DEVELOPMENT OF A CANINE COCCIDIOSIS MODEL AND THE ANTICOCCIDIAL EFFECTS OF A NEW CHEMOTHERAPEUTIC AGENT

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ABSTRACT

Coccidia are obligate intracellular parasites belonging to the phylum Apicomplexa. Many coccidia are of medical and veterinary importance such as *Cystoisospora* species and *Toxoplasma gondii*. The need to discover new anticoccidial therapies has increased due to development of resistance by the parasite or toxicity issues in the patient. The goals of this work were to develop a model for canine coccidiosis while proving that *Cystoisospora canis* is a true primary pathogen in dogs and to determine the efficacy of a new anticoccidial agent. A canine coccidiosis model would be useful in evaluating new anticoccidial treatments. Oral infections with 5×10^4 (n=2) and 1×10^5 (n=20) sporulated *C. canis* oocysts were attempted in 22 purpose bred beagle puppies. Clinical signs associated with disease were observed in all dogs. Bacterial and viral pathogens were ruled out by transmission electron microscopy (TEM) and bacterial growth assays. Development of *C. canis* in cell culture was also evaluated.

The efficacy of ponazuril, a new anticoccidial drug, was examined in *T. gondii*. *In vitro* studies were conducted to determine the activity of ponazuril on tachyzoites and how this agent affects development of apicomplexcan parasites. The tachyzoite production assay was conducted. Ponazuril at a dose of 1.0 µg/ml had a significant affect on tachyzoite reproduction. Comparisons were made on

how ponazuril affects *T. gondii* and *Neospora caninum*. Inhibition of *T. gondii* tachyzoites occurred after the second round of replication and with *N. caninum* tachyzoites after 4 rounds of replication. Results of TEM revealed ponazuril affects replication of *T. gondii* and *N. caninum* differently.

The efficacy of ponazuril to prevent and treat acute and chronic toxoplasmosis was investigated. Mice treated prophylactically with ponazuril were completely protected from developing an acute *T. gondii* infection. Fatal toxoplasmosis was prevented in mice starting treatment 3 and 6 days post infection at a dose of 20 mg/kg. Immunohistochemistry was used to evaluate ponazuril's effect on chronic toxoplasmosis. Sections of brain were scored according to the number of tissue cysts present. Ponazuril also proved to be highly active against toxoplasmic encephalitis in an interferon-gamma knockout mouse model.

DEDICATION

I dedicate this dissertation to my family, especially my niece; River Cheyenne Poncin.

"Nine tenths of education is encouragement"
- *Anatole France*

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LIST OF ABBREVIATIONS

ATTC	American Type Culture Collection
BT	Bovine Turbinate Cells
CAPC	Companion Animal Parasite Council
CSF	Cerebrospinal Fluid
CV-1	African green monkey kidney cells
dPI	Days Post Infection/Inoculation
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-linked immunosorbent assays
EPM	Equine Protozoal Myeloencephalitis
HCT-8	Human Ileocecal Adeno Carcinoma Cells
HBSS	Hanks Balanced Salt Solution
HEP-2	Human Larynx Carcinoma
Hs68	Human Foreskin Fibroblast Cells
IgG	ImmunoGlobulin
IFAT	Indirect Fluorescent Antibody Test
INFG-KO	Interferon γ Gene Knockout
ISAGA	Immunosorbent Agglutination Assay
LM	Light Micrsocopy
MAT	Modified Agglutination Test
Mo	Month
NC-1	Neospora caninum isolate
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PI	Post Infection/Inoculation

PV	Parasitophorous vacuole
SD	Sodium Sulfadiazine
TE	Toxoplasmic encephalitis
TEM	Transmission Electron Microscopy
TP	Tachyzoite Production
TSA	Trypticase-Soy Agar
Wk	Week

CHAPTER 1

INTRODUCTION

The phylum Apicomplexa contains many organisms of veterinary and medical importance. Coccidia make up a large portion of this phylum. In particular, *Cystoispsora* species (syn. *Isospora*), specifically *Cystoisospora canis* and *Toxoplasma gondii* are two coccidia which differ in many ways but are both important in causing disease in animals. Domestic and wild canids are host to four species of *Cystoisospora*; *Cystoisospora canis* Neméseri, 1959; *Cystoisospora ohioensis* Dubey, 1975; *Cystoisospora burrowsi* Trayser and Todd, 1978; and *Cystoisospora neorivolta* Dubey and Mahrt, 1978 with *C. canis* proposed to be more pathogenic than the others. Coccidial disease caused by *Cystoisospora* species in dogs is commonly seen in veterinary clinics. Diagnosis is based upon patient history, clinical signs, and the presence of oocysts in feces. Typical clinical signs of disease are diarrhea either with or with out blood, anorexia, weight loss, and vomiting. Death can occur in severe untreated cases. However, asymptomatic dogs may excrete oocysts. Puppies and immunosuppressed dogs are more susceptible to disease than healthy dogs.

True Pathogenicity of *Cystoisospora* has been debated for many years. Laboratory studies have not clearly demonstrated a pathogenic role for *Cystisospora* spp in the canine host (Dubey, 1978, Dubey and Fayer, 1976, Dubey et al., 1978, Trayer and Todd, 1978, Lepp and Todd, 1974). The presence of oocysts in diarrheic stool, alone is not conclusive that *Cystoisospora* is the primary cause of clinical disease unless other potential pathogens are rigorously

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ruled out. The lack of a reliable and reproducible canine coccidiosis model makes it hard to determine if anticoccidal treatments are specifically acting on the *Cystoisospora* parasite since many anticoccidials also have antibacterial properties. This means a positive response to current drug therapies does not equal proof that this parasite is the cause of clinical coccidiosis. Canine *Cystoisospora* species are not considered to infect humans.

Toxoplasma gondii is one of the more important coccidial parasites due the threat of zoonosis and is a superior model for the coccidia. Infection consists of two stages in the warm-blooded host, first is the acute stage, which is associated with tachyzoites, a rapidly dividing stage of the organism. The second stage is a chronic or latent stage which is a tissue cyst containing bradyzoites, a slowly dividing stage (waiting stage) that is commonly found in the brain or CNS of an infected host and may be associated with toxoplasmic encephalitis. Animals such as goats, sheep and wild game become infected through ingesting sporulated oocysts. Pigs can become infected through oocyst ingestion and/or tissue cysts ingestion. Humans can become infected through many different routes. In some countries, humans are most likely infected through ingesting tissue cysts containing bradyzoites in raw or undercooked meat (Luft and Remington, 1992). Humans are also infected through ingesting sporulated oocysts or though congenital transmission. Therapeutic agents would ideally be able to treat both tachyzoites and tissue cyst stages; however the current standard treatment does not seem to have an effect on tissue cysts stages. Many

therapy options are associated with considerable toxicity leading to discontinuation of drug treatment.

Current drug therapies to treat various coccidia are either inadequate or suffer from drug resistance accelerating the need for discovery of new treatment options. The following studies were undertaken to determine if *Cystoisospora* species can be a primary cause of clinical disease while also providing a reproducible canine coccidiosis model that could be used in anticoccidial studies. In addition, a new anticoccidial agent, ponazuril, was evaluated using *T. gondii*: a reliable and reproducible apicomplexan model. Currently, no one has studied the effect of ponazuril on *T. gondii* or similar coccidial parasites. Ponazuril is a major metabolite of toltrazuril, a coccidiostat used to control coccