. Overview of phytoproducts tested against *Histomonas meleagridis*

	Phytoproducts	Components	References
plant extract	Natustat TM	red yeast rice extract, garlic and vitamins	65, 66, 77
	saponin extract	saponin extract from the cortex of <i>Quillaja saponaria</i>	94
	PE1	coated grapefruit seed extracts	111
	PE2	extracts from cinnamon, garlic, lemon and rosemary	104
essential oil mixture	<i>Cassia</i> oil	extracted from <i>Cinnamomum cassia</i> of the <i>Lauraceae</i> family (false or Chinese cinnamon or cassia lignea)	94
	Protophyt TM	oils extracted from cinnamon, garlic, lemon and rosemary	292, 77
	EO1	extracted from thyme and rosemary	94
	EO2	blend of microencapsulated cinnamon, thyme, citrus fruit extract and capsicum	111
	Repaxol TM	blend of microencapsulated oregano, cinnamon, thyme, citrus fruit extract and capsicum	111
	Carvacrol	extracted from the essential oils of oregano, thyme, pepperwort or wild bergamot	94
ethanol extracts	EE1	ethanol extracts from <i>Peganum harmala</i>	5
	EE2	Pumpkin fruit, grape seed, saw palmetto and thyme	95

In addition, loss of pathogenicity can also be caused by irradiation of *H. meleagridis* cultures. Depending on the dose of gamma radiation, this technique can kill *H. meleagridis* cultured *in vitro*, stimulate or retard its growth, or, interestingly, attenuate the protozoa [243,244].

PREVENTION AND TREATMENT

There have been many chemical compounds and phytoproducts tested *in vitro* and *in vivo* for their efficacy against *H. meleagridis*. Some of these pharmaceuticals (i.e. nitroimidazoles, nitrothiazoles, and nitrofurans) can be used both as a chemoprophylactic and as a therapeutic.

Some of these pharmaceuticals are prodrugs which are processed into active compounds in the caeca by the anaerobic metabolism of the protozoa. The use of prodrugs is particularly appealing because of their selective toxicity for microorganisms which possess these specific enzymes to activate the compounds [254].

Inhibition of Nucleic Acid Function

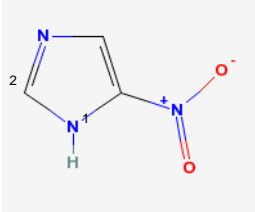
Nitroimidazoles

In general, nitroimidazoles prodrugs (table 2) are reduced by anaerobe protozoa into cytotoxic anion radicals within the hydrogenosomes [129]. The knowledge by which these highly active radicals kill the protozoa is incomplete, although there is evidence indicating that one of the targets is DNA [78 152].

Dimetridazole, metronidazole, ipronidazole, ornidazole, ronidazole, and tinidazole are all found to suppress the *in vitro* growth of *H. meleagridis* with varying efficacy (dimetridazole being the most effective) [17 132 292]. Dimetridazole, metronidazole, ornidazole, ronidazole, ipronidazole, tinidazole, flunidazole, and MF nitroimidazole are also highly protective in *in vivo* experiments [24,86,132,170,198,199,217,223,249,252]. Dimetridazole and ipronidazole also significantly increase weight gain and feed conversion rates, although dimetridazole only does so up to 8 weeks of age [6,223]. Furthermore, ipronidazole shows remarkably low toxicity and high safety levels [223]. In addition, nitroimidazoles are highly effective for the treatment of blackhead. Treatment of infected turkeys with dimetridazole often results in complete regeneration of the liver lesions with a noticeable remission of the disease within the first 24 to 48 hours after medication [86,171,217,226]. However, after withdrawal, birds can suffer from a relapse of the disease [171,252]. Nevertheless, increasing the dosage above the therapeutic dosage can lead to a decrease in weight gain [217]. Ipronidazole, ronidazole, and metronidazole can also be used as therapeutics against histomonosis [113,223,252,266]. After treatment with ronidazole or dimetridazole, *H. meleagridis* is still present in the caeca of the treated turkeys, which could lead to a relapse [252] and transmission of the parasite [29].

However, due to their carcinogenic, mutagenic, and genotoxic effects, not only on the parasites but also on laboratory animals, all nitroimidazoles are considered unsafe as pharmaceuticals for treatment of animals of the food chain. Therefore, these products have been banned in the European Union and the United States since 1996 [32,74].

Table 2. Overview of the nitroimidazoles active against *H. meleagridis*.

Nitroimidazoles			
	Drug	Substituent at position 1	Substituent at position 2
	Dimetridazole	Methyl	Methyl
	Metronidazole	β -hydroxyethyl	Methyl
	Ipronidazole	Methyl	Isopropyl
	Ornidazole	2-hydroxy-3-chloropropyl	Methyl
	Ronidazole		Carbamoyloxymethyl
	Tinidazole	Methyl	Methyl
	Flunidazole	2-(ethylsulfonyl)ethyl	p-fluorophenyl
	MF nitroimidazole	2-hydroxyethyl	p-fluorophenyl
		Methyl	

Chemical structure based on <http://pubchem.ncbi.nlm.nih.gov/>

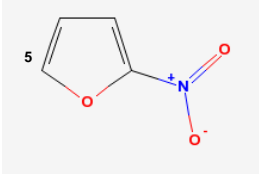
Disruption of Energy-transfer Reactions

Nitrofurans

Reduction of the nitro group of the nitrofurans prodrugs (table 3), results in the activation of the drugs to their free radical states, which are mutagenic, although less than those of the nitroimidazoles. The basic mechanism of action of the nitrofurans is however not fully understood, although some hypotheses have been made. Protozoa can be destroyed because of the competition for reducing enzymes and disrupting electron transfer reactions, essential for the anaerobic energy metabolism of the parasites. However, it is also possible that new unknown intermediate products are formed which have another detrimental effect on certain cell functions [232,254,290].

Although furazolidone does not show an *in vitro* anti-*H. meleagridis* activity [128], it can prevent blackhead in turkeys [126,141,214,215,217]. Nevertheless, furazolidone has not been used extensively in the control of blackhead due to competition with other, more efficient drugs and because of economical reasons [297]. Furthermore, nidrafur, a drug which can be used in the control of several poultry diseases, provides an effective protection against naturally-induced histomonosis [107]. However, both compounds are considered to exhibit carcinogenic and genotoxic activity [309]; these nitrofurans are not used as prophylactics against blackhead in poultry.

Table 3. Overview of the nitrofurans active against *H. meleagridis*

Nitrofurans		
	Drug	Chemical formula with indication of the substituent at position 5
	Furazolidone	N-(5-nitrofurfurylidene)-3-amino-2-oxazolidone
	Nidrafur	Acetic acid, 5-nitrofurfurylidene hydrazide
	Nifursol	3,5- dinitrosalicylic acid, 5-nitrofurfurylidene hydrazine

Chemical structure based on <http://pubchem.ncbi.nlm.nih.gov/>

Although nifursol only inhibits the *in vitro* growth of *H. meleagridis*, regardless of the concentration [17], this nitrofuran provides protection against histomonosis in turkeys and in chickens by reducing mortality and morbidity to a minimum [267,296,297]. Little or no relapse incidences of blackhead can be observed following withdrawal of nifursol from the feed. Also, no negative effects on weight gain or feed efficiency can be established [267,296,297]. Nevertheless, as for the other nitrofurans, the use of this compound in animals of the food chain poses carcinogenic, mutagenic, and genotoxic risks for the consumer, which has led to the ban of nifursol as a feed additive in the EU and the USA [37,74].

Nitrothiazoles and Related Compounds

The mechanism of action of nitrothiazole prodrugs has not been clarified fully, although the same hypotheses as for the nitrofuran drugs can be made [253].

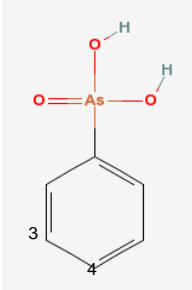
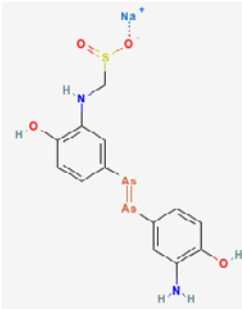
Both 2-amino-5-nitrothiazole and its derivate, 2-acetylamino-5-nitrothiazole, can inhibit the growth of *H. meleagridis in vitro* [8,128,210,299]. *In vivo*, both of them are able to prevent blackhead in turkeys [15,53,57,100,102,142,144,170,251,300,305], although relapse of the disease after withdrawal from 2-amino-5-nitrothiazole can occur when the treatment is begun too late or the dosage is too low [58,212,300,302]. In addition, 2-amino-5-nitrothiazole has an anthelmintic effect upon the larvae of *Heterakis*, which contributes to its role in the control of histomonosis [270,272]. However, treatments with 2-amino-5-nitrothiazole for longer periods or at higher dosages can cause toxic effects with symptoms such as growth retardation and disturbances of the reproductive system [53,57,101,139,142,237,251]. This means that a carefully designed schedule is necessary. In contrast, no toxic effect of treatment with 2-acetylamino-5-nitrothiazole could be observed on hatchability or fertility [15,101]. The related compounds, 5-nitropyrimidin-2-amine and nithiazide (or 1-ethyl-3-(5-nitro-2-thiazolyl) urea), also have a highly prophylactic effect on *H. meleagridis*. The latter has no detrimental effect on growth rate or feed conversion rate [38,39,170,299,300]. At a curative level, 2-amino-5-nitrothiazole, 2-acetylamino-5-nitrothiazole, and nithiazide are all able to treat turkeys from blackhead [144,213,217,271].

Nevertheless, over the years, all nitrothiazoles and related products have been replaced with new chemical compounds which have less toxic effects, are more effective against blackhead, or are more readily available and cheaper.

Arsenical Compounds

The arsenical drugs (table 4), or metabolites, formed by the host are involved in inhibition of different enzymes of the energy metabolism of the parasite [108].

Table 4. Overview of the arsenical compounds active and/ or used against *H. meleagridis*

Arsenical compounds			
	Drug	Substituent at position 3	Substituent at position 4
	carbarsone	/	carbamoylamino
	tryparsamide	/	glycineamide
	nitarsonsone	/	nitro
	sodium acetarsol	acetamido	hydroxy
	roxarsone	nitro	hydroxy
		Drug	Chemical formula
	Nearsphenamine		Sodium 3,3'-diamino-4,4'-dihydroxy-arsenobenzene-N-methylene sulphinate

Chemical structures based on <http://pubchem.ncbi.nlm.nih.gov/>

The compounds carbarsone and tryparsamide are both found to protect birds against blackhead [203,217,233,234,248,268,280,304,305]. In addition, carbarsone has little or no effect on growth and feed conversion rate [203,233,304]. Conflicting results are found regarding the prophylactic properties of sodium acetarsol, as there is protection when turkeys are infected intracloacally [82,248], but no protection against oral administration of infected embryonated eggs of *Heterakis* [274]. Besides its prophylactic activity, tryparsamide also shows a curative effect as it reduces the mortality caused by blackhead quite strongly without any signs of toxicity when administered intravenously and/or subcutaneously [280].

Treatment of blackhead with nearsphenamine led to conflicting results. Moreover, due to its high costs, its instability and its high toxicity, there are too many disadvantages to its usage [118,280].

Occasionally, roxarsone, an anticoccidial compound, is currently used as an anti-*H. meleagridis* prophylacticum, although no *in vivo* experiments have proven its efficacy [130]. However, *in vitro* tests have established that roxarsone has an anti-*H. meleagridis* activity, but only at high concentrations and after a 48 hours incubation period [17]. Moreover, high

dosages of this compound tend to have a negative effect on avian motoric functions and the birds become depressed [307]. Therefore, roxarsone should not be used as a measure to control blackhead.

Nitarsonsone (HistostatTM) is proven to be effective in the prevention of histomonosis in turkeys [77,217,219,305, observed by M. De Gussem]. At the moment, this is the only drug approved by the FDA for the prevention of blackhead in the USA, and recently it was registered in Canada, Morocco, and Israel as well. At FDA-approved levels, this drug has no significant effect on growth or feed efficiency [6], although one study observed a positive effect on weight gain [219]. When administered in higher dosages, growth retardation and other toxic effects can manifest itself such as leg weakness, head tremors, and paralysis [265,269].

Antibiotics

Inhibition of Protein Synthesis

Paromomycin, an aminoglycoside antibiotic, is known to inhibit the protein synthesis of protozoan organisms [20,99,145,263]. In an *in vitro* study, this antibiotic has proven its activity against *H. meleagridis* [132], and, in all but one *in vivo* study [132], this compound also showed its preventive effect on *H. meleagridis* infections [76,169, observed by K. De Gussem]. Registration of this compound (as HistoBlocTM) as a prophylactic drug against histomonosis is currently being requested in the EU.

Elimination of Accompanying Caecal Bacteria

Antibiotics like bacitracin, apramycin, penicillin, tylosin, and sarafloxacin are effective at FDA-approved dosages in reducing morbidity of histomonosis in chickens, probably by eliminating some of the caecal bacteria. Higher levels of bacitracin and apramycin are required to obtain a further decrease of liver lesions [130]. However, as prophylactic treatment of turkeys with bacitracin does not appear to have a significant effect upon blackhead in turkeys [210], the results obtained in chickens cannot be extrapolated to turkeys [130]. Nevertheless, elimination of the essential caecal microflora and, thus, prevention by antibiotics seems to be quite ambitious.

Anthelmintic Compounds

Benzimidazoles

Sometimes, anthelmintic compounds are used in the control of histomonosis. These chemicals do not have an effect on the protozoa themselves, but only on the intermediate host and thus will only have an outcome on the transmission via *Heterakis* [132]. *In vitro* tests with some of these compounds, such as albendazole (methyl N-(6-propylsulfanyl-1H-benzimidazol-2-yl)carbamate) and fenbendazole (methyl N-(6-phenylsulfanyl-1H-benzimidazol-2-yl)carbamate), are therefore negative [17]. On the other hand, *in vivo* experiments in turkeys demonstrate that both drugs have a prophylactic effect if administered for at least 14 consecutive days. In addition, both drugs are associated with a significant

increase in weight gain, which is probably due to the elimination of nematodes [113]. In chickens, benzimidazoles can also be used to successfully eliminate *Heterakis* and will therefore lead to the end of the carrier status of the birds [observed by M. De Gussem].

However, as *H. meleagridis* appears to be transmitted in the absence of the intermediate host by direct transmission (especially for birds in intensive animal production facilities), complete elimination of blackhead by these anthelmintic compounds is not possible.

Compounds with unknown Mechanisms of Action

Phytoproducts

As all available prophylactics and therapeutics against *H. meleagridis* have been banned from the EU market since 2003 and are limited in the USA and other turkey-producing countries, phytotherapy has become an interesting domain regarding the development of new prophylactics against histomonosis. An overview of all the phytoproducts tested against *H. meleagridis* is given in table 1.

NatustatTM, a natural, plant-derived product, significantly lowers the morbidity caused by blackhead in *in vivo* experiments where birds were infected on contaminated litter. However, as the mortality rate of the untreated infected control group was very low [65,66], the results are very difficult to interpret. Nevertheless, when the turkeys are severely infected by intracloacal inoculation, which produces consistent experimental infections, NatustatTM does not protect the birds against blackhead [77].

A saponin extract derived from the cortex of the Soap bark tree exhibits anti-*H. meleagridis* activity in *in vitro* experiments [94] as does coated grapefruit seed extracts, although it is not yet clear whether or not this is due to their broad antimicrobial properties, which thereby diminish the essential microbial flora in the culture [111]. *In vitro*, ethanol extracts of *Peganum harmala*, or its β -carboline alkaloid components harmaline and harmane, all inhibit *H. meleagridis* [5]. As harmane, in contrast to harmaline, is considered to have genotoxic activities, only harmaline should be tested in *in vivo* experiments [5].

An ethanol extract of pumpkin fruit, grape seed, saw palmetto, and thyme (EE2) has proven to completely inhibit *in vitro* growth of *H. meleagridis*, although no effect except for a delayed mortality is noticed when these products are tested in *in vivo* experiments in turkeys where birds are either infected intracloacally or through contact. This could be due to the high virulence of the strain, which causes a very high mortality rate and could mask any positive effects. Another possible reason is that the active compounds do not reach the caeca [95].

Plant extracts derived from cinnamon, garlic, lemon, and rosemary (PE2 and ProtophytTM, oil-based) have a negative effect upon *H. meleagridis*. ProtophytTM has been found to inhibit the growth of the parasite *in vitro* [292] and both have already proven to be an effective prophylactic measure against experimental histomonosis in turkeys [77,104,293]. However, when using PE2 in a field trial, it does not give a satisfactory outcome, indicating that this compound is probably not suitable for use in field conditions [104].

Carvacrol, *Cassia* oil, and an essential oil mixture of thyme and rosemary (EO1) all inhibit the *in vitro* growth of *H. meleagridis* [94]. Furthermore, *in vitro* tests with essential oil mixtures of cinnamon, thyme, citrus fruit extracts, and capsicum (EO2 and RepaXolTM, with added oregano) proved that these herbal products exhibit anti-histomonad activity, although this could also be due to their broad antimicrobial properties [111].

Quinolines

Iodochloroxyquinoline and chlorohydroxyquinoline both have a protective effect against blackhead in turkeys [53,55,56,82,211,248]. Moreover, *in vitro* iodochloroxyquinoline also exerts an anti-*H. meleagridis* activity [8]. Nevertheless, the prophylactic effect of chlorohydroxyquinoline does not occur in birds infected by contaminated soil, the imitation of a natural infection [53]. Moreover, these substances have toxic side effects in poultry and therefore should not be used for the control of blackhead [153].

Other Compounds

Vitamins A and E alone are not effective against histomonosis in terms of reducing mortality and morbidity, regardless of the dose. However, supplementing high levels of vitamin A in combination with dimetridazole, carbarson, 2-acetylaminonitrothiazole, and ipronidazole (although not statistically significant) will enhance the prophylactic effect of these compounds in terms of mortality and morbidity caused by a severe blackhead infection [249,305]. In addition, vitamin E improved the performance of ipronidazole [249].

Treatment of blackhead by intramuscularly injecting adrenomone (adrenocorticotrophic hormone), a hormone in the past commonly used in treatments of a variety of animal and human diseases, was only able to induce a transitory delaying effect on the course of the disease [216].

Future Compounds

Due to the way of transmission, good hygienic practices are important in the control of blackhead. However, management practices alone are rarely adequate, such as in some cases in which the farms implementing the highest hygienic standards had a higher incidence of blackhead than in farms with lower standards [76]. Moreover, as the use of conventional indoor housing facilities (i.e. cages) for laying hens will be drastically reduced in the European Union by 2012 due to animal welfare considerations and rearing of the hens in alternative systems, such as enriched cages and outdoor systems [36], risks for contracting the nematode and therefore also *H. meleagridis* will be increased [81]. Therefore, new pharmaceuticals are necessary in the battle against histomonosis, and the need for a new measure becomes rather urgent.

Since blackhead is not detected until the first symptoms are showing, therapeutic treatment of the birds is quite often too late. Indeed, when the *H. meleagridis* strain is highly virulent and the liver damage is already too severe, treatments generally are not able to elicit a full recovery. Therefore, vaccination becomes a more and more appealing strategy.

IMMUNITY AGAINST *H. MELEAGRIDIS*

After *H. meleagridis* invades the lamina propria, heterophils start to accumulate around the protozoa (figure 5). The tissue-dwelling parasites appear to secrete histolytic enzymes to attack these leukocytes and phagocyte them, indicating that the heterophils do not impede the progression of the parasites. A few days later, as parasites migrate to the submucosa and later

on to the liver, the amount of heterophils in the lamina propria declines, although the total amount of these leukocytes increases as they follow the parasites to the new infection sites [159,218]. When the parasites reach the caecal submucosa or are migrating to the liver through the portal vein, the lymphoid tissue in the caecal wall increases. Within this tissue, degenerating parasites can be found. The amount of lymphocytes in the blood increases from this point on, although it turns back to normal just before death. Macrophages surround the parasites throughout the infection process and, in a later stage, so do giant cells and plasma cells. However, the latter cells appear to be attacked by the phagocytizing action of the parasites. Internalization and subsequent destruction of *H. meleagridis* by giant cells and macrophages is probably an important part of the immune response [159,218].

When survivors of an initial infection are reinfected, protective immunity is only established in certain cases but is hardly consistent [259,288]. In addition, immunization by intramuscular or intravenous injection with diseased tissue emulsions or by intramuscular injection with lysed cloned *H. meleagridis* fails to induce protection against histomonosis [11,117,289].

When blackhead in turkeys is treated with nitroimidazoles, liver lesions start to heal and appear microscopically as foci of lymphocytes surrounded by regenerating hepatocytes [247], suggesting a role for lymphocytes in the immunity against this disease. In addition, immunity induced by infection and treatment does result in protection when the infection is allowed to go through the early pathological processes in the caeca before being stopped by the treatment [11,30]. This immunity, however, cannot be transferred to naive turkeys and chickens by antibodies, indicating that antibodies as a sole source of immunity are not able to protect birds against histomonosis [11,30]. Therefore, further investigation concerning mucosal and cellular immunity is necessary.

Recently, the differential outcome of histomonosis in chickens and turkeys has been linked to the differential immune responses in these galliform species when infected with *H. meleagridis*. When the infection starts in the caeca, chickens build up an effective pro-inflammatory innate immune response. This is followed by an IL-13-driven Th2 response in the caecal tonsils, which hinders the migration of the parasites to the liver. Parasites that do move to the liver induce an efficient Th2 immune response that causes elimination of the protozoa and thus prevents further development of lesions. In contrast to chickens, turkeys do not develop such an efficient innate immune response in the caecal tonsils, which therefore results in extensive caecal lesions and a greater dissemination of the parasite to the liver and liver lesions [239].

H. meleagridis propagated for a prolonged period either *in vitro* [117,282,283,284] or *in vivo* [3, observed by J. De Gussem] is found to lose their pathogenicity. These attenuated strains are able to immunize both chickens and turkeys by intracloacal inoculation and protect them against subsequent intracloacal inoculations with pathogenic strains [117,282,283,284] observed by J. De Gussem]. *H. meleagridis* is still detectable in the caeca of the challenged birds after vaccination with *in vitro*-attenuated strains, indicating that the birds have in fact become carrier animals [117,282,283,284]. Even birds reared with birds vaccinated with (and thus carrier of) the *in vitro*-attenuated strain were protected against a subsequent infection [117]. Nevertheless, continuous exposure to pathogenic *H. meleagridis* is necessary to maintain this immune status at a high level [283,284]. Still, when these attenuated cultures are cultured for even longer periods, their immunization properties appear to diminish [191,192,283,284,285]. Possibly, due to longer *in vitro* attenuation, the attenuated culture

loses some of its transient invasive properties, which might lead to its incapability to invade the mucosa and hence to evoke an immune response [283]. Or, *H. meleagridis* cannot multiply to a sufficient amount to induce a protective immune reaction due to the nutritional changes they undergo when cultured for a longer period of time [191,192,284]. Furthermore, as *H. meleagridis* does not survive well at room temperatures, it is difficult to transport this life-attenuated vaccine. Moreover, even an attenuated strain may be regarded as a potential risk, especially considering that the pathogenicity differs between avian species [284]. These obstacles should be overcome before vaccination with an attenuated strain takes place on a commercial basis.

In contrast, the immunization of turkeys with radio-attenuated histomonads does not protect birds against subsequent infection with un-attenuated *H. meleagridis*, indicating that no protective immunity has been achieved [243].

Attempts were also made to protect chickens against *Heterakis* in order to control histomonosis by oral administration of infected *Heterakis* eggs. Although protection against the nematode was developed, a challenge of these birds with *Heterakis* ova containing *H. meleagridis* showed only a slight reduction of the incidence of blackhead. Probably the induced death of the nematodes would in most cases liberate the protozoan parasite and thereby initiate histomonosis [176].

CONCLUSION

Over the years, detailed information has been gathered about the life cycle of *H. meleagridis*, its morphology, and its pathogenesis. Many investigations have been performed to search for the optimal prophylactic or therapeutic compound against this disease; however, after the ban in the EU and the limited use in the USA and other turkey-producing countries, the discovery of a new measure against blackhead is rather urgent. Possibly, after registration of HistoBloc™ in the European Union, this antibiotic could be of assistance in the control of histomonosis.

Although histomonosis was discovered many years ago, little is known about the molecular characteristics of the protozoan parasite. Nevertheless, this information is very important for vaccine development, drug targeting, and developing new diagnostics.

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Chapter II

PARASITE CONTROL AND LIVESTOCK PRODUCTION IN NIGERIA

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ABSTRACT

Parasitic infestations constitute about 70-80% of animal diseases in Nigeria. Their control is increasingly becoming difficult because of the ever-growing parasite resistance against veterinary drugs. Moreover, the low social profile of most Nigerian farmers, who rear livestock essentially on a small scale, has made improved livestock health through parasite control almost a mirage. The overall effect of this is decreased livestock production, leading to poor animal protein supply and intake. The nomadic Fulani pastoralists of rural Nigeria, who own livestock resources in the country, prefer the use of herbal remedies to treat animal diseases. It is believed that the development of potent parasite vaccines will ameliorate the menace of veterinary drug resistance. In this chapter, the biochemical, molecular, and genetic mechanisms of parasite resistance against veterinary pharmaceuticals and the prospects of emerging biotechnologies in successful parasite control for optimal livestock production are discussed.

INTRODUCTION

Nigeria's Livestock Resources

The most reliable data on Nigeria's livestock population put the country's livestock resources at an estimate of 82.4 million chickens, 34.5 million goats, 22.1 million sheep, 13.9 million cattle, 0.9 million donkeys, 0.2 million horses, 90,000 camels, 31.9 million other poultry (pigeons, ducks, guinea fowl, and turkeys), 3.5 million pigs, 1.7 million rabbits, 0.5 million guinea pigs, and 60,000 giant rats (RIM, 1992). The nomadic Fulani pastoralists of

rural Nigeria own about 70-80% of the country's livestock resources and are said to be the custodians of livestock production in the biggest country in Africa, south of the Sahara. They move from one place to another within the country and its neighbours, in search of pasture for their livestock (especially cattle) during the dry periods of the year when feed supply is usually scarce.

Poultry outnumbers all other forms of livestock in Nigeria, and, not surprisingly, are found throughout the country wherever there is human settlement. Although pigeons, ducks, guinea fowl, and some turkeys are also kept in substantial numbers, chickens are by far the most common. Typically, they are maintained under traditional, low-input, free-range systems of management, but a great number are also reared intensively on a commercial basis. Commercial holdings account for some 10 million chickens, or 11 percent of the total estimated population of 82.4 million. There was a boom in intensive chicken production in the early 1980s, when the government subsidized the prices of day-old chicks and feed ingredients. As the subsidies have now been withdrawn, both extensive and intensive commercialized production have tended to decline, especially in urban areas, despite the continued demand for chicken meat and eggs.

Small ruminants are almost as ubiquitous as poultry, though not so numerous. There is estimated to be a total of 56.6 million head throughout the country, with goats outnumbering sheep by three to two. Although some seasonal movement of pastoral sheep does take place, the great majority of small ruminants are sedentary village livestock, and their patterns of distribution mirror those of human settlement.

There are three main varieties of goat in Nigeria: the West African Dwarf, the Sokoto Red, and the Sahel. Goats are renowned for their hardiness and can survive in most environments. West African Dwarf goats are kept in the forest zones and in the Middle Belt, Sokoto Red goats are kept throughout the north, and Sahel goats are restricted to a strip along the frontier with the Niger. Although pastoral Sahel goats are found in the northern semi-arid zone, most goats are kept in villages. The most common production system is that of seasonal confinement. Northern goats were found to be markedly more productive than West African Dwarf goats, with lower ages at first kidding and shorter kidding intervals, although they produced fewer kids per kidding.

There are four main types of sheep native to Nigeria: the Balami, Uda, Yankasa, and West African Dwarf. Balami and Uda sheep are kept in the semi-arid regions, West African Dwarf sheep in the south, and Yankasa sheep are found throughout the country. Sheep are the second most numerous pastoral species and small flocks accompany many cattle herds in the north and in the Middle Belt. A comparison of pastoral and village stock shows that pastoral animals are generally more productive. The productivity of West African Dwarf sheep was substantially lower than that of other breeds. All Nigerian sheep are used for wool, but they are rarely milked. In the north, they are eaten regularly and form part of the daily protein supply, but there is also a marked variation in demand coinciding with religious festivals. As a result, there are dramatic seasonal price fluctuations, and, in some areas, the household fattening of sheep for sale is a major economic activity.

Cattle are found throughout Nigeria, but they are most common in the northern two-thirds of the country. Seasonal transhumance does take place. Humped Zebu cattle are by far the most common, but limited numbers of Keteku, Muturu, and Ndama cattle occur in the south-western and southern parts of the country, respectively.

The traditional Nigerian black hairy pig is gradually being replaced by various exotic breeds, including the Large White, Landrace, Hampshire, and Duroc breeds. Pigs are generally kept under systems of seasonal confinement in the north and Middle Belt, but they are usually confined all year-round in the south, except in the Niger Delta region. Pigs must be given supplementary feeds, and, in village systems, the lees of beer are often combined with household scraps for food. The production of pigs is obviously profitable and continues to spread in many parts of non-Muslim Nigeria. Intensive pig rearing is economically viable on the periphery of large cities because of the availability of industrial by-products, particularly brewers' grain. Units of between 50 and 200 pigs kept in concrete pens, although not advisable, are common, especially in the densely populated regions of the south. Commercially managed piggeries with more than five breeding sows account for about 3 percent of the total estimated pig population of 3.5 million.

Constraints of Livestock Production in Nigeria

The constraints of livestock production in Nigeria include disease, lack of adequate feed, and adverse climatic factors (Jagun, 1985; Useh, 2007). Of the reported diseases of livestock in the country, 70-80% of them are parasitic and retard production (Useh *et al.*, 2003; Useh, 2005). The parasitic diseases found to be devastating in Nigeria's livestock are caused by haemo, helminthic (gastro-intestinal), or ectoparasites (Useh and Du-Sai, 2003; Useh *et al.*, 2006a). Haemoparasites of indigenous livestock are transmitted by ectoparasites, such as ticks, and, hence, the control of the latter is central to parasitic disease control in Nigeria. In one study, gastro-intestinal helminthiasis was found to be prevalent in pigs, and although the diseases reported were not of any zoonotic significance, they retarded growth, causing losses in production (Useh and Du-Sai, 2003). Some Nigerian authors also reported that parasitic diseases are prevalent and constitute the greatest problem faced by sheep farmers in the country (Useh and Esievo, 2006). Haemoparasites of livestock have been reported to cause anaemia, leading to death, if not treated (Chae *et al.*, 1999; Useh *et al.*, 2005). In Nigeria, the illegal involvement of unlicensed practitioners in animal disease management, control, and prevention has created many decades of setback in the quest for livestock disease control. These people are usually patronized by the nomads, since their services are cheaper, although not supported by the rational use of veterinary drugs. Hence in most cases, animals treated by this group of untrained persons never recovered, either as a result of wrong diagnosis or sub-therapeutic doses of the veterinary drugs administered. There is a sustained abuse of the broad spectrum of parasitic drugs, most times at lower than therapeutic doses. The overall effect of the above is that the parasites being treated become resistant to the drugs, thus creating a bigger problem, as the entire world of parasitology is today grappling with the problem of resistant species. The nomadic Fulani pastoralists of rural Nigeria on one hand prefer the use of herbal remedies to treat livestock diseases, most of which are parasitic, and would only report such diseases to veterinary authorities if their treatment approach fails, accompanied by an upsurge in mortality of the animals involved (Abdu *et al.*, 2000). The resolve to use the herbal remedies may either be attributed to the low income profile of the nomads or their lack of faith in modern veterinary drugs. The side effects of herbal remedies have been identified namely: inappropriate dosing (Gueye, 1999), partial efficacy (Sonaiya, 1990), and deaths due to intoxication (Nomoko, 1997).

PARASITE RESISTANCE IN NIGERIA AND OTHER AFRICAN COUNTRIES

In domestic animals, parasites are an important cause of disease and production loss and in some cases, these pathogens act as an important source of disease to humans. For these reasons, there has been much investment in research in the treatment and control of parasites of veterinary importance. Many of these pathogens are currently, or have been, controlled using specific therapeutic agents. However, for many species, drugs have been rendered ineffective by the development of resistance (Matthews, 2006). Resistance, therefore, is an important limiting factor in the use of drugs to control veterinary parasites. There are many reports of parasite resistance against drugs elsewhere (Anderson, 1977; Geerts *et al.*, 1987; Prichard, 1990), but in Nigeria, although parasite resistance is a major problem, there are not many scientific reports about parasite resistance. This is because not much research is currently on-going about parasite resistance in the country. Mbah *et al.* (1992) reported benzimidazole resistance in strongyles of sheep in Nigeria. A recent study which investigated ivermectin resistance in three farms in Oyo state, Nigeria showed there was no ivermectin resistance in the sheep investigated (Ademola, 2002). Obviously, the data volume was small that it had no chance of producing a positive result. Fashanu and Fagbemi (2003) reported the resistance of strongyles to albendazole, febantel, levamisole, and morantel in 10 herds of cattle in Shaki, Oyo state, Nigeria.

In the 37 African countries with endemic animal trypanosomiasis, including Nigeria, trypanocides play a key role in the control of the disease. There are currently only three trypanocides available for controlling tsetse-transmitted trypanosomiasis in domestic ruminants (caused by *Trypanosoma congolense*, *Trypanosoma vivax*, and, to a lesser extent, *Trypanosoma brucei* and *Trypanosoma evansi*). These are isometamidium and homidium, which have both prophylactic and therapeutic effects, and diminazene, which has only therapeutic properties. It is estimated that 35 million doses of these drugs are used in Africa each year, with about 50–70 million animals at risk from trypanosomiasis (Geerts and Holmes, 1998). All three drugs have been on the market for >40 years and, for much of this time, they have been provided by a few European manufacturers. However, generic forms of these compounds from a variety of sources have recently become available. In the past, the availability of trypanocides was strictly controlled in most African countries by government veterinary departments. However, in recent times, with the privatization of veterinary services and a general trend towards the deregulation of markets, trypanocides, along with many other pharmaceutical products, have become more freely available through local pharmacists, agroveterinary suppliers, and the informal sector, and many are now purchased directly by farmers. In this way, availability has increased, but so have the risks of misuse. Trypanocidal drugs are probably the most commonly used veterinary products in sub-Saharan Africa (SSA), with the possible exception of anthelmintics and traditional remedies. They are often the first drugs tried by farmers in SSA when their cattle develop (any) symptoms of disease because they are affordable, at approximately US\$1 per treatment. As a result, they are frequently used without an accurate diagnosis in tsetse-infested areas. There is also an understandable trend for farmers to restrict treatments to the more valuable animals in the herd, such as work oxen and milking cows.

The development of resistance to therapeutic agents has been well-documented for antibiotics, anthelmintics, and insecticides. Thus, it is not surprising that drug resistance has also emerged in the three commonly used trypanocides given their long use (Geerts and Holmes, 1998). Trypanocide resistance has been demonstrated conclusively under laboratory conditions by inoculation of trypanosome isolates into bovines and treatment with correct drug dosage regimens, or by the administration of prophylactic drug dosages followed by challenging it with tsetse-infected, well-characterized trypanosome populations at regular intervals (Peregrine, 1994). An ELISA test for isometamidium has demonstrated the presence of high drug levels in the blood of cattle harbouring trypanosomes, whereas the same drug concentrations were shown to be prophylactic for drug-sensitive isolates (Eisler *et al.*, 1997). Furthermore, characterization of numerous trypanosome isolates in rodents has revealed significant differences in drug sensitivity that correlate well with the drug resistance patterns observed for these populations in cattle.

Although drug resistance has now been reported in at least 13 African countries (Peregrine, 1994; Geerts and Holmes, 1998), the reports have usually been of isolated cases, and there has been little attempt to undertake spatial or temporal assessments of the scale of the problem. An important prerequisite to undertaking such studies is the availability of reliable and simple tests for drug resistance, as the failure of drug treatments might have causes other than drug resistance. These might include significant under-dosing, treatment with bogus drugs, or rapid re-infection after treatment with a short-acting therapeutic drug. Although a variety of tests for trypanocide resistance have been developed over the years, using animals (ruminants and laboratory animals) or *in vitro* approaches, there have been few attempts to compare the reliability and acceptability of these different techniques. Unfortunately, in most African laboratories, *in vitro* cultivation of trypanosomes is not yet readily available and one has to turn to less sophisticated tests using animals (Geerts *et al.*, 2001).

PARASITE VACCINES VERSUS DRUG RESISTANCE

Parasite vaccines have been reviewed by Crampton and Vanniasinkam (2007). Vaccination against parasitic infections is relatively unsuccessful despite decades of research and millions of dollars spent. One reason is that parasitic infections, unlike those caused by bacterial or viral pathogens, tend to be chronic in nature. This is primarily because parasites often elicit inappropriate and ineffective immune responses in the host, or dampen the host's immune system, thereby preventing a robust and effective immune response by the host (Cox, 1997). Moreover, parasites often exhibit various immune evasion strategies such as antigenic variation, molecular mimicry, and sequestration at both the individual and infective population levels. Additionally, they exhibit complex lifecycles and other biological characteristics which complicate vaccine development (Good *et al.*, 2004). Nevertheless, in most cases, parasitic infections do confer immunity to subsequent infections by the same parasite in the host, thus demonstrating the potential for a vaccine strategy (Scott, 2005). Parasite vaccines have been developed utilizing a range of strategies from crude whole organism preparations to peptide antigens (Liddell *et al.*, 1999; Woollard *et al.*, 1999). More recently, novel molecular-based strategies have been employed. These include DNA

vaccines, viral vector-based vaccines, and combinations of DNA and viral vector-based modalities (Da'Dara *et al.*, 2003; Nielsen *et al.*, 2006). For example, a multitude of vaccine modalities have been evaluated against a single pathogen, *Leishmania* spp.

The early vaccines against parasites were often based on crude whole organism preparations, either intact irradiated larvae or ground-up parasites of various stages (Sharma *et al.*, 1988; Delgado and McLaren, 1990; Eberl *et al.*, 2001; Ploeger, 2002). More recently, vaccines against parasites have sometimes been based upon protein, glycoprotein or carbohydrate antigens (Law *et al.*, 2003; Vervelde *et al.*, 2003). Recombinant proteins in particular are popular as vaccine antigens as they are easy to identify and easily produced commercially. Immunogenic surface antigens are the most common antigens used for the construction of protein-based vaccines. Tran *et al.* (2006) reported the success of a vaccine based on the *Schistosoma mansoni* surface antigen tetraspanin (TSP1 and 2). Studies revealed that the presence of antibodies directed towards these *S. mansoni* membrane proteins in the human host conferred protection from chronic infection. Recombinant TSP-based vaccines were evaluated in mice and TSP-2 in particular was effective in the mouse model reducing worm burdens by 57% and liver egg burdens by 64% in *S. mansoni*-challenged mice (Tran *et al.*, 2006). Another example of a protein-based vaccine is a commercial vaccine that was developed for use in dogs, the Leishmunel vaccine. Approaches involving the use of more than one antigen from the parasite have also been adopted with success, such as the vaccine for *T. saginata* (based on the TSA-9 and TSA-18 antigens) (Lightowers *et al.*, 1996).

In addition to protein and carbohydrate antigen-based vaccine technology, many vaccinologists are now focussing on developing genetic and viral vector-based vaccines for parasitic diseases. The application of a broad range of technologies to vaccine development is perfectly illustrated by the range of vaccine candidates that have been developed against *Leishmania* spp. with vaccines ranging from purified whole organisms to DNA vaccines and a commercialized protein-based vaccine (Leishmunel). Interestingly, some whole organism vaccines have been found to be at least as effective, if not more effective than some of the more recent technologically-advanced vaccine formulations (Boulter and Hall, 1999; Matthews *et al.*, 2001; Suo *et al.*, 2006). It has been suggested that while the whole organism vaccine and the recombinant vaccine may contain the same key antigens, the process by which the recombinant protein is produced could result in the loss of crucial structural features associated with the protein's immunogenicity in its native state (Hein and Harrison, 2005). Hence, the recombinant vaccine may be less effective than the whole organism-based vaccine. Consequently, it appears that when it comes to parasites, some vaccinologists are moving towards refining the traditional whole parasite-based methodologies; for example a whole organism vaccine approach is being pursued to develop an effective vaccine against *S. mansoni* (Kariuki *et al.*, 2006) and a phase III clinical trial of an attenuated parasite-based vaccine against *Leishmania* is currently underway in Iran (www.ClinicalTrials.gov). Still though, such whole organism vaccines have only been effective for a handful of parasitic diseases, hence the continued focus on other vaccine modalities such as genetic and viral vector-based vaccines.

In the years since Wolff *et al.* (1990) first demonstrated that naked DNA injected into mammalian muscle cells resulted in protein expression, DNA or genetic vaccines have been developed against a wide range of pathogens including *S. mansoni* (Ganley-Leal *et al.*, 2005), Mycobacteria (Kamath *et al.*, 2000), *Rhodococcus equi* (Vanniasinkam *et al.*, 2004), Hepatitis C virus (Encke *et al.*, 2007), *Trypanosoma cruzi* (Dumonteil *et al.*, 2004), and *Taenia solium*

(Guo *et al.*, 2007). The ease of construction, preparation, and administration, as well as the potential for its use in remote locations, contributes to the overall attractiveness of this vaccine modality. Furthermore, DNA vaccines can be used to generate either strong Th1 or Th2 type immune responses in the host. This makes them ideal for use against parasitic infections that require specifically-tailored immune responses in order to confer protection. For example, studies on leishmaniasis reveal that a Th1 type response is protective while a Th2 response is detrimental, eventually resulting in death of the host (Campos-Neto, 2005). Conversely, recent findings on schistosomiasis suggest that a Th2-biased cytokine response is essential for resistance to infection (Leenstra *et al.*, 2006). Studies on *Neospora caninum* have also shown that a Th1/Th2 cytokine balance is critical to determining host control of the infection (Nishikawa *et al.*, 2003). Interestingly, studies on *Leishmania* spp. demonstrate that although Th1 responses in the host are protective, *Leishmania* antigens that typically elicit a Th1 response in the host are not necessarily protective when used as a vaccine. Conversely, antigens such as LACK (*Leishmania* homologue of receptors for activated C kinase) that usually promote a Th2 type immune response in the host when manipulated to induce a protective Th1 type response in the host using immunomodulating adjuvants have been found to elicit a protective response (Campos-Neto, 2005). The fact that DNA vaccine efficacy is largely dependent upon the route of vaccine application and choice of gene is well known (Li *et al.*, 2004). Studies on *Brugia malayi* and *Plasmodium* spp. underscore the importance of the route of vaccine delivery, with the same antigen administered by two different routes resulting in diverse immune responses (Li *et al.*, 2004; Weiss *et al.*, 2000).

In recent years, large scale screening of parasite genomes is being used extensively to identify genes that may be potential DNA vaccine candidates (Bhatia *et al.*, 2004). However, due to the differences in codon usage between mammals and parasites, the codon preference of the DNA used in the vaccine must first be optimised to ensure maximum efficacy (Gaucher and Chadee, 2002). Genes used in the DNA vaccine construction range from those involved in the adhesion and invasion process within the host to genes that encode heat shock proteins, antioxidant enzymes, and paramyosins (LoVerde *et al.*, 2004; Beghetto *et al.*, 2005; Solis *et al.*, 2005). Not surprisingly, the majority of genes used in the construction of anti-parasitic vaccines are those which encode immunogenic surface antigens (Da'Dara *et al.*, 2003). DNA vaccine technology also led to the construction of multi-epitope vaccines. Scorza *et al.* (2005) have described a multi-epitope malaria vaccine (derived from a *Plasmodium chabaudi adami* DS DNA expression library) that when used to vaccinate BALB/c mice generated opsonizing antibodies and an antigen-specific interferon gamma response. Importantly, the vaccine conferred protection against a heterologous (*P. chabaudi adami* DK) parasite challenge (Scorza *et al.*, 2005). On the other hand, some researchers have demonstrated the ineffectiveness of the multivalent DNA vaccine approach. Li *et al.* (2004) developed DNA vaccines based on four antigens of *Brugia malayi* (paramyosin (BM5), heat shock protein (BMHSP-70), intermediate filament (BMIF), and an immunogenic antigen (BM14)). Subsequent studies on mice indicated that a mixture of all four vaccines induced a significant antibody response to all antigens. Interestingly, the response to each of the four antigens was varied. Importantly, the response to the polyvalent vaccine regimen was less effective than the response induced when each component was administered by itself, suggesting that a multi-epitope vaccine approach may not be feasible in some cases (Li *et al.*, 2004). Many researchers have demonstrated variable successes using DNA vaccines. In one study, a vaccine against *S. Mansoni*, based upon the 23 kDa gene (Sm23) that encodes a membrane

protein, was developed and tried in mice as both a DNA vaccine and a recombinant protein vaccine (Da'Dara *et al.*, 2003). Three weeks after two doses of vaccines, administered 21 days apart, the animals were challenged with *S. mansoni* cercariae. The results indicated that the best level of protection, which was a 44% reduction in worm burden, was obtained in mice primed and boosted with the DNA vaccine (Da'Dara *et al.*, 2003). Other investigators have developed DNA vaccines based on antigens expressed during the chronic stage of the parasitic infection; this approach is particularly relevant to diseases such as toxoplasmosis which can occur as relapsing chronic conditions. A DNA vaccine based on the bradyzoite antigens (BAG1 and MAG1) of *T. gondii* in the C3H/HeN mice elicited a 62% reduction in *T. gondii* cyst burden upon oral challenge 4 weeks after the final dose of three DNA vaccine doses was administered (Nielsen *et al.*, 2006). These examples further highlight the difficulty in obtaining sterilized immunity using DNA vaccines against parasites. Overall, DNA vaccines have not been seen as being universally successful parasite vaccines. Despite showing promise in the mouse model, clinical trials of DNA vaccines (parasite and non-parasite specific) indicate that overall, these vaccines are not efficacious in large mammals, including humans (Smooker *et al.*, 2004; Laddy and Weiner, 2006). Furthermore, some studies have shown that DNA vaccines are not as effective as other vaccine modalities. For instance, a comparison between the schistosomal membrane antigen encoding gene (sm23)-based DNA vaccine with a vaccine comprising irradiated cercariae showed that, although the DNA vaccine induced significant parasite-specific immune responses in mice, it did not induce optimal vaccine efficacy (Ganley-Leal *et al.*, 2005). The reasons for the lack of efficacy of DNA vaccines have been discussed in detail in other reviews (Ertl, 2003; Vanniasinkam and Ertl, 2004). In order to improve overall efficacy, DNA vaccines are now often used as part of prime boost vaccination schedules with either recombinant protein or viral vector-based vaccines (Goonetilleka *et al.*, 2006; Liang *et al.*, 2006). However, this strategy does not always lead to the efficacy levels required of an effective vaccine or vaccine strategy. Iborra *et al.* (2003) developed a DNA vaccine based upon the immunodominant *L. infantum* acidic ribosomal protein P0 (LiPO). The efficacy of this vaccine was ascertained in the BALB/c mouse model. Animals were immunized with either a DNA vaccine alone or as a prime followed by a recombinant protein (rLiPO) boost. Results indicated that protection was achieved by the DNA vaccine alone and not when used with an rLiPO boost or when mice were immunized with rLiPO alone. In this study, the DNA vaccine elicited a protective Th1-type response and the rLiPO vaccine elicited a Th2 response. Consequently the heterologous prime boost protocol resulted in a mixed Th1/Th2 response which was not as protective as the un-mixed Th1 response. Interestingly, other studies have found no significant difference between a recombinant protein-based vaccine and its DNA counterpart. For example, Solis *et al.* (2005) constructed a DNA vaccine based on the *Taenia solium* paramyosin gene VW2-1. Mice immunized with the DNA vaccine or recombinant VW2-1 vaccine, were challenged intraperitoneally with *Taenia crassiceps* cysticerci. The results of this study revealed that there was no significant difference in the level of protection (43–48%) afforded by the DNA vaccine when compared with the recombinant VW2-1 vaccine. These results were replicated in the porcine model (Solis *et al.*, 2005). Other attempts to enhance the efficacy of a DNA-based vaccine strategy have included co-administering DNA vaccines with cytokine-expressing vaccines in order to augment the efficacy of the DNA vaccine, and some researchers have found this approach to be reasonably successful. The success of this approach was illustrated in a study which showed that the co-application of IL-12 (as a DNA

vaccine) together with a DNA vaccine based on the *S. japonicum* triose-phosphate isomerase gene resulted in approximately a further 30% decrease in liver egg burdens in the porcine model (65.8% reduction in liver egg burden) than when the triose-phosphate isomerase-based DNA vaccine was used alone (49.4% reduction in eggs found in the vaccinated animal's liver) (Zhu *et al.*, 2006). Some researchers have found that DNA vaccines are most effective when administered in a heterologous prime boost protocol comprising a DNA vaccine prime and viral vector-based vaccine boost, with both vaccine modalities based on the same antigen. Pe´rez-Jime´nez *et al.* (2006) demonstrated a significant immune response (increase in interferon gamma secreting CD8+ T cells) in mice primed with a LACK -expressing DNA vaccine and boosted with a LACK-expressing modified vaccinia Ankara (MVA). Recent studies suggest that the co-administration of cytostatic drugs, such as trichostatin A, may help up-regulate viral promoter-driven DNA vaccine activity, thus improving the vaccine's performance. Further studies are required to determine if this tactic will improve DNA vaccine efficacy in large animals (Vanniasinkam *et al.*, 2006).

Viral vectors based on adenovirus and pox virus are being increasingly used in the construction of effective vaccines (Xing *et al.*, 2005; Hanke *et al.*, 2007). Overall, studies have shown them to be far more effective than DNA vaccines (Prieur *et al.*, 2004). Viral vector-based vaccines are capable of inducing potent, highly effective immune responses in the vaccinated hosts (Pe´rez-Jime´nez *et al.*, 2006; Vanniasinkam and Ertl, 2005). Furthermore, studies have shown that these vaccines may indeed be used to elicit strong Th1-type immune responses which are particularly useful when developing a vaccine against a disease such as leishmaniasis, which typically requires a strong Th1-type protective response (Campos-Neto, 2005). One disadvantage associated with some viral vaccine vectors (e.g. human adenovirus serotype 5-Ad5) is that they may not work effectively in the host if there is pre-existing immunity to the carrier. This is particularly a concern with some human serotype adenovirus-based vectors as extensive research in this area has revealed that a significant percentage of the population possess neutralizing antibodies to some of these viruses (Fitzgerald *et al.*, 2003). Consequently, research involving alternative adenovirus serotypes such as those of simian origin is being undertaken (Roy *et al.*, 2006). Some researchers have focused their attention on developing replication defective viral vaccine vectors which are considered to be safer than replication competent vectors (Farina *et al.*, 2001).

BIOCHEMICAL BASIS OF PARASITE RESISTANCE

Parasitic infestations are a medical and public health problem of high magnitude both in humans and domestic animals. In Nigeria, where 70-80% of livestock production employs native practices that involve extensive grazing of animals, parasites cause serious economic losses due to emaciation, reduced/low growth rates, anaemia, and deaths (Useh *et al.*, 2006a). An up-to-date report of the economic losses of livestock to parasitic diseases has yet to be documented in most countries of the world, but in the United States of America (USA), parasites cost the livestock industry an estimated \$2 billion per year in lost productivity and increased operating expenses (Köhler, 2001).

The biochemical mechanisms underlying anthelmintic resistance are not well understood, but appear to be complex and vary among different helminth species and even isolates. The

major mechanisms helminths use to acquire drug resistance appear to be through receptor loss or decrease of the target site affinity for the drug (Köhler, 2001) and a change in metabolism that activates or removes the drug, or prevents its activation (Wolstenholme *et al.*, 2004). The majority of modern anthelmintics exert their effects within three biochemical/ physiological areas. The known target sites are solely proteins and include ion channels, enzymes, structural proteins, and transport molecules. The mechanisms of action and biochemical basis of anthelmintic resistance have been summarized (Köhler, 2001). Benzimidazole-based compounds appear to be the most widely used anthelmintics available. The story of this large class of extremely successful and potent drugs started with the introduction of thiabendazole in 1961, the first broad spectrum anthelmintic produced (Brown *et al.*, 1961). This compound heralded the development of a variety of structurally-related drugs and prodrugs, which turned out to be of lower mammalian toxicity and even more effective against a wide range of helminth species than their predecessor, thiabendazole (McKellar and Scott, 1990). Earlier studies on the effect of these compounds in helminths have shown that they cause ultrastructural alterations in both intestinal cells of nematodes and tegumental cells of cestodes, in particular a redistribution of cytoplasmic vesicles and other organelles (Borgers and De Nollin, 1975). As these changes coincided with the disappearance of cytoplasmic microtubules, it was suggested that benzimidazoles act by inhibiting the microtubule-mediated transport of secretory vesicles in the helminth absorptive tissues with the released digestive enzymes being responsible for the observed tissue damage. These proposed drug-induced physiological reactions were later supported by the observation that the lethal effect of fenbendazole in *Haemonchus contortus* is associated with the inhibition of secretory vesicle transport in the intestine, followed by dispersal of the vesicle contents throughout the intestinal cytoplasm (Jasmer *et al.*, 2000). It appears quite clear that the biochemical basis of benzimidazole's action is its ability to bind with high affinity, in a pseudo-irreversible fashion, to the microtubule subunit protein, tubulin, thereby disrupting microtubule structure and functions (Friedman and Platzer, 1978; Köhler and Bachmann, 1981; Lacey, 1988). Studies with the classical antimetabolic agent colchicine have shown that this compound binds to tubulin prior to its polymerisation (Sackett and Varma, 1993; Uppuluri *et al.*, 1993). Inhibition of microtubule assembly appears to be achieved through addition of colchicine-bearing tubulin to the end of a growing microtubule and loss of the ability of these subunits to accept other tubulin molecules for further microtubule growth. Data obtained from fluorescence spectroscopy and labelling experiments have indicated that the colchicine binding site is on the β -tubulin monomer, obviously close to the α,β subunit interface (Uppuluri *et al.*, 1993). Other spectroscopic analyses suggest that inhibition of tubulin polymerisation is due to a drug-induced local unfolding of a small region within the β -tubulin monomer (Sackett and Varma, 1993). A similar mechanism may be responsible for the inhibition of tubulin polymerisation by benzimidazoles, as the binding site of these compounds appears also to be located on the β -tubulin monomer (Kwa *et al.*, 1994; Nare *et al.*, 1996; Hollomon *et al.*, 1998). Microtubules are highly dynamic, ubiquitous cellular organelles serving a variety of vital functions including mitosis, motility, and transport in all eukaryotes. Many of these structures exist in a dynamic steady state in which assembly and disassembly of the soluble subunits are balanced. In such systems, the drug-tubulin interaction results in a shift of this steady state with a net loss of microtubules and accumulation of free tubulin. In view of the crucial roles microtubules play in many cellular processes, their drug-induced destruction eventually leads to the death of the organism. The principle of the high selective toxicity of

benzimidazole anthelmintics is not entirely clear, but it appears primarily to be due to the much stronger and irreversible binding interaction of the drugs with helminths as compared with mammalian tubulins (Lacey, 1988). The mechanism of benzimidazole resistance in helminths has been extensively researched primarily by Roos and collaborators (Roos *et al.*, 1995; Roos, 1997) and is to a large extent understood. Phenotypically, resistant worms are characterized by the loss of the high affinity binding sites for these drugs on the microtubule subunit protein as shown by radio-ligand-binding assays (Lacey, 1988).

Most of the commercially available anti-nematodal drugs exert their effect on the nervous system of the parasites. Members of one of this drug category act as acetylcholine agonists and include levamisole, the tetrahydropyrimidines (e.g. pyrantel and morantel) and some other structurally related compounds. Studies using electrophysiological techniques have shown that the surface of somatic muscle cells of nematodes possess nicotinic acetylcholine receptors (nAChR) that can be opened by the nicotinic anthelmintics (Martin *et al.*, 1996, 1998). Binding of these compounds to the recognition site of the excitatory receptor produces depolarisation and spastic paralysis of the nematode muscle that can result in parasite expulsion. The nAChR of vertebrates is a thoroughly investigated receptor operated cation channel, composed of a pentameric structure built up of a combination of different subunits (Unwin, 1995). Each chain of the channel contains a binding site for acetylcholine. The subunit composition and stoichiometry of this receptor can vary between different subtypes, resulting in a functional diversity of nAChRs. Details of the biochemical nature of the nematode nAChR have not yet been revealed, but the subunit sequence features and pharmacological profile of this channel resembles vertebrate nAChRs (Martin *et al.*, 1997). Work on *Caenorhabditis elegans* has been very useful in describing the structural and pharmacological properties of the invertebrate nAChR, but it remains unclear to what extent this information can be extrapolated to parasitic nematodes (Fleming *et al.*, 1997). Molecular cloning and co-expression in *Xenopus* oocytes of several genes encoding nAChR subunits of the free-living nematode produces functional channels that can be gated by levamisole and suppressed by classical nAChR antagonists (Fleming *et al.*, 1997). Three genes (lev-1, unc-29, and unc-38) associated with levamisole resistance were found to encode nicotinic ion channel subunits of the nematode with unc-38 encoding an α subunit (UNC-38) and the other genes encoding non- α subunits (UNC-29 and LEV-1). The sequence characteristics of these subunits and their comparison with corresponding homologous polypeptides in other organisms have been described in great detail by Fleming *et al.* (1997). In this analysis, the lev-1 and unc-29 encoded subunits showed close homology to vertebrate neuronal subunits and were, like the latter channels, insensitive to α -bungarotoxin. A unique aspect of the nematode receptor appears to be that its α -subunit lacks the conserved target amino acid residue for lophotoxin present in all muscle and neuronal AChRs of other invertebrates and vertebrates tested (Bai and Sattelle, 1993). The two unc-encoded subunits of *C. elegans* are required for levamisole-sensitive channel function, while the lev-subunit is normally dispensable from AChR composed of unc-29 and unc-38 encoded proteins. The levamisole binding site is suggested to be located at the α /non- α subunit interface of the channel, whereas the natural transmitter binds to the α -subunit. Studies by Robertson and Martin (1993) on *Ascaris* muscle nAChR at the single-channel level have shown a considerable variation in the conductance of the channels, suggesting that these receptors are composed of different subunit combinations, having different pharmacological properties. A similar heterogeneity was seen with the *C. elegans* nAChR subunit genes of which varying combinations could produce

functional channels when co-expressed in *Xenopus* oocytes, but also the subunits of neuronal vertebrate nAChRs may combine to generate a large number of subtypes (Fleming *et al.*, 1997; Martin *et al.*, 1997). In conclusion, the target site of levamisole, and most likely also pyrantel, morantel, and other structurally related compounds, is a pharmacologically distinct ion channel that forms a nicotinic AChR on the body muscle of nematodes. The selective toxicity of these compounds appears to be based on the unique properties of the nematode nAChR, which appear pharmacologically distinct from those of the homologous receptors in higher animals.

Comparatively little is known on the biochemical mechanisms of resistance to nicotinic anthelmintics. However, the experimental evidence suggests that resistance to these drugs is, like that to the benzimidazoles, associated with alterations in the target site (Martin *et al.*, 1998; Robertson *et al.*, 1999; Sangster and Gill, 1999; Martin and Robertson, 2000). Radioligand-binding assays have shown that binding of tritiated amino-levamisole to the nAChR of *H. contortus* involves two sites and that levamisole-resistant worms bound the drug less tightly at the low affinity site than susceptible worms (Sangster *et al.*, 1998; Sangster and Gill, 1999). In *C. elegans*, several nAChR subunit encoding genes with strong levamisole resistance have been identified (Fleming *et al.*, 1997). Some of these mutant genes were expressed in *Xenopus* oocytes and found to be associated with deficient or modified receptor binding properties (Fleming *et al.*, 1997). Better insight into the mechanism of levamisole resistance in parasitic nematodes was achieved by looking at properties of individual nAChRs using the patch-clamp technique (Robertson *et al.*, 1999). In these studies, changes in levamisole-activated receptor channel currents were identified in muscle patches between levamisole-sensitive and resistant isolates of the pig nematode, *Oesophagostomum dentatum*. Although the number of receptors present in these patches was similar in both worm populations, the patches of resistant isolates, when analyzed at estimated chemotherapeutic drug concentrations, contained a lower number of active channels as compared to sensitive isolates, suggesting an increased desensitisation of levamisole receptors in the resistant isolates. Other parameters, including the mean channel open times and the probability that the channel is in the open state, were also reduced in resistant isolates, indicating a smaller contractile response produced by the receptors to levamisole in the resistant parasites than in the sensitive parasites. In the same study, channel-conductance histograms indicated that one of the levamisole receptor subtypes present in *O. dentatum* is missing in the resistant isolate. From these analyses, it was concluded that the physiological differences observed between levamisole-resistant and susceptible worm populations may be explained by a shift in the relative proportion of the heterogeneous nAChR subtype population in favour of receptors less sensitive to the drug. The biochemical mechanism underlying the reduced drug sensitivity of nAChR subtypes is still unknown, but may involve alterations in the levamisole binding UNC-29 and LEV-1 subunit structures, although modifications in subunit stoichiometry or variations in the orientation of subunits towards their adjacent channel components may also contribute to the development of nicotinic anthelmintic resistance. There is evidence to suggest that the biochemical basis of resistance in nematodes to other nicotinic agonists is similar to that observed with levamisole. In a study using the patch-clamp technique, a reduction of both the percentage of active patches and the mean probability of nAChR channel opening values were found in *O. dentatum* membrane patches of pyrantel resistant, compared to drug sensitive worms (Robertson *et al.*, 2000). Molecular studies are now required to analyze possible changes in the levamisole-binding nAChR α subunits and the

subunit composition of the receptors in worms selected for resistance to nicotinic agonist anthelmintics.

The macrocyclic lactones comprise the avermectins (e.g. ivermectin and doramectin) and the more recently developed milbemycins (e.g. moxidectin) that lack the glycosidic substitution. Representatives of both classes of antiparasitics are extremely potent antinematodal drugs, insecticides, and acaricides. These drugs are widely used to treat nematode infections in animals, but are also the drugs of choice to control human onchocerciasis. A study on the effects of ivermectin and moxidectin on pharyngeal pumping in *H. contortus* has shown that both drugs may share a common mechanism of action, but that there may be subtle differences in the response to the target site between these compounds (Paiement *et al.*, 1999). Macrocyclic lactone antiparasitics produce a flaccid paralysis of the somatic worm musculature and inhibit feeding of the parasite by blocking pharyngeal pumping (Geary *et al.*, 1993; Martin *et al.*, 1996; Kotze, 1998; Sangster and Gill, 1999). The latter effect is exhibited at chemotherapeutically-relevant levels, and it has therefore been suggested that disruption of ingestive activity and worm starvation is the real nematocidal action of these compounds (Sangster and Gill, 1999; Paiement *et al.*, 1999). However, the somatic musculature, though less sensitive to ivermectin than the pharynx, remains a potential target for this drug as inhibitory concentrations still fall into the therapeutic range. Molecular genetic work on the *C. elegans* model, together with electrophysiological studies on expressed receptors using the micro-electrode current clamp technique, has clearly shown that the physiological site of action of these drugs is a family of invertebrate-specific, glutamate-gated chloride channels (GluCls) that contain a-type subunits (Cully *et al.*, 1996; Martin, 1996; Dent *et al.*, 1997; Vassilatis *et al.*, 1997). These channels are inhibitory ionotropic membrane-spanning protein complexes that are phylogenetically related to the vertebrate GABA-gated chloride channels and are likely to exhibit a similar subunit assembly. Ivermectin appears to act as an agonist of glutamate by increasing the open times of the receptor. At low concentrations it potentiates the effect of the natural transmitter and at higher levels opens the channel directly. The binding of ivermectin results in irreversible chloride ion currents followed by hyperpolarisation of the cell membrane and muscle paralysis. Two subunit encoding genes of the GluCl family, GluCla and GluClb, were previously cloned from *C. elegans* by Cully *et al.* (1994). One expression of these genes is in *Xenopus* oocytes, the subunits co-assembled to form glutamate-activated chloride channels that are highly sensitive to ivermectin. The results from these expression experiments also suggest that the GluCl a-type subunits carry the binding site for ivermectin, whereas the glutamate binding site is located on the b-subunit, with a possible second site on the a-subunits not coupled to channel opening. In the meantime, a family of genes encoding a-type GluCl subunits has been identified in *C. elegans*, including *glc-1* and *avr-14* (syn: *gbr-2*)-encoding neuronal GluCla1 and GluCla3 subunits, respectively, and *avr-15*-encoding pharyngeal muscle cell GluCla2 subunits (Dent *et al.*, 1997; Vassilatis *et al.*, 1997; Dent *et al.*, 2000). Data from expression studies has indicated that the combination of GluCla2 and b-subunits may constitute the physiologically occurring nematodal GluCl (Vassilatis *et al.*, 1997). The mechanism of ivermectin sensitivity in nematodes is highly complex, involving the interplay between different a-types of GluCl channels both in the pharyngeal muscle and extra-pharyngeal neurons (Dent *et al.*, 2000). On the basis of results obtained from mutation studies on *C. elegans* by Dent *et al.* (2000), a physiological mechanism for ivermectin sensitivity was proposed in which an important component is the spread of hyperpolarisation from extra-

pharyngeal neurons back to the pharynx. The data shows that ivermectin acts independently on GluCl channels containing different types of α -subunits. GluCl α 2 functions in pharyngeal muscle, while the ivermectin target in neurons is GluCl α 3 and possibly GluCl α 1 channels. However, other, non-GluCl-type components appear to be required for high level ivermectin toxicity and worm killing. These include gap junctions that link the extra-pharyngeal with the pharyngeal nervous system and, thus, ivermectin sensitivity conferred by the different α -type containing GluCl channels (Dent *et al.*, 2000). Studies have provided evidence to suggest that the severe central nervous system side effects seen in various vertebrates following ivermectin treatment may be due to the lack or functional deficiency of a P-glycoprotein membrane pump (Mdr1a protein) in their blood brain barrier (Kwei *et al.*, 1999). Resistance to macrocyclic lactone antiparasitics has been reported worldwide (Sangster and Gill, 1999; Dent *et al.*, 2000). Field and *in vitro* studies have shown that parasites resistant to avermectins are also resistant to the chemically related milbemycins, suggesting a common mode of action (Paiement *et al.*, 1999; Sangster and Gill, 1999). However, data obtained from other work is controversial and has indicated that macrocyclic lactone action and resistance mechanisms are far from being understood (Martin *et al.*, 1996; Sangster and Gill, 1999; Dent *et al.*, 2000; Le Jambre *et al.*, 2000). For example, moxidectin, a milbemycin type of compound, was found to be effective against *H. contortus* when ivermectin failed treatment at recommended doses (Coles *et al.*, 1994). Clear differences in response to ivermectin inhibition of pharyngeal pumping between ivermectin selected and susceptible *H. contortus* have been reported (Kotze, 1998; Sangster and Gill, 1999), indicating that resistance to ivermectin may be associated with changes in the putative major physiological target site of this drug, the pharyngeal muscle.

Praziquantel is the drug of choice for the treatment of schistosomiasis today. An enormous amount of research data and clinical experience has accumulated in the last 20 years to demonstrate that this drug is a safe and effective treatment not just for schistosome, but also cestode infections (Cioli *et al.*, 1995). The pyrazino-isoquinoline ring system of praziquantel represents a completely novel structure in anthelmintic therapy with no relationship to other anti-parasitic drug structures. Despite considerable research efforts, the precise mechanism by which praziquantel affects the target worms has yet to be elucidated. Earlier studies have shown that exposure of schistosomes and cestodes to the drug cause an immediate muscle contraction and vacuolation of the tegument (Mehlhorn *et al.*, 1981; Prichard *et al.*, 1982; Xiao and Catto, 1989; Day *et al.*, 1992). These alterations were found to be linked to an induction of calcium efflux across the tegumental membrane (Prichard *et al.*, 1982; Redman *et al.*, 1996; Cioli, 1998). As the tegument is electrically coupled to muscle cells, an increase in tegumental calcium levels could result in elevated calcium within the sarcoplasmic reticulum, which may then produce muscle contraction. The obvious relationship between praziquantel and calcium redistribution among worm tissues and the environment suggests that the target site of the drug could be a calcium-permeable membrane channel. However, so far none of the mechanisms known to be responsible for changes in calcium efflux rates across membranes seem to be a possible explanation of the praziquantel-induced effect (Redman *et al.*, 1996). Various alternative mechanisms have also not been substantiated (Xiao and Catto, 1989; Cioli *et al.*, 1995). In any case, the target of praziquantel has to be unique to the parasite and pharmacologically very distinct from any equivalent structure in the host, otherwise the highly selective toxicity of this drug could not be explained.

Perhaps with the exception of oxamniquine and related compounds, the target sites and mechanisms of action of other anthelmintics are less extensively investigated than of those discussed above. The old antinematodal drug, piperazine, acts as a GABA agonist by opening GABA-gated chloride channels present on nematode somatic muscle cells (Martin *et al.*, 1997). Binding to these receptors induces an increase in chloride permeability of the muscle cell membrane that eventually results in a relaxation of the body musculature and flaccid worm paralysis. Oxamniquine and the structurally-related hycanthonone act on *Schistosoma mansoni* by binding covalently to nucleic acids, resulting in an irreversible inhibition of the synthesis of these macromolecules (Cioli *et al.*, 1995). The binding reaction is initiated by an enzyme, presumably a sulfotransferase, which is unique to the schistosome and can catalyze an esterification of the drug's hydroxymethyl group. The resulting products spontaneously dissociate to form electrophilic molecules capable of binding to schistosome DNA. The primary target of the salicylanilides and nitrophenols and their selective toxicity in liver flukes, cestodes, and blood-sucking nematodes is not well understood. Members of these compounds have been shown to disrupt ATP generation by uncoupling mitochondrial oxidative phosphorylation (Van den Bossche, 1985). Other studies using ³¹P-NMR and intrategumental pH measurements have indicated that this action may not be the primary mechanism of these drugs, but that, at least in trematodes, interference with processes regulating intracellular pH may explain, in part, their anthelmintic action (Rohrer *et al.*, 1986; Pax and Bennett, 1989). The potent fasciolicidal sulphonamide derivative, clorsulon, inhibits the activity of two glycolytic enzymes, phosphoglycerate kinase and phosphoglyceromutase, preventing carbohydrate utilisation and its associated substrate-level ATP production in the liver fluke (Schulman *et al.*, 1982). The selective action of this drug remains to be determined. A definitive statement on the primary mode of action of triclabendazole, a chlorinated thio-benzimidazole compound with potent activity against all stages of *Fasciola hepatica* within the vertebrate host, awaits further studies. In earlier work, inhibition of colchicine binding to tubulin purified from *F. hepatica* suggested an action of this drug similar to that known for the other benzimidazole-based anthelmintics (Bennett and Köhler, 1987). Other actions of triclabendazole and its sulfoxide metabolite have been described, including inhibition of protein synthesis and uncoupling of oxidative phosphorylation, which may become synergistic over long treatment periods (Carr *et al.*, 1993; Stitt *et al.*, 1995; Stitt and Fairweather, 1996).

Several lines of evidence obtained from studies on *S. mansoni* have indicated the possibility of selecting parasites that are relatively refractory to treatment with praziquantel (Fallon *et al.*, 1996; Kusel and Hagan, 1999). However, whether or not these experiments generate truly resistant parasites, defined as the genetically transmitted loss of sensitivity in worms that were previously sensitive to the drug, is not known. In the last years, various efforts have been undertaken to clarify whether or not the emergence of possible praziquantel-resistant schistosomes has also occurred in the field (Pica-Mattoccia *et al.*, 1992; Fallon *et al.*, 1996). Among those are studies in Senegal and Egypt, countries with reports of low praziquantel cure rates. However, the judgement of these observations has turned out to be very difficult since alternative explanations for the observed decrease in praziquantel sensitivity of schistosomes do exist, based primarily on infection intensity, prepatent infections, and, possibly, immunological factors. Currently, studies are undertaken to test whether the poor cure rates observed in some geographic areas are due to a decreased susceptibility of adult worms or to the presence of immature stages known to be insensitive to

praziquantel (Ismail *et al.*, 1999). However, the various field and laboratory studies suggest that the suspicion that praziquantel-resistant or tolerant schistosomes might exist appears to be justified by the available data (Fallon *et al.*, 1996; Ismail *et al.*, 1999). The major findings supporting this are: (1) the good cure rates seen with patients insensitive to praziquantel treatment using oxamniquine as an alternative drug; (2) the overall correlation between the requirement for repeated treatments and the ED50 values of particular schistosome isolates; (3) the survival of resistant worm isolates exposed to praziquantel *in vitro* at concentrations that result in the killing of susceptible isolates (Ismail *et al.*, 1999); and (4) the possibility for selection of praziquantel resistance in laboratory animals. The observed resistance in *S. mansoni* against oxamniquine and hycanthone is due to the absence in resistant worm isolates of an enzyme activity mediating the covalent binding of these drugs to DNA (Pica-Mattoccia *et al.*, 1992; Cioli *et al.*, 1995; Pica-Mattoccia *et al.*, 1997).

Anthelmintic resistance in human and animal pathogenic helminths has been spreading in prevalence and severity to a point where multidrug resistance against the three major classes of anthelmintics—the benzimidazoles, imidazothiazoles, and macrocyclic lactones—has become a global phenomenon in gastro-intestinal nematodes of farm animals. Hence, there is an urgent need for an anthelmintic with a new mode of action. Amino-acetonitrile derivatives (AADs) active against parasitic nematodes also exerted marked effects on the movement, growth, and viability of the free-living nematode *C. elegans*, inducing a pleiotropic combination of phenotypes distinct from the effects of any single known anthelmintic. The AADs cause hypercontraction of the body wall muscles leading to paralysis, spasmodic contractions of the anterior portion of the pharynx, and ultimately death (phenotypic effects are observed at AAD 1336 or AAD 1470 concentrations of \$50–100 ng ml⁻¹, with full lethality occurring above 1 mg ml⁻¹). Similar effects were observed for *H. contortus* (adults). AAD-exposed *C. elegans* also exhibited moulting defects and growth-arrested nematodes frequently developed large vacuoles characteristic of necrosis. Similar phenotypes were also observed after exposure of *C. elegans* to the general nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) but not to the anthelmintic levamisole, which agonizes a specific subtype of nicotinic acetylcholine receptor (nAChR). AAD 1470 showed similar activity against ivermectin-, benzimidazole- and levamisole-resistant *C. elegans* strains as against the wild-type strain. Notably, resistance to the AADs was not conferred by the loss of any of the three required nAChR subunits of the levamisole receptor (UNC-29, UNC-38, and UNC-63). AADs 450 and 1470 cured sheep infected with drug-resistant field isolates of *H. contortus*, *T. circumcincta*, and *T. colubriformis*, completely inhibiting egg shedding within 2 days after treatment, whereas the recommended doses of the three major classes of anthelmintics failed. AAD 1470 was effective against levamisole-, benzimidazole-, macrocyclic-lactone-, and multidrug-resistant pathogenic nematodes at 10 mg race mate kg⁻¹. The resistance-breaking activities of the AADs and the unique suite of phenotypes induced in *C. elegans* suggest that these compounds act by a novel mode of action, different from those of the currently used anthelmintics (Kaminsky *et al.*, 2008).

Parasitic protozoa are responsible for some of the most devastating and prevalent diseases of humans and domestic animals. Protozoan parasites threaten the lives of nearly one-third of the world's human population and also result in considerable losses of life and productivity of domesticated animals. The biochemical basis of most parasitic infestations, although studied mainly in humans, may provide insight on what resistance to such drugs may look like in animals. Analysis of resistance mechanisms in several organisms is warranted as their general

biochemical framework of resistance is often similar. Cells may evade drug action by hiding in sanctuaries. Drug uptake may be thwarted by loss of uptake systems or alteration of membrane composition; once inside, drugs may be inactivated, excreted, modified and excreted, or routed into vacuoles. Drug activation mechanisms may be suppressed or lost; the interaction of the drug with the target may be made less effective by increasing the level of competing substrates or by altering the target to make it less sensitive to the drug. The cell may learn to live with a blocked target by bypassing the block. Parasitic protozoa are using most of these strategies to achieve resistance. Resistance in clinical samples is less easily defined in biochemical terms. Parasite populations may be heterogeneous; the exact parental strain is often not available for comparison and the culture of the parasites, often necessary to get sufficient material for analysis, may change resistance. Chloroquine acts by inhibiting polymerization of the toxic haeme that is released during haemoglobin degradation within the digestive vacuole of the parasite (Sullivan *et al.*, 1996). Resistance to chloroquine shares several phenotypic features with multidrug resistance (MDR) of mammalian tumour cell lines. Chloroquine efflux is increased in resistant parasites (Krogstad *et al.*, 1987), and verapamil, the classical agent to reverse MDR in animal cells, can restore chloroquine sensitivity in resistant cells (Martin *et al.*, 1987). In anti-folate resistance, reduced folates serve as co-factors in a variety of one carbon transfer reactions including the biosynthesis of thymidylate. Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) catalyze consecutive reactions in the *de novo* synthesis of thymidine monophosphate (dTMP). In protozoa and plants, these two enzymes are fused, resulting in a DHFR-TS protein (Ferone & Roland, 1980). The enzyme DHFR is the target for the action of antifolates, such as pyrimethamine (PYR). Folates are made of three building blocks: a pteridine, p-aminobenzoic acid (PABA), and glutamic acid. Malaria parasites are capable of synthesizing folates *de novo*; others, such as kinetoplastid parasites, rely primarily on folates from the environment and require specific transport systems for their folate requirements. Resistance to suramin is rare and not a problem in clinical practice. Resistance to the pentavalent arsenical melarsoprol does occur (Legros *et al.*, 1999), which leaves no alternatives for treatment. Resistance to melarsoprol and other arsenical drugs has been studied mostly *in vitro*. Wild-type trypanosomes have two adenosine transporters, P1 and P2, and P2 transports melanophenyl arsenicals (Carter & Fairlamb 1993). Resistant parasites lack the P2 transporter, suggesting that resistance to arsenicals can be the result of loss of uptake (Carter and Fairlamb, 1993).

Trypanosomes exhibit a variety of biochemical peculiarities, several of which have pharmacological importance (Oppendoes, 1985). They possess a single mitochondrion whose genome is organized into a network of over one thousand catenated DNA circles of variable size, called kinetoplast DNA (kDNA). Hence their name, kinetoplast protozoa or kinetoplastids. This feature renders mitochondrial replication a promising drug target (Shapiro and Englund, 1995). Another unique feature of kinetoplastids is trypanothione, a covalent complex of two glutathiones linked via spermidine (Fairlamb *et al.*, 1985). Trypanothione and trypanothione reductase constitute an essential defence against oxidative stress and radicals (Penketh and Klein, 1986). The presence of spermidine in trypanothione attracts pharmacological attention also to polyamine synthesis (Bacchi and Yarlett, 1993), in particular to *S*-adenosylmethionine decarboxylase (SAMDC) and ornithine decarboxylase. Suramin is still used for the treatment of early-stage sleeping sickness and for nagana in livestock. The cellular target of suramin is not known. In mammalian cells, suramin was shown to inhibit topoisomerase II (Bojanowski *et al.*, 1992) and to interfere with polyamine

salvage (Gritli-Linde *et al.*, 1998; Sandgren and Belting, 2003). The biochemical mechanisms of suramin resistance have been reviewed (Delespaux and Koning, 2007). Resistance of *Trypanosoma evansi* to suramin has been reported in isolates from Sudan (El Rayah *et al.*, 1999) and China (Zhou *et al.*, 2004), and suramin is not effective against *T. vivax* and *T. congolense* (Leach and Roberts, 1981). Little is known, however, about the mechanism of suramin resistance. It has been argued that suramin resistance was unlikely to be coupled to a drastic reduction in drug uptake (De Koning, 2001). This was primarily deduced from the fact that the molecule is too highly charged and too large to be taken up by a specific plasma membrane transporter, yet in the presence of serum proteins, taken up faster than could be explained by simple endocytosis (Fairlamb and Bowman, 1980). It was subsequently shown that suramin enters trypanosomes through receptor-mediated endocytosis with LDL (Coppens *et al.*, 1987; Vansterkenburg *et al.*, 1993). Since LDL uptake is essential for proliferation in trypanosomes, which cannot synthesise their own fatty acids and cholesterol *de novo* (Coppens and Courtoy, 2000), LDL uptake could not be seriously impaired. However, Pal *et al.* (2002) have presented strong evidence that, at least in procyclics, suramin uptake, while proceeding by receptor-mediated endocytosis, is not coupled to LDL uptake rates.

There are numerous reports of resistance to the veterinary diamidine drug, diminazene aceturate (DA, Berenil®), in various *Trypanosoma* species and from many different parts of Africa (Chitambo and Arakawa, 1992), though, on the whole, this still seems to be limited to highly endemic areas where use of the drug is highest. Given the highly charged nature of diamidine compounds, specific carriers are required to translocate the drugs across biomembranes. Conversely, the absence or loss of these transport activities would render cells impervious to this class of drugs, explaining both their selective toxicity and the probable resistance mechanism.

The amphiphilic cationic phenanthridine, isometamidium chloride, has been used in the field for several decades prophylactically or therapeutically for livestock suffering from trypanosomiasis due to infection with *Trypanosoma congolense* and other *Trypanosoma* spp. (Leach and Roberts, 1981). It was first synthesized by coupling homidium (Ethidium®) with *p*-amino phenyl diazonium chloride (Wragg *et al.*, 1958; Berg, 1960; Berg, 1963) or, in other words, by coupling homidium with a part of the diminazene (Berenil®) molecule. The first reports of ISM use dates to 1963 and the first case of resistance to homidium and cross-resistance between homidium and ISM was reported in 1967 (Na'isa, 1967). Resistance to ISM is mostly associated with cross-resistance to homidium (Peregrine *et al.*, 1997), and it could be speculated that these structurally-related compounds might share the same uptake mechanism albeit that their distributions within the trypanosome are slightly different. ISM is mainly concentrated in the kinetoplast, whereas homidium is spread much more diffusely throughout the trypanosome (Boibessot *et al.*, 2002). The first study of ISM and diminazene uptake mechanisms (Girgis-Takla and James, 1974) concluded an energy dependent process for diminazene, but not for ISM. Complete glucose deprivation induced a significant decrease of diminazene uptake and an increase in ISM uptake, which is in contradiction with some studies which conclude that the transport of ISM was energy dependent, as it was reduced in the presence of metabolic inhibitors such as SHAM/glycerol (Sutherland *et al.*, 1992; Sutherland and Holmes, 1993). The observed increase in ISM uptake in the absence of glucose was very likely due to cellular damage and loss of the integrity of the mitochondrial membrane, allowing the trypanocide direct access to the kinetoplast DNA, for which it shows a high affinity (Boibessot *et al.*, 2002). The main mode of action of ISM chloride is thought

to be the cleavage of kDNA-topoisomerase complexes, causing the desegregation of the mini-circle network within the kinetoplast (Shapiro and Englund, 1990), though Kaminsky *et al.* (1997) later showed that dyskinetoplastic trypanosomes are at least as sensitive to isometamidium as the kinetoplastic line. Induction of resistance to ISM seems not to be an easy process as, in most cases, the drug is still effective in the field even though it was first marketed nearly half a century ago. However, more and more cases of therapeutic failure have been reported (Kinabo and Bogan, 1988; Chitambo and Arakawa, 1992; Clausen *et al.*, 1992; Geerts and Holmes, 1998; McDermott *et al.*, 2003; Sinyangwe *et al.*, 2004; Gall *et al.*, 2004; Awa and Ndamkou, 2006). The authenticity of the resistance phenotype in some of these field isolates of trypanosomes has been confirmed by *in vivo* testing of individual clones derived from the isolates (Codjia *et al.*, 1993; Zhang *et al.*, 1993; Sinyangwe *et al.*, 2004; Delespaux *et al.*, 2005). Resistance occurs where a large proportion of the trypanosome population is exposed frequently to the drug, such as in commercial ranches, for instance, after government policies based on large-scale block treatments (Sinyangwe *et al.*, 2004), or where frequent under-dosing of the trypanocide occurs (Delespaux *et al.*, 2002). Eleven months of repeated sub-curative treatments in mice were necessary to induce resistance in one cloned isolate (Peregrine *et al.*, 1997).

Reduced drug uptake as a mechanism of drug resistance in African trypanosomes has also been reported by several laboratories, using different methods and different mutants. The recurrent association of reduced drug uptake with drug resistance stresses the importance of drug transport in African trypanosomes. Reduction of net drug uptake can be caused by either decreased drug import (chemoreceptor hypothesis) or increased drug export. Either mechanism implies mutations in transporters since most trypanocides do not freely diffuse through the plasma membrane (Mäser *et al.*, 2003).

MOLECULAR AND GENETIC MECHANISMS OF PARASITE RESISTANCE

The molecular and genetic mechanisms of drug resistance in veterinary helminths have been reviewed (Wolstenholme *et al.*, 2004). BZ act by inhibiting the polymerization of tubulin to form microtubules, and it is clear that resistance is associated with mutations in β -tubulin genes that prevent drug binding. However, several different polymorphisms of the β -tubulin genes have been correlated with BZ resistance (Prichard, 2001). The well-known Phe-Tyr polymorphism at codon 200 of β -tubulin isotype 1 was the first described (Kwa *et al.*, 1994), and it has frequently been considered the most important mutation conferring resistance to these compounds. However, even in the early studies, highly resistant populations of *Haemonchus contortus* were also known to possess a deletion in β -tubulin isotype 2. More recently, a second Phe-Tyr polymorphism, at codon 167 of β -tubulin isotype 1, was detected in BZ-resistant populations of *H. contortus*. The same two polymorphisms also occur in the β -tubulin isotype 2 gene of *H. contortus*, and they too can confer BZ resistance (Prichard, 2001). The codon 167 polymorphism was also present in BZ-resistant *Teladorsagia circumcincta*, but not in *Trichostrongylus colubriformis* (Silvestre and Cabaret, 2002). Mutations at codon 167, but not 200, were found in several highly BZ-resistant *Cyathostomin* species from horses (Drogemüller *et al.*, 2004). Binding studies with

recombinant *H. contortus* β -tubulins indicated that mutations at codon 167 of isotype 1 or 2 reduce affinity for BZ (Prichard, 2001). However, genotyping two *H. contortus* field populations showed that Tyr at codon 200 is required for BZ resistance. This was not true for *T. circumcincta*: worms homozygous for Phe at codon 200, but heterozygous or homozygous for Tyr at codon 167, survived BZ treatment (Silvestre and Cabaret, 2002). Of these surviving *T. circumcincta*, a similar proportion was heterozygous at codon 167 as were homozygous for Tyr. This implies that the genetics of BZ resistance in *T. circumcincta* are different from those of *H. contortus*, and investigators using molecular techniques to evaluate field resistance levels should bear this in mind. In horse *Cyathostomins*, the β -tubulin isotype 1 codon 200 polymorphism is not the only, and probably not even the most important, mutation with respect to resistance (Pape *et al.*, 2003). However, it remains to be seen to what extent codon 167 mutations contribute to resistance in these worms. The codon 200 polymorphism has been described in the cattle nematode, *Cooperia oncophora*, and found to occur in BZ-resistant populations (Njue and Prichard, 2003). Apart from such target gene changes, multiple data suggest that modulation of the activity of the cell-membrane efflux pump p-glycoprotein (Pgp) could also contribute to BZ resistance in trichostrongyles (Kebauf *et al.*, 2003). The fasciolicide, triclabendazole, is an atypical BZ with a very narrow spectrum of activity. Observations by microscopy indicate a microtubule-directed action typical of BZs. The active form of the drug, triclabendazole sulfoxide (TCBZ.SO), blocks the movement of tegumental secretions, leading to widespread sloughing of the tegument. This is reminiscent of early ultrastructural studies on mebendazole involving nematodes and cestodes. In addition, TCBZ.SO inhibits mitotic division of the vitelline and spermatogenic cells and causes a reduction in the intensity of tubulin immuno-staining, changes typical of microtubule disruption that were not seen in a triclabendazole-resistant isolate. However, resistance to triclabendazole does not appear to be associated with mutations in β -tubulin. Tyrosine is present at position 200 in β -tubulin from both susceptible and resistant isolates (Robinson *et al.*, 2002), and no amino acid polymorphisms are present in any *Fasciola* β -tubulin isolated to date.

Studies on resistance to the avermectin and milbemycins (AM anthelmintics) present a more confusing picture. Genetic studies have found that ivermectin resistance is dominant in *H. contortus*, perhaps reflecting a gain-of-function mutation, although it could be that true resistance results from polymorphisms in several closely linked genes. There are suggestions that p-glycoproteins (Pgp) are involved in AM resistance (Kebauf *et al.*, 2003). Population genetics studies in *H. contortus* found evidence for the association of Pgp genes with AM resistance (Blackhall *et al.*, 1998ab, 2008), but failed to establish consistent associations (Sangster *et al.*, 1999), and a segregation study indicated that a particular Pgp gene was not the major determinant of resistance in one isolate (Blackhall *et al.*, 1998ab). Other studies found selection at GluCl and GABACl genes in individual resistant isolates (Blackhall *et al.*, 2003). Ivermectin increased the GABA response in cells transfected with an unselected, wild-type allele of a *H. contortus* GABACl subunit gene, whereas in cells transfected with the AM selected allele, ivermectin attenuated the GABA response (Feng *et al.*, 2002). Resistance to the AM anthelmintics could be caused by a gain-of-function mutation in a Pgp gene, leading to the more rapid removal of the drug from the worm. Such mutations could either: (i) cause increased expression of a pump capable of carrying the AM, which might lead to changes in gene expression that could be detected by microarray or proteomics experiments; or (ii) increase the affinity for these substrates, detection of which might require careful studies on

reconstituted worm Pgp preparations. Alternatively, parasites could become resistant by the accumulation of one or more mutations in the GluCl and other genes. Such multigenic resistance would be slower to appear, requiring tens of generations rather than four or five, and the genes involved would vary between species and even isolates, depending on their relative importance in drug action and the genetic constitution of the populations.

Drug resistance is now a severe and increasing problem in trypanosomes, but molecular details of mechanisms of resistance are only beginning to be unveiled. Reports on mechanisms associated with resistance to trypanocides have revealed that genetic mechanisms leading to either loss of gene function through mutations (Matovu *et al.*, 2001) or specific gene expression up-regulation leading to active drug extrusion might play significant roles. It is proposed that one approach to understanding the molecular mechanisms of trypanosomal drug resistance could be by cloning and functional characterization of genes that are differentially expressed between the drug-resistant and the sensitive clones. Witola *et al.* (2005) cloned and characterized a novel gene, TeDR40, whose expression was found to be highly up-regulated in berenil-resistant *T. evansi*. The gene was over-expressed in an isogenic clone of wild-type *T. evansi* and found to have a possible correlation between TeDR40 expression with reduced sensitivity to berenil. Up-regulated expression of the TeDR40 gene in berenil-resistant *T. evansi* was validated by real-time PCR and Northern blotting and Western blotting analyses. The results indicated dramatic up-regulation of the gene expression in the resistant parasites, but the gene was almost quiescent in the drug sensitive parasites. This expresses the point that expression of the TeDR40 gene in *T. evansi* is under some form of regulatory mechanism at the transcription level. Regulated gene transcription of VSGs in *T. brucei* at the promoter level has been shown to be life-cycle and stage-specific (Graham, 1995). Induced over-expression of the TeDR40 gene in transgenic, berenil-sensitive *T. evansi* led to a reduced level of sensitivity to berenil trypanocidal effects. However, the level of resistance provoked by induced over-expression in transgenic cells was minimal compared to the level of resistance depicted by the *T. evansi* parasites that had up-regulated TeDR40 gene.

THE ROLE OF EMERGING BIOTECHNOLOGIES IN PARASITE CONTROL

It has been reported that indigenous Nigerian breeds of cattle, dogs, and poultry are highly resistant to disease, compared to their exotic counterparts imported into the country. The concept of trypanotolerance in Nigeria's Ndama cattle that withstood trypanosomal challenge more than trypanosusceptible Zebu cattle was reported a long time ago (Stephen, 1966). It took two decades for Esiebo *et al.* (1986) to document that Ndama cattle had higher sialic acid levels on their erythrocytes, compared to trypanosusceptible Zebu cattle and that the sialic acid was highly O-acetylated, hence resistant to trypanosomal neuraminidase that is released in the peripheral blood circulation during infection to cause anaemia, retarded growth, emaciation, and death. Recently, Useh *et al.* (2006b) reported a high sialic acid complement on the erythrocytes of indigenous Nigerian poultry species, compared to the exotic shaver breed that had a low sialic acid complement. The report corroborated earlier studies that indigenous Nigerian ducks were resistant to Newcastle disease virus infection

(Echeonwu *et al.*, 1993). Another study in the same laboratory (Useh *et al.*, 2006c) showed that Nigeria's indigenous mongrel dog had a higher sialic acid complement on its erythrocytes, compared to exotic German shepherd (Alsation) and Terrier breeds. Based on these reports, the authors proposed that the high sialic acid level may be responsible for the ability of the former to withstand diseases caused by parasites that predispose dogs to anaemia compared to the latter. They concluded that since sialic acid appears to be a significant index of tolerance or susceptibility to diseases, whose aetiological agents produce neuraminidase, concerted efforts should be made to improve the genetic potentials of indigenous animals by boosting their erythrocyte sialic acid complement through selective breeding. Abenga and Uza (2005) posited that the innate ability of trypanosome-infected animals to control anaemia and development of parasitaemia are some of the indicators of trypanotolerance, although trypanotolerance is a genetically-defined complex mechanism involving factors which are not yet well known. According to these authors, although biotechnologies have not been able to identify the complete gene pool involved in trypanotolerance, they have raised the hope of producing synthetic breeds of animals with higher trypanotolerance levels, and enhancing the tolerance of susceptible breeds. It is believed generally that genetic engineering will improve the welfare of animals by imparting resistance to disease and enhancing overall health and well being. The politics of genetically engineered animals in ameliorating disease has been reviewed (Sullivan *et al.*, 2008). Improvements in tools used for genetic engineering and transgenesis over the past 20 years have opened new opportunities for the commercialization of products from genetically engineered animals (Robl *et al.*, 2007). Recently, the European Union approved the first genetically-engineered animal product in the world, a biopharmaceutical product called A Tryn from GTC Biotherapeutics (Framingham, MA, USA). A Tryn, which is indicated for the prophylaxis of venous thromboembolism in surgery of patients with congenital anti-thrombin deficiency, was approved for commercialisation in Europe in 2006 (EMA 2006a, 2006b). Rhucin (Pharming Group N.V., Leiden, The Netherlands), a recombinant human C1 esterase inhibitor produced in the milk of transgenic rabbits and used to treat acute attacks of hereditary angioedema (HAE—a rare disease characterised by painful swelling of soft tissue), is also in clinical trial in Europe (van Doorn *et al.*, 2005). Several other genetically-engineered animal applications are in various stages of research, including monoclonal antibodies, polyclonal antibodies, single-chain antibodies, xenotransplantation, human serum albumin, and other human proteins (Keefer *et al.*, 2007). Other applications of genetically-engineered animals are related specifically to food applications, both for agronomic and nutritional benefit. These food animal applications include swine that produce phytase in their saliva to reduce environmentally hazardous phosphorus emissions in manure (Golovan *et al.*, 2001) and swine that produce beneficial omega-3 fatty acids in their meat (Prather, 2006). Whatever the application, research and development associated with genetically-engineered animals to produce useful products continues to move forward. Interestingly, when the terms 'research' and 'development' are used, we often mean 'research only' and may have a limited understanding of the 'development and commercialization' of a product. As with all technological advances, the process of moving from research through development to commercialization is not without significant challenges. These challenges are often referred to as the 'valley of death' (Auerswald and Branscomb, 2003) and may be associated with a lack of understanding or resources to provide financial support, organize development processes, create a regulatory strategy, provide manufacturing controls, and undertake a market analysis,

all of which are required to commercialize any product. Because of these challenges, many excellent and innovative research projects may end their life cycle in the myriad of published journal articles and academic theses, even those that may have tremendous merit for commercialization (Sullivan *et al.*, 2008).

CONCLUDING REMARKS

Parasitic diseases are a major problem of livestock in Nigeria, and, hence, their control is central to improved livestock production in the country. The production of healthy, genetically-engineered animals capable of withstanding disease and adverse environmental factors will certainly be of tremendous advantage to Nigeria's drive towards food sufficiency by year 2020 AD as this will revolutionize the livestock production system, which currently involves 70-80% native practices and remains open to the risks of disease.

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Chapter III

COMMON AVIAN PARASITES AND EMERGING DISEASES

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ABSTRACT

Avian parasites have been well-known for a long time and form a major part of avian diseases. This chapter includes information about susceptible species, transmission routes and distribution of the most common avian parasites. It also focuses on clinical symptoms and therapeutic approaches. Moreover, it highlights the new, emerging parasitic diseases in birds.

Parasitic diseases are among the most common infections in birds and may pave the way for other secondary infections due to immunosuppression if being unrecognized or left untreated.

Among the most common avian parasites are ectoparasites like arthropods, but also endoparasites, including protozoa, and the large groups of helminths. Helminths can be differentiated into the classes of trematodas (flukes), cestodas (tapeworms), and nematodas (roundworms). Moreover, blood parasites like *Plasmodium sp* or *Haemoproteus sp.* are present in birds and can cause considerable problems for them. However, several parasites are host-specific like *Serratospiculum seurati* or *Caryospora sp.*, which is found in falconiformes. Other parasites have very low species-susceptibility like trematodes or roundworms. Apart from common diagnostic methods like flotation or smear tests, blood smears, and staining, more sophisticated diagnostic methods like molecular analysis have found their way into modern veterinary parasitology diagnostics.

However, apart from the common avian parasites, new parasitic diseases are emerging like microsporidiosis caused by *Enterocytozoon bieneusi* or *Cryptosporidium* infections. Moreover, parasites like mosquitos may even transmit zoonotic viruses like the West-Nile Virus to birds. Evolutionary changes and mutations in parasites like *Plasmodium sp* pose new challenges for veterinarians. Due to their possible implications on human health and a possible transmission of avians to humans, these new, emerging parasitic diseases may pose a considerable and highly underestimated threat to both human and avian health.

INTRODUCTION

Parasites are a common feature in birds. They can be differentiated according to their location as ectoparasites and endoparasites, as well as by their life cycle and hosts. Their size may also vary considerably from intracellular organisms to blood parasites and larger visible helminths or arthropods. Regional differences as well as seasonal changes may be observed in several parasites. Immune suppression in the case of overcrowding, poor hygienic conditions, contaminated soil or litter, and contact between wild birds and cage birds can serve as predisposing factors for parasite invasion. The damage they cause to their avian host depends largely on the amount of parasites. In the case of only a few parasites being present, the damage to the host may be of minor importance. However, large parasite burdens may cause considerable damage and thus pave the way to secondary infections either by bacteria or viruses. Moreover, the clinical picture shows changes in the lumen of affected organs and can cause anemia in the case of blood-sucking endoparasites and ectoparasites.

Diagnostic Examinations

Several different methods can be used for detection of parasites, their eggs, and intermediate stages. However, not all routine methods show the same sensitivity for different parasites.

Ectoparasites can be detected by skin scrapings. This is the preferred method for the detection of *Knemidokoptes* mites because they are located in the tunnels of the skin scrapings. This also helps to secure the diagnosis range as the differential diagnosis is the pox lesion that may resemble earlier *Knemidokoptes* lesions (Greve, 1996b). Other ectoparasites can be seen visually like hippoboscid flies.

The most common routine method, the direct flotation, is commonly used for detection of avian helminths in feces. Different flotation solutions do exist, with the most common one being saturated saline nitrate, which is the result of 568g sodium nitrate being mixed with 1000ml water. This flotation solution is used to detect nematodes, cestodes, and acanthocephala. Another solution is saturated zinc sulfate solution, which is 336g zinc diluted with 1000ml water and is most suitable for the detection of *Giardia* cysts and spiruoid eggs (Greiner and Ritchie, 1994). Trematodes or flukes cannot be detected through flotation methods. For them, sedimentation with a soap-in-water solution is the method of choice (Greiner and Ritchie, 1994).

The size of the different *Cryptosporidium* stages is very small and ranges from 4-6 μm (Fayer, 1997). Due to their small size and ability to float in a higher plane, the likelihood to miss them in the routine fecal examination is relatively high. However, they can be concentrated in a sugar flotation in which they appear a pinkish color. The sugar flotation makes oocysts visible. It can be easily made of 454g (1lb) cane or beet sugar and 335ml boiled water. 6ml of 10% formalin helps to preserve the solution (Patton, 2000). Another possibility for the Sheather's sugar solution is the dilution of 500g table sugar in 320ml water and 6.5g phenol crystals, which is helpful in identifying coccidian oocysts (Greiner and Ritchie, 1994). Although *Cryptosporidium spp.* can be identified through acid-fast staining methods, precise examinations are required because the staining material might be mistaken

as *Cryptosporidium* and the real parasite might be overlooked. ELISA and immunofluorescent assays can be performed, but do not seem to be as useful in avians as in mammals (Patton, 2000). Advanced diagnostic methods like molecular analysis give more precise results (Sreter and Varga, 2000). However, the direct comparison of ELISA techniques, molecular analyses, and immunofluorescence techniques showed the highest number of positive results conclusive with the clinical symptoms of affected birds of 15 different species for the ELISA technique (Cray et al., 2002).

Motile protozoan trophozoites like *Giardia*, *Hexamita*, and *Trichomonas* can be identified in direct smears. For *Giardia* and *Hexamita*, the feces material is mixed with Lactated Ringer's Solution or normal saline. Another possibility to detect *Giardia* is the staining of fresh feces material with carbol fuchsin or iodine. Sterile tissue swabs are soaked with warm saline or warm Lactated Ringer's Solution and must be examined under the microscope within ten minutes after sample taking in order to detect *Trichomonas sp.* (Greiner and Ritchie, 1994).

Enzyme-linked immunosorbent assays (ELISA) methods have found their way into avian parasite diagnostics. The ELISA tests detect an antigen of the parasite through a parasite-specific coated antibody membrane. Especially for detection of *Giardia* and *Cryptosporidium*, ELISA methods have proved to be helpful although they are not especially developed for avian parasite testing (Patton, 2000). Furthermore, immunofluorescent antibody (IFA) tests have been added to avian parasite diagnostics. They are mainly used for testing of *Giardia* and *Cryptosporidium spp.* (Patton, 2000).

Polymerase-chain-reaction methods have also been used for the detection of avian parasites like *Enterocytozoon bieneusi* (Muller et al., 2008). However, PCR might not be used as a routine diagnostic tool due to cost and time consideration, but it is extremely helpful for detection of uncommon or new parasitic diseases.

Avian hematozoa can be identified in blood smears on microscope slides. It is helpful to cover the microscope slides with cover slips to keep the parasites in place. The smears can be stained with Wright/Giemsa or Giemsa stain (Greiner and Ritchie, 1994).

ECTOPARASITES

Ectoparasites are frequently found on the feathers and integument of birds. In small numbers, they do not cause major problems; in large quantities, however, they may lead to a major disturbance of the affected birds and might result in anemia in the case of blood-sucking ectoparasites. Moreover, ectoparasites can become vectors for the transmission of infectious diseases. However, not all ectoparasites are commonly found in pet birds due to their in-house keeping, but the ectoparasites of pet birds have clinical significance. Therefore, only the most clinically-relevant ectoparasites are mentioned in this chapter.

Mallophaga

Mallophaga, or chewing lice, can be differentiated into two subspecies, *Ischnocera* and *Amblycera*. Both are strictly host-specific and can be isolated in a large number of avian species. Their development cycle takes six weeks (Gylstorff and Grimm, 1998).

Disease transmission takes place through contact and contaminated litter. The parasite's life takes up to 2-3 months, but is reduced if it does not find a host. In ducks, death cases through Mallophaga infestation have been observed (Gylstorff and Grimm, 1998).

Mallophaga can be frequently found in raptors with reduced immune systems (Muller et al, 2008). Moreover, *Ischnocera* and *Amblycera* can serve as vectors for the transmission of the nematode *Eulimdana* spp in charadriiform birds (Bartlett, 1993).

Hippoboscidae

Louse flies, or Hippoboscids, are frequently found in wild birds, psittacines, and raptors (Gylstorff and Grimm, 1998), which are kept in open-air aviaries (Greve, 1996b). The main hippoboscid species that are affected for those birds are *Ornithomyia*, *Lynchia*, and *Pseudolynchia*. They have a characteristic dorsoventrally flattened body (Greiner, 1997). *Pseudolynchia canariensis* are found in pigeons and doves. It is relatively difficult to detect louse flies as they move very quickly between the feather and stick to them. Adult louse flies are blood-sucking and can lead to anemia and death of juvenile birds. Moreover, they may serve as vectors for the transmission of viruses, bacteria, and *Haemoproteus* sp (Gylstorff and Grimm, 1998). The disease can be controlled by removing the potential source of transmission, the wild birds, as well as cleaning and disinfecting the aviary (Greve, 1996b).

In birds with injuries and traumas causing open wounds, myiasis can be found. The larvae of blow flies and flesh flies can invade those open wounds (Greve, 1996b).

Knemidokoptes

The *Knemidokoptes* spp. mites can be found in the leg region or face of various bird species. They lead to mange, known under the names of "scaly face" or "scaly legs". The mites burrow into the cornified layer of the epidermis. This is the place where they lay eggs (Greve, 1996b). In budgerigars and cage birds, *Knemidokoptes pilae* infections start with tiny wart-like swellings (Greve, 1996b). The mange later leads to hyperkeratosis in the face and especially the cere up to the conjunctiva of affected birds. In severe cases, the beak might even get deformed (Gylstorff and Grimm, 1998). The disease develops slowly in psittacines and may break out after months during which the birds do not show clinical symptoms (Greve, 1996b). In canaries, finches and passerines, *K. jamaicensis* is commonly the cause of mange especially in the legs (Greve, 1996b). In canaries, the disease is localized in the leg region with thick hyperkeratotic crusts especially on the plantar foot area and deformities of the toes and talons. In some cases, lameness can occur in birds (Gylstorff and Grimm, 1998). Cracked skin and fissures can pave the way for secondary bacterial infections (Greve, 1996b). A transmission from *Knemidokoptes* sp. to other birds does not seem likely (Greve, 1996b).

In pigeons, *K. leavis* is the causative agent for mange, which is known as “depluming scabies”. The mites burrow a tunnel under the feather follicles and shafts, which leads to massive itching and irritation for the affected pigeons. They start plucking the feathers until they are deplumaged. The loss of the plumage results in shivering of the pigeons, which is associated with weight loss and reduced egg production (Greve, 1996b).

Treatment can be performed with Ivermectin (Greve, 1996b).

Flies

In waterfowls, ectoparasites can lead to a fatal myiasis via the infestation of tissue by fly larvae in wild ducklings. The larvae of *Wohlfartia opaca* enters in newly-hatched ducklings through the umbilicus and moves to the abdominal subcutis (Wobeser, 1981).

ENDOPARASITES

Protozoa

Common avian protozoa or single-celled parasites are the varied group of coccidians (e.g. *Eimeria*, *Isospora*, and *Caryospora*), microsporidians (*Enterocytozoon*), as well as flagellates (*Trichomonas* and *Giardia*). Moreover, protozoa include blood parasites like *Plasmodium* and *Haemoproteus*.

Trichomonas

Trichomonads are transmitted by direct contact through contaminated food or water. They are also present as commensal species. The most common pathogenic trichomonad species is *Trichomonas gallinae*. It has several strains ranging from avirulent to highly virulent (Greve, 1996a). *T. gallinae* affects mainly pigeons, either free-range or captive, as well as Passeriformes, Psittaciformes, Falconiformes, and Phasianiformes (Gylstorff and Grimm, 1998). Moreover, *Trichomonas* infections have been reported in budgerigars, cockatiels, and blue-fronted amazon parrots. Often, parental birds transmit the protozoa to their chicks with the crop milk of regurgitated food or while feeding (Greiner and Ritchie, 1994). In raptors, infected prey like doves and pigeons (Greve, 1996a) play an important role in the disease transmission (Gylstorff and Grimm, 1998). Trichomonads are found as motile trophozoites with four free anterior flagellates and one undulating membrane. Their size range from 8 to 14 μm length (Greiner and Ritchie, 1994) and they cannot survive for a long time in the environment. Latent carriers are often found among adults birds. Water bowls as well as small lakes can serve as reservoirs of the pathogen (Gylstorff and Grimm, 1998).

Clinical symptoms are weight loss, caseous lesions up to ulcers in the mouth, oropharynx, esophagus, and crop where they can reach walnut size. *Trichomonas* lesions may also be found in the choana. Birds have problems eating, they show ruffled feathers, and get emaciated in more advanced stages. The diseases might be acute or chronic (Greve, 1996a; Gylstorff and Grimm, 1998). Spreading of *Trichomonas* lesions up to the sinuses and conjunctivae is possible (Greve, 1996a).

In raptors, the feeding of pigeons infected with *Trichomonas gallinae* may transmit the disease. *Trichomonas* infection might be associated with *Pseudomonas aeruginosa* infections (Samour, 2000). Often, trichomonads pave the way for the *Pseudomonas* infection, which cannot be detected clinically in smears anymore. In such cases, raptors often suffer from swollen tongues, sometimes with caseous, whitish lesions inside the tongue. Cheesy lesions can be found in the mouth, oropharynx, choana, and up to the esophagus and crop. The lesions can be disinfected with chlorhexidine or iodine povidone solution (Samour, 2000). Great care should be taken not to remove lesions that are still fully attached as they can lead to considerable bleeding. Detaching lesions can be carefully removed with forceps or curettes. In the oral cavity, a good healing process can be observed. However, large masses in the mouth or choana may lead to large holes after removal which might require surgical closure. This can be performed with thin absorbable sutures 4-0 or 5-0 in order to reduce the artificial trauma in the highly vascularized oral cavity.

Metronidazole application for at least 5 consecutive days is useful for the treatment of trichomoniasis. However, not all available brands seem to have the same effects. The *P. aeruginosa* infection can be treated with Piperacillin as the first drug of choice until an antibiotic sensitivity test is available (Muller et al., 2006).

Giardia

Giardia are protozoa which can be found in many bird species, although the major clinical problems are caused in psittacines (Greve, 1996a). They are characterized by a motile trophozoite and a cyst stage (Greiner and Ritchie, 1994) which invade the lumen of the small intestines (Greiner, 1997). The cysts can be transmitted directly when the host digests food with contaminated feces. Moreover, the cysts can stay alive in the environment, thus serving as a potential source of re-infection for birds. This protozoal disease can be found in various psittacine species like budgerigars, cockatiels, lovebirds, and parakeets (Greiner and Ritchie, 1994), as well as in herons, toucans, raptors, and Anseriformes (Zucca, 2000).

Adult budgerigars and cockatiels seem to be largely asymptomatic carriers as *Giardia* spp. are frequently shed with the feces without causing significant clinical signs. Predisposing factors for giardiasis are stress, molting, poor ventilation of the rooms and malnutrition. Some disease signs affect the integument with dry skin and feather plucking, especially in cockatiels. They pluck their feathers up to a stage of self-mutilation. In juvenile and neonate birds of those species, *Giardia* infestation can lead to weakness, poor plumage, skin contamination with feces (Greve, 1996a), reduced growth, and a high mortality rate of up to 50% (Greiner and Ritchie, 1994). Other disease symptoms in psittacines are watery, whitish diarrhea, enteritis, anorexia, depression, yeast infection, and changes in the blood patterns like eosinophilia and hypoproteinemia (Greiner and Ritchie, 1994; Greve, 1996a). Malabsorption is likely to cause hypoproteinemia as well as diarrhea. Birds that enter newly into bird groups may serve as subclinical carriers (Greve, 1996a).

In post mortem examinations, scrapings of the small intestine mucosa may lead to the detection of *Giardia* sp. being located in the mucous between the villi. Often dilatation and milky-whitish mucous can be found in the small intestine (Greve, 1996a).

Giardia is not only limited to avians, but transmission of avian *Giardia* isolates to mammals like mice was successfully performed. This may have serious implications for contamination of watersheds (Upcroft et al., 1997), especially as the presence of *Giardia* in infected feces and contaminated drinking water cause important implications for human

health as it is regarded as the most important cause for protozoan diarrhea worldwide (Fayer, 2008).

Treatment with metronidazole or nitrofurazone for a minimum of 5 days can be done in drinking water. Even after successful treatment, diarrhea can persist for another one or two weeks as it takes time for the mucosa to heal completely (Greve, 1996a). Probiotics can be helpful in restoring the intestinal flora.

Coccidia

Eimeria and *Isospora* are part of the coccidian species and are distributed worldwide (Zucca, 2000). The maturation of *Coccidia sp.* goes through three phases. The first phase is schizogony followed by the second phase of gamogony. Both phases develop in the host cells. The third stage develops in the environment and is called sporogony (Gylstorff and Grimm, 1998). The infectious stage of the coccidia is during the maturation process when the sporulated oocysts get divided into sporocysts with sporozoites (Greiner and Ritchie, 1994).

The strictly host-specific (Gylstorff and Grimm, 1998) *Eimeria sp.* are commonly found in poultry, Galliformes, and Columbiformes, and can be regarded as one of the most important protozoal pathogens in the poultry industry (Zajac and Conboy, 2006). Severe infections with *Eimeria sp.* can be diagnosed in juvenile Anatiformes (Gylstorff and Grimm, 1998). Moreover, two *Eimeria* species have been reported in psittacines (Greiner and Ritchie, 1994). Oocysts have a size of 10–45 μm (Zajac and Conboy, 2006) and a direct life cycle. In *Eimeria sp.*, sporulated oocysts have four sporocysts with two sporozoites each (Greiner and Ritchie, 1994).

Isospora sp. can be diagnosed in psittacines, passerines, and Piciformes (Zajac and Conboy, 2006). In *Isospora sp.*, four sporozoites are located in two sporocysts (Greiner and Ritchie, 1994)

Both coccidian species can be either asymptomatic in birds or cause melena, depression, or diarrhea (Greiner and Ritchie, 1994). Typical signs for coccidiosis are also soiled vents, distended intestines and swollen abdomen, fluffed feathers, and emaciation (Greve, 1996a). Advanced disease stages lead to anorexia and death (Greiner and Ritchie, 1994) usually 1 week after the beginning of the coccidial infection (Greve, 1996a). Nervous symptoms have been reported, although they are not a common feature of coccidiosis. These nervous symptoms can be caused by an electrolyte imbalance, hypoglycemia, and debilitation (Greve, 1996a).

Coccidiosis can be treated with amprolium in the drinking water (Greve, 1996a) or the coccidiostat toltazuril administered orally. It is advisable to give supplementary multivitamins (Greve, 1996a). Moreover, probiotics can be helpful to restore the intestinal bacterial flora after finishing the antiparasitic therapy.

Caryospora

Caryospora sp. can be isolated from raptors and, hereby, mainly Falconiformes. Although being described as a disease affecting mainly juveniles and sub-adult birds (Zucca, 2000), this coccidia can be identified as well in older falcons. More than seven different *Caryospora* species have been described (Heidenreich, 1997). In falcons with a heavy *Caryospora* burden, clinical symptoms like changes in the fecal consistence and weight loss can be observed. Moreover, in these severe cases, enteritis with thickened intestinal walls can be visualized in the X-ray. A heavy *Caryospora* infestation also leads to a suppressed

immune system and can pave the way for secondary infections like clostridiosis and microsporidiosis with *Enterocytozoon bieneusi*. Toltazuril has been used effectively against *Caryospora sp.* Moreover, in case of a heavy *Caryospora sp.* infestation, the application of probiotics after the anticoccidial treatment is recommended to restore the intestinal flora (Muller at al., 2008).



Figure 1. *Caryospora sp.* in a Gyr-Saker falcon.

HELMINTHS

Trematoda (Flukes)

Trematoda, or flukes, belong to the *Platyhelminthes* and can live either in the liver of the blood vasculature of Psittaciformes (Greiner and Ritchie, 1994), Passeriformes, Anseriformes, and poultry (Zucca, 2000). The flukes in the blood are called *Schistosomatidae*. All flukes possess a simple digestive system and are hermaphroditic apart from the *Schistosomatidae* (Zucca, 2000). The liver flukes are part of the family *Dicrocoelidae*. The life cycle of flukes contains one or two intermediate hosts (Zucca, 2000), the first one usually a snail (Kassai, 1999) and the second one an arthropod (Greiner and Ritchie, 1994), dragonfly, or dragonfly larvae (Kassai, 1999). The transmission of flukes happens via a second intermediate host which is eaten by the bird. Due to its intermediate host range, infections arise more frequently in aquatic bird species, or Anseriformes (Zucca, 2000).

Fluke infection affecting the liver can lead to major clinical symptoms like hepatomegaly, hepatic necrosis, elevated liver enzymes, anorexia, and diarrhea, as well as weight loss. In the advanced stage, the liver fluke infection may be lethal for the infected bird. In cockatoos, the liver and bile ducts can get infected by flukes. This leads to major clinical

signs like hepatomegaly. In the histopathology, fibrotic changes of the liver as well as the bile duct hyperplasia can be seen (Greiner and Ritchie, 1994).

Treatment with anthelmintics like fenbendazole or praziquantel may not lead to clinical improvement, but might reduce the number of fluke eggs (Greiner and Ritchie, 1994). The use of praziquantel, albendazole, flubendazole, and fenbendazole has been suggested (Kassai, 1999). In raptors, rafoxanide can be used (Zucca, 2000).

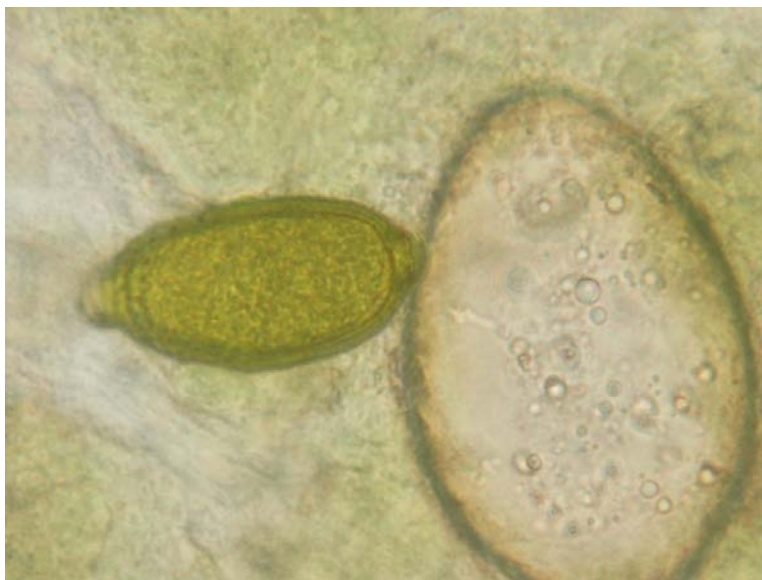


Figure 2. Capillaria (left) and trematode (right) of a Gyr-Peregrine falcon.

Cestoda (Tapeworms)

Tapeworms can be found in Psittacines and, hereby, especially in African grey parrots, cockatoos, finches, and Eclectus parrots. Newly-imported African grey parrots and kakadus can suffer from heavy tapeworm burdens (Gylstorff and Grimm, 1998). In some psittacine species, 10-20% of birds can be infected with tapeworms (Greve, 1996a). Intermediate hosts are required for the infection with tapeworms which leads to the infection of birds that mainly have contact with the ground. Water and land arthropods as well as molluscs can serve as intermediate hosts (Gylstorff and Grimm, 1998). The tapeworm eggs have hexacanth larvae and six hooks on oncosphere (Greiner and Ritchie, 1994). They can be found in the small intestines (Greiner, 1997).

The infection can remain asymptomatic, but can also show clinical signs like diarrhea, weight loss, and death in the case of heavy parasite burden. A possible eosinophilia might be present but does not reflect a direct relationship with the parasite infection (Greiner and Ritchie, 1994). Compared to the relatively low infection rate in companion birds, tapeworms can be frequently observed in raptors in the Middle East living in captivity.

Cestode infections can be treated with praziquantel. In parrots, the praziquantel treatment should be repeated after 2 weeks (Gylstorff and Grimm, 1998).

Nematoda (Roundworms)

Ascaridia

The roundworms of the species *Ascaridia* can be frequently found in birds (Greiner, 1997) and are moderately common in cage and aviary birds (Greve, 1996a). The difference between females and males is the large precloacal sucker in the male worms (Mines and Green, 1983). *Porrocaecum sp.*, especially *Porrocaecum ensicaudatum*, can be found in passerine birds (Greve, 1996a). The ascarids *Contraecum spp.* are often found in fish-eating birds. They have two intermediate hosts, the first one being small crabs and the second being fish (Gylstorff and Grimm, 1998). In *P. ensicaudatum*, earthworms serve as intermediate hosts (Greve, 1996a). *Ascaridia* have a direct life cycle. The infective larvae need 2-3 weeks to develop in the egg. After hatching, the larvae migrate in the mucosa of the small intestine and then to the lumen to mature (Greve, 1996a). They can also develop in the oral cavity, esophagus, and proventriculus where they might get attached to the proventricular mucosa. Especially in young pelicans, a heavy *Ascaridia* burden can be found in the mouth and esophagus. In penguins, *Contraecum spiculigerum* can cause inflammations of the proventriculus. Other symptoms might be local inflammation and weight loss (Gylstorff and Grimm, 1998).

Ascaridia spp. eggs are ellipsoidal, smooth, and colorless, whereas *Porrocaecum spp.* eggs are more round-shaped with pitted and brown shells. The eggs can survive in the environment for several months (Greve, 1996a).

In necropsies, ascarids can be easily identified with female worms reaching up to 40 mm in size and smaller males (Greve, 1996a).

Ascarid infections can be treated with levamisole, fenbendazole, mebendazole, ivermectin, and pyrantel tartrate. However, fenbendazole should not be used in molting birds as they affect the feather formation. Pigeons and doves may react with toxicosis to the application of benzimidazoles (Greve, 1996a).

Capillaria

Capillaria sp. are trichuroid nematodes (Greiner, 1997). They are very small, thread-like nematodes with the typical two poles of the eggs. *Capillaria sp.* are located in the gastrointestinal tract and especially in the crop, esophagus, and small intestine (Greiner and Ritchie, 1994; Zucca, 2000). This nematode can be found in poultry, psittacines like budgerigars, macaws, and canaries, as well as in raptors like Gyr falcons, peregrine falcons, American kestrel (Trainer et al., 1968), Gyr-peregrine hybrid falcons, and Gyr-Saker hybrid falcons. In pigeons, *Capillaria columbae* can be isolated (Greiner, 1997).

Having a direct life cycle, it takes approximately two weeks to develop adult *Capillaria* larvae. The eggs show the very characteristic pattern of bipolar eggs (Greiner and Ritchie, 1994). The eggs can survive up to several months in the environment, especially in humid conditions and moderate temperatures (Zucca, 2000). The adult *Capillaria* worms have a beaded esophagus (Greiner, 1997). They can invade the crop and esophagus mucosa, as well as the intestinal mucosa (Greiner and Ritchie, 1994), mainly of the small intestines (Greiner, 1997). This results in clinical symptoms like regurgitation, dysphagia, weight loss, diarrhea, and melena. In cases of heavy *Capillaria* burden, hemorrhages may arise in the upper

intestinal tract (Greiner and Ritchie, 1994). *Capillaria* infestation in raptors may also lead to vomiting and a reduced appetite.

Whereas in poultry severe infections in the upper digestive tract can be observed, the clinical pattern in other avian species is not unified (Gylstorff and Grimm, 1998).

Hygienic measures have to be strictly enforced to avoid re-contamination. Treatment with fenbendazole, levamisole, and mebendazoles is usually successful. A repeated treatment is to be done in case of a heavy *Capillaria* burden (Gylstorff and Grimm, 1998).



Figure 3. *Capillaria* sp. in Peregrine falcon.

Serratospiculum

Being a diplotriaenoid nematode of the respiratory tract, *Serratospiculum* spp. can be identified in air sacs. Its adult filaroid worms can be found in connective tissue, meninges, heart, blood vessels, and under the skin (Greiner, 1997). *Serratospiculum* sp. can be frequently found in Falconiformes. It has been detected in North America, some European countries, and tropical and subtropical countries (Zucca, 2000). Moreover, the *Serratospiculum* infection associated with bronchopneumonia and airsacculitis was identified in New Zealand falcons (Green et al., 2006). *Serratospiculum seurati* is frequently found in the Middle Eastern countries (Samour and Naldo, 2001).

Serratospiculum seurati has an indirect life cycle. The larvae stages of *S. seurati* can be found in 7 intermediate hosts, namely beetles and the wood louse. Falcons in captivity eat the infected intermediate hosts. The L3 larvae move out from their capsule, penetrate the proventriculus and ventriculus walls, and migrate directly to the airsacs. Inside the airsacs, the L3 larvae undergo direct mold two times. The resulting L5 larvae are regarded as the immature adult filarial worms. The adult worms can breed and produce large numbers of embryonated eggs. Those ova can be coughed up through the trachea, swallowed in the digestive tract, and then shed with the feces. Histopathological changes include the different *S.seurati* stages like larvae, adult worms and eggs, but also mild focal hemorrhages, focal necrosis, and macrophage infiltration. *S.seurati* infections are associated with airsacculitis,

pneumonia and early Aspergillosis lesions (Samour and Naldo, 2001). In prairie falcons, the heavy *Serratospiculum* burden of more than 100 worms has led to acute respiratory problems and subsequent death (Ward and Fairchild, 1972). In the same falcon species, another fatal infection due to *Serratospiculum amaculata* was reported (Kocan and Gordon, 1976).



Figure 4. *Serratospiculum seurati* in Gyr-Saker falcon.

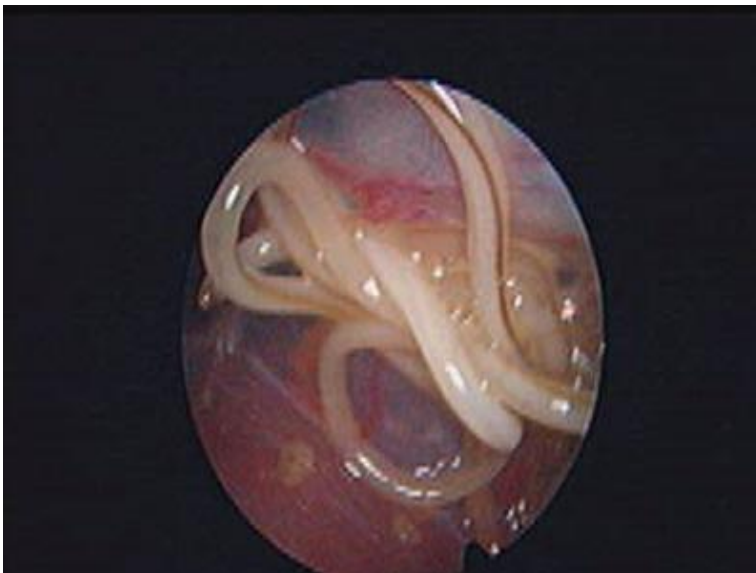


Figure 5. Adult *Serratospiculum seurati* worms in endoscopic view.

However, *S. seurati* infections can be present even though no embryonated worm eggs might be detected in the feces. On the other hand, *S. seurati* eggs can be present in the feces examination, but no eggs, larvae, or adult worm stages might be visible in the air sacs when performing endoscopic examinations. Other features of the endoscopic examination are

present, yellowish-colored egg clutches that can be easily mistaken with fat deposits. The air sac membrane might show for *Serratospiculum seurati* typical whitish, spotty discoloration, which is usually produced by larvae or worm stages even if they are not present anymore (Muller, 2009). Discussions among avian veterinarians are on-going if the *S. seurati* worms should be removed during endoscopy before or after treatment with anthelmintics. If the worms have not been treated yet but are detected during routine endoscopy, it is more advisable to remove them carefully with the help of biopsy forceps. However, it is not indicated to remove adult *S. seurati* worms if they are fully covered with large numbers of blood vessels, as the subsequent hemorrhage would be more problematic for the falcon. Large numbers of *S. seurati* can be frequently found in older Saker falcons (*Falco cherrug*) (Muller, 2009).

Ivermectin and Moxidectin can be used to treat Serratospiculosis (Samour and Naldo, 2001).

Syngamus Trachea

The nematode *Syngamus trachea* belongs to the genus *Strongyloidea* and family *Syngamidae*. This bright red-colored gapeworm can be found in the respiratory tract of birds like in the trachea, bronchi, and bronchioles. Bird species that are commonly affected are chicken, quails, turkeys, guinea fowl, geese, peafowl, and pheasants (Ruff, 1991). Other avian species susceptible to *Syngamus trachea* infections are Columbiformes and Psittaciformes, especially cockatoos, birds of prey, and Passeriformes, and mainly Corvidae and starlings (Gylstorff and Grimm, 1998). Typically, *S. trachea* worms are found in pairs. The females and males are attached on a permanent basis through copula (Greiner, 1997) in a Y-form position (Rosskopf and Woerpel, 1996).

Disease transmission can arise through shedding of thin-shelled *Syngamus* eggs in the feces of infected older birds. Eggs can remain infectious for up to four years in cages or pens. Wild birds are also a source for infections. Eggs develop in the moist warm environment and can mature up to larva stages. The eggs or larvae are eaten by birds either directly or through an intermediate host like earthworms, snails, or insect larvae. *Syngamus trachea* larvae migrate through the blood to the trachea. Disease symptoms are severe respiratory diseases with coughing, opening of the beak, and respiratory sounds. The general conditions of the infected birds declines and death arrives through asphyxiation (Gylstorff and Grimm, 1998).

Treatment can be performed with the manual removal of the gapeworms through endoscopy or tracheotomy with air sac infusion anesthesia. Medication combines application of Ivermectin as well as antibiotics to cover up the tracheal damage (Rosskopf and Woerpel, 1996) and to prevent secondary infections.

Blood Parasites

Haemosporidian parasites are distributed worldwide. Three main species are isolated in birds: *Haemoproteus*, *Plasmodium*, and *Leukocytozoon*. *Haemoproteus* is the most commonly found blood parasite and along with *Leukocytozoon* host specific. In contrast, *Plasmodium* has a wide host spectrum and can be isolated in several avian species (Atkinson and Van Riper, 1991). The occurrence of blood parasites in avians are known to have an impact on the flight performance by reducing the flight distance. Moreover, the number and species of

blood parasites are also determining components for the reduced flight distance. Therefore birds infected with haemoparasites get more vulnerable to fall prey to raptors (Møller, 2008). Furthermore, acute *Plasmodium* infections lead to reduced oxygen transport and impact the thermoregulation (Hayworth et al., 1987).

Plasmodium

Plasmodium infections can be found in canaries, small passerine birds, pigeons, waterfowl, penguins and poultry. In those species, the infection with *Plasmodium* can result in major clinical problems which can lead to increased morbidity and mortality. Passeriformes and Psittaciformes are often asymptomatic carriers. Mainly *P. relictum* and *P. elongatum* have been isolated in the peripheral erythrocytes, thrombocytes, leucocytes and in the cells of the endothelium. The disease is usually transmitted through sporozoites that are located in the saliva of blood sucking mosquitoes (Gylstorff and Grimm, 1998). In waterfowl, malaria caused by *Plasmodium sp.* exists, however, their pathogenicity is not clear (Wobeser, 1997). Golden or black colored refractile pigment granules in the schizonts and gametocytes can be identified in the cytoplasm of *Plasmodium sp.* (Greiner, 1997).

First schizonts stages can be found in the reticulo-endothelial cells of visceral organs and bone marrow (Gylstorff and Grimm, 1998). Schizogony occurs in the peripheral blood where gametocytes and schizonts are visible (Van der Heyden, 1996). However, extra-erythrocytic stages were detected in the endothelial cells of brain and lung capillaries of hiking throattles (*Merula migratoria*). Schizonts and gametocytes develop at later stages. Gametocytes located in the erythrocytes are transmitted to blood sucking insects which pose the intermediate host for the further *Plasmodium* development (Gylstorff and Grimm, 1998).

The infection e.g. to passerines can be transmitted through mosquitoes. This leads to a seasonal occurrence of *Plasmodium* infections, especially in spring and autumn in North America (Fudge, 2000). Such a seasonal infection distribution pattern can also be found in blue tits *Cyanistes caeruleus*, with a bimodal occurrence in spring and autumn peaks, especially in *Plasmodium circumflexum* infections. However, in winter, no infection could be observed. In contrast, infections with *Plasmodium relictum* showed reduced seasonal patterns. The age also might play a role in the seasonal occurrence of the infection as in the blue tits, the seasonal infection was found only in one-year-old birds. Older birds did not show the seasonal *Plasmodium* infection patterns (Cosgrove et al., 2008).

Newly-infected birds show more clinical symptoms. Clinical symptoms may not correlate with the number of parasites in the peripheral blood (Van der Heyden, 1996). Anemia can occur as disorders of erythrocytes, including intravascular hemolysis in passerines and penguins. In the latter, *P. relictum* can cause a relative lymphocytosis, anorexia, vomiting, and cramps with mortality, especially in the months of July and August (Gylstorff and Grimm, 1998). A high parasitaemia can be observed in penguins, too. In gyrfalcons, a high parasitaemia can be seen associated with lethargy, anorexia, and jade-green fecal (Van der Heyden, 1996). Moreover, prominent poikilocytosis can be diagnosed in the avian blood smears. Intraerythrocytic gametocytes with nuclear displacement can be identified in the blood smears (Fudge, 2000). Other disease symptoms, especially in Falconiformes, are ballooning with the erythrocytes and eosinophils, as well as jaundice and elevated AST parameters. Moreover, dyspnea, vomiting, and cramps, especially in gyrfalcons have a more severe manifestation than in peregrine falcons, for example (Gylstorff and Grimm, 1998). Canaries and finches show dyspnea, lethargy, and anorexia with high parasitaemia. Sudden

death cases have been observed in those species. In other species, death may arise immediately or within 1 to 2 days after occurrence of clinical symptoms (Van der Hayden, 1996). Post-mortem often reveals hepatosplenomegaly, pulmonary edema, and pericardial diffusion (Van der Heyden, 1996).

Despite being host-specific haemoparasites, *Plasmodium sp.* can also affect other bird species, e.g. *Plasmodium sp.* of passerines was detected in birds of prey and owls in Europe. This leads to the conclusion that a much broader and non-host specific presence of *Plasmodium sp.* exists which is not confined to one avian species only (Krone et al., 2008).

As therapeutic agents, most human anti-malarial medications like chloroquine and primaquine are effective in birds. Supportive therapy with fluids is helpful (Van der Heyden, 1996).

Haemoproteus

Haemoproteus spp. can be frequently found in wild-caught birds like white cockatoos, green-winged macaws (*Ara chloroptera*), and halfmoon conures (Fudge, 2000), as well as in Psittaciformes, Accipitriformes, Falconiformes, and Strigiformes (Gylstorff and Grimm, 1998). It is the most common haemoparasite in birds (Van der Heyden, 1996). Pigmented gametocytes are visible in erythrocytes and can be used as differentiation to the other haemoparasites (Greiner, 1997). Blue staining is characteristic for macrogametocytes, whereas pale blue and pink staining is indicative for microgametocytes (Van der Heyden, 1996). In North American waterfowl, *Haemoproteus nettionis* is the most commonly distributed haemoparasite (Wobeser, 1997). They have been regarded to be the least pathogenic of all haemoparasites (Atkinson and Van Riper, 1991). However, if more than 10% of the erythrocytes are affected, severe disease symptoms like inappetence, apathy, and anemia might arise and could be lethal, especially in pigeons and quails. In Muscovy ducks, lameness and restlessness might be observed before their death (Gylstorff and Grimm, 1998). In other waterfowl, no major disease effects have been described (Wobeser, 1997).

The *Haemoproteus sp.* life cycle is similar to the life cycle of *Plasmodium sp.* and *Leukocytozoon sp.* However, the schizonts develop exclusively in the endothelium of the different visceral organs, whereas the gametocytes mature in the circulating erythrocytes (Gylstorff and Grimm, 1998). Intermediate hosts can be *Culicoides spp.* or *Hippoboscidae spp.* The acute parasitaemia occurs usually 7 to 10 days after infection and can remain up to several weeks. Recurrence of the disease is caused by stress and in spring time (Gylstorff and Grimm, 1998).

Although being usually host-specific (Atkinson and Van Riper, 1991), *Haemoproteus sp.* switches not only between the same species, but also between species of different families (Bensch et al., 2000). Moreover, a host switching at a familial level has been observed in a promiscuous genotype of *Haemoproteus sp.* in six different exotic passerine birds in California. The infection led to severe morbidity and high mortality, although clinical signs were either few or not observed. The blood smears did not reveal any *Haemoproteus* parasites. However, the pathological picture included hemocoelom, hemorrhages, and hepatocellular necrosis. Various stages of degenerated protozoal megaschizonts were isolated in the affected liver areas. Moreover, peripheral non-suppurative inflammation was present. The molecular analysis resulted in avian malarial mitochondrial cytochrome B. Its sequencing identified the close relation to *Haemoproteus sp.* of North American passerine birds that were asymptomatic (Donovan et al., 2008)

Leukocytozoon

Malaria caused by *Leukocytozoon sp.* can be found in poultry as well as 100 other wild-living avian species. More than 70 species can be isolated, mainly in juvenile birds (Gylstorff and Grimm, 1998). In poultry and waterfowl, malaria caused by *Leukocytozoon sp.* is known as reason for serious diseases. In other avian species, the pathogenicity is not fully researched yet (Atkinson and Van Riper, 1991). *Leukocytozoon* is the largest haemoparasite in birds (Van der Heyden, 1996).

Transmission occurs through the black flies of the family *Simuliidae*. The schizogony happens in the liver parenchyma, whereas the merozoites develop in erythrocytes to gametocytes. In contrast to *Plasmodium* and *Haemoproteus*, pigments are not produced by *Leukocytozoon sp.* (Gylstorff and Grimm, 1998). Syncytia of the liver parenchyma gets released and ingested through phagocytosis in the liver, spleen, heart, kidneys, and brain. In these organs, megaloschizonts develop of up to 200µm and can contain millions of merozoites. The megaloschizonts get encapsulated through fibrotic tissue and macrophages, heterophils, plasma cells, and red blood cells as a hostinflammatory response. Necrosis and calcification of megaloschizonts can happen. A direct correlation between the presence of megaloschizonts and the pathogenicity of *Leukocytozoon sp.* is present (Atkinson and Van Riper, 1991).

Leukocytozoon sp. infections cause severe anemia (Atkinson and Van Riper, 1991). Young turkeys, raptors, and waterfowl suffer from hemolytic anemia and hemoglobulinemia (Van der Heyden, 1996). In turkeys, pneumonia, lung congestion, and blockage of alveolar capillaries can arise (Atkinson and Van Riper, 1991). Other disease symptoms are inappetence, dyspnea, cough, diarrhea, infertility, and high mortality (Gylstorff and Grimm, 1998). Furthermore, necrosis of the liver, spleen enlargement, and lymphocytic infiltration of the liver and heart, as well as haemosiderosis, can be present (Atkinson and Van Riper, 1991).

Emerging Diseases

Microsporidiosis Caused by Enterocytozoon Bieneusi

The diverse group of Microsporidians is eukaryotic, single-cell organisms closely related to fungi (Thomarat et al., 2004). Microsporidians are intracellular parasites of vertebrates and invertebrates (Wasson and Peper, 2000), but have been isolated from birds as well.

Enterocytozoon bieneusi has been reported in Germany in 2 out of 8 chickens examined. The genome sequencing revealed the genotype J (Reetz et al., 2002). Moreover, this microsporidian parasite was identified in 17 of 124 (13%) healthy pigeons examined in Spain, but the genotype was not identified (Haro et al., 2005). Furthermore, in Portugal, *E. bieneusi* was recently detected in 24 of 83 (28.9%) faecal droppings from birds of the orders Columbiformes, Passeriformes, and Psittaciformes (Lobo et al., 2006).

A large microsporidian outbreak in falconiformes has been reported in the United Arab Emirates where 70 falcons were infected by *E. bieneusi* (Muller et al., 2008). The pathological picture of the infected falcons revealed multiple yellowish 3-5 mm large plaques that were found mainly on the small intestine and colon. On the liver and kidneys, several yellowish foci of 1-5 mm in diameter were identified. Lesions also occurred on the pancreas and spleen. In an advanced stage, the infection leads to high mortality. The epidemiology

indicates the lymphatic spreading of spore-infected macrophages from the gallbladder reservoir, inducing proliferative serositis. No significant changes in hematological and biochemical blood parameters are symptomatic for this disease except that elevated white blood cell counts were detected in the majority of infected falcons. Underlying diseases like coccidiosis, aspergillosis, and hepatopathy pave the way for microsporidian infections (Muller et al., 2008; Muller 2009).

The histopathology findings included extensive thickening of the intestinal serosal surfaces. They were characterized by proliferation of mesothelial cells and non-suppurative inflammatory cell infiltrate which involves all intestinal layers from the serosa to the mucosa. The liver lesions showed large areas with foamy hepatocytes and bile duct proliferation, fresh necrosis, and microabscesses in the adjacent areas. On the pancreas and kidneys, multifocal, severe diffuse degeneration with pyogranulomatous inflammation, with most renal tubuli containing protein cylinders, were found. Immunohistochemistry is another laboratory examination that can be performed for *E. bienewsi*. The examined liver, kidney, and intestinal lesions were tested positive for the microsporidian antigen in the immunohistochemistry. The microsporidian antigen was identified as a brownish-stained material in the cytoplasm of numerous cells in and around necrotic areas (Muller et al., 2008; Muller 2009).

Detection of microsporidian stages in faecal samples might be difficult due to the small size (1-2 μm) in some species (Canning, 1993), which can make a reliable visualization by light microscopy difficult (Franzen and Müller, 1999). Faecal identification methods include simple salt flotation followed by cytospin and Giemsa staining (Van Gool et al., 1990) and PAS staining (Canning, 1993). Another fecal staining method is Weber's chromotrope stain, as well as Gram chromotrope-based stains (Weber et al., 1992; Moura et al., 1996). In recent years, microsporidia were detected through the use of monoclonal antibody-based fluorescence assays (Singh et al., 2005) and multiplexed fluorescence in situ hybridisation (FISH) assays (Graczyk et al., 2007). Other laboratory methods include transmission electron microscopy (TEM) (Franzen and Müller, 1999), as well as in situ hybridization (ISH) on formalin-fixed, paraffin-embedded tissues (Chalifoux et al., 2000). Furthermore, molecular analysis through PCR has gained increasing importance in recent years for the detection of microsporidia as a diagnostic method for in-clinical samples and different protocols have been published (Franzen and Müller, 1999). The *E. bienewsi*-specific PCR showed the typical 607 bp fragment for falcons being identical to the AF023245 strain isolated from a macaque and to the INDRE01, the AF024657, and the L16868 strain, all three isolated from patients with AIDS (Muller et al., 2008).

The route of microsporidian and *E. bienewsi* transmission is still not fully researched yet. Transmission is possible through contaminated chicken meat or water (Reetz et al., 2002). The spores can also contaminate water in areas with infected pigeons. This possibility of an air- and waterborne transmission route through infected pigeon faeces can cause infections in falcons as well (Muller et al., 2008). However, the zoonotic potential of *E. bienewsi* is well-known (Mathis et al., 2005). *E. bienewsi* spores of infected urban pigeons' faeces can be aerosoled, which might lead to an airborne transmission of humans. This infection of humans might arise through inhalation of 4 times higher *E. bienewsi* spore concentrations than potentially viable, as well as by direct contact through oral mucosa and ingestion of the microsporidian spores (Haro et al., 2005; Graczyk et al., 2007). Moreover, a possible transmission from infected humans to birds could not be ruled out (Muller et al., 2008).

Treatment of *E. bienewsi* in falcons was performed with dimetronidazole 50mg/kg p.o. once daily for 10 days. Falcons with intestinal abscesses were treated after one week of rest again with dimetronidazole in the same dosage for another 10 days. The application of probiotics after the end of the treatment period helps to restore the damaged intestinal flora. Underlying diseases have to be treated as appropriate (Muller, 2007; Muller 2009).



Figure 6. Necropsy view of intestinal tract affected with an advanced *Enterocytozoon bienewsi* infection in a Gyr-peregrine falcon.

Cryptosporidia

Cryptosporidiosis is a parasitic protozoan disease of the phylum *Apicomplexa* that is regarded as one of the most prevalent infections in more than 30 species of domestic, wild, cage, and pet birds (Fayer, 1997; Sreter and Varga, 2000; Ng et al., 2006). Chicken, quails, ducks, turkeys, geese, pheasant, as well as ostriches and swans, are among the species that can be affected. Finches, cockatiels, macaws, budgerigars, and canaries had reported cases of cryptosporidiosis (Fayer, 1997). In avians, three main species can be found—namely *C. baileyi*, *C. meleagridis*, and *C. galli*. Their life cycle develops from un-sporulated oocysts, young schizonts, and mature schizonts to sporulated schizonts. This leads to the longtime survival of the oocysts (Fayer, 1997). In contrast to other coccidian species, the *Cryptosporidium* oocysts are immediately infective (Patton, 2000).

Although cryptosporidiosis mainly infects the intestinal tract, renal and respiratory *Cryptosporidium* infections have also been diagnosed. The main clinical symptoms for enteric cryptosporidiosis are diarrhea and enteritis. Respiratory infections show unspecific signs like coughing, sneezing, and even dyspnea, whereas renal cryptosporidiosis leads to enlarged and pale, discolored kidneys (Fayer, 1997). In cockatiels, five- to thirty-days-old juveniles had been affected with enteric *Cryptosporidium* infections. The main symptoms were severe diarrhea, dehydration, depression, ruffled feathers, and a high mortality rate. Histological

changes included villous atrophy and detachment of erythrocytes (Kwon et al., 2005) and purulent inflammations and necrosis of the Bursa Fabricii (Gylstorff and Grimm, 1998).

The treatment of cryptosporidiosis in avians poses considerable challenges as most anticoccidial medicines are not very effective, especially against enteric and renal infections (Fayer, 1997).

Moreover, another *Cryptosporidium*, *C. parvum*, is commonly found in humans with special significance in immunosuppressed humans, but it has been detected in immunocompetent humans as well (Xiao et al., 2000). The genome structure of *C. parvum* differs from other eukaryotes as its sequencing revealed that the mitochondria do not seem to contain DNA (Abrahamsen et al., 2004). Being the most important contaminant in drinking water in the USA, its oocysts contaminate 90% of the surface water in the U.S. (Fayer, 2008). Cryptosporidiosis can cause massive lethal outbreaks like in Milwaukee with 403,000 deaths (MacKenzie et al., 1994). It was also possible in clinical studies to inoculate Peking ducks (*Anas platyrhynchos*) where the parasite was shed in infective stages. This raises concerns about the epidemiological potential of this parasite in human hosts, as well as its zoonotic potential (Graczyk et al., 1996). These concerns are supported by the finding that waterfowl is able to act as mechanical carriers of *C. parvum* by disseminating infectious oocysts in the environment (Fayer et al., 1997). Another avian-specific *Cryptosporidium* species, *C. meleagridis*, also has zoonotic potential and is known to infect humans (Xiao and Ryan, 2004). Furthermore, one case of an AIDS patient infected with *C. baileyi* has been reported (Ditrich et al., 1991). As both species, *C. meleagridis* and *C. baileyi*, have been isolated in cockatiels, it raises even more concerns of possible avian-to-human disease transmission due to the close relationship between pet birds and their owners (Abe and Iseki, 2004).

However, it seems to be highly likely that a much larger diversity of *Cryptosporidium* spp. exists in humans, mammals, and avians (Xiao et al., 2002). This raises questions regarding the extent to which further zoonotic potential exists and what further epidemiological interactions might not have been identified yet.

Mosquito-borne Parasitoses

West-Nile Virus

An arthropod-borne virus (arbovirus) is the West-Nile Virus (WNV), which belongs to the genus *Flavivirus* and family *Flaviviridae*. Its first detection was in the eastern part of the United States in 1999 from where it spread quickly to the western regions. Transmitted mainly by mosquitoes, the WNV has been reported to affect more than 325 avian species, among them magpies, jays, crows, birds of prey, as well as psittacines (Shivaprasad et al., 2007)

Clinical symptoms of the West-Nile Virus infection in psittacines include general symptoms like ruffled feathers, lethargy, anorexia, depression, weight loss, and sudden death. The gross post-necropsy results only revealed atrophy of the pectoral muscles, as well as mild to moderate liver and spleen enlargement. However, the histopathological changes are more obvious with myocarditis, non-suppurative interstitial nephritis, hepatitis, spermatitis, enteritis, pancreatitis, and, in a few cases, encephalitis (Shivaprasad et al., 2007).

CONCLUSION

Parasitic diseases have been common in avian medicine for a long time. Causing major symptoms in debilitated, immuno-suppressed, and weak birds, it is highly important to keep birds healthy and enhance their immune systems. Moreover, new parasitic diseases have recently emerged that had not been identified in birds before. This leads to the assumption that a wider range of parasitic diseases of other animal species or even humans might have to be taken into consideration as causative parasitic agents in birds from now on. It can be expected that new parasitic diseases will be detected in the coming years due to enhanced diagnostic methods like molecular diagnostics and sequencing.

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Chapter IV

PRIMARY IMMUNE RESPONSE AND PARASITE DISSEMINATION IN CANINE VISCERAL LEISHMANIASIS

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ABSTRACT

Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum/L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

Keywords: *Leishmania chagasi*, canine visceral leishmaniasis, macrophage, spleen, parasite dissemination.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a chronic and frequently lethal disease caused by *L. (L) donovani* and *L. (L.) infantum* in the Old World and *L. chagasi* in the Americas (Desjeux, 2001a, b, 2004; Palatnik-de-Sousa *et al.*, 2001; WHO, 1999). Recent evidence shows that the *L. (L) chagasi* strains could not be distinguished from *L. (L.) infantum*, indicating a recent geographical separation, and that *L. (L) infantum* and *L. (L) chagasi* are two names for the same species (Mauricio *et al.*, 2000). VL is mostly a rural disease, with a domestic or peridomestic occurrence. In Brazil, however, urbanization of visceral disease has been found in towns or in the outskirts of large cities such as Belo Horizonte, Montes Claros, Rio de Janeiro, Salvador, and Fortaleza (Palatnik-de-Sousa *et al.*, 2001).

The disease is lethal if not treated early after the onset of the symptoms. Dogs present several signs, and progressive suppression of the cellular immune response. These parasites live inside monocytes and macrophages of lymphoid organs such as the spleen, lymph nodes, bone marrow, and liver. Their biological cycle alternates between the amastigote form in vertebrate host and the promastigote form in the gut of the insect sand fly vector (Baneth and Aroch, 2008). The present review aim to discuss the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL. These mechanisms probably account to development of T-cell unresponsiveness, and disease progression in susceptible dogs.

EPIDEMIOLOGY

The American and European forms of zoonotic VL show several similarities, as both are canid zoonoses that affect mainly children and young human adults (Palatnik-de-Sousa *et al.*, 2001); however, higher seroprevalences have been reported in South America (Ashford *et al.*, 1998; Zerpa *et al.*, 2000). It is well accepted that canids are the most common reservoirs of viscerotropic species causing zoonotic VL in the Mediterranean region, Asia, North Africa, and South America (Moreno and Alvar, 2002), playing a central role in the transmission cycle to humans by phlebotominae sand flies. CVL is a major veterinary and public health problem, and it has also been suggested as a good model for investigating the pathogenesis of human VL (Quinnell *et al.*, 2001b).

CVL has a high prevalence of infection, involving as much as 63%–80% of the dog population, and it is accompanied by a lower rate of apparent clinical disease (Berrahal *et al.*, 1996; Leontides *et al.*, 2002; Solano-Gallego *et al.*, 2004). Previous epidemiologic studies have indicated that about half of the dogs possessing anti-leishmanial antibodies exhibit no clinical signs of disease (Abranches *et al.*, 1991; Acedo-Sanchez *et al.*, 1998; Fisa *et al.*, 1999; Mauricio *et al.*, 1995). Interestingly, in a recent study in Northeast Brazil, a higher prevalence of positive dogs presenting no clinical signs of the disease was detected in the

metropolitan when compared to a rural area (Alvar *et al.*, 2004; Berrahal *et al.*, 1996; Queiroz *et al.*, 2008; Solano-Gallego *et al.*, 2001). Cumulative evidence shows that: (1) infection in the canine population in endemic areas is widespread, and the rate of infected dogs is much higher than the fraction that shows clinical illness; (2) infection spreads quickly and extensively among the dog population when environmental conditions for transmission are optimal (Baneth *et al.*, 2008); and (3) removal or elimination of infected dogs from endemic areas would be followed by immediate substitution of susceptible dogs (Dye, 1996).

Dye (1996) and Burattini and others (1995) have shown that the dog population in endemic areas is composed of four mutually exclusive groups: those susceptible, those resistant, those susceptible that become latent after sand fly bite (asymptomatic), and those infectious to sand flies that emerge from latent dogs at a constant rate (Dye, 1996). Dogs born resistant do not become infectious to sand flies or develop the disease, but do become seropositive after sand fly bite. Such animals include seropositive, non-infectious, asymptomatic dogs from endemic areas that are able to maintain an effective cellular immune response against the parasite. Alvar and others demonstrated that naturally infected asymptomatic seropositive dogs (resistant or latent) are infectious to sand flies (Alvar *et al.*, 1994). Dye also refers to a previous cohort study that used xenodiagnosis to show that infected dogs became infectious to sand flies after a median period of approximately 200 days; although several dogs died of clinical VL, the data indicate that infectiousness was unrelated to the severity of symptoms (Dye, 1996). This model demonstrated that targeting control measures at infectious dogs has a potential impact to reducing transmission. Conversely, infectiousness to sand flies has been shown to be positively associated with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) and to the intensity of skin disease (dermatitis, alopecia, and chancres) (Courtenay *et al.*, 2002).

In the natural history of *L. chagasi* infection in dog, following transmission the parasites initially multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can disseminate via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis *et al.*, 2006). In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites (Cardoso *et al.*, 1998; De Luna *et al.*, 2000; Martinez-Moreno *et al.*, 1995; Pinelli *et al.*, 1994; Pinelli *et al.*, 1999; Santos-Gomes *et al.*, 2002; Solano-Gallego *et al.*, 2000). After infection and before seroconversion, dogs infected with *L. chagasi* present with enlarged lymph nodes and dermatitis, without signs of visceral leishmaniasis or changes in behavior. This phase is followed by dissemination of the infection and clinical findings, including loss of appetite, fever, weight loss, alopecia, skin ulceration, onychogryphosis, keratoconjunctivitis, uveitis, bleeding, diarrhea, neuralgia, polyarthritis, interdigital ulceration, and kidney insufficiency (Abranches *et al.*, 1991; Bettini *et al.*, 1986; Molina *et al.*, 1994).

INITIAL IMMUNE RESPONSE

L. chagasi infection initiates when the parasite is inoculated into the skin by a female phlebotominae that probes the skin for blood (Rogers *et al.*, 2004). Incoming and resident phagocytes exit the blood vessels and become infected with the parasites (Moll *et al.*, 1993; Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). Based on *in vitro* and *in vivo* animal models of visceral leishmaniasis, it is widely accepted that macrophages play a central role in the control of *Leishmania* infection. Most of these studies have involved human or murine monocytes/macrophages (Bodman-Smith *et al.*, 2002; Gomes *et al.*, 2000; Murray, 2001), and only a few *in vitro* studies used canine macrophages and *L. chagasi* (Bueno *et al.*, 2005; Goncalves *et al.*, 2005; Sampaio *et al.*, 2007). Previously, Gonçalves and collaborators (2005) demonstrated that the frequency of peritoneal macrophages from naturally infected dogs expressing the monocyte surface molecules CD11b or CD18 significantly drops upon interaction with *L. chagasi*. More recently, Sampaio and collaborators (2007) showed that monocytes from naturally-infected animals compared to those from experimentally-infected ones are significantly more capable of binding to *Leishmania* promastigotes. Using peripheral monocytes from these naturally *L. chagasi*-infected dogs, the authors demonstrated that these cells display a higher frequency of CD11b-positive monocytes when obtained from peripheral blood. Similar to the previous study performed by Gonçalves and collaborators (2005) the frequency of macrophages expressing CD11b or CD18 has been shown to drop significantly upon interaction with *Leishmania*, and this decrease is more accentuated when *Leishmania* is incubated with exogenous serum (Sampaio *et al.*, 2007). The authors propose that downregulation of these receptors may be related to two mechanisms, they can be occupied by *Leishmania*, or the receptor complexes can be internalized after *Leishmania*-macrophage interaction (Sampaio *et al.*, 2007).

After phagocytosis, *Leishmania* promastigotes transform into amastigotes, which can survive inside macrophages. Some genetic alterations have been related to this event. The *Slc11a1* (NRAMP) protein acts as a proton/divalent cation antiporter, which controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome (Gruenheid *et al.*, 1997). The *Slc11a1* gene also regulates macrophage function – including upregulation of chemokine and cytokine genes such as TNF and interleukin-1 β and increased expression of inducible nitric oxide synthase (iNOS) (Blackwell *et al.*, 2001). Polymorphisms in the *Slc11a1* gene have been associated with CVL in dogs of different breeds (Sanchez-Robert *et al.*, 2005). In 164 dogs, 24 polymorphisms were found in the *Slc11a1* gene and 3 polymorphisms were associated with an increased risk for CVL (Sanchez-Robert *et al.*, 2008). Among these, two were single nucleotide polymorphisms (SNP) in the *Slc11a1* promoter region that disrupted putative transcription factor binding sites. These types of SNPs in the canine *Slc11a1* gene promoter suggest a possible role of differential *Slc11a1* gene expression that can interfere with *Slc11a1* function and/or its interaction with many other genes, contributing to CVL susceptibility (Sanchez-Robert *et al.*, 2005; Sanchez-Robert *et al.*, 2008).

COMPARTMENTAL IMMUNE RESPONSE

Skin

From the original site of infection in the skin, amastigotes disseminate throughout the body, causing lesions in different tissues such as the lymph node, liver, spleen, gut, bone marrow and, in dogs, mainly other sites of the skin (Barrouin-Melo *et al.*, 2004; dos-Santos *et al.*, 2004; Reis *et al.*, 2006). In the last few years, a growing number of systematic works provided important contributions to our understanding of the histopathological alterations that occur in these target organs (Brachelente *et al.*, 2005; Giunchetti *et al.*, 2007; Giunchetti *et al.*, 2008a; Giunchetti *et al.*, 2008b; Lage *et al.*, 2007; Santana *et al.*, 2008; Solano-Gallego *et al.*, 2007; Strauss-Ayali *et al.*, 2007).

Some studies evaluated histological alterations that occur in dog skin in response to *Leishmania* infection (dos-Santos *et al.*, 2004, Brachelente *et al.*, 2005; Solano-Gallego *et al.*, 2007). In a previous study, the histological pattern and parasite load were investigated in clinically normal skin of *Leishmania*-infected dogs (Solano-Gallego *et al.*, 2004). Two groups of *Leishmania*-infected dogs, symptomless animals that, although seronegative or only mildly seropositive, provided positive PCR results for *Leishmania* in the skin and a group of clinically affected dogs that were highly seropositive and PCR-positive were compared. The muzzle skin of symptomless dogs had no demonstrable microscopic lesions or amastigotes. This, together with the positive PCR results for *Leishmania*, indicates that the number of parasites in skin samples from the muzzle must have been very low. The most severe lesions and the greatest parasite loads were located around hair follicles, mainly around the isthmus, associated with the middle vascular plexus of the dermis. This finding suggested hematogenous dissemination of the parasite and tropism for the skin (Solano-Gallego *et al.*, 2007). In conclusion, the results of this study cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniasis (Solano-Gallego *et al.*, 2007).

In another recent study (Brachelente *et al.*, 2005), the question of whether a correlation exists between the number of parasites, the histological response, and the expression of cytokines produced by CD4⁺ Th (Thelper)-2 and Th-1 lymphocytes in lesional skin of naturally infected dogs was assessed. To achieve this objective, the authors evaluated the mRNA expression of canine cytokines such as IL-4, IL-13, TNF- α , and IFN- γ by real-time RT-PCR (qRT-PCR) to determine the cellular immune response in lesional skin biopsies from naturally infected dogs. qPCR was used to determine the number of *Leishmania* in paraffin-embedded tissue sections, and this was compared with cytokine expression in tissue lesions. In *Leishmania*-infected dogs, IL-4, TNF- α , and IFN- γ mRNA production were significantly higher than in controls. Furthermore, dogs with a high *Leishmania* burden demonstrated significantly higher IL-4 expression, whereas no difference was noted with regard to expression of other cytokines. By comparing the pattern of inflammation and cytokine expression, a clear trend became evident, that levels of IL-4, TNF- α , and IFN- γ were elevated both in biopsies with a periadnexal nodular pattern and in biopsies where the severity of the periadnexal infiltrate was equivalent to that of the perivascular interstitial infiltrate. Expression of IL-4, IL-13, and TNF- α was slightly increased in biopsies in which plasma cells prevailed over lymphocytes, whereas expression of IFN- γ was moderately higher when lymphocytes were predominant. In summary, this study demonstrates that the local immune

response in naturally occurring leishmaniasis includes Th-1 as well as Th-2 cytokine subsets. Furthermore, the increased expression of the Th2-type cytokine IL-4 associated with both severe clinical signs and a high parasite burden in skin lesions connects severity of the disease to a Th-2-type of immune response (Brachelente *et al.*, 2005).

From Skin to other Organs

A variety of adhesion molecules are involved in phagocyte adherence to the extracellular matrix and cells of the connective tissue (Carlos and Harlan, 1994). Using adhesion blocking assays, our group observed that adherence of non-infected mononuclear phagocytes to the inflamed connective tissue is mediated by beta-1 and beta-2 integrins (Carvalho *et al.*, 2004). Flow cytometry experiments showed no consistent changes in the expression of several integrins on the surface of infected murine phagocytes (Carvalho *et al.*, 2004; Pinheiro *et al.*, 2006). These data suggest that infected and non-infected phagocytes expressed similar amounts of integrins, even though adherence of the former cells to the connective tissue was diminished (Carvalho *et al.*, 2004). CCR1 and CCR7 have been shown to be upregulated about two-fold compared to the control group (Steigerwald and Moll, 2005) and, after infection, there is a decrease in CCR4 and CCR5 expression on phagocytes infected with *L. amazonensis* (Pinheiro *et al.*, 2006). Another factor that may differentially interfere with the migration capability of infected cells is parasite burden. There is an inverse relationship between the degree of infection and the adhesive capability of infected cells. Although infection with small numbers of *Leishmania* does not reduce phagocyte adhesion to connective tissue, connective-tissue adhesion by infected phagocytic cells reaches levels of 20-30% that observed for non-infected cells under conditions of high parasite burden (Pinheiro *et al.*, 2006). These data are in accordance with evidence suggesting that heavily *Leishmania*-infected phagocytes present a wide spectrum of suppressive changes, including changes in B7 expression, impaired integrin function, and inhibition of the expression of a variety of genes in mononuclear phagocytes (Buates and Matlashewski, 2001).

We still have a long way to go to understand the mechanisms that control parasite dissemination in dogs. It appears, however, that animals exhibiting more strict control of parasite burden develop a more limited pattern of differential dissemination of infected cells. As recently shown by Reis and collaborators (Reis *et al.*, 2006), higher parasite burden is found in the skin and spleen than in the bone marrow, liver, and lymph nodes of infected healthy animals (Reis *et al.*, 2006). The parasite distribution tends to be more uniform among tissues of unhealthy animals, and parasitism tends to be more intense in the spleen of animals with more severe disease.

In some visceral leishmaniasis-endemic areas of Brazil, the skin of dogs is prone to be in an almost permanent inflammatory state, not always related to *Leishmania* infection. In a series based on the study of stray dogs from the streets of Jacobina (Bahia state, Brazil), 81% of the dogs without evidence of *Leishmania* infection had inflammatory infiltrates in the ear skin (dos-Santos *et al.*, 2004). Pucheu-Haston and collaborators (Pucheu-Haston *et al.*, 2006) showed that inflammation causes dog skin to function as a source of chemoattractants and favors phagocyte adherence (Carvalho *et al.*, 2004) to the connective tissue. This may, therefore, constitute an important incentive to infected phagocytes remaining or disseminating to the skin of dogs during different phases of visceral leishmaniasis. A pattern of parasite

dissemination to inflammatory sites has been confirmed in an experimental model of cutaneous leishmaniasis (Bertho *et al.*, 1994).

In fact, it is not well established how *Leishmania* parasites are transported from the original infection site in the skin to other organs. Transport of amastigotes by cutaneous Langerhans cells from the skin to the draining lymph nodes was demonstrated by Moll and collaborators (1993) in murine models of infection, and Fiorini and collaborators (2002) detected myeloid cells containing *Leishmania* in human blood (Fiorini *et al.*, 2002). In all lesions caused by *Leishmania*, the parasite is found inside mononuclear phagocytes, which maintain close contact with the extracellular matrix and cells of the connective tissue (Abreu-Silva *et al.*, 2004). In only a few instances have parasites been found free in the tissues (Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). These data suggest that the main interface between *Leishmania* and the host may be the mononuclear phagocyte cell surface. It also suggests that the relevant information for *Leishmania* to remain within or to leave tissues may be expressed on the surface of these phagocytes.

Another interesting aspect of cell migration that affects the tissue in leishmaniasis is related to the loss of lymphoid tissue structure in the spleen, as reported by some authors for visceral leishmaniasis. Such alterations have been described in human beings by Veress and collaborators in 1977 (Veress *et al.*, 1977), and have recently been re-examined in a number of important studies performed by the group led by Paul Kaye. These studies have shown that the observed changes in the lymphoid tissue of the spleen are due to impaired leukocyte migration into the white pulp induced by TNF (Engwerda *et al.*, 2002) and IL10 (Ato *et al.*, 2002). Basically, a number of interactions between lymphocytes and mononuclear phagocytes may be disrupted in the marginal zone of the spleen, disturbing the entry of cells into the white pulp and follicle organization (Ato *et al.*, 2002; Engwerda *et al.*, 2002). We recently observed a similar pattern of lymphoid tissue disorganization in the spleens of dogs with visceral leishmaniasis. Such changes were more prominent in animals with a susceptibility pattern in response to *L. chagasi* infection (negative leishmanin skin test and positive spleen culture for *Leishmania*) than in non-infected animals or in animals with a positive leishmanin skin test (Santana *et al.*, 2008). These alterations of the white pulp appear to be associated with the disappearance of a population of marginal zone macrophages defined by an H11 monoclonal antibody staining (Aguiar *et al.*, 2004). Whether this represents a cause or consequence of tissue disorganization remains unclear. Such a loss of lymphoid tissue structure may, however, underlie the increased susceptibility of these animals to bacterial infection and enhanced dissemination of *Leishmania* during late stages of the disease.

Recently, the cellular response in spleen was investigated (Lages *et al.*, 2008; Strauss-Ayali *et al.*, 2007). In both works, the mRNA expression levels for a wide panel of cytokines, transcription factors, and chemokines were examined. Both studies clearly show that Th1-1 and Th-2 immune responses occur simultaneously in the spleen during canine *L. infantum* infection (Lage *et al.*, 2007; Strauss-Ayali *et al.*, 2007). The frequency of IL-12 and IFN- γ expression within symptomatic dogs was significantly different from that of the uninfected group, although there were no significant differences between the symptomatic groups with respect to the expression of these cytokines (Lage *et al.*, 2007). In accordance, the other study identified higher IFN- γ , T-bet, IP-10, and RANTES mRNA levels in infected dogs during both oligosymptomatic and polysymptomatic stages of the disease (Strauss-Ayali *et al.*, 2007). These results agree with those reported by Quinnell and collaborators (Quinnell *et al.*, 2001b), who suggested that IFN- γ expression is not an appropriate indicator of resistance

since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues, as is the case in humans, mice, and hamsters (Lage *et al.*, 2007). On the other hand, the Th-2 immune response in dogs was differentially described by these works. In one study, positive correlations between the levels of IL-10 expression with respect to the progression of the disease were observed. The other authors identified increased IL-4 and IL-5 expression during oligosymptomatic disease instead of enhanced expression levels of the Treg (T regulatory)-associated cytokines, IL-10 and TGF- β (Strauss-Ayali *et al.*, 2007).

Blood Compartment

The results of the serological analysis of VL-infected dogs also identified mixed Th-1 and Th-2 responses in the serum of infected dogs, with detectable expression levels of IFN- γ , TNF- α , and IL-12 together with IL-4 and IL-10. However, when clinical indications are considered alongside the biochemical data, the Th-2 response appears to be predominant, since the expression of IL-4 increased within the symptomatic group while the expression of IL-12 increased within the asymptomatic group (Santos-Gomes *et al.*, 2002).

The nature of the dog's PBMC responses to *Leishmania* is not completely understood. Asymptomatic dogs show protective immunity, which has generally been associated with a strong proliferative response of peripheral blood lymphocytes to leishmanial antigens (Cabral *et al.*, 1992; Pinelli *et al.*, 1995; Pinelli *et al.*, 1994). However, development of a Th-1 and Th-2 mixed response by antigen-stimulated PBMCs from asymptomatic dogs expressing IL-2, IFN- γ , and IL-10 mRNA transcripts has also been reported. Although, in these studies, IL-2 and IFN- γ predominated in asymptomatic dogs, the development of symptomatic infections could not be related to IL-10 expression (Carvalho *et al.*, 1994; Chamizo *et al.*, 2005). Thus, in contrast to what occurs in human visceral leishmaniasis, the role played by PBMC-expressed IL-10 in *L. chagasi*-infected dogs is not well established (Carvalho *et al.*, 1994).

Dogs with symptomatic CVL (Berrahal *et al.*, 1996) present with depressed T cell-mediated functions and high levels of specific antibodies (Abranches *et al.*, 1991; Barbieri, 2006; Killick-Kendrick *et al.*, 1994; Oliva *et al.*, 2004; Santos-Gomes *et al.*, 2002). These animals present immunological changes involving T cells, including absence of delayed type hypersensitivity (DTH) to *Leishmania* antigens (Berrahal *et al.*, 1996; Oliva *et al.*, 2004; Quinnell *et al.*, 2001b), decreased T cell numbers in the peripheral blood (Cabral *et al.*, 1998; Killick-Kendrick *et al.*, 1994; Oliva *et al.*, 2004), and absence of IFN- γ and IL-2 production by PBMCs *in vitro* (Alvar *et al.*, 2004; Oliva *et al.*, 2004; Pinelli *et al.*, 1994). Interestingly, the Th-1 cytokine profile in bone marrow aspirates positively correlates with humoral, but not with lymphoproliferative responses to *Leishmania* antigen. It is noteworthy that increased accumulation of IL-4, IL-10, and IL-18 mRNA was not observed in infected dogs, and the mRNA for these cytokines did not correlate with antibody or proliferative responses. However, infected dogs with detectable IL-4 mRNA display significantly more severe symptoms (Quinnell *et al.*, 2001b). These data suggest that clinical symptoms are not due to a deficiency in IFN- γ production.

It is well established that early events are considered to be a determinant of infection outcome in humans and mice (Gomes *et al.*, 2000; Rogers and Titus, 2004; Shankar and Titus, 1993; Veras *et al.*, 2006). Prediction of dog immune responses *in vivo* early after

exposure to *L. chagasi* is a difficult task (human models). We have established an *in vitro* priming system (PIV) using naïve canine PBMCs in order to assess dog PIV immune response to *L. chagasi* (Rodrigues *et al.*, 2008). We co-cultivated PBMCs primarily stimulated with *L. chagasi in vitro* with autologous infected macrophages and found that IFN- γ mRNA is upregulated in these cells compared to control unstimulated cells. IL-4 and IL-10 mRNA expression in *L. chagasi*-stimulated PBMCs was similar to control unstimulated PBMCs when incubated with infected macrophages. Surprisingly, correlation studies showed that a lower IFN- γ /IL-4 expression ratio correlates with a lower percentage of infection. We proposed that the direct correlation between the IFN- γ /IL-4 ratio and parasite load is dependent on the positive correlation of both IFN- γ and IL-4 expression with lower parasite infection. This PIV system was shown to be useful in evaluating the dog immune response to *L. chagasi*, and the results indicate that a balanced expression of IFN- γ and IL-4 by these naïve cells is associated with control of parasite infection *in vitro* (Rodrigues *et al.*, 2008).

In experimental infections, intradermal inoculation of promastigotes triggers asymptomatic infections, and PBMCs from these dogs stimulated with soluble leishmanial antigens (SLA) *in vitro* express both Th-1 cytokines, such as IL-12, IFN- γ , TNF- α , and IL-18, and Th-2 cytokines, such as IL-4, IL-6, and IL-10. Despite the fact that PBMCs from these asymptomatic dogs present such apparently mixed Th-1 and Th-2 responses, they predominantly produce IL-12 and IFN- γ . In accordance with a previous observation (Pinelli *et al.*, 1994), these data support the protective immune response observed in these animals (Chamizo *et al.*, 2005). We recently observed that PBMCs from immunized dogs and than subcutaneously challenged with *L. chagasi* promastigotes are still asymptomatic. PBMCs from these apparently protected dogs liberate IFN- γ into the cell supernatant upon *L. chagasi* stimulation *in vitro* (Rodrigues *et al.*, 2007). Moreover these cells express IFN- γ but not IL-4 mRNA (Rodrigues *et al.*, 2007), showing that these dogs display a predominant Th-1 type of immune response. On the other hand, dogs experimentally infected by intravenous inoculation of amastigotes develop progressive symptomatic infections. PBMCs from these dogs produce reduced levels of both Th-1 and Th-2 cytokines (IFN- γ , IL-2, IL-12, IL-6, and IL-10) during the active phase of the disease (Santos-Gomes *et al.*, 2002).

In murine models, it is well established that macrophages participate in parasite killing via reactive oxygen and nitrogen intermediate-dependent mechanisms. However, mechanisms involved in Leishmania killing by canine macrophages have not been as thoroughly investigated. There are cumulative data implicating canine macrophages in parasite killing by a NO-dependent mechanism. NO produced by macrophages has been found to be the principal effector molecule responsible for mediating intracellular killing of Leishmania (Holzmuller *et al.*, 2006; Panaro *et al.*, 2001; Pinelli *et al.*, 2000). A canine macrophage cell line incubated with supernatant (containing IFN- γ , IL-2, and TNF- α) produced significant amounts of NO, sufficient to mediate *L. infantum*-killing (Pinelli *et al.*, 2000). PBMCs from vaccinated dogs were also able to reduce macrophage infection via an NO-dependent mechanism upon *in vitro* stimulation with both Leishmania promastigotes and concanavalin A (ConA). This effect was potentiated by the addition of LPS (Panaro *et al.*, 2001). Using a macrophage cell line, Pinelli and collaborators (Pinelli *et al.*, 2000) showed that parasite burden is reduced upon activation of cells with cytokine-rich supernatants. These supernatants were obtained from a Leishmania-specific T cell-line generated from dogs immunized with soluble Ag (Panaro *et al.*, 2001). Infected canine macrophages incubated with autologous

lymphocytes of immunized dogs also induced IFN- γ with increased NO production (Holzmuller *et al.*, 2005). The increased IFN- γ production and NO release by macrophages suggest a role for this cytokine in iNOS induction. We recently established an *in vitro* model to test whether PBMC supernatants from asymptomatic dogs immunized with promastigote lysates and infected with *L. chagasi* promastigotes were able to stimulate PBMC-derived macrophages from healthy dogs to control parasite infection (Rodrigues *et al.*, 2007). Using our system, we demonstrated for the first time that PBMCs from these asymptomatic dogs stimulated exclusively with *L. chagasi in vitro* reduce macrophage infection by the parasite (Rodrigues *et al.*, 2007). Moreover this effect is associated with high IFN- γ , but not IL-4, mRNA expression and release of this Th1 cytokine into the PBMC supernatant via an NO-dependent mechanism, as AMG reversed this effect (Rodrigues *et al.*, 2007). In contrast to other works (Panaro *et al.*, 2001), PBMCs in our system were exclusively stimulated with *L. chagasi in vitro*. Additionally, the protective response of these dogs to *L. chagasi* was demonstrated by the positive proliferative response to *Leishmania* antigens exhibited by PBMCs from these dogs *in vitro* (Rodrigues *et al.*, 2007). In addition, PBMCs from the majority of these immunized and experimentally infected dogs expressed IFN- γ mRNA and released IFN- γ upon LSA stimulation. These data suggest that lymphocytes from apparently protected dogs produce cytokines related to a protective immune response (Rodrigues *et al.*, 2007). A recent study demonstrated that, although Th-1 and Th-2 cytokines are produced in asymptomatic *Leishmania*-infected dogs, there is a prevalent Th-1 cytokine response that confers immunity against the parasite (Chamizo *et al.*, 2005). Finally, our data reinforce the notion that the leishmanicidal effect of canine macrophages is NO-dependent (Rodrigues *et al.*, 2007).

It was recently demonstrated that there are some differences in iNOS expression in lesion macrophages *in situ*. iNOS-negative dermal and splenic macrophages contain numerous *Leishmania* amastigotes. In contrast, dermal and splenic macrophages, which present high iNOS expression, contain few or no amastigotes, suggesting that iNOS-positive activated macrophages are able to destroy and/or do not allow multiplication of intracellular amastigotes (Zafra *et al.*, 2008). PBMC-derived macrophages infected with *L. infantum* produce a significantly higher amount of NO than uninfected macrophages *in vitro* (Panaro *et al.*, 2008; Rodrigues *et al.*, 2007). In a comparison between infected dogs, the levels of NO in supernatants of *Leishmania*-infected macrophages were significantly higher in symptomatic than in asymptomatic animals. However, four months after diagnosis, the addition of autologous lymphocytes significantly decreased NO production only in symptomatic dogs, while NO production by macrophages co-cultured with autologous lymphocytes was significantly reduced eight months after diagnosis in *Leishmania*-infected macrophages from both asymptomatic and symptomatic dogs (Panaro *et al.*, 2008). These higher levels of NO observed during follow-up of symptom-free (only 8 months) animals may suggest a protective role for this molecule in long-term asymptomatic parasitism.

CONCLUSION

The nature of the dog's cellular immune response is not completely understood. Evidence points that although CVL dogs develop a mixed Th-1 and Th-2 cellular immune response,

asymptomatic dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection, as well as predominance of Th-1 cytokines. On the other hand, as the disease progresses in susceptible dogs, the protective responses diminish with involvement of either IL-4 or IL-10 in uncontrolled infection. Disease progression occurs together with parasite dissemination. *Leishmania*-infected mononuclear phagocyte may stay or leave inflammatory sites disseminating the parasite through the host tissues. The migration of these cells depends upon the leukocyte phenotype and is modulated by parasite burden that results in changes in integrin function and in the expression of chemokine receptors.

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Chapter V

EPIDEMIOLOGIC ASPECTS OF BOVINE NEOSPOROSIS IN HIGH-PRODUCING DAIRY HERDS IN NORTH-EASTERN SPAIN (REVIEW)

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ABSTRACT

Since the first identification in dogs in 1984, the intracellular protozoan parasite *Neospora caninum* has been found to infect a wide range of animals worldwide. Cattle, horses, goat, sheep, and deer have been described as intermediate hosts, and dogs and coyotes, and possibly red foxes, as definitive hosts. In cattle, *N. caninum* has emerged as an important cause of abortion. Reduction in milk yield, increased culling rate, decreased growth rate in calves, and birth of calves with congenital abnormalities have also been related to *Neospora*-infection in dairy herds. Neosporosis can persist for a long time in a herd since the parasite can be transmitted transplacentally. The efficiency of vertical transmission has been estimated to be as high as 95%. In Spain, the mean seroprevalence registered for *N. caninum* was 32.5% for individual cows and 83.2% for herds. We revise here several studies on epidemiological aspects of neosporosis performed in high-producing dairy herds in North-eastern Spain. Points to be highlighted should include: that the irrespective of the herd level of *Neospora*-seroprevalence, plasma antibody titration against *N. Caninum*, is a good indicator of risk of abortion; *Neospora*-infection does not affect fertility nor compromises the subsequent maintenance of gestation during the first trimester of gestation; the use of beef semen, especially derived from the Limousin bulls, reduces dramatically the incidence of abortions; and that *Neospora*-

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seropositivity can be very stable throughout years so that chronically-infected cows can show a high rate of repeat abortions.

INTRODUCTION

Neospora caninum is an obligate intracellular protozoan closely related to *Toxoplasma gondii* that was first described in dogs in 1984 (Bjerkas et al., 1984) and identified since then in a wide range of warm-blooded animals (Dubey et al., 2007). Before 1988, when Dubey et al (1988) described a new genus and species, *Neospora caninum*, this parasite was probably misdiagnosed with *T. gondii* (Dubey et al, 1988). Since then, bovine neosporosis has emerged as a disease of international concern, being a significant cause of abortion in cattle in many countries (reviewed Anderson et al., 2000; Dubey et al., 2007).

Neospora caninum infection has been reported worldwide. Among the European countries included in a recent supranational comparison of *N. caninum* seroprevalence in cattle (Germany, The Netherlands, Spain, and Sweden), Spain had the highest animal true prevalence for dairy cattle (16.2%), with more than 10% of the herds showing within-herd prevalence between 50 and 100% (Bartels et al., 2006). Herd prevalence was 63% and 46% for dairy herds and beef herds, respectively (Bartels et al., 2006). More importantly, in Spain, depending on the diagnostic criteria, between 32% and 57% of the bovine foetuses analysed were considered to be infected by *N. caninum* (Gonzalez et al., 1999; Mainar-Jaime et al., 1999; Pereira-Bueno et al., 2003).

We here review epidemiologic aspects and prospects for the control of neosporosis in cattle based on our studies on dairy cattle in North-east Spain, where seropositive cows had 12-19 times more risk of abortion than seronegative dairy cows, with abortion occurring from 30% to 44% of seropositive cows (López-Gatius et al., 2004a,b).

LIFE CYCLE AND TRANSMISSION OF THE PARASITE

Neospora caninum has a wide host range (including goats, sheep, water buffalo, white-tailed deer, and other wild animals), but it is primarily a disease of cattle and dogs (For more detailed information on host range and geographic distribution, see Dubey et al., 2007). Serological evidence in multiple species, including domestic, wildlife, and zoo animals provides evidence that many species have been exposed to this parasite. Another species, *N. hughesi*, has been described from horses (Marsh *et al* 1998). Although there is a concern about the zoonotic potential of *N. caninum*, as of yet, there is no firm evidence that *N. caninum* successfully infects humans (reviewed Dubey et al., 2007).

In the life cycle of *N. Caninum*, there are three known infectious stages: tachyzoites and bradizoites in tissue cysts are found intracellular in the intermediate hosts (IH) and oocysts are excreted by the definitive hosts (DH). Bradizoites inside tissue cysts are primarily found in the central nervous system of IH, but has also been described in cattle muscle (Peters et al, 2001).

Neospora caninum can be transmitted post-natally (also named as exogenous transplacental transmission (Trees and Williams, 2005)) by ingestion of tissues infected with tachyzoites or tissue cysts, by ingestion of food or drinking water contaminated with

sporulated oocysts, or transplacentally (vertically, congenitally, or endogenous transplacental transmission), when tachyzoites are transmitted from an infected dam to her fetus during pregnancy. The ingestion of oocysts is the only demonstrated mode for horizontal transmission in herbivores (Dubey et al., 2007). To date, cow-to-cow transmission of *N. caninum* has not been observed. Neonatal calves may become infected after ingestion of milk contaminated with tachyzoites (Davison et al, 2001), and *N. caninum*-DNA in milk, including colostrum, has been demonstrated (Moskwa et al, 2003, Moskwa et al., 2007). However, there is no conclusive evidence that lactogenic transmission of *N. caninum* occurs in nature (Dijkstra et al, 2001). There is no evidence that venereal transmission can occur, although the presence of the parasite in semen has been demonstrated (Ortega-Mora et al, 2003; Ferre et al, 2005).

POSSIBILITY OF SYLVATIC CYCLE IN SPAIN

Many aspects of the life cycle of *N. caninum* are still unknown and the role of wildlife in the life cycle of *N. caninum* is still uncertain. The dog and the coyote (*Canis latrans*) are the only proven definitive hosts (DH) known to excrete the environmentally-resistant oocysts (McAllister et al., 1998; Gondim et al., 2004a). In dogs, oocysts are excreted in an unsporulated stage (McAllister et al, 1998; Lindsay et al, 1999) and sporulate outside the host in as few as 24 hours (Lindsay et al, 1999). Recently, *N. caninum*-like oocysts were found in the feces of free-ranging red foxes (*Vulpes vulpes*) in Canada (Wapenaar et al., 2006), and wolves (*Canis lupus*) are suspected to be a DH for *N. caninum* (Gondim et al., 2004a). In North America, there is data consistent with a sylvatic transmission cycle of *N. caninum* between cervids and canids, and a sylvatic cycle involving the white tailed-deer and canids (Gondim et al., 2004b, Rosypal and Lindsay, 2005). White-tailed deer have shown elevated seroprevalence of infection and viable *N. caninum* has been isolated from this host (Gondim et al., 2004b; Vianna et al., 2005), and, more importantly, dogs shed *N. caninum* oocysts after being fed with the brain of naturally-exposed white tailed-deer (Gondim et al., 2004b).

A similar sylvatic cycle among deer and wild canids in Europe is still unknown. Recently, we have reported presence of antibodies to *N. caninum* in non-carnivore wildlife including red deer (*Cervus elaphus*), barbary sheep (*Ammotragus lervia*), roe deer (*Capreolus capreolus*), and occasionally in wild boar (*Sus scrofa*) in Spain (Almería et al., 2007). In Europe, presence of antibodies in red deer, roe deer, and alpine chamois has also been reported in the Italian Alps (Ferroglia and Rossi, 2001), and in the Czech Republic in roe deer, red deer, sika deer (*Cervus nippon*), fallow deer (*Dama dama*), mouflon (*Ovis musimon*) (Bartova et al., 2007), and in wild boars (Bartova et al., 2006) in higher prevalence than our study (18.1%). We did not detect antibodies in rabbits, while low prevalence was observed in hares imported in Italy from East Europe (Ezio Ferroglia and Anna Trisciuglio, 2003)

A further study in wild carnivorous species has shown antibodies in red foxes (*Vulpes vulpes*), wolves (*Canis lupus*), Iberian lynx (*Lynx pardinus*), European wildcats (*Felis silvestris*), Eurasian badgers (*Meles meles*), stone martens (*Martes foina*), pine martens (*M. martes*), and polecats (*Mustela putorius*) in Spain (Sobrino et al., 2008). Red foxes are the main wild canid species in Europe where coyotes are not found, and some studies have considered the presence of foxes a risk factor for transmission of *N. caninum* to cattle

(Simpson et al., 1997; Barling et al., 2000). Although, presence of oocysts has been observed in naturally-infected red foxes in Canada (Wapenaar et al., 2006), to date, red foxes have not been proven to be a definitive host of the parasite by experimental inoculation (Schaes et al., 2002). In Europe, reports of antibodies in red foxes are very numerous (reviewed Sobrino et al., 2008) and *N. caninum*-DNA has been demonstrated in the brains of red foxes in Catalonia, North-East Spain (Almería et al., 2002), and the Czech Republic (Hurková and Modrý, 2006). In particular, a recent study in an area of Catalonia showed very high seroprevalence in red foxes (Marco et al., 2008). The prevalence levels of *N. caninum* antibodies in wildlife species in our studies were related to the geographical areas analyzed (Almería et al., 2007; Sobrino et al., 2008). These results indicate that in Spain, *N. caninum* infection in wild carnivores and red deer is localized to certain areas, where, on the other hand, it can be present in moderate to high prevalence (Almería et al., 2007; Sobrino et al., 2008). These results could have important implications in both sylvatic and domestic cycles, since sylvatic cycles may influence the prevalence of infection in cattle farms in the same areas.

SEROLOGIC AND DIAGNOSTIC PROCEDURES AT FARM LEVEL

1. Pregnancy Diagnoses

In the farms under our surveillance, pregnancy is diagnosed by trans-rectal ultrasonography on day 40 post-insemination and by palpation per rectum on days 90, 120, 150, 180, and 210, or until abortion. Abortion is recorded in the case of a negative pregnancy diagnosis after day 90, or when signs of abortion were observed. The outcome of pregnancy is recorded for all non-aborting animals. Animals are observed daily for signs of abortion from 120 days of gestation.

2. Serology

Blood samples are collected from each animal immediately before each pregnancy diagnosis or until the time of abortion detection. These blood samples are centrifuged (10 min, 1600 x g) and the sera or plasma store at -20°C until further analysis.

For serologic analysis of *N. caninum* in cows, a great variety of assays are available such as indirect fluorescent antibody test (IFAT), *Neospora* agglutination test (NAT), enzyme-linked immunosorbent assay (ELISA), or immunoblotting, among others. Of those, the ELISA test is very commonly used at herd level. It can be performed on sera, plasma or milk samples. An avidity-ELISA has the advantage of distinction between acute and latent infections (Björkman et al., 1999). In our lab we compared two commercial ELISA techniques for *N. caninum* antibody analysis in cattle (López-Gatius et al., 2004b). Blood samples were collected from 3,400 animals from 6 herds. Whole herds were sampled, with the exception of calves less than 6 months of age to avoid colostral antibodies. Sera were tested for specific detection of antibodies to *N. caninum*, using two commercial ELISA kits based on the whole tachyzoite lysate of *Neospora* NC-1, according to the instructions of the manufacturers (Herd

check® anti-*Neospora*, IDEXX laboratories, Madrid, Spain; CIVTEST® anti-*Neospora*; Hipra, Girona, Spain). An animal was considered to be seropositive when at least one of the two tests denoted seropositivity. Analysis of agreement between both ELISA techniques showed a Kappa value of 0.94 (0.90-0.96), indicating a very high level of agreement between both tests when a value of ≥ 6.0 units is taken as the threshold for seropositivity for CIVTEST. This kit has also been compared to other ELISA kits in a recent European study and found to have good sensitivity and specificity (Bartels et al., 2006), and it is the one we currently use in our studies.

We use mean *N. caninum* antibody titres tested by ELISA on days 40, 90, and 120 of gestation to classify the cows as showing high (≥ 30 units) or low (< 30 units) titration (Bech-Sabat et al., 2006).

3. Analysis of Aborted Fetuses

In bovine fetuses, multifocal areas of necrosis surrounded by inflammatory cells are commonly observed in the brain, spinal cord, heart, lung, and placenta (Barr et al., 1991). The most common observation is multifocal non-suppurative encephalitis and myocarditis (Barr et al., 1991; Wouda et al., 1997). Confirmatory diagnosis of *N. caninum* infection relies on immunohistochemical staining of tissue sections using antibodies specific for the parasite (Lindsay and Dubey, 1989) and on demonstrating *N. caninum*-specific DNA by PCR (Baszler et al., 1999). Given the low sensitivity of immunohistochemical staining, several researchers have applied PCR to amplify *N. caninum* sequences from DNA extracted from fresh, frozen, formalin-fixed, or paraffin-embedded foetal tissues (Baszler et al., 1999). *N. caninum* has not yet been identified in stained histological sections of tissues of cattle older than 2 months. Therefore, specificity of *N. caninum*-associated lesions has not been verified (Dubey et al., 2007).

In the farms under our surveillance, when possible, aborted fetuses are submitted for laboratory analyses. We look for evidence of protozoal lesions in hematoxylin-eosin stained tissues consistent with multifocal necrosis associated with mononuclear inflammation. The presence of *N. caninum* is established by a specific immunohistochemical procedure (Lindsay and Dubey, 1989) and specific Nc5 PCR performed in brain tissue (Liddell et al., 1999; Almería et al., 2002). Briefly, half the brain is fixed in 10% neutral buffered formalin and the remaining unpreserved brain tissue stored at -20°C for DNA analysis. Paraffin-embedded histological sections are prepared and examined microscopically after staining with hematoxylin and eosin. For immunohistochemical staining, paraffin sections react with anti-*N. caninum* polyclonal antibodies. When the submitted fetuses have undergone autolysis and lesions cannot be examined, specific PCR analysis is always performed.

The presence of *N. caninum*-DNA in an aborted fetus is, however, not necessarily an indication of *N. caninum* as the cause of abortion and epidemiologic data about the herd need to be taken into account to reach that conclusion.

ENDOGENOUS VERTICAL TRANSMISSION IS THE MAIN TRANSMISSION OF *N. CANINUM* IN DAIRY HERDS IN NORTH-EASTERN SPAIN

Neospora caninum is one of the most efficiently, transplacentally transmitted organisms in cattle (Dubey et al., 2007). Up to 95% of calves can be born infected (Paré et al., 1996; Schares et al., 1998; Davison et al., 1999a; Moen et al., 1998). The majority of calves born from infected mothers are clinically normal, but they are infected for life, and a seropositive cow is more likely to abort than is a seronegative cow (Davison et al., 1999a; Hietala and Thurmond, 1999; Moen et al., 1998). In our geographical area of study in North-east Spain, the risk of abortion is 12-19 times greater in *Neospora*-seropositive dairy cows than in their seronegative counterparts (López-Gatius et al., 2004a, López-Gatius et al., 2004b). In addition, *N. caninum* can cause repeated abortion in cattle (Anderson et al., 1995; Williams et al., 2003; Pabón et al., 2007).

In a retrospective study performed on a *Neospora*-associated abortion episode taken place over a 12-month period in a dairy herd in north-east Spain (López-Gatius et al., 2004a), the overall abortion rate for the herd was 23.2% (38 abortions of 164 diagnosed pregnancies). Antibodies to *N. caninum* were found in 35.4% of the cattle (84 seropositive animals out of 237) and 44% of seropositive pregnant animals aborted over the 1 year period. The presence of *N. caninum* antibodies in the animals was the only variable included in the final logistic regression model for abortion. *Neospora caninum* infection increased the probability of abortion by an odds ratio of 12.2 ($P < 0.0001$). Parity, season of pregnancy, and common risk factors associated with pregnancy loss in the geographical area of the study had no effect on this episode of abortion.

Age-related differences in *N. caninum* seroprevalence were not statistically significant, indicating vertical transmission as the main route of infection. Indeed, a high percentage of congenitally-infected offspring was observed (90.6%). A strong association was noted between the serostatus of dams and their progeny. Out of 32 seropositive cows with descendants in the herd at the time of sampling, 29 seropositive cows had 40 seropositive descendants and no seronegative offspring, while the remaining 3 seropositive cows had 4 seronegative calves. Thus, 90.6% of the seropositive dams had seropositive offspring and 90.9% of the calves born to the 32 seropositive dams were seropositive. Of note, was the birth of 2 seropositive offspring to 2 seronegative dams that may indicate a small degree of horizontal transmission in the herd. The farm had been free of dogs for the last 7 years, but contact with wild canids, such as red foxes, was possible. In a subsequent study (López-Gatius et al., 2004b) based on analysis in 6 herds, a very low incidence of seroconversion and horizontal transmission occurring in the herds during the study period was also observed.

In our area, *N. caninum*-associated abortions have shown an endemic pattern, since the abortions persists in the herds for several months or years probably related to the vertical transmission. In addition, avidity-ELISA results for animals undergoing seroconversion in another study indicated that most of these animals were chronically-infected (Pabón et al., 2007). In several studies, a low rate of seroconversion in endemically-infected herds has been observed, suggesting a low level of horizontal infection (Paré et al., 1996; Paré et al., 1997; Schares et al., 1998; Wouda et al., 1998; Davison et al., 1999a; Hietala and Thurmond, 1999;

López-Gatius et al., 2004a). In some of those animals, antibody fluctuations in *Neospora* titres could account for the seroconversions detected.

It has to be taken into account that postnatal transmission, although low, is necessary for the persistence of *N. caninum* infection in the herds since vertical transmission is not 100% effective.

NEOSPORA CANINUM INFECTION IN ANIMALS CHRONICALLY-INFECTED PRIOR TO PREGNANCY EXERT A SIGNIFICANT ABORTIFACIENT EFFECT AFTER 90 DAYS OF GESTATION BUT NOT BEFORE

Abortion is the main clinical manifestation of bovine neosporosis in both dairy and beef cattle. Cows of any age may abort from 3 months of gestation to term (Anderson et al, 1991; Barr et al, 1991; Otter et al, 1995; Wouda et al, 1997; Hattel et al 1998), with most abortions occurring at 5-7 months of gestation (Anderson et al, 1991; Wouda et al, 1997). Fetuses may die *in utero* or be reabsorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected (reviewed by Dubey, 2003). *Neospora* infection has also been associated with increased culling and reduced milk yield, and it is responsible for a reduction in the value of female breeding cattle (reviewed by Trees et al., 1999).

In farm conditions, early *N. caninum* abortion may be missed by the farmer, and, therefore, not evaluated by the diagnostic laboratories. Hence, the effects of *N. caninum* infection in cattle during the first trimester of pregnancy are not yet clearly understood.

In a retrospective study mentioned above (López-Gatius et al., 2004a), the risk of abortion was significantly higher during the second term of gestation ($P<0.01$) than during the first and third terms. To further analyze the effect of *N. caninum* abortion before and after 3 months of gestation, *N. caninum* antibodies were analyzed in 2,773 pregnant animals (2,022 parous cows and 751 heifers) from 6 herds. The mean seroprevalence of antibodies to *N. caninum* in the herds was 15.1% ($n=419$). One hundred eighty-three abortions (6.6% of total pregnancies) were recorded from gestation day 34 to the 90th day of pregnancy (23 in *Neospora* positive animals), and 146 abortions (5.3%) were registered after 90 days of pregnancy, 126 during the second and 20 during the third trimester of pregnancy (105 in *Neospora*-positive animals). Logistic-regression analyses were performed and no significant effects of *Neospora* positivity and herd were found on abortion rate before 90 days of pregnancy, while significant effects of season and parity were observed on abortion rate at that time (Table 2). Based on the odds ratio, the abortion rate was 4 times higher ($P<0.0001$) in animals that became pregnant in the warm than in the cool period, and 3.7 times higher ($P<0.0001$) in parous than in non-parous animals. On the other hand, *Neospora* positivity was the only variable included in the logistic-regression model for abortions suffered after 90 days of pregnancy. Seropositivity in an animal increased the probability of abortion by an odds ratio of 18.9 ($P<0.0001$; 95% confidence interval 12.9 to 27.8). Season, parity, and herd showed no effect (Table 1). The results of the present study suggest that chronic *Neospora*

caninum infections prior to pregnancy are not associated with abortion during the early fetal period, but they exert a significant abortifacient effect after 90 days of pregnancy.

Table 1. Odds ratios of variables included in the final logistic-regression models for abortions

Abortion before gestation Day 90^a

Factor	Class	N	Odds ratio	95%	Confidence Interval	P
Season	0	1648				
	1	1125	4	2.9	5.6	<0.0001
Parity	0	751				
	1	2022	3.7	2.3	6.1	<0.0001
Intercept			0.011			<0.0001

Abortion after gestation day 90^b

Factor	Class	N	Odds ratio	95%	Confidence Interval	P
Seropositivity	0	2194				
	1	396	18.9	12.9	27.8	<0.0001
Intercept			0.019			<0.0001

^a Likelihood ratio test = 1237.5; 2df, P<0.0001;

Hosmer and Lemeshow Goodness-of-fit test = 1.75; 2 df, P = 0.42 (the model fits).

^b Likelihood ratio test = 865.6, 1df, P<0.0001.

(From López-Gatius et al., 2004b, used with permission from Elsevier)

Other epidemiological studies have similarly observed no indication that *N. caninum* is able to cause early pregnancy losses (Bjorkman et al., 1996; Jensen et al., 1999; López-Gatius et al., 2005a; Romero et al., 2003). However, some studies, 4 of them in beef cattle in Canada, have indicated that *N. caninum* is not only associated with abortion but also with early pregnancy losses (Muñoz-Zanzi et al., 2004; Waldner, 2005; Waldner et al., 1998; Waldner et al., 2001), and cattle experimentally-infected at 70 days post-insemination with high doses of *N. caninum* tachyzoites are more susceptible to abortion than those infected with the same doses at day 140 or 210 of insemination (Williams et al., 2000). These early losses are probably due to first contact with the parasite in cows whose fetuses are immunologically immature and more susceptible to abortion early in gestation, while when cows are congenitally-infected prior to gestation, our results show no indication that *N. caninum* causes early pregnancy losses.

During the first third of pregnancy, the fetus is particularly vulnerable, when the thymus, spleen, and peripheral lymph nodes are forming, but these tissues start to recognize and respond to microorganisms in the middle third of pregnancy (Osburn, 1986). Thus, in the first trimester, the fetus is much more vulnerable to *N. caninum* infection and is unlikely to survive. In the middle third of pregnancy, fetuses may be able to mount an immune response to *N. caninum* infection (Andrianarivo et al., 2001; Almería et al., 2003; Innes et al., 2005).

ANNUAL SEROLOGICAL SCREENING FOR *N. CANINUM* CAN BE AN EFFECTIVE AND RAPID METHOD OF DETECTING *N. CANINUM* INFECTION AND RELATED RISK OF ABORTION

As mentioned above, a strong association between *N. caninum* seropositivity and abortion has been observed in our studies (López-Gatius et al., 2004a, b), and similar results have been reported in numerous studies worldwide (Anderson et al., 1995; Corbellini et al., 2006a; Davison et al., 1999b; Schares et al., 2002; Moore et al., 2003; Waldner, 2005; Weston et al., 2005), among others. This increase in risk of abortion among seropositive cows indicates a causal link between natural *N. caninum* infection and abortion in the studied herds, where *N. caninum* was confirmed in aborted fetuses. Maternal antibody levels during gestation have been shown to predict congenital infection of calves and abortion of fetuses of infected cows (Paré et al., 1997). Interestingly, in herds with different seroprevalence (4-28%), the percentages of seropositive animals that aborted for the different herds remained similar (approximately 30%) (Table 2) (López-Gatius et al., 2005a).

When titration was considered, the *N. caninum* antibody titre showed a significant relationship with abortion odd ratios 1.01 in abortion risk ($p=0.004$) (Lopez Gatius et al., 2005b), but only if the data of parous cows was considered. In agreement with this finding, in a recent study (Yániz et al., 2009), the likelihood of abortion was 3.2 times lower for pregnancies of parous cows with low antibody titres against *Neospora caninum* (6-29 units) as compared to those with high antibody titres (≥ 30 units), whereas in heifers this variable had no effect (Yániz et al., 2009).

Table 2. *Neospora caninum* seropositivity and abortion rates in three dairy herds over a 2-year period^a

Herd	n	Seropositive (%)	Seropositive aborting animals (%)	Seronegative aborting animals (%)
A	180	47 (26) ^b	18/64 (28) ^c	4/182 (2) ^d
B	440	123 (28) ^b	60/192 (31) ^c	21/646 (3) ^d
C	1350	60 (4) ^b	19/58 (33) ^c	68/1398 (5) ^d
Total	1970	230 (12) ^b	97/314 (30) ^c	93/2226 (4) ^d

^aBased on yearly serological screening for neosporosis and on the confirmation of *N. caninum* infection in aborted foetuses;

^bWith respect to the total number of animals;

^cWith respect to the total number of pregnancies in seropositive animals;

^dWith respect to the total number of pregnancies in seronegative animals;

The abortion rates (c versus d) for all three herds were different ($P<0.0001$) when compared in a 2x2 contingency table using the Chi-square test.

(Copyright (2005) Wiley. Used with permission from López-Gatius et al 2005a. *Neospora caninum* infection does not affect the fertility of dairy cows in herds with high incidence of *Neospora*-associated abortions. J. Vet. Med. B., Wiley Publishers).

In recent studies (Yániz et al., 2009; Almería et al., submitted), an important finding was that *N. caninum*-associated abortion rates were dependant on the antibody titration.

Significantly lower abortion rates were observed if the seropositive AI dams had low antibody titres. Several studies have related increased risk of abortion with increased antibody titres (Kashiwazaki et al., 2004, López-Gatius et al., 2005b, Waldner, 2005), as well as with the occurrence of lesions in aborted fetuses (De Meerschman et al. 2002). These results indicate that in an increased incidence of abortions due to neosporosis in dairy cattle, maternal serology, including titration levels, can be a good indicator of abortion risk (López-Gatius et al., 2005b), and our results support the proposal by Quintanilla-Gozalo et al. (2000) that the antibody titre (and not only seropositivity) could be used as a cost-effective predictive tool to identify animals carrying a high risk of abortion in herds with a high seroprevalence for *Neospora*.

Currently, in the herds under our surveillance, if seroprevalence is higher than 20%, annual samples are collected from the whole herd, with the exception of calves less than 6 months of age to avoid colostral antibodies. Usually, this sampling coincides with the annual herd check-up for Brucellosis and other disease control. In herds with low seroprevalence, animals older than 6 months not previously analyzed are sampled also annually.

NEOSPORA SEROPOSITIVITY CAN BE VERY STABLE THROUGH TIME

When seroprevalence levels were analyzed during 3 years in 122 animals that remained in a herd with high *Neospora*-seroprevalence in our area of study (Pabón et al., 2007), we could observe that *Neospora* seropositivity can be very stable through time. Initial seroprevalence of *Neospora* in the 122 animals was 18% the first year, seroconversion only occurred in 4 animals during the second and third years of the study and seroprevalence increased to 21.3% in the second and third years (Pabón et al., 2007). *N. caninum*-associated abortion occurred only in seropositive individuals. Similarly, Hasler et al. (2006a) observed that only 2 out of 30 seropositive animals and 1 out of 83 seronegative animals changed their serological status during pregnancy, indicating minor transient instability of anti-*N. caninum* antibody reactivity in adult cattle. These results reinforce the idea that annual serological screening for *Neospora* can be an effective and rapid method of detecting *N. caninum* infection, such that control measures can be established at the farm level.

REPEATED ABORTION RATES IN N. CANINUM-INFECTED COWS

A chronically *N. caninum*-infected cow passes the infection to the fetus in successive pregnancies and may experience repeated abortions over her lifetime (Barr et al., 1993; Anderson et al., 1995). Anderson et al. (1995) concluded that a low proportion of cows (<5%) may have repeat abortions due to *Neospora caninum*. However, it has been observed that cattle repeatedly transmit the infection to their offspring and the re-abortion rate is similar to the abortion rate (Barr et al., 1993; Guy et al., 2001). In our study in a dairy herd with high seroprevalence of abortion analyzed during 3 consecutive years (31.7%), a high repeat

abortion rate in *Neospora*-seropositive chronically-infected dairy cows was observed. In fact, with respect to the total number of seropositive animals with a previous history of abortion, 36.8% of abortions were recorded in the whole herd and 26.7% of abortions were registered in animals remaining in the herd over the three-year study period (Table 3). Thurmond and Hietala (1997) showed that *N. Caninum* congenitally-infected cows that had aborted previously carried a higher risk of subsequent abortion compared with congenitally-infected cows that had not aborted previously. Similarly, Corbellini et al. (2006b) recently observed that cows with a history of previous abortion were more likely (2.4 times) to abort a *N. caninum*-infected fetus than cows with no prior history of abortion. These results indicate that *N. caninum*-infected cows that abort are not able to develop adequate protective immunity (Thurmond and Hietala, 1997; Corbellini et al., 2006b), and that the immunity provided by an initial infection is inadequate to prevent repeated abortion in mature cows. The elimination of cows undergoing two or more abortions could further reduce the abortion rate in the herd.

Table 3. Seroprevalence of *N. caninum* infection and abortion and repeat abortion rates recorded in a dairy herd over three consecutive years

Year	Number of animals	Seropositive animals (%)	Total no. of pregnancies (seropositive) ^a	Total no. of abortions (%)	Abortions in seropositive animals (%) ^b (%) ^c	Seropositive animals with a previous history of abortion	Repeat abortions in seropositive animals (%) ^d (%) ^e
1	259	82 (31.7)	165 (65)	34 (20.6)	31 (47.7) (91.2)	35	18 (58.1) (51.4)
2	222	55 (24.8)	128 (32)	7 (5.5)	7 (21.9) (100)	23	4 (57.1) (17.4)
3	231	46 (19.9)	121 (27)	12 (9.9)	11 (40.7) (91.7)	10	3 (27.3) (30)
Total	712	183 (25.7)	414 (124)	53 (12.8)	49 (39.5) (92.5)	68	25 (51) (36.8)

^aTotal number of seropositive pregnant animals;

^bWith respect to the total number of seropositive pregnant animals;

^cWith respect to the total number of abortions;

^dWith respect to the total number of abortions in seropositive animals;

^eWith respect to the total number of animals with a previous history of abortion.

(From Pabón et al., 2007, used with permission from Elsevier)

ANTIBODY DYNAMICS IN DAIRY CATTLE CHRONICALLY-INFECTED WITH *N. CANINUM*

We examined the dynamics of antibody production against *Neospora caninum* during the gestation period in 86 pregnant parous chronically-infected dairy cows, 21 of which had suffered abortion (Nogareda et al., 2007). The cows belonged to two herds in which a diagnosis of *N. caninum* infection had been previously confirmed in aborted fetuses. The non-aborting cows were divided into two groups according to whether their antibody values in the

second half of gestation had increased or not, while aborting cows were classified as those showing an antibody peak before abortion or those not showing a pre-abortion peak. Differences in antibody values throughout pregnancy in each group of non-aborting and aborting cows were analyzed by GLM repeated measures of analysis of variance. While 32 non-aborting cows (49%) showed a significant and consistent increase in anti-*Neospora* antibody values during the second half of gestation, antibody values in the remaining 33 non-aborting cows were practically constant throughout gestation (Figure 1). An antibody peak around abortion was observed in 11 aborting cows (52%), while antibody values in the remaining 10 aborting cows were similar before and at abortion (Figure 2). Seroprevalence fluctuations, defined as seronegative blood samples at some point during the gestation period, were, furthermore, observed in 2 aborting and 11 non-aborting cows. Our results indicate two clearly distinguishable types of humoral immune dynamics throughout gestation: an increased or flat production of antibodies during the second half of gestation in non-aborting animals and before abortion in aborting cows. The observation that some *Neospora*-infected dams can exhibit negative antibody values at any time during gestation, particularly at parturition or abortion, is of importance from a diagnostic point of view (Nogareda et al., 2007).

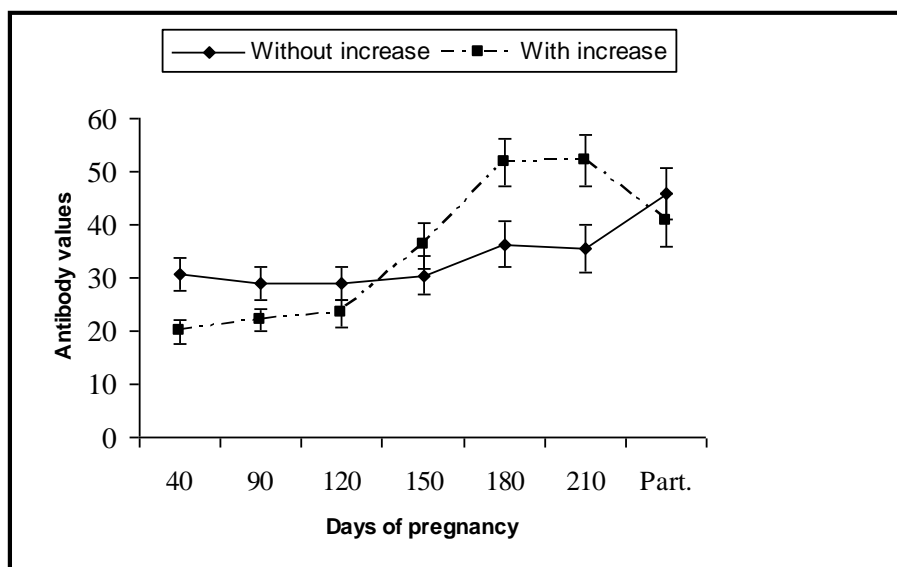


Figure 1. Mean (units \pm SEM) plasma anti-*N. caninum* antibody values recorded from Day 40 of gestation to parturition in non-aborting cows showing (n = 32) or not showing (n = 33) an antibody value increase during the second half of the gestation period. (From Nogareda et al., 2007, used with permission from Elsevier)

In our studies, some dams that aborted showed maximum antibody titres just before the abortion took place. The consistent pattern of rise in antibody titres observed during pregnancy in most of the cows indicated a re-activation rather than a re-infection of the parasite at mid-gestation.

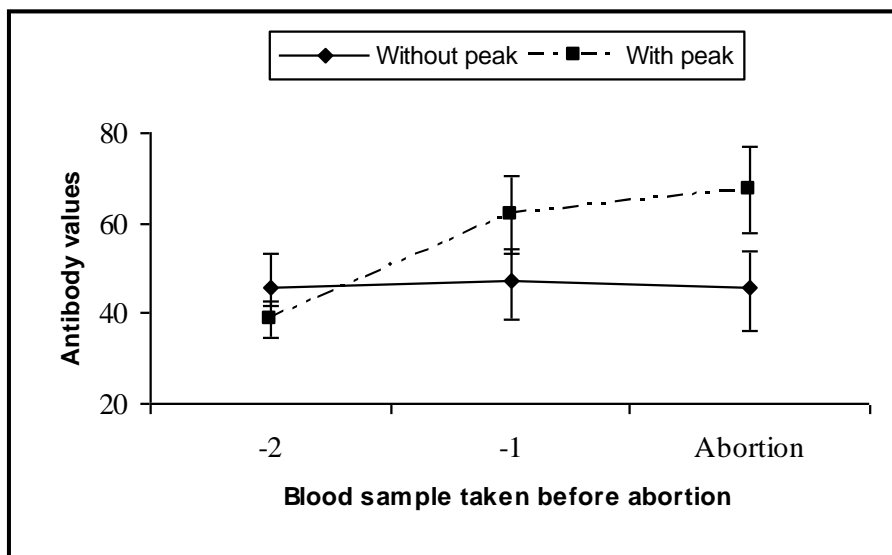


Figure 2. Mean (units \pm S.E.M.) plasma anti- *N. caninum* antibody values in pre-abortion sample -2 for aborting cows showing (n = 11) or not showing (n = 10) a peak in antibody values before abortion. Samples -2 and -1 were taken 30 days apart and abortion occurred between 7 and 32 days after the collection of sample -1. (From Nogareda et al., 2007, used with permission from Elsevier)

NEOSPORA CANINUM INFECTION DOES NOT AFFECT THE FERTILITY OF HIGH-PRODUCING DAIRY COWS

In a study performed in high-producing dairy cows with high risk of abortion associated to *N. caninum* (30%), the factors affecting fertility were herd, season, lactation number, Artificial insemination (AI) number, and bull and AI technician, but *Neospora* seropositivity was not a risk factor (López-Gatius et al., 2005a). Based on an assessment of 7,518 artificial inseminations (applied to parous cows) performed in 3 herds, of these inseminations, 2,540 (33.8%) resulted in pregnancy: 34% of which corresponded to seronegative cows (2,226 of 6,556 AI performed in seronegative cows), and 32.6% to seropositive animals (314/962). Abortion occurred in 97 (30.1%) of the 314 pregnancies recorded in dams seropositive for *N. caninum*, while only 93 (4.2%) of the 2,226 pregnancies in seronegative animals ended in abortion (López-Gatius et al, 2005a). This lack of effect has two possible explanations:

- In early stages of pregnancy, tachyzoites may not have attained sufficient numbers in the placenta or fetus to cause damage, but are able to harm the fetus later after further replication;
- The sudden migration of the protozoa into the bloodstream at mid-gestation might increase the chances of their detection at that time. Long and Baszler (1996) were unable to identify tachyzoites in placental and fetal tissues during the pre- and early pregnancy stages in mice experimentally-infected with *N. caninum*, but could detect them in late mid-gestation.

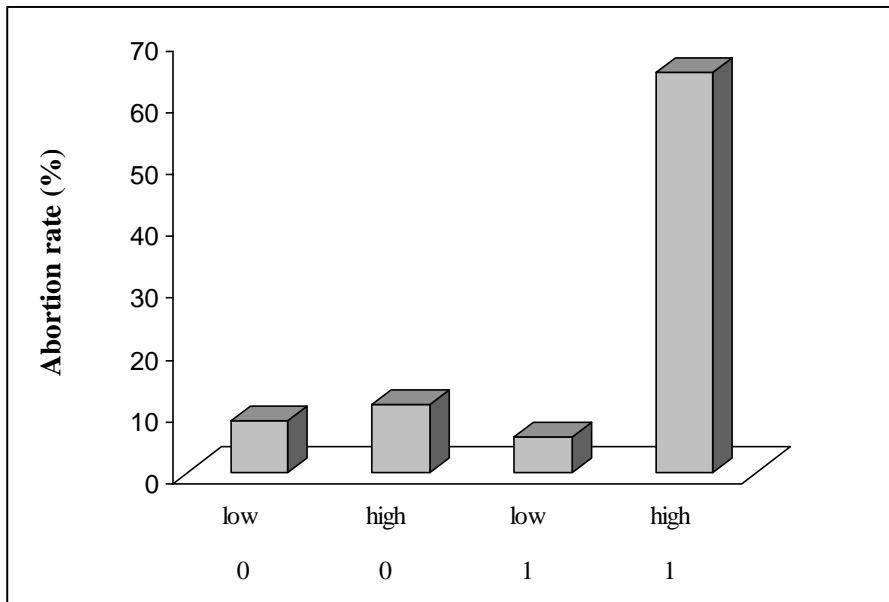


Figure 3. Abortion rates in cows with low (<30 units) or high (≥30 units) *N. caninum* antibody titration at the onset of treatment.

IN COWS WITH HIGH *N. CANINUM* ANTIBODY TITRES, SUPPLEMENTARY PROGESTERONE AT MID-GESTATION INCREASES ABORTION

Th1 immune responses are considered protected in protozoan infections, including *N. caninum* (reviewed Innes et al., 2005). However, during pregnancy a considerable level of immune-modulation seems to exist (Raghupathy 1997; Innes et al., 2005), and responses such as those induced by IFN- γ (Th1) can be potentially damaging and may cause rejection or abortion of the fetus for excessive inflammatory response in the placenta and/or fetus. Progesterone, in the natural immunomodulation of gestation, has been implied to reduce the level of Th1 response (Szekeres-Bartho et al. 2001; Druckmann and Druckmann, 2005). We hypothesized that immune rejection of the conceptus and therefore the abortion rate should be reduced following progesterone supplementation of pregnant *Neospora*-infected cows with exogenous progesterone during the mid-gestation period. Seropositive animals were assigned on day 120 of gestation to Control (n=33) or Treatment (n=34) groups (Bech-Sabat et al., 2006). Treatment consisted of progesterone supplementation by fitting a progesterone-releasing intravaginal device containing 1.55 g of progesterone for 28 days. Mean *N. caninum* antibody titres tested by ELISA on days 40, 90, and 120 of gestation were used to classify the cows as showing high (≥30 units) or low (<30 units) titration. Abortion rates showed a significant increase ($P<0.01$) in the Treatment group (35%) compared to the Control group (9%). Using logistic regression procedures, there was a significant ($P<0.0001$) interaction between progesterone treatment and antibody titration. Cows with high titration had a likelihood of abortion 14.3 times higher after treatment than those without treatment, whereas

in cows with low titration, exogenous progesterone had not effect (Figure 3). Our results did not support the established hypothesis and suggest that in cows with high *N. caninum* antibody titres, supplementary progesterone increases abortion and might affect cell-mediated immune responses (Bech-Sabat et al., 2006).

NEOSPORA CANINUM DOES NOT SEEM TO AFFECT PLACENTAL FUNCTION IN THOSE ANIMALS SEROPOSITIVE THAT DO NOT ABORT

Pregnancy-associated glycoproteins (PAG) are a multigene family belonging to the aspartic proteinase superfamily (Xie et al., 1991). These proteins are abundantly expressed in the outer-cell layer of the placenta of artiodactylids such as porcine and ruminants (Szafranska et al., 1995; Xie et al., 1997; Garbayo et al., 2000). Although their function is still unknown, PAG levels in maternal blood have been used for pregnancy diagnosis and as a marker for placental/fetal well-being (Skinner et al., 1996; Zarrouk et al., 1999a,b). Indeed, a protective mechanism against rejection has been suggested based on the finding that peripartum PAG levels in cows carrying fetuses of a different breed were higher than levels in cows bearing fetuses of their own breed (Zoli et al., 1992). PAG concentrations were not affected by persistent *N. caninum* infection in dairy cows in a study based on data derived from 22 multiparous cows: 16 *N. caninum*-seropositive and 6 *N. caninum*-seronegative animals (used as controls), in which three of the 16 seropositive cows aborted during the study period (Table 4). The corresponding data of aborted cows was analyzed separately. There was a significant 52% increase ($P < 0.0001$) in *N. caninum* antibody titres during the second half of gestation compared to the first half, but the increased humoral immune response observed during the second half of gestation in seropositive cows could not be related to variations in PAG concentrations. Of interest was the fact that in the cows with mummified fetus, PAG concentrations were low or undetectable when the diagnosis was made (López-Gatius et al., 2007a). These findings suggest that *N. caninum* infection has no effect on placental function in chronically-infected cows not suffering abortion, while PAG measurements in aborting animals provide a useful indication of feto-placental status.

Similar results were obtained in a recent study involving a high number of animals (Serrano et al., 2008). It was interesting to observe that Limousin-cross breeds had higher levels of PAG-1 compared to pure breed animals (Figure 4). There were no significant effects of herd, sex of fetus, milk production, lactation number, and plasma progesterone concentrations. In twin pregnancies, the use of Limousin semen and conception during the cool period were found to be factors increasing significantly the plasma PAG-1 concentrations throughout gestation. Our data indicates that cow well-being during the early placenta development, probably due to less heat stress during the cool season of conception, and crossbreed pregnancies favour PAG-1 production throughout the gestation period.

Table 4. Mean (\pm S.D.) PAG concentrations (ng/ml) recorded during gestation in *N. caninum* seropositive and seronegative cows

	Seropositive	Seronegative	Total
Days of gestation	n = 13	n = 6	n = 19
40	1.87 \pm 1.27	3.95 \pm 2.28	2.53 \pm 1.87
90	4.84 \pm 3.24	5.59 \pm 2.36	5.08 \pm 2.95
120	17.44 \pm 14.67	18.84 \pm 17.52	17.88 \pm 15.14
150	29.52 \pm 14.59	34.89 \pm 15.91	31.21 \pm 14.79
180	47.77 \pm 20.38	61.73 \pm 26.57	52.18 \pm 22.75
210	75.03 \pm 57.48	83.86 \pm 40.1	77.82 \pm 51.65
Parturition	852.92 \pm 676.34	963.42 \pm 474.17	887.82 \pm 608.44

(From López-Gatius et al., 2007a, used with permission from Elsevier)

Cross-breed pregnancies seem to favour placental function since a lower risk of abortion has been demonstrated in *Neospora*-seropositive dairy cows inseminated with beef semen (López-Gatius et al. 2005b). Since immunosuppressive properties have been attributed to the secretion of PAGs, at least into the uterine zone (Wooding et al. 2005), high plasma PAG-1 concentrations could reflect placental mechanisms needed to avoid maternal rejection, and, thus, to compensate the genetic distance with the fetus. This process may be particularly important for reinforcing the barrier effect of the placenta against transplacental infectious agents such as the parasite *N. caninum*.

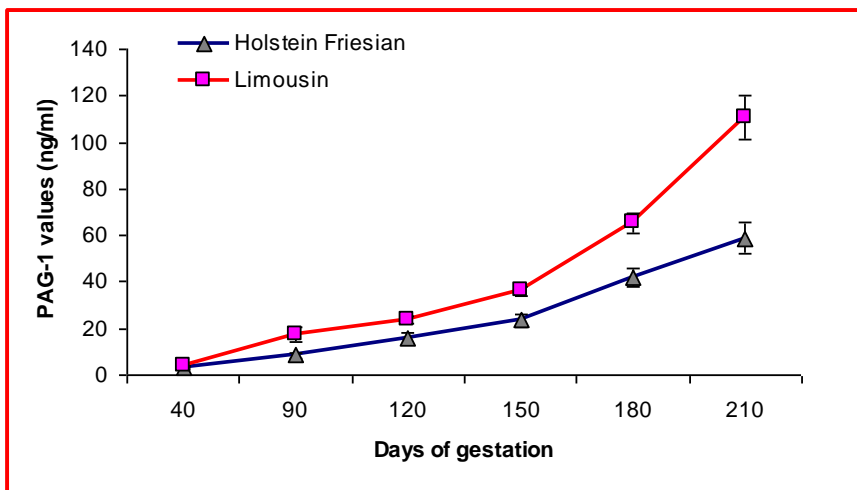


Figure 4. Mean plasma concentrations (\pm S.E.M.) of pregnancy-associated glycoprotein-1 (PAG-1) throughout gestation for cows pregnant by Friesian and Limousin semen. (From Serrano et al., 2008)

ROLE OF INTERFERON-GAMMA (IFN- γ) IN PROTECTIVE IMMUNE RESPONSES IN NATURALLY- INFECTED *N. CANINUM* COWS

Since progesterone supplementation at mid-gestation did not improve abortion rates in *N. caninum*-infected animals, we hypothesized a possible role of IFN- γ production in the maintenance of gestation. IFN- γ production in plasma was detected at some point along the pregnancy in 16 (19%) of 86 *Neospora*-seropositive cows, yet was undetectable in the 40 seronegative animals (López-Gatius et al., 2007b). Of the 126 pregnancies examined, 22 (17.5%) ended in abortion. Abortion occurred in 24.4% of seropositive cows (21/86), and in 2.5% of seronegative animals (1/409). Significant ($P < 0.0001$) interaction was observed between *Neospora*-seropositivity and IFN- γ production (Table 5). Based on the odds ratio, the risk of abortion was 15.6 times higher in seropositive cows not producing IFN- γ than in seronegative animals, whereas neosporosis had no effect in seropositive cows with IFN- γ production. A significant ($P = 0.001$) negative effect of IFN- γ production on the *Neospora* titre was furthermore observed in the 65 non-aborting seropositive animals (Figure 5). These results indicate that IFN- γ production is associated with protection against abortion in *Neospora*-infected cows and also point to a reduced humoral immune response to *N. caninum* during gestation in cows producing IFN- γ (López-Gatius et al., 2007b).

Table 5. Effects of IFN- γ production on abortion rate in *Neospora*-seropositive cows

IFN- γ production	<i>Neospora</i> seropositivity	Abortion rate		Odds ratio	95%CI ^a	P-value
		n	%			
Negative	Seropositive	20/70	28.6	15.6 ^b	2-121	0.009
Positive	Seropositive	1/16	6.3	2.6	0.2-44.6	0.51
Negative	Seronegative	1/40	2.5	Referent		

^aConfidence Interval;

^bOdds of a *Neospora*-seropositive cow without IFN- γ suffering abortion, compared to seronegative cows.

(From López-Gatius et al., 2007b, used with permission from Elsevier)

When we further analyzed the IgG isotypes (IgG1 and IgG2) related to production of IFN- γ in those animals (Almería et al., 2009a), a significant negative effect of IFN- γ production on IgG1 antibodies was observed, while IFN- γ production did not affect IgG2 antibody levels. In contrast, higher levels of IgG2 antibodies compared to IgG1 antibodies were observed over the whole of gestation in aborting animals, both in those not producing IFN- γ and in the single aborting cow that produced the cytokine. Our findings indicate that a Th1 immune response, in which IgG2 antibodies prevail, could be protective against *N. caninum* abortion, but only in the presence of IFN- γ production. On their own, elevated IgG2 antibody titres appear to be insufficient to protect dams chronically-infected with *N. caninum* against abortion (Almería et al., 2009a).

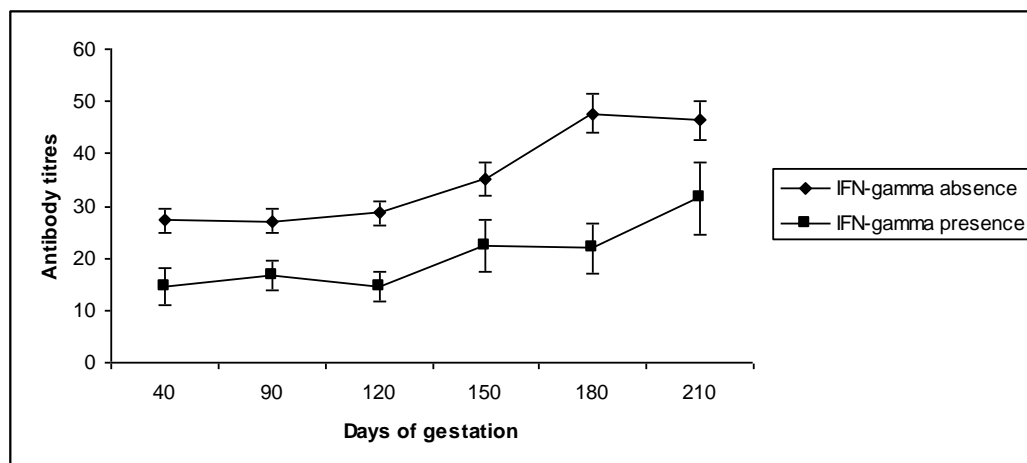


Figure 5. Mean anti-*N. caninum* antibody titres (units \pm S.E.M.) recorded during the gestation period in non-aborting seropositive cows producing ($n = 15$) or not producing ($n = 50$) IFN- γ . (From López-Gatius et al., 2007b, used with permission from Elsevier)

CONTROL AND PREVENTION: THE USE OF BEEF BULL SEMEN REDUCES THE RISK OF ABORTION IN *N. CANINUM* SEROPOSITIVE DAIRY CATTLE

In a prospective cohort study using dairy or beef bull semen to inseminate *N. caninum*-seropositive dairy cows in our area, it was observed that the use of beef bull semen reduced the risk of abortion (López-Gatius et al., 2005b). Recently, a study based on a large number of animals (1,115 *N. caninum*-seropositive lactating dairy cows inseminated), provided further confirmation showing a significant reduction on the incidence of abortion in *N. caninum* seropositive dairy cows using beef bull semen compared to the use of Holstein-Friesian semen (Almería et al., 2009b). In the latter study, abortion rates were 15.2% (96/633) and 32.2% (155/482) for seropositive cows inseminated with beef breed and with Holstein-Friesian semen, respectively. The analysis of the effect of different cross-breed pregnancies on the risk of abortion of *Neospora*-infected dairy cows showed abortion rates of 32% of 482 cows inseminated with Holstein-Friesian semen, 22% of 49 cows inseminated with Charolais semen, 20% of 191 cows inseminated with Belgium Blue semen, 19% of 89 cows inseminated with Piedmontese semen, and 10% of 304 cows inseminated with Limousin semen (Almería et al., 2009b). If the cows inseminated with Limousin semen had low titration of antibodies, the results were even more striking and showed an abortion rate similar to that in seronegative animals (2.1%, 3/145, and 3.2%, 239/7432, respectively). Therefore, different cross-breed pregnancies have different risks of abortion in *Neospora*-infected dairy cows, so that the use of beef bull semen dramatically reduces the risk of abortion in dairy cows, especially if Limousin breed semen is used.

We hypothesized that placental function might be favoured in cross-breed pregnancies possibly via an increased concentration of pregnancy-associated glycoproteins (PAG). Effectively, when PAG-1 levels were analyzed in cross-pregnancies, it was found that levels

of PAG-1 in Limousin-cross breed pregnancies were significantly higher than those of pure Holstein-Friesian (Serrano et al., 2008).

These results could be indication that certain breeds, e.g., Limousin, are less susceptible to *N. caninum* infection than others. Differences in susceptibility to infection have been observed among breeds in some recent surveys. Cattle breeds in Spain, mainly native breeds pasturing in highlands at very low stocking densities were found to be significantly less likely to test seropositive compared to other breeds, while in Sweden, Swedish Red and White breed cattle were more likely to test *N. caninum* positive compared to other breeds (Bartels et al., 2006). Similarly, the Limousin breed showed seroprevalence levels 6.7 times lower than that noted in other dual-purpose, or beef cattle, breeds in the same extensive management (Table 9) (Armengol et al., 2007).

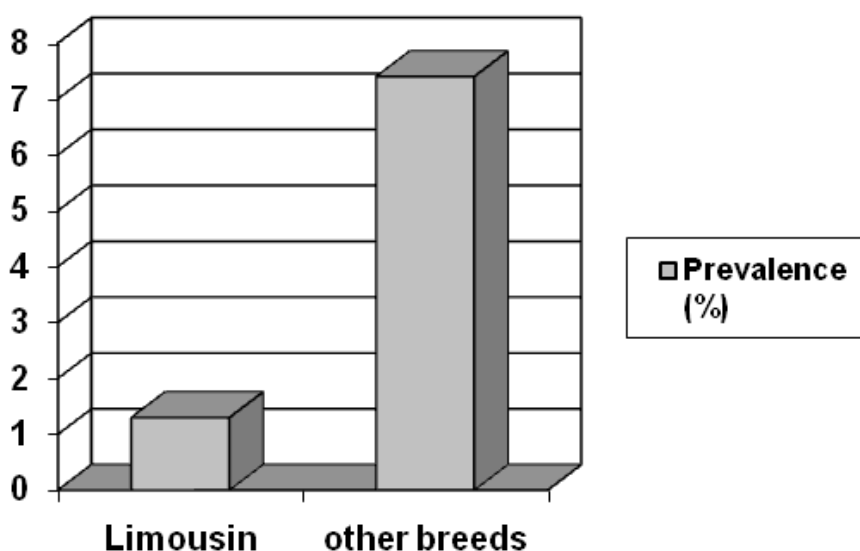


Figure 6. Prevalence in Limousin breed cattle compared to the remaining breeds in the same extensive management system

In *N. caninum*-free herds, prevention of the introduction of the infection through standard biosecurity measures is the primary goal, whereas in *N. caninum*-infected herds, control programs are based on reducing vertical transmission of mother-calf by reduction of the number of seropositive cattle and decreasing the risk of horizontal transmission of *N. caninum* principally by controlling the definitive host population as a source of oocyst contamination (reviewed Dubey et al., 2007). Regardless of farm level of *N. caninum* seropositivity, a quarantine and testing of replacement should be done in all new animals due to the importance of vertical transmission on the maintainance of infection. Prevention of dogs and others definitive hosts from contaminating pastures is recommended. In fact, the presence of dogs in cow herds should be avoided (Dubey et al., 2007).

Control measures recommended to decrease vertical transmission in *N. caninum*-infected herds include, among others, testing and culling of seropositive animals, discontinued breeding with offspring from seropositive cows, chemotherapeutic treatment of calves from

seropositive cows, vaccination of susceptible and infected animals, and insemination with beef bull semen (Dubey et al., 2007). The control measure “not breeding replacements from infected cattle”, is an effective method in the short term, particularly in herds with a higher turnover of cattle (French et al., 1999; Hasler et al., 2006b), and it has been considered the best control strategy currently available in economic terms (Hasler et al., 2006c). Artificial insemination of *N. caninum* seropositive cows with beef bull semen would combine the “not breeding replacements from infected cattle” control measure to reduce *N. caninum* prevalence, and, in addition, would allow lower abortions rates, higher number of complete lactations in seropositive cows, and the additional economic benefit from selling cross-breed offspring that will not remain in the herd. Therefore, the use of beef bull semen in seropositive cows could help to overcome some of the economic expenses of the yearly serological testing for *N. caninum*. We consider that the use of Limousin semen is highly recommended for AI of *N. caninum* seropositive cows in herds with high prevalence of *N. caninum*.

Transfer of embryos of *N. caninum*-infected cows to uninfected receptors is an interesting way to maintain important seropositive animals in the herd. *Neospora caninum* infection was not demonstrable in any of 70 fetuses or calves born to seronegative cows that received embryos from seropositive donors, whereas 5 of 6 calves resulting from embryo transfer from seronegative donors to seropositive recipients were infected with *N. caninum* (Baillargeon et al., 2001). In agreement with the results of Baillargeon et al. (2001), in our conditions, the transfer of embryos of *N. caninum*-infected cows to uninfected seronegative cows has always produced seronegative heifers (at least 18 heifers to date) (unpublished data).

In summary, based on our studies, practical recommendations include the control of the cow environment during the second trimester of gestation, the priority of culling for parous cows with higher antibody titres against *N. caninum*, preferably animals that suffered two or more abortions (Pabón et al., 2007), and the insemination of *Neospora*-seropositive cows with semen from the Limousin breed. Of course, it needs to be taken into account that the long-term effectiveness of these control measures also would depend on the amount and source of horizontal infection (French et al., 1999), and that current control strategies should consider to apply strict dog-management measures as well as to minimize within-herd seroprevalence by monitoring serostatus of animals (Bartels et al., 2007). Considering the possible implication of wildlife, some control measures such as protection of feedstuff from contamination with canine faeces and the correct disposal of deer carcasses, that if not most probably would be available for consumption by a variety of carnivores, should be considered in red deer estates to avoid possible transmission to wild and domestic carnivores (Almería et al., 2007, Sobrino et al., 2008).

A general strategy to control neosporosis worldwide is not applicable because of regional differences in the epidemiology of bovine neosporosis, and it is prudent to thoroughly study the regional epidemiology of neosporosis, as has been performed in our area, before embarking on a control program (Dubey et al., 2007).

REDUCTION OF SEROPREVALENCE AND ABORTION RATES RELATED TO *N. CANINUM* IN NORTH-EAST SPAIN AFTER ADVISED CONTROL MEASURES AT FARM LEVEL

When the control measures: 1) culling of animals in the herd (37), mainly *Neospora*-seropositive aborted animals, and 2) the use of beef bull semen to inseminate *Neospora*-seropositive dams to exclude their progeny of the herd were used in a dairy herd followed during three consecutive years, we observed that seroprevalence of *N. caninum* infection of the herd decreased from 31.7% in the first year to 24.8% in the second year, and, more importantly, abortion rate dropped from 20.6% in the first year to 5.5% in the second year. *Neospora* seroprevalence underwent a further decrease in the third year (19.9%) although the total number of pregnancies in the herd decreased slightly while the number of abortions increased, rendering an abortion rate of 9.9% (Pabón et al., 2007).

As indicated above, when insemination of chronically-infected cows with low titres against *N. caninum* was performed with Limousin semen, the abortion rate was similar to that in seronegative animals (Almería et al., 2009b).

In summary, our results indicated a close relationship between mother seropositivity to *N. caninum* and abortion risk and that irrespective of the herd level of *Neospora*-seroprevalence, plasma antibody titration against *N. caninum* is a good indicator of risk of abortion; *Neospora*-infection does not affect fertility nor compromises the subsequent maintenance of gestation during the first trimester of gestation in animals chronically-infected prior to pregnancy. *Neospora*-seropositivity can be very stable throughout years so that chronically-infected cows can show a high rate of repeat abortions and that the use of beef semen, especially that derived from the Limousin bulls, reduces dramatically the incidence of abortions. The latter approach has been included in the control measures for neosporosis in dairy herds with high prevalence of *N. caninum* in our area and significantly reduced both abortion and seroprevalence levels in those herds.

Future studies should focus on the analysis of the immune-endocrin changes that take place in *N. caninum*-infected dairy cattle, which could help to understand why some animals abort and other do not. These studies could also help to find some markers for occurrence of *N. caninum* abortion in chronically-infected cows.

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Chapter VI

GENE EXPRESSION IN THE BOVINE GASTRO-INTESTINAL TRACT DURING NEMATODE INFECTION

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ABSTRACT

Gastro-intestinal nematodes have been ranked among the top three diseases that may have a significant economic impact on the American cattle industry with an estimated annual cost in excess of \$2 billion. At least 41 different species of parasitic nematodes have been described in the bovine gastrointestinal tract with predilection sites in the abomasum, small intestine and large intestine. Understanding molecular mechanisms that contribute to acquired immunity, immunosuppression and innate resistance is an important prerequisite for developing sound alternative nematode control strategies, such as vaccination and breeding for resistant populations based on genetic variations in the host genome. Evidence suggests that parasitic nematode infection elicits drastic changes in gene expression patterns in host cells. Gene expression profiling represents the first step in understanding of the mechanism underlying protective immunity and host resistance. In this chapter, we organize our discussion into four topics: 1) A summary of recent advances on alterations in cytokine expression profiles during nematode infection; 2) A discussion of gene expression patterns of cell adhesion molecules, such as collectins, galectins, and cadherins, and their possible roles in host immune responses; 3) The effect of mucins and mucin biosynthesis during nematode infection and cell-specific patterns of expression of select genes; and 4) Perturbations in pathways and regulatory networks during nematode infection. The gene expression data accumulated over years will provide insight into cattle-parasite interactions and protective immunity against gastro-intestinal nematodes.

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INTRODUCTION

Internal parasites, especially gastro-intestinal (GI) nematodes, have been ranked among the top three diseases or conditions that may have a significant economic impact on the American cattle industry. The estimated annual cost in reduced productive efficiency and increased operating expenses for parasite control is in excess of \$2 billion in the US alone. At least 41 different species of nematodes have been reported to reside in the bovine GI tract, with predilection sites in the abomasum, small intestine and large intestine (Table 1). While the majority of nematodes summarized in this table are found worldwide, some are rare and have a restricted geographic distribution. Additionally, some nematodes, such as *Haemonchus contortus* and *Trichuris ovis*, can be economically important in small ruminants such as sheep and goats, but may not be major parasites in cattle under certain climatic and management conditions. The severity of GI nematodiasis, which is considered a herd disease, is determined by the rate of parasite transmission among herd members. The combination of environmental and host immune factors results in transmission patterns characterized by periods of high levels of transmission and periods of little or no transmission. GI nematodes are highly adapted for survival in their hosts. One example of this adaptation is that environmental factors for optimal nematode transmission parallel optimal conditions for forage growth. This synchrony coupled with the ubiquitous distribution of parasites, the variability in host susceptibility and the ability of wild ungulates to serve as reservoirs of infection makes eradication of GI nematodes in grazing ruminants virtually impossible.

Approximately 25-35 years ago, anthelmintic agents with both low toxicity for the mammalian host and high efficacy against a broad spectrum of parasites were introduced. Since their introduction into the marketplace, producers have grown increasingly dependent on anthelmintics for parasite control. However, this complete reliance on anthelmintics, especially when used as a “suppressive” agent, has resulted in a number of negative impacts on agriculture. First and foremost is the appearance of parasites resistant to anthelmintics throughout the world. While resistance has been less common in parasites of cattle than in small ruminants, there has been an increasing number of reports in cattle from New Zealand (Vermunt et al, 1995), England (Stafford and Coles 1999), South America (Anziani et al. 2004), to the US (Gasbarre, personal communication). The initial high efficacy and broad spectrum of modern drugs has also resulted in a stagnation of research to identify alternative methods of parasite control. This has left producers who do not wish to use anthelmintics with no practical or adequate means of controlling parasite-induced losses. Alternative nematode control strategies are urgently needed due to increasing numbers of organic producers resulting from a growing insistence by consumers for chemical residue-free meat and milk products. Other strategies, such as proper management practice using extremely low stocking rates, carefully constructed rotational programs, and fungus-based biological control, can be effective in some geographical areas, but are usually impractical due to economical considerations.

Table 1. Nematodes in the bovine gastro-intestinal tract and their predilection sites*

Species	Host	Major Predilection Site/Habitat	Reference
PART 1: Common/Major Cattle Parasites			
<i>Bunostomum phlebotomum</i>	cattle, sheep	Small intestine	Leite, 1992; Borgsteede et al., 2000
<i>Capillaria bovis</i>	cattle, sheep, goats, deer	Small intestine	McKenzie and Davidson, 1989; Borgsteede et al., 2000
<i>Cooperia oncophora</i>	cattle, sheep goats	Small intestine	Lichtenfels et al., 1994; Borgsteede et al., 2000
<i>Cooperia pectinata</i>	cattle, sheep, buffalo, camels	Small intestine	Berrie et al., 1988
<i>Cooperia punctata</i>	cattle, axis deer	Small intestine	McKenzie and Davidson, 1989
<i>Cooperia spatulata</i>	cattle, sheep	Small intestine	Lima 1998
<i>Cooperia surnabada</i>	cattle, sheep, goats	Small intestine	Rehbein et al., 2003
<i>Haemonchus contortus</i>	sheep, goats, cattle, axis deer	Abomasum	Lichtenfels et al., 1994
<i>Haemonchus placei</i>	cattle, sheep goats	Abomasum	Lichtenfels et al., 1994; Achi et al., 2003
<i>Haemonchus similis</i>	cattle, sheep goats	Abomasum	Lima 1998; Achi et al., 2003
<i>Nematodirus battus</i>	sheep, goats, cattle	Small intestine	Hoberg et al., 2005
<i>Oesophagostomum radiatum</i>	cattle, buffalo	Small intestine (also in cecum and colon)	Gasbarre et al., 1985; Berrie et al., 1988
<i>Oesophagostomum venulosum</i>	cattle	Large intestine	Charles and Baker 1988; Newton et al., 1998
<i>Ostertagia lyrata</i>	cattle	Abomasum	Lichtenfels et al., 1994, 1997
<i>Ostertagia ostertagi</i>	cattle, goats, rarely sheep, horses	Abomasum	Lichtenfels et al., 1994, 1997
<i>Setaria cervi</i>	cattle, buffalo, bison, yak, deer	Peritoneal cavity, occasionally in other organs	Griffiths, 1978
<i>Strongyloides vituli (S.papillosus)</i>	cattle, sheep, pig	Small intestine	Aumont et al., 1991; Eberhardt et al., 2008
<i>Toxocara vitulorum</i>	cattle, buffalo, sheep, goats, cats, dogs	Small intestine	Ferreira et al., 2005
<i>Trichostrongylus axei</i>	cattle, sheep, goats, horse, deer, pig	Abomasum but occasionally in duodenum)	Kates 1965; Palcy et al., 2008;
<i>Trichuris discolor</i>	cattle	Large intestine (cecum)	Knight, 1971; Perdrizet and King, 1986

Table 1. (Continued)

Species	Host	Major Predilection Site/Habitat	Reference
PART 2: Other Species Reported From Cattle			
<i>Bunostomum trigonocephalum</i>	sheep, goats, cattle	Small intestine	Torina et al., 2004; Makovcova et al., 2008
<i>Capillaria brevipes</i>	cattle, goat, sheep,	Small intestine	Stoll, 1936; Read, 1949
<i>Capillaria longipes</i>	cattle, goat, sheep,	Small intestine	Stoll, 1936; Read, 1949
<i>Chabertia ovina</i>	Sheep, goats, cattle	Large intestine	Torina et al., 2004; Sevimli et al., 2007
<i>Cooperia bisonis</i>	cattle, sheep, bison, antelopes	Small intestine	Malczewski et al., 1996
<i>Marshallagia marshalli</i>	sheep, cattle, many other vertebrates	Abomasum	Lichtenfels and Pilitt 1991; Lichtenfels and Hoberg 1993;
<i>Nematodirus filicollis</i>	sheep, goats, cattle	Small intestine	Hoberg et al., 2005
<i>Nematodirus helvetianus</i>	cattle, goats, sheep	Small intestine	Hoberg et al., 2005
<i>Nematodirus spathiger</i>	sheep, goats, cattle	Small intestine	Hoberg et al., 2005
<i>Ostertagia bisonis</i>	cattle, wild ruminants	Abomasum	Lichtenfels et al., 1994, 1997
<i>Ostertagia leptospicularis</i>	cervid deer, cattle	Abomasum	Lichtenfels et al., 1994, 1997
<i>Ostertagia orloffii</i>	Barbary sheep, cattle, deer	Abomasum	Lichtenfels et al., 1994, 1997
<i>Ostertagia podjapolskyi</i>	cattle, sheep, moufflon	Abomasum	Lichtenfels et al., 1994, 1997
<i>Ostertagia trifurcata</i>	sheep, goats, cattle	Abomasum	Lichtenfels et al., 1994, 1997
<i>Physocephalus sexalatus</i>	pig, camel, occasionally cattle & horse	Abomasum	Griffiths, 1978; Humbert and Henry, 1989
<i>Stephanofilaria stilesi</i>	cattle, especially beef cattle	Abomasum (skin epithelium)	Griffiths, 1978
<i>Teladorsagia circumcincta</i>	sheep, goats, cattle, cervids	Abomasum	Lichtenfels and Hoberg, 1993; Torina et al., 2004
<i>Trichostrongylus colubriformis</i>	sheep, goats, cattle, camels, horse, deer	Small intestine but also in abomasum	Lima 1998, Riffkin et al., 2000; Audebert et al., 2003
<i>Trichostrongylus extenuatus</i>	cattle, sheep, goat	Small intestine	Kreis 1960;
<i>Trichostrongylus vitrinus</i>	sheep, goats, cattle, camel, humans	Duodenum	Borgsteede, 1981; Audebert et al., 2003
<i>Trichuris ovis</i>	sheep, goats, cattle	Large intestine (cecum and colon)	Knight ,1984; Nogareda et al., 2006

*Notes:

The list may not be comprehensive.

Host specificity and habitats of many species have not been well-defined.

The taxonomy of some species is controversial and many described species could be synonymous.

Immunological tools such as vaccines or immunomodulators seem to be an attractive alternative control strategy and can protect cattle from infection. This control approach may be feasible for certain nematode parasites (Gasbarre and Canals, 1989; Claerebout et al., 2005); however, at the present time, the value of this approach is limited by the ability of nematodes to evade host immune responses (Gasbarre, 1997). As these evasive mechanisms are better understood, procedures to ablate these immunoregulatory activities can be devised. A continuing problem with immunotherapy (either vaccines or immunomodulators) is the need for the treatment to confer protection against a broad range of important parasite species in a given locality, and, at the same time, fit within producer guidelines regarding cost and labor management.

Understanding the mechanism that contribute to protective immunity, immunosuppression and resistance will have a huge impact on alternative control strategies including development of vaccines, breeding and selection of resistant populations based on genetic variations within the host genome. Approximately a decade ago, attempts were made to understand cytokine profiles during the primary infection with *O. ostertagi* in cattle (Canals et al., 1997). A significant number of research projects on host immune responses, molecular mechanisms of host resistance and vaccine development have since been initiated. This has allowed for the use of high throughput genomic technologies, which leads the expansion of our knowledge on host protective immunity and immunosuppression. However, our understanding of host-parasite interactions has been derived largely from a small number of nematode species of high economical or medical impacts, but not necessarily from parasites with the most biological relevance. Molecular data on even very basic aspects of host-parasite interactions are lacking for the majority of the 41 nematodes in the bovine GI tract. In this study, we discuss recent progress on gene expression in the bovine GI tract during nematode infection from four aspects. First, we summarize recent advances in alterations in cytokine expression profiles during infection. We then discuss gene expression of cell adhesion molecules and their roles in host immune response. Mucins and mucin biosynthesis affected during nematode infection are also discussed. Finally, we review recent advances on perturbations in pathways and regulatory networks during nematode infection. While certain species of nematodes have a broad range of natural hosts and are major parasites of many ruminants or non-ruminant species, we attempt to limit our discussion to data relevant only to cattle-parasite systems.

ALTERATIONS IN CYTOKINE PROFILES DURING INFECTION

Cytokines are signaling molecules involved extensively in cellular communication. By acting as immunomodulating agents, cytokines are critical to the development and functionality of both innate and adaptive immune responses, especially during defense against unwanted pathogens. They play a major role in the generation of immune responses by regulating inflammation as well as T and B cell growth and differentiation. For example, Type-2 cytokine responses have been labeled as a hallmark of helminthic infections with a characteristic polarization of the cytokine response towards the secretion of IL-4, IL-5, IL-10 and IL-13. However, molecular mechanisms involved in the polarization of type-2 responses and their biological significance in helminthic infections are still unclear. Understanding these

issues, including defining cytokine responses to nematode infection, may contribute to the development of successful immunization strategies and provide insight into protective immunity. The interest in understanding the role of type-2 responses in parasitic infections also lies in the possibility of modulating these responses through the regulatory functions of cytokines; for example, an ineffective type-2 response might be turned into a protective response by immune intervention. For these reasons, expression profiles of several classical cytokines have been extensively investigated. In the cattle-*O. ostertagi* system, a drastic increase of IL-4 is observed by 11 days post-infection (dpi) in un-stimulated abomasal lymph nodes (ABLN) compared to those from naïve un-infected control calves and remains elevated through 28dpi (Canals et al., 1997). In contrast, IL-2 mRNA level is down-regulated 5 fold at 11 dpi and remains down-regulated. Cytokine gene expression in lamina propria lymphocytes of cattle after primary infection follows a similar trend: a strong up-regulation of both IL-4 and IFN- γ in the infected animals at 10dpi as well as at 60dpi. The primary site of antigen presentation, ABLN, undergoes a drastic shift in population profiles of lymphocytes and a rapid expansion of certain cell types after *O. ostertagi* infection. For example, an increase in both IgM⁺ (B cells) and TcR1⁺ cells, as well as a reduction in the percentage of T cells after infection, is documented (Baker et al., 1993). An increase in B cell populations is consistent with strong serum antibody responses (Canals and Gasbarre 1990). The increase in TcR1⁺ cell populations may be responsible for observed up-regulation of IFN- γ mRNA. While changes elicited in cattle by *O. ostertagi* infection in both cell types and cytokine are consistent with a Th2 immune response, observation of a strong increase of IFN- γ mRNA raises the question that the Th1 and Th2 paradigm may not be as clearly delineated in the cattle-*Ostertagia* system as in murine models. Earlier studies also attempt to identify *O. ostertagi* antigens able to modulate bovine lymphocyte proliferation (De Marez et al., 1997; Gomez-Munoz et al., 2004). L3 soluble extracts (SE), regardless of their concentrations, have no inhibitory effects on mitogen-induced proliferation of lymphocytes isolated from naïve calves. In contrast, L4-soluble extracts cause a significant suppression of lymphocyte proliferation in a dose-dependent manner. Underlying the inhibition of lymphocyte proliferation by L4SE is a significant reduction in mRNA expression of most cytokines tested, including IL-2, IL-4, IL-13, TNF α , IFN- γ , and a slight up-regulation (2-3 fold) of IL-10 and TGF- β . TGF- β has been described as a parasite escape mechanism for *Leishmania* (Barral-Netto et al., 1992) and limits T cell clonal expansion by acting as an autoregulatory cytokine (Sporn and Roberts 1990). TGF- β expression is significantly elevated in calves during a primary infection; however, its expression is decreased in immune calves after repeat drug-attenuated infections (Almeria et al., 1998). It would be intriguing to understand possible roles of these 2 cytokines in developing protective immunity. Cytokine responses are also investigated in immunized calves after *O. ostertagi* infection (Claerebout et al., 2005). Compared to the uninfected group, ABLN from immunized bull calves after weeks of trickle infections displays a significant reduction in Th1 cytokines, such as IL-12 and IFN- γ , and a strong increase in Th2 cytokines, such as IL-4 and IL-13. However, this distinction between Th1 and Th2 cytokines is not obvious in the abomasal mucosa from these animals. As discussed previously, IL-4, IL-10 and IFN- γ are up-regulated by infection. While vaccination with both *O.ostertagi* adult excretory-secretory products (ES thiol) and polyprotein allergens (OPA) seems efficacious in reducing cumulative fecal egg counts up to 60%, it does not alter cytokine profiles in either ABLN and abomasal mucosa after infection. No significant correlation between levels of Th2 cytokines and any of the common effector mechanisms in the mucosa, such as *Ostertagia*-

specific IgA, IgG, and IgM and the number of mast cells, or eosinophils, is observed. There is also no significant correlation between IL-5 and the number of eosinophils or between IL-5 and *Ostertagia*-specific IgA levels in the abomasal mucosa.

In the small intestine of *C. oncophora*-infected calves, expression of IL-4, IL-5 and IL-13 is up-regulated at 14dpi while expression of IL-1 β , IL-6, MIP α and TNF α remains unchanged during infection. Our previous results also indicate that, unlike what is observed in *O. ostertagi*-infected cattle, the IFN- γ expression is not significantly altered during *C. oncophora* infection (Li and Gasbarre, 2009).

In order to understand mechanisms of resistance to gastrointestinal nematodes, we have examined cytokine profiles of selected Angus cattle from a resource population consisting of approximately 600 animals with complete pedigree records and well-characterized phenotypes for parasite resistance (Li et al., 2007). Expression profiles of 17 cytokines, cytokine receptors and chemokines are investigated in animals demonstrating resistance or susceptibility to pasture challenge at the site of infection (fundic and pyloric abomasa and small intestine) as well as their respective draining lymph nodes after an experimental challenge with both *O. ostertagi* and *C. oncophora*. While most of the cytokines examined are indistinguishable in expression profiles between resistant and susceptible animals, resistant heifers exhibit elevated expression of inflammatory cytokines, such as IL-1 β , IL-10, MIP-1 α and TNF α , greater than 2 fold in both fundic and pyloric abomasa at 7dpi. The expression levels of IL-6, IL-10 and MIP-1 α are also elevated in the small intestines of resistant animals. Cytokine gene expression is also compared in selected Nelore cattle in order to dissect the molecular mechanism of resistance to *Cooperia punctata* natural infection (Bricarello et al., 2008). Among a panel of 9 classical cytokines tested in the small intestine, gene expression of 2 classical Th2 cytokines, IL-4 and IL-13, is significantly higher in the resistant group, whereas expression of Th1 cytokines, IL-2, IL-12p35, IFN- γ and MCP-1 in the susceptible group is also up-regulated. Immune response to *C. punctata* natural infection is probably mediated by Th2 cytokines in the resistant group and by Th1 cytokines in the susceptible group (Bricarello et al., 2008).

A significant and negative correlation between the mucosal IgA levels and fecal egg counts and the number of eggs per female parasite has been reported, suggesting mucosal IgA may be associated with reduced *Ostertagia* fecundity (Claerebout and Vercruyse, 2000). An association between increased local IgA levels and decreased adult female worm length in sheep is also observed (Smith et al., 1985; Strain and Stear, 1999). Although reduced worm length can result from inhibited growth or selective expulsion of large worms, worm length is often an indicator of fecundity. Expulsion of adult *Cooperia* worms appears to be mast-cell independent and was associated with a significant increase in mucosal IgA and an influx of eosinophils (Kanobana et al., 2002). PIGR is the gene responsible for the trans-epithelial transport of polymeric immunoglobulins, such as IgA dimers and IgM pentamers, into mucosal and glandular secretions. Its expression is essential for achieving mucosal immunity (Verbeet et al., 1995). Indeed, evidence demonstrates that PIGR knockout mice become more susceptible to *Mycobacteria bovis* infection (Tjarnlund et al., 2006). In mice absent of both passive and active immunity, expression of PIGR is significantly attenuated (Jenkins et al., 2003). Up-regulation of PIGR at both mRNA and protein levels following virus infection in human intestinal epithelial cells appears to be an innate immune response against invading pathogens (Pal et al., 2005). PIGR is over-expressed in the abomasa of resistant heifers (Li et al., 2007). WSX-1 displays the same expression profile as PIGR: over-expressed 3.6 and

19.9-fold in fundic and pyloric abomasa of resistant heifers, respectively (Li et al., 2007). WSX-1, together with gp130, constitutes a functional receptor for IL-27, a heterodimeric cytokine related to IL-12 (Pflanz et al., 2002). Although its downstream signaling pathway has not been elucidated, WSX-1 is known to be required for IFN- γ production by naive CD4⁺ T cells, is essential for resistance to *Leishmania major* infection (Yoshida et al., 2001) and plays a critical role in promoting Th1 development. WSX-1 knockout mice show higher levels of protective immunity against *Mycobacteria tuberculosis* infection than wild-type mice (Holscher et al., 2005) with a concomitant increase of chronic inflammatory responses. WSX-1 has been suggested as an inhibitory regulator of pro-inflammatory cytokine production (Yamanaka et al., 2004). The expression levels of WSX-1 and two components of its ligand IL-27, EBI3 and IL-27p28, are examined in draining mesenteric lymph nodes following *Trichuris muris* infection (Artis et al., 2004). WSX-1 remains unchanged 7 dpi, which is consistent with our results in the draining lymph nodes. However, as controversial as it may seem, WSX knockout mice exhibit accelerated parasite expulsion mediated by enhanced production of Th2 cytokines (Artis et al., 2004). WSX-1 expression is markedly up-regulated in inflammatory CD4⁺ T cells in an experimental disease model and regulation of its expression is cell-type dependent (Li et al., 2005). In the abomasum, WSX-1 is up-regulated up to ~20 folds in resistant heifers, suggesting a potential role in rendering host resistance to nematodes. However, whether or not WSX-1 up-regulation is a cause or a consequence of over-expression of pro-inflammatory cytokines remains unclear.

Although a significant amount of gene expression data on cytokines has been accumulated over the years, many unanswered questions still remain. For example, biological functions of cytokines in the host-parasite interaction remain unknown in many infections. Cytokine expression does not correlate well with resistance in many cases (Finkelman et al., 1991; Maizels and Yazdanbakhsh, 2003). The biochemical property of parasite molecules that stimulate cytokine production is still unclear. From a technical stand point, considerable variations exist in cytokine profiles among individuals and between populations due to large variations in host genetics and parasitological parameters, such as worm burden and EPG, at both basal and infected levels, especially in out-bred populations used in experiments. The problem is further compounded by generally small sample sizes due to space and budget constraints for large animal research. Host genotype and heterogeneity in cell populations due to sampling errors also contribute to variations in cytokine profiles, which makes direct comparisons between published reports difficult if not impossible. Using homogeneous cell populations enriched by LCM or flow cytometry and cell sorting is recommended. A significant amount of data in cytokine responses have also been accumulated from non-cattle host-parasite systems, such as in *H. contortus*-infected (Lacroux et al., 2006; Terefe et al., 2007; Rowe et al., 2008) and *Trichostrongylus colubriformis*-infected sheep (Pernthaner et al., 2006; Ingham et al., 2008). These studies may be valuable in defining host immune responses, characterizing cytokine profiles and providing insight into protective immunity and mechanisms of host resistance. Cattle are also one of several natural hosts of these parasitic nematodes. However, due to the scope of this chapter, the data obtained from those non-cattle hosts are excluded from discussion.

CELL ADHESION MOLECULES

Cell adhesion molecules, including lectins, cadherins, integrins, neural cell adhesion proteins, and some members of proline-rich proteins, play essential roles in mediating cell-cell adhesion and interaction in virtually every aspect of a multicellular organism's life, including cell proliferation and differentiation, tissue construction and wound repair, embryonic development, pathogen recognition and host defense. The interaction of the cell with its surroundings profoundly influences cell shape, strength, flexibility, motility and adhesion. Lectins comprise a ubiquitous family of extracellular glycoproteins which bind cell surface carbohydrates specifically and reversibly, resulting in the agglutination of cells (Drickamer and Taylor, 1993). Lectins are important activators of immune responses, and also mediate agglutination and mitogenic stimulation of lymphocytes at sites of inflammation (Lasky, 1991). Among the 4 groups of extracellular lectins, galectins and Ca^{++} -dependent lectins (C-type lectins) are the most important in host responses to parasite infection. Galectins are widely expressed, tightly regulated and play a number of roles in diseases associated with cell-cell interactions. For example, certain galectins are associated with inflammation at sites of infection and bind to cell surface IgE molecules. Collectins, members of the C-type lectin family, act as sentinels of innate immunity via their capability to recognize pathogen-associated molecular patterns (PAMPs) on foreign organisms (Gupta and Surolija, 2007). Upon recognition of PAMPs, collectins inhibit infection by direct opsonization, neutralization, agglutination and phagocytosis, as well as acting as modulators of inflammatory responses as well as apoptosis and activating certain pathways in innate immunity such as the complement system. Indeed, the plasma concentration of conglutinin is found to be genetically determined and has a negative correlation with infection frequency, suggesting that low levels of conglutinin predispose cattle for infectious diseases (Holmskov et al., 1998). Several collectins have been identified in the bovine genome, including SP-A (SFTPA), SP-D (SFTPD), MBL-A (MBL1) and bovidae-specific collectins, such as conglutinin (COLEC8), CL-43 (COLEC9) and CL-46 (COLEC13). A novel conglutinin-like gene (COLEC14) has been described recently (Gjerstorff et al., 2004). These collectins are co-localized on BTA28 at position q1.8-1.9. Homologous arrangements of collectin genes in humans, mice and cattle indicate a common ancestral origin (Gjerstorff et al., 2004).

Our recent results demonstrate that at least five cell adhesion molecules are strongly up-regulated in the bovine small intestine during *C. oncophora* infection (Li and Gasbarre, 2009). These molecules include the $\alpha 4$ integrin (ITGA4), a molecule partially responsible for eosinophil homing (Rothenberg et al., 2001), COLEC13, CDH26, LGALS3 and LGALS15. COLEC13, which is expressed mainly in the bovine liver, thymus, mammary gland and gastrointestinal tract (Hansen et al., 2002), is strongly up-regulated in the bovine small intestine during *C. oncophora* infection starting at 14 dpi and reaching a plateau at 28 dpi. The expression of this gene along the bovine GI tract during *O. ostertagi* infection has also been investigated. As depicted in Figure 1, COLEC13 expression in the abomasal lymph node at 14dpi is largely unchanged compared to those from age-matched naïve controls. Its expression level is actually decreased in blood by *O. ostertagi* infection. Similar to what is observed in the bovine small intestine during *C. oncophora* infection, COLEC13 expression is strongly up-regulated at the sites of *O. ostertagi* infection, fundic and pyloric abomasa, and the tissues closer to the infection sites, such as the duodenum and jejunum.

Galectin LGALS15 shares sequence homology with *ovga11*, a gene recently cloned from sheep abomasa infected with *H. contortus* (Dunphy et al., 2000). This common parasitic nematode has a similar enteric life cycle as *C. oncophora*. However, the difference in expression pattern between galectin *ovga11* in the sheep abomasum induced by *H. contortus* and LGALS15 in the bovine small intestine infected induced by *C. oncophora* is obvious. Galectin *ovga11* is up-regulated by *H. contortus* as early as 2 dpi, while LGALS15 is not up-regulated until 14 dpi. In addition, adult worms are unable to induce galectin *ovga11* expression. LGALS15 remains strongly up-regulated in the bovine small intestine as late as 42 dpi, when the parasites become fully mature. Another molecule that demonstrates a similar expression profile is CDH26, whose expression is up-regulated several hundred fold as detected by real-time quantitative PCR at 28 dpi in the bovine small intestine during *C. oncophora* infection. Interestingly, molecule numbers of CDH26 are positively correlated with both the number of eosinophils and adult parasites in the tissue (Li and Gasbarre, 2009). Strong up-regulation of CDH26 at the sites of infection is also confirmed in the cattle-*O. ostertagi* system where CDH26 expression is significantly induced in both the fundic and pyloric abomasa, as well as the duodenum (data not shown). CDH26 belongs to the cadherin superfamily, which is comprised of a number of Ca⁺⁺-dependent adhesion molecules mediating cell-cell binding critical to the maintenance of tissue structure and morphogenesis. A large number of cadherins and cadherins-like genes (>80) have been identified in the human genome (Yagi and Takeichi 2000). These cadherins belong to several well-defined subfamilies such as classical cadherins (type I), desmocollins, desmogleins and protocadherins, as well as less-defined and isolated cadherin subfamilies (Nollet et al., 2000). Studies reveal that genes from different cadherin subfamilies tend to have a unique tissue or cell-type-specific distribution pattern and their expression appears to be spatially and temporally regulated during development. The unique expression patterns and gene clustering of different cadherins may reflect their functional difference. Because of their roles in cell adhesion and as signaling and regulatory molecules, cadherins have diverse functions in normal physiology, such as central nervous system or synaptic functions and diseases, such as cancers. However, the function of many cadherins and cadherins-like molecules remains unknown. Associations between certain cadherins and disease conditions have been observed, but the underlying molecular mechanism has yet to be revealed. For example, cadherin-like molecule 26 (CDH 26) is strongly up-regulated in patients with rheumatoid arthritis (Nakamura et al., 2006) and eosinophilic esophagitis, an inflammatory condition in which the wall of the esophagus becomes filled with large numbers of eosinophils (Blanchard et al., 2007). Most recently, two functional estrogen response elements (ERE) were identified in the 20 kb promoter region around the transcription start site (TSS) of CDH26; and its regulation is controlled by ER α (Bourdeau et al., 2008).

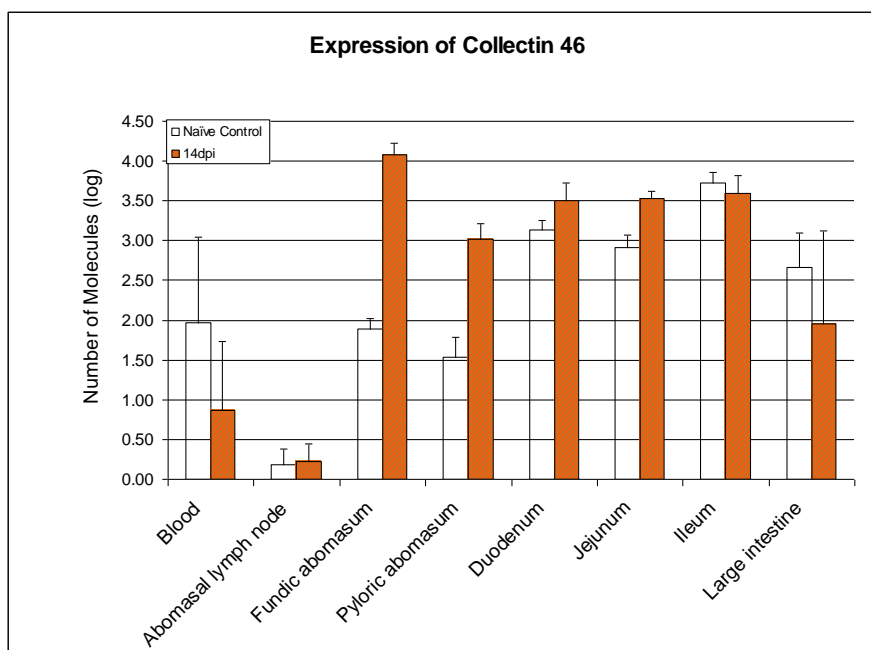


Figure 1. Expression of collectin 46 (COLEC13) along the GI tract during *Ostertagia ostertagi* infection detected by real-time quantitative PCR. The samples were taken from 3-months-old bull calves infected with 100,000 L3 larvae of *O. ostertagi* for 14 days. Age-matched, uninfected animals (naïve) served as controls. Molecule numbers (copy numbers) of collectin 46 per 100ng of total RNA were first log₁₀ transformed. Mean log₁₀ molecule numbers ($N = 3$) were presented with SEM as error bars. The difference of COLEC13 mRNA levels between the infected (14dpi) and naïve animals in the fundic abomasum ($P < 0.001$), the pyloric abomasum ($P < 0.01$), and jejunum ($P < 0.05$) was statistically significant based on unpaired t -test.

In summary, similar overall expression patterns in response to nematode infection suggest that cell adhesion molecules may indeed be involved in the recognition of carbohydrate moieties on the cell surface of nematodes in ruminants. These molecules, along with parasite-specific carbohydrate moieties, could be exploited in vaccine development.

MUCINS AND MUCIN BIOSYNTHESIS

As a group of high molecular weight glycoproteins consisting of a mucin peptide backbone and *O*-linked oligosaccharides, mucins are the major protein components of the protective mucus barrier that covers epithelial surfaces in the GI tract, which represents the first line of defense against pathogens. At least 13 membrane-associated and 8 secretory mucins have been cloned in humans (Voynow et al., 2006; Hatstrup and Gendler 2008). Mucins have a broad range of biological functions. As major components of mucus layers, mucins are the major contributor to the viscoelastic property of mucus and participate in protection from proteases. Successful penetration of the mucus barrier allows pathogens to invade the host. Mucins possess molecular properties for optimum binding and trapping of inhaled bacteria and particles for lung clearance (Knowles and Boucher 2002). Mucins also play an important role in several cellular signaling pathways. For example, mucin 1 (MUC1)

functions as an oncoprotein and activates fibroblast growth factor (FGF) and receptor (FGFR) signaling (Ren et al., 2006). MUC1 also affects EGFR- and ERK-signaling pathways and plays an important role in tumorigenesis and cancer metastasis. In addition, mucins are involved in immune functions and inflammation. Membrane-bound mucins serve as ligands for many cell adhesion molecules involved in cell-cell interaction (Tsuboi and Fukuda, 2001). The host could alter the structure of their mucins as a form of active immune responses against pathogens. Mucins and their roles in normal human physiology and diseases have been well reviewed (Callaghan and Voynow, 2006; Thai et al. 2008).

Mucins have long been recognized to play significant roles in host-parasite interactions. Gastro-intestinal nematode infection has been shown to induce goblet cell hyperplasia and mucus hypersecretion. Expression of mucin 2 (MUC2) and trefoil factor family 3 (TFF3) at mRNA levels is up-regulated as early as 2 to 4 dpi in rat intestinal epithelium during *Nippostrongylus brasiliensis* infection, suggesting that these genes may be associated with an early protective response (Yamauchi et al., 2006). The expression of these two genes is induced similarly in both euthymic and athymic rats, indicating that the regulation of these genes is independent of thymus-derived T cells (Kawai et al., 2007). Immune-mediated changes in secretion of gastric mucins are responsible, at least partially, for expulsion of these intestinal nematodes. Enhanced goblet cell hyperplasia and increased mucin production are also observed in rat-*Hymenolepis diminuta* (Webb et al., 2007), sheep-*H. contortus* (Newlands et al., 1990), and mouse-*Trichinella spiralis*, as well as in many other host-parasite systems (Theodoropoulos et al., 2001). Mucins and their roles in innate immune response in cattle during nematode infection have only recently been characterized (Li and Gasbarre, 2009). We have examined cell-specific expression of mucins and enzymes involved in *O*-linked glycosylation in mucin biosynthesis at mRNA levels using LCM in the Cattle-*Cooperia oncophora* system. At the mRNA level, MUC 2 expression is significantly higher in both the lamina propria and goblet cells at 28 dpi compared to naïve controls. MUC5B expression at the mRNA level is also higher in the lamina propria in infected animals. Expression of MUC1, MUC4, MUC5AC and MUC6 is extremely low or not detectable in goblet cells, columnar epithelial cells and the lamina propria from both naïve control and infected animals. Among the enzymes involved in post-translational *O*-link glycosylation of mucins, glucosaminyl (N-acetyl) transferase 3, mucin type (GCNT3) mRNA expression is up-regulated in goblet cells, columnar epithelial cells, and the lamina propria as well as the whole small intestine tissue during the course of infection. Western blot analysis reveals that MUC2 glycoprotein is strongly induced by infection in both the gross small intestine tissue and its mucosal layer. In contrast, the higher MUC5B protein expression is observed only in the mucosal layer. MUC1 expression is also examined in resistant and susceptible Nelore cattle infected with *C. punctata* (Bricarello et al. 2008). MUC1 mRNA level is relatively higher in whole small intestine samples of susceptible cattle. However, cells specialized in mucin biosynthesis (goblet cells, mucosal epithelial cells, or submucosal glandular cells) account for only a small fraction of total cells in the whole small intestine. Thus, mRNA expression detected using mixed cell populations from whole tissues may not reflect the true picture of mucin biosynthesis. GCNT3 and COLEC13 share a very similar expression pattern in the bovine GI tract at 14 dpi by *O. ostertagi* (Figure 2). GCNT3 expression is strongly up-regulated in the fundic abomasums during *O. ostertagi* infection.

Both galectins (LGALS3 and LGALS15) and MUC2 are up-regulated in the bovine GI tract during *C. oncophora* infection. The relationship between these two classes of genes may

not be coincidental. MUC2 is one of the major ligands for LGALS3 (Bresalier et al., 1996; Dudas et al., 2002). Alterations in LGALS3 and MUC2 expression have been correlated with malignant behavior of colon cancers. LGALS3 modulates expression of its own ligand MUC2 (Dudas et al., 2002) and up-regulation occurs at the level of transcription through AP-1 binding sites in the MUC2 promoter region (Song et al., 2005). It would be intriguing to understand LGALS3-MUC2 relationships and their roles in host-parasite interactions.

PERTURBATION IN PATHWAYS AND REGULATORY NETWORKS

High-throughput genomic technologies for gene expression analysis, such as high density microarrays and sequencing-based tools such as SAGE (serial analysis of gene expression) or mRNA-seq, have been used to identify differentially-regulated genes or depict changes in expression patterns across different classes of biological samples and/or experimental conditions. While these tools effectively organize experimental samples that are biologically-similar based on their significant gene lists or genes that are co-expressed and may be functionally-related, they provide limited insight into biological pathways underlying changes in gene expression patterns. Holistic analysis of pathways and regulatory networks help us gain insight into mechanisms underlying physiological and pathological processes and understand biological implications of observed molecular events.

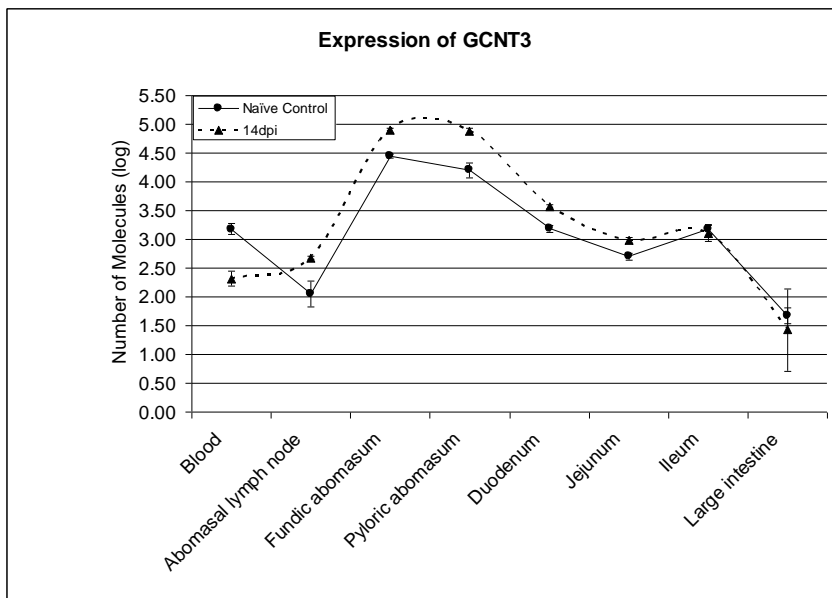


Figure 2. Expression of glucosaminyl (N-acetyl) transferase 3, mucin type (GCNT3 in the bovine GI tract during *Ostertagia ostertagi* infection detected by real-time quantitative PCR. The samples were taken from 3-months-old bull calves infected with 100,000 L3 larvae of *O. ostertagi* for 14 days ($N=3$). Age-matched uninfected animals (naïve) served as controls. Molecule numbers (copy numbers) of GCNT3 per 100ng of total RNA were first log10 transformed. Mean log10 molecule numbers ($N=3$) were presented with SEM as error bars. The difference of GCNT3 mRNA levels between the infected (14dpi) and naïve animals in the fundic abomasums was statistically significant ($P < 0.05$) based on unpaired t -test.

Pathways and regulatory networks impacted in the bovine small intestine during *C. oncophora* infection have been systematically analyzed recently (Li and Gasbarre, 2009). Temporal shifts in expression patterns during the course of infection are apparent. Parasite infection affects host immune responses significantly at all four time points tested, but underlying mechanisms are rather different. At the early stages of infection, the major immune responses include leukocyte homing and complement activation. Infection by *C. oncophora* influences two pathways in the host: the acute phase response and the complement system, as indicated by the down-regulation of key genes involved. As the infection progresses, immune responses shift to neutrophil release as well as migration of dendritic and natural killer cells. At 42 dpi, some of the dominant immune mechanisms include granulocytes-trafficking and eosinophil-rolling and egression. Coinciding with these different mechanisms of immune responses, different sets of genes linked to inflammation, such as ITGA4, are regulated during the course of infection.

Besides immune responses, lipid metabolism is also significantly ($P < 0.05$) regulated at all four time points during *C. oncophora* infection. At 7 dpi, the genes regulating two major classes of molecules, cholesterol and retinoic acid, such as serum-amyloid A-like (SAA1) and mannosidase alpha-like 1 (EDEM1), as well as squalene epoxidase (SQLE), are significantly impacted. At 14 dpi, the top canonical pathway related to lipid metabolism impacted by infection is sphingolipid metabolism. Fatty acid transporter SLC27A6, a gene involved in the uptake of polyunsaturated fatty acids (PUFA), linoleic acid and oleic acid, is down-regulated. At 28 dpi, the majority of the pathways impacted are related to lipid metabolism, including phospholipid degradation, eicosanoid signaling and metabolism of arachidonic acid, linoleic acid metabolism and glycerophospholipid, as evidenced by the up-regulation of the key genes in the pathways, such as arachidonate 5-lipoxygenase (ALOX5) and arachidonate 15-lipoxygenase (ALOX15). In addition, membrane-bound or lipid raft-associated phospholipids are beginning to be degraded to form arachidonic acid (a PUFA). Subsequently, eicosanoids, such as leukotrienes and prostaglandins, which are known to induce inflammation, are generated. By 42 dpi, the gene responsible for biosynthesis of triacylglycerol (DGAT2) is down-regulated. Because phospholipids in host cells are utilized to synthesize eicosanoids and enhance inflammatory responses during *C. oncophora* infection, it is possible that the lipid balance in the bovine gastrointestinal tract during *C. oncophora* infection is disrupted and that lipids, especially PUFA, are in short supply. Because of this, supplementary PUFA could enhance host immune responses. PUFA, especially those in omega-3 (n-3) and omega-6 (n-6) families, such as docosahexaenoic acid (DHA), arachidonic acid and linoleic acid, have long been known to have strong immunomodulatory effects (Hwang, 2000). Dietary supplements of these molecules have been shown to alter lymphocyte functions in rats. It has been suggested that omega-3 PUFA may serve as a potent inhibitor for Th1 response (Wallace et al., 2000). In ruminants, the effect of dietary PUFA (fish oil) on calves infected with both *O. ostertagi* and *C. oncophora* has been investigated (Muturi et al., 2005). Total fecal egg counts after the infection are decreased 24% by omega-3 PUFA treatment. More importantly, the number of intestinal immature parasites is significantly higher in the infected group fed with fish oil, suggesting that omega-3 fatty acids may indeed enhance protective immunity against *C. oncophora* by affecting worm development, resulting in an inhibition of L3 maturation and a reduction in egg production.

Several regulatory networks have been identified during *C. oncophora* infection (Li and Gasbarre, 2009). These networks are generally associated with certain predominant molecular

functions. The major cellular functions identified at 28dpi are depicted in Fig. 3 and include cell-cell signaling and interaction, inflammation and cell death.

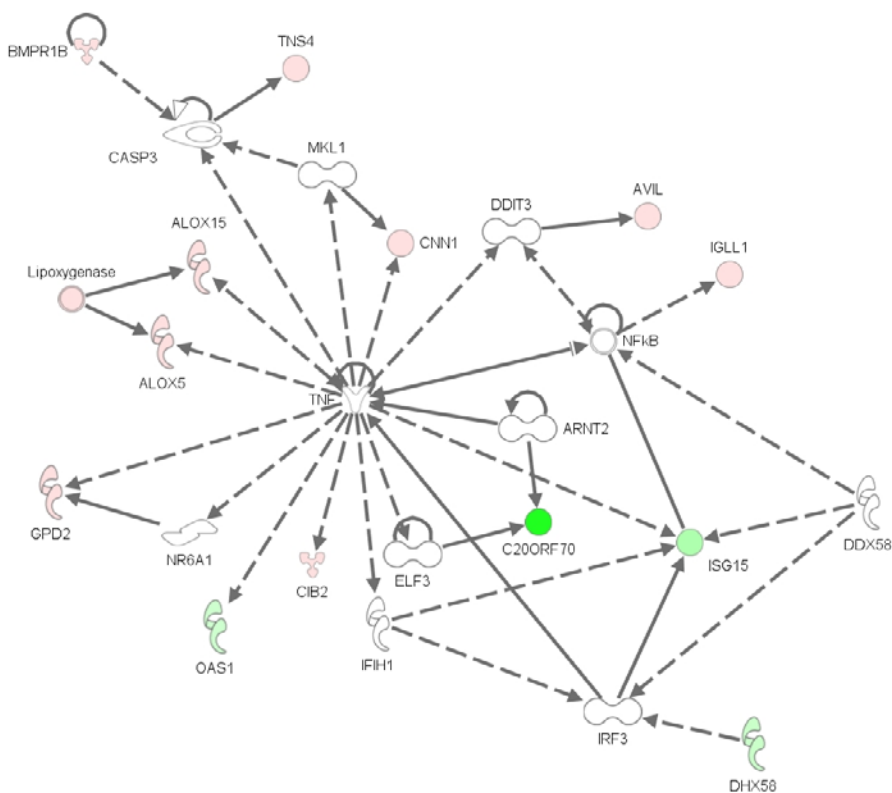


Figure 3. One of three major regulatory networks identified in the bovine small intestine at 28 days post-infection by *C. oncophora*.

In conclusion, parasitic nematode infection elicits drastic changes in gene expression patterns in host cells. The types of host immune responses are nematode species-dependent and cytokine profiles are associated with specific infection patterns. Certain nematodes, such as *H. contortus* and *C. oncophora*, seem to induce an unequivocal Th2 immune response whereas, in the cattle-*Ostertagia* system, the Th1 and Th2 paradigm may not be as clearly delineated as in murine models. While more direct evidence is still needed, it seems clear that cell adhesion molecules, such as galectins and collectins, may be involved in recognition of pathogen-associated molecular patterns in gastrointestinal nematodes. The evidence we have recently gathered suggests that local complement activation may be involved in the development of long term protective immunity against *Ostertagia ostertagi* in cattle. Mucins may play a significant role in host-parasite interactions in cattle and that immune-mediated changes in mucin secretion are responsible, at least partially, for the expulsion of intestinal nematodes. In addition, perturbations in regulatory networks and pathways during the course of *C. oncophora* infection in the bovine small intestine may have a profound impact on host-parasite interactions. The host activates different mechanisms of immune responses at various time points post-infection in response to changes in parasite cells as the parasite life cycle

progresses. Gene expression data will undoubtedly help us gain insight into mechanisms of protective immunity against gastro-intestinal nematode infection in cattle.

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Chapter VII

**AN UPDATE ON *LIBYOSTRONGYLUS*,
A GASTRO-INTESTINAL
NEMATODE OF OSTRICHES**

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ABSTRACT

Ostriches are commercially-reared mainly in South Africa and are spreading rapidly all over the world. Among the factors that lower the productivity of this poultry are endo- and ectoparasites. In this aspect, the nematode of the *Libyostrongylus* genus deserves attention. Although this parasite has been described for a long time (since 1882), only recently attention has been given to this genus, and a new species was identified in 1995. The occurrence of this new species has been neglected in the word probably due to its small dimension, less known diagnostic methods, and lack of knowledge on how to collect and differentiate the species. This article reviews the *Libyostrongylus* genus as a gastro-intestinal pathogen, principally its characteristics, distribution worldwide, control, biology, diagnosis, and future perspectives.

Keywords: *Libyostrongylus douglassii*, *Libyostrongylus dentatus*, *Struthio camelus*, Nematoda, Trichostrongylidae, world distribution, and diagnosis.

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1. INTRODUCTION

The nematode *Libyostrongylus* (“wireworm”) belong to the *Trichostrongylidae* family and is one of the most important pathogens for ostriches. This parasite produces a disease known as “vrootmag”, or “rotten stomach”, causing high mortalities among juvenile ostriches (Nel, 1980; Reinecke 1983; Sotiraki et al., 2001) and, occasionally, adults (Jansson & Christensson 2000, cited by Ponce Gordo et al 2002). Three species of *Libyostrongylus* have been described: *Libyostrongylus douglassii* (Cobbold, 1882) Lane, 1923, *L. magnus* Gilbert, 1937, and *L. dentatus* Hoberg, Lloyd, and Omar, 1995. *L. douglassii* is reported as being the most common; however, lack of knowledge on species diagnosis suggests that this might not be true. *L. magnus* has been reported in the Ukrain from Ethiopian ostriches (Gilbert 1937), and, since then, *L. douglassii* has been reported in most of the countries that are raising ostriches (Hoberg, et al, 1995; Ponce Gordo, et al, 2002, Huchzermeyer, 1998; Barton & Seward, 1993), and *L. dentatus* was only described in the USA (Hoberg et al., 1995) and recently reported in Brazil (Bonadiman et al., 2006, Ederli et al., 2008a; Ederli et al., 2008b, Ederli & Oliveira, 2008) where mixed infections were reported.

Although *Libyostrongylus* pathology has been said to be caused by *L. douglassii* (Soulsby, 1982; Reinecke, 1983; Malan et al., 1988; Huchzermeyer, 1998; Pennycott & Patterson, 2001; Bastianello, et al. 2005), there is no available data to determine if *L. dentatus* or *L. magnus* might also damage the infected tissue. Because mixed infection was reported recently as common, a suggestion has been proposed that some clinical manifestations attributed to *L. douglassii* may actually be caused by *L. dentatus* or both species (Ederli et al., 2008a; Ederli et al., 2008b). More recently, information concerning *L. dentatus* has been generated (Bonadiman et al., 2006; Ederli et al., 2008a; Ederli et al., 2008b; Ederli & Oliveira 2008). However, the role and pathogenicity of these species remain to be determined. Thus, this review will focus on *L. douglassii* and *L. dentatus*. However, most of the information concerning the biology of this genus is said to be from *L. douglassii*.



Figure 1. Eggs of *Libyostrongylus*. Bar: 20µm.

2. LIBYOSTRONGYLUS PARASITE

2.1. Morphology of *L. douglassii* and *L. dentatus*

The eggs from the *Libyostrongylus* genus are similar in size and structure to other strongyliform; thus, it cannot be used for diagnosis of the genus or species (Figure 1). However, differences in infective larvae can be used to differentiate both species (Ederli et al., 2008a). Both infective larvae have at their tail tip a peculiar spiny knob that allows the differentiation of the genus (Figures 2a–d). *L. dentatus* infective larvae possess a tail with filamentous termination (Figures 2a, c and e), while *L. douglassii* have an acute tail ending (Figures 2b, d and f). Infective larvae of *L. douglassii* have a higher total length when compared to the *L. dentatus*. However, a more secure significance value to differentiate both larvae is the ratio of the total length by the length of the sheath tail (Ederli et al., 2008b).

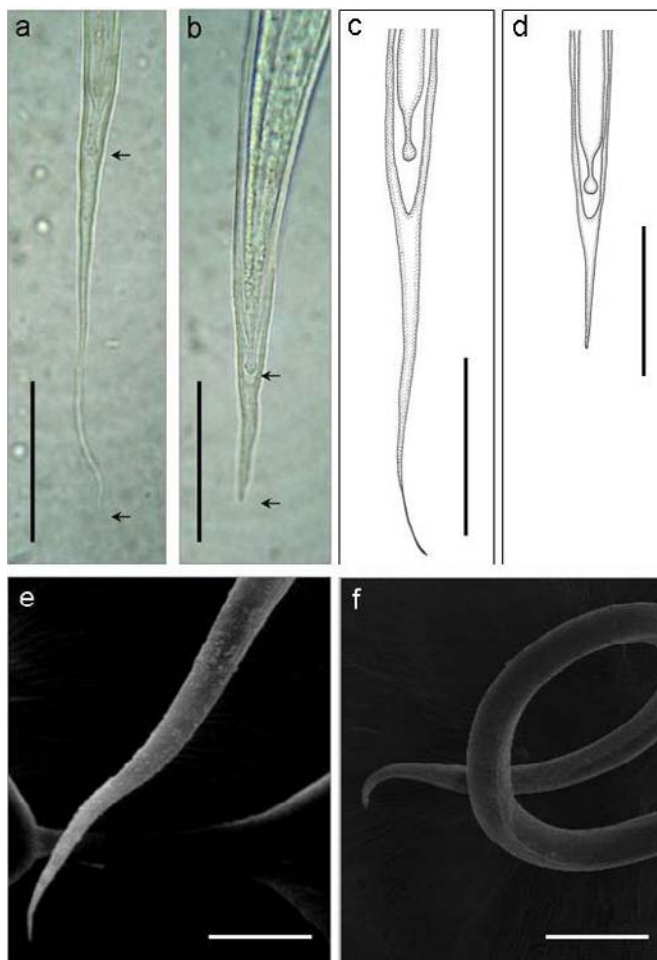


Figure 2. Infective larvae of *Libyostrongylus douglassii* and *L. dentatus*. (a and b) Light microscopy; (c and d) hand drawings; (e and f) scanning electron microscopy. a, c, and e: *L. dentatus*. b, d, and f: *L. douglassii*. Arrows indicate the beginning of the sheath tail. Bar: 30 mm. Ederli et al., (2008b)

The morphological differences of adults from *L. douglassii* and *L. dentatus* has been established by Hoberg et al. (1995) and complemented by Ederli et al. (2008a). Briefly, the adult nematodes of *L. douglassii* have no esophageal tooth (Figure 3A); females present a short ovejector (Figure 3B) and a rounded tip tail with no cuticular inflammation (Figure 3C). Males have spicules with a main shaft ending in a point (Figure 3D). A prominent esophageal tooth at the cephalic extremity is found in *L. dentatus* (Figure 1E), thus deriving its name (Hoberg et al., 1995). Differently from *L. douglassii*, females have a long ovejector (Figure 3F) and a tail with prominent cuticular swelling at the anus and a digitate tip (Figure 3G). Males have the main shaft of their spicules terminating in a rounded point with a hyaline sheath (Figure 3H) (Ederli et al., 2008a).

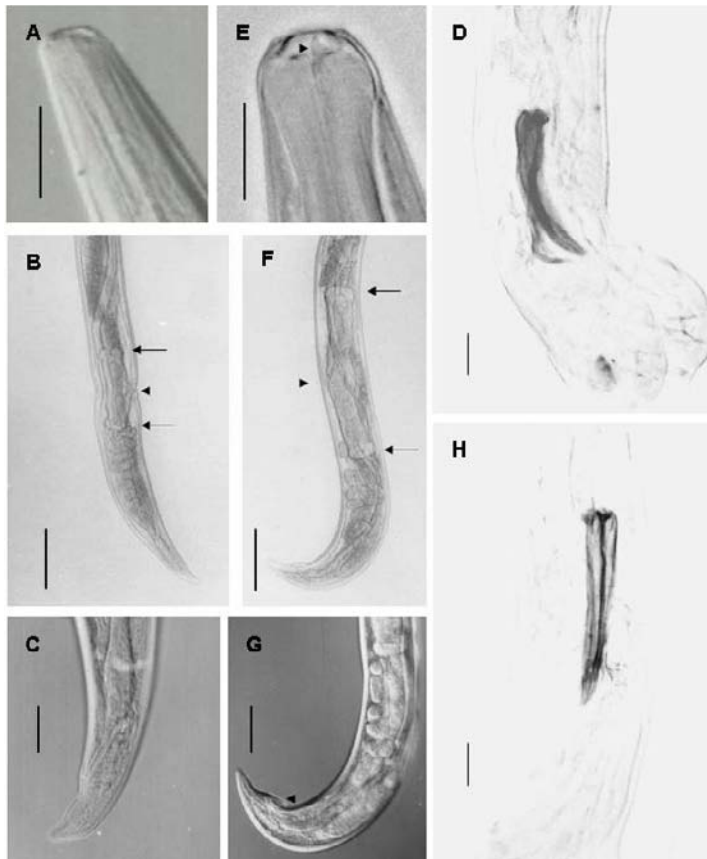


Figure 3. Images by differential interference contrast microscopy of *Libyostrongylus*. (A–D) *L. douglassii*. (A) Cephalic extremity showing the absence of prominent esophageal tooth; Bar: 50 mm; (B) Short ovejector showing region of sphincters and vestibule (between arrows) and position of vulva (arrowhead); Bar: 260 mm; (C) Female tail lacking cuticular inflation with rounded tip; Bar: 30 mm. (D) Spicules showing main shaft and pointed end; Bar: 40 mm. (E–H) *L. dentatus*. (E) Cephalic extremity showing prominent esophageal tooth (arrowhead); Bar: 30 mm. (F) Relatively long ovejector showing region of sphincters and vestibule (between arrows) and position of vulva (arrowhead); Bar: 260 mm. (G) Tail in female with prominent cuticular swelling at level of anus (arrowhead) and digitate tip; Bar: 30 mm. (H) Spicules with main shaft with rounded tip and hyaline cap at apex of primary shaft; Bar: 35 mm. Ederli et al., (2008a).

Scanning electron microscopy confirmed that *L. dentatus* have a buccal orifice with an oval shape, thick lips, four externo-labial papillae, six round cephalic papillae with two pairs near the lateral amphids, and dorsal and ventral notches (Figure 4A). As described above, this species has a tail, which is strongly curled ventrally with a rounded digitate tip and a ventral cuticular inflammation at the level of the anus (Figure 4B). For *L. douglassii*, scanning electron microscopy confirmed a buccal orifice with an oval shape, no apparent tooth, fine lips, six lengthened cephalic papillae with two pairs near the lateral amphids, and four externo-labial papillae (Figure 4C). It was observed that the tail of this species is curled ventrally, lacks distension of the cuticle at the level of the anus, and has a simple rounded tip (Figure 4D) (Ederli et al., 2008a). These authors also reported that males and females of *L. dentatus* are larger (4954 and 9347 μm , respectively) than those of *L. douglassii* (3411 and 4229 μm , respectively).

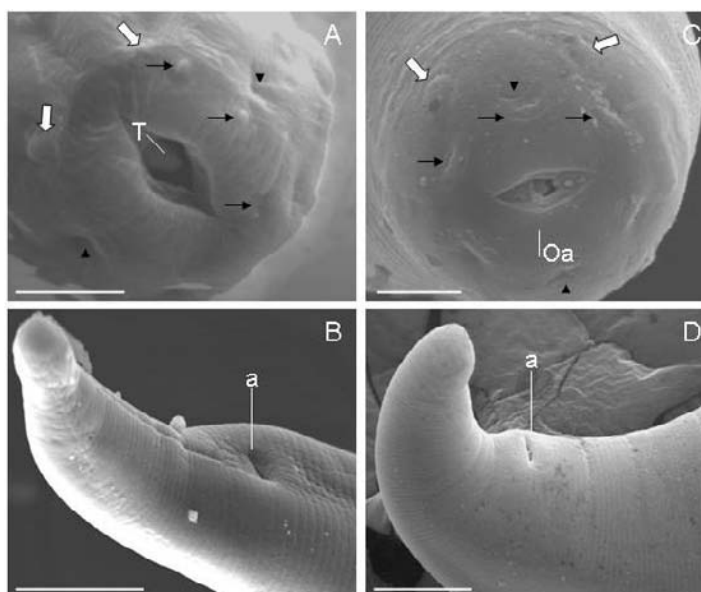


Figure 4. Scanning electron microscopy of *Libyostrongylus*. (A and B) *L. dentatus*. (A) Apical view of the female cephalic end showing outer papillae (large arrows), inner papillae (arrows), amphids (arrowheads) and esophageal tooth (T). (B) Ventrolateral view of the caudal end of a female with prominent cuticular swelling at the anus (a) level and digitate tip; Bars: 5 and 20 mm. (C and D) *L. douglassii*. (C) Apical view of the female cephalic end, showing outer papillae (large arrows), inner papillae (arrows), amphids (arrowheads) and the oral aperture (Oa) centrally located. (D) Tail in female with rounded tip lacking cuticular inflation. Anus (a). Bars: 5 and 20 mm Ederli et al., (2008a).

2.2. Life Cycle

Nematodes of the *Trichostrongylidae* family are localized at the gastro-intestinal tract of mammals and birds with similar life cycle (Dunn, 1978). They are direct, rapid and include only the host and the pasture. The cycle is classically divided in two parts: a free living and a parasitic stage. The free living stage occurs at the pasture and is characterized by development of the eggs until infective larvae. The parasitic stage begins with the ingestion of the larvae by the vertebrate host. The larvae evolves until it reach the adult stage, copulation

occurs, and egg production begins. Most trichostrongyles mature and start egg production about 3 weeks after infection. The period between the infection of an ostrich (ingestion of infective larvae) and the first egg production by adult female is called the pre-patent period (Charles, 1992). In *L. douglassii* the pre-patent period is approximately 33 days (Theiler & Robertson 1915), which is long for a Trichostrongylid. Because this period is different for the species of this family, it might differ between *L. dentatus* and *L. magnus*. The life cycle is shown in Figure 5.



Figure 5. Life cycle of *Libyostrongylus*.

2.2.1. Free Living Stages

Being from an ostrich, a bird from arid zones, *Libyostrongylus* is an extremely resistant nematode. Under dry conditions, the infective larvae develop from eggs in approximately 3 days (Theiler & Robertson, 1915; Soulsby, 1982). *Libyostrongylus* eggs in the feces are quite resistant to climatic conditions (hot, dry, and freezing). Eggs can also survive desiccation for 3 years (Theiler & Robertson, 1915). Eggs hatch into the first larval stage (L₁), which feeds on bacteria found in feces. Later, L₁ enter into lethargy, preparing themselves for the first change emerging as second-stage larval (L₂). The process of feeding and growing is repeated, followed by lethargy and change to the third stage larval (L₃), which is the infective larvae. In this larval stage the cuticle of the L₂ does not shed and remains surrounding the larvae. The habits of the larvae are different from the first two stages. Its cuticle does not allow it to feed, thus, it uses the nutrient reserve in the form of granules stored in intestinal cells (Dunn, 1978). This larval stage remains viable under dry conditions for 9 months or longer (Theiler & Robertson, 1915). This happens at minimum temperatures of 7 to 10° C and maximum of 37° C. Under optimal conditions, at 36°C, development to the infective larvae occurs in about 60 hours (Barton & Seward 1993; Jansson et al., 2002). Infective larvae will climb the blades of grass in moisture films (Dunn, 1978). As described above this stage is very resilient, allowing great chance to be ingested by an ostrich.

2.2.2. Parasitic Stages

Infection of an ostrich is by the ingestion of pasture containing the third larvae stage. After 4 to 5 days, the fourth larvae stage (L₄) is produced in the proventriculus. The molt from the fourth to the fifth stage occurs at about 20 days, and eggs are produced after 33 days of infection (Theiler & Robertson, 1915).

At the proventriculus, the third larvae stage penetrates deeply into secretory glands and originates L₄, which emerges as young adults that later become mature adults. Adults live on the surface where they suck blood and cause severe inflammation (Soulsby 1982; Barton and Seward 1993; Craig & Diamond 1996). It has been described that *L. dentatus* and *L. douglassii* localizes differently at the proventriculus, respectively inserted in the koilin layer with a reeled formation and at the inner surface of the koilin layer (Ederli et al., 2008b; Ederli & Oliveira 2008). This difference in localization suggests that the pathology of both species may be different. New studies are necessary to further investigate this possibility. The adult worms copulate and produce eggs. An infected bird might produce up to 3 million parasite eggs per day (Theiler & Robertson 1915).

3. DISTRIBUTION AND PREVALENCE

Libyostrongylus has been stated to occur only in ostriches (Craig & Diamond, 1996). However, its pathogenicity for other ratites has not been systematically investigated. Nonetheless, a work done in Sweden reported the occurrence of *Libyostrongylus* in Emus (*Dromaius novaehollandiae*) (Jansson & Christensson 2000, cited by Ponce Gordo et al 2002). Ostriches's importation from African countries to other continents, as well as the mixing of ostriches of different subspecies, has lead to *Libyostrongylus* spreading worldwide, as well as the development of mixed species wireworm infections (Huchzermeyer, 1998).

Libyostrongylus infections have been recorded in ostriches in several countries in Africa (Cobbold, 1882), North America (Hoberg et al., 1995), Europe (Ponce Gordo, et al., 2002), Australia (Huchzermeyer, 1998; Barton & Seward, 1993; McKenna, 2005), and, more recently, in Brazil (Bonadiman et al., 2006; Ederli et al., 2008a; Ederli et al., 2008b; Ederli & Oliveira 2008). The first finding of this parasite in Europe was in 1998 from ostriches raised in Spain (Ponce Gordo et al., 2000, cited by Ponce Gordo, et al., 2002). This parasite has been reported in Portugal, Belgium, and The Netherlands (Ponce Gordo et al., 2002). The nematode has also been identified in ostriches raised in Scotland (Pennycott & Patterson, 2001). Studies show that the infective larvae of *L. douglassii* probably have the ability to remain viable on pastures during winter in Sweden (Jansson et al., 2002). However, this is probably an underestimation of its distribution range, and it may increase as further investigations are carried out.

In the north region of the state of Rio de Janeiro, Brazil, the parasite is well-adapted. The strongiliform eggs were detected in five of the six farms inspected and the prevalence reached 100% (Bonadiman et al., 2006). On 12 farms investigated in eastern Australia, the prevalence was 33% (More, 1996). In Zimbabwe, eggs of *L. douglassii* were detected in samples from breeder stock in eight of 11 farms surveyed. Based on fecal culture, *L. douglassii* was detected in 49, 9% breeder stock samples and 100% of the farms tested were positive (Mukaratirwa et al., 2004). *Libyostrongylus* infection in New Zealand was also detected. Sixteen farms were identified as having sent live birds to the index farm, and 20 farms were known to have received live birds from the index farm (Mackereth, 2004).

4. PATHOLOGY

All information concerning the pathology of *Libyostrongylus* is said to be caused by *L. douglassii*; however, as mentioned above, there is no available data to discriminate the pathology caused by the three species. This genus is a hematophagous parasite that invades the proventricular glands (Figure 6), causing a diphtheritic proventriculitis (Reinecke, 1983). Necropsy of ostriches with libyostrongylosis clinical signs shows proventricular stasis or impaction, characteristic necrotic proventriculitis, and subsequent fermentation of the proventricular contents (Malan et al., 1988). Infected birds may also develop secondary impaction of the proventriculus and gizzard, as well as secondary bacterial and/or mycotic infections of the gastro-intestinal tract (Malan et al., 1988; Huchzermeyer, 1998; Jansson & Christensson, 2000 cited by Ponce Gordo, et al, 2002).

Adult ostriches with good health can harbor high parasite burdens with no clinical signs (Barton & Seward 1993). These birds develop an immunity response against these parasites that control parasite load. However, these birds continue to shed eggs and have no apparent change in productivity. In that situation, these ostriches might have parasite burden outbreaks due to stress, and clinical manifestations may appear. Because little chicks have a non-mature immune response, high death rates are observed after the infection. Thompson and Mason (2004) stated that a satisfactory immune response probably develops when the birds reach 30-40 kg. Worm infestation may persist in untreated bird for many years, possibly for life (Smit, 1963, cited by Huchzermeyer, 1998).



Figure 6. *Libyostrongylus* attached to the ventricular mucosa after removal of the koilin layer. Bar: 1mm.

5. DIAGNOSIS

5.1. Clinical Examination

Clinical diagnosis is not well-indicated because other diseases cause similar symptoms, including other nematodiasis. Thus, anorexia, weight loss, diarrhea, and paleness of the ocular and oral mucous membranes are suggestive but not specific and should be interpreted with care. Infections with *Libyostrongylus* include these signs, with the exception of diarrhea. The symptoms can be very similar to gastric paralysis, constipation, and “megabacteriosis” (Huchzermeyer et al., 1998).

5.2. Laboratorial Examination

Definitive diagnosis of this nematode requires either microscopic examination of adult worms or the infective larvae recovered from fecal culture.

5.2.1. Feces examination: Eggs and Infective Larvae Observations

Of the egg counting methods in feces, the Gordon & Withlock (1939) method is the most simple and most widely used. This test identifies eggs that are classified according to its nematode group. Because eggs of the *Strongyloidea* superfamily are similar, only the family is identified. Therefore, the diagnosis needs to be complemented with a feces culture.

The infective larvae diagnosis is performed after fecal cultivation by microscopical analysis and identifies the genus (Ederli et al., 2008b). It was first introduced in the diagnosis of ostrich nematodes because this bird is also infected with another genus, *Codiostomum* (a Strongylidae nematode), thus, with similar egg morphology as *Libyostrongylus*. Theiler & Robinson (1915) described “a small knob on the point of the tail having a prickly appearance and resembling somewhat the fruit capsule of *Datura*” being a crucial feature in being able to distinguish the genus of the ostrich’s nematode. *C. struthionis* does not have a knob on the extremity of the larva tail (Theiler & Robinson 1915; Ederli et al., 2008c). Although *C. struthionis* has been described as non-pathogenic (Huchzermeyer, 1998b), recent work has shown lesions at the caeca where this parasite is localized (Ederli et al., 2008c). Thus, it confirms previous concerns that this species might be pathogenic (Craig & Diamond, 1996). Attention must be taken in future surveys to also determine if this nematode species is infecting the bird.

Recently, Bonadiman et al. (2006) suggested and Ederli et al. (2008b) confirmed that it is possible to differentiate morphologically-infective larvae of *L. douglassii* and *L. dentatus*. Both larval types finished their tails in a spiny knob. However, *L. dentatus* has a long sheath tail with a filamentous termination, while *L. douglassii* has a short sheath tail. This new diagnosis tool is being used to differentiate both species in a population dynamic study in the north region of the Rio de Janeiro State, Brazil (unpublished data).

5.2.2. Necropsy

Besides finding the adult, necropsy reveals typical lesions due to the infection. During necropsy, the ventriculus and proventriculus are analyzed. When the koilin layer is removed, it is easy to observe, with the aid of a stereomicroscope, *L. douglassii* at the inner surface of this membrane and at the tissue of the proventriculus. On the other hand, *L. dentatus* are preferentially inserted in the koilin layer with a reeled formation (Ederli et al., 2008b, Ederli & Oliveira 2008). Thus, necropsy must be done with care.

6. METHODS OF CONTROL

Libyostrongylus control might be curative or preventive similar to what is applied to other production animals. The curative control is used when clinical cases are detected or susceptible animals demonstrate symptoms. The preventive control aims to decrease the number of the infective larvae in the pastures. Anthelmintics are the principal elements of control and are used to prevent clinical cases, minimize sub-clinical losses, and reduce pasture contamination. Preventive programs allow a contamination threshold that induces immunity without interfering on the productivity (Charles, 1992). Application of anthelmintics, taking in consideration the epidemiological status of the region and the management care of the animals, is defined as strategic anthelmintic treatment. Application of the chemical alone or a strategic anthelmintic treatment leads to drench resistance, unless it is combined with other forms of control to reduce current dependence on anthelmintics (Barger, 1999). For that integrated control has as a principle the use of the management action by which anthelmintics is not used alone. Among these are:

Flock Management

After examining the birds and defining the prevalence and the level of parasitemia, all new animals should be quarantined for at least 5 weeks (Barton and Seward, 1993). During this period, eggs count in feces must be monitored; if the animal is negative, it can be united with the other birds. Always avoid contact of juvenile birds (born without the parasite) with infected adults or infected pasture. Units that are not infected should have restricted barriers including human transit (Barton and Seward, 1993).

Grazing Management and Use of Safe Pastures

It has been known for a long time that an efficient pasture management in livestock results in a low pasture contamination (Michel, 1985). Safe pastures can be produced and should be used by juvenile birds. Pastures that have low infective larvae or that have never had ostriches are safe. New pastures are regarded as safe by cultivation and fallow; the number of eggs and larvae that survive is greatly reduced. Another action that reduces infective larvae is to rotate different production animals between the pastures. This only works if the pasture grows to a minimal size of 8-10 cm creating the conditions that are proportionate to the migration of infective larvae to the sward. *Libyostrongylus* will not affect cattle or sheep, thus, the larvae are naturally removed by the grazing of these animals. This management must be done several times to make sure all eggs have hatched and infective larvae migrated to the top of the pasture; it is recommended from 4 to 12 months of rotation grazing (Thompson and Mason, 2004).

Grazing management might be impossible to be implemented due to small size and the lack of simultaneous production animals in a farm (Miller & Jackson, 2006). Thus, another important measure to prevent contamination of paddocks, especially the ones used by chicks, is regular egg counting and testing anthelmintics efficacy, which needs to be done by a veterinarian or other qualified technical professional. Dosage of anthelmintics should be recommended by a veterinarian and a change of the active principle should be performed after some applications. All new birds must be tested, quarantined, and treated with the anthelmintic before transport to an uninfected farm. Feeding adult feces to newly hatched chicks should not be done. If owners want to continue this practice, feces must be from *Libyostrongylus*-free birds (Button et al., 1992).

6.1. Anthelmintics Treatment

Levamisole and the benzimidazoles are effective anthelmintics against *Libyostrongylus*. Treatment with fenbendazole, levamisole, or ivermectin can be effective (Tully & Shane, 1996). Cooper (2005) suggests administration to chicks every 3 weeks until 4 months of age. However, this practice must be viewed with care since drench resistance can arise as already reported. Levamisole is no longer effective for some strains of *Libyostrongylus* (Malan et al., 1988). It has been shown that *Libyostrongylus* was not eliminated from South Africa ostriches that received levamisole at 30 mg/kg. This is apparently the first reported case of drench resistance in ostriches (Malan et al., 1988). However, due to difficulties in specifying dosages and a lack of appropriate management, resistance might be common among *Libyostrongylus*. Furthermore, a dosage of 15 mg/kg of fenbendazole was 99.7% effective against adult *Libyostrongylus*, but 82.5% against L₄ (Fockema et al., 1985). It has been reported that an

oral dose of 0.2 mg/kg of ivermectin or (Kloek & Smith, 1995 cited by Huchzermeyer, 1998) subcutaneous injection of 0.3 mg/kg is also effective against *Libyostrongylus* (Stewart, 1989 cited by McKenna, 2005). Furthermore, ostriches treated with moxidectin (Cydectin) at a dose of 0.2 mg/kg were all negative for eggs shedding (Bastianello et al., 2004).

7. CONCLUSION

Libyostrongylus have been co-imported with African ostriches, and they are now established in different countries. *L. dentatus* and *L. douglassii* found in farms from the USA and Brazil demonstrate that the mixed infection can be present in other continents besides America. This shows the importance of quarantine for animals, establishment of pasture management, and anthelmintics treatments. These parasites have a direct life cycle, thus, facilitating their transmission within and between farms. The economic impact of these parasites on ratite farming is still undetermined. Further detailed analyses are needed to determine their precise host-specific status. The population dynamic of this parasite is also lacking. Thus, further work is needed to better understand pathological aspects caused by *Libyostrongylus* and their dynamics as populations. Only after the basic biological aspects of this nematode are studied, will we be able to better prevent this disease, with further economical reflection to ostrich farming.

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Chapter VIII

GENITAL LESIONS AND VENEREAL TRANSMISSION OF CANINE VISCERAL LEISHMANIASIS

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ABSTRACT

Visceral leishmaniasis (VL) is a major zoonotic disease endemic in several regions in the world, particularly tropical and Mediterranean countries. The dog is the most important reservoir for human VL. The disease is caused by *Leishmania chagasi* in the Americas, *L. infantum* in Europe, and *L. donovani* in Southern Asia. Transmission of VL is heavily dependent on the biologic vector, which are sand flies belonging to the genus *Lutzomyia* (in the Americas) or *Phlebotomus* (in the Old World). However, several cases of VL transmission in the absence of the invertebrate vector have been documented. Although VL in the dog is characterized by a chronic debilitating disease, with splenomegaly, hepatomegaly, lymphadenopathy, and emaciation among other classical signs, a diverse range of atypical clinical manifestations has also been reported. This prompted us to study genital lesions associated with VL in the dog. Interestingly, *L. chagasi* has a tropism to the male genital system, where it is located preferentially in the epididymis and external genitalia, and is associated with epididymitis, balanitis, and posthitis. Conversely, bitches naturally infected with *L. chagasi* do not develop significant genital lesions. In addition, the organism is shed in the semen, and the natural mating of dogs shedding *Leishmania* in the semen with *Leishmania*-free susceptible bitches may result in the venereal transmission of the disease.

INTRODUCTION

Leishmaniasis is caused by protozoa of the genus *Leishmania*, family *Trypanosomatidae*, order *Kinetoplastida*. These parasites develop distinct forms during their life cycle. The

flagellated and motile promastigote and paramastigote forms develop and multiply in the gut of phlebotomine sand fly vectors, whereas the intracellular non-motile amastigote form is found within macrophages of the vertebrate host [26,30]. *Leishmania* is adapted to replicate in the sand fly gut, where it differentiates into infective metacyclic promastigotes that are capable of infecting a susceptible vertebrate host [26]. Metacyclic promastigotes transform into amastigotes soon after being internalized by a vertebrate host macrophage [26,30]. *Leishmania* is internalized and it is able to survive and replicate within the vertebrate host macrophages [8,9].

Approximately 30 different species of phlebotomine sand flies have been identified as biological vectors capable of transmitting *Leishmania* [16]. Localization of the parasite within the gut varies according to the species of phlebotomine and the strain of *Leishmania*. This feature had been used as a criterion for classification of *Leishmania* spp. [35,38]. Incrimination of a particular sand fly species as a biological vector is difficult since the presence of *Leishmania* in the gut does not necessarily mean that the insect is able to transmit the organism to a susceptible vertebrate host [23]. Five genera belonging to the subfamily *Phlebotominae* are distributed throughout the tropics and subtropics: *Sergentomyia* spp., *Plebotomus* spp., *Lutzomyia* spp., *Brumptomyia* spp., and *Warileya* spp. [23]. Two of these genera have been identified as vectors of *Leishmania*, namely *Phlebotomus* and *Lutzomyia*, which comprises the insect vectors of *Leishmania* in the Old and New World, respectively [19,28].

Leishmaniasis is associated with variable clinical manifestations, depending on the parasite and host species involved. In man, visceral, cutaneous, and mucosal forms are clinically distinct and result from replication of the parasite in macrophages in lymphoreticular tissues (i.e. lymph nodes, spleen, and liver), dermis, or naso-oropharyngeal mucosa, respectively [3]. These syndromes are caused by a total of 21 different *Leishmania* species [16]. Visceral leishmaniasis (VL), also known as kala-azar, is a major zoonotic disease caused by species grouped into the donovani complex, including *L. donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World [38].

VL is endemic in areas of the tropics, subtropics, and Southern Europe, including geographic areas ranging from rain forests in the Americas to deserts in Western Asia, and rural or urban areas. This broad distribution of the disease is associated with the distribution of the sand fly vector [16]. In developing countries, environmental changes have resulted in habitat destruction, favoring adaptation of the vector population to anthropogenic environmental condition. Therefore, VL that was originally restricted to rural and peri-urban areas has become a major public health issue in urban areas [19]. Human VL is still endemic in the Mediterranean region, and epidemic in India, Brazil, Sudan, South America, North Africa, and Central Africa [4]. Humans are considered accidental hosts, whereas the dog is the most important reservoir for human VL in urban areas [35].

Prevalence of VL in the Mediterranean region is much higher in dogs than in humans, and the disease is only sporadically reported in humans in endemic areas for canine VL [6]. In many cases, more than one mammalian species is found to be infected with a given species of *Leishmania*, making the identification of a specific reservoir host difficult. In the New World, although the domestic dog is the most important reservoir in an urban area, numerous wild and domestic animals may serve as reservoir hosts, allowing transmission of leishmaniasis in sylvatic and rural areas [1,5,15,17]. Among wild canids, there are reports of VL infection in

crab-eating fox (*Cerdocyon thous*), maned wolf (*Chysocyon brachyurus*), hoary zorro (*Lycalopex vetulus*), and bush dog (*Speototus venaticus*) [14,27].

GENITAL LESIONS AND VENEREAL TRANSMISSION OF CANINE VL

Dogs develop an intense cutaneous parasitism, favoring infection of sand flies, thereby playing an important role in the epidemiological chain of VL since the dogs act as a source of infection for blood-sucking sand flies [43]. Importantly, most dogs remain asymptomatic for long periods of time, which contribute to the maintenance and transmission of the disease [19]. Clinical manifestations of canine VL range from unapparent sub-clinical infections to a systemic disease, which is usually chronic, associated with fever, anemia, cachexia, cutaneous lesions, renal failure, hepatomegaly, splenomegaly, and lymphadenopathy [28,32,33]. Interestingly, a diverse range of atypical clinical manifestations have been reported in canine VL, such as the osteoarticular and ocular manifestations that may or may not be associated with the systemic disease [12,41].

Although vector-borne is the most common mode of VL transmission, other routes have been reported in humans, such as congenital (or vertical) and parenteral by blood transfusion, and needle sharing or laboratory accident [24,42]. Transmission by blood transfusion has also been documented in a dog [34], and there is a documented case of transmission by packed RBC transfusion [22]. Moreover, autochthonous cases of canine VL have been described in areas free of known invertebrate vectors [21,24]. In one of these reports, a prevalence of 41% in a foxhound kennel housing 112 dogs was observed in the Northeastern United States, where there is no known suitable biological vector [21]. In these cases, exposure to an alternative insect vector and direct or vertical transmissions were considered possible routes of infection [21].

In spite of a previous report of venereal transmission of human VL [42], it has only recently been experimentally demonstrated by our group that venereal transmission of canine VL in the absence of the biological vector also occurs [39]. Our previous studies have demonstrated that the *L. chagasi* has a tropism for the canine male genital system, particularly to the epididymis, prepuce, and glans penis, resulting in inflammation of these organs and shedding of *Leishmania* in the semen [18]. A very large proportion of naturally-infected dogs shed *Leishmania* in the semen as assessed by PCR detection of *Leishmania* kDNA in semen samples, but shedding is intermittent in most of the cases [39].

Naturally-infected male dogs usually do not develop gross lesions in the genital system. However, these dogs do develop microscopic changes, particularly in the epididymis, glans penis, and prepuce, with a frequency that is significantly higher than in their *Leishmania*-negative cohort [18]. These findings support the notion that *L. chagasi* has a tropism for the canine male genital system. There are several published reports of genital lesions associated with *Leishmania* infection in man [10,11,25,37], although *Leishmania*-associated genital lesions in women have not been reported, with the exception of one case of sexual transmission [42]. This tropism for the human male genital system parallels our previous findings in dogs [18,39].

Although amastigotes are often present in the testis, there is not an increase in the frequency of orchitis in association with VL in dogs. Amastigotes are observed mostly within macrophages in the interstitium in association with a lympho-plasmacytic interstitial infiltrate with mild to moderate testicular degeneration [18]. In contrast, VL in dogs is associated with a significant increase in the frequency of epididymitis, which is characterized by a lympho-histio-plasmacytic infiltrate (Figure 1) that is more intense in symptomatic than asymptomatic dogs [18]. Inflammatory infiltrate in the glans penis and prepuce of naturally-infected dogs are predominantly histiocytic, with morphological features of a granulomatous reaction in some cases. Large numbers of *Leishmania* amastigotes were observed intracellularly in macrophages associated with these lesions (Figure 2) [18].

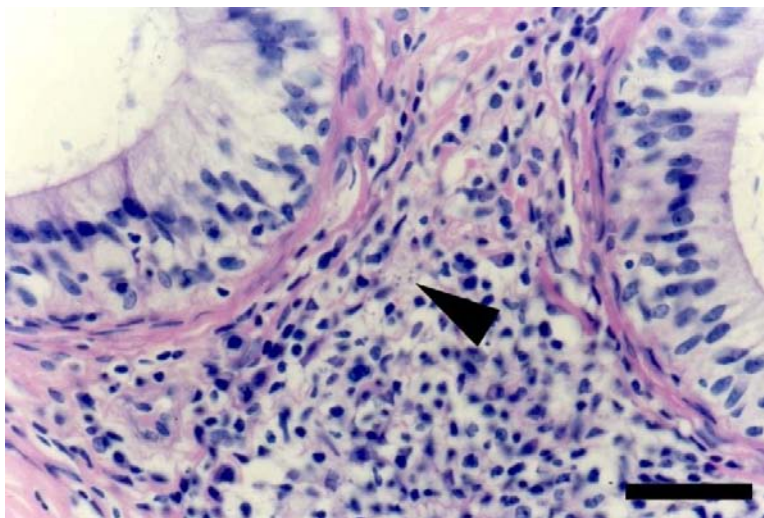


Figure 1. Dog naturally-infected with *Leishmania chagasi*. Lympho-histio-plasmacytic epididymitis with several amastigote-containing macrophages (arrowheads). Hematoxylin and eosin; bar = 40 μ m.

In contrast to male dogs, a recent study has demonstrated that *L. chagasi* does not have a tropism for the genital tract in naturally-infected bitches [40]. These results support the notion that venereal transmission in dogs is likely to be unidirectional, preferably from infected dogs to susceptible bitches [39]. Additional studies are needed to determine the source of infectious organisms during venereal transmission, i.e. whether *Leishmania* secreted in the semen or amastigotes from the male external genitalia are the source of infection for susceptible bitches. The traumatic copulatory behaviour of dogs would favour the prepuce and glans penis as a likely source of infection. Importantly, the recent study in which we demonstrated venereal transmission [39] assessed parameters of infection such as seroconversion and amplification of *Leishmania* kDNA by PCR, but not development of clinical disease. Therefore, it would be interesting to compare the infective potential of *Leishmania* transmitted through the classical vector-born route or the venereal route. Presumably vector-born metacyclic promastigotes inoculated into the mammalian host through the bite of sand flies should be more infective than sexually-transmitted amastigotes since the phlebotomine saliva contains several factors that favor infection [2,13].

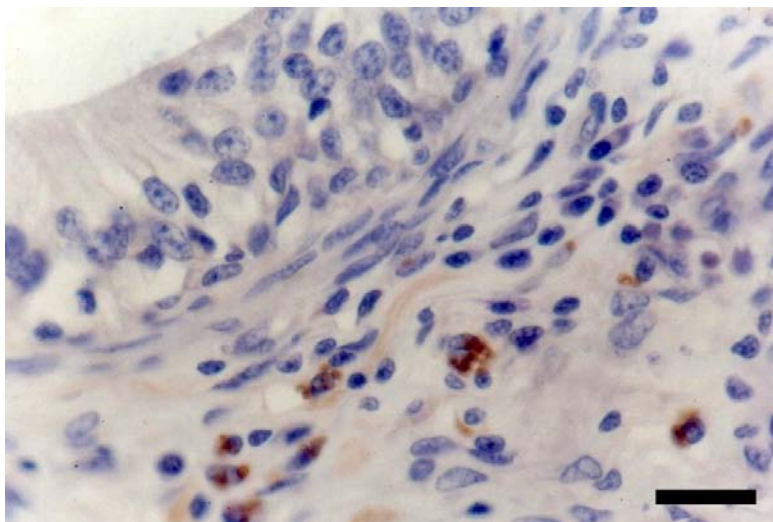


Figure 2. Dog naturally-infected with *Leishmania chagasi*. Lympho-histio-plasmacytic epididymitis with immunolabelled amastigotes associated with macrophages (brown staining). Streptavidin-biotin-peroxidase; bar = 20 μ m.

Although VL in bitches is not associated with genital lesions [39], congenital transmission of *Leishmania* sp. has already been demonstrated in an experimentally-infected Beagle whose infected fetuses were removed from the uterus by c-section, thus preventing the possibility of transvaginal transmission [36]. Abortion associated with a necrotizing placentitis with large amounts of *Leishmania* amastigotes in the placenta but not in fetal organs was also reported [20], as well as infection in newborn dogs [29]. Congenital transmission has also been demonstrated in humans [7,31] and in experimentally-infected BALB/c mice [36]. In spite of these clear indications of vertical transmission of VL in dogs, the frequency of transmission through the placenta remains unclear, as well as a possible difference in transmission potential of symptomatic and asymptomatic bitches. We are currently conducting a study to address those questions and to assess the distribution of *Leishmania* amastigotes in fetal organs and histopathologic changes associated with parasitism. Our preliminary data indicates that significant amounts of amastigotes may be detected in fetal tissues (Pangrazio and Avalos, et al., unpublished data).

CONCLUSION

In conclusion, the fact that the canine VL may be venereally and vertically transmitted in the absence of the biological insect vector should be taken into account for establishing eradication programs. The use of infected dogs for reproductive purposes should definitely be avoided. In addition, these alternative routes of transmission may have a significant impact in areas with very low prevalence of VL or under conditions where a status of eradication is achievable.

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Chapter IX

THE RELEVANCE OF *GIARDIA* INFECTIONS IN VETERINARY MEDICINE

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ABSTRACT

Worldwide prevalence studies indicate a high prevalence of *Giardia* in domestic animals, mainly in livestock and in companion animals. In mammals, *Giardia duodenalis* is the most prevalent species. Phylogenetic analysis revealed that *G. duodenalis* is in fact a species complex, comprising 7 so-called assemblages (assemblage A to G). Some of these assemblages are host-specific and others are zoonotic. In domestic animals, infections with only one assemblage are most often reported, and to a lesser extent mixed infections. Until recently, most research on *Giardia* in domestic animals has been inspired by the concern for zoonotic transmission, and not from a veterinary perspective. Despite the high prevalence and the alleged pathogenicity, *Giardia* is often neglected as a pathogen in veterinary medicine. This is mainly due to the vagueness of the clinical symptoms, ranging from growth retardation and ill thrift to more overt symptoms, such as acute or chronic diarrhea. Although several clinical studies do indicate an association between *Giardia* infection and clinical signs, other studies report the presence of *Giardia* trophozoites or cysts in fecal samples from apparently healthy animals. Whether there is a difference in clinical outcome when animals are infected with one particular assemblage or another or with multiple assemblages has not yet been determined, and certainly warrants further studies. At present, only fenbendazole/febantel is registered in dogs for the treatment of *Giardia*. Several other chemotherapeutics are efficacious, but none are registered. Whether chemotherapeutic treatment is useful in the prevention of infection is uncertain, and additional hygienic or management measures seem to be needed.

Several questions still surround the relevance of *Giardia* infections in domestic animals. Is treatment and control of giardiasis in livestock economically justified? From a

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public health point of view, should chemotherapeutic treatment be advocated to prevent environmental contamination or direct zoonotic transmission? Is vaccination a valid alternative for chemotherapeutic treatment?

Keywords: *Giardia duodenalis*, veterinary medicine, clinical symptoms, diagnosis, treatment, and control.

INTRODUCTION

Although *Giardia* has been known since its discovery by Anthony van Leeuwenhoek in 1681, the clinical relevance was not acknowledged until late in the 20th century. Since, *Giardia* is increasingly considered in human medicine as the most common parasitological cause of gastro-intestinal disorders, with an average of 280 million human infections per year. Giardiasis is also a frequently diagnosed waterborne infection and a major concern to drinking water authorities. Because of the impact on socio-economic development, especially in developing countries, *Giardia* has since 2004 been included in the 'Neglected Disease Initiative' of the World Health Organization (Savioli *et al.*, 2006).

In veterinary medicine, the increased interest in *Giardia* since the 1990s was mainly driven by public health concerns, and to a lesser extent from a veterinary perspective. Research therefore focused on parasite prevalence and on the molecular characterization of isolates from different hosts to elucidate the zoonotic hazard, as livestock and companion animals are considered as a potential reservoir for human infections. Due to this focus on transmission patterns, the clinical relevance of a *Giardia* infection in animals was somewhat neglected. The aim of the present review is therefore to present an overview of the current knowledge on *Giardia* in veterinary medicine, with emphasis on prevalence, clinical outcome, diagnosis, treatment, and control.

PARASITE LIFE CYCLE

There are two main stages in the life cycle of *Giardia*: a cyst stage which is resistant in the environment, and a trophozoite stage which colonizes the intestinal lumen of the host. After oral ingestion, the infective cysts release the trophozoite in the upper part of the small intestine. For the colonization of the duodenum and the jejunum, attachment to epithelial cells of the intestinal mucosa is essential, for which the trophozoites use a ventral adhesive disk. The trophozoites multiply by binary fission in the lumen of the small intestine, although sexual reproduction has been suggested (Meloni *et al.*, 1989). Finally, exposure to biliary salts leads to encystation of trophozoites in the jejunum. Cysts are passed in the feces and are immediately infectious upon excretion, allowing completion of the life cycle within 72h (Thompson and Monis, 2004).

PARASITE TAXONOMY

A taxonomy describing over 50 different *Giardia* species was primarily based on host specificity and was later replaced by a taxonomy based on morphological characteristics, such as shape and length of the trophozoite and median bodies (Filice, 1952). Three distinct groups or *Giardia* species were described, including *G. duodenalis* with a wide mammalian host range. Molecular characterization further revealed that *G. duodenalis* is in fact a species complex, comprising 7 assemblages (ass. A to G), some of which have distinct host preferences or a limited host range (Thompson and Monis, 2004). Next to the zoonotic assemblages A and B, several host-specific assemblages have been identified, of which assemblage E is mainly identified in production animals, assemblages C & D in dogs, and assemblage F in cats.

PREVALENCE

Giardia has worldwide been reported in production and in companion animals. In tables 1, 2, and 3, an overview of the most recent prevalence studies in different animal species around the world is provided. Overall, the prevalence reported in these studies varies considerably. In cattle, for example, the animal prevalence ranges from 9 to 73% and the farm prevalence from 45 to 100%. Although management, geographical, and climatological parameters partially account for this variation, differences in study design also need to be considered, such as the number of animals included in the study and the assay used for diagnosis. Since there is no gold standard reference test for the diagnosis of *Giardia*, the use of diagnostic techniques with specific sensitivity and specificity, might thwart comparison between prevalence studies. In calves, the prevalence estimate using three different diagnostic assays resulted in a different estimate for each assay (Geurden et al., 2004). Next to study design, the age of the animals needs to be taken into account, as in all species the prevalence peaks in young animals.

EPIDEMIOLOGY

Infection starts by oral intake of infectious cysts, and excretion of cysts via the feces is observed as soon as 3 days later. The maximum cyst excretion can be as high as 10^6 cysts per gram of feces and peaks in young animals (Nydam et al., 2001), probably due to the slow development of adaptive immunity by the host. Direct contact with an infected host, primarily a young animal, should therefore be considered as a major source of infection to susceptible hosts. The development of adaptive immunity results in an intermittent or reduced cyst excretion in older animals (Xiao and Herd, 1994; Nydam et al., 2001). Adult hosts should however not be excluded as sources of infection, since a limited number of cysts suffices for infection (Bernander et al., 2001). Furthermore, a periparturient rise of the cyst excretion has been suggested in sheep, goats, pigs, and cattle, although not conclusively confirmed (Xiao and Herd, 1994; Wade et al., 2000a; Castro-Hermida et al., 2005).

Table 1. The animal prevalence (P_A) and farm prevalence (P_F) of *Giardia* in cattle in different countries. The age of the animals, the number of animals ($\#_A$) and farms ($\#_F$) is presented along with the diagnostic assay (Diag) used in the study (IFA: Immunofluorescence assay, PCR: polymerase chain reaction or ME: microscopical examination)

Country	Diag	$\#_A$	$\#_F$	age	P_A	P_F	Reference
Dairy < 6m							
Belgium	IFA	499	100	<2.5m	22	48	Geurden et al., 2008b
Canada	IFA	386	20	<6m	73	100	Olson et al., 1997
Canada	ME	-	505	<6m	-	45	Ruest et al., 1998
Denmark	IFA	377	50	<1m	24	82	Maddox-Hyttel et al., 2006
New Zealand	IFA	715	12	<2m	41	100	Hunt et al., 2000
New Zealand	IFA	-	10	<2m	-	31	Winkworth et al., 2008
Norway	IFA	1386	136	<6m	49	93	Hamnes et al., 2006
Spain	IFA	734	60	<6m	29-57	67	Castro-Hermida et al. 2006a
USA	ME	2943	109	<6m	20	70	Wade et al., 2000b
USA	PCR	407	14	<2m	40	100	Trout et al., 2004
Vietnam	IFA	68	8	<3m	50	88	Geurden et al., 2008c
Dairy > 6m							
Denmark	IFA	518	50	1-12m	43	100	Maddox-Hyttel et al., 2006
Denmark	IFA	255	50	>12m	40	60	Maddox-Hyttel et al., 2006
Spain	IFA	734	60	>6m	25-40	67	Castro-Hermida et al. 2006a
Spain	IFA	379	60	>36m	27	97	Castro-Hermida et al. 2007
Spain	ME	199	30	<24m	26	53	Quilez et al., 1996
USA	PCR	456	14	3-11m	52	100	Trout et al., 2005
USA	PCR	571	14	12-24m	36	100	Trout et al., 2006
USA	PCR	541	14	>24m	27	100	Trout et al., 2006
Beef							
Belgium	IFA	333	50	<2.5m	45	64	Geurden et al., 2008b
Canada	IFA	193	10	<2.5m	36	100	McAllister et al., 2005
Canada	IFA	495	9	<3m	34	100	Appelbee et al., 2003
Canada	IFA	605	100	<6m	23	48	Gow and Waldner, 2006
Canada	IFA	605	100	>24m	17	69	Gow and Waldner, 2006
Canada	IFA	669	39	>24m	9	64	McAllister et al., 2005

- = not known.

Infection can also spread indirectly through a contaminated environment, such as animal housing. Animals, especially production animals, reared indoors are three times more likely to acquire infection compared to outdoor housing (Ruest *et al.*, 1998). Particularly group housing, such as stables or kennels, is considered to favor transmission. However, infections are known to spread in individual housing, too, due to the subsequent use of these facilities

without proper disinfection. Infection can also spread through utensils or animal care-takers. In general, intensive management systems with a high density of young animals, such as the dairy cattle management system or kennels, favor transmission of infection, due to the concentration of animals able to excrete high numbers of infective cysts and the continuous introduction of susceptible animals.

Similar to other protozoan parasites, several parasite characteristics facilitate infection with *Giardia*, such as the high excretion of cysts by infected animals and the low dose needed for infection. Furthermore, *Giardia* cysts are immediately infective upon excretion and do not need to sporulate in the environment. Cysts are also very resistant and able to survive for several weeks in the environment, resulting in a gradual increase in environmental infection pressure (Xiao *et al.*, 1993; Olson *et al.*, 1999; Wade *et al.*, 2000a).

Table 2. The animal prevalence (P_A) and farm prevalence (P_F) of *Giardia duodenalis* in other production animals in different countries. The number of animals ($\#_A$) and farms ($\#_F$) is presented along with the diagnostic assay (Diag) used in the study (IFA: Immunofluorescence assay, PCR: polymerase chain reaction or ME: microscopical examination, cELISA: copro-antigen Elisa)

Country	Diag	$\#_A$	$\#_F$	P_A	P_F	Reference
Sheep						
Australia	ME	1647	-	9	-	Ryan <i>et al.</i> , 2005
Belgium	IFA	137	10	36	100	Geurden <i>et al.</i> , 2008d
Canada	IFA	89	6	38	100	Olson <i>et al.</i> , 1997
Italy	ME	325	20	1.5	10	Giangaspero <i>et al.</i> , 2005
Spain	IFA	446	38	19	92	Castro-Hermida <i>et al.</i> 2006a
Spain	IFA	575	68	33	97	Castro-Hermida <i>et al.</i> 2006b
Goats						
Belgium	IFA	148	10	53	80	Geurden <i>et al.</i> , 2008d
Brazil	ME	105	6	14	66	Bonfim <i>et al.</i> , 2005
Spain	IFA	116	20	20	90	Castro-Hermida <i>et al.</i> 2006a
Spain	ME	574	-	4	-	Diaz <i>et al.</i> , 1996
Spain	ME/Elisa	315	40	42	95	Ruiz <i>et al.</i> , 2008
Pigs						
Canada	IFA	236	6	9	66	Olson <i>et al.</i> , 1997
Denmark	IFA					Maddox-Hyttel <i>et al.</i> , 2006
weaners		504	50	38	84	
piglets		488	50	3	22	
sows		245	50	4	18	
Norway	IFA	684	100	1.5	10	Hamnes <i>et al.</i> , 2007
Croatia	cELISA	-	38	-	66	Bilic <i>et al.</i> , 2006
Water buffalo						
Italy	cELISA	347	90	18	30	Rinaldi <i>et al.</i> , 2007

- = not known.

Table 3. The prevalence (Prev) of *Giardia* in different dog populations in different countries, with number of dogs included in the study (N°)

Country	N°	Prev	reference
Household dogs			
UK	432	2-13	Guest et al., 2007
The Netherlands	92	15.2	Overgauw et al., 2008
France	93	12.9	Beugnet et al., 2000
Switzerland	505	2.4	Sager et al., 2006
Italy	156	16.7	Capelli et al., 2006
Brazil	138	16.9	Katagiri et al., 2008
Brazil	100	9	Meireles et al., 2008
Brazil	122	4.1	Mundim et al., 2007
South Korea	430	7.6	Liu et al., 2008
Kennel dogs			
UK	117	3	Guest et al., 2007
Czech Republic	524	9.5	Dubna et al., 2007
Slovak Republic	164	4.3	Szabova et al., 2007
Italy	64	20.3	Capelli et al., 2006
Italy	1400	20.9	Scaramozzino et al., 2008
Brazil	100	24	Meireles et al., 2008
Brazil	199	49.7	Mundim et al., 2007
Spain	1800	1	Martinez-Moreno et al., 2007
Spain	1161	7	Miro et al., 2007
Australia	1400	9.3	Palmer et al., 2008
South Korea	42	47.6	Liu et al., 2008
Stray or shelter dogs			
Italy	87	16.1	Capelli et al., 2006
Brazil	89	16.8	Mundim et al., 2007
Italy	183	55.2	Papini et al., 2005

PATHOGENESIS OF *GIARDIA*

Although clinical symptoms associated with a *Giardia* infection have been documented in several animal species, the pathogenesis is not clearly understood. Whether or not *Giardia* should be considered as a primary pathogen or only as a concurrent infection is still uncertain, although the intrinsic pathogenicity of *Giardia* is increasingly suggested (Aloisio *et al.*, 2006; Geurden *et al.*, 2006a; O'Handley *et al.*, 1999). Studies in human epithelial cell lines (Buret *et al.*, 1990a), in laboratory animals (Buret *et al.*, 1990b; Buret *et al.*, 2002; Scott *et al.*, 2002), in goat kids (Koudela and Vitovec, 1998), and in calves (Taminelli *et al.*, 1989; Ruest *et al.*, 1997) indicate that giardiasis essentially leads to microvillus alterations, including a decreased crypt to villus ratio and brush border enzyme deficiencies. As suggested by the absence of these particular alterations in response to a *Giardia* infection in B and T cell-deficient mice (Scott *et al.*, 2000), they are not only a direct consequence of the interaction

between trophozoites and epithelium, but are also mediated by the host's immune response. The pathogenesis of giardiasis can therefore be considered as a multifactorial process, involving both parasite characteristics and the host immune response. Furthermore, an increase in epithelial permeability has been described, which appears to result from enterocyte apoptosis (Cevallos *et al.*, 1995; Chin *et al.*, 2002) and from cytoskeletal reorganization induced by trophozoite toxic products (Buret *et al.*, 2002; Scott *et al.*, 2002), leading to local disruption of tight-junctional proteins. Whether *Giardia* secretory-excretory products induce direct proteolytic degradation of tight-junctional proteins is uncertain (Buret, 2007). The increased epithelial permeability leads to a higher number of intraepithelial lymphocytes (IEL) and to activation of T-lymphocytes. Trophozoite toxins and T cell activation also initiates a diffuse shortening of brush border microvilli and a decreased activity of the small intestinal brush border enzymes, especially lipase, some proteases and the disaccharidases lactase, and maltase and sucrase (Buret *et al.*, 1990a; Scott *et al.*, 2000).

In human patients, in calves and in goat kids, malabsorption due to an infection with *Giardia* has been associated with an increased number of IEL and a decreased villus to crypt ratio (Farthing, 1997; Ruest *et al.*, 1997; Koudela and Vitovec, 1998). The diffuse microvillus shortening leads to a decrease in the overall absorptive area in the small intestine and an impaired intake of water, electrolytes, and nutrients (Buret, 2007). The combined effect of this decreased resorption and the brush border enzyme deficiencies results in malabsorptive diarrhea and lower weight gain, both in murine models (Buret *et al.*, 1990a) and in ruminant experimental models (Ruest *et al.*, 1997; Koudela and Vitovec, 1998). The reduced activity of lipase and the increased production of mucine by goblet cells may explain the steatorrhea and mucous diarrhea which has been described in *Giardia*-infected hosts (Zajac, 1992; Moncada *et al.*, 2003).

CLINICAL SYMPTOMS

As discussed above, the clinical outcome is determined by several factors, including parasite virulence and host immune response. The subsequent symptoms vary considerably between individual animals and animal species, and this lack of consistency in clinical outcome, along with the diagnosis of the parasite in apparently healthy animals, may result in the perception that *Giardia* is not a major cause of clinical disease in veterinary medicine. Next to the above-mentioned factors, the environmental infection pressure contributes to a large extent to the spread of giardiasis, and is largely dependent on the management/housing system with a higher impact on prevalence, animal health, and production in intensively-reared systems with indoor housing compared to extensive systems (Geurden *et al.*, 2006e). In general, infection results in diarrhea, which does not respond to antibiotic or coccidiostatic treatment. The excretion of pasty to fluid feces with a grey, mucoid appearance is indicative of giardiasis, especially when the diarrhea occurs in young animals.

In calves, lambs, and goat kids, several studies reported clinical symptoms caused by *Giardia* both in natural (StJean *et al.*, 1987; Xiao *et al.*, 1993; O'Handley *et al.*, 1999; Aloisio *et al.*, 2006; Geurden *et al.*, 2006b) and in experimental conditions (Koudela and Vitovec, 1998; Olson *et al.*, 1995; Geurden *et al.*, 2006a). An acute diarrhea can occur, although chronic and intermittent symptoms are observed more often. In pigs, a significant association

between *Giardia* infection and the occurrence of clinical symptoms has not yet been demonstrated (Hamnes et al., 2007), although an experimental infection to study the detrimental effects of *Giardia* is still to be performed. Next to diarrhea, there is a potential impact on production due to giardiasis in production animals. In goat kids and in lambs, an experimental infection resulted in a decreased feed efficiency and, subsequently, a decreased weight gain (Olson et al., 1995; Koudela and Vitovec, 1998). In calves there are at present no similar experimental data to conclusively indicate an economical impact. In one study, a decreased weight gain was observed (Geurden et al., 2006a) in contrast to the vaccination trial conducted by Uehlinger et al. (2007). In general, *Giardia* can be considered as a cause of diarrhea and ill-thrift in production animals, although there is a lack of consistent symptoms.

In companion animals, there is too few data to conclusively confirm the relevance of *Giardia* as an important pathogen in the aethiology of gastro-intestinal disease. Although a recent efficacy trial reported the occurrence of unformed to diarrhetic feces in the untreated group (Montoya et al., 2008), experimental infections have yet to be conducted to confirm the intrinsic pathogenicity. Nevertheless, *Giardia* is considered by several authors as an important pathogen in companion animals (Kirkpatrick, 1987; Zajac, 1992). As discussed by Irwin (2002), several issues complicate a conclusive diagnosis of *Giardia* in companion animals. Given the high prevalence of *Giardia* in the feces of animals without overt clinical symptoms, it is not surprising that they are occasionally detected in stools of diarrhetic patients as well, without necessarily a causal association. The high overall prevalence might, hence, lead to a false positive diagnosis. On the other hand, the intermittent excretion of *Giardia* cysts in the feces might lead to a false negative result, especially when using a diagnostic assay with a low sensitivity. A *Giardia*-positive diagnosis (made on stool samples from 3 consecutive days, see also below) from a patient with chronic, small bowel diarrhea is consistent therefore with a diagnosis of giardiasis. It is advised in those cases to treat the *Giardia* infection initially. If the diarrhea recurs, treatment resistance, reinfection from the environment, or another infection/disease process should be considered.

In human patients, infections with assemblage A are more likely to result in clinical symptoms than infections with assemblage B (Read et al., 2002), although once established, infections with assemblage B seem to result in more persistent diarrhea (Homan and Mank, 2001). Furthermore, allergic skin disease and increased visceral sensitivity are thought to be associated with giardiasis in human patients (Giacometti et al., 2003, Dizdar et al., 2007). Whether a similar difference in clinical outcome occurs between the livestock-specific assemblage E and the zoonotic assemblage A and B in production animals or whether allergic conditions are associated with infection in production animals is not known and should be further studied.

DIAGNOSIS

Due to the vagueness of the symptoms, the clinical diagnosis of giardiasis is not straightforward, and is mainly based on clinical history, background data infection pressure, such as housing and the exclusion of other infectious diseases. The clinical diagnosis needs to be confirmed by the detection of the parasite in a fecal sample, either by microscopical examination, antigen detection, or polymerase chain reaction (PCR). Given the intermittent

excretion of cysts, especially in the chronic phase of infection, multiple samplings are advised, either from the same animal for 3 consecutive days (O'Handley *et al.*, 1999) or from several animals within the same housing facility. Especially young animals should be included in the sampling since the peak excretion is observed in those animals, even if they do not yet display clinical symptoms. The diagnosis of a *Giardia* infection by antibody detection in the blood is not readily performed, as the antibody titers are not significantly increased after infection (Yanke *et al.*, 1998; O'Handley *et al.*, 2003).

Microscopical Examination

Both the trophozoites and the cysts of *Giardia* can be detected by microscopy, either directly or after concentration with sucrose, zinc sulphate, or formalin. Steathorrhoea, which is observed in giardiasis, can interfere with sucrose flotation (Xiao and Herd, 1993). Trophozoites can sometimes be detected in fecal samples with diarrhoea due to the increased peristalsis. Given the characteristic movement of the trophozoites, they are preferably visualized in a native smear using recently-obtained feces.

More frequently, the detection of cysts in the feces is preferred for diagnosis. Prior to examination, cysts can be stained. Frequently used stains are iodine (Zajac, 1992) and trichrome (Addiss *et al.*, 1991). The major advantage of a microscopical examination is the limited cost of consumables. The major disadvantage is the need for a skilled and experienced microscopist and the allegedly lower sensitivity compared to immunological assays (Geurden *et al.*, 2004, 2008a).

Immunological Diagnosis

For the detection of parasite antigen immunofluorescence assays (IFA) (Xiao and Herd, 1993), enzyme-linked immunosorbent assays (ELISA) (Boone *et al.*, 1999) and rapid solid-phase qualitative immunochromatography assays (Garcia *et al.*, 2003) are commercially available. Most tests were developed and evaluated for use in human stool samples. IFA and copro-antigen ELISA use monoclonal antibodies against cyst wall proteins. IFA can be used as a quantitative test, with a detection limit estimated around 1,000 cysts per gram of feces (CPG) (Xiao and Herd, 1993). Both IFA and ELISA were found to be sensitive and specific assays for the diagnosis of infection, compared to microscopical examination (Geurden *et al.*, 2004, 2008a).

Partially due to the requirement of laboratory settings and trained personnel, the main disadvantage of IFA and ELISA is the high cost compared to microscopical examination and the time lost by transport to and analysis in the laboratory. This could be circumvented by the use of immunochromatography enabling on-site diagnosis within 15 minutes. Immunochromatography uses monoclonal antibodies directed against specific trophozoite or cyst wall proteins. In human medicine, several assays are commercialized, including dipsticks and rapid membrane assays. Similarly, in veterinary medicine, the SNAP®*Giardia* test (IDEXX Laboratories, Inc., Westbrook, Maine, USA) is commercialized for use in dogs, and has recently been proven to be a valuable alternative in the clinical diagnosis of giardiasis

(Geurden *et al.*, 2008a). In production animals, an immunochromatographic assay is available for calves (Speed[®] *Giardia*, BioVetoTest, La Seyne-sur-Mer, France), but has not yet been properly evaluated.

Polymerase Chain Reaction (PCR)

PCR is primarily used for the identification of different species and genotypes of *Giardia* for taxonomical and epidemiological research, although there is potential for diagnostic use. Several genes are commonly used for genotyping, and for clinical diagnosis, the 18S rDNA (Read *et al.*, 2004) seems most suitable. In theory, the detection limit of PCR is 1 cyst (Amar *et al.*, 2002), which improves considerably diagnostic sensitivity. However, several factors can interfere with PCR such as inhibition, which is known to occur frequently in DNA extracted from fecal samples. Furthermore, the extraction of parasite DNA from feces needs to be standardized for diagnostic use. At present, PCR is considered too expensive and cumbersome for use in veterinary diagnostics (da Silva *et al.*, 1999), and has yet to be evaluated as a diagnostic assay.

TREATMENT AND CONTROL

Several compounds have a known efficacy against *Giardia*, both *in vitro* and in laboratory animals. For sheep, goats, and pigs, there are no studies available on the efficacy of treatment against *Giardia*. In calves and dogs, a number of studies evaluated the efficacy of different compounds, both in experimental and natural conditions. At present, no drug is however licensed for the treatment of giardiasis in ruminants. In dogs, fenbendazole is licensed in the USA for the treatment of *Giardia*.

Chemotherapeutic Treatment

Nitro-imidazoles (NZs) such as metronidazole and tinidazole, quinacrine or furazolidine, are frequently used to treat giardiasis in human patients. Although therapy with these compounds is effective, considerable side-effects can occur. Metronidazole is even considered to be carcinogenic (Morgan *et al.*, 1993; Harris *et al.*, 2001). Furthermore, resistance to treatment has been described both for metronidazole and furazolidine (Upcroft *et al.*, 1990). In veterinary medicine, metronidazole (St. Jean, 1987; Xiao *et al.*, 1993) and dimetridazole (St. Jean, 1987) have been used in companion animals and in calves, achieving symptomatic improvement. Furthermore, in several countries the NZs are no longer approved for use in livestock. More recently, nitazoxanide has been shown *in vitro* to be a promising new drug against *Giardia* (Cedillo-Rivera *et al.*, 2002), but no data on *in vivo* activity in animals is available. In calves and in other production animals, benzimidazole compounds, or paromomycin, are currently considered as most suitable for treatment of giardiasis.

(Pro)Benzimidazoles

An option for treatment of giardiasis is the benzimidazole compounds (BZs) which are well-known broad spectrum anthelmintics. BZs are believed to have a high safety margin and a selective toxicity (Xiao *et al.*, 1996). *In vitro* studies indicated that BZs are more efficacious against *Giardia* than either metronidazole or tinidazole (Edlind *et al.*, 1990; Meloni *et al.*, 1990; Morgan *et al.*, 1993), and that this anti-*Giardia* effect is irreversible (Morgan *et al.*, 1993). The BZs interfere with the polymerization of tubulin and are believed to induce mutations in the tubulin-encoding gene. Tubulin is a major component of the *Giardia* trophozoite cytoskeletal structures. Therefore all functional activities of tubulin-dependent structures, such as the median body and the ventral disk, are inhibited. As a result, BZs interfere with trophozoite attachment to the intestinal mucosa and prevent intestinal colonization. BZs seem not to affect flagellar tubulin, which has a different tubulin subunit structure. The BZ mode of action might also include binding to giardins, which are *Giardia*-specific proteins restricted to the ventral disk (Edlind *et al.*, 1990; Meloni *et al.*, 1990).

In dogs, fenbendazole (Zajac *et al.*, 1998), albendazole (Barr *et al.*, 1993), and a combination of pyrantel embonate and febantel (Barr *et al.*, 1998; Montoya *et al.*, 2008) were all found to be efficacious when administered during 3 to 5 days. In calves, data on reduction in cyst excretion is available for treatment with fenbendazole (Xiao *et al.*, 1996; O'Handley *et al.*, 1997) and albendazole (Xiao *et al.*, 1996). Both have been shown to significantly reduce the peak and the duration of cyst excretion and to result in a clinical benefit (O'Handley *et al.*, 2000), although the dosage of both BZs needed for *Giardia* treatment (5 to 20 mg per kg bodyweight per day during three consecutive days) is higher compared to helminth treatment. Furthermore, the cyst-suppressing effect of BZ treatment was either not complete or short-lasting in field conditions, despite the high *in vitro* efficacy of both drugs. This might be either due to a high environmental infection pressure which counters the effect of treatment or to the lack of persistent efficacy of BZs against *Giardia* in calves, resulting in a rapid re-infection shortly after the end of the treatment. To prevent re-infection from the environment and to improve long term production parameters, it has been hypothesized that calves should be treated with a continuous low dosage of BZs (O'Handley *et al.*, 2000), since the treatment duration seems to be more important than treatment dosage (O'Handley *et al.*, 1997). However, there are no data on the safety of a long-term treatment with BZs, and there might be a risk for developing BZs-resistant *Giardia* field strains.

Paromomycin

Paromomycin, or aminosidin, is a broad-spectrum amino-glycoside antibiotic, with well-known efficacy against several protozoan parasites, such as *Cryptosporidium* in calves (Fayer and Ellis, 1993; Mancassola *et al.*, 1995; Chartier *et al.*, 1996; Viu *et al.*, 2000; Grinberg *et al.*, 2002), *Histomonas meleagridis* in chickens (Hu and McDougald, 2004), and *Giardia* in rats (Awadalla *et al.*, 1995) and humans (Wright *et al.*, 2003). Paromomycin binds to the small subunit rRNA and inhibits protein synthesis, which has either a direct effect on *Giardia* or an indirect effect of nutrient withdrawal caused by the inhibition of bacterial protein synthesis and destruction of the bacterial flora (Edlind *et al.*, 1990; Harris *et al.*, 2001). Paromomycin is poorly absorbed from the gastro-intestinal tract and is therefore well-tolerated by calves (Grinberg *et al.*, 2002). In calves, paromomycin was shown to effectively reduce the *Giardia* cyst excretion in experimentally-infected calves during at least 2 weeks, when administered at 50 and 75mg/kg/day during 5 consecutive days (Geurden *et al.*, 2006a).

In other production animals or in dogs, there is no experimental data on the efficacy of paromomycin.

Alternative Approaches

Several compounds were shown to affect parasite growth, adhesion capacity, and morphology *in vitro*, including curcumin (Pérez-Arriaga et al., 2006) and peppermint (Vidal et al., 2007). In mice, extracts from medicinal plants used in Mexican traditional medicine were shown to effectively kill *Giardia* trophozoites (Barbosa et al., 2006). However, none have so far been evaluated in animals with a continuous exposure of animals to infection. The most promising alternative approach is probably vaccination.

Vaccination

Since both paromomycin and BZs do not have a persistent efficacy, the effect of treatment is often short-lasting in natural conditions (O'Handley et al., 2000; Geurden et al., 2006 a and b). Vaccination could provide an alternative for chemotherapeutic treatment, achieving a prolonged protection against infection and preventing the excretion of cysts in order to break the transmission by reducing environmental contamination. In the United States, a *Giardia* vaccine is commercially available for use in cats and dogs (Fel-O-Vax *Giardia* or *Giardia* Vax, Fort Dodge Animal Health), but the efficacy of a preventive (Olson et al., 1996) or curative (Olson et al., 2001; Payne et al., 2002; Stein et al., 2003; Anderson et al., 2004) vaccination seems to be variable. In calves, a similar vaccination protocol did result in a higher humoral immune response, which was, however, not protective as the cyst excretion was not reduced and clinical symptoms could not be prevented (Uehlinger et al., 2007). As the *Giardia* vaccine consists of sonicated trophozoites from axenic reference strains, the vaccine might not reflect the specific *G. duodenalis* assemblages infecting production animals, resulting in a poor vaccine efficacy. However, injection of whole trophozoites from one particular strain did induce protection when challenged by a different strain in dogs (Olson et al., 2000).

Several issues need to be considered as far as vaccination against *Giardia* is concerned. As the current vaccine consists of sonicated trophozoites, the production is cumbersome (Olson et al., 2001), and the use of a recombinant vaccine containing one or more protective antigens has been advocated. Some *Giardia* proteins seem however to be assemblage-specific, while others are common for all assemblages (Steuart et al., 2008). Although several *Giardia* antigens have been proposed as potential vaccine candidates (Olson et al., 2000; Abdul-Wahid and Faubert, 2007), more research is needed to identify proteins which are both commonly expressed in all assemblages and are known to induce a specific and protective immune response.

Giardia is a luminal parasite and does not infect intestinal cells, resulting in a limited contact between parasite and host. As an increase in serological antibodies is not well correlated with protection against infection (Yanke et al., 1998; O'Handley et al., 2003), a local immune response seems to be essential to prevent colonization of the gut by *Giardia* trophozoites, along with cellular mechanisms (Müller and von Allmen, 2005). Vaccination through the subcutaneous injection of an antigen (Uehlinger et al., 2007) might therefore not be a suitable route to induce the localized immune response in the lumen of the small intestine

in production animals, and an appropriate method to deliver the protective antigen to the mucosa of the small intestine is needed. In mice, oral immunization with a live vector expressing *Giardia* cyst wall protein resulted in a significant reduction of the cyst excretion (Lee and Faubert, 2006). However, oral vaccination implies the passage of the hostile gastrointestinal environment, especially in ruminants, which might alter the antigenic structure of the protective protein. Vaccination is therefore a promising alternative control, although in need of further research.

Control

Measures to Support Curative Treatment

Although compounds such as fenbendazole, albendazole, and paromomycin are effective against *Giardia*, most animals are re-excreting cysts within 2 to 3 weeks after treatment. Since *Giardia* cysts can survive for at least 1 week in feces and up to 7 weeks in soil (Olson *et al.*, 1999), the effective treatment period of most protocols (3-5 days) may be too short to prevent re-infection from a contaminated environment shortly after treatment. Furthermore, a high environmental infection pressure can result in an efficacy less than 100% from 1 week after treatment onwards, as in the albendazole trial in calves (Xiao *et al.*, 1996). The short term cyst-suppressing effect of treatment in a contaminated environment (Xiao *et al.*, 1996; O'Handley *et al.*, 2000) emphasizes the need for an integrated control program combining both treatment and cleaning-disinfection of the environment at the end of the treatment period to minimize the risk of re-infection after treatment.

Giardia cysts are known to be resistant to commonly-used disinfectants, such as chlorine. Alternative disinfectants, including chlorine dioxide, ozone, and ultra violet irradiation have been the focus of research in drinking water treatment processes, although there are practical objections against most of these disinfection procedures for use in calf facilities. Alternatively, heat or desiccation (Olson *et al.*, 1999) and disinfection with quaternary ammonium (Xiao *et al.*, 1996; O'Handley *et al.*, 1997) can be used in calf facilities. In a recent study in calves, the efficacy of a combination of animal treatment with fenbendazole and environmental cleaning and disinfection with ammonia 10%, or relocation, was evaluated on commercial dairy farms and found to achieve a significant reduction both in the number of calves excreting cysts and in the number of cysts excreted. The environmental infection pressure was, thus, decreased by effectively breaking the transmission cycle of *Giardia*, resulting in a long-term efficacy of treatment (Geurden *et al.*, 2006b).

Measures to Prevent Infection

As discussed above, management measures are essential to achieve clinical improvement in a curative treatment regime. Similarly for the prevention of infection, proper management may contribute to a decreased infection rate, and aims to break the parasite's transmission cycle through specific measures countering the parasite's ability to spread easily (see also table 3). These management measures include a low stocking rate (Wade *et al.*, 2000a), combined with regular cleaning and disinfection of the housing facilities (Bomfin *et al.*, 2005; Maddox-Hyttel *et al.*, 2006). A well thought-over housing is also important, as animals reared indoors are three times more likely to acquire infection than those reared outside

(Ruest *et al.*, 1998), and a solid floor type is known to favor transmission (Maddox-Hyttel *et al.*, 2006).

Preventing giardiasis by strategic or metaphylactic treatment (Dauguschies *et al.*, 2007) is probably not an option as all active compounds against *Giardia* lack persistent efficacy. Hence, animals in an infected environment are re-infected shortly after the end of the treatment period. As *Giardia* cysts are immediately infective upon excretion and the prepatent period is only 3-5 days, the environmental infection pressure increases rapidly, and repeated metaphylactic treatment would probably be necessary to prevent giardiasis.

CONCLUSION

Many studies indicate that worldwide *Giardia* occurs frequently in animals, although the prevalence reports vary markedly. Besides substantial differences between regions or countries, the variation in prevalence can also be ascribed to differences in study design, the technique used for parasite diagnosis, in the number of animals, or to differences in age and breed of the animals under study.

The clinical relevance of an infection differs between animal species. In dogs, calves, goat kids, and in lambs, *Giardia* is able to induce diarrhea and a reduction in weight gain. In pigs, however, there are at present no indications that *Giardia* is of clinical importance. Moreover, the symptoms differ considerably between individual animals, as the clinical outcome is the combined result of the parasite's virulence and the host immune response. Due to the vagueness of the symptomatology in production animals, infection needs to be confirmed by laboratory diagnosis on fecal material. Most often, diagnosis is performed through IFA or ME, but a promising new diagnostic tool seems to be immunochromatography, allowing on-site and, hence, quick diagnosis of infection.

At current, there are no drugs registered for the treatment of *Giardia* in production animals, although several benzimidazole compounds and paromomycin were shown to be efficacious in experimental conditions. In dogs, only fenbendazole is licensed in the USA. A promising alternative for chemotherapeutic treatment is vaccination. However, many technical questions still need to be answered prior to the development and evaluation of a vaccine against *Giardia* in production animals. Last, but not least, the economical effects of a *Giardia* infection in production animals need to be further studied, in order to know if research into vaccine development is warranted.

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SEROLOGICAL DIAGNOSIS OF *PARELAPHOSTRONGYLUS TENUIS* IN CERVIDS OF NORTH AMERICA: A REVIEW

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ABSTRACT

The meningeal worm (*Parelaphostrongylus tenuis*; Family: *Protostrongylidae*) of white-tailed deer (*Odocoileus virginianus*) in eastern North America is a cause of neurologic disease and mortality in co-habiting, native cervid species and certain livestock. The traditional method of identifying animals exposed to the parasite relies on detecting excreted larvae in feces and has been found inadequate. New enzyme-linked immunosorbent assays (ELISAs) using excretory-secretory antigens of the infective larvae of *Parelaphostrongylus tenuis* to detect antibodies in infected white-tailed deer, elk (*Cervus elaphus*), and moose (*Alces alces*) have been developed. Subsequently, the tests were rigorously evaluated to assess their fitness-for-use as tools for preventing the spread of the parasite through animal translocation and for clinical diagnosis. For the purpose of developing reliable performance parameters for the ELISAs, serum samples obtained from the three cervids species, either naturally- or experimentally-infected, were tested. All three tests showed high sensitivity ($\geq 97\%$) and specificity ($\sim 100\%$), good repeatability, and reproducibility, and are deemed useful for detecting cervids with either early exposure to *P. tenuis* or with long-standing infections. The tests are now commercially available for diagnostic use. The application of the ELISA test in a free-ranging elk showing characteristic clinical signs was instrumental in achieving the first antemortem laboratory diagnosis of *P. tenuis* infection.

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INTRODUCTION

The meningeal worm, *Parelaphostrongylus tenuis*, (Family *Protostrongylidae*, Subfamily *Elaphostrongylinae*), is the cause of “moose sickness”, a neurologic disease associated with declines in moose populations where they share a habitat with increasing populations of infected white-tailed deer. The parasite is also thought responsible for failed or depressed introductions of caribou and elk, respectively, onto deer ranges. It is also pathogenic to certain livestock, particularly llamas and sheep (Lankester 2001). The parasite is capable of spreading from its geographical range in eastern North America, i.e., Canada and the United States, where it is very prevalent in the white-tailed deer (WTD), to the western parts of the continent. The lack of reliable methods to detect animals infected with the meningeal worm has increased the risk of its spread and has led to restrictions on the westward translocation of wild- and game-farmed cervids. A desire to have these restrictions removed, inspired mainly by the growth in the game-farming industry, prompted research aimed at developing more reliable methods of identifying infected cervids. The need for improved husbandry of certain domestic and zoological animals, as well as better management of susceptible wild cervid hosts has also motivated the development of a reliable serological test.

The interest in *Parelaphostrongylus tenuis* spans many disciplines including parasitology, wildlife biology, game-farming, epidemiology, immunology, and veterinary practice. Probably for this reason, *P. tenuis* is the most studied protostrongylid parasite of cervids. Two other congeneric family members infect North American cervids, namely, *Parelaphostrongylus odocoilei* in mule and black-tailed deer (*Odocoileus hemionus columbianus*) of western North America and *P. andersoni* which occurs in white-tailed deer as well as in caribou (*Rangifer tarandus caribou*) throughout much of the range of these two hosts (Lankester, 2001). These three *Parelaphostrongylus* species, along with three members of the related genus *Elaphostrongylus* (*E. cervi*, *E. alces*, and *E. rangiferi*), comprise the subfamily *Elaphostrongylinae*, and are of varying importance to the cervid industry and wildlife managers, worldwide.

The only known natural host of *P. tenuis* is the WTD. The life cycle of the parasite in the WTD has been well-documented from the seminal work of Anderson and colleagues (Anderson, 1965; Anderson and Prestwood, 1981). Briefly, fawns living in areas endemic for the parasite are typically infected during the first few years of life after ingesting terrestrial gastropods harboring the infective, third larval stage (L3). In the deer's abomasum, the L3 are freed from the gastropod tissue and migrate through the gut wall. Once in the peritoneal cavity, the larvae find nerve fibres originating from the spinal cord and, by this means, enter the central nervous system where they undergo two molts into the fourth and fifth larval stages (L4 and L5). As juvenile adults, the L5 leave the spinal cord and migrate anteriorly in the subdural space, becoming associated with venous blood sinuses and vessels in the meninges of the cranial cavity. In this location, female and male adult worms mate. Females lay eggs which are carried by the venous circulation and are often found in large numbers in the lungs. The eggs hatch in the lungs and emerging first stage larvae (L1) migrate up the respiratory tree. Inevitably, they are carried by the pulmonary mucociliary escalator system, coughed, and swallowed. Within 82 to 137 days after infection, the L1 are first excreted in the feces. Upon contact with a gastropod, the L1 pierce through the foot tissue and undergo two molts into the second (L2) and third, or infective, larval stage (L3). Following infection,

WTD, which is the normal host of this parasite, rarely show clinical signs but are responsible for disseminating *P. tenuis* in the field to susceptible host species including a number that may die as a result of the infection (Lankester, 2001). At least 20 susceptible ungulate species, excluding subspecies and naturally-occurring hybrids, have been identified from studies of natural and experimental infections (Anderson, 2000; Anderson and Prestwood, 1981; Lankester, 2001). Three groups of susceptible animals are known, namely: cervids, [moose, elk, reindeer (*Rangifer tarandus tarandus*), caribou (*Rangifer tarandus caribou*), fallow deer (*Dama dama*), and black-tailed deer]; domestic livestock, [goats, sheep, and cattle]; and exotic, zoo, or non-cervid, wild animals, [llama (*Lama glama*), bighorn sheep (*Ovis canadensis*), pronghorn antelopes (*Antilocapra americana*), and eland (*Taurotragus oryx*)]. *P. tenuis* was incriminated as the cause of setbacks and some failures of the translocated cervid species and game-farmed animals (reviewed by Lankester, 2001). Infection can result in acute death due to peritonitis because of migrating worms, e.g., in goats and fallow deer, or more commonly in the field, from neurological lesions resulting in death, predation, or increased susceptibility to collision with moving vehicles.

The peculiar restriction of *P. tenuis* to the eastern half of North America (Anderson and Prestwood, 1981; Wasel et al., 2003) has led to increased vigilance amongst wildlife managers determined to prevent its westward incursion from translocated WTD and other susceptible hosts that can transmit larvae (Samuel et al., 1992). This determination has led to restrictions by western provinces in Canada on the movement of elk, WTD, and other cervids originating from eastern North America. For example, the province of Alberta imposed a moratorium on the importation of elk in September 1988 and British Columbia banned the importation of live deer, moose, and elk in May 1991 (Samuel et al., 1992). Saskatchewan also restricts the entry of cervids from the eastern Canadian provinces. These policies preceded any awareness of chronic wasting disease in cervids in Canada, and remain in place pending the development and acceptance of reliable tests for *P. tenuis*.

Previously, the only available antemortem test for diagnosing *P. tenuis* infection was the Baermann procedure which relies on the recovery of dorsal-spined, first-stage larvae (L1) from the feces of infected animals, followed by microscopic examination. This method does not permit elaphostrongyline L1 to be reliably distinguished from one another. Conclusive identification of recovered *P. tenuis* L1s did require complicated and expensive infection experiments (Pybus et al., 1989). More recently available alternatives employ molecular diagnostic methods such as a polymerase chain reaction (PCR) test (Gajadhar et al., 2000) or single-strand conformational polymorphism (Huby-Chilton et al., 2006). Nonetheless, larvae are not readily recovered from the feces of susceptible hosts such as elk, moose, caribou, and llama, which may shed intermittently or not at all (Karns, 1977; Ogunremi et al., 2002a; Rickard et al., 1994; Samuel et al., 1992). As well, infections with a single worm or worms of the same gender do not lead to larval production, yet may result in the death of the host. Neither multiple Baermann testing protocols (Welch et al., 1991), nor the use of a superior Baermann apparatus (Forrester and Lankester, 1997) has significantly improved the probability of detecting larvae in feces. Furthermore, the Baermann test works best with pelleted feces which are typically difficult to obtain from cervids under chemical or physical restraint. As with many other helminths, traditional parasitological methods of diagnosis and estimation of prevalence are inadequate when dealing with *P. tenuis*.

EARLY EFFORTS AT DEVELOPING AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR *P. TENUIS*

As an alternative to the parasitological method and because of its sensitivity, the enzyme-linked immunosorbent assay or ELISA became an attractive option to researchers exploring improved methods to diagnose *P. tenuis* infections in cervids. The earliest attempted serological diagnosis of *P. tenuis* was performed on experimentally-inoculated goats and WTD (Dew et al., 1992) by means of an ELISA which utilized a test antigen crude parasite extract prepared by sonicating adult worms in isotonic buffer. Anti-*P. tenuis* IgG antibodies were detected in the cerebrospinal fluids of both WTD and goats, but only in the serum of the goats. Failure to detect serum antibody response to the parasite in infected WTD was attributed to the poor sensitivity of the assay (Dew et al., 1992). It was soon afterwards that Duffy et al. (1993) used a similar crude antigen preparation to detect serum antibodies at 75 days post-inoculation (dpi) in two WTD experimentally-inoculated with 20 L3. The length of time that elapsed between infection and detection corresponded with the time required for adult worms to mature in the host, but shorter than the time larvae are expected in the feces (i.e., prepatent period). Despite the limitations, both groups of workers concluded that antigens derived from adult worms were useful for serodiagnosis of *P. tenuis* infection. In contrast, the work of Neumann et al. (1994) revealed extensive cross-reactivity between adult *P. tenuis* antigens and those of two phylogenetically-distant nematodes, i.e., *Dictyocaulus* and *Trichinella*. The authors speculated that antigens of the L3 stage, instead of those from adults, may be more suitable for the serodiagnosis of meningeal worm infections. Their rationale was based on the fact that infective larvae migrate extensively in host tissue, resulting in their exposure to the immune system and followed by antibody production by the host. The authors suggested that the L1 which hatch within, or close to, rich vascular supplies in the lungs may produce useful diagnostic antigens. As it turned out, antigens derived from the L3 proved to be the most useful diagnostic antigens but for reasons other than the ones advanced above.

The inherent properties of the immune system dictate that factors other than extensive parasite migration or habitation close to rich vascular beds play critical roles in the induction of an antibody response. Intact nematodes, even if migrating, are not able to stimulate an antibody response until they, or their products, can be processed by an antigen-presenting cell (APC) or bind to the receptors on an antibody-forming cell. The requirement for antibody production is met, at least in part, by the ability of the worm to produce and secrete metabolites that can be ingested by an APC or be exposed to antibody-producing cells leading to the production of specific and detectable antibody levels.

EXCRETORY-SECRETORY (ES) PRODUCTS: CHOICE ANTIGEN FOR *P. TENUIS* SERODIAGNOSIS

Materials secreted or excreted by parasites into their environment are termed excretory-secretory (ES) products (Fife, 1971; De Savigny, 1975). They consist of proteases, metabolites, and various parasite molecules produced during the course of normal parasite

growth and function (Yatsuda et al., 2003). Some of the molecules are required for the development and survival of the parasite in the host (Vercauteren et al., 2003). The development of techniques to culture parasites *in vitro*, and to generate appreciable quantities of ES products has led to the availability of reagents that can be exploited for the serodiagnosis of parasitic helminths. Due to poor sensitivity and specificity of earlier described *P. tenuis* serological tests based on adult worm antigens, it became necessary to investigate ES products from the L3 for use in serodiagnosis. Superior performance of ES products in ELISAs over two other *P. tenuis* antigen preparations, namely, somatic antigens of L3 and of adult worms (Ogunremi et al., 1999a), made it a good choice of an antigen preparation for serodiagnosis. The superiority of ES products was subsequently demonstrated for the diagnosis of infected elk and moose (Ogunremi et al., 2002a,b). Work done to date showed that all necropsy-confirmed, infected elk (n=12), WTD (n=17), and moose (n=6) were detected within one month of experimental inoculation with the parasite, usually as early as 15 days post-inoculation (Ogunremi et al., 1999a, 2002a,b; Ogunremi et al., 2008). A single exception was a moose from which only one adult worm was recovered postmortem at 96 dpi. The animal had been seronegative at 16 dpi but tested seropositive at the next bleeding date, 46 dpi. All seropositive animals remained so until the termination of infection, 2 – 8 months later. Also, larval ES products proved useful in detecting all of 12 necropsy-confirmed, naturally-infected WTD between 4 - 8+ years of age (Ogunremi and Yabsley, manuscript under preparation). Since most WTD living in endemic areas become infected within their first year of life and fail to acquire new infections with time (Slomke et al., 1995), the only tenable inference from the positive test results is that the ELISA was useful in detecting animals that acquired their infections several years previously, and continued to harbor the parasite. The use of infective larval ES products to successfully detect exposed animals, including those with long-standing infections harboring only the adult worm stage, suggest significant antigenic similarity between the ES products of the larval and adult stages. In this regard, the two ES products may mirror observed antigenic similarities between the somatic antigen fractions of the two parasite stages (Ogunremi et al., 1999a).

In contrast to the ES products, use of unfractionated somatic antigen of adult worms in ELISA resulted in reduced test sensitivity in necropsy-confirmed, infected WTD (Ogunremi et al., 1999a) and moose (Ogunremi et al., 2002b). Also, high levels of cross-reactivity were routinely observed between the somatic antigen of the adult worm and sera from animals not harbouring *P. tenuis*. Yet, upon fractionation of larval or adult worm somatic antigen preparation, individual antigens (e.g., a 37 kDa antigen) could be identified in each preparation that allowed for sensitive and reliable diagnosis using the Western blot technique (Ogunremi et al., 1999a, b).

The entire complement of the somatic molecules of the adult *P. tenuis* is a complex antigen. When used as a coating antigen in ELISA, the unfractionated somatic *P. tenuis* antigen leads to poor test specificity and sensitivity. The presence of cross-reactive antigen molecules in the unfractionated somatic antigens of the adult *P. tenuis* worm is the major reason for the poor specificity (Bienek et al., 2002; Neumann et al., 1994; Ogunremi et al., 1999b). The poor sensitivity may be attributable to the induction of low levels of anti-somatic antibodies (Ogunremi et al., 2002a, b) due to limited exposure of parasite somatic molecules to the immune system. Apart from antigens sloughing off the surface of intact parasites, the only other means of exposure of somatic antigens to the immune system will require death of the parasites. Detectable antibody levels may be produced if enough numbers of parasites die

following infection of the host. Yet, two field observations lead to the conclusion that somatic antigens of the adult worm may not readily induce significant antibody responses. First, *P. tenuis* is a long-lived parasite (Duffy et al., 2002a). Second, only few parasites are present in the infected host, mean = 2.5 worms (Slomke et al., 1995). Thus, it is clear that there are both quantitative (i.e., amount of antigen) and qualitative (nature of antigen) restrictions imposed on the usefulness of somatic antigens as a diagnostic reagent.

In contrast, the combination of antigens secreted by live parasites and molecules sloughing off the surfaces as part of the process of normal and essential parasite metabolism (= ES products) would result in exposure to the immune system in quantities high enough to generate a measurable antibody response and serological detection of infected animals.

Because the secreted and excreted antigens are products of the essential physiological processes in a parasite, ES products are likely to display parasite-specific characteristics, which should translate to a specific laboratory diagnosis of the infected host. The technology to carry out an *in vitro* culture of helminths and to generate ES products in sufficient quantities has become available, and refined, over the years (Smyth, 1990). This provides a means of overcoming the problem of availability of antigen for serodiagnosis. It is important to appreciate that the laboratory generation of ES products poses a formidable set of logistical hurdles.

The generation of the L3 of *P. tenuis* and the subsequent production of ES products are very labor-intensive, in part because the effort requires *ab initio* the availability of large numbers of viable L1 from an infected WTD. The animal has to be acquired as a neonate or from an environment free of any of the protostrongylid parasites and transported under strict regulatory provisions. It is then held in a snail-free pen for a number of months (\geq three months) following inoculation with *P. tenuis*, and long enough for the animal to excrete L1 in the feces. The L1 will need to be recovered in relatively large numbers and be passaged through previously parasite-free, gastropod intermediate hosts to generate L3. Extracting larvae from feces or the gastropod host is also labor-intensive, but the development of a technique for extracting clean larvae from feces that have been subjected to the Baermann procedure (Ogunremi and Gajadhar, 2000) and its adaptation in recovering L3 by digestion from inoculated snails (Ogunremi, unpublished) has simplified the task and reduced, but not eliminated, the requisite labor. Recovered larvae need to be cultured under sterile conditions, fed and monitored frequently, and maintained for about three weeks. The duration of parasite culture before harvesting ES products has to be optimal to ensure a good balance between the generation and secretion of large amounts of ES products on one hand, and normal catabolic degradation of parasite-secreted material on the other (Riga et al., 1995; Ogunremi et al., unpublished). The harvested antigen needs to be batch-tested under a rigorous quality assurance system to ensure that test results are repeatable within a laboratory, and reproducible between laboratories, for the purpose of drawing reliable and valid inferences from test results.

Before outlining the effort made to standardize the use of ES products of *P. tenuis* L3 for serodiagnosis, it is worth noting that other antigens have been evaluated. Duffy and Burt (2002) used ES products derived from adult worms to detect red deer infected with *P. tenuis* by the Western blot technique. As expected, animals exposed to doses that approximate the numbers of L3 typically ingested in the field failed to develop antibodies until after three months post-inoculation. However, the use of this technique for field *P. tenuis* serodiagnosis poses some difficulty because of the observations that the antibody levels waned and became

undetectable by 11 months post-inoculation. A cloned recombinant *P. tenuis* antigen (Ogunremi and Gajadhar, 2002), later described as the aspartyl protease inhibitor (Duffy et al., 2002b), was expressed in *Escherichia coli* and used in an ELISA to detect all 109 necropsy-confirmed, *P. tenuis*-infected WTD while at the same time correctly identifying 54 of 59 negative WTD (Ogunremi et al., 2008; Ogunremi and Yabsley, manuscript under preparation).

STANDARDIZATION AND OPTIMIZATION OF *P. TENUIS* ELISA TESTS AND REAGENTS, ESPECIALLY LARVAL EXCRETORY-SECRETORY PRODUCTS AND CONJUGATES

The complex biological processes involved in a helminthic infection are important considerations when assessing the indices of a standardized/optimized test, namely high analytical sensitivity, high analytical specificity or good repeatability. As is the case with other pathogens that undergo multistage development or antigenic variation, helminths can induce the production of stage-specific antibodies in the infected host during the course of their development from L3 to the adult stage. It was therefore important, as part of the standardization and optimization process of the *P. tenuis* serological tests, to ensure that antibodies of diagnostic importance are present in the host during the entire course of infection and are detectable in a repeatable manner. For this reason, serially-obtained serum samples from experimentally-inoculated animals were tested. Each animal was bled once before inoculation with the parasite and many times afterwards. Bleeding was done usually every 1 - 2 weeks until the end of the experiments (Ogunremi et al., 1999a, b; 2002a). The testing of samples collected at regular intervals over time provided an early indication about the potential utility of a test for field use.

Because *P. tenuis*-infected animals can harbor the parasite for many years, it is critical that persistent, long-term infections are detected. The testing of many samples collected over time from the same infected animal becomes more important if only a few exposed animals are available to the researcher. This is usually the case when the test is to be used on rare parasites or wild, mammalian hosts that are difficult to acquire or access. The serially-collected serum samples tested during this phase were obtained from 6 - 10 individual animals from each target host that had been experimentally-inoculated with doses of *P. tenuis* larvae judiciously chosen to approximate field exposures in a majority of the animals (Ogunremi et al., 1999b, 2002a, 2008).

The assessment of antibody production in the experimental host over time should provide a critical piece of information for eventual application in disease control and regulatory veterinary medicine relating to live animal trade. The earliest time when circulatory antibodies are detectable in an animal exposed to the parasite of interest, will determine the length of time required between a pre- and a post-embarkation testing of animals. This information will also determine the length of quarantine before an imported animal, which is found to be free of the parasite, can be released to the owner. Because of its inherent robustness and the relative ease at troubleshooting, ELISA enjoys a widespread familiarity and use by workers in many immunodiagnostic laboratories worldwide. However,

unstandardized test reagents (e.g., antigen and conjugate) will negatively affect the validity of test results and their interpretation.

Without a doubt, the most important reagent of a *P. tenuis* ELISA test, from the technical and analytical perspective, is the coating antigen, i.e., the ES products. Pre-treatment of the antigen with reducing agents before coating the ELISA plate has led to increased analytical specificity by reducing the optical density (OD) values of negative serum samples without affecting the high OD values of positive serum samples (Ogunremi and McDonald, unpublished observations). Rigorous evaluation of the different batches of ES products was critical to define conditions of parasite culture necessary to ensure repeatable ELISA results. As measured by the co-efficient of variation (C.V.) of the absorbance value, which is defined as the standard deviation of the OD values of replicates divided by the mean OD of the replicates, repeatability of the ELISA results were good with C. V. typically $\leq 10\%$ when ES products harvested from 3- to 21-day-old larval cultures were used as coating antigen. C. V. estimates computed from untransformed OD results of at least five infected animals from each of the three species tested over n number of days were as follows: C. V. for elk = 8.3%, $n = 3$ days; C.V. for WTD = 8.4%, $n = 7$ days; C. V. for moose = 9.9%, $n = 4$ days (Ogunremi, Vidal and MacDonald, unpublished). Once generated and stored, batches of ES products were found to be stable for 272 days at 4°C, and longer (≥ 5 years), or probably indefinitely at - 20°C or - 70°C (Ogunremi, MacDonald and Vidal, unpublished).

There is an advantage in using commercially available conjugates in an ELISA test. They undergo pre-release quality assurance testing, details of which are provided to the buyer. However, homologous reagents are not always available for many species of interest, e.g., elk and moose. For this reason, the *P. tenuis* ELISA has benefited from the use of in-house prepared reagents (Ogunremi et al., 2002a, b). Analytical sensitivity was found to be low when a commercially available, non-homologous conjugate (anti-WTD IgG) was used to test elk sera and this problem was overcome with the preparation of an anti-elk IgG conjugate (Ogunremi et al., 2002a). Similarly, a mouse anti-moose conjugate used for testing moose was found to provide excellent results (Ogunremi et al., 2002; Lankester et al., 2007). Steps were taken to rigorously evaluate and optimize each reagent and every test condition. Different ELISA plates, concentrations of the coating antigen and conjugates, type of diluents and buffers, and the incubation conditions for each step were all evaluated, analyzed by checkerboard titration when necessary, and changes made as required to ensure a good dose-response curve with each titrated serum sample (Ogunremi et al., 1999a, 2002a and b, 2008). The optimization led to tests with high analytical sensitivity, specificity, and repeatability, paving the way to evaluate the performance of the test on samples similar to those expected to be submitted for routine field testing.

VALIDATION OF *P. TENUIS* ELISAS

The goals of the validation exercise for the *P. tenuis* tests were to ensure that test results provided a reliable basis for correctly classifying test animals as “exposed” or “non-exposed” to the parasite, at a specified confidence level (Jacobson, 1998). Samples were chosen to approximate field situations as much as possible and to ensure that they could be obtained at a reasonable cost. With the collaboration of another laboratory, Prairie Diagnostic Services,

Inc. (PDS), which participated in blind, parallel testing of elk and moose samples, a number of parameters for the *P. tenuis* tests were established; namely, sensitivity, specificity, repeatability, reproducibility, as well as negative and positive predictive values.

The test sensitivity of the *P. tenuis* ELISA for elk was estimated at 100% (95% confidence interval, CI = 73.6 - 100%) on 12 animals sampled from day 28 after experimental inoculation with *P. tenuis* larvae (total number of serum samples = 68; Ogunremi et al., manuscript under preparation). Infection was confirmed in all the animals at necropsy. A specificity estimate of 97.3% (95% CI = 95.8 - 98.6%) was determined from the test results on serum samples obtained from 579 elk including a number known to harbor other helminths such as *Dictyocaulus* and intestinal strongyles. Sensitivity was determined to be 100% from the results of tests performed on 99 naturally-infected and 17 experimentally-infected WTD (95 CI = 96.9 - 100%). Test specificity was also found to be 100% from test results of 64 non-infected WTD (95% CI = 94.4 - 100%). Among 16 moose presenting clinical signs characteristic of parelaphostrongylosis and/or from which adult worms were recovered, sensitivity was estimated to be 100% (95% CI = 79.5 - 100%) and specificity was 100% (95% CI = 88.5 - 100%) among 30 non-infected moose (Lankester et al., 2007; Ogunremi et al., 2002b; Ogunremi, unpublished observations). The validated test for elk was recently used to carry out the first antemortem detection of *P. tenuis* in any species (McIntosh et al., 2007). Similarly, the tests for moose and WTD have been used to investigate field infections in the respective species with reliable results that were confirmed in a number of cases by postmortem findings (Lankester et al., 2007; Ogunremi and Yabsley, manuscript under preparation).

The establishment of predictive values for the *P. tenuis* tests requires knowledge of the prevalences of the parasite in different host species. Published prevalence data of the parasite in eastern North America are variable and depend on the method of diagnosis, i.e., larval or adult worm recovery. From an assembly of data generated from field studies of WTD tested using both larval and adult worm recovery methods, an overall prevalence rate of 50% was estimated for the southeastern US (Anderson and Prestwood, 1981). It is known, however, that the L1 recovery method consistently underestimates the occurrence of the parasite because of unisex infections or the presence of non-fecund female parasites. Studies of vehicle-killed WTD in Minnesota found that 53% had L1 in the feces (Slomke et al., 1995), a prevalence rate that compared favourably with an earlier estimate of 49% (Karns, 1967) using the same method on a different population of WTD in the same state (Minnesota). However, a prevalence rate of 82% was found when the heads of WTD were examined for adult *P. tenuis* worms (Slomke et al., 1995). A similar value of 80% prevalence based on adult worm recovery was observed in another, more recent study in Minnesota (Gogan et al., 1997). The negative and positive predictive values of the *P. tenuis* ELISA for WTD at 50% prevalence are approximately 95 -100%. At 80% prevalence, the positive predictive value range is 99 - 100%, while the negative predictive value is 88 - 96% (sensitivity = 97 - 100%; specificity = 94 - 100%).

Estimating the prevalence of *P. tenuis* in moose is difficult; however, its occurrence has been shown to mirror the density of sympatric WTD (Dumont and Crête, 1996; Lankester 2001). The use of fecal larval detection is limited by the possibility that moose infected with only *P. andersoni* larvae can also pass dorsal-spined larvae (Lankester and Fong, 1989), and moose showing clinical signs from *P. tenuis* infection may fail to pass larvae (Karns, 1977). Similarly, prevalence rates based on adult worm recovery can lead to an underestimation

because of the difficulty of finding the worms (Lankester, et al., 2007) or overestimation, if the entire sampled population is derived from vehicle- or hunter-killed animals because of possible over-representation of infected moose. For instance, Karn (1977) found a prevalence of 0.6% in 361 moose fecal samples in Minnesota, while Gilbert (1974) recovered worms from the heads of 25% of poacher-killed moose in Maine. In a study of 66 moose in Nova Scotia mostly derived from legal hunting, but also including accidentally-killed animals and clinically-sick moose, Thomas and Dodds (1988) found adult worms in 8 animals, i.e., 7.6%. Using a prevalence of 10% estimated from a recent seroprevalence study in a population of Minnesota moose (Ogunremi et al., 2002b), positive predictive value of the *P. tenuis* ELISA for moose was estimated at 43 - 92%, and the negative predictive value at 97 - 100% (sensitivity = 78 - 100%; specificity = 89 - 100%).

Although there are a number of reports of mortality due to *P. tenuis* in elk (Carpenter et al., 1973; Olsen and Woolf, 1979), including assertions that the parasite was responsible for failure of introduction into certain enzootic areas (Severinghaus and Darrow, 1976; Raskevitz et al., 1991), there are only a few documented studies on the prevalence of the parasite in elk. One study reported an average infection rate of 45.4% over three years, but clinical signs were reported in only 4.5% (Woolf et al., 1977). A more recent study of elk translocated into Kentucky found that 23% of non-capture related mortalities among radio-collared elk were due to *P. tenuis* (Larkin et al., 2003). Nevertheless, elk in enzootic areas with low WTD densities are expected to acquire low numbers of *P. tenuis* L3 without displaying clinical signs (Samuel et al., 1992). Assuming prevalences of 5, 20, or 45%, the positive predictive value ranges are 48 - 79%, 66 - 89%, and 93 - 98%, respectively, while the negative predictive value ranges are 99 - 100%, 97 - 100%, and 82 - 100%, respectively.

The variability in ELISA test results were measured by assessing the measure of agreement in (a) the results obtained by a single operator using the same set of instruments on different days, i.e., repeatability, and (b) results of different operators using the different equipment in different laboratories on different days, i.e., reproducibility. For both attributes, different ES products were used as ELISA coating antigen. Intratest C.V. estimated from 100 samples per each host species were also used to assess repeatability of the results of replicate samples, and estimated to be 5.8% for the WTD test, 2.6% for elk, and 4.3% for moose. Results of tests on elk and moose in our laboratory and a collaborator's (i.e., PDS Inc.), provide evidence of strong reproducibility of the *P. tenuis* ELISAs: concordance correlation coefficient, ρ_c , for elk = 0.98, $n = 15$ samples; for moose $\rho_c = 0.93$, $n = 6$ samples.

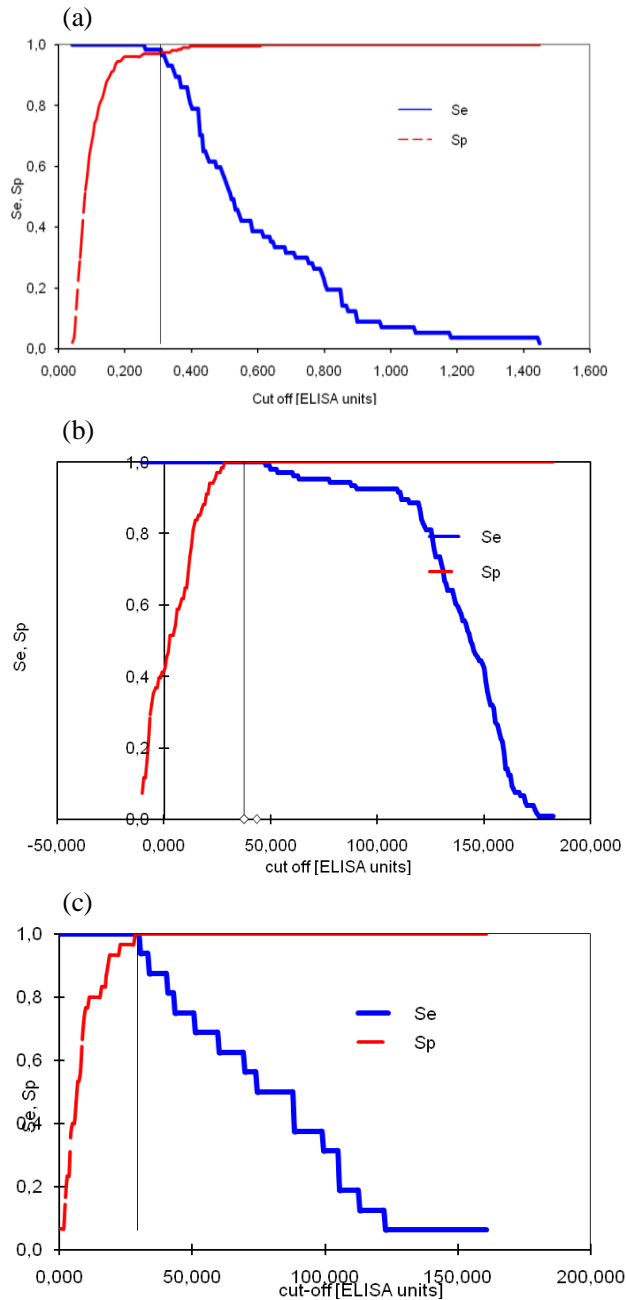


Figure 1. Two-graph receiver operating curves (TG-ROC) for cervid populations tested *Parelaphostrongylus tenuis* by newly developed and validated ELISA tests. Negative and positive populations were tested and analyzed as follows (a) elk, positive = 12; negative = 201; (b) white-tailed deer, positive = 106; negative = 68; and (c) moose, positive = 16, negative = 30). ELISA cut off values (units) and sensitivity/specificity (%) for each test were determined from the TG-ROC as follows: elk = 0.31×10^1 units; 99.5%; white-tailed deer = 37.7 units, 100%; moose = 29.4 units, 100%. The maximum allowable number of entries used by the software to calculate each parameter is 201. For the elk negative population, 201 animals were randomly selected from a total of 579 test results.

REGULATORY AND MANAGEMENT IMPLICATIONS

The *P. tenuis* serological tests are intended to be used as regulatory tools in preventing the introduction of the parasite into areas of North America currently free of the parasite. For this purpose, the highest possible test sensitivity is desirable (i.e., approximately 100%), and an appropriate, statistically justifiable cut-off value has been used for each test/species to ensure that all exposed animals are detected. For clinical diagnosis, it may be desirable to place equal weights on test sensitivity and specificity as a decision criterion for an appropriate cut-off value. The development of a receiver operating curve (ROC) for *P. tenuis* in elk, WTD, and moose provides a means for altering the cut-off values according to any intended use of the test. By computing the two-graph ROC curve and assigning equivalent weight on test sensitivity and specificity (Greiner, 1995), the following values were obtained for the two parameters of each of the tests: elk = 99.5%; white tailed deer = 100%; moose = 100% (Figure 1a - c). The validated *P. tenuis* ELISA tests meet the goal of *ensuring* that at a 95% confidence level or higher, a positive test result accurately reflects the *P. tenuis* exposure status of a WTD or an elk tested one month or more after exposure, or two months or longer for a moose. The establishment of each performance characteristic, as shown above, and its communication to each user, is a key component of assuring test validity.

CAN A SEROLOGICAL TEST, SUCH AS A *P. TENUIS* ELISA TEST, DIFFERENTIATE BETWEEN A CURRENTLY-INFECTED HOST AND ANOTHER THAT HAS OVERCOME THE PARASITE?

The use of serological tests for clinical diagnosis is typically constrained by the inability to distinguish between an infected animal that actively harbors an infection, and a previously-exposed host that has since overcome the infection. Many studies have observed lowered antibody levels following the elimination of an infection, e.g., by chemotherapy, prompting several authors to identify serological testing as a means of monitoring the success of chemotherapy (Noya et al., 1995). However, antibodies can persist for many months following elimination of a parasite (Doenhoff et al., 2003), thereby making the detection of antibodies, or the lack thereof, a less precise tool to assess parasite elimination. Detailed analyses of the immune response in helminth-infected humans have conclusively shown that while antibodies recognizing certain antigenic determinants persist following the elimination of infection, other types of antibodies experience a rapid decay (Kanamura et al., 1997). Thus, it should be possible to overcome the constraints of sustained seropositivity seen in animals that no longer harbour an infection, by using the appropriate type of test antigen in the test format such that antibody detection correlates with the presence of an infection. During the course of this study, some inoculated animals appeared to have spontaneously overcome their infection allowing assessment of antibody persistence in animals that no longer harbored the infection. An experimentally-inoculated moose which showed transient clinical symptoms suggestive of infection, also displayed only a transient seropositive status. In time, the animal became seronegative, and was shown at necropsy to be free of any adult worms despite a meticulous search. The anti-*P. tenuis* antibody level diminished and became undetectable

following elimination of the infection (Ogunremi et al., 2002b, Lankester, 2002). Similar observations, namely transient seropositivity and inability to recover adult *P. tenuis* worms, were made in an experimentally-inoculated elk (Ogunremi et al., 2002a). These observations are consistent with a correlation between the elimination of parasites in an infected host and the decay of antibodies directed against the ES products of the parasite. As metabolites of a living organism, ES products will inevitably disappear following the death of the parasite. In the absence of metabolites, the stimulation for new antibody synthesis (directed against the metabolites) will be lost, and the detection of antibody molecules against the metabolites will only be determined by the half-life of the existing antibodies, e.g., half-life for IgG molecules = 2 - 4 weeks. The *P. tenuis* tests which employ ES products, appear to be useful and precise tools for mapping the development of the immune response as a result of infection, and, in a case where infection is followed by the death of the parasites, to demonstrate the decay of the anti-parasite antibody response. In contrast to the ES products, the use of somatic antigens of the adult worms to test the serum samples in an ELISA did not provide the same quality of results for monitoring the development or decay of the antibody response (Ogunremi et al., 2002a, b), thereby reinforcing the utility of the ES products in serodiagnosis. Therefore, an adequately-refined and validated antibody detection method such as the *P. tenuis* tests developed during the course of the present study can differentiate between an active and an aborted helminthic infection.

CONCLUSION

Detection of *P. tenuis*-infected cervids has until now been difficult: the movement of susceptible cervid species from an enzootic area to one free of the parasite has remained under severe restrictions. Using the excretory-secretory products of the L3, new reliable serological tests for *P. tenuis* have been developed for elk, white-tailed deer, and moose. With this work, tools have now become available for the antemortem diagnosis of cervids exposed to *P. tenuis*, for use in reviewing current restrictions on the movement of live cervids, and to mitigate the risk of translocating *P. tenuis* into areas currently free of the parasite. These tools are expected to work well if applied in conjunction with a proper and adequate quarantine protocol. The tests should also be useful for the laboratory confirmation of meningeal worm infection in elk and moose when clinical signs are suggestive of parelaphostrongylosis. The use of the test on susceptible wild cervid populations should help to accurately assess exposures to the parasite and a better understanding of the parasite's impact, for example, on moose populations. It can also be used to investigate observations that suggest recently introduced animals, such as elk, tend to show greater susceptibility to parelaphostrongylosis when compared to populations established for some time in an enzootic WTD area (Larkin et al., 2003; Bender et al., 2005). The testing of more samples from animals with an independently-confirmed infection status, e.g., by necropsy, can be expected to result in the narrowing of the confidence-interval estimates for the three validated tests and thereby further improve the available validation parameters of the *P. tenuis* tests.

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Chapter XI

TICK G PROTEIN-COUPLED RECEPTORS AS TARGETS FOR DEVELOPMENT OF NEW ACARICIDES

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ABSTRACT

The GPCR class of receptors is a source of many pharmacologicals in human medicine and is still being pursued in research programs as promising targets for drug development. GPCRs have been identified in arthropods, and with the advent of expressed sequence tag (EST) and genome projects in the ticks *Ixodes scapularis*, *Rhipicephalus (Boophilus) microplus*, and *Rhipicephalus appendiculatus*, there is an opportunity for mining these sequence databases to identify tick GPCRs. These tick GPCRs can be identified using GPCR-targeted bioinformatic approaches and investigated for their potential in the development of novel tick control chemical technologies. There is precedence for developing acaricides targeting membrane-associated proteins like the GPCR family, as the pyrethroid, formamidine, and macrocyclic lactone families of acaricides target membrane-associated proteins. Current acaricides are becoming less efficacious due to problems with resistance and new chemistries which attack new targets are desperately needed by the cattle industry. We have used bioinformatics approaches to identify and classify putative tick-specific GPCRs in our *R. microplus* gene index of assembled EST sequences. Opportunities for using high throughput screening approaches are identified and discussed which can facilitate discovery of chemical compounds which inactivate the GPCR and can serve as an acaricide.

INTRODUCTION

Among the arthropods of veterinary importance, ticks are certainly among those with the most serious economic impact, as they parasitize animals in most parts of the world, causing blood loss and physical damage and transmitting disease to the animals they infest. In fact, the tick-disease complex of *Boophilus* spp.-*Babesia* spp.-*Anaplasma marginale* has been cited as the most important complex affecting worldwide cattle production (de Castro 1997). Annual losses in cattle production attributable to *Boophilus* ticks have been estimated to be approximately \$2 billion in Brazil (Grisi et al. 2002) and over \$100 million in Australia (Angus 1996). Annual losses to the United States' cattle industry were estimated at over \$130 million in 1906 (Graham and Hourrigan 1977), equivalent to approximately \$3 billion in 2008 dollars. Those losses were such that a *Boophilus* tick eradication program was initiated in 1906 and essentially completed in 1943. The eradication status of the United States is currently maintained by an extensive U. S. Department of Agriculture quarantine program at an annual cost of approximately \$4 million (Dietrich and Adams 2000). Tick control efforts by cattle producers are centered around the use of chemical pesticides, generally of the pyrethroid, organophosphate, formamidine, or macrocyclic lactone classes. However, pesticide resistance has become a serious impediment to tick control in many parts of the world (Angus 1996, Kemp et al. 1998, Miller et al. 1999), and novel control technologies are sorely needed to maintain control over these parasites and the diseases they transmit.

Current acaricides generally target membrane-associated or receptor-type membrane proteins as their site of activity. Organophosphate and carbamate acaricides act on acetylcholinesterase, a membrane-anchored enzyme (Fournier et al. 1988) critical to the arthropod nervous system. DDT and the pyrethroid class of acaricides target the sodium channel (Soderlund and Knipple 1995), a voltage-gated intrinsic membrane protein also critical to the arthropod neural system. Other ion channel-intrinsic membrane proteins which are targets of acaricides are the gamma-aminobutyric acid (GABA)- and glutamate-gated chloride channels which are the targets of fipronil (Wen and Scott 1999) and macrocyclic lactones (Kane et al. 2000), respectively. All of these acaricides have been used for control of *Boophilus* ticks; however, the problem of acaricide resistance is impacting their effectiveness and new acaricides acting against new target sites would be welcomed by the cattle industry.

G protein-coupled receptors (GPCRs) are an attractive family of membrane proteins to target in searches for candidate acaricides with novel modes of activity. Members of this large protein family perform a range of critical biological functions in eukaryotes, with roles in signal transducing processes such as chemoreception, photoreception, hormonal responses, and neural activity. Interestingly, around 40% of the prescription pharmaceuticals on the market target GPCRs (Filmore 2004). However, unlike human pharmaceuticals where the specific aim of the GPCR-targeting drug is to enhance the chances for survival or quality of life of the individual taking the drug, our ultimate aim would be the identification of a suitable tick GPCR whose perturbation by a specific small molecule would cause death of ticks infesting the animal receiving treatment. Arthropods possess a large family of GPCRs and approximately 270, including representatives from all five classes of GPCRs, have been described from the *Drosophila melanogaster* (Brody and Cravchik 1999) and *Anopheles gambiae* genomes (Hill et al. 2002). A few GPCRs have been reported from ticks, including an octopamine-like receptor (Baxter and Barker 1999), a myokinin receptor (Holmes et al.

2003), and a serotonin receptor (Chen et al. 2004), all from the Southern cattle tick, *Rhipicephalus (Boophilus) microplus*. Genome-wide searches for GPCRs have not been specifically reported in ticks; however, the *Ixodes scapularis* genome is available for analysis (Van Zee et al. 2007), as are annotated EST databases for *R. microplus*, *R. appendiculatus*, *I. scapularis*, and *Amblyomma variegatum* (<http://compbio.dfci.harvard.edu/tgi/>). Identification of tick candidate GPCR genes from EST databases would be a first step to the evaluation of the corresponding gene products as potential targets for the development of novel acaricides. We have used our annotated *R. microplus* EST sequence database to identify candidate tick GPCRs by using the Gene Ontology (GO) Term annotation associated with individual sequence entries. We also discuss the necessary subsequent steps to identify candidate-small molecules with acaricidal activity resulting from their interaction with the tick GPCR target.

MATERIALS AND METHODS

The construction of a pooled *R. microplus* normalized cDNA library and generation of ESTs has been described (Guerrero et al. 2005). Briefly, a single normalized cDNA library was synthesized from pooled RNA samples that had been individually purified from ticks subjected to various environmental exposures, including heat shock, cold shock, permethrin, coumaphos, amitraz, host odor for 24 hr, and infection with *Babesia bovis*, the apicomplexan causative agent of cattle fever. RNA purified from eggs, nymphs, adults, and dissected adult ovaries, guts, salivary glands, and synganglia was also used for the library synthesis. The acaricide exposed tick samples utilized several *R. microplus* strains which varied in their susceptibility to pyrethroid, organophosphate, and formamidines. This pooled library was the source of 42,512 of the 51,438 ESTs in the final assembled *R. microplus* database used in this report.

Three other subtracted libraries which were synthesized under contract by Express Genomics, Inc. (Frederick, Maryland) also served as source material for our EST database. The strain of *R. microplus* used as the source for these subtracted libraries was the La Minita strain maintained since 1999 on Holstein calves 5-6 months of age at the University of Idaho Holm Research Center. Ticks for the colony were originally collected in Starr Co. Texas in 1996 and the colony was in approximately the f20 laboratory generation at the time of our study. Libraries were synthesized from cDNA of ticks which were infected with *B. bovis*, subtracting with cRNA from uninfected ticks. Larvae and dissected ovary and gut from replete adult females were the source material for these three libraries. To obtain uninfected ticks, *R. microplus* larvae from 1.0 gram of eggs were placed on a calf on day 1. On day 22, the first replete tick females were collected and dissections were done 4 days post-repletion, while females were actively ovipositing. To obtain *B. bovis*-infected ticks, *R. microplus* larvae from 1.0 gram of eggs were placed on a calf on day 1 and again 2 days later. The calf was infected on day 14 with *B. bovis* by inoculating it intravenously with 1.8 ml of *B. bovis* blood stabilate (T2Bo strain of *B. bovis*, prepared November 14, 1989 at 5.5% parasitized erythrocytes and stored in liquid nitrogen). Peak level of infection in the calf was determined by rectal temperature to be day 22. The first replete females were collected on day 21 and dissected 9 days post-repletion, while actively ovipositing. To confirm infection, hemolymph smears were examined from 66 females collected on this day: 46 females had ≥ 5 kinetes per

high power field; the remaining 20 females had ≥ 3 kinetes per high power field (Howell et al., 2007). Ticks were held in place for dissection with melted wax in petri dishes and dissected under phosphate-buffered saline (PBS). The ventral cuticle was excised with a scalpel and the ovaries and gut dissected, rinsed in sterile PBS, and placed in RNAlater (Ambion, Inc., Austin, TX) at room temperature until the material from 5 ticks was accumulated. Excess RNAlater was then removed and tissues placed at 4°C for 2-5 days then frozen at -80 °C. Larvae were collected in similar fashion and used without dissection and frozen immediately on dry ice. Total RNA was isolated with the TotallyRNA Kit (Ambion, Inc.) according to the kit's standard protocol utilizing the lithium chloride precipitation step to remove contaminating genomic DNA. The subtracted larval, gut, and ovary libraries contributed 791, 4,077, and 4,058 ESTs, respectively, to the assembled database used in this report.

The EST sequences were annotated by mapping to functional classification systems, including Gene Ontology (Ashburner et al. 2000), KEGG pathways (Ogata et al. 1999), and Swiss Prot Protein Keywords (Bairoch et al. 2005), through the use of the High Throughput Gene Ontology and Functional Annotation Toolkit which is a functional annotation engine based upon WND-BLAST (Dowd et al. 2005) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis Jr. et al. 2003). To find sequences annotated as GPCRs in other tick EST databases, we used data from The Gene Index Project (<http://compbio.dfci.harvard.edu/tgi/>) and the molecular function gene ontology classifications associated with each species, searching for GO term 0004930, which is G-protein-coupled receptor activity.

RESULTS AND DISCUSSION

The *R. microplus* EST database was assembled from a total of 51,438 ESTs, and, using Blast analysis cutoff E-values of 10, 0.001, and 1e-25, we identified 14,464; 6,377; and 3,745 entries, respectively, which have hits to the UniProt database (The UniProt Consortium 2008). The average length of the 14,464 entries is 989 bp. We utilized the GO Term annotations to determine which contigs contained the Molecular Function GO Term 0004930 G-protein-coupled receptor activity and had Blast E-values of <0.001. In this manner, we identified 30 contigs which meet these criteria and are potential GPCR-encoding sequences (Table 1). We also found 232 other contigs which had the G-protein-coupled receptor activity GO Term 0004930 in their annotations and a Blast E-value for their top UniProt database hit of between 0.001 and 10. Among this group of tick contigs are sequences with similarity to metabotropic glutamate receptor 4, various prostaglandin receptors, methuselah, vomeronasal 1 receptor, various olfactory and gustatory receptors, muscarinic cholinergic receptor 1, and others (data not shown).

Table 1. *R. microplus* EST database contigs with GO Term 0004930 G-protein-coupled receptor activity and E-Values <0.001

Contig ID	Length bp	UniProt Acc. No.	UniProt Blast Hit ID	E-Value
Class A Rhodopsin-like				
15709	1652	O57422	OPSIN 4 (MELANOPSIN)	2.4 E-25
17291	818	Q9DDN6	NEUROPEPTIDE Y RECEPTOR Y2	2.9 E-20
16527	1470	Q5WA50	CEPHALOTOCIN RECEPTOR-2	1.5 E-12
4975	1277	P17152	TRANSMEMBRANE PROTEIN 11	4.7 E-28
5090	1114	Q9BXB1	LEUCINE-RICH REPEAT-CONTAINING G PROTEIN-COUPLED RECEPTOR 4	2.6 E-08
Class B Secretin-like				
11215	772	O75575	CALCITONIN GENE-RELATED PEPTIDE-RECEPTOR COMPONENT PROTEIN	1.0 E-17
7646	1483	O88923	LATROPHILIN 2	4.7 E-06
16110	984	O88923	LATROPHILIN 2	0.6 E-2
5369	1187	O95490	LATROPHILIN 2	9.2 E-07
16412	853	Q9NYQ7	CADHERIN, EGF LAG SEVEN-PASS G-TYPE RECEPTOR 3 (FLAMINGO)	0.2 E-3
15940	470	Q9VXD9	METHUSELAH-LIKE 1	1.7 E-10
12804	990	Q9W4Y2	PROTEIN GROOM-OF-PDF	6.9 E-34
Class C Metabotropic glutamate/pheromone				
5957	662	P31422	GLUTAMATE RECEPTOR, METABOTROPIC 3	1.7 E-23
16629	1228	P41594	GLUTAMATE RECEPTOR, METABOTROPIC 5	8.0 E-62
7684	1321	P91685	METABOTROPIC GLUTAMATE RECEPTOR	7.7 E-66
17600	809	P91685	METABOTROPIC GLUTAMATE RECEPTOR	1.4 E-44
15372	951	Q14416	GLUTAMATE RECEPTOR, METABOTROPIC 2	4.1 E-73
6036	1219	Q9Z0U4	GAMMA-AMINOBUTYRIC ACID (GABA) B RECEPTOR 1	1.6 E-09
4699	1375	Q9Z0U4	GAMMA-AMINOBUTYRIC ACID (GABA) B RECEPTOR 1	2.7 E-08
2353	1663	Q8C419	G PROTEIN-COUPLED RECEPTOR 158	4.5 E-13
Frizzled/Smoothed Family				
212	633	O00144	FRIZZLED HOMOLOG 9	1.8 E-24
5986	1549	Q8BKG4	FRIZZLED HOMOLOG 10 (DROSOPHILA)	9.3 E-114
Miscellaneous				
17428	967	Q8K4M9	OXYSTEROL BINDING PROTEIN-LIKE 1A	1.0 E-90
15438	2033	Q8K4M9	OXYSTEROL BINDING PROTEIN-LIKE 1A	2.2 E-68
13924	1355	Q8K4M9	OXYSTEROL BINDING PROTEIN-LIKE 1A	1.2 E-34
7686	1447	Q9BXS9	SOLUTE CARRIER FAMILY 26, MEMBER 6	4.6 E-59
11000	934	Q9BXS9	SOLUTE CARRIER FAMILY 26, MEMBER 6	5.6 E-51
12381	933	Q9GZR5	ELONGATION OF VERY LONG CHAIN FATTY ACIDS -LIKE 4	1.5 E-51
13609	903	Q9GZR5	ELONGATION OF VERY LONG CHAIN FATTY ACIDS -LIKE 4	3.3 E-24
11501	931	Q9WC54	POL PROTEIN	1.8 E-4

It is likely that some of the contigs from this group with relatively high E-values (0.001-10) will prove to be authentic GPCRs because this family of proteins is well known for having members which show very little sequence similarity to each other even when grouped into subfamilies and classes according to similarity of transmembrane topology or function (Smith 1999). In fact, this problem of low sequence similarity among related family members has led to a number of bioinformatics approaches being developed to help identify new members of the GPCR class. Inoue et al. (2004) developed a binary topology pattern analytical approach which was more effective than a Blast search for finding mammalian-type GPCRs in non-mammalian genomes. Included in their study were the arthropod genomes of *D. melanogaster* and *A. gambiae*. Another approach was reported by Ono et al. (2005), who developed an automated bioinformatic pipeline which integrated gene finding, sequence alignment, motif and domain assignment, and transmembrane helix-predicting software. It is likely that these non-Blast based approaches will be most valuable for identification of tick-specific GPCRs not only because of the sequence divergence typically seen in the GPCR family but also because of the general sequence divergence seen between ticks and other sequenced organism's genomes (Van Zee et al. 2007). Although the genome sequence of *R. microplus* is not available, translation of the *R. microplus*-assembled ESTs would be a source of open reading frames which could be analyzed by these non-Blast methodologies. Additionally, as open reading frames from the *I. scapularis* genome sequencing project become identified, either group's approach could be used to identify *I. scapularis* GPCRs with the assumption that orthologues occur in *R. microplus* which could be isolated by cDNA library hybridization techniques. Using the Gene Index Project's annotation system (<http://compbio.dfci.harvard.edu/tgi/>), a number of putative GPCRs can be found from four tick species which are included in the Project's list of species. The numbers in Table 2 were derived by a simple query of the Gene Index with GO Term 0004930, G-protein-coupled receptor activity, and include Gene Index members with subtree TCs branching from Term 0004930.

Table 2. Putative G-protein-coupled receptor annotated sequences from other tick EST databases

Species ^a	Database size No. ^b	Putative GPCRs No. ^c
<i>Ixodes scapularis</i> Ver. 3.0	192,746	170
<i>Rhipicephalus appendiculatus</i> Ver. 2.1	18,509	36
<i>Amblyomma variegatum</i> Ver. 1.1	3,992	16
<i>Boophilus microplus</i> Ver. 2.1	42,651	70

^aData from The Gene Index project (<http://compbio.dfci.harvard.edu/tgi/>).

^bNumber of ESTs used to assemble the Gene Index.

^cNumber of annotated assembled contigs or singletons with annotation containing GO Term 0004930, G protein-coupled receptor activity or listed as subtree TCs under the GO Term 0004930 in the Gene Index annotating system.

We have now identified several candidate tick GPCRs based on Blast analysis E-Values, and more are likely to be identified in the future by structurally-based (as opposed to sequence similarity-based) analytical methods such as discussed above. As resources are generally limited, it seems prudent to prioritize tick- or arthropod-specific GPCRs from the

group of candidates for further studies. At the very least, to minimize off-target organism effects, GPCR candidates can be prioritized based on minimum similarity to mammalian orthologues. Although based on sequence similarity among open reading frames, the Blast Score Ratio analysis (Rasko et al. 2005) is one such approach, as this comparative genomics tool allows comparison of a collection of translated tick GPCR open reading frames against two selected translated proteomes. Tick GPCRs which were found to have close mammalian orthologues by this analysis could be eliminated from further consideration.

On the progression from gene sequence to acaricide product, the next step beyond selection of a target GPCR involves the identification of a small molecule, which is able to be applied to the tick in a manner consistent with current application technologies, will penetrate the individual's cuticle, and cause the tick's death. Although *in silico* screening methods are being developed (Jacob et al. 2008), cell-based assays are the standard high-throughput screening methods currently employed. Future studies of putative *R. microplus* GPCRs identified from our EST database will generally follow the standard methodologies, beginning with stable expression of the GPCR's open reading frame in a eukaryotic cell line compatible with high-throughput screening. Holmes et al. (2003) used Chinese hamster ovary cells to stably express and functionally characterize the myokinin GPCR from *R. microplus*. This group used fluorescent techniques to measure differences in intracellular calcium induced by agonist challenge. GPCRs which are coupled to the class of G proteins, known as G_q, are naturally suited for using calcium ion monitoring in high-throughput screening for small molecule agonists/antagonists. Rhodopsins are coupled to G_q proteins and there are 5 members of the Rhodopsin-like class of GPCRs in our database (Table 1). The class 1 and 5 metabotropic glutamate receptors are also coupled to G_q proteins and at least one of those receptor types is in the *R. microplus* EST database. For GPCRs which do not interact with G_q proteins, there are generally other means to couple receptor activation to calcium-based methods of detection, including the use of promiscuous or chimeric G proteins which can couple to a range of GPCRs and link them into a signal transduction pathway which involves calcium release (Kostenis 2001). Monitoring of cyclic adenosine monophosphate (cAMP) is an alternative to calcium-based screening, as cAMP is an important second messenger in the GPCR activation process. There are commercially available GPCR assays which use luminescent tags which bind to cAMP and are useful to monitor changes in cAMP in GPCR-expressing cells in response to external addition of small molecules from a library. Most high-throughput screening methods use either calcium- or cAMP-based detection protocols to screen candidate peptide ligands as in Holmes et al. (2003), random peptide mRNA display (Ja et al. 2007) or phage display libraries (Szardenings et al. 1997; Hessling et al. 2003), or commercially-available libraries of small molecules.

CONCLUSION

GPCRs are a rich source of biologically-active proteins which have been aggressively studied by the pharmaceutical industry for human drug development, but are largely unexplored for their potential as targets for novel pesticide development. We have used our annotated *R. microplus* EST sequence database to identify 30 candidate tick GPCRs by using the Molecular Function Gene Ontology annotation associated with individual sequence

entries possessing significant sequence similarity to known GPCRs. We also discuss the necessary subsequent steps to identify molecules with acaricidal activity resulting from binding to their tick GPCR target.

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Chapter XII

BIOTRANSFORMATION OF XENOBIOTICS IN LANCET FLUKE (*DICROCOELIUM DENDRITICUM*)

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ABSTRACT

Biotransformation of xenobiotics serves as an efficient defense against potential negative action of foreign compounds in an organism. The activities of biotransformation enzymes determine desired, as well as undesired, effects of drugs and knowledge of drug metabolism is necessary for safe and effective pharmacotherapy. While human and mammalian biotransformation enzymes have been intensively studied for many years, the ability of helminth parasites to metabolize xenobiotics has been relatively little investigated so far. The aim of the present study was to test the activities of biotransformation enzymes towards model substrates and to study the biotransformation of albendazole (ABZ) in lancet fluke (*Dicrocoelium dendriticum*). Dicrocoeliosis, lancet fluke infection, is a frequent parasitosis of small ruminants and ABZ belongs among anthelmintics effective in control of this parasitosis. *In vitro* experiments (subcellular fractions of fluke homogenate), as well as *ex vivo* experiments (adult flukes cultivated in medium), were performed in the study. Biotransformation of many model xenobiotics was tested and activities of peroxidases, catalases, aldo/keto reductases, glutathion S-transferases, and UDP-glucuronosyl transferases were found. On the other hand, no activities towards model substrates of cytochromes P450 and flavine monooxygenases were detected. LC-MS analyses of ABZ metabolites revealed that lancet fluke enzymes metabolized ABZ via sulfoxidation, but they were not able to form any conjugate of ABZ.

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INTRODUCTION

Many xenobiotic compounds, e.g., environmental contaminants, drugs or food additives, permanently enter animal bodies. In an organism, most of the xenobiotics are metabolized by biotransformation enzymes into more polar metabolites that are easier to excrete. Xenobiotic biotransformation is considered to occur in two phases. Oxidation, reduction or hydrolysis of drugs, represent phase I of metabolism. In this step, reactive and hydrophilic groups are inserted or uncovered in the structures of xenobiotics. In phase II, xenobiotics, or their phase I metabolites, can undergo conjugation reactions with endogenous compounds. Glutathione, glucuronic acid, amino acids, and sulphates represent the main conjugation agents.

The most important enzymes involved in phase I biotransformation are cytochromes P450 (CYPs), heme-proteins ubiquitous in living organisms. Thousands of different CYPs have been identified and classified into several families and subfamilies on the basis of the sequence homology of the genes. CYPs commonly catalyze monooxygenation of substrates, but they are also able to act as peroxidases or reductases. In addition to xenobiotic biotransformation, CYPs play a very important role in the endogenous metabolism of steroids, fatty acids, and prostaglandins. Flavin-containing monooxygenases (FMO) mediate the oxidation of a large number of xenobiotics, preferring substrates with nucleophilic nitrogen or sulphur atoms. The FMO gene family is much smaller than the CYP family, and the FMO physiological functions are poorly understood. In spite of this fact, FMO activities are important in biotransformation of many xenobiotics (Krueger 2005). Peroxidases, monoamine oxidase, and xanthine oxidase represent other enzymes which can catalyze the oxidation of xenobiotics (Utrecht, 2007). Alcohols, aldehydes, and ketones are often metabolized via the action of reductases/dehydrogenases. These enzymes are classified into three protein classes: medium chain dehydrogenases (MDR), short chain dehydrogenases (SDR), and aldo-keto reductases (AKR) (e.g., Jez, 2001). The substrates of reductases/dehydrogenases include not only xenobiotics but also a number of endogenous substances. Reductive biotransformation is less frequent than the oxidative one, but in case of ketones, aldehydes, quinones, and nitro compounds, N-oxides or S-oxides, the reduction can represent the main metabolic pathway (Testa, 2003, 2007).

Most of the metabolites formed in phase I and appropriate functional groups containing xenobiotics are conjugated with certain endogenous compounds. Conjugation usually introduces hydrophilic ionizable functional groups to the molecule of a xenobiotic making it more polar and facilitating renal excretion. Glucuronidation is the most common conjugation pathway in mammals. The enzymes involved are UDP-glucuronosyl transferases (UGT) of two gene families. Typical UGT substrates are xenobiotic or eobiotic alcohols, phenols or carboxylic acids. Electrophilic compounds are mostly conjugated with glutathione through the action of glutathione S-transferases (GST). These ubiquitous conjugation enzymes, found in all organisms which were tested for their presence, protect biomolecules against reactive electrophiles (Utrecht, 2007).

All biotransformation enzymes serve for detoxification of potentially harmful xenobiotics, and their activities are crucial for the effect and/or efficacy of drugs and other xenobiotics. Generally, the parent substance and the metabolite or conjugate differ in both physico-chemical properties and pharmacodynamic effect, as well as in toxicity and pharmacokinetic behaviour (Testa, 2007). The occurrence and activity of biotransformation

enzymes determines the way and extent in which a drug or a xenobiotic is metabolized. Hence the biotransformation enzymes fundamentally affect the biological effects (both desired and undesired) of an administered drug. In the case of anthelmintics, the activity of biotransformation enzymes of both the helminth parasite and the host substantially affects the efficacy of pharmacotherapy of a helminthosis. While biotransformation enzymes in mammals have been intensively studied for many years, the knowledge of helminth biotransformation enzymes remains insufficient (Cvilink et al., 2009a).

Lancet fluke (*Dicrocoelium dendriticum*, synonym *D. lanceolatum*, *Dicrocoelidae*, *Platyhelminthes*, *Scolecida*) is a flat worm with a transparent body reaching the length of 5 to 15 mm and width of 1.5 to 3 mm. It particularly parasitizes in the biliary duct, the gallbladder, and at the outlet of pancreas of its host. Dark brown eggs are oval and extremely small (36-45×20-30 µm). They can survive even at very unfavourable conditions such as a dry and cold spell of one year without losing their infectivity. Eggs contain the first developmental stage – a miracidium. The life cycle of a fluke is indirect (with two interhosts and one final host) and its duration is approximately six months. The final host is infected by ingestion of the second interhost – an ant. The adult flukes live in the bile ducts and the gallbladder for many years – until the host's death (Otranto and Traversa, 2002). Dicrocoeliosis – a helminthosis caused by lancet fluke – is at present considered a globally significant, but little investigated disease of farm, domestic, and wild animals (Otranto and Traversa 2003), and a relatively rare illness of man. The occurrence of the helminthosis is worldwide, i.e., everywhere where there are suitable conditions for the life of interhosts (terrestrial calciphilic gastropods and ants), and where, at the same time, the final hosts live (Eckert and Hertzberg 1994). Clinical symptoms of dicrocoeliosis in animals are not specific, and the course of infection is mostly unapparent. In the cases of massive infection (more than several thousands of adult dicrocoelia in the attacked individual), the illness is manifested by anaemia, icterus, body weight loss, and disturbance of reproduction indicators of the breed (Jurášek and Dubinsky, 1993). Dicrocoeliosis negatively affects, in particular, the growth and development of young animals. In the final hosts, flukes cause an acute inflammation of intrahepatal biliary ducts, which is gradually transformed into chronic form with typical symptoms such as widening of the ducts, their glandular hyperplasia, proliferation, and desquamation of mucosis (Rahko, 1972). Pathological changes are caused by both the action of toxic products formed by the parasite and mechanical irritation of the walls of ducts by the parasite bodies (Hillyer and Hopla 1998). The damage of the host's liver tissue, connected with many pathological changes, is apparent not only macroscopically, but also by the means of physiological and biochemical markers (Sanchez-Campos et al., 1996, 1998, 2000).

The only generally accepted tool to control lancet fluke so far is pharmacotherapy and pharmacoprophylaxis practised in endangered or attacked breeds using suitable anthelmintics (antitrematods). From among the spectrum of accessible antitrematods, the preference is given to benzimidazole anthelmintics (e.g., albendazole, ABZ). However, a predominant part of adult fluke population is found in narrowed biliary ducts changed by the chronic process. Hence, a single administration of a drug usually fails to reach the desired effect, and the drug must be administered repeatedly and in higher doses. The situation is further complicated by limited knowledge of the parasite's ability to biotransform the administered anthelmintics. There is lack of information on ABZ metabolism in *D. dendriticum*, although this anthelmintic is an important tool for the control of lancet fluke infections in ruminants.

The presented projects were designed to bring out new information on biotransformation enzyme activities and ABZ metabolism in lancet fluke.

MATERIAL AND METHODS

Chemicals

Albendazole was purchased from Sigma-Aldrich (Pobřežní 46, Prague 8, Czech Republic). Albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSO₂) were obtained from Toronto Research Chemicals Inc. (2 Brisbane Rd., North York, ON, Canada). Liquid sterile-filtered RPMI medium (Roswell Park Memorial Institute medium) and all other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Pobřežní 46, Prague 8, Czech Republic).

Collection of Parasite Material

D. dendriticum adults were isolated from naturally-infected mouflon ewes (*Ovis musimon*, n = 5, age 5-7 years) bred in game-enclosure Vlkov (Czech Republic). The infected mouflon ewes were culled and exsanguinated immediately in agreement with Czech law. After the removal of the liver from the abdominal cavity (up to 5 min.), the liver was coated with a polyethylene sac, immersed in the warm saline solution (0.9% NaCl in water, 38°C), and transported to the laboratory (up to 60 minutes). There the liver tissue was cut and repeatedly washed with a saline solution (38°C) in an attempt to collect the fluke adults. In the following experiments, the mixture of flukes isolated from individual mouflons was used.

Preparation of Subcellular Fractions

Freshly-isolated *D. dendriticum* adults were washed repeatedly in 0.1 M phosphate-buffered saline (pH 7.4) and quickly homogenized in 0.1 M sodium phosphate buffer (pH 7.4, ratio 1:6, w/v), using a Potter-Elvehjem homogenizer and sonication with Sonopuls (Bandelin, Germany). The subcellular fractions were isolated by fractional ultracentrifugation of the homogenate in the same buffer. The first centrifugation removed un-homogenized pieces and the nuclei. Fraction A (20 000 g sediment of the second centrifugation) corresponds to the mitochondrial fraction in vertebrate tissue fractionation. Fraction B and Fraction C correspond to 105 000 g sediment and supernatant (microsomes like and cytosol like). A re-washing step in the same buffer (followed by the second ultracentrifugation) was included at the end of the Fraction B preparation procedure. Fractions A and B were finally re-suspended in a 0.1 M sodium phosphate buffer (pH 7.4) and in a 0.1 M sodium phosphate (pH 7.4) buffer containing 20% glycerol (v/v), respectively. All fractions were stored at -80°C. Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

Cultivation of *D. Dendriticum* in Medium

Freshly-isolated living flukes were washed three times with a phosphate-buffered saline (pH 7.4) and cultivated in plastic flasks with 5 mL RPMI medium (pH 7.4, containing 10 U mL⁻¹ penicillin and 10 µg mL⁻¹ streptomycin) at 38°C under humid atmosphere with 5 % CO₂.

Biotransformation of ABZ and ABZSO In Vitro

The Fractions A, B, and C were incubated in the presence or absence of ABZ or ABZSO (10 µM). The total volume of the reaction mixture (0.3 mL) contained 50 µL of Fractions (containing 0.4-0.6 mg of proteins), 3 µL of substrate pre-dissolved in dimethylsulfoxide (DMSO), NADPH or NADH (1.0 mM); and 0.1 M sodium phosphate buffer (pH 7.4). The blank samples contained 50 µL of 0.1 M sodium phosphate buffer (pH 7.4), instead of Fractions or 50 µL of 10-minute-boiled Fractions. The concentration of DMSO in all reaction mixtures and blank samples was 1% (v/v) in order to obtain the precise concentration of ABZ and ABZSO, which are only slightly soluble in water. All incubations were carried out at 37°C for 30 minutes under aerobic conditions. The formation of products was linear up to 60 minutes. After the incubation, 30 µL of ammonium solution (concentrated) and 700 µL of cooled ethyl acetate were added, the mixture was shaken (3 min, vortex), and centrifuged (10 min, 10000 g). Supernatants were evaporated and stored under -20°C until HPLC analysis.

Biotransformation of ABZ and ABZSO Ex Vivo

At the beginning of the incubation, 2.5 mL of medium was removed from each flask with flukes and the same volume of fresh medium containing ABZ or ABZSO was added. ABZ was pre-dissolved in DMSO. The concentration of DMSO in the medium was only 0.1% (v/v) to prevent any harmful effects on the living flukes. The flukes were incubated in the medium with ABZ (10.0 µM) or ABZSO (10.0 µM) for 48 hours. In blank samples, the medium with ABZ (or ABZSO) but without flukes (or the medium with flukes but without ABZ or ABZSO) was incubated. After the incubation, the medium was placed into plastic tubes, frozen, and stored under -80°C. More than 60% of the flukes were alive after 48h experiments (vivid flukes were suckered onto the flask walls). In the next step, the flukes were repeatedly washed up, transferred into plastic tubes, frozen, and stored under -80°C. Prior to the analysis, the flukes were quickly homogenized in 0.1 M phosphate buffer (pH 7.4) using Sonopuls. The medium and flukes homogenate were alkalized and liquid-liquid extracted under the same conditions as described in the previous section.

HPLC analysis of ABZ and Its Metabolites

The HPLC analysis was carried out using a Shimadzu LC-10ADvp solvent delivery module, a Shimadzu SIL-10ADvp autoinjector, a Shimadzu RF-10Ax1 fluorescence detector

($\lambda_{\text{EX}}=290$ nm, $\lambda_{\text{EM}}=320$ nm), and a Shimadzu CTO-10ACvp column oven fitted with a LiChroCART 250-3 (LiChrospher 60 RP-select B, 250 mm x 3 mm, 5 μ m) reverse-phase HPLC column (Hewlett Packard, USA) equipped with a LiChroCART 4-4 (LiChrospher 60 RP-select B, 4 mm x 4 mm, 5 μ m) guard column (Merck, Germany). The mobile phase A consisted of an acetonitrile - 25 mM potassium phosphate buffer (pH 3.0, 35:65, v/v). The flow rate was 0.5 mL/min in isocratic mode. All experiments were carried out at 25°C. Data was processed using the Shimadzu Class VP integrator software, version 6.12 SP2. The compounds were identified according to the retention times of reference standards. Under these chromatographic conditions, the retention times were 5.1 min (ABZSO), 7.1 min (ABZSO₂), and 17.2 min (ABZ). The limit of quantification for ABZ, ABZSO, and ABZSO₂ was 120.0, 2.0 and 0.07 pmol mL⁻¹, respectively. The linear calibration curves in range 10-60 nmol mL⁻¹ (ABZ), 1.5-10.0 nmol mL⁻¹ (ABZSO), and 0.09-0.7 nmol mL⁻¹ (ABZSO₂) served for quantification.

Solid Phase Extraction and Sample Preparation for LC-MS

Samples (medium and fluke homogenate) were extracted using two solid phase extraction methods: the method A was suited for extraction of cationic metabolites, and the method B was designed to extract the anionic metabolites. In the method A, one mL of the sample was acidified with 1 μ L 98% formic acid and centrifuged at approximately 3000 g for 3 minutes. The supernatant was loaded on to the Waters Oasis MCX (1cc, 30 mg, 30 μ m particles; Waters, Dublin, Ireland) extraction cartridge previously conditioned by washing it with 1 mL acetonitrile and 1 mL purified water. In the next step, the cartridge was washed with 1 mL 2% (v/v) aqueous formic acid. Compounds of interest were eluted with 1 mL 4% aqueous ammonia in 80% (v/v) acetonitrile. Similarly, in the method B, 4 μ L 25% (v/v) aqueous ammonia was added to 1 mL of the sample, centrifuged, and loaded onto the pre-conditioned (1 mL acetonitrile and 1 mL water) Waters Oasis MAX (1cc, 30mg, 30 μ m particles; Waters, Dublin, Ireland) extraction cartridge. The cartridge was rinsed with 2% (v/v) aqueous ammonia and the retained analytes were eluted with 2% (v/v) formic acid in 80% (v/v) aqueous acetonitrile. The eluates were evaporated to dryness using an Eppendorf 5310 concentrator (30°C, Hamburg, Germany), reconstituted in 100 μ L 10% (v/v) aqueous acetonitrile and 20 μ L of the final solution was injected into the analytical column.

LC-MS

The liquid chromatographic system comprised a Surveyor MS pump and a Surveyor autosampler (both ThermoFinnigan, San Jose, CA, USA). The column employed was SymmetryShield RP18 (2.1x100 mm, 3.5 μ m, Waters, Milford, USA). Two kinds of mobile phases were used. For the MS measurements in positive ion mode, the mobile phase consisted of solvent A (0.1% (v/v) aqueous formic acid) and solvent B (0.1% (v/v) formic acid in acetonitrile). For the MS measurements in the negative ion mode, the mobile phase consisted of solvent C (0.1% (v/v) aqueous ammonia) and solvent D (0.1% (v/v) ammonia in acetonitrile). A 120 μ L min⁻¹ flow rate gradient was developed over 25 minutes. Column compartment temperature was set to 40°C.

Mass spectrometry analyses were performed with an LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an electrospray ion source. Optimized ion source parameters were set for benzimidazole drug analysis during sample data acquisition in positive ion mode (negative ion mode values in brackets): spray voltage: 5.3 kV; capillary voltage: 25.0 V (-11.0 V); heated capillary temperature: 200°C; tube lens offset voltage: 30.0 V (-19.0 V); sheath gas flow rate: 20.0 arbitrary units; auxiliary gas flow rate: 10.0 arbitrary units. Ion optics settings were as follows: multipole 1 offset voltage: -3.0 V (4.0 V); lens voltage: -16.0 V (10.0 V); multipole 1 offset voltage: -8.0 V (2.0 V); multipole RF amplitude: 550 V. Nitrogen was used as both sheath and auxiliary gas, and helium was used as the damping gas. Data acquisition and processing were carried out using Xcalibur software (version 1.2).

For testing of ABZ fragmentation, a working ABZ standard was introduced into the mass spectrometer at a flow rate of 5 $\mu\text{L min}^{-1}$ using an inbuilt syringe pump. Stock standard solutions were prepared in a mixture of DMSO:acetonitrile:water (5:40:55; v/v) and stored at 4°C. Working standard solutions were prepared by diluting the corresponding volume of stock standard solution with 10% (v/v) aqueous acetonitrile. The final concentration of each working standard solution was 1 μg benzimidazole drug per mL. The spectra were recorded over the mass range of m/z 100–650 Da in MS experiments, for MS^n experiments the range was variable. The MS and MS^n spectra were measured in order to obtain fragmentation patterns of ABZ to facilitate the spectra interpretation of novel, unknown metabolites.

The screening for metabolites was carried out by comparing the chromatograms obtained from incubation samples with those of their respective biological and chemical blank samples. Full-scan data was acquired over the mass range m/z 50–650 Da and processed with Metabolite I.D. software (ThermoFinnigan, CA, USA). The selected ions, present in incubation samples and absent in blank samples, were then subjected to MS^n analysis. The product ion spectra obtained were examined and compared with the product ion spectra of the standards (if available). The structures of metabolites were tentatively assessed by the combination of their corresponding full-scan spectra, MS^n spectra, and chromatographic behavior.

Enzyme Assays

Enzyme activities were assayed in the cytosol, mitochondria, or microsomes like fractions of *D. dendriticum* homogenate. Each enzyme assay was performed in triplicates. The amount of organic solvents in the final reaction mixtures did not exceed 0.1% (v/v).

The activities of several isoforms of CYPs were tested. The 7-ethoxyresorufin (EROD; specific for human/rat CYP1A), 7-methoxyresorufin (MROD; CYP1A), 7-pentoxoresorufin (PROD; CYP2B) O-dealkylases, and 7-benzoyloxyresorufin O-dearylase (BROD; CYP3A) activities were determined at 37°C using fluorimetric determination of resorufin (Burke et al., 1994; Weaver et al., 1994). Each substrate (dissolved in DMSO) was added at a final concentration of 5 μM . Assays were conducted using the Perkin-Elmer luminescence spectrophotometer LS 50B with the excitation and emission wavelengths of 530 nm and 585 nm, respectively. The 7-methoxy-4-trifluoromethylcoumarin demethylase (MFCD; CYP2C9) activity was measured using fluorimetric determination of 4-trifluoromethylumbelliferone. The final concentration of substrate (dissolved in DMSO) was 20 μM . The excitation and

emission wavelengths of 410 nm and 510 nm, respectively, were used (Crespi and Stresser, 2000; Price et al., 2000). The chlorzoxazone hydroxylase (CXOH; CYP2E1) assay was performed using the method of Peter et al. (1990) as detailed elsewhere (Skalova et al., 2001). The final concentration of substrate (dissolved in 60 mM KOH) was 1 mM. The amount of 6-hydroxychlorzoxazone was determined by HPLC.

The activity of flavine monooxygenases (FMO) towards thiobenzamide (TBSO) was assayed using the method of Cashman and Hanzlik (1981). The final concentration of substrate (dissolved in acetonitrile) was 1mM. The formation of the metabolic product (S-oxide of thiobenzamide) was followed using the spectrophotometer at 370 nm.

Peroxidase activity was determined using the method based on oxidation of o-phenyldiamine dihydrochloride (OPD) in the presence of hydrogen peroxide (Pérez et al., 2002). The output 2,2'-diaminoazobenzene was detected spectrophotometrically with microplate reader (Biorad 550) at 490 nm. The measurement was performed in a 96-well microplate. The reaction mixture consisted of 50 μ L of subcellular fraction (5 times diluted with 0.1 M Na-phosphate buffer pH 7.40) and 50 μ L of substrate, which was composed of 10 mM OPD and 1 mM H₂O₂ in 50 mM Tris/HCl buffer pH 8.0 with 0.1% Triton. The samples with specific peroxidase inhibitor - salicylhydroxamic acid (SHA) - contained 10 μ L of 100 mM SHA (Ikeda-Saito et al., 1991). Blank samples contained 50 μ L of Na-phosphate buffer instead of the biological fraction. The samples were incubated at 37°C for 30 minutes being continuously mixed. Reaction was stopped by the addition of 25 μ L of 4 M H₂SO₄. The enzymatic activity was calculated using the molar extinction coefficient ($\epsilon = 1.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Catalase activity was determined using Europium(III)-Tetracycline (Eu-Tc)-derived substrate, which forms a fluorescent system with H₂O₂. The samples contained 545 μ L of 10 mM Mops buffer, 195 μ L of Eu-Tc standard solution (prepared from 10 mL of 6.3 mM solution with Eu³⁺ and 10 mL of 2.1 mM solution of tetracycline, both in Mops buffer) and 60 μ L of H₂O₂. After 10 minutes, 100 μ L of fractions, 100 μ L of standard bovine liver catalase (50 U mL⁻¹), or 100 μ L of 0.1 M Na-phosphate buffer pH 7.4 (blanks) were added. The samples were incubated at 37°C for 30 minutes and then the fluorescence was measured (excitation wavelength 378 nm, emission wavelength 613 nm). The decrease in fluorescence was calculated from the difference between fluorescence of the samples and respective blank samples. For inhibition, 60 μ L of a specific catalase inhibitor, 3-amino-1,2,4-triazole (3-AT, 50 mM; Wu et al. 2003), was added into the reaction mixture instead of the corresponding volume of Mops buffer. The activity was determined according to the known number of units (U = μ mol/min) in samples with a catalase standard.

The activities of carbonyl group reductases were tested using the following substrates: 1 mM metyrapone (dissolved in redistilled water) (METR; specific for human/rat 11 β -HSD 1, AKR1C, CBR), and 0.1 mM daunorubicin (dissolved in redistilled water) (DAUR; AKR1A at pH 8.5; AKR1C2, CBR at pH 6.0). Spectrophotometric determination (detection wavelength 340 nm, 25°C) of NADPH consumption (0.3 mM) in the reaction mixture served for the assessment of reductase activities (Felsted and Bachur, 1980; Maser and Oppermann, 1997; Kawamura et al., 1999; Palackal et al., 2001; Ohara et al., 1995).

Reductases of oracin were assayed as described (Wsol et al., 2003). Briefly, fractions A, B, or C (100 μ L) were incubated with 0.3 mM oracin and 1 mM NADPH in a total buffer volume of 0.3 mL. Incubations (37°C, 30 min) were terminated by alkalization and incubates were extracted into ethyl acetate. The extracts were evaporated to dryness and dry samples were dissolved in the mobile phase prior to HPLC analysis. The HPLC separation of

dihydrooracin enantiomers was performed using a 250 x 4.6 mm ODR Chiralcel column. The mobile phase was prepared by mixing buffer (0.3 M sodium perchlorate, pH 3.00 set by HClO₄) with acetonitrile (69:31 v/v). HPLC separation was performed at 25°C with a flow rate of 0.5 mL/min. Dihydrooracin was detected with a fluorescence detector using an excitation wavelength of 340 nm, and an emission wavelength of 418 nm. Oracin was detected with a UV-VIS detector at a detection wavelength of 280 nm.

Enzyme activities for the model substrate 1-acenaphthenol (substrate of AKR1C) were determined using methods described by Palackal et al. (2002) with modifications. The velocity of substrate dehydrogenation was determined spectrophotometrically by measuring the change in absorbance of a cofactor (NADP⁺) at 340 nm. A final 1.0 mL system contained 1 mM acenaphthenol dissolved in DMSO (1% of organic solvent in final mixture), 2.0 mM NADP⁺, 50 µL of fractions A, B or C and 0.1 M TRIS-HCl buffer (pH 8.9).

The microsomal UDP-glucuronosyl transferase activity was assayed following the method by Mizuma et al. (1982). Microsomes were pre-incubated with the Slovasol detergent at 4°C for 20 minutes. The reaction mixture (total volume of 0.1 mL) contained 10 µL of microsomes (0.12-0.14 mg of protein), 0.33 mM UDP-glucuronic acid, and 166.8 µM p-nitrophenol (dissolved in redistilled water) in 0.1 M Tris/HCl buffer (pH 7.4). After 20 minutes of incubation at 37°C, the reaction was stopped by an addition of 50 µL 3% trichloroacetic acid (v/v). After shaking and centrifugation (3 min., 2000 g), 50 µL of the supernatant were mixed with 50 µL 1M NaOH. The absorbance was measured using a BioRad microplate reader (detection wavelength 415 nm). The same procedure was used for the testing of microsomal UDP-glucosyl transferase activity using UDP-glucose instead of UDP-glucuronic acid.

The cytosolic glutathione S-transferase (GST) activities were assayed using 1-chloro-2,4-dinitrobenzene as a substrate (dissolved in ethanol). The reaction mixture (total volume 1 mL) contained 10 µL of cytosol (0.14-0.18 mg of protein), 1 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M Na-phosphate buffer (pH 6.5). The reaction mixture was shaken and the absorbance was measured spectrophotometrically at 340 nm four times in 60 second intervals. For the kinetic study, the substrate in concentration range 0.2-4.0 mM was used.

RESULTS AND DISCUSSION

Activities of Oxidation Enzymes in Lancet Fluke In Vitro

Activities of oxidation biotransformation enzymes were tested in subcellular fractions from *D. dendriticum* using several model substrates. 7-Ethoxyresorufin, 7-methoxyresorufin, 7-pentoxyresorufin, 7-benzyloxyresorufin, 7-methoxy-4-trifluoromethylcoumarin, and chlorzoxazone are typical substrates for individual cytochromes P450 (CYP) isoforms in mammals, while thiobenzamide is a good substrate mainly for FMO. No activities towards these compounds were detected in *D. dendriticum* fractions. These results indicate that CYP and FMO do not play important role in xenobiotics oxidation in *D. dendriticum*.

In other helminth species, the finding of CYP activities has been reported. Kotze et al. (1997) found typical CYP activities, such as epoxidation of aldrin and deethylation of

ethoxycoumarin in microsomes isolated from *Haemonchus contortus* larvae. Microsomes isolated from adult *H. contortus* showed only a very low level of aldrin epoxidation activity, 10000-fold less intensive than that reported for rat liver microsomes (Kotze, 1997). Low aldrin epoxidase activity was also detected in *H. contortus ex vivo* (Kotze, 2000). However, in *Schistosoma mansoni*, adult homogenates high CYP-like activities towards aminopyrine, ethylmorphine, benzphetamine, and N-nitrosyldimethylamine were observed. Kerboeuf et al. (1995) demonstrated very low activities of aminopyrine N-dealkylase in L₁ and L₂ larvae of *Heligmosomoides polygyrus*. This activity was inhibited by benzylimidazole, a CYP inhibitor, which indicated the participation of CYP in this reaction. In other experiments by Kotze et al. (2006), the toxicity of rotenone in adults and larvae of *H. contortus* and larvae of *Trichostrongylus colubriformis* was increased in the presence of piperonyl butoxide. In mammals and insects, the detoxification of rotenone is catalyzed by CYP. A CYP inhibitor, piperonyl butoxide, possessed no toxicity to helminths when used alone. Synergism of rotenone and piperonyl butoxide toxicity is in line with the assumption that CYP takes part in rotenone detoxification in helminths (Kotze et al., 2006).

In contrast to CYP activities, the activities of peroxidases and catalase were found in all subcellular fractions from *D. dendriticum* homogenate. The highest specific activity of peroxidase was found in cytosol-like fraction, and the lowest one in microsomes like fraction (78.5 ± 4.4 nmol/min/mg and 22.1 ± 3.3 nmol/min/mg, respectively). The specific activity in mitochondria-like fraction was 51.2 ± 6.9 nmol/min/mg. The specific peroxidases inhibitor, salicylhydroxamic acid (SHA, 10 mM), caused a significant decrease in peroxidases activities. The results are recorded in Figure 1.

The highest specific activity of catalase was found in microsomes like fraction, and the lowest one in cytosol-like fraction (30.2 ± 0.4 μ mol/min/mg and 10.2 ± 0.2 μ mol/min/mg, respectively). The specific activity in mitochondria-like fraction was only a little higher than in cytosol (13.8 ± 0.4 μ mol/min/mg). The addition of the catalase inhibitor 3-amino-1,2,4-triazole (3-AT, 3.3 mM) caused mild but significant decrease in catalase activity in all fractions (see Figure 2)

Peroxidases represent an additional group of enzymes that may be able to oxidize xenobiotics in helminths (Kotze, 1999), as several peroxidases catalyze the oxidation of xenobiotics in mammals (Testa, 1995, 2007). The involvement of peroxidases in the detoxification of xenobiotics in helminths has already been entertained in a review by Barrett (1997), but helminth peroxidases have been mostly studied only from the view of their antioxidant function, and the information about oxidation of xenobiotics via peroxidases in helminths is insufficient. Participation of catalase in xenobiotics oxidation has been also described in mammals, although catalase main function lies in hydrogen peroxide decomposition (Testa 1995). Presence of significant peroxidases and catalase activities in *D. dendriticum* indicate that those enzymes might substitute CYP role in oxidative biotransformation of xenobiotics in this species.

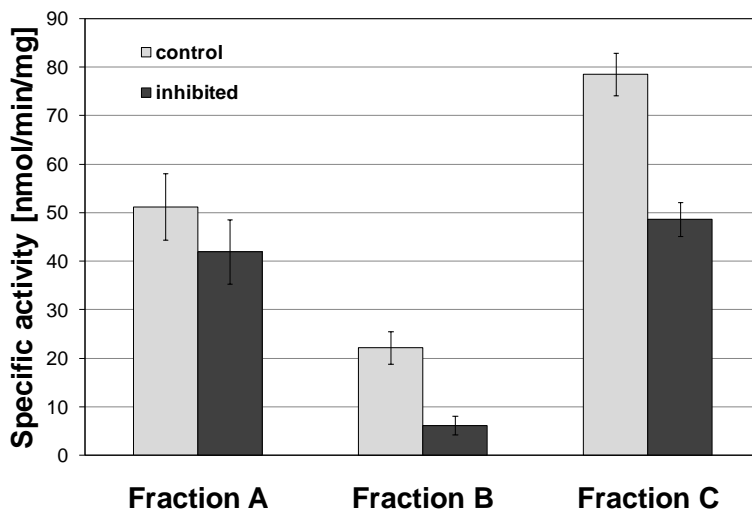


Figure 1. Comparison of peroxidases specific activity in subcellular fractions of *D. dendriticum* homogenate incubated without (control) or with peroxidases inhibitor salicylhydroxamic acid (inhibited).

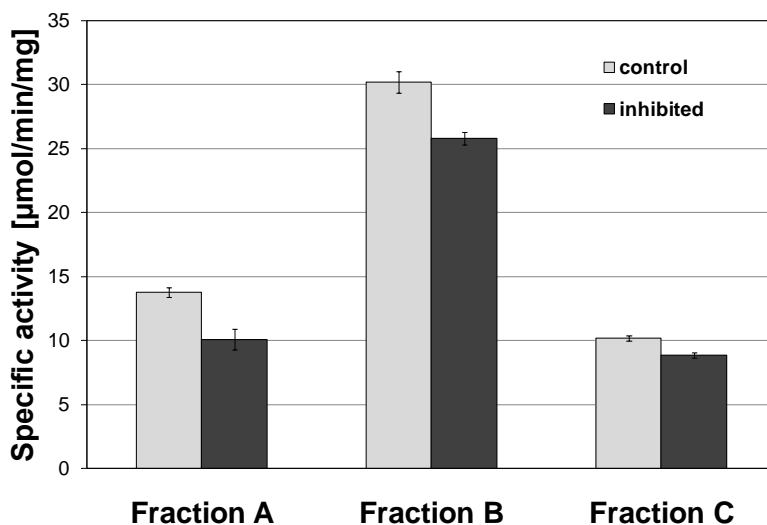


Figure 2. Comparison of catalase specific activity in subcellular fractions of *D. dendriticum* homogenate incubated without (control) or with catalase inhibitor 3-amino-1,2,4-triazole (inhibited).

Activities of Reduction Enzymes in Lancet Fluke In Vitro

With the aim to detect the activity of reduction biotransformation enzymes, several model substrates were used in *D. dendriticum* subcellular fractions. Daunorubicin (at pH 8.5) is a good substrate for human AKR1A enzymes, while daunorubicin at pH 6.0 is preferentially-reduced by action of carbonyl reductase and AKR1C enzymes. Acenaphthenol represents the typical substrate for enzymes from the AKR1C subfamily. Metyrapone is reduced by 11β -hydroxysteroid dehydrogenase in microsomes and by AKR1C, AKR1A, and carbonyl

reductase in cytosol (Kawamura et al. 1999; Maser and Oppermann 1997; Ohara et al. 1995; Palackal et al. 2002). Almost all carbonyl-reducing enzymes are able to reduce oracin in human (Wsol et al. 2003; Martin et al. 2006).

Table I. Specific activities of reductases/dehydrogenases toward selected substrates and UDP-glukuronosyl transferases (UGT) toward p-nitrophenol tested in subcellular fractions B (microsomes like) and C (cytosol like) of *Dicrocoelium dendriticum* homogenate

	Specific activities [nmol.min ⁻¹ .mg ⁻¹]	
	Fraction B	Fraction C
Metyrapone reductase	ND	6.2 ± 0.8
Daunorubicin reductase (pH 6,0)	ND	1.4 ± 0.2
Daunorubicin reductase (pH 8,5)	ND	4.3 ± 0.4
Oracin reductase	0.03 ± 0.01	1.8 ± 0.1
Acenaphthenol dehydrogenase	ND	14.5 ± 1.1
UGT	0.08 ± 0.02	ND

The data represent the mean ± S.D. from 3-5 samples. ND = not detected.

In *D. dendriticum* cytosol-like fraction, all model substrates were metabolized. In microsomes like fraction, only oracin was slightly reduced. The results are presented in Table I. The specific activities of *D. dendriticum* carbonyl-reducing enzymes were relatively high, similar to the activities found in farm animal species (Szotakova et al. 2004). These results prove that *D. dendriticum* is able to effectively reduce xenobiotic aldehydes or ketones. In this way, carbonyl-reducing enzymes can protect lancet flukes against the toxic effect of carbonyl group-containing xenobiotics. In our previous project, the activities of carbonyl-reducing enzymes were found in *H. contortus* subcellular fractions (Cvilink et al. 2008a). Comparing both helminth species, *D. dendriticum* possesses significantly higher activities of carbonyl reducing enzymes (3-10 folds) than *H. contortus*.

Activities of Conjugation Enzymes in Lancet Fluke In Vitro

Glutathione S-transferase (GST) activities towards 1-chloro-2,4-dinitrobenzene were assayed in *D. dendriticum* cytosolic fraction. The UDP-glucuronosyl transferase (UGT) and UDP-glucosyl transferase (UGlT) activities towards p-nitrophenol were tested in *D. dendriticum* microsomal fractions.

In cytosol-like fraction, significant GST activity was found. The results of kinetic study are shown in Figure 3.

Apparent kinetic parameters, maximal velocity $V_{\max} = 1.64 \pm 0.25 \mu\text{mol/l/min}$ and Michaelis' constant $K_m = 2.57 \pm 0.38 \text{ mM}$, were calculated using GraphPad Prism software. These results indicate a relatively high activity and a low affinity of lancet fluke GSTs towards 1-chloro-2,4-dinitrobenzene. The specific activity of GST in lancet fluke was similar to this activity in hepatic cytosol of sheep and goats (Szotakova et al. 2004), and significantly higher than those in *H. contortus* (Skalova et al. submitted).

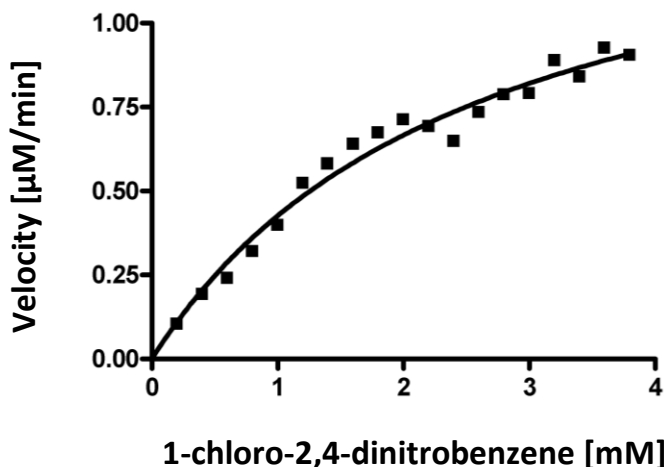


Figure 3. Michaelis-Menten kinetics for conjugation of 1-chloro-2,4-dinitrobenzene with glutathione in fraction C.

In microsomal fractions, a low UGT activity was observed (Table I), but when UDP-glucose was used as the conjugation agent, no activities of UDP-glucosyl transferase were detected. Inverse results were found in *H. contortus*. This helminth was able to conjugate p-nitrophenol with UDP-glucose, but not with UDP-glucuronic acid (Skalova et al., submitted). This finding was interesting as glucose conjugation, common metabolic pathway in plants, occurs rarely in the animal kingdom.

While little is known about helminth UGT, GSTs have been studied intensively and the present knowledge of structure and localization of helminth GSTs is large (e.g., Torres-Rivera and Land 2008). In spite of this fact, information about GSTs-mediated xenobiotics conjugation is rare. Lo and colleagues (2007) showed that extracts from *S. mansoni* efficiently conjugated synthetically-prepared electrophilic compounds with glutathione. Vande Waa et al. (1993) found GST activity towards model substrate (1-chloro-2,4-dinitrobenzene) in *S. mansoni*. While GSTs catalyze conjugation of many drugs in mammals, no information about the role of helminth GST in drug metabolism is available.

Biotransformation of Albendazole (ABZ) in Lancet Fluke In Vitro and Ex Vivo

In *D. dendriticum*, phase I biotransformation of ABZ was tested both *in vitro* and *ex vivo* (Cvilink et al. 2009b). Structure of ABZ and its two phase I metabolites, ABZSO and ABZSO₂, is presented in Figure 4.

Sulfoxidation of ABZ occurred in Fractions A (mitochondria-like) and B (microsomes-like). The specific activities of ABZ oxidases were significantly higher in mitochondria than in microsomes (see Table II). No ABZSO formation was observed in *D. dendriticum* Fraction C (cytosol-like), while in a related species, *Fasciola hepatica*, sulfoxidation of ABZ in both microsomal and cytosolic fractions occurred (Solana et al. 2001). Triclabendazole sulfoxidation by *F. hepatica* microsomal fraction has also been reported (Mottier et al. 2004, Alvarez et al. 2005). The specific activity of triclabendazole sulfoxidation was comparable

with the specific activities of *D. dendriticum* ABZ oxidases determined in our *in vitro* experiments.

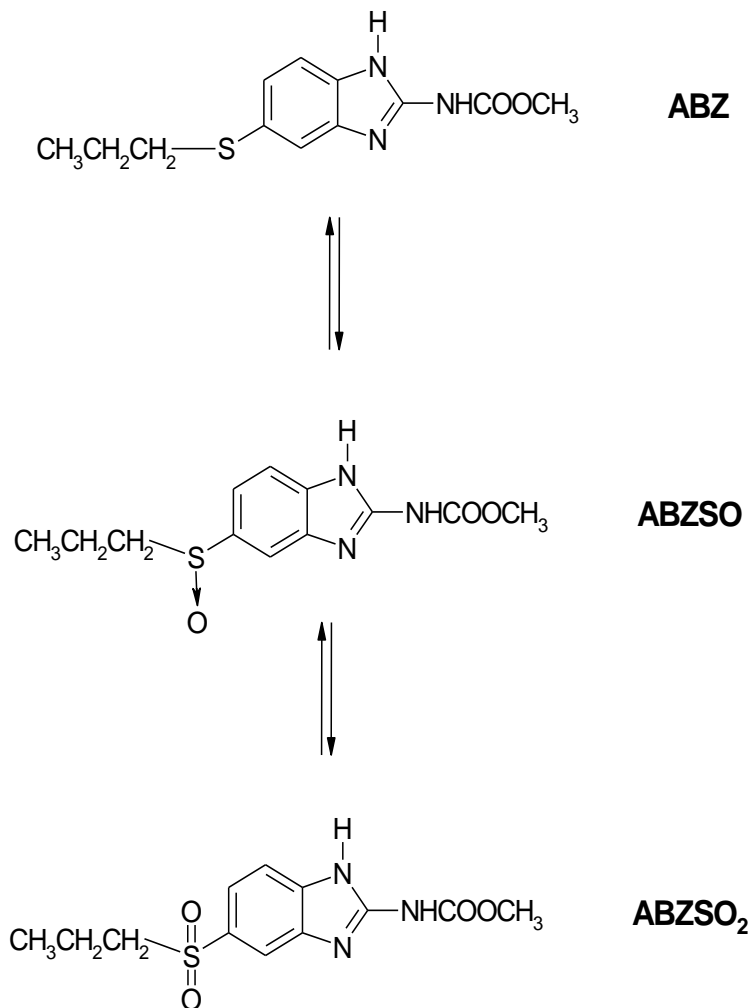


Figure 4. Structure of albendazole (ABZ) and its two metabolites ABZ sulfoxide (ABZSO) and ABZ sulfone (ABZSO₂).

Table II. Specific activities of ABZ oxidases tested in subcellular fractions A (mitochondria like) and fractions B (microsomes like) of *Dicrocoelium dendriticum* homogenate

	Specific activities [pmol.min ⁻¹ .mg ⁻¹]	
	Fraction A	Fraction B
ABZSO formation	47.00 ± 4.00	37.00 ± 4.00
ABZSO ₂ formation	0.11 ± 0.02	ND

The data represents the mean ± S.D. from 4 samples. ND = not detected.

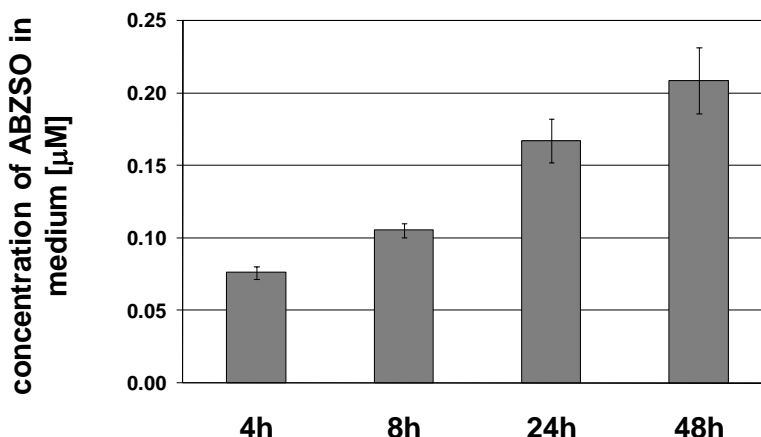


Figure 5. Time-dependent formation of albendazole sulfoxide (ABZSO) in lancet flukes incubated *ex vivo* with 10 μM albendazole (ABZ).

Ex vivo incubations of living *D. dendriticum* adults with ABZ resulted in the formation of ABZSO. Time-dependent efflux of ABZSO into the medium is demonstrated in Figure 5.

The extent of ABZSO formation was low as only 2.5 % of the initial ABZ concentration was metabolized during the incubation. Since ABZSO itself is an anthelmintically-active compound, any extent of ABZSO formation would not decrease the ABZ efficacy. The structure of ABZSO was confirmed using LC-MS (Cvilink et al., 2009c). Protonated albendazole, the parent drug, was observed at m/z 266 (elution time 12.6 min). A possible metabolite of ABZ formed in *ex vivo* experiments was detected at m/z 282 eluting at 9.5 min. The MS/MS analysis of m/z 282 resulted in product ions at m/z 159, 191, 208, and 240, which were typical for the product spectrum of ABZSO (see Figure 6). The retention times of the metabolite and ABZSO were identical; the metabolite was assessed as ABZSO.

The second step of ABZ oxidation, sulfonation, was observed in *D. dendriticum* fraction A (mitochondria like) incubations. This is the first piece of evidence that helminth enzymes are able to deactivate ABZ via formation of the biologically-inactive ABZSO₂. However, the activity of ABZSO oxidases was so low that there cannot be assumed any significant protective role of this reaction. Moreover, ABZSO₂ was formed only *in vitro* experiments, no sulfonation was detected in *ex vivo* experiments. In the case of triclabendazole, much more intensive sulfonation was reported in *F. hepatica* (Robinson et al. 2004). *F. hepatica* was able to effectively convert triclabendazole sulfoxide to inactive triclabendazole sulfone and the extent of the reaction was significantly higher in the triclabendazole-resistant than in the susceptible strain. In contrast to *Moniezia expansa* (Solana et al. 2001), *F. hepatica* specimens were unable to reduce ABZSO to ABZ. Neither in *in vitro* nor in *ex vivo* experiments with *D. dendriticum* was the sulforeduction of ABZSO observed. It appears that reverse metabolism of ABZSO does not occur in lancet flukes.

Phase II ABZ metabolites formed by conjugation enzymes of lancet fluke were searched for using LC-MS in medium as well as in homogenate from fluke bodies. No ABZ or ABZSO conjugates were detected. It is a distinct result from our previous experiments with *Haemonchus contortus* (Cvilink et al., 2008b) where glucose conjugates of ABZ were found. This fact shows the significant inter-species differences in drug metabolism among helminths.

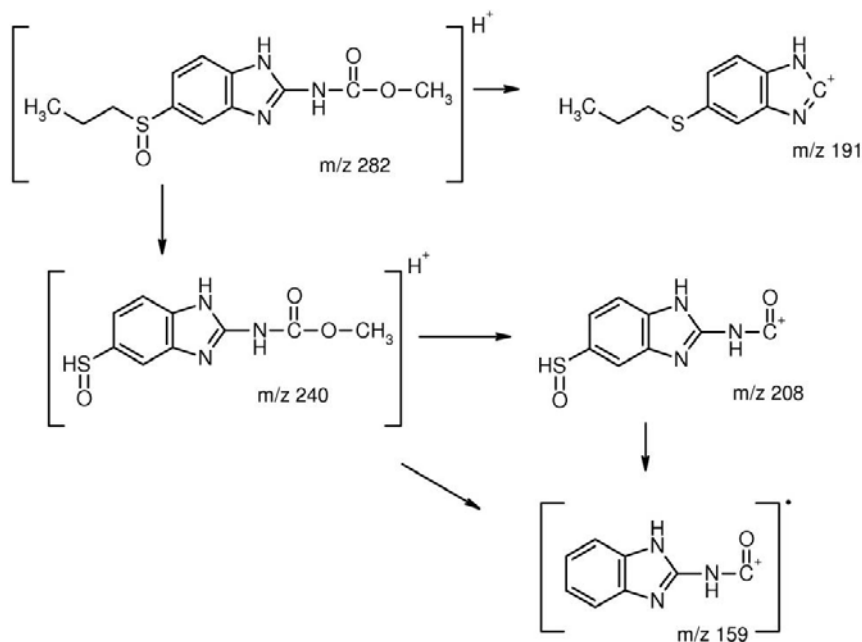


Figure 6. Fragmentation of albendazole sulfoxide (ABZSO) found in medium after 24-hour incubation of vivid lancet flukes with 10 μ M albendazole (ABZ).

CONCLUSION

All presented results demonstrate that *D. dendriticum* is able to metabolize a wide range of xenobiotics. High activities (comparable to those of mammals) of carbonyl-reducing enzymes and GSTs were found in cytosol-like fraction from lancet fluke homogenate. While CYP or FMO activities were not observed, significant activities of peroxidases and catalase were detected and these enzymes might participate in oxidation of xenobiotics, including ABZ. Formation of ABZSO by lancet fluke enzymes was observed *in vitro* as well as *ex vivo*. ABZ sulfonation took place only *in vitro* in the mitochondria-like fraction. No conjugates of ABZ were found.

Comparison of results obtained in *D. dendriticum* and those from other helminth species shows significant differences in activities of biotransformation enzymes. This fact should be taken into account when trying to understand the variable efficacies of anthelmintics against different helminth parasites. Complete knowledge of drug metabolism in helminths is necessary so that the control of parasitoses, including dicrocoeliosis, becomes more effective.

ACKNOWLEDGMENT

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Chapter XIII

**CROSS-IMMUNITY AND INTERPRETATION OF
THE DIAGNOSTICS OF PARASITIC
TREMATODOSIS IN RUMINANTS BY MEANS OF
IMMUNOENZYMATIC PROBES**

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ABSTRACT

Trematodosis caused by *Fasciola hepatica* or *Paramphistomum* spp are responsible for severe lesions in ruminants which affect production and cause important economical losses. These are parasitic infections with an analogous external phase in their life cycle and periods of high risk also. The occurrence of fasciolosis and/or paramphistomosis needs for the presence of an aquatic snail acting as the intermediate host, so the ruminants grazing in humid areas are at a highest risk for developing these diseases.

The routine procedure for the detection of infections by parasitic trematoda is based on the coprological sedimentation, and by the detection of the presence of eggs in the feces the existence of mature adult flukes in the definitive host is concluded. The main disadvantages are that a period longer than 10 weeks after infection is required, and that during many chronic infections fluke eggs are erratically excreted.

Immunoenzymatic techniques focused on the detection of antibodies against several antigens have been frequently applied in the last decade. Although good results can be

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achieved, the presence of cross-reaction might difficult the exact diagnosis of these diseases.

To gain more knowledge about the influence of the possible cross-immunity against *F. hepatica* and *Paramphistomum* on the interpretation of the diagnostics of fasciolosis or paramphistomosis, fecal and blood samples were individually obtained from Rubia Gallega autochthonous cattle during the 2007 year. With the purpose to select cattle with a single infection by *F. hepatica* or *C. daubneyi*, the liver, rumen, and abomasum from 492 slaughtered autochthonous cattle were examined. In addition, 1,158 fecal samples belonging to Rubia Gallega animals were analyzed by the coprological sedimentation technique. Blood samples from all of these ruminants were collected and the analyzed by means of an ELISA and different antigens of *F. hepatica* (FES) and *C. daubneyi* (CES). After collecting their excretory/secretory crude products, purification was carried out under nature conditions by using liquid chromatography (FPLC), and under reducing conditions by electrophoresis (SDS-PAGE) and electroelution.

Five peaks were collected from the fractionation of the FES and CES under an FPLC system. The molecular weight was similar in four peaks and differed in the fourth only. After the electroelution the presence of three proteins common to both FES and CES, with a molecular mass of 106, 41, and 23 kDa was observed.

The ELISA showed that cattle with fasciolosis reacted strongly against CES-peaks P3 and P1, whereas the lowest cross-reaction against P4 (<13.7 kDa) was reached. Sera from cattle with paramphistomosis recognized FES-peaks P2 and P3, and the weakest reaction against P4 (<13.7 kDa) was obtained. By means of the electroelution procedure one protein belonging to CES (Cd2; 52 kDa) was identified, which was recognized only by the 15% of sera from cattle with fasciolosis. Furthermore, only 14% of the sera from bovine with paramphistomosis reacted with an 18-kDa protein from the FES (F8).

The elevated similarity between the excretory/secretory antigens from these parasitic trematoda under native and reducing conditions points the existence of some antigenic determinants being shared by them, hypothesis supported by the observation of an important cross-immune reaction among sera from cattle monoinfected by *Fasciola* or *Paramphistomum*. It is concluded that the application of immunoenzymatic diagnostic probes such as the ELISA requires the investigation of the possibility for a cross-immune reaction to enhance the right interpretation of the achieved results. The purification of antigens by electroelution allows the production of proteins with low cross-immunity.

Keywords: *Fasciola hepatica*, *Paramphistomum* spp, ruminants, cross-immunity, serological probes, FPLC, electroelution

INTRODUCTION

Paramphistomum spp and *Fasciola hepatica* are parasitic trematoda affecting a wide range of herbivorous mammals, such as sheep, cattle, and goats, although *Fasciola* can infect horses, pigs, and men (Sánchez-Andrade et al., 2008).

These are trematoda with a similar external phase in their life cycle and the periods of high risk are also analogous. Fasciolosis is well-known for its geographical distribution and the serious economic losses this parasite causes in the animal industry; on the other hand, paramphistomosis is still underestimated (Mage et al., 2002). Although the highest frequency of this infection has been registered in tropical and subtropical regions of the world, recent reports indicate this prevalence is increasing in other areas like central France (Szmidt-Adjidé et al., 2000).

Values of prevalence have been reported for paramphistomosis of 10-20% in several European countries like Italy, France, or Spain (Agosti et al., 1980; Szmidi-Adjidé et al., 2000; Díaz et al., 2006). Rangel-Ruiz et al. (2003) reported a prevalence of 39% in Mexico.

LIFE-CYCLE

The adult flukes produce operculated eggs which are expelled into the external environment with the feces of the host. In a humid milieu and adequate temperatures, the miracidia hatch from the eggs, and, after a brief free-swimming existence, penetrate the snail host *Galba truncatula*.

Within the snail, the polyembryony gives rise to cercariae several weeks later. These escape from the snail and encyst on aquatic vegetation to become metacercariae.

Infection by *Paramphistomum* and *Fasciola* is caused by the ingestion of metacercariae. There are two main vehicles of ingestion of metacercariae: water from streams and plants like grass or watercress (Esteban et al., 2002).

Excystment takes place in the intestine. *Fasciola* newly-excysted juveniles pass across the peritoneum and arrive at the liver. After migrating through the parenchyma, the trematoda reaches the bile ducts, attains sexual maturity, and passes eggs by feces after three or four months post-infection.

When ingested by the definitive host, *Paramphistomum*-metacercariae excyst and develop into juvenile worms which feed on the intestinal mucosa before migrating to the upper rumen where eggs are passed three to four months post-infection. The total life cycle requires a minimum of 6 months.

Most of the pathological damage occurs during the migratory phase, and the release of parasitic antigens happens at this time. Exposure to these antigens serves as a stimulus to the production of antibodies by the host, so some diagnostic tests have focused on the detection of antibodies against the trematoda.

EPIDEMIOLOGY

Paramphistomosis and Fasciolosis are both a parasitosis caused by the ingestion of metacercariae. As mentioned above, trematode-transmission depends on a snail intermediate host (*Lymnaea* spp), which is enzootic primarily in regions with high annual rainfall and large areas that are poorly drained. The use of irrigated pastures may increase the distribution and prevalence of liver flukes (Sánchez-Andrade et al., 2002).

Oceanic climates are characterized by a narrower annual range of temperatures, and differ from Mediterranean climates in that significant amounts of precipitation are received in summer, so rainfall is both adequate and reliable at all times of the year (Kottek et al., 2006). This is the climate typically found along the west coasts at the middle latitudes (40°–60°N) of all the world continents and in southeastern Australia; similar climates are also found at coastal tropical highlands and tropical coasts on the leeward sides of mountain ranges. Several investigations showed that mild temperatures and frequent rainfall (frequent in oceanic climate areas) throughout the year are the suitable conditions for the infection of

cattle with parasite trematoda like *Fasciola hepatica* (Digenea: Fasciolidae) or the ruminal fluke *Calicophoron daubneyi* (Digenea: Paramphistomidae) (Dinnik 1962; Eduardo 1983) (Ollerenshaw and Smith 1969).

The annual variations on the climatic pattern in a typical region with oceanic climate are drawn in Figure 1 (Díaz et al., 2006). Mean temperatures around 5°C are recorded from December to April. The rainfall peaks in April and in November, whereas the greater maximum and minimum temperatures were observed from June to September. Christensen and Nansen (1976) proved that the minimum temperature for lymaneid host-finding is 5–6°C. These results led us to consider the possibility for emission of cercariae was enhanced in two periods, May-June and November-December, becoming the periods of high risk for infection by the trematoda.

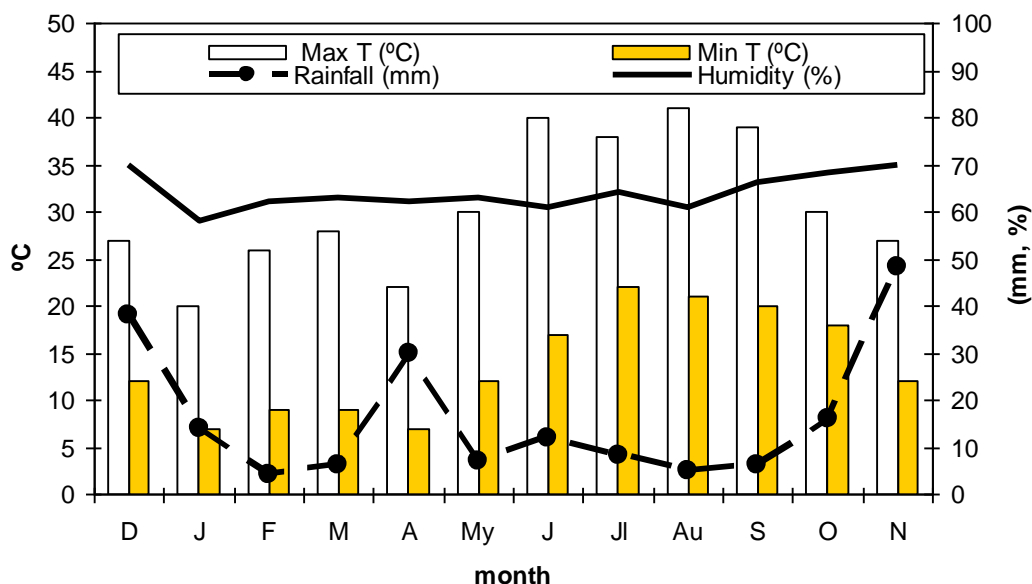


Figure 1. Annual climatic patterns in a region with oceanic climate.

PATHOLOGY

The immature stages of Paramphistomidae can cause severe pathology and even kill the host in heavy infections because they are attached to the wall of the small intestine causing important inflammation, characterized by focal infiltration of macrophages and lymphocytes in the lamina propria and in the interstitial tissue of Brunner's gland (Singh et al., 1984; Silvestre et al., 2000). Digestion and absorption are damaged, and appetite is also depressed, resulting in diarrhea, anorexia, anemia, and weakness (Spence et al., 1996). There is little evidence regarding the pathogenicity of the adult worms to their hosts, but severe damage to the mucosa of the rumen is provoked in heavy infections (Rolfe et al., 1994). The injury caused by this infection in cattle affects production, since these parasites cause a lower feed conversion, a loss of weight and/or a decrease in milk production, responsible for economic losses (Rangel-Ruiz et al., 2003).

The main clinical disease after the infection by *F. hepatica* is caused by extensive damage to the hepatic parenchyma produced during 6–8 weeks by migration of juvenile flukes. Adult liver flukes reside in the bile ducts of host animals, and eggs are passed onto the pasture in the feces. Liver lesions predispose animals to infectious necrotic hepatitis and bacillary hemoglobinuria; also, host fertility can be decreased (López-Díaz et al., 1998).

DIAGNOSIS

Current efforts for the control of trematodosis need diagnostic techniques which allow the early detection of active infections (Sánchez-Andrade et al., 2002). The coprological sedimentation probe is the routine procedure used for the detection of infections by parasitic trematoda.

Traditionally, it is accepted that the presence of trematoda-eggs in the feces indicates the presence of mature adult flukes in their definitive localization (bile ducts and gall-bladder for *Fasciola* and rumen and abomasum in the case of *Paramphistomum*). This implies a period longer than 10 weeks after infection is required, so most of the pathological damage has already occurred (Pérez et al., 2005; Díaz et al., 2007). Likewise, many chronic infections are in individuals who excrete fluke eggs erratically, whereby eggs are often not seen, thus leading to false-negative results. Another disadvantage relies on the differential diagnostics between the eggs of *Fasciola* and *Paramphistomum*, due to the high similarity between them.

A novel multivalent fecal egg count method, the so-called FLOTAC technique, has been described recently both for human and veterinary medicine (Cringoli, 2006). This method is facilitated by the FLOTAC® apparatus, which has been designed to carry out flotation in a centrifuge, followed by a transversal cut (i.e. translation) of the apical portion of the floating suspension. The FLOTAC technique allows quantification of eggs and/or larvae of nematodes and trematodes as well as cysts and oocysts of intestinal protozoa in up to 1 g of feces. It should therefore considerably improve the sensitivity of the aforementioned diagnostic tools.

Research on several parasitic trematode infections, such as dicrocoeliosis, paramphistomosis, or fasciolosis, have shown that the immune system was stimulated to produce antibodies against the parasites, and this has been applied for its detection by means of enzyme-linked immunoassays (Hillyer et al., 1996; Paz et al., 1998; Sánchez-Andrade et al., 2000; Sánchez-Andrade et al., 2003; Díaz et al., 2006).

Immunoenzymatic techniques are mainly focused on the detection of antibodies against parasitic antigens by ELISA and are useful to solve the disadvantages of coprological examinations (Cornelissen et al., 1992; Hillyer et al., 1996; Dalton et al., 2003). Some investigations on the suitability of immunological probes, such as the ELISA, have been carried out. This technique provides a useful method for the diagnosis of fasciolosis or the evaluation of an anthelmintic treatment because many sera can be processed and reactants are reasonably priced (Arriaga de Morilla et al., 1989).

There are few studies about the immunological diagnosis of paramphistomosis. The immunodiagnosis tests are an important adjunct for the diagnosis of fasciolosis because an earlier detection than coproscopic is allowed. Nevertheless, the presence of antibodies does not always correlate with the existence of active infection, but only with exposure to the parasite (Espino et al., 1998; Sánchez-Andrade et al., 2001). It has been shown that antibody

levels diminish slowly after the cure (Hillyer, 1999; Sánchez-Andrade et al., 2002). Another disadvantage consists of the persistence of IgG antibodies for long periods (Sánchez-Andrade et al., 2000) and how it makes interpretation of the results difficult.

Other ELISA-probes have been developed for the detection of parasite antigens in blood, milk, or feces from naturally- and infected animals, based on the use of both monoclonal and polyclonal antibodies (Duménigo et al., 1999, Almazán et al., 2001; Sánchez-Andrade et al., 2001). It has been shown that this procedure provides a feasible and correct diagnostics of fasciolosis in serum or fecal samples. Duménigo et al. (1999) and Almazán et al. (2001) proved that in ovine experimental fasciolosis, antigenaemia was detected 1 week after infection, whereas positive antibody values were obtained from the second week after infection. Regardless of successful results having been matched, the highly time-consuming protocol of these probes and its economical cost reduces its application.

More recently, developments in molecular biology have been applied to many areas of the biomedical sciences, and the number of applications of molecular biology in veterinary parasitology is increasing rapidly (Prichard, 1997).

Most of these applications have been focused on searching for an efficient vaccine to prevent fasciolosis (Sexton et al., 1994; Dalton et al., 1996; Muro et al., 1997; Smooker et al., 1999; Casanueva et al., 2001; Knox et al., 2001).

Likewise, one of the main uses consists in the expression of recombinant proteins, which improves the possibilities for the standardization of the techniques used to detect fasciolosis because a homogenous antigen is easily obtained when necessary, without dependence of collecting trematoda at the slaughterhouse (Carnevale et al., 2001; Cornelissen et al., 2001; Silva et al., 2004; Arias et al., 2006, 2007, 2009). Therefore, the comparison among the results obtained from different laboratories might also be easier by getting a homogenous antigen to develop an ELISA.

RECENT APPROACHES IN SEROLOGICAL METHODS TO THE DETECTION OF PARASITIC TREMATODOSIS

The introduction of techniques of immunoassay focused on antibody detection against parasite-antigens represented an important contribution to the detection of infection by trematoda. Nevertheless, these techniques have some disadvantages, like the possible cross-immunity against different parasites like *Paramphistomum* or *Fasciola* specimens.

To gain more knowledge about the influence of the possible cross-immunity against *F. hepatica* and *Paramphistomum* on the interpretation of the diagnostics of fasciolosis or paramphistomosis, fecal and blood samples were individually obtained from Rubia Gallega autochthonous cattle during the 2007 year. The present investigation was developed in Northwest Spain (42°20' to 43°45'N, 6°49' to 8°00' W) where mild temperatures and high precipitation favor the proliferation of lymneid snails, the intermediate host for Fasciolae and Paramphistomidae trematoda (Augot et al., 1996; Abrous et al., 1999).

Rubia Gallega autochthonous cattle are kept under field conditions and most herds are maintained outdoors and are only brought into the paddocks during the night. This allows the possibility of infection and challenge. The grazing herd is generally fed on natural pastures, characterized mainly by annual grass species.

Only cattle with a single infection by *F. hepatica* or *C. daubneyi* were selected to be included in the current work. For this purpose, we examined the liver, rumen, and abomasum from 492 slaughtered autochthonous cattle.

Besides this, 1,158 fecal samples belonging to Rubia Gallega animals were analyzed by the coprological sedimentation technique. Professional veterinary clinicians were responsible for collecting all the samples.

a) Coprological Analysis

Trematoda-eggs were observed by means of the coprological sedimentation technique, using individual samples of 5 g of feces. The egg-counts were presented as the number of eggs per gram of feces (epg). The eggs were identified according to Tienpont et al. (1979).

b) Blood Samples Collection

All the animals were bled by jugular venous puncture, and two samples were collected, one with anticoagulant and the other without to obtain serum.

c) ELISA

For the analysis of the humoral IgG response, *F. hepatica* and *C. daubneyi*-excretory/secretory antigens (FES and CES, respectively) were prepared from live adult flukes obtained from bovine slaughtered at a local abattoir. Fasciolae were collected in the liver and paramphistomids in the rumen and abomasum of infected animals.

The parasites were incubated for 24 h at 37°C and 5% CO₂ in RPMI medium containing 0.5 mM PMSF. The medium was removed every 3 h, and then centrifuged and concentrated (Sánchez-Andrade et al., 2000; Díaz et al., 2006).

ELISA procedures were performed on serum samples by using U-bottom microtiter plates (Costar[®], Barcelona, Spain). The antigen concentration, the sera, and immunoconjugate dilutions were assessed by a checkboard titration.

Sera were tested in duplicate, and the absorbances read using a Titertek Multiskan spectrophotometer at 492 nm.

Negative serum samples utilized as controls were obtained from 53 animals negative both to necropsy and sedimentation.

The cut-off value was estimated as the mean of negative absorbances from G-C plus three standard deviations (Sánchez-Andrade et al., 2001). Thus, positive absorbance values were 0.4435 or higher for fasciolosis, and ≥ 0.396 for paramphistomosis.

All the blood samples collected from the autochthonous cattle were analyzed by means of an ELISA with the FES or CES antigens.

d) Analysis of the Cross-immune Reaction

The analysis of cross-immunity was conducted by purifying *F. hepatica* and *C. daubneyi* antigens by liquid chromatography (FPLC), electrophoresis under reducing conditions (SDS-PAGE), and electroelution.

1) Gel-Filtration Chromatography

The native *F. hepatica* and *C. daubneyi* excretion/secretion antigens (FES and CES, respectively) were fractionated by size-exclusion FPLC (fast protein liquid chromatography) on a Duo-Flow (Bio-Rad[®], Madrid, Spain) system and a Sepharose S-200 HR 10/30 column. For this purpose, the following protocol was employed:

Table 1. Gel-Filtration chromatography protocol

Step	Volume (ml)	Characteristics
1	0.00	Collection fractions of size 4 ml during entire run
2	0.00	Lamp (UV detector) Turn ON
3	0.00	Zero baseline
4	0.00	Isocratic flow
5	5.00	Load/Inject 2.5 ml sample
6	7.5	Isocratic flow
7	600	End of protocol

Fractionation of the antigens was carried out by the injection of 2.5 ml antigen at a 5 mg ml⁻¹ protein concentration. The buffer was composed of bisodium phosphate, monosodium phosphate and sodium chloride (pH 7.4). All the antigens and the buffers were passed through a 0.22 µm filter before the utilization into the FPLC system.

The serodiagnostic value of the antigens contained in each one of the peaks obtained was tested by using an ELISA-test and sera from cattle with monoinfection by *Fasciola* or *Calicophoron*. The presence of infection was assessed by necropsy or coprological methods.

2) SDS-PAGE

The trematode excretory/secretory antigens and molecular weight markers from 205 to 8 kDa (Bio-Rad[®], Madrid, Spain) were separated using polyacrilamide gel electrophoresis in a discontinuous buffer system in the presence of sodium dodecyl sulphate (SDS-PAGE) and β-mercaptoethanol. Resolving gels were 12% acrylamide and measured 7 cm high (Romasanta et al., 2003).

3) Electroelution

The FES and CES separated using SDS-PAGE were eluted from the gels by using a Whole Gel Eluter (Bio-Rad), according to the manufacturer's instructions. For this purpose, gels were soaked in a buffer elution composed by HCl-Trizma and boric acid (pH 8.8), and then ran under a 90 mA intensity during 30 min.

The different fractions collected were concentrated under a Speed Vac until a 200 µg ml⁻¹ concentration was reached and kept at -85°C until used.

e) Results

1) Chromatography

As drawn in Figure 2, five peaks were collected from the fractionation of the FES and CES under an FPLC system. The molecular weight was similar in four peaks and differed in the fourth only (Table 2).

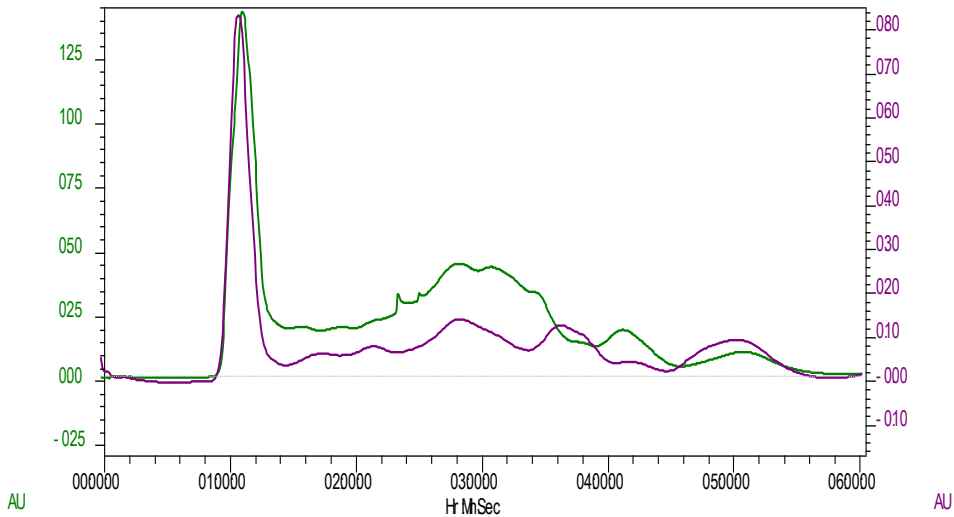


Figure 2. Peaks collected after fractionating excretory/secretory antigens from *C. daubneyi* (CES) and *F. hepatica* (FES) by gel-filtration chromatography.

Table 2. Peaks collected after the chromatography of FES and CES

	FES		CES	
Peak	Time (min)	MW (kDa)	Time (min)	MW (kDa)
P1	56	56	56	56
P2	84	46	84	46
P3	140	26	140	26
P4	240	<13.7	204	<13.7
P5	276	<13.7	276	<13.7

2) Electroelution

After the electroelution of *F. hepatica* excretory/secretory products, 8 protein fractions were collected, with molecular weights of 106, 93, 81, 63, 59, 41, 23, and 18 kDa (Figure 3). These fractions were identified as F1 to F8.

Seven fractions were obtained after the electroelution of *C. daubneyi* excretory/secretory products, with molecular weights of 106, 52, 41, 27, 23, 12, and <8 kDa (Figure 4). These polypeptides were identified as Cd1 to Cd7.

By contrasting the molecular weights of the different fractions, we observed the presence of three proteins common to both FES and CES, with a molecular mass of 106, 41, and 23 kDa.

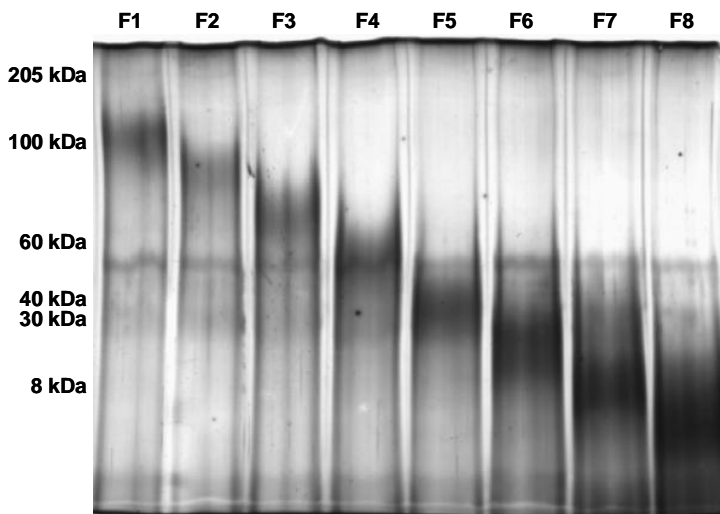


Figure 3. Fractions collected after the electroelution of FES.

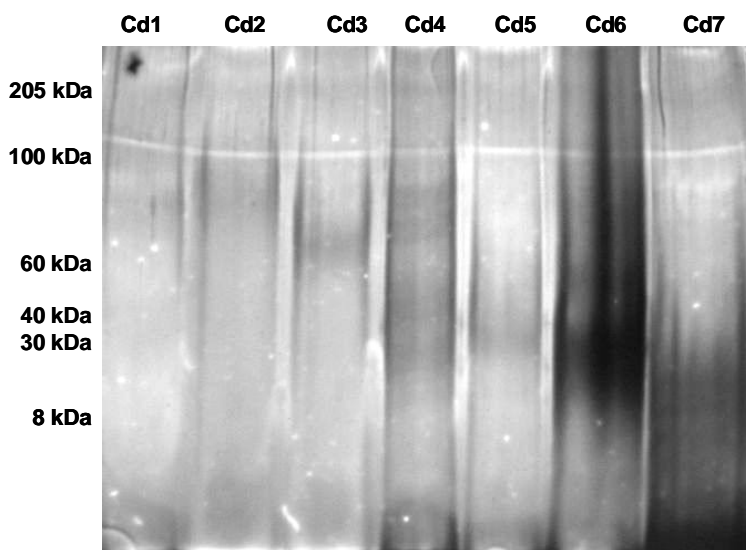


Figure 4. Fractions collected after the electroelution of CES.

3) ELISA

From the 492 cattle examined at the slaughterhouse, we found 72 with monoinfection by *F. hepatica* as shown by the presence of fasciolae in their livers and the FES-ELISA results also; the number of animals positive to paramphistomosis by necropsy and CES-ELISA was 58.

After the analysis of 1,158 fecal samples, we observed 98 positive to *F. hepatica*-eggs and FES-ELISA, and 133 to *C. daubneyi* and CES-ELISA.

According to these results, two groups of cattle were considered: G-F, 170 animals positive to fasciolosis, and G-P, 191 cattle with paramphistomosis.

4) Cross-immunity

Crude excretory/secretory antigens

To the evaluation of cross-immunity against CES and FES, the sera from G-F and G-P were tested against these antigens (Table 3). The greatest presence of cross-reaction was obtained in cattle with fasciolosis (55%).

Table 3. Analysis of cross-immunity against FES and CES

Group	N	FES +	CES +
G-F	170	170 (100%)	94 (55%)
G-P	191	42 (22%)	191 (100%)

Chromatography peaks

Table 4 represents the cross-immune reaction against the peaks obtained after the chromatography of *F. hepatica* and *C. daubneyi* excretory/secretory antigens. It is interesting to remember that the sera were collected from cattle with a single infection by one of these trematoda only.

Table 4. Analysis of cross-immunity against the peaks collected after the chromatography of FES and CES

G-F (n= 170)		G-P (n= 191)	
CES peaks	Positive	FES peaks	Positive
P1	102 (60%)	P1	55 (29%)
P2	66 (39%)	P2	80 (42%)
P3	119 (70%)	P3	107 (56%)
P4	36 (21%)	P4	34 (18%)
P5	76 (45%)	P5	57 (30%)

The ELISA showed that cattle with fasciolosis reacted strongly against CES-peaks P3 and P1, whereas the lowest cross-reaction against P4 (<13.7 kDa) was reached (Table 4). We observed that sera from G-P recognized FES-peaks P2 and P3, and the weakest reaction against P4 (<13.7 kDa) was obtained.

Electroeluted fractions

Once the different polypeptides by electroelution of FES and CES under reducing conditions were obtained, the possible cross-immunity was assessed by an ELISA procedure. As represented in Table 5, a strong cross-reaction was observed among the sera from G-F and G-P, and the proteins eluted from CES and FES, respectively.

It is remarkable that in the sera from cattle infected by *F. hepatica*, the percentage of cross-reaction to the CES-fractions ranged between 68% and 92%. By using the sera from bovine with paramphistomosis, cross-immunity to the FES-fractions varied from 57% to 100%.

Table 5. Analysis of cross-immunity against the fractions electroeluted from FES and CES

G-F (n= 170)			G-P (n= 191)	
Positive	CES fraction	MW (kDa)	FES fraction	Positive
53 (80%)	Cd1	106	F1	164 (86%)
		93	F2	191 (100%)
		81	F3	136 (71%)
		63	F4	191 (100%)
		59	F5	136 (71%)
10 (15%)	Cd2	52		
49 (74%)	Cd3	41	F6	164 (86%)
45 (68%)	Cd4	27		
50 (76%)	Cd5	23	F7	109 (57%)
		18	F8	27 (14%)
47 (71%)	Cd6	12		
61 (92%)	Cd7	<8		

As represented in Table 5, the electroelution procedure allowed us to identify one protein belonging to CES (Cd2; 52 kDa), which was recognized only by the 15% of sera from cattle with fasciolosis. Furthermore, only 14% of the sera from bovine with paramphistomosis reacted with an 18-kDa protein from the FES (F8).

DISCUSSION

Paramphistomosis and fasciolosis are infections that affect ruminant livestock. However, the infection by *F. hepatica* has been widely studied while the infection with *Paramphistoma* has not been thoroughly evaluated, in spite of the damage caused by this infection and its effect on production (Urquhart et al., 1996; Rangel-Ruiz et al., 2003).

Estimation of the prevalence of parasitic trematodosis has classically been done by coprological analysis (Bouvry and Rau, 1986; González-Lanza et al., 1989; Düwell and Reisenleiter, 1990). However, this method has disadvantages because a prepatent period of over 12 weeks elapses after infection before it is possible to detect eggs of *F. hepatica*, *Calicophoron*, or *Dicrocoelium* spp in feces, by which time most of the pathological damage has already happened (Guobadia and Fagbemi, 1996; Campo et al., 2000; Díaz et al., 2006).

Another inconvenience resides in the similarity between the eggs of *F. hepatica* and *Calicophoron* spp, which makes difficult the differential diagnosis.

It has been demonstrated that most of pathological damage takes place when flukes are migrating through peritoneal cavity and liver parenchyma before their establishment in the bile ducts for *F. hepatica* flukes, and through the intestine for *Calicophoron*. Likewise, it is very important to develop right and early diagnostic techniques to reduce the great losses due to these infections.

During the migratory phase of infection, *F. hepatica* antigens are released and presented to the immune system, thus enhancing the production of antibodies (Langley and Hillyer,

1989). After the parasite is established in the bile ducts, a lesser amount of antigen is then available to the immune system.

The immunoenzymatic techniques such as ELISA have been found to be very suitable for the diagnosis of fasciolosis due to their high sensitivity and the possibility of processing many sera samples (Arriaga de Morilla et al., 1989). These probes focused on the detection of antibodies against parasitic antigens by ELISA and seem very useful to solve the disadvantages of coprological examinations (Cornelissen et al., 1992 and Hillyer et al., 1996). Rodríguez-Pérez and Hillyer (1995) and Duménigo et al. (1999) proved that in sheep experimentally-infected with *F. hepatica* metacercariae IgG against crude excretory/secretory, antigens were detected from the second to third weeks after infection. By means of an ELISA technique and excretory/secretory antigens of *Dicrocoelium dendriticum*, experimentally-infected lambs reached IgG values higher than the cut-off point from day 30 post-infection (p.i.), showing positive infection (González-Lanza et al., 2000).

The presence of antibodies does not correlate with the existence of active infection, but only with exposure to the parasite (Espino et al., 1998 and Sánchez-Andrade et al., 2001). Another trouble consists of that long persistence of high levels of immunoglobulins, even though animals have been successfully treated (Ibarra et al., 1998).

The potential of immunodiagnosis assays for early detection of helminth infections is marred by non-specificity and cross-reactions because of shared common antigenic epitopes in several trematoda (Ghosh et al., 2005).

The possibility for mixed infections developed by *Fasciola* and *Paramphistomum* trematoda is very elevated in areas with moderate temperature and high humidity where the survival of their intermediate host is guaranteed (Díaz et al., 2006). According to Mage et al. (2002), the prevalence of bovine natural fasciolosis is decreasing simultaneously to the increase in that of natural paramphistomosis in central France. Likewise, specific treatments for cattle fasciolosis are usually employed, and control of paramphistomosis is not practiced, which enhances the need for new methods to evaluate the distribution of this trematodosis.

Furthermore, a more accurate knowledge of the status of infection could be very helpful to choose the suitable anthelmintic for chemoprophylaxis. There are significant differences in activity and cost of the different commercial anthelmintics available, depending on their activity against the immature stages mainly.

Another problem that derives from chemoprophylaxis that needs to be considered is the appropriate period(s) of suppression of anthelmintics on milk and meat, so administration of any anthelmintic would imply reduction on animal production, increasing the losses due to the parasite infection.

The study of cross-immunity against the antigens of *Fasciola* and *Paramphistomum* is necessary to the right diagnosis of these trematodosis. In the current investigation, an elevated similarity between the excretory/secretory antigens from these parasitic trematoda under native conditions has been shown. The observation of an important cross-immune reaction among sera from cattle monoinfected by *Fasciola* or *Paramphistomum* and the protein peaks collected by liquid chromatography suggests the existence of some antigenic determinants being shared by them.

The analysis of the antigenic products from both trematoda under reducing conditions revealed the presence of three proteins belonging to the antigens of both trematoda with the same molecular weight, which were highly recognized by sera from cattle with a single-

trematode infection. This supports the hypothesis about the presence of several antigenic fractions common to the excretory/secretory products of *Fasciola* and *Paramphistomum*.

In the present study, we proved that the problem due to the cross-immunity can be minimized by using purified antigens. We obtained successful results when an 18-kDa protein from the *F. hepatica* excretory/secretory antigens was employed, which agree with data from previous investigations (Hillyer and Soler de Galanes, 1988; Arafa et al., 1999; Mousa, 2001; Kim et al., 2003). A 52-kDa protein collected from the *Calicophoron* excretory/secretory antigens showed low cross-reaction.

Current efforts for the control of parasitic diseases need diagnostic techniques which allow their early and accurate detection. We concluded that the application of immunoenzymatic diagnostic probes such as the ELISA requires the investigation of the possibility for a cross-immune reaction to enhance the right interpretation of the achieved results. The purification of antigens by electroelution allows the production of proteins with low cross-immunity.

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Chapter XIV

ANIMAL MODELS SUITABLE TO STUDY COMPLEX POLYGENIC PROCESSES

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ABSTRACT

Parasitic infections are chronic processes difficult to eradicate, but studies both in animals and in humans indicate that the host genetic background contributes to resistance/susceptibility and to the acquisition of the infection. Despite the high global prevalence of enteroparasitic infections in humans and the economic damage they cause in livestock, our understanding of the host-parasite relationship at the molecular level is still rudimentary, and the elucidation of the genetic basis of resistance is a major challenge in parasitology research. Laboratory animal models have the advantage of facilitating the study of the interaction among multiple genes, characteristic of complex diseases such as a parasitic infection. Furthermore, genetic heterogeneity and a range of variation of exposure to environmental agents can be controlled, allowing the identification of the different susceptibility loci. Differences in the quality and quantity of natural enteroparasitosis on the chronic phase, observed in adult mice of the CBI-IGE stock in a previous study, led us to analyze the pattern of inheritance of these variables in the reciprocal crosses of the inbred lines CBI/C and CBI-, differing in resistance. The hybrids were similar to the resistant parent, though a maternal effect was observed in the proportion of mice infected with protozoans. The helminth burden showed dominance of the resistance and a sex effect, differences among genotypes being higher in males than in females. Susceptibility to enteroparasites in the F1 differed from that of the parental lines, evincing that the host genotype is crucial in modulating the host-parasite relationship. Control of parasitic diseases based on the host genetic resistance is currently

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being considered as a complement or replacement of existing technologies. For this approach to be successful, a good understanding of the genes involved and the molecular pathways they control should be achieved from studies of animal models, since an organism's phenotype is the result of complex interactions between its genotype and the environment. Animal models generated through targeted gene deletion or transgenesis, though useful, involve manipulation of single major genes, making these models inappropriate to analyze interactions among genes. Contrariwise, lines of mice generated by long-term artificial selection, a powerful technique to modify the genetic structure of a population, are valuable resources to obtain populations suitable for QTL-mapping studies to increase our understanding of the many biological processes underlying resistance to parasitic infections.

INTRODUCTION

Gastro-intestinal parasites constitute a severe problem for both human and animal health; they infect a considerable proportion of the human population and some, such as helminths, are characterized by developing resistance to antiparasitic treatments. Though they rarely cause death, these infectious agents are usually associated to high morbidity levels and impose a major economic burden in areas where the infection is endemic. Not only are parasites common and widespread in populations throughout the world, but they frequently bring on chronic infections, and these infections may occur repeatedly throughout the lifetime of an individual. Their abundance and long-term survival present a paradox, since parasites are highly immunogenic and their environment, the gastro-intestinal tract, is adequately prepared to mount potent immune and inflammatory responses.

Several investigations indicate that the result of a parasitic infection depends, to a great extent, on the host's ability to produce antibodies, mount a cellular immune response, develop NK activity, and produce cytokines; in other words, to develop a normal immune response, which is clearly under genetic control. The variation in responsiveness can be related to non-immunologic or non-specific resistance mechanisms (Ruwende C et al, 1995; Mockenhaupt et al, 2004), or to mechanisms mediated by the immunologic system, either innate (De Veer et al, 2007; Knight et al, 2008; Perrigoue et al, 2008) or adaptive (Grencis, 2001; Johnston et al, 2005), and its control depends on both the genes linked (Stewart and Chapes, 2003; Reiner et al, 2007) and not linked to the major histocompatibility complex (Else and Wakelin, 1988; Artis, 2006). In this line of thought, studies in human populations allowed the identification of chromosomal regions whose genes would have a major effect in controlling parasitic infections; some of them would code for cytokines that regulate T lymphocyte function (Traore et al, 1999), while others would be quantitative trait loci (QTL), regulating activity and differentiation of B cells (Williams-Blangero et al, 2008).

Infectious agents, and among them parasites, induce a wide spectrum of changes in their hosts. The course of an infection shows frequent variations that would be genetically-determined (Wakelin, 1978; Quinnell, 2003; Behnke et al, 2006), and several genes of interest have been identified both in human population studies and mouse models (Campino et al, 2006). In domestic animals, resistance is a hereditary trait with an heritability above 0.3 (Nejsum et al, 2009). Also, it has been possible to show in some cases that a single gene or group of related genes determines resistance to parasitic infections; this has been demonstrated in several mouse strains infected with nematodes (Menge et al, 2003), whereas

resistance to *Toxoplasma gondii*, *Trichinella spiralis*, *Trypanosoma musculi*, and *Trypanosoma congolense* appear to be under polygenic control (Iraqi et al, 2000). In certain experiments, it has been also possible to identify specific genetic loci in the host, as well as interactions between the infectious agent and its host, that define resistance to infection. It has been reported that part of the variability of the host's response to infections with nematode parasites, like *Heligmosomoides polygyrus*, is based on the different ability to mount an effective immune response (Ben-Smith et al, 2003), a genetically-determined trait. Another aspect of the host-parasite interaction, the relationship between intestinal pathology and immune expulsion of gastro-intestinal nematodes, has not been completely elucidated. Parasite expulsion is associated with intestinal pathology in several model systems and both are T cell-dependent processes. Though immune expulsion of gastro-intestinal helminth parasites is usually associated with Th2 responses (Lawrence et al, 1998), the effector mechanisms directly responsible for parasite loss have remained elusive and are currently being investigated (Artis, 2006). Bell et al (1982), studying the ability of expulsion of adult *Trichinella spiralis* in lines of mice of the Swiss strain differing in the character, reported that crosses between responder and non-responder strains showed that rapid expulsion behaves as an autosomal dominant trait. These authors were also able to identify the mendelian locus, not linked to the major histocompatibility complex (MHC, chromosome 17) or the gene for albinism (*c/c* locus, chromosome 7) (Bell et al., 1984). They named this locus, not previously identified as conferring resistance to any infectious agent, *Ihe-1* (intestinal helminth expulsion 1).

More recently, Knight et al (2000) showed that ablation of the effector molecule derived from the gene *mMCP-1* (mouse Mast Cell Protease-1) delays the host's capability to expel the parasite; mMCP-1 has also been associated with the intestinal inflammation that usually accompanies gastro-intestinal helminth infections (Lawrence et al, 2004). Clearly, murine models of intestinal nematode infection have allowed the definition of several of the many effector molecules and cells participating in the regulation of resistance and susceptibility to infection.

Studies in human populations suggest that genetic factors may have a protective role (Williams-Blangero et al, 2002), while others (Chan et al, 1991) propose that the effect of the genetic background is overcome by the environment or habits of the individual. Undoubtedly, if resistance is a quantitative character determined by polygenes, the environmental component should play a major role in the evolution of the parasitic infection. Though our current understanding of the underlying mechanisms of resistance to parasitic diseases is not complete, experimental data indicate that they are often regulated by multiple genes that control different aspects of the host-parasite interaction. Genetically-distinct inbred strains of mice that differ in their susceptibility to specific pathogens are invaluable for dissecting such complex patterns and have allowed the identification of several host-resistance loci that regulate natural and acquired immunity in response to infection (Malo and Skamene, 1994). Cloning these genes, as well as identifying their products, is the first step in elucidating their functional roles in host defense.

Infectious diseases are a major selective pressure and genes involved in the immune response are the most numerous and the most diverse in the vertebrate genome (especially mammals), reflecting the evolutionary advantages of a diverse immunological response to a wide range of infectious pathogens. This is most evident at the Major Histocompatibility Complex (MHC) loci, the typical genetic region associated with infectious disease

susceptibility. The MHC is a gene-dense region found in all jawed vertebrates studied to date, and contains a great proportion of the immune genes that are highly polymorphic (Kelly et al, 2005). It has been proposed that heterozygosity in this region is associated with a slower progress to disease and lower mortality, whereas the converse scenario, increased MHC homozygosity, may contribute to the increased susceptibility to infection in genetically-isolated populations (Burgner et al, 2006).

Susceptibility to many common diseases (such as diabetes and ischemic heart disease in humans) originates in the complex interaction of environmental and host genetic factors, whereas susceptibility to parasitic infections is determined by a more complex equation, since it arises from the interplay of the environment, host genotype, and parasite genotype (Nino Incani et al, 2001). Furthermore, parasites such as helminths have acquired the remarkable ability to down-regulate host immune response, protecting themselves from elimination and minimizing pathology in the host (Maizels and Yazdanbakhsh, 2003). Susceptibility should then be regarded as a genetically-complex trait, in which many genetic loci participate, each with a modest contribution to the phenotype; most of the focus in the field has been on identifying these loci and their effects on infection and in other conditions. Besides this quantitative genetic determination, there are also many single genes or Mendelian disorders affecting immune function and determining primary immunodeficiencies; more than 300 of these have been identified in humans. Although generally rare, these severe immunodeficiencies, which stem from major functional aberrations at single genes, can be highly informative about immunological mechanisms and protection against specific infections.

Since host-parasite adaptation is a consequence of a mutual genetic interaction aiming to attain a balanced relationship, the host genotype certainly possesses genes that harm or benefit the parasite: the host genotype plays a major role in controlling parasitic infections and conditions its resistance, or susceptibility, to the establishment of the infection; genetic variation in these genes increases fitness in the population by limiting the number of possible hosts. On the other hand, genetic diversity at parasite-recognition loci increases the range of potentially susceptible hosts (Tellicer and Brown, 2007).

According to Råberg et al (2009), two conceptually different components of the host's response against parasites, are the ability to limit parasite burden (resistance, which reduces the probability of being infected or attacked) and the ability to limit the harm caused by a given burden (tolerance, which reduces the loss of fitness caused by the infection or attack). Closely linked to the latter is the concept of resilience that is defined as the ability to recover from or adjust easily to change. Together, resistance and tolerance (or resilience) determine how well a host is protected against the effects of parasitism. As pointed out by Råberg et al (2009), this distinction is useful because it recognizes that hosts that are best at controlling parasite burdens are not necessarily the healthiest. Moreover, resistance and resilience can be expected to have different effects on the epidemiology of infectious diseases and host - parasite coevolution and are, probably, determined by different sets of genes. Restif and Koella (2004) in a theoretical study describing the simultaneous evolution of these two strategies of defense in a population of hosts submitted to a pathogen, assuming that the two traits are determined by two unlinked genes, conclude that (a) resistance and tolerance do not necessarily exclude each other; (b) they should respond in different ways to changes in parameters that affect the relative costs and benefits of defenses. Their results highlight the problems of estimating tradeoffs and costs of defense among natural populations without

knowledge of the underlying mechanisms. To date, studies of defense in animals have focused on resistance, and tolerance/resilience and its implications in animal well-being and productivity have been largely overlooked.

A key to understanding these diseases is then the study of the interactions between the host and the parasite. This is a complex relationship that has evolved over millennia and focuses on the host immune response to infection. This is further complicated by the finding that, while some host's defense mechanisms may protect against infection, other responses are the reason why the disease develops: schistosomiasis is one of such diseases, in which most of the pathology is secondary to the host immune response to parasite antigens (Bethony and Quinnell, 2008). Since an important proportion of the variability observed in the host - parasite relationship is of host genetic origin, one of the goals is the genetic analysis of this complex trait to understand its "genetic architecture," that is the number and position of loci affecting the trait, the magnitude of their effects, allele frequencies and types of gene action. Natural selection may have also maintained a certain combination of resistant loci, most of which, having small individual effects, are more difficult to detect. Again, the characteristics of parasitic infections undoubtedly emphasize the need to develop new and diverse experimental animal models to analyze host-parasite relationships.

ANIMAL MODELS

By definition, an animal model is a biological system useful to study normal biology and behavior, as well as to investigate spontaneous or induced pathological processes in a given species, and in which the phenomenon analyzed reflects, wholly or in part, the same phenomenon observed in another species of interest (Infante et al, 1998). Traditionally, numerous life processes related to humans and species of economic interest have been studied in rodent model systems, mainly rats and mice. Animal models, and particularly mouse models, have an extraordinary potential to break down the complex genetic system of host-parasite interaction into simpler components. The main approaches used to generate mouse models for infectious diseases are: (a) genetic manipulation using transgenesis or generation of targeted mutations in candidate loci; (b) identification by positional cloning of genes associated with resistant/susceptible phenotypes in spontaneous or induced mouse mutants; and (c) mapping and characterization of quantitative trait loci (QTL) governing the complex aspects of the host - pathogen interaction.

In the context of QTL identification, the many lines of mice obtained by applying different artificial selection criteria have acquired such a significance that they are currently considered an irreplaceable resource to map genes as Bünger et al (2001) emphasized while studying the genetic control of growth. Though the conclusions obtained from these studies cannot be directly extrapolated to other species, they are useful to solve certain basic aspects of the process such as the biochemical or physiological determinants (metabolism, physiology, and cell signaling) common to different species. Also, there are an important number of animal models with significance in biomedical investigation, since they allowed the development of a more precise and detailed knowledge of the physiology of the different apparatuses and systems, as well as the pathogenesis of infectious and degenerative diseases, favoring the advancement of knowledge in their prevention and treatment. These

experimental models may have had different origins such as spontaneous or induced mutations, or transgenesis both in zygotes or stem cells, and, with the typical limitations of interspecific extrapolation previously mentioned, may contribute to the development of therapies that replace the defective function of certain genes. Although animal models for genetic diseases have arisen spontaneously in a variety of species (e.g., mice, cats, and dogs), the use of new methods to mutate genes in embryonic stem cells can produce mice with engineered alterations in any given gene. Numerous mouse strains with mutations in genes relevant to human diseases have already been created in this manner, and also by injection of human genes into fertilized mouse eggs. In some instances, mice with such mutations in a Mendelian gene exhibit a phenotype similar to that seen in humans. It has to be remembered, however, that mouse models do not always mimic faithfully the relevant conditions of the trait in the species of interest. For example, hypoxanthine phosphoribosyltransferase deficiency associated with Lesch-Nyhan disease in humans is benign in mice due to the presence of an alternative metabolic pathway. Another limitation of these approaches is that genetic manipulations in mice would usually allow the study of single major or Mendelian gene effects whereas common diseases are typically polygenic.

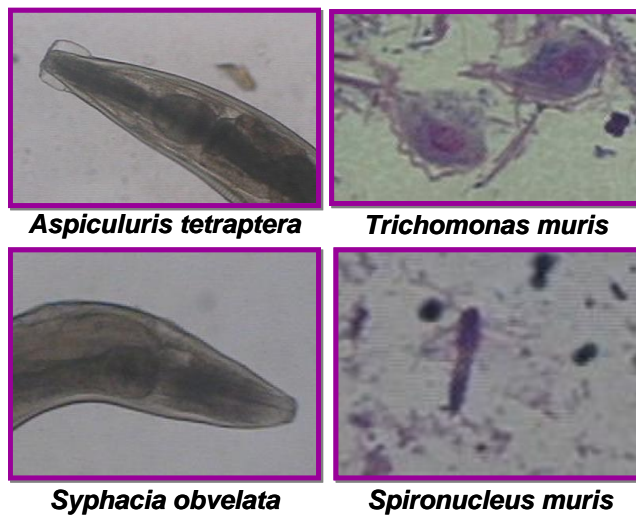


Figure 1. Enteroparasites naturally inhabiting the intestine in mice of the CBi stock. The intestine was excised and the length of each of its segments measured. Each segment was longitudinally cut and an aliquot of its content was examined microscopically with a magnification of X100 and X400, with and without wet stain, to assess the presence of trophozoites, oocysts, cysts, eggs and/or larvae of enteric parasites. The protozoan load was semi-quantified by crosses (number of organisms per microscopic field, magnification X400). The remaining content was fixed with SAF and examined microscopically with an X40 magnification, to identify and count helminths. The results of counting the worms in all stages of development were expressed as total and relative number of helminths (total number of helminths per unit of large intestine length, count/cm).

When using mouse models of parasitic diseases, one of the advantages is the ability to control pathogen effects such as dose, strain, and route of infection. Also, the mouse constitutes an ideal model system for genetic studies as many inbred, congenic, recombinant inbred, naturally-occurring or experimentally-induced mutant strains have been developed. In

addition, mice have a relatively short generation time, their genome has been sequenced and high density marker maps are available

Considerations of the complexity of the host-parasite relationship are critical when one thinks about new drugs or vaccine development for any parasitic disease, and they underline the need for models that allow identifying which components, if any, of the host immune response are protective against infection. The challenge then becomes one of finding the ways to recognize which pathways in the host response to the parasite are protective and when stimulated do not induce a pathological reaction. In this respect, animal models of infections have been a central tool in studying the immunopathogenesis of parasitic diseases, since specific hypotheses and experimental therapies should generally be tested extensively in small animals prior to human experiments. Studying the differences of the animal model phenotypes may provide insights into disease pathogenesis that may, in turn, be exploited either by gene therapy or pharmacological approaches.

Despite the potential phenotypic differences in the trait between the animal model and the species of interest, its study for the design of gene therapy approaches in a pre-clinical setting is important and should not be undervalued.

THE CBI-IGE MODEL

We recently described phenotypic differences in the quality and quantity of natural enteroparasites in lines of mice of the CBI-IGE colony (Vasconi et al, 2008). These lines were obtained by divergent selection for body conformation (Di Masso et al., 1991), and are currently in their 110th generation of selection. They show both phenotypic and genotypic differences in characters related (Di Masso et al, 1997; Hinrichsen et al, 1999; Di Masso et al, 2004) and unrelated (Suárez C et al, 2006) to the selection criteria. The CBI-IGE lines (CBI+, CBI-, CBI/C and CBI/L and CBI) constitute an interesting model that originated by selection from a common base population (CBI-Fm, Central Animal Facilities, Facultad de Ciencias Médicas, Universidad Nacional de Rosario); four of the lines were obtained by disruptive selection for body conformation (CBI+, CBI-, CBI/C and CBI/L), while CBI is the unselected control line. Due to the joint effects of selective breeding for more than 100 generations, correlated responses to selection, non-directional effects of inbreeding and genetic drift, and the appearance of new mutations, each line fixed different genic combinations. When natural enteroparasitosis was studied in these lines, protozoans (*Trichomonas muris* and *Spiroplasma muris*) and nemathelminths (*Syphacia obvelata* and *Aspiculurus tetraptera*) were found naturally-parasitizing the mice's intestines (Figure 1). CBI/C and CBI were the only genotypes in which *T. muris* was found. CBI- was the least resistant to *S. muris*. The helminth parasitic burden showed differences between sexes within genotypes (males had a higher burden than females) and among genotypes (CBI/L males had the lowest burden). In addition, since both the protozoans (*T. muris* and *S. muris*) and nematodes (*S. obvelata* and *A. tetraptera*) found in this study were present in the CBI-Fm colony (Zdero M, personal communication), the differences in the parasitic burden quality and quantity in the lines of the CBI-IGE stock may be viewed as resulting from the co-evolution of host and parasite during the course of selective breeding. We also found that these distinct genotypes showed differences in their resistance to both natural and experimental parasitosis. CBI/L animals

were also the most resistant to experimental challenge with *Heligmosomoides polygyrus* and *Trypanosoma cruzi*. Thus, infection in these mouse strains showed a range of response phenotypes similar to the variation observed in the wild, under natural conditions. To further analyze the relative contribution of host genotype and environment to resistance to parasitosis, we analyzed the quality and quantity of enteroparasites in mice derived from the reciprocal crosses of lines CBi/C and CBi- of the CBi-IGE stock, differing in resistance to natural enteroparasitosis in the chronic phase of the infection; we also estimated the pattern of inheritance of the character. Males and females of lines CBi/C and CBi- and of the F1 reciprocal crosses were studied, mice of the parental lines being contemporaneous of the progenitors of the F1 (Charts 1 and 2; in the reciprocal crosses the maternal genotype is indicated in first place). Mice were sacrificed during the first half of the light cycle, at 90 ± 10 days of age (see Figure 1 for brief description of the methodology).

**Chart 1. Natural enteroparasites in adult females of the CBi-IGE stock.
Effect of genotype and maternal line**

Variables Genotypes	Protozoans				Nematodes	
	<i>Trichomonas muris</i>		<i>Spiroucleus muris</i>		Parasitic burden*	
	Proportion of mice with parasites (%)‡	Parasitic burden‡, *	Proportion of mice with parasites (%)‡	Parasitic burden‡, *	Total count (N) ‡	N/large intestine length (count/cm) §
CBi/C (n = 15)	100 ^a	5 ^a (1-5)	40 ^a	3 ^a (2-5)	50 ^a (11-147)	5.2±0.92 ^a
F1 (C x -) (n = 14)	50.0 ^c	4 ^a (1-5)	7.1 ^c	1 ^a (1-1)	32 ^b (6-80)	3.2±0.68 ^b
F1 (- x C) (n = 9)	0 ^b	---	33.3 ^{a,c}	1 ^a (1-1)	21 ^b (12-42)	2.1±0.31 ^b
CBi- (n = 18)	0 ^b	---	100 ^b	5 ^b (3-5)	36 ^{a,b} (14-127)	5.8±0.88 ^a

§ Data are expressed as mean \pm standard error;

‡ Data are expressed as median (range);

* Only mice with positive fecal smears were used in the calculation;

Differences among genotypes were analyzed with parametric or non parametric ANOVA, as appropriate. Comparisons between genotypes within sex or between sexes within genotype were done with Bonferroni or Dunns post-tests. Fisher's exact probability test was used to analyze differences in qualitative variables. Differences were considered significant if $p < 0.05$.

For each variable, differences between groups not sharing the same superscript are significant at the 0.05 level or higher.

Charts 1 and 2 show the proportion of mice harboring the different parasites and the parasitic burden in females and males, respectively. *T. muris* was found parasitizing all CBi/C animals and approximately half of the F1 (C x -) mice, in both sexes. This flagellate was absent in the CBi- and the F1 (- x C). *S. muris* was observed in all the genotypes, but the percentage of parasitized mice and the burden were different among groups: all CBi- animals were positive for the parasite and had the highest burden while only half CBi/C mice carried

the parasite and had a lower burden; both reciprocal crosses showed a lower proportion and burden. The adult nematode form of *S. obvelata* was found in all mice studied, while *A. tetraptera*, though present in all the lines, showed a non-significant variation in the proportion of parasitized animals per group (results not shown). The absolute and relative parasitic burdens showed significant genotype and sex effects ($P < 0.01$); CBI- had the highest values in the two measurements. An overall sex effect was seen in burden, males showing the highest values in both variables ($P < 0.01$) when compared within line. This sex difference was significant only in CBI- ($P < 0.01$).

**Chart 2. Natural enteroparasites in adult males of the CBI-IGE stock.
Effect of genotype and maternal line**

Variables Genotypes	Protozoans				Nematodes	
	<i>Trichomonas muris</i>		<i>Spirotrichomonas muris</i>		Parasitic burden*	
	Proportion of mice with parasites (%)‡	Parasitic burden‡, *	Proportion of mice with parasites (%)‡	Parasitic burden‡, *	Total count (N)‡	N/large intestine length (count/cm) §
CBI/C (n = 15)	100 ^a	5 ^a (4-5)	53 ^a	1.5 ^a (1-5)	75 ^a (22-151)	7.2±1.07 ^a
F1 (C x -) (n = 14)	69.2 ^c	4 ^a (1-5)	23.1 ^a	2 ^a (2-3)	104 ^a (14-578)	15.0±3.40 ^a
F1 (- x C) (n = 9)	0 ^b	---	42.8 ^a	1 ^a (1-2)	160 ^a (15-355)	17.0±2.90 ^a
CBI- (n = 18)	0 ^b	---	100 ^b	5 ^b (3-5)	427.5 ^b (47-938)	46.8±7.51 ^b

§ Data are expressed as mean ± standard error;

‡ Data are expressed as median (range);

* Only mice with positive fecal smears were used in the calculation;

For each variable, differences between groups not sharing the same superscript are significant at the 0.05 level or higher.

These results, although preliminary, indicate that the genotype plays an important role in the host-parasite interaction, since F1 showed a different susceptibility to parasitosis when compared with the parental lines. A maternal effect was observed in the host - *T. muris* relationship affecting burden and proportion of parasitized mice. Notwithstanding the high infective capacity of this parasite and the time the pups stayed with the mother (from birth to weaning), the hybrid (C x -) had a parasitic burden lower than that of the maternal line CBI/C, which could be ascribed to a heterotic effect. Maternal and heterotic effects were also observed in the relationship between hosts and *S. muris*, since both crosses were resistant to this flagellate, at variance with the parental lines that were susceptible and/or had very high burdens as the parental line CBI-. Absolute and relative helminth burden showed a clearly marked sex effect. Males showed dominance of the maternal line CBI/C; females differed with the maternal line CBI/C in the absolute burden, whereas the relative burden showed negative heterosis. The present data highlights the functional importance of gender and background strain in interpretation of studies using experimental animal models. Confronted

with a natural, continuous exposure to helminths, no genotype was able to resist totally the infection. Since mice of the different genotypes shared the same environmental factors, and helminths do not reproduce inside the host, the burden would estimate the host's resistance. The differences in parasitic burden among genotypes and between sexes within a genotype suggest a host genetic control to limit the settlement of ingested larvae and regulate expulsion of the adult worms and female fecundity, thus modifying the parasite capacity to disseminate; the degree of susceptibility would be related to the type of immune response the host develops to defend itself from the parasite. Interestingly, during this study no signs associated to the extent of the parasitic load were observed, suggesting that the phenotypes would also differ in the degree of tolerance and/or resilience to these parasites, or that the maximum load found in these animals would be below the threshold needed to produce detectable signs.

Genetic analysis in animal models can identify host genes that play critical roles at the interface of host - pathogen interactions. Genetic effects detected as variations in susceptibility in inbred, recombinant, and mutant strains of mice can be mapped as simple traits or quantitative trait loci followed by identification by positional cloning. Though this approach has been used to discover genes and proteins that are important to defend against such infectious agents, overall orchestration of the protective intestinal response of the host against enteroparasites is a complex process that is still being unraveled at the molecular level. This emphasizes the need to develop animal models that will allow dissection of the host-parasite relationship. Inbred, extreme-selected lines of mice derived from long-term selection experiments like the CBI-IGE lines could be conceived of as a model for characterizing host - parasite relationships, in terms of QTL or gene-mapping, as well as for candidate gene identification (Bünger et al., 2001).

Natural populations show a wide spectrum of phenotypes, ranging from asymptomatic infection to severe manifestations. Host - parasite interactions often occur in nature with distinct hosts and elaborate life cycles. An experimental model, on the contrary, confines its analysis to a specific host-parasite interaction and omits all the other elements of the epidemiological chain that may be important in the establishment of the disease. Nevertheless, models are widely used in science and, by definition, they are simplifications of reality, constructed with a defined purpose. The purpose guiding the makeup of a model determines what is included and what is excluded: the way in which modelers approach a problem will depend upon their objectives, and these will depend on the nature of the problem they are addressing (France and Thornley, 1984). That limitation in the scope of a model can be turned into an advantage, provided the model allows that the genetic complexities of a trait be broken down into simpler and more manageable gene effects.

CONCLUSION

The onset, progression, and outcome of infections are determined by performance of the host's defense mechanisms and expression of pathogen virulence determinants. Infection is the result of the interaction between host, parasite, and environment, superimposed on the continual evolution and adaptation of the parasite and host genomes to each other. As appropriately stated by Nuismer and Otto (2005), "Hosts and parasites are locked in a continual co-evolutionary race, which generates persistent selection for resistant hosts and

infectious parasites.” This characteristic generates a need to develop new drugs and strategies for prevention and treatment of parasitic diseases. It is important to improve our understanding of the mechanisms set in motion by a host to defend itself against a parasite for these new approaches to be successful. Currently, the mechanisms implicated in resistance and susceptibility to infection and/or development of a parasitic disease in mammals are not completely understood because of the complexity of the interactions among host, parasite, and environmental factors. Furthermore, the response to resist parasitosis and/or disease is under genetic control and varies among species and among individuals within species. To begin unraveling this complexity, the genetic basis of resistance/susceptibility to parasitosis and parasitic disease should be revealed. In this context, mice have played a very important role in elucidating the molecular pathways that contribute to disease, and are invaluable to dissect the effect of the host’s genes in a complex character (Peters et al, 2007), such as parasitic diseases, using, among other approaches, well-defined inbred lines.

Although gene knockout lines (obtained by targeted gene deletions) and transgenic, as well as congenic, lines generated by introgressing a chromosomal region in a particular genetic background offer a wide spectrum of models to study individual genes and gene products, they require previous knowledge about the genes associated with the phenotype under study. On the contrary, anonymous genes underlying complex traits can be identified by positional cloning based solely on their position in the genome without any knowledge about their functions (Corva and Medrano, 2001). In this sense, lines generated by long-term artificial selection are valuable resources to create suitable mapping populations. The number of QTL mapped in a particular study is limited to those at which different alleles are fixed in the two parental strains (Mackay, 2001). The lines herein described have apparently fixed several different alleles associated with resistance/susceptibility to parasitosis. The results herein described permit us to conclude that this animal model could be a useful resource for mapping genes that will improve our understanding of the mechanisms (such as molecules involved in signaling pathways and induction of transcription factors) that control the complex and dynamic relationship established between parasite and host.

