BIOLOGY OF SELECT ZOONOTIC PROTOZOAN INFECTIONS OF DOMESTIC ANIMALS

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Keywords: protozoa, flagellates, apicomplexa, *Cryptosporidium*, *Eimeria*, *Theileria*, *Giardia*, African trypanosomes, epidemiology, diagnosis, treatment, life cycle, immune response, pathology, zoonosis.

Contents

Summary

In this overview, the basic biology of select protozoan parasites of veterinary importance was covered. These include the Apicomplexans, *Eimeria*, *Theileria*, *Cryptosporidium*, and the Flagellates *Giardia* and African trypanosomes. Available information was synthesized into a relatively simple information guide of potential interest to a wide reading audience. Where appropriate, references are made to human disease and zoonoses, but the main focus is on the biology of these infections in domestic animals. A more detailed and comprehensive treatise on different aspects of the biology of these protozoan infections can be obtained from several scientific review articles which have been listed and annotated in the bibliography section of this article.

1. Introduction

Protozoan parasitic infections of domestic animals have a significant economic impact on agriculture. These infections often results in serious losses due to mortality, reduction in protein and milk quality, emaciation and in some cases reduced reproductive rate. Some of the diseases caused by these parasites have been neglected allowing persistence and re-emergence. Inadequate diagnostic methods, lack of inexpensive and effective drugs and lack of vaccines to control outbreaks have also contributed to persistence of these infections in livestock. In some cases, the available drugs are too old, inefficient and toxic with severe side effects, and the emergence of drug resistant parasites has become a serious problem. Thus, there is an urgent need for development of new drugs and vaccines for control of these infections. In this overview,

we provide information about the epidemiology, basic biology, diagnosis and control of select protozoa of agricultural importance. We focus on select protozoa belonging to the two major groups, the apicomplexans and the flagellates.

2. The Apicomplexa

Apicomplexans are obligate intracellular protozoan parasites that are responsible for numerous diseases in humans and economically important livestock. Members of the Apicomplexa, which contains over 5000 species, are the *Plasmodium* species, which cause malaria that results in significant morbidity and mortality in the developing countries, *Cryptosporidium* an enteric pathogen of animals and humans well known for causing waterborne outbreaks of enteric disease, *Eimeria* species that infect chickens and cattle, and *Neospora* and *Theileria* parasites, which are important veterinary pathogens. This overview will focus mostly on apicomplexan parasites that infect domestic animals, although on occasion, reference will be made to human disease where appropriate since the biology of the parasites and pathology of the diseases they cause are similar.

2.1. Eimeria

2.1.1. Epidemiology

Eimeria spp. cause disease called coccidiosis that is responsible for severe enteritis in poultry and can result in death of the susceptible hosts. Seven species of this obligate intracellular protozoan parasite have been identified in chickens: *E. tenella*, *E. maxima*, *E. acervulina*, *E. brunetti*, *E. mitis*, *E. necatrix*, and *E. praecox*, of which the first three are believed to be the principle species responsible for majority of infections in poultry. However, other species of *Eimeria* have been found to infect cattle, sheep, rabbits, mink and pika. It has been estimated that at least $2.4 billion dollars per year are lost throughout the world due to coccidiosis, and this infection continues to impose a great economic burden on poultry farming. This huge economic loss is the result of (1) the weight loss, and in some cases death of poultry due to either the infection and/or the methods used to control the infection, (2) the continued cost associated with the need for research and development of new prophylactic drugs due to the emergence of drug resistance in *Eimeria* and (3) the relatively high cost associated with the production of live vaccines.

2.1.2. Biology and Life Cycle

The infectious stage in the life cycle of *Eimeria* spp. are environmentally resistant oocysts that are released in the feces of the infected host and once released into the environment the oocysts are initially non-sporulated and non-infective. After incubation for one to two days outside the host, the parasites undergo asexual reproduction called sporogony and the oocysts became sporulated because they now contain infectious sporozoites. The time from initial exposure of the host to the release of oocysts is referred to as the prepatent period, and is usually 4-7 days depending on the *Eimeria* species. The ingestion of sporulated oocysts will cause an infection in the naïve host. Upon ingestion, the oocysts undergo excystment which is believed to be mechanical

rather than chemical disruption of the oocyst wall that results in the release of the motile and infectious sporozoites which actively penetrate the intestinal epithelium. The exact site of intestinal epithelium invasion varies between species of *Eimeria*, and may also vary within a species depending on the age of the host. In the case of *E. tenella*, the sporozoites invade the caeca, whereas *E. acervulina* invades the duodenum of the small intestine. It is well established that apicomplexans such as *Plasmodium* invade the host cells using a specialized structure called the "glideosome" complex via a calcium dependent mechanism. Recently, the invasion of epithelial cells by *E. tenella* sporozoites was also shown to be calcium dependent and involves the utilization of lipid rafts and serine proteases that are believed to be involved in the secretion of microneme proteins. The microneme proteins EtMIC4 and EtMIC5 form complexes that in turn interact with the host cell ligands/receptors. It has also been demonstrated that *E. tenella* can adhere to chicken duodenal mucins which interfere with host cell invasion, suggesting a possible infection blocking strategy that requires further study.

Once intracellular, the sporozoites of *Eimeria* transform into trophozoites and undergo asexual reproduction called schizogony. The end result of schizogony are infectious merozoites which are initially released into the lumen of the small intestine after which they promptly reinvade the nearby columnar epithelial cells. Depending on the species of *Eimeria*, two to five additional schizogony cycles occur during the infection, increasing the number of infected epithelial cells and causing significant pathology in the host. Like the sporozoite stage of the parasite, the merozoites invade the epithelial cells using a gliding movement and secretion of specialized parasite-derived microneme proteins.

The development of the parasites inside epithelial cells has been documented using a fluorescent dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) employing an *in vitro* assay. After schizogony, the sexual phase of the life cycle is initiated where the merozoites differentiate into microgametocytes and macrogametocytes. The microgametocutes and macrogametocytes combine to form a zygote and eventually the zygote differentiates into an environmentally resistant oocyst that is shed in the feces.

The discovery and characterization of *Eimeria* strains with reduced asexual proliferation capacity in the gut due to early maturity (pre-cociousness strains) has become a useful tool for the development of vaccines against this infection. The analysis of the genetic linkage map of *E. tenella* identified a linkage group on chromosome 2 of the parasite that encodes for the traits of precocious development.

2.1.3. Host Defense and Pathology

Upon infection with *Eimeria*in chickens, the hosts develop characteristic intestinal lesions, anorexia, and weight loss due to malabsorption of nutrients. In more severe infections, severe hemorrhage in the intestine and mortality of poultry, have also been observed. Most of the pathologenesis is due to the columnar epithelial cell damage caused by the proliferation and reinvasion by the merozoite stage of the parasite. The parasite has also evolved immune evasion mechanisms which allow it to prolong its stay in the host that results in even greater pathology. For example, studies have identified a macrophage migration inhibitory factor (MIF) produced by *E. acervulina* and *E. tenella*

merozoites, which may suppress T-cell activation and promote anti-inflammatory responses.

The host mucosal immune response towards the parasite has been measured in terms of cytokine expression, antibody response, and cell mediated response. Macrophage and T-cells are believed to be the primarily immune cells involved in the elimination of the infection and resistance to reinfection in avian host. Seven days post infection of chickens with *E. tenella* or *E. maxima* various cytokines are upregulated *in vivo*, but only at the site of infection. For example, *E. tenella* upregulates the expression of interleukin-1-beta (IL-1β), myelomonocytic growth factor (MGF), inducible nitric oxide synthase (iNOS), interferon-gamma (IFNγ), COX-2, and the CC chemokines K203 and MIP-1β These cytokines/chemokines are believed to be produced by infiltrating macrophages. *E. maxima* infection upregulate IL-1β, iNOS, IFNγ, and K203. Additionally, the number of CD3+ intraepithelial lymphocytes (IELs) increase in *E. maxima* infection, and these CD3+ cells may be responsible, in part, for the production of the pro-inflammatory cytokine IFNγ.

The expression of different genes by IELs during *E. maxima* or *E. acevulina* infection was examined using cDNA microarray. These studies confirmed that IL-1β, IFNγ, and iNOS genes were upregulated, as well as interleukins (IL-2, IL-8 and IL-15), and lymphotactin. *In vitro* studies where the avian HTC macrophage cell line was exposed to *E. tenella*, *E. acervulina*, or *E. maxima* for up to 48 hours showed similar gene expression patterns. Similarly, the mRNA levels of various cytokines and chemokines in IELs following *E. maxima* infection were upregulated, including IFNγ, IL-1β, interleukins (IL-3 IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17 and IL-18), granulocyte-macrophage colony stimulating factor (GM-CSF), lymphotactin, macrophage migration inhibitory factor (MIF), and chemokine K203. In addition, an increase in CD3+, CD4+ and CD8+ lymphocyte subpopulations, seven days post infection was reported.