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INDEX

A

- AAV, 143
abdomen, 121
abnormalities, x, 171
abortion, x, 171, 172, 175, 177, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206
absorption, 365
academic, 92
accidental, 259
acetate, 18, 341, 345
acetonitrile, 83, 342, 343, 344, 346
acetylcholine, 76, 84, 94, 99, 107
acetylcholinesterase, 322
acid, 16, 21, 28, 45, 56, 59, 62, 77, 85, 89, 91, 94, 98, 102, 110, 115, 227, 322, 332, 336, 340, 342, 343, 345, 346, 348, 351, 357, 359, 371, 379, 381
acidic, 73, 101
acidity, 6, 7
acquired immunity, xi, 207, 231, 389
ACS, 332
activation, 27, 48, 57, 75, 84, 159, 185, 226, 229, 236, 237, 277, 329, 358
activators, 220
active radicals, 26
acute, xii, 50, 92, 119, 128, 130, 132, 175, 200, 226, 241, 265, 267, 278, 301, 331, 338
acute infection, 331
acute lymphoblastic leukemia, 265
adaptation, 144, 209, 259, 306, 390, 399
additives, 40, 336
adenosine, 85, 95, 329
adenovirus, 74, 98, 108, 112
adhesion, xi, 72, 153, 163, 208, 214, 219, 220, 221, 223, 224, 229, 234, 283
administration, 7, 30, 36, 60, 68, 71, 74, 251, 339, 377
adrenocorticotrophic hormone, 34, 54
adults, 45, 83, 106, 119, 149, 234, 240, 242, 246, 251, 253, 301, 303, 318, 324, 339, 340, 347, 354
aerobic, 41, 341
age, 5, 11, 14, 26, 41, 56, 59, 109, 131, 175, 178, 182, 199, 205, 221, 251, 270, 271, 286, 292, 305, 324, 339, 382, 395, 401
agglutination, 175, 220
agonist, 79, 80, 81, 84, 329
agriculture, 209, 401
aid, 17, 54, 250
AIDS, 136, 138, 141, 144
air, 117, 127, 129, 130, 136
airways, 233
albinism, 388
albumin, 16
alcohols, 337
aldehydes, 337, 350
alkali, 7
alkaloids, 37
allele, 89, 391, 399
allergens, 216
alopecia, 150
alpha, 227
alternative, viii, xi, xiii, 2, 34, 74, 81, 83, 208, 209, 213, 214, 260, 263, 266, 268, 281, 284, 285, 287, 303, 329, 358, 392
alters, 107
amine, 29
amino, 18, 28, 29, 42, 45, 46, 48, 49, 54, 56, 59, 77, 78, 86, 89, 94, 102, 105, 283, 336, 345, 348, 349
amino acid, 18, 77, 89, 102, 105, 336
ammonia, 286, 342, 343
ammonium, 286, 341
amoeboid, 3, 17
amorphous, 19

amplitude, 343
 amylase, 16
 amyloid, 227
 anaemia, 66, 75, 91, 110, 163, 338
 anaerobe, 23, 26
 anaerobic, 18, 26, 27, 41, 60
 analysis of variance, 184
 anemia, 114, 116, 117, 132, 134, 260, 365
 angioedema, 92, 111
 animal care, 273
 animal diseases, viii, 63
 animal health, 278, 330, 386
 animal models, xvi, 151, 385, 391, 392, 394, 397, 398
 animal welfare, 34
 annotation, 323, 325, 328, 330
 anorexia, 120, 122, 123, 131, 139, 249, 365
 antagonist, 77, 94, 290, 329
 anthropogenic, 259
 antibacterial, 233
 antibiotic, 31, 37, 44, 120, 278, 283
 anticancer drug, 360
 anticoagulant, 369
 antigen, 70, 71, 72, 107, 111, 112, 116, 135, 157, 166, 215, 231, 274, 279, 280, 285, 287, 303, 304, 305, 306, 307, 308, 309, 311, 314, 316, 319, 368, 369, 370, 376, 380, 381, 383
 antigen-presenting cell, 304
 antioxidant, 72, 104, 348
 ants, 338
 anus, 242, 243, 244
 apathy, 132
 APC, 304
 APHS, 330
 apoptosis, 165, 220, 277, 290
 appetite, 126, 365
 application, xiii, xvi, 40, 70, 71, 73, 92, 109, 120, 122, 125, 130, 136, 141, 163, 206, 300, 308, 328, 358, 362, 367, 377
 arachidonic acid, 227
 arid, 246
 ARS, 321
 arsenic, 62
 arthropods, ix, xiv, 44, 113, 114, 124, 255, 321, 322
 aspergillosis, 135
 assessment, 186, 308, 314, 345
 assignment, 327
 asymptomatic, x, 111, 120, 121, 124, 130, 133, 148, 149, 156, 157, 158, 159, 160, 162, 163, 166, 168, 259, 261, 262, 265, 288, 291, 398
 atmosphere, 340
 atoms, 336
 ATP, 18, 82

atrophy, 138, 139
 attachment, 269, 282
 attacks, 92
 attractiveness, 71
 authenticity, 87
 autoimmune, 234
 autolysis, 177
 autosomal dominant, 388
 availability, 65, 68, 304, 306, 319
 avermectin, 89, 96, 103, 104, 111
 awareness, 302

B

B cell, 169, 215, 387
 Bacillus subtilis, 24
 bacteria, 13, 17, 22, 23, 24, 31, 38, 40, 42, 44, 46, 51, 58, 114, 117, 223, 246
 bacterial, 23, 24, 46, 51, 69, 118, 122, 156, 248, 283
 bacterial infection, 118, 156
 balanitis, xii, 257
 barrier, 80, 190, 223, 290, 296
 barriers, 251
 beating, 17
 beef, x, 172, 178, 180, 190, 192, 193, 194, 196, 198, 199, 202, 205, 213, 231, 235, 288, 293, 295
 beer, 65
 beet sugar, 115
 beetles, 127
 behavior, 21, 150, 225, 344, 391
 benefits, 109, 390
 benign, 392
 bile, 11, 123, 135, 338, 359, 364, 366, 376
 bile duct, 124, 135, 338, 364, 366, 376
 bilirubin, 16, 47
 binding, 49, 76, 77, 78, 80, 82, 83, 88, 106, 108, 110, 151, 152, 164, 168, 221, 223, 225, 231, 282, 330, 379, 381
 bioinformatics, xiv, 322, 327
 biological control, 209
 biological processes, xvii, 307, 386
 biomechanics, 401
 biomolecules, 337
 biopsies, 153
 biopsy, 129
 biosecurity, 194
 biosynthesis, xi, 85, 208, 214, 223, 224, 227, 234
 biotechnologies, viii, 63, 91
 biotechnology, 107
 biotin, 23, 262
 biotransformation, xiv, 335, 336, 337, 339, 347, 348, 349, 352, 356, 357, 358, 359
 bipolar, 126

birth, x, 171, 178, 397
 bison, 211, 212
 bladder, 366
 bleeding, 119, 150, 304
 blocks, 12, 89, 94
 blood monocytes, 168
 blood smear, ix, 114, 116, 131, 133
 blood transfusion, 260, 265
 blood vessels, x, 13, 127, 129, 147, 150, 151
 bloodstream, 13, 98, 187
 blot, 225, 305, 307, 381
 body temperature, 4, 7, 23, 142
 body weight, 338
 bone marrow, x, 13, 131, 147, 148, 150, 152, 154, 157, 235
 boric acid, 371
 bowel, 279
 brain, 13, 15, 80, 99, 131, 133, 174, 176, 177, 206, 331
 branching, 328
 breeder, 48, 247
 breeding, vii, xi, 66, 91, 179, 194, 208, 214, 394
 broad spectrum, 66, 75, 209, 282
 broilers, 50
 bronchioles, 129
 bronchopneumonia, 127
 brucellosis, 330
 buffalo, 173, 210, 211, 232, 275
 buffer, 303, 340, 341, 342, 345, 346, 370, 371
 building blocks, 85
 bursa, 7, 13, 15, 40, 53, 58
 buyer, 309
 by-products, 65

C

cachexia, 260
 CAD, 326
 cadherin, 221, 236
 cadherins, xi, 208, 219, 221
 caecum, 9, 11, 19, 62
 calcification, 133
 calcium, 81, 107, 329
 calibration, 342
 cancer, 223, 231
 cancer cells, 237
 candidates, 70, 71, 94, 284, 328
 capillary, 343
 capsule, 127, 249
 carbohydrate, 23, 70, 82, 223
 carbon, 85
 carboxylic acids, 337
 carcinogenesis, 404

carcinogenic, viii, 2, 26, 28, 282
 carcinogens, 358
 carcinoma, 108, 358
 carrier, 8, 10, 32, 36, 60, 74
 caspase, 290
 cast, 153
 catabolic, 306
 catalase, 345, 347, 348, 349, 355, 360
 category a, 76
 cathepsin B, 103
 cation, 77, 151
 cats, 211, 270, 284, 288, 295, 296, 392
 cecum, 47, 210, 211, 213
 cell adhesion, xi, 208, 214, 219, 220, 222, 223, 224, 229, 234
 cell death, 165, 228
 cell growth, 108
 cell line, 85, 159, 166, 276, 329
 cell organelles, 19
 cell signaling, 228, 392
 cell surface, 155, 219, 223, 232
 cellular immunity, 35, 163
 cellular signaling pathway, 223
 central nervous system, 80, 173, 222, 301, 316
 centriole, 18
 cercaria, 102
 CES, xv, 362, 369, 370, 371, 372, 373, 374, 375
 cestodes, 75, 81, 82, 89, 115
 channels, 75, 77, 78, 79, 81, 96, 104, 323, 332
 charcoal, 23
 chemical properties, 337
 chemicals, 32, 59, 339
 chemokine receptor, 160, 167, 169
 chemokines, 156, 217
 chemotherapeutic agent, 54, 61
 chemotherapy, 48, 59, 103, 161, 314, 316, 318, 380
 Chemotherapy, 44, 45, 46, 55, 57, 61, 95, 100, 101, 106, 293
 chewing, 117
 chicken, 8, 14, 23, 24, 41, 53, 58, 60, 64, 129, 136, 263
 chicks, 39, 40, 64, 119, 248, 251
 children, 149, 318, 380, 403
 chimpanzee, 98
 chloride, 79, 81, 86, 94, 96, 97, 102, 104, 111, 323, 325, 332, 370
 chlorine, 285
 chloroquine, 85, 102, 104, 109
 cholesterol, 86, 227
 cholinergic, 326
 cholinesterase, 16, 105
 chromatograms, 343
 chromatography, 357, 370, 372, 374

- chromosome, 388, 404
circulation, 91, 301
citrus, 25, 33
classes, ix, 79, 83, 113, 205, 225, 227, 322, 323, 327, 337
classical, xii, 76, 77, 85, 215, 217, 221, 257, 262, 330
classification, 3, 39, 258, 325, 331, 381
cleaning, 117, 285, 286
cleavage, 87, 108
climatic factors, 66
clinical diagnosis, xiii, 279, 281, 299, 313
clinical symptoms, ix, xii, 113, 115, 118, 122, 123, 126, 131, 137, 157, 267, 268, 276, 278, 279, 284, 314
clinical trial, 70, 73, 92
cloaca, 7
clone, 90, 106
cloning, 77, 90, 200, 330, 391, 398, 399
closure, 120
clustering, 222
CNS, 234
CO₂, 340, 369
codon, 71, 88, 99, 106
coenzyme, 21
cohort, 150, 163, 167, 192, 202, 260
collaboration, 309, 315
colon, 134, 210, 213, 225, 232, 237
colon cancer, 225, 232, 237
colonization, 40, 269, 282, 285
colostrum, 173, 199, 202, 232
combined effect, 277
commercialization, 91
communication, 209, 215, 313
compatibility, 53
competition, 23, 27, 28
complement, xvii, 91, 220, 226, 229, 264, 305, 386
complement system, 220, 226
complementary DNA, 319
complex interactions, xvii, 386
complexity, 393, 399
components, 22, 33, 56, 79, 80, 130, 218, 223, 390, 391, 393
composition, 77, 79, 84
computing, 313
concentration, 6, 9, 16, 28, 193, 220, 273, 280, 341, 343, 344, 346, 354, 369, 370, 371
conception, 189
concordance, 312
concrete, 66
conductance, 77, 78
confidence interval, 179, 309
confinement, 65
conjugation, 336, 337, 350, 351, 355
conjunctiva, 118
connective tissue, 127, 153, 154, 155, 163
consolidation, 11
constant rate, 149
constipation, 249
constraints, 66, 219, 314
construction, 70, 71, 72, 74, 219, 324
consumers, 209
consumption, 5, 16, 195, 345
contaminant, 138
contaminated food, 119
contamination, xiii, 120, 121, 126, 194, 195, 250, 251, 268, 284
contingency, 182
contractions, 83
control group, 32, 154
conversion, 26, 29, 30, 366
conversion rate, 26, 29, 30
copulation, 5, 244
correlation, 83, 90, 133, 153, 158, 216, 217, 220, 293, 312, 314
correlation coefficient, 312
cortex, 25, 32
cost-effective, 182
costs, 30, 390
cough, 134
coughing, 130, 137
coupling, 86
covalent, 83, 85, 106
covering, 12, 20
crab, 259
CRC, 140, 320
C-reactive protein, 162
cross-sectional study, 165
crystalline, 19
crystals, 115
CTA, 109
cultivation, 41, 42, 43, 50, 51, 69, 249, 251, 320
culture, 9, 22, 23, 24, 33, 36, 40, 45, 47, 50, 51, 57, 60, 61, 84, 155, 249, 304, 306, 308
culture media, 22
curcumin, 283, 295
cuticle, 244, 246, 325, 328
cycles, 175
cyclosporine, 102
cyst, viii, 2, 8, 19, 55, 72, 120, 199, 269, 270, 280, 281, 282, 283, 284, 285, 288, 292, 294
cytochrome, 133, 357, 359, 360
cytokine, xi, 71, 73, 103, 151, 153, 156, 157, 159, 163, 165, 167, 191, 208, 214, 215, 216, 218, 219, 229, 230, 231, 232, 236
cytokine receptor, 217

cytokine response, 71, 103, 159, 167, 215, 219, 232
 cytokines, 153, 156, 157, 158, 159, 160, 215, 217,
 218, 238, 387
 cytology, 290
 cytometry, 154, 219
 cytoplasm, 14, 17, 18, 19, 75, 131, 135
 cytosol, 340, 344, 346, 347, 348, 349, 350, 351, 352,
 355
 cytosolic, 346, 350, 352
 cytostatic drugs, 74
 cytotoxic, 26, 295
 cytotoxicity, 238

D

dairies, 201
 damping, 343
 database, 94, 323, 324, 325, 326, 329, 330, 331
 de novo, 85, 86
 dead birds, 21
 death, 16, 35, 37, 66, 71, 76, 83, 91, 93, 117, 122,
 124, 128, 130, 132, 139, 140, 248, 301, 302, 305,
 314, 323, 328, 338, 386
 death rate, 248
 deaths, 67, 75, 138
 debt, 378
 decay, 314
 decomposition, 348
 defects, 84
 defense, xiv, 215, 219, 223, 233, 293, 335, 358, 389,
 390, 391, 399
 defense mechanisms, 391, 399
 defenses, 390
 deficiency, 80, 92, 157, 392, 403
 definition, 388, 391, 398
 deformities, 118
 degenerative disease, 392
 degradation, 85, 227, 277, 306
 dehydration, 6, 16, 137
 dehydrogenase, 15, 295, 349, 350, 358, 360
 dehydrogenation, 346
 delivery, 71, 106, 111, 287, 292, 341
 dendritic cell, 161, 169
 density, 164, 167, 198, 225, 273, 311, 393
 Department of Agriculture, 41, 55, 58, 229, 322
 deposition, 11
 deposits, 129
 depressed, 30, 157, 300, 365
 depression, 120, 121, 137, 139
 deregulation, 68
 derivatives, 47, 83, 99
 dermatitis, 150
 dermis, 153, 258

desiccation, 246, 285
 destruction, 16, 35, 76, 259, 283
 detachment, 138
 detoxification, 337, 347, 348, 356, 358
 detoxifying, 359
 developing countries, 259, 268, 330
 dexamethasone, 235
 diabetes, 389
 diagnostic criteria, 172
 diarrhoea, 295
 diet, 17
 dietary, 42, 43, 58, 228, 235
 differential diagnosis, 49, 115, 376
 differentiation, 37, 45, 132, 141, 215, 219, 241, 292,
 387
 diffusion, 43, 132, 358
 digestion, 306
 digestive enzymes, 75
 digestive tract, vii, 2, 6, 8, 126, 127
 dimethylsulfoxide, 340
 disaster, 43
 discharges, 7, 9, 22, 23
 disease model, 218
 disease progression, 148
 disinfection, 285
 displacement, 131
 disseminate, 150, 152, 397
 distribution, ix, xii, 16, 64, 94, 106, 113, 131, 154,
 173, 205, 208, 222, 235, 237, 239, 240, 247, 259,
 262, 265, 266, 295, 363, 364, 377, 400
 divergence, 327
 diversity, 77, 138, 206, 238, 390
 division, 3, 19, 47, 89
 docosahexaenoic acid, 227
 dominance, xvi, 386, 397
 donors, 195
 dosage, 26, 29, 68, 136, 252, 282
 dosing, 67, 68, 87
 down-regulation, 226
 drinking water, 121, 122, 138, 173, 268, 285
 drought, 6
 drug action, 84, 90, 104
 drug interaction, 356
 drug metabolism, xiv, 335, 351, 355, 356, 357, 359
 drug resistance, viii, 50, 63, 68, 75, 87, 88, 90, 95,
 97, 100, 103, 104, 106, 109, 254
 drug treatment, 68
 drug-induced, 75
 drug-resistant, 84, 90, 98, 102, 105, 110
 dry ice, 325
 duodenum, 211, 221, 269, 383
 duration, 58, 282, 306, 338
 dyspepsia, 290

dysphagia, 126
dyspnea, 131, 134, 137

E

earthworms, 4, 6, 49, 125, 129
eating, 119, 125, 259
ecological, 161
ecology, 320
economic activity, 65
economic development, 268
economic losses, 75, 363, 366
economic problem, 14
ectoparasites, ix, xi, 66, 113, 114, 115, 116, 118, 239
education, 298
egg, 5, 9, 45, 70, 73, 84, 118, 125, 129, 216, 217, 228, 245, 249, 251, 316, 318, 366, 369, 379
eicosanoid, 227
electrolyte imbalance, 122
electrolytes, 277
electron, 18, 19, 27, 55, 105, 205, 243, 244
electron microscopy, 135, 242, 243, 244
electrophoresis, xv, 362, 370, 371
electrophysiology, 96
elk, xiii, 299, 300, 301, 302, 304, 309, 311, 312, 313, 314, 315, 316, 318, 319, 320
email, 207
embryonic development, 219
embryonic stem cells, 392
embryos, 195
emission, 344, 345, 346, 365
emulsions, 35
enantiomers, 345
encapsulated, 133
encephalitis, 139, 176
encephalomyelitis, 199, 234
encoding, 73, 77, 78, 80, 103, 112, 237, 282, 287, 325, 383
encyst, 363
endocytosis, 86, 96
endoscopy, 129, 130
endothelial cell, 131
endothelium, 130, 132
energy, 18, 27, 29, 87
enlargement, 134, 139
enteritis, 56, 120, 122, 137, 139
environment, vii, xvii, 2, 4, 6, 15, 18, 41, 81, 85, 119, 120, 121, 125, 126, 129, 138, 151, 195, 269, 273, 274, 279, 283, 285, 286, 304, 306, 386, 388, 389, 395, 399
environmental change, 259
environmental conditions, 6, 149
environmental contaminants, 268, 284
environmental factors, 8, 93, 209, 397, 399
enzymatic, 106, 345
enzymatic activity, 106, 345
enzyme-linked immunoassay, 367
enzyme-linked immunosorbent assay, xiii, 150, 175, 280, 287, 288, 299, 303, 380, 381, 382
enzymes, xiv, 13, 17, 18, 26, 27, 29, 35, 37, 54, 72, 75, 82, 85, 104, 123, 224, 277, 335, 336, 337, 347, 348, 349, 350, 354, 355, 356, 358, 359
eosinophilia, 120, 124, 233, 382
eosinophils, 131, 216, 217, 221, 236
epidemic, 205, 206, 259
epidemiologic studies, 149
epidemiology, 21, 134, 140, 144, 153, 166, 195, 235, 263, 264, 265, 288, 296, 300, 390, 400
epidermal growth factor, 289
epidermis, 117
epididymis, xii, 257, 260
epididymitis, xii, 257, 261, 262
epithelial cell, 218, 224, 233, 269, 276, 288
epithelial transport, 217
epithelium, 11, 15, 20, 213, 224, 238, 276, 402
epitope, 72
epitopes, 100, 376, 383
erythrocyte, 91, 163
erythrocytes, 15, 16, 17, 91, 130, 131, 132, 133, 138, 141, 324
esophagitis, 222, 230
esophagus, 119, 125, 126, 222
essential oils, 25
EST, xiv, 321, 323, 324, 325, 326, 328, 329
estates, 195
esterase, 92
esterases, 16
esterification, 82
estimating, 390
estrogen, 222
ethanol, 25, 33, 346
ethyl acetate, 341, 345
etiology, 45, 50, 58
eukaryotes, 76, 138, 323
eukaryotic cell, 329
europium, 360
evolution, 44, 144, 166, 388, 390, 395, 399, 403
examinations, 114, 115, 120, 129, 367, 376
excitation, 344, 345, 346
exclusion, 235, 279, 370
excretion, 269, 270, 274, 278, 279, 282, 283, 284, 285, 286, 290, 294, 337, 370
exercise, 309
experimental autoimmune encephalomyelitis, 234
experimental condition, 225, 278, 287

exposure, xiii, xvi, 23, 36, 59, 62, 81, 84, 157, 260, 269, 283, 300, 303, 305, 306, 313, 367, 376, 385, 397
 expressed sequence tag, xiv, 321
 expulsion, 6, 77, 217, 224, 229, 233, 388, 397, 400, 401, 402
 external environment, 363
 extinction, 345
 extracellular matrix, 153, 155
 extraction, 56, 281, 290, 342
 extrapolation, 392
 extrusion, 90
 eyes, 10

F

faecal, 21, 134, 135, 230, 379
 failure, 49, 68, 87, 99, 103, 311
 faith, 67
 false negative, 279
 false positive, 279
 familial, 133
 family, xiv, 3, 24, 25, 47, 79, 123, 129, 133, 139, 188, 204, 206, 219, 224, 234, 240, 244, 249, 258, 300, 321, 323, 327, 336, 380
 family members, 300, 327
 FAO, 99, 332
 farmers, viii, 63, 66, 68, 97
 farming, 252, 255, 297, 300
 farms, 5, 34, 67, 175, 176, 199, 247, 252, 271, 274, 286, 295, 297, 378
 Fas, 364
 fat, 129
 fatalities, 95
 fats, 238
 fatty acids, 86, 92, 228, 336
 fax, 239, 267, 385
 FDA, 31
 fecal culture, 247, 249
 fecal smear, 396, 397
 feces, xiii, xv, 44, 46, 55, 115, 120, 121, 127, 129, 144, 174, 205, 246, 249, 251, 269, 270, 278, 280, 281, 285, 287, 294, 299, 301, 302, 303, 306, 310, 317, 362, 363, 364, 366, 367, 369, 375, 378
 feeding, 7, 45, 49, 50, 56, 58, 79, 119, 204, 246, 264
 females, xvi, 125, 129, 242, 244, 324, 386, 394, 395, 396, 397
 femur, 401
 fermentation, 248
 fertility, x, 29, 46, 172, 182, 186, 196, 201, 203, 366
 fetal, 179, 187, 188, 205, 262
 fetal tissue, 187, 263
 fetus, 173, 177, 180, 183, 186, 188, 189, 190

fetuses, 176, 180, 181, 184, 189, 195, 197, 198, 199, 203, 262
 fever, 150, 260, 324, 330
 fibroblast growth factor, 223, 236
 fibrosarcoma, 95
 filament, 17, 72
 films, 246
 filtration, 372
 financial support, 92
 financing, 93
 fish, 125, 228, 135
 fish oil, 228
 fishing, 332
 fission, 269
 fitness, xiii, 299, 390
 flagellum, 14, 17, 20
 flatworms, 402
 flavonoid, 359
 flexibility, 219
 flight, 130
 float, 115
 floating, 366
 flora, 24, 33, 46, 51, 121, 122, 136, 283
 flotation, ix, 114, 115, 135, 280, 366
 flow, 11, 164, 219, 342, 343, 346, 370
 flow rate, 342, 343, 346
 fluctuations, 65, 178, 185, 204
 fluid, 15, 232, 278, 316
 fluorescence, 76, 135, 341, 345, 346
 focusing, 101
 folate, 85
 follicle, 155
 food, 4, 10, 14, 16, 17, 18, 19, 27, 28, 56, 65, 92, 93, 119, 120, 173, 336
 food additives, 336
 foodstuffs, 40
 forceps, 119, 129
 foreign organisms, 220
 fowl, 38, 40, 49, 59
 fractionation, xv, 305, 340, 362, 371
 fragmentation, 343
 free radical, 27
 freezing, 6, 246
 fungal, 6, 23, 144, 253
 fungi, 134
 fungicides, 101
 fungus, 209
 fusion, 18, 101

G

G protein, vi, 321, 323, 328, 329, 330, 331
 GABA, 79, 81, 89, 98, 322, 326, 327

- galectin-3, 231
 gallbladder, 134, 338
 gametocytes, 131, 132, 133
 gamma radiation, 25, 57
 gamma-aminobutyric acid, 322
 gas, 343
 gastric, 224, 235, 249
 gastrointestinal, x, xi, 147, 150, 207, 216, 221, 227, 229, 231, 232, 234, 235, 237, 238, 400, 402, 403
 gastrointestinal tract, x, xi, 147, 150, 207, 221, 227, 235
 geese, 53, 129, 137
 gel, 43, 371, 372
 gender, 302, 397
 gene expression, xi, 89, 90, 100, 152, 162, 167, 197, 208, 214, 215, 217, 218, 225, 229, 230, 231, 233, 236, 403
 gene pool, 91
 gene promoter, 152
 gene therapy, 394
 gene transfer, 112
 generation, 82, 96, 111, 215, 306, 324, 391, 393, 394
 genes, xi, xvi, 44, 71, 77, 78, 80, 88, 89, 90, 99, 144, 151, 154, 206, 208, 220, 221, 224, 225, 226, 227, 235, 238, 281, 323, 336, 385, 387, 388, 389, 390, 391, 392, 398, 399, 400, 401
 genetic alteration, 151
 genetic control, 387, 392, 397, 399
 genetic disease, 392
 genetic diversity, 390
 genetic drift, 394
 genetic factors, 388, 389, 404
 genetic marker, 97
 genetics, 88, 89, 97, 219, 401
 genome, xi, xiv, 85, 134, 138, 140, 144, 208, 214, 220, 221, 232, 321, 323, 327, 332, 333, 389, 393, 399, 400
 genome sequencing, 134, 327
 genomes, 71, 323, 327, 399
 genomic, 214, 225, 238, 325, 328, 332, 333, 379, 402
 genotoxic, 26, 28, 33
 genotype, xvi, 106, 133, 134, 219, 292, 295, 386, 389, 390, 395, 396, 397
 genotypes, xvi, 21, 143, 281, 295, 296, 297, 386, 394, 396, 397
 gestation, x, 172, 175, 176, 178, 179, 180, 181, 184, 185, 187, 188, 189, 190, 191, 192, 195, 196, 197, 198, 201, 202, 203, 205
 gizzard, 6, 7, 8, 248
 gland, 220, 319, 365
 glucocorticoids, 230
 glucose, 16, 87, 346, 351, 355
 glutamate, 15, 79, 94, 96, 104, 106, 295, 323, 326, 329, 332
 glutamatergic, 97
 glutamic acid, 85
 glutathione, 98, 337, 346, 351, 358, 360, 382, 383
 glycans, 97
 glycerol, 87, 340
 glycogen, 16
 glycoprotein, 69, 89, 94, 190, 202, 204, 206, 225, 380
 glycoside, 283
 glycosylation, 224, 238
 goals, 309, 391
 goblet cells, 224, 277, 402
 gold, 270
 gold standard, 270
 government, 64, 68, 87, 315
 G-protein, 325, 326, 328, 332
 grain, 17, 23, 65
 granules, 17, 18, 131, 246
 granulomas, 13
 grapefruit, 25, 32
 graph, 313, 317
 grass, 246, 363, 368
 grazing, xv, 75, 209, 230, 236, 251, 253, 361, 368
 groups, ix, 9, 113, 120, 149, 152, 156, 184, 188, 220, 269, 292, 301, 303, 336, 337, 373, 396, 397
 growth, x, xii, 10, 12, 20, 22, 23, 24, 25, 26, 28, 29, 30, 31, 33, 38, 39, 41, 46, 48, 51, 53, 57, 59, 66, 75, 76, 83, 91, 98, 108, 120, 164, 171, 209, 215, 217, 223, 230, 236, 237, 267, 283, 289, 290, 300, 304, 338, 392, 400, 401
 growth factor, 22, 23, 24, 223, 230, 236, 237, 289
 growth hormone, 39
 growth rate, x, 29, 75, 98, 171
 GST, 337, 350, 351
 guidelines, 214
 guinea fowl, 10, 39, 52, 64, 129
 gustatory, 326
 gut, 20, 148, 152, 233, 258, 285, 301, 324

H

- habitat, 259, 300, 319
 habitation, 303
 haemoglobin, 85
 hair follicle, 153
 half-life, 314
 harbour, 314
 harm, 187, 390
 harmful effects, 341
 harvesting, 306
 healing, 119

- health, vii, viii, ix, 63, 91, 114, 121, 141, 248, 264, 278, 330, 386, 403
- health status, vii
- heart, 13, 15, 107, 127, 133, 134, 176, 206
- heart disease, 389
- heat, 23, 24, 72, 189, 285, 324
- heat shock protein, 72
- helium, 343
- helix, 327
- hematological, 134
- hematoxylin-eosin, 176
- heme, 336
- hemisphere, 103
- hemoglobin, 379
- hemolymph, 324
- hemolytic anemia, 134
- hemorrhage, 129, 140
- hemorrhages, 15, 126, 127, 133
- hepatic necrosis, 123
- hepatitis, 49, 56, 57, 58, 98, 139, 238, 366
- hepatitis C, 98
- hepatocytes, 35, 135, 359
- hepatomegaly, xii, 123, 257, 260
- hepatosplenomegaly, 132
- herbal, viii, 21, 33, 46, 61, 63, 66
- herbivores, 173
- herbivorous, 363
- heritability, 387, 403
- heterogeneity, xvi, 77, 108, 219, 385, 403
- heterogeneous, 84
- heterosis, 397
- heterozygosity, 389
- heterozygotes, 403
- high risk, xv, 182, 186, 361, 363, 365
- highlands, 193, 364
- high-level, 39
- high-throughput screening, 329
- histochemical, 62
- histogram, 78
- histological, 152, 153, 176, 177
- histopathology, 42, 124, 135, 170
- homeostasis, 96
- homogenized, 7, 9, 340, 341
- homogenous, 368
- homology, 77, 221, 336
- homozygosity, 389
- hormone, 34, 39, 54
- horse, 22, 23, 48, 88, 211, 212, 213
- horses, x, 64, 88, 171, 173, 211, 363
- host population, 194
- host tissue, 49, 160, 204, 303
- household, 65, 293
- housing, 5, 34, 260, 273, 278, 279, 286
- HPLC, 339, 341, 344, 345
- human genome, 221
- human immunodeficiency virus, 100, 101
- humans, vii, ix, xvi, 2, 6, 13, 67, 73, 74, 84, 114, 136, 138, 139, 149, 156, 157, 168, 173, 213, 220, 223, 259, 260, 262, 283, 297, 314, 385, 389, 391, 392
- humidity, 376
- humoral immunity, 163
- hunting, 311
- husbandry, 143, 291, 300
- hyaline, 242, 243
- hybrid, 126, 397
- hybridization, 22, 51, 135, 328
- hybrids, xvi, 301, 386
- hydrazine, 28
- hydro, 336, 337
- hydrocarbon, 358
- hydrogen, 18, 344, 348
- hydrogen peroxide, 344, 348
- hydrolysis, 336
- hydrophilic groups, 336
- hygienic, xiii, 34, 114, 268, 291
- hyperkeratosis, 117
- hyperplasia, 124, 224, 238, 338
- hypersensitivity, 157
- hypoglycemia, 16, 122
- hypothesis, xvi, 21, 88, 188, 362, 377

I

- ice, 325
- icterus, 338
- identification, x, xvi, 21, 45, 51, 94, 135, 162, 171, 206, 236, 259, 281, 302, 319, 323, 327, 328, 331, 356, 357, 385, 387, 389, 391, 392, 398
- identity, 263
- IFA, 116, 271, 272, 273, 274, 275, 280, 287
- IFN, 101, 153, 156, 157, 158, 159, 163, 165, 167, 188, 190, 191, 192, 215, 216, 217, 218
- IgE, 220
- IgG, 111, 191, 199, 216, 303, 309, 314, 367, 369, 376, 379, 380, 381, 382
- imitation, 33
- immune cells, 403
- immune function, 223, 389
- immune reaction, xvi, 36, 362, 370, 374, 377
- immune system, 69, 117, 122, 139, 232, 303, 305, 306, 367, 376
- immunization, 35, 36, 38, 101, 215, 235, 285, 292
- immunoassays, 367
- immunodeficient, 140
- immunofluorescence, 115, 297

- immunogenicity, 70, 99, 103, 111
 immunoglobulin, 236, 237, 316
 immunohistochemical, 21, 22, 164, 169, 176, 205, 266
 immunohistochemistry, 135
 immunological, 83, 100, 157, 280, 293, 367, 389, 390, 403
 immunology, 104, 163, 200, 300
 immunomodulation, 188
 immunomodulatory, 204, 228
 immunopathogenesis, 394
 immunosuppression, ix, xi, 113, 207, 214
 immunotherapy, 214
 immunotherapy, 97
 in situ, 61, 135, 159
 in situ hybridization, 135
 in utero, 179
 in vivo, 3, 9, 11, 21, 24, 26, 30, 31, 32, 33, 36, 45, 87, 95, 98, 112, 151, 157, 282, 358
 inactive, 206, 354
 inbreeding, 394
 incentive, 154
 incidence, xi, 34, 37, 45, 172, 178, 182, 192, 196, 202, 203, 206
 income, 67
 incubation, 23, 30, 309, 341, 343, 346, 354, 355
 incubation period, 30
 indication, 28, 177, 180, 189, 193, 307
 indicators, 91, 338
 indices, 307
 indigenous, 66, 90, 110
 indirect effect, 283
 induction, 81, 101, 159, 290, 303, 305, 359, 400
 industrial, 65
 industry, viii, xi, xiv, 2, 14, 43, 75, 121, 207, 208, 300, 301, 315, 321, 322, 323, 329, 363
 ineffectiveness, 72
 infectious disease, 50, 57, 103, 116, 165, 220, 279, 317, 389, 390, 391, 400
 infectious diseases, 50, 103, 116, 220, 279, 317, 390, 391, 400
 inferences, 306
 infertility, 134
 infestations, viii, 63, 74, 84
 inflammation, 13, 125, 133, 135, 153, 154, 176, 215, 220, 223, 227, 228, 234, 242, 243, 246, 260, 338, 365, 388
 inflammatory, 11, 36, 135, 154, 160, 164, 176, 188, 217, 218, 220, 222, 227, 234, 238, 387
 inflammatory cells, 176, 234
 inflammatory response, 188, 218, 220, 227, 387
 inflation, 243, 244
 informal sector, 68
 ingest, 6, 18
 ingestion, 4, 5, 7, 20, 102, 136, 173, 199, 244, 246, 269, 338, 363, 364
 inhalation, 136
 inheritance, xvi, 386, 395
 inhibition, 22, 29, 75, 81, 82, 108, 154, 167, 216, 228, 281, 283, 345, 357
 inhibitor, 92, 95, 111, 228, 230, 307, 317, 331, 345, 347, 348, 349
 inhibitory, 22, 79, 97, 163, 216, 218
 initiation, 238
 injection, 35, 111, 252, 284, 285, 370, 392
 injuries, 117
 innate immunity, 220, 232
 innovation, 93
 inoculation, 7, 8, 9, 32, 36, 60, 68, 158, 165, 168, 174, 303, 304, 306, 307, 310
 inoculum, 9, 41
 iNOS, 151, 159, 170
 insecticide, 14, 62, 68, 79, 332
 insects, 131, 264, 347
 insemination, 175, 180, 186, 194, 195, 196
 insight, xi, 78, 84, 208, 215, 219, 225, 229
 inspection, 299, 315
 instability, 30, 183
 instruments, 311
 integrin, 154, 160, 167, 220
 integrity, 87
 integument, 116, 120
 interaction, xvi, 53, 76, 84, 151, 152, 188, 191, 218, 219, 224, 228, 276, 323, 385, 388, 389, 390, 391, 397, 398, 399
 interactions, vii, xi, xvii, 61, 138, 140, 155, 208, 214, 220, 224, 225, 229, 237, 265, 356, 386, 387, 391, 398, 399, 403
 interface, 76, 77, 155, 398
 interference, 82, 243
 interferon, 72, 101, 105, 197, 202
 interferon gamma, 72
 interleukin, 105, 151, 296
 interstitial, 139, 153, 261, 365
 interstitial nephritis, 139
 interval, 315
 intervention, 215
 intestinal flora, 121, 122, 136
 intestinal tract, 5, 6, 126, 137, 207, 209, 244, 248, 283, 386
 intestine, 4, 75, 95, 101, 121, 210, 211, 212, 213, 217, 221, 225, 269, 285, 364, 376, 393
 intoxication, 67
 intramuscular, 35
 intramuscular injection, 35
 intramuscularly, 34

intravascular, 131
 intravenous, 35, 158
 intravenously, 8, 30, 324
 intrinsic, 276, 278, 322
 inulin, 106
 invasive, 17, 18, 36
 invertebrates, 4, 6, 77, 96, 134
 investigations, 255, 319
 investment, 67
 iodine, 116, 119, 280
 ion channels, 75, 332
 ions, 24, 344, 354
 iron, 21
 irradiation, 25, 285
 irrigation, 380
 irritable bowel syndrome, 290
 irritation, 118, 338
 ischemic, 389
 ischemic heart disease, 389
 island, 235
 isoenzymes, 359
 isoforms, 344, 347
 isolation, 41, 54, 231

J

jaundice, 16, 131
 jejunum, 13, 221, 223, 269, 295
 juveniles, 122, 137, 364

K

keratoconjunctivitis, 150
 ketones, 337, 350, 358
 kidney, 51, 135, 150
 kidneys, x, 11, 13, 15, 133, 134, 135, 137, 147, 150
 killing, 80, 83, 158
 kinase, 71, 82, 108, 296
 kinetic parameters, 350
 kinetics, 49, 288, 351
 knockout, 217, 237, 399

L

labor, 214, 306
 laboratory method, 135
 laboratory studies, 83
 labor-intensive, 306
 lactase, 277
 lactate dehydrogenase, 15
 lactating, 192
 lactation, 186, 189

lactones, 79, 83, 102, 323
 lakes, 119
 lamina, 11, 35, 215, 224, 230, 365
 land, 124
 laparotomy, 8
 large intestine, xi, 207, 208, 393, 395, 396
 large-scale, 87
 larva, 129, 249
 larval, 93, 235, 246, 250, 301, 302, 304, 305, 307, 308, 310, 311, 316, 320, 325
 laser, 208
 law, 339, 378
 LDH, 15
 LDL, 86, 106
 lectin, 99, 220
 legislation, viii, 2
 leishmaniasis, vi, ix, xii, 71, 74, 95, 106, 108, 147, 148, 150, 151, 153, 154, 155, 157, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 257, 258, 259, 263, 264, 265, 266
 lens, 343
 lesions, vi, vii, xii, xv, 1, 2, 8, 9, 11, 12, 15, 16, 18, 21, 22, 26, 31, 35, 36, 37, 56, 115, 119, 128, 135, 152, 153, 155, 176, 182, 249, 250, 257, 259, 260, 261, 262, 264, 266, 302, 361, 366
 lethargy, 131, 139, 246
 leukemia, 164, 265
 leukocyte, 155, 160, 226
 leukocytes, 35, 161
 leukotrienes, 227
 liberation, 52
 lice, 117
 life cycle, vii, xv, 20, 37, 92, 114, 121, 123, 125, 126, 127, 132, 137, 173, 174, 221, 229, 244, 252, 258, 269, 288, 301, 338, 361, 363, 364, 398
 life span, 331
 life-cycle, 90, 96
 lifetime, 183, 386
 ligand, 76, 78, 218, 225, 231, 329, 332
 light cycle, 395
 likelihood, 115, 181, 188
 limitation, 392, 398
 limitations, 109, 303, 392
 linear, 341, 342
 linoleic acid, 227
 lipase, 277
 lipid, 163, 227
 lipid metabolism, 227
 lipids, 227
 lipoxigenase, 227
 liquid chromatography, xv, 362, 370, 377
 liquid nitrogen, 22, 47, 324
 lithium, 325

liver cells, 16
 liver damage, 13, 34
 liver enzymes, 123
 livestock, vii, viii, xii, xiii, xvi, 63, 64, 66, 74, 86,
 92, 93, 99, 102, 107, 251, 254, 267, 268, 279,
 282, 299, 300, 301, 330, 331, 375, 385
 living conditions, 293
 localization, 57, 246, 351, 366
 location, 114, 301
 locomotion, 17
 locus, 295, 388
 long period, 260, 367
 loss of appetite, 150
 losses, xv, 66, 75, 84, 180, 202, 209, 322, 361, 376,
 377
 low-density, 111
 low-density lipoprotein, 111
 LPS, 159
 LTD, 253
 lumen, vii, 1, 5, 10, 11, 12, 16, 17, 20, 114, 120, 125,
 269, 285
 luminal, 18, 284
 luminescence, 344
 lung, 131, 134, 176, 223, 233, 358
 lupus, 174
 lymph, x, xii, 11, 147, 148, 150, 152, 154, 155, 162,
 164, 166, 169, 180, 215, 217, 218, 221, 236, 257,
 258, 260, 381
 lymph node, x, 147, 148, 150, 152, 154, 155, 162,
 164, 166, 169, 180, 215, 217, 218, 221, 258, 381
 lymphadenopathy, xii, 257, 260
 lymphatic, x, 134, 147, 150, 230
 lymphocyte, 163, 216, 228, 230, 231
 lymphocytosis, 131
 lymphoid, 13, 35, 148, 155, 168, 169, 230
 lymphoid organs, 148
 lymphoid tissue, 35, 155, 168, 169, 230
 lymphoreticular, 258

M

macromolecules, 82, 106
 macrophage, 128, 148, 151, 159, 162, 166, 238, 258
 macrophages, x, 18, 22, 35, 61, 133, 134, 147, 148,
 150, 151, 155, 158, 159, 162, 164, 165, 166, 168,
 170, 258, 261, 262, 264, 365
 maintenance, x, 172, 191, 196, 221, 260, 316
 major histocompatibility complex, 166, 169, 387,
 388, 402, 404
 malabsorption, 277
 malabsorptive, 277
 malaria, 72, 100, 108, 111, 130, 133, 403
 malate dehydrogenase, 15

males, xvi, 125, 129, 244, 386, 394, 396
 malic, 21
 malignant, 225
 malnutrition, 120
 mammalian cell, 86
 mammalian genomes, 327
 mammals, xii, 71, 115, 121, 138, 236, 244, 267, 337,
 338, 347, 348, 351, 355, 363, 389, 399
 management, xiii, 34, 41, 64, 66, 99, 110, 142, 193,
 194, 195, 208, 209, 214, 250, 251, 252, 253, 268,
 270, 273, 278, 286, 291, 292, 300, 313
 management practices, 34
 manipulation, xvii, 386, 391
 man-made, 380
 manufacturer, 371
 manufacturing, 92
 manure, 92, 100
 mapping, xvii, 314, 325, 383, 386, 392, 398, 399,
 400, 401, 402
 market, 32, 67, 92, 323
 marketplace, 209
 markets, 68
 mask, 33
 mast cell, 402
 maternal, xvi, 182, 188, 190, 200, 386, 395, 396, 397
 matrix, 153, 155, 161
 maturation, 121, 228
 maturation process, 121
 MCP, 217
 MCP-1, 217
 MDH, 15
 MDR, 85, 337
 measurement, 344
 measures, xiii, 126, 150, 183, 184, 194, 195, 196,
 268, 286, 291
 meat, 46, 64, 92, 136, 209, 377
 media, 22, 41
 median, 150, 269, 282, 396, 397
 mediation, 238
 medication, 26, 58, 132
 medicinal plants, 283
 medicine, xiii, 100, 139, 141, 142, 143, 145, 268,
 269, 280, 283, 321
 Mediterranean, xii, 149, 257, 259, 263, 295, 364
 Mediterranean climate, 364
 Mediterranean countries, xii, 257
 melanoma, 100
 melena, 121, 126
 membranes, 19, 81, 331
 memory, 108
 men, 363
 meninges, 127, 301
 mentoring, 315

- merozoites, 133
mesothelial cells, 135
metabolic, 87, 142, 231, 337, 344, 351, 356, 392
metabolic rate, 142
metabolism, xiv, 26, 27, 29, 75, 94, 100, 106, 227, 306, 335, 336, 339, 351, 354, 355, 356, 357, 358, 359, 360, 392
metabolite, 82, 109, 337, 354
metabolites, xiv, 29, 95, 304, 314, 336, 337, 341, 342, 343, 352, 353, 355, 356, 357, 358
metabolizing, 356
metabotropic glutamate receptor, 326, 329
metastases, 162
metastasis, 223
methylene, 30
metropolitan area, 293
MHC, 388, 389
microarray, 89, 208
microbial, 33, 56
microflora, 31
microorganisms, 26, 58, 180
micro-organisms, 13, 22
microscope, 116, 205
microscopy, 89, 135, 242, 243, 244, 280
microsomes, 340, 344, 346, 347, 348, 349, 350, 352, 353, 360
microtubule, 19, 75, 89
microtubules, 18, 20, 75, 88
migration, 11, 13, 36, 154, 155, 160, 187, 227, 251, 303, 366
milk, x, 92, 119, 171, 173, 175, 179, 189, 202, 209, 366, 367, 377, 383
mining, xiv, 321, 379
MIP, 217
miracidium, 338
mirror, 64, 305, 311
mites, 115, 117, 118
mitochondria, 18, 138, 344, 347, 348, 352, 353, 354, 355
mitochondrial, 82, 85, 87, 95, 133, 140, 340
mitochondrial membrane, 87
mitogen, 216
mitogenic, 220
mitosis, 76
mitotic, 89
mixing, 247, 346
modalities, 69, 71, 73
modality, 71
model system, 388, 391, 393
models, vi, xvi, 151, 155, 157, 158, 179, 200, 216, 229, 277, 385, 387, 388, 391, 392, 393, 397, 398, 399, 401
modulation, 89, 99, 187
moieties, 223
moisture, 246
mold, 127
molecular biology, 367, 381, 382
molecular mass, xv, 362, 372
molecular mimicry, 69
molecular weight, xv, 223, 362, 371, 372, 377
molting, 120, 125
monkeys, 42
monoamine, 337
monoamine oxidase, 337
monoclonal antibodies, 92, 161, 280
monoclonal antibody, 135, 155
monocyte, 151, 162, 168, 264
monocytes, 18, 148, 151, 168
monolayers, 289
monomer, 76
mononuclear cell, 167, 168, 235
moratorium, 302
morbidity, 10, 28, 31, 32, 34, 130, 133, 386
morphogenesis, 221
morphological, 18, 234, 242, 253, 261, 269
morphology, vii, 3, 37, 249, 283, 289, 380
morphometric, 401
mortality, viii, xiii, 2, 4, 7, 9, 10, 11, 14, 28, 30, 32, 33, 34, 56, 67, 120, 130, 131, 133, 134, 137, 203, 299, 311, 389
mortality rate, viii, 2, 7, 10, 11, 14, 32, 33, 120, 137
mosquitoes, 130, 131, 139
mothers, 177
moths, 251
motion, 399
mouse model, 70, 72, 387, 391, 392, 393
mouth, 119, 125
movement, 17, 64, 83, 89, 280, 302, 315
mRNA, 49, 153, 156, 157, 158, 159, 215, 218, 223, 224, 225, 226, 329
mucin, xi, 208, 214, 223, 224, 226, 229, 231, 235, 237, 238
mucoid, 278
mucosa, vii, 1, 5, 12, 15, 16, 36, 120, 121, 125, 126, 135, 136, 216, 233, 248, 258, 269, 282, 285, 364, 365
mucous membrane, 249
mucus, 223, 224
mucus hypersecretion, 224
multidrug resistance, 83, 85
multiplication, 11, 19, 23, 58, 160
murine model, 111, 155, 158, 216, 229, 277, 388
muscle, 11, 71, 76, 78, 80, 81, 104, 107, 112, 173
muscle cells, 71, 76, 81
muscle contraction, 81
muscles, 83, 139

mutagenic, 26, 27, 28
 mutant, 78, 393, 398
 mutation, 80, 88, 89, 102
 mutations, ix, 88, 89, 90, 105, 114, 282, 391, 392, 394
 MVA, 74
 mycobacterial infection, 101, 237
 myeloid cells, 155
 myeloperoxidase, 357
 myocarditis, 139, 176
 myosin, 296
 myositis, 199

N

N-acety, 224, 226
 NADH, 340
 National Academy of Sciences, 99, 109
 National Research Council, 161
 natural killer cell, 227
 necrosis, 12, 13, 15, 84, 123, 127, 133, 134, 135, 138, 164, 176
 neonatal, 289
 neonate, 120, 306
 nephritis, 139
 nerve, 301
 nervous system, 76, 80, 322
 nested PCR, 46
 neuralgia, 150
 neuraminidase, 91
 neurons, 80
 neurotransmission, 97
 neutralization, 220
 neutrophil, 227
 neutrophils, 13
 nitrate, 115
 nitric oxide, 13, 151, 165, 166
 nitric oxide synthase, 151
 nitrogen, 22, 47, 158, 324, 336
 nodes, 150, 218
 non-infectious, 149
 norepinephrine, 357
 normal, 16, 35, 49, 116, 152, 169, 177, 179, 222, 224, 301, 304, 306, 387, 391
 nuclear, 131
 nuclei, 340
 nucleic acid, 45, 82
 nucleus, 17, 18, 19
 nutrient, 246, 283
 nutrients, 18
 nymphs, 324

O

oat, 212, 213
 obligate, 38, 172
 observations, vii, 13, 14, 20, 38, 42, 44, 47, 54, 59, 60, 83, 249, 305, 307, 308, 310, 314, 315
 odds ratio, 177, 179, 191
 oedema, 11
 oil, 25, 33, 228
 olfactory, 326
 oligodeoxynucleotides, 103
 oligosaccharides, 223, 237
 omega-3, 92, 227
 onchocerciasis, 79
 oocyte, 20
 oocytes, 20, 77
 operator, 311
 optical, 308
 optical density, 308
 optics, 343
 optimization, 307, 309
 oral, 7, 8, 30, 36, 50, 56, 72, 94, 105, 119, 125, 136, 244, 249, 252, 269, 270, 285, 290
 oral cavity, 119, 125
 oral transmission, 7, 56
 orchestration, 398
 orchitis, 261
 organ, 11
 organelles, 14, 18, 75
 organic, 5, 45, 209, 344, 346
 organic compounds, 45
 organic solvent, 344, 346
 organism, xii, xiv, xvii, 3, 5, 14, 17, 20, 23, 41, 48, 69, 70, 76, 219, 257, 258, 314, 327, 328, 335, 336, 386
 orientation, 17, 79
 ornithine, 86
 oropharynx, 119
 osteomyelitis, 266
 ovaries, 5, 20, 324, 325
 ovary, 5, 324, 329
 oxidation, 336, 344, 347, 348, 354, 355, 356
 oxidative, 82, 85, 95, 337, 348, 357, 359
 oxide, 13, 151, 165, 166, 344
 oxides, 337
 oxygen, 16, 23, 130, 158
 oysters, 140
 ozone, 285

P

palpation, 175

- pancreas, 13, 134, 135, 338
pancreatitis, 139
paradox, 386
paraffin-embedded, 57, 135, 153, 176, 266
paralysis, 31, 77, 79, 82, 83, 99, 249
parameter, 313
parasitaemia, 91, 131, 132, 205
parasitemia, 250
parasitic diseases, ix, xvii, 66, 70, 75, 96, 113, 114, 116, 139, 255, 317, 377, 386, 388, 393, 394, 399
parasitic infection, xv, xvi, 69, 71, 72, 215, 253, 361, 385, 387, 388, 389, 390, 391, 401, 404
parasitic infestation, 84
parasitosis, xiv, 335, 364, 395, 397, 399
parenchyma, 133, 364, 366, 376
parenteral, 260
particles, 18, 23, 223, 342
passive, 218, 233, 378
pastoral, 64, 65, 96
pasture, 64, 217, 230, 244, 246, 250, 251, 252, 366
pastures, 194, 231, 247, 250, 251, 364, 368
pathogenesis, 15, 21, 24, 37, 44, 46, 149, 276, 290, 392, 394
pathogenic, 5, 24, 36, 83, 119, 132, 249, 300
pathogens, 67, 69, 71, 121, 164, 215, 218, 223, 240, 307, 389
pathology, 2, 9, 38, 44, 53, 62, 143, 240, 246, 247, 359, 365, 388, 389, 391
pathways, xi, xvii, 165, 208, 214, 220, 223, 225, 226, 227, 229, 234, 325, 357, 386, 394, 399
patients, 83, 92, 103, 111, 136, 144, 222, 235, 263, 277, 278, 279, 281, 318
PBMC, 156, 159, 160, 167
PCR, 22, 38, 45, 46, 48, 55, 56, 90, 99, 116, 135, 139, 152, 153, 167, 169, 176, 177, 198, 201, 221, 222, 226, 260, 262, 271, 272, 274, 279, 281, 287, 295, 302
pedigree, 217
penguins, 125, 130, 131
penicillin, 31, 340
penis, 260, 261, 266
pentamidine, 96
peptide, 69, 223, 329, 331, 332
peptides, 112
perception, 278, 292
perchlorate, 346
pericardial, 132
peripheral blood, 91, 131, 151, 157, 162, 167, 168, 264
peripheral blood lymphocytes, 157
peripheral blood mononuclear cell, 167, 168
peristalsis, 4, 7, 9, 280
peritoneal, 151, 164, 301, 376
peritoneal cavity, 301, 376
peritoneum, 16, 364
peritonitis, 13, 301
peri-urban, 259
permeability, 16, 81, 277, 289
permeable membrane, 81
permit, 8, 302, 399
peroxide, 106
personal communication, 209
perturbation, 323
perturbations, 214, 229
pesticide, 322, 329
pesticides, 322
P-glycoprotein, 80, 102
phage, 329, 331
phagocyte, 35, 153, 154, 155, 160, 163
phagocytic, 154
phagocytosis, 17, 18, 133, 151, 162, 220
pharmaceutical industry, 43, 329
pharmaceuticals, viii, 26, 27, 34, 63, 323
pharmacists, 68
pharmacokinetic, 337
pharmacological, 77, 85, 394
pharmacology, 102
pharmacotherapy, xiv, 335, 338, 339
pharynx, 79, 83, 99
phenol, 115
phenotype, xvii, 87, 160, 386, 389, 392, 399
phenotypic, 83, 85, 394
pheromone, 326
phosphate, 73, 112, 325, 340, 341, 342, 345, 346, 370
phospholipids, 227
phosphorus, 92, 100
phosphorylation, 82, 95
phylogenetic, 141
phylogeny, 39
phylum, 137
physico-chemical properties, 337
physiological, 7, 75, 78, 79, 226, 306, 337, 339, 392
physiology, 222, 224, 392
phytotherapy, 32
pig, 65, 78, 211, 212, 359
pigments, 133
pigs, 64, 65, 66, 100, 110, 112, 270, 278, 281, 287, 292, 363, 403
placenta, 176, 186, 188, 189, 190, 199, 262
placental, 187, 188, 190, 193
plantar, 118
plants, 85, 283, 351, 363
plaques, 134
plasma cells, 35, 133, 153
plasma levels, 233

- plasma membrane, 86, 88
plasmid, 101, 109, 111, 112
plasmids, 103
plastic, 340, 341
platyhelminthes, 123, 338
play, 6, 13, 67, 76, 90, 119, 131, 151, 215, 219, 223, 224, 229, 303, 336, 347, 388, 398
plexus, 153
ploidy, 288
PMSF, 369
pneumonia, 128, 134
polarization, 215
politics, 91
polyamine, 86, 94, 100
polycyclic aromatic hydrocarbon, 358
polyethylene, 339
polygenes, 388
polymerase, 162, 165, 271, 274, 279, 302, 317
polymerase chain reaction, 162, 165, 271, 274, 279, 302, 317
polymerization, 84, 88, 282
polymorphism, 88, 287, 302, 317
polypeptides, 77, 372, 374
polysaccharides, 55
polyunsaturated fat, 227, 235
polyunsaturated fatty acids, 227, 235
poor, viii, 10, 23, 63, 83, 114, 120, 284, 303, 304, 305
population, xvii, 39, 47, 64, 66, 69, 74, 78, 84, 87, 106, 140, 149, 155, 194, 198, 201, 215, 217, 250, 252, 259, 310, 311, 313, 318, 319, 320, 339, 386, 387, 390, 394
population density, 47
pork, 107
portal vein, 12, 13, 35
positive correlation, 156, 158
postmortem, 304, 310
post-translational, 224
posture, 10
potassium, 342
poultry, viii, xi, 2, 4, 6, 23, 28, 33, 38, 43, 44, 49, 50, 52, 53, 54, 56, 61, 64, 90, 100, 109, 110, 121, 123, 126, 130, 133, 143, 239
powder, 23, 98
power, 325
precipitation, 325, 364, 368
pre-clinical, 394
predators, 6
predisposing factors, 114
pre-existing, 74
preference, 71, 339
pregnancy, 173, 175, 177, 178, 179, 180, 183, 184, 185, 186, 187, 188, 190, 191, 193, 196, 200, 201, 202, 203, 204, 205, 206
pregnancy test, 205
pregnant, 177, 179, 184, 188, 190, 197, 200, 202, 206
prepuce, 260, 261
pressure, 274, 278, 279, 282, 285, 286, 389
prevention of infection, xiii, 268, 286
preventive, 31, 38, 42, 59, 62, 250, 284
prices, 64
primaquine, 132
priming, 157, 164, 169
privatization, 68
probability, 78, 177, 179, 302, 390, 396
probe, 22, 293, 359, 366
prodrugs, 26, 27, 29, 75
producers, 209, 322
productive efficiency, 208
productivity, xi, 65, 75, 84, 239, 248, 250, 390
progenitors, 395
progeny, 178, 196, 206
progesterone, 187, 188, 189, 190
program, 195, 285, 322
proinflammatory, 238
pro-inflammatory, 36, 218
prolactin, 16, 39
proliferation, x, 86, 100, 135, 147, 163, 216, 219, 231, 236, 338, 368
promoter, 74, 90, 152, 222
promoter region, 152, 222
prophylactic, viii, 2, 29, 30, 31, 32, 33, 34, 37, 41, 42, 44, 54, 58, 59, 67, 68, 93
prophylaxis, 91, 290
prostaglandin, 326
prostaglandins, 227, 336
proteases, 223, 277, 304
protection, 28, 30, 35, 37, 70, 71, 72, 96, 98, 100, 103, 111, 191, 195, 214, 223, 284, 390
protective role, 160, 354, 388
protein family, 323
protein synthesis, 31, 82, 109, 283
proteinase, 188, 204, 206, 379
proteolytic enzyme, 13
proteomes, 328
proteomics, 89
protocol, 73, 284, 315, 325, 340, 367, 370
protocols, 136, 166, 285, 302, 329
protozoan parasites, 274, 283, 296
pseudo, 76
pseudocoel, 5, 20
Pseudomonas aeruginosa, 119, 143
pseudopodia, 17

public, vii, xiii, 74, 142, 144, 149, 259, 268, 293
 public health, vii, xiii, 74, 144, 149, 259, 268, 293
 pulmonary edema, 132
 pumping, 79
 purification, xv, xvi, 362, 377
 pyruvate, 18

Q

quail, 42, 50
 quality assurance, 306, 309
 quality of life, 323
 quarantine, 252, 308, 315, 322
 quaternary ammonium, 286
 query, 328
 quinacrine, 281
 quinone, 46
 quinones, 337

R

race, 84, 112, 399
 radiation, 25, 57, 97
 radio, 36, 76, 311
 radiolabeled, 293
 rain, 259
 rain forest, 259
 rainfall, 364
 random, 329
 rat, 95, 224, 238, 289, 290, 344, 345, 347, 356, 358, 359, 360
 rats, 64, 224, 228, 233, 238, 283, 357, 381, 391
 reactants, 367
 reactive oxygen, 158
 reactivity, 183, 303, 305, 382
 reading, 327, 328, 329
 reagent, 305, 308, 309
 reagents, 304, 307, 308, 309
 reality, 398
 receptors, vi, xiii, 71, 76, 78, 79, 81, 108, 151, 160, 195, 217, 293, 304, 321, 323, 326, 329, 330, 331, 332
 reciprocal cross, xvi, 386, 395, 396
 recognition, 77, 219, 223, 229, 237, 320, 390, 403
 recovery, 6, 12, 15, 16, 34, 144, 302, 310, 311
 rectal temperature, 324
 rectum, 175
 red blood cell, 133
 redistribution, 75, 81
 reductases, xiv, 336, 345, 349, 357
 refining, 70
 reflection, 252

refractory, 82, 104, 141
 regeneration, 26, 55
 regional, 195
 regression, 177, 179, 188
 regular, 68, 251, 286, 307
 regulation, 90, 215, 218, 221, 224, 225, 226, 227, 235, 388, 401, 402
 rejection, 188, 189, 190
 relapse, 26, 28, 29
 relationship, xvi, 3, 20, 38, 81, 125, 138, 154, 181, 196, 197, 200, 201, 203, 225, 385, 388, 390, 391, 393, 397, 398, 400, 404
 relationships, vii, 43, 206, 225, 235, 318, 391, 398, 401
 relaxation, 81
 relevance, xiii, 153, 214, 268, 269, 278, 286
 reliability, 69
 remission, 26
 remodeling, 164
 renal, 135, 137, 138, 260, 337
 renal failure, 260
 repair, 12, 15, 219
 repeatability, xiii, 300, 307, 308, 309, 311
 replication, 74, 85, 99, 108, 151, 187, 258
 reproduction, 269, 338
 reproductive organs, 5
 research and development, 92, 93
 reservoir, ix, xii, 134, 147, 257, 259, 264, 268, 296
 resilience, 390, 398
 resources, viii, xvii, 63, 64, 92, 107, 328, 386, 399, 400
 respiration, 18, 98
 respiratory, 127, 128, 129, 130, 137, 237, 301
 respiratory problems, 128
 responsiveness, 387
 restriction fragment length polymorphis, 287
 retardation, xii, 10, 29, 31, 56, 267
 retention, 5, 52, 342, 354
 reticulum, 81
 retinoic acid, 227
 rheumatoid arthritis, 222, 235
 rheumatoid factor, 382
 ribosomal, 48, 49, 73, 101, 139, 235
 ribosomal RNA, 49, 139
 rice, 14, 23, 25
 risk factors, 163, 177, 199, 288, 291, 292
 risk perception, 292
 risks, viii, 2, 28, 34, 68, 93, 193
 RNA, 17, 18, 222, 226, 324, 325
 robustness, 308
 rocky, 316
 rodent, 391
 rolling, 227

room temperature, 17, 36, 325
 roundworms, ix, 113, 125
 ruminant, 101, 214, 277, 294, 375
 rural, viii, 63, 64, 66, 109, 148, 149, 167, 259, 290, 382
 rural areas, 167, 259, 290, 382

S

safeguard, 141
 safety, 26, 53, 282, 283
 saline, 115, 116, 325, 339, 340
 saliva, 92, 130, 262, 264
 salivary glands, 324
 salt, 135, 269
 sample, 22, 116, 186, 219, 279, 309, 342, 343, 370
 sampling error, 219
 sanctuaries, 84
 sand, ix, xii, 147, 148, 149, 168, 257, 258, 259, 262, 264
 saponin, 25, 32, 164
 savannah, 110
 scabies, 118
 scanning electron microscopy, 242, 243
 scar tissue, 15
 schistosomiasis, 71, 81, 95, 110, 318, 391, 400
 Schmid, 98, 102, 104
 SCS, 21
 SDS, xv, 362, 370, 371
 SEA, 316, 382
 search, 37, 64, 94, 314, 327
 searches, 323
 searching, 233, 325, 367
 seasonal pattern, 131
 secrete, 17, 18, 35, 304
 secretion, 11, 190, 215, 224, 229, 238, 306, 370
 sedentary, 64
 sediment, 340
 sedimentation, xv, 115, 361, 362, 366, 368, 369
 seed, 25, 32, 33
 segregation, 89
 selecting, 82
 self-mutilation, 120
 SEM, 185, 222, 226
 semen, x, xii, 172, 173, 189, 190, 192, 194, 195, 196, 200, 202, 203, 258, 260, 261, 264
 semi-arid, 65
 sensitivity, xiii, 46, 68, 78, 80, 82, 85, 90, 97, 114, 120, 176, 270, 279, 280, 281, 290, 300, 303, 304, 305, 307, 309, 310, 311, 313, 366, 376
 separation, 148, 345
 sequencing, 133, 134, 138, 139, 225, 263, 327
 series, 154
 serology, 165, 167, 169, 182
 serotonin, 323, 330
 serum, xiii, 15, 16, 22, 23, 40, 48, 50, 86, 92, 97, 151, 156, 164, 206, 215, 227, 232, 300, 303, 307, 308, 309, 310, 314, 316, 367, 369, 380, 381
 serum albumin, 92
 severity, 11, 14, 15, 83, 150, 153, 205, 208
 sexual reproduction, 269
 shape, 11, 17, 219, 243, 269
 shares, 85, 221
 sharing, 260, 396, 397
 shelter, 276, 293, 295
 shock, 72, 324
 short supply, 227
 sialic acid, 91, 110
 side effects, 33, 67, 80
 signal transduction, 329
 signaling, 215, 218, 222, 223, 227, 228, 236, 392, 400
 signaling pathway, 218, 223, 400
 signs, xii, xiii, 6, 10, 11, 30, 120, 121, 124, 133, 137, 148, 149, 150, 153, 175, 179, 248, 249, 257, 267, 294, 300, 301, 310, 311, 315, 398
 similarity, xvi, 20, 305, 326, 327, 328, 330, 362, 366, 376, 377
 single nucleotide polymorphism, 152
 sinuses, 119, 301
 sites, xi, 35, 75, 78, 81, 104, 152, 155, 160, 207, 208, 209, 220, 221, 225, 323, 332
 skeletal muscle, 203
 skin, x, 10, 115, 118, 120, 127, 141, 147, 148, 150, 151, 152, 153, 154, 155, 160, 163, 164, 166, 169, 213, 279, 291
 skin diseases, 291
 SLA, 158
 sleeping sickness, 86
 small intestine, xi, 120, 124, 125, 126, 134, 207, 208, 216, 217, 220, 221, 224, 226, 229, 234, 269, 277, 285, 365
 SNAP, 281
 SNP, 152
 115, 322, 332, 340, 341, 346, 370, 371
 software, 313, 327, 342, 343, 344, 350
 soil, 8, 10, 33, 49, 51, 52, 114, 285, 294
 solid phase, 342
 solvent, 341, 343
 sorting, 219
 sounds, 130
 spastic, 77
 spatial, 68
 specificity, xiii, 46, 140, 176, 213, 230, 269, 270, 300, 304, 305, 307, 308, 309, 310, 311, 313, 376
 spectroscopy, 76

- spectrum, 66, 75, 89, 130, 154, 163, 209, 282, 283, 339, 354, 387, 398, 399
- spermatids, 5
- spermidine, 85, 98
- spinal cord, 176, 301
- spleen, x, 13, 133, 134, 139, 147, 148, 150, 152, 154, 155, 156, 165, 169, 180, 258
- splenomegaly, xii, 257, 260
- sporadic, 205
- spore, 134, 136
- standard deviation, 308, 369
- standard error, 396, 397
- standardization, 307, 368
- standards, 34, 40, 342, 344
- starch, 14, 17, 23
- starch granules, 17, 23
- starvation, 79
- stasis, 248
- steady state, 76
- steatorrhea, 277
- stem cells, 392
- sterile, 306, 325, 339
- steroids, 336, 381
- stimulus, 364
- stock, xvi, 65, 247, 343, 386, 393, 395, 396, 404
- stoichiometry, 77, 79
- stomach, 232, 233, 240
- strain, 5, 11, 14, 24, 33, 34, 36, 39, 41, 84, 108, 136, 258, 284, 289, 292, 324, 333, 354, 388, 393, 397, 400
- strains, 21, 24, 36, 38, 55, 60, 84, 94, 105, 119, 148, 236, 252, 283, 284, 324, 387, 388, 392, 393, 395, 398, 399, 400
- S-transferase (GST), 346, 350
- strategies, xi, 69, 84, 195, 201, 208, 209, 214, 215, 390, 399
- streams, 363
- strength, 219
- stress, 14, 85, 120, 133, 189, 248
- structural changes, 168
- structural characteristics, 20
- structural protein, 75
- subcutaneous injection, 252, 285
- submucosa, 11, 12, 15, 35
- subsidies, 64
- substances, 22, 33, 337
- substitution, 79, 149
- substrates, xiv, 84, 89, 335, 336, 337, 345, 347, 349, 350
- success rate, 8
- sucrose, 280
- suffering, 86, 189, 191
- sugar, 115
- sulfate, 39, 115
- sulfur, vii, 1, 60
- sulphate, 51, 280, 288, 291, 371
- sulphur, 10, 21, 144, 336
- summer, 364
- superiority, 304
- supernatant, 158, 159, 340, 342, 346
- supplements, 228
- suppliers, 23, 68
- supply, viii, 63, 64, 65, 142
- suppression, 114, 148, 162, 216, 231, 377
- surface water, 138
- surfactant, 232
- surgery, 92, 141Sry, 143, 253
- surgical, 120
- surplus, 22
- surveillance, 175, 176, 182
- survival, 39, 44, 51, 52, 56, 83, 137, 162, 168, 205, 209, 233, 254, 294, 304, 323, 376, 386
- surviving, 88
- survivors, 35
- suspensions, 23, 48
- swelling, 92, 242, 243, 244
- switching, 133
- symptom, 2, 160
- synergistic, 82
- synthesis, 31, 82, 85, 86, 107, 109, 283, 314, 324, 358
- systematics, vii, 144, 234
- systems, 20, 34, 64, 65, 76, 84, 106, 214, 219, 224, 273, 278, 291, 325, 379, 392

T

- T cell, 61, 74, 100, 157, 159, 165, 166, 169, 215, 218, 224, 233, 236, 276, 388
- T lymphocyte, 232, 296, 387
- tapeworm, 124
- target organs, 152
- targets, vi, xiii, 26, 60, 165, 321, 322, 323, 329, 332, 379
- taxonomic, 3
- taxonomy, vii, 2, 17, 144, 213, 269, 296, 380
- T-cell, x, 100, 101, 148
- technical assistance, 229, 315
- technician, 186
- technology, 70, 72, 306
- TEM, 135
- temperature, 4, 7, 17, 23, 142, 343, 365, 376, 379
- temporal, 68, 234
- testis, 261
- tetracycline, 345, 360
- TGF, 156, 216

therapeutic agents, 67, 68, 132
 therapeutic approaches, ix, 113
 therapeutics, 21, 26, 32
 therapy, 81, 122, 132, 281
 threat, ix, 114
 three-dimensional model, 383
 threshold, 176, 250, 398
 thrombin, 92
 thromboembolism, 91
 thymidine, 85
 thymus, 13, 180, 220, 224, 232
 ticks, xiv, 66, 321, 322, 323, 324, 327, 330, 332
 tight junction, 289
 timing, 58
 tissue, x, 5, 12, 15, 17, 18, 21, 22, 35, 50, 57, 75, 92,
 116, 118, 127, 133, 147, 153, 154, 155, 163, 167,
 168, 173, 176, 177, 201, 203, 219, 221, 224, 240,
 250, 301, 303, 338, 340, 365
 titration, x, 172, 176, 181, 182, 187, 188, 193, 196,
 309, 369
 T-lymphocytes, 277
 TNF, 151, 153, 155, 156, 158, 159
 tolerance, 91, 390, 398, 403
 tonsils, 11, 36, 58
 topology, 327, 331
 toxic effect, 29, 31, 350
 toxic products, 277, 338
 toxic side effect, 33
 toxicity, 26, 30, 50, 51, 58, 62, 75, 78, 80, 81, 82, 86,
 209, 282, 333, 337, 347
 toxins, 277
 toxoplasmosis, 72
 trachea, 127, 129, 130, 319
 trade, 229, 308
 traits, 390, 398, 399, 401, 402
 transcription factor, 152, 156, 400
 transcripts, 157
 transfer, 27, 85, 104, 112, 195, 198
 transforming growth factor, 237
 transfusion, 260, 265
 transfusions, 265
 transgenesis, xvii, 91, 386, 391, 392
 transgenic, 90, 92, 102, 107, 399
 transition, 93
 translation, 327, 366
 translocation, xiii, 299, 300, 319
 transmembrane, 327
 transmission electron microscopy, 135
 transparent, 338
 transport, 36, 75, 85, 86, 87, 104, 109, 130, 166, 217,
 251, 280
 transportation, 58
 trauma, 120

trial, 33, 70, 92, 109, 278, 285, 297
 trichloroacetic acid, 346
 trichostatin, 74
 triggers, 158
 trophoblast, 206
 tropism, xii, 153, 257, 260, 261
 troubleshooting, 308
 trypanosomiasis, 67, 96, 97, 99, 100, 103, 106, 109,
 402
 TSA, 70
 TSP1, 70
 tuberculosis, 112, 218, 233
 tumor necrosis factor, 164
 tumorigenesis, 223
 tumour, 85, 238
 turnover, 194

U

UGT, 337, 349, 350, 351
 ulceration, 150
 ultrasonography, 175
 ultrastructure, 3
 underlying mechanisms, 226, 388, 390
 UNDP, 169
 unification, 330
 uniform, 154
 urban areas, 64, 259, 264
 urbanization, 148
 urea, 29, 40
 urine, 169
 uterus, 5, 262
 uveitis, 150

V

vaccine, 21, 36, 37, 69, 70, 71, 74, 94, 95, 98, 99,
 100, 101, 102, 107, 108, 109, 112, 164, 214, 223,
 284, 287, 294, 295, 367, 379, 393
 vacuole, 14, 85
 validation, 309, 315
 validity, 308, 313
 values, 79, 83, 184, 185, 186, 308, 309, 310, 313,
 317, 325, 327, 343, 367, 369, 376, 396
 variability, 107, 209, 311, 387, 391
 variables, xvi, 179, 386, 396
 variance, 184, 397
 variation, xvi, 65, 69, 77, 140, 270, 286, 307, 308,
 320, 385, 387, 390, 395, 396, 400
 vas deferens, 20
 vasculature, 123

vector, xii, 6, 20, 69, 70, 73, 74, 98, 106, 108, 109,
148, 162, 257, 258, 259, 260, 262, 263, 285
vegetation, 363
vehicles, 302, 363
velocity, 346, 350
ventilation, 120
verapamil, 85, 104
vertebrates, 77, 80, 134, 212, 316, 389
vesicle, 75
vessels, 13, 301
veterinarians, ix, 114, 129
veterinary medicine, xii, 3, 267, 268, 278, 281, 282,
308, 366
village, 64, 65
villus, 276, 277
viral promoter, 74
virulence, viii, 2, 5, 11, 14, 21, 24, 33, 43, 60, 277,
287, 399
virus infection, 91, 98, 203, 218
viruses, ix, 74, 114, 117
visible, 114, 115, 129, 131, 132
visualization, 135
vitamin A, 34, 62
vitamin E, 34
vitamins, 25, 57
vomeronasal, 326
vomiting, 126, 131
vortex, 341
vulva, 243

W

war, 400
warrants, xiii, 268
water, 11, 58, 115, 119, 121, 122, 136, 138, 142,
173, 268, 277, 285, 294, 295, 339, 341, 342, 343,
345, 346, 363
waterfowl, 130, 132, 133, 134, 138, 140, 144
watersheds, 121
wavelengths, 344
weakness, 10, 31, 120, 365
weapons, 400

weight gain, 26, 28, 31, 32, 277, 278, 286
weight loss, 10, 118, 119, 122, 123, 124, 125, 126,
139, 150, 249, 287, 338
welfare, 91
well-being, 188, 189, 390
white blood cell count, 135
WHO, 148
wild animals, 173, 301, 338
wildlife, vii, 173, 174, 195, 197, 300, 302
wild-type allele, 89
winter, 6, 131, 247
withdrawal, 26, 28, 29, 40, 283
women, 260
wood, 127
wool, 65
workers, 303, 308
wound repair, 219

X

xenobiotic, 336, 337, 338, 350
xenobiotics, vi, xiv, 335, 336, 337, 347, 348, 350,
351, 355, 356, 358, 360
xenotransplantation, 92

Y

yeast, 22, 25, 120
yield, x, 61, 171, 179
young adults, 246

Z

zinc, 115, 280
zinc sulfate, 115
zoonosis, 161, 265
zoonotic, ix, xii, xiii, 66, 114, 136, 138, 149, 173,
257, 258, 267, 268, 269, 279, 292, 295, 296
zygotes, 392