The reported upregulation of numerous T-helper 1 cytokines is indicative of the importance of cell-mediated immune responses in host defense against *Eimeria*. Similarly, early studies using thymectomy, blocking of T-cell responses, or transfer of peripheral blood lymphocytes from immune to non-immune chickens, demonstrated the essential role of cell mediated immunity in host defense of chickens against *Eimeria*. These studies also reported an increase in the proportion of CD4+ and CD8+ T-lymphocytes in the IEL population at the site of intestinal invasion in *E. acervulina*, *E. tenella* and *E. maxima* infections in chickens, further supporting the central role of cell mediated immunity in host defense against these parasites.

The protective role of antibodies in coccidiosis is controversial, because the passive transfer of serum antibodies to naïve chickens confers only partial protection against infection. However, much like the expression of genes encoding specific cytokines, antibody levels against specific merozoite proteins have been shown to be the highest at the site of infection (caeca for *E. tenella* and duodenum for *E. acervulina*). Anti-parasite immunoglobulin M (IgM) was first observed seven days post infection and immunoglobulin IgA, 14 days post infection. The production of parasite specific antibodies is used as an indicator of host protective response against different species of *Eimeria*. Regardless of the immune mechanisms responsible for the control and

eventual elimination of the parasite, hosts that have successfully eliminated the first infections are protected against secondary exposure to the same species of *Eimeria*.

2.1.4. Diagnosis and Treatment

The diagnosis of different species of *Eimeria* is dependent upon a number of different factors that include the size and morphology of the oocysts, host specificity, the site of intestinal invasion, the morphology of the different life cycle stages, the pathological manifestations during the infection and the duration of the prepatent and patent periods. Current advances in diagnosis of *Eimeria* include the determination of species-specific serum antibodies using enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (Q-PCR) assays that can differentiate betwen species of *Eimeria*.

Various approaches have been used to control *Eimeria* infections in poultry. Studies have been conducted on the efficacy of disinfectants such as formol and sodium dodecylbenzene sulfonate, sodium hypochlorite, and orthodichlorobenzene and xylene, to either destroy or inhibit sporulation of *E. tenella* oocysts. However, while these treatments are partially effective (65% to 79%), the impractical nature of disinfection on a large scale, makes these control strategies less than ideal. Chemotherapeutic agents such as sulfaquinoxaline, sulfaguanidine, nitrofurazone and nitrophenide pyrimthamine and sulphadimethypyrimidine, imidazopyridine, and cyclosporine A have all been used as anti-coccidial agents. These drugs are administered in either food or drinking water. Although these anti-coccidal agents are very effective, drug rotations are frequently used in order to prevent the build up of drug resistance in the parasite.

Another approach that has been successfully used to control coccidiosis is vaccination. Early attempts to vaccinate chickens against *E. tenella* involved the inoculation of small numbers of sporulated oocysts or graded doses of oocysts prior to challenge infection. While these methods confered protection to avian livestock, the cost associated with manufacturing live vaccines was very high. At present, most of the commercial vaccines are based on this principle, whether the innoculum is live or attenuated (precocious mutants). Usually the commercial vaccine preparations contain more than one species of *Eimeria* in the innoculum. For example, Paracox® vaccine is a cocktail of *E. acervulina, E. tenella*, two strains of *E. maxima, E. necatrix, E. brunetti*, *E. mitis*, and *E. praecox*. Often, these vaccines are delivered either by ovo injection, food, drinking water, or through spraying of the chickens which then ingest the attenuated oocysts in vaccine preparation while grooming. The complex composition of the "cocktail" coccidial vaccines is required due to the species-specific immunity that is induced, since cross-species protection is relatively weak.

The development of new vaccines for coccidiosis focuses on the identification of species-specific antigens and the generation of the recombinant vaccines. Several stages of the *Eimeria* life cycle are being targeted for the identification and characterization of protective antigens. For example, the antibodies against the gametocyte GAM56 protein of *E. tenella* were shown to impair excystation *in vitro*, suggesting that this antigen may be a useful vaccine candidate. However, in order to be protective, the vaccination with GAM56 must evoke an antibody production in the host, and that antibody must be

continuously secreted and transported to the mucosa of the small intestine to prevent excystation of the oocysts, making this vaccination approach impractical. For this reason, the coccidial antigens of sporozoites and merozoites that are used in invasion of columnar epithelial cells have been identified and characterized and examined for their ability to evoke protective immune responses in chickens.

Synthetic peptides as vaccine candidates were developed based on *E. acervulina* antigens from sporozoites and merozoites, and the chickens immunized with these peptides exhibited a significant anti-parasite antibody response and enhanced antigen-specific lymphocyte proliferation, not only to the synthetic peptides but also native oocyst antigens of *E. acervulina* and *E. tenella*. The antibody and cell mediated responses observed *in vitro*, were related to a statistically significant decrease in oocyst production in immunized versus non-immunized chickens. Another developmentally regulated *E. tenella* antigen SO7, expressed only on non-sporulated oocysts and motile sporozoites has been shown to confer protection against a challenge infection.

Perhaps the most promising vaccination approach is the one that delivers protective antigens using the intracellular bacterium, *Salmonella typhimurium*. Researchers used *S. typhimurium* carrying a DNA vaccine encoding for a portion of the EtMIC4 protein (believed to be invovlved in sporozoite invasion) and *Salmonella enterica* carrying the *E. acervulina* sporozoite antigen EASZ240 and the merozoite antigen EAMZ250. The vaccination trials in both of these studies indicated significant protection against a challenge infection. Further refinement of the *Salmonella* delivery system in which multiple plasmids, each encoding antigen from a different species *Eimeria*, may be developed in the future and will undoubtedly confer significant protective immunity in the vaccinated hosts.

2.2. Theileria

2.2.1. Epidemiology

The tick-transmitted protozoa of the genus *Theileria* is an obligate intracellular parasite that causes lymphoproliferative disease in mammalian hosts. There are several species of *Theileria* that infect cattle, sheep, goats, horses, and small ruminants found in various regions of Europe, Australia, Japan, Korea, Africa, and Asia : *T. parva*, *T. annulata*, *T. mutans*, *T. velifera*, *T. tarurotragi*, *T. sergenti*, *T. buffeli*, *T. lestoquardi*, *T. ovis*, and *T. separata*. These parasites are transmitted by ixodid ticks of the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphyalis*.

The *Theileria* species are of particular economic importance, and thus the main foci of this section of the overview are *T. parva* and *T. annulata*, which infect cattle and often cause significant morbidity and mortality. *T. parva* is transmitted by *Rhipicephalus* ticks and cause a disease known as the East Coast Fever (ECF) in cattle. The primary host for this parasite is the African Cape buffalo (*Syncerus caffer*) and *T. parva* infection is non-pathogenic in these animals. It is estimated that the economic loss due to this infection is $169 million/year. *T. annulata* is transmitted by *Hyalomma* ticks and causes tropical theileriosis of cattle and it is estimated that greater than 250 million cattle are at risk. Like *T. parva*, *T. annulata* also has a primary host in which it is non-pathogenic, the

Asian water buffalo (*Bulbulus bubulis*). The parasite genomes for both of these species of *Theileria* have been completed, which will undoubtedly help in devising strategies for protecting the livestock against these infections.

2.2.2 Biology and Life Cycle

The life cycle stages of *Theileria* within the tick host and how *Theileria* sporozoites enter their mammalian host cells has been comprehensively described. Consequently, only a brief overview will be provided here. Ticks acquire the sexual stages of the parasite, the gametocytes (microgametocytes and macrogametocytes) present in the blood of the infected hosts. In the mid gut of the tick, the sexual reproduction occurs where the gametocytes fuse to form a zygote as identified by fluorochrome Hoechst 33258 staining. The zygote then transforms into a motile ookinete which migrates to the salivary gland and enters the salivary gland cells. In this intracellular environment, the parasite undergoes asexual reproduction called sporogony. Sporogony is initiated approximately 64 days post infection and is triggered by increased temperature and nutrients from the blood meal. However, in older ticks infected with *T. parva* longer periods are required for the initiation of sporogony in salivary gland cells. The *in vitro* models for *T. parva* life cycle using *Rhipicephalus* ticks have been developed using artificial membranes that allow the ticks to reach engorgement.

The infection is transmitted to the warm-blooded hosts during tick feeding, when the infectious sporozoites present in the saliva are injected into the host. Studies using mice demonstrated that *Rhipicephalus* ticks need to be attached to the host for at least 72 hrs before transmission of *T. parva* would occur and the successful transmission of the parasite was observed up to seven days post attachment.

Once the non-motile sporozoites are released into the mammalian host they are capable of recognizing, binding and entering lymphocytes (*T. parva*), or monocytes/macrophages and B-cells, cells that express major histocompatibility complex class II (MHC II) (*T. annulata*). For *T. parva*, it is believed that the major surface antigen of the sporozoites is p67, and the host cell MHC class I molecule, possibly with another co-receptor, mediate the recognition of p67 and binding of the parasites to the host cell. The entry of sporozoites into the host cell occurs by a continuous zippering of the host and parasite membranes and this process is rapid and temperature dependent. Unlike other apicomplexans such as *Plasmodium*, the rhoptries and microneme secretory vesicles of *Theileria*, remain intact throughout the entry process into the cell, rather than being used during the entry. This passive entry mechanism is distinct from the process of phagocytosis because (1) the sporozoite loses part of its surface coat during the zippering of the parasite and host membranes, (2) there are no major surface remodeling events, and (3) actin filaments do not appear to be involved in the entry of the sporozoite into the host cell. Once enveloped in the host membrane inside the cell, the sporozoites quickly discharge their secretory vesicles in a process that appears to be calcium independent, and escape the parasitophorous vacuole and remain free within the cytoplasm of the host cell where they undergo asexual reproduction. This is in contrast to other apicomplexans such as *Plasmodium* that survive and multiply within the parasitophorous vacuole.

The sporozoites that are free within the cytoplasm of the host cell evoke a response which is first manifested by the host microtubles polymerization around the sporozoite. This process appears to be induced by parasite secreting a 37kDa TaSE protein which was originally identified in *T. annulata*. The association of the schizont stage of the parasite with the nuclear spindle of the host cell provides a means for the parasite to ensure that daughter cells remain infected. Once infected, the lymphocytes become transformed, but much of the parasite and host cell molecules involved in this process remain to be characterized. Infected lymphocytes/myeloid cells are the site of asexual reproduction called merogony and eventual release of infectious merozoites which enter red blood cells and transform into piroplasms in the host cell cytoplasm. Ticks become infected after feeding on red blood cells containing piroplasms.

2.2.3. Host Defense and Pathology

The severity of ECF is dependent on the number of sporozoites that are injected into the mammalian host. Symptoms include fever, weakness, lethargy, lymphoproliferation, enlarged lymph nodes, anorexia and eventually death of the host. In cases where the host recovers, the host harbors low numbers of piroplasms in the red blood cells. The cattle that either naturally recover from theilerosis, or those that are drug-cured from the infection, develop long-lasting immunity to reinfection. Both innate and humoral immunity are thought to be involved in host defense against *Theileria*. The cytotoxic CD8+ T cells that kill *Theileria*-infected lymphocytes via an MHC class I-dependent mechanism are of central importance for host defense. It has been shown that the passive transfer of CD8+ T cells conferred protection to naïve cattle to lethal *T. parva* challenge, and this host defense mechanisms appear to be *Theileria* strain-specific. Furthermore, primed CD4+ cells are crucial for the activation of the specific CD8+ T cells subsets that confer host resistance to theileriosis. The role of various cytokines such as IFNλ and tumor necrosis factor alpha (TNFα) in host resistance to *T. parva* infection is controversial. Current research is focusing on the identification of target T-cell antigens from *T. annulata* and *T. parva* which is important for the development of new vaccines. Humoral immunity may also play a role in host defense against *Theileria*. For example, the sera obtained from cattle that have recovered from the infection often contain antibodies that are specific for p67 or SPAG-1, sporozoite surface antigens of *T. parva* and *T. annulata*, respectively, that prevent sporozoites from entering lymphocytes or monocytes/macrophages. Not only are these antibodies effective in limiting the spread of parasites during a primary infection but can also prevent sporozoite entry upon reinfection.

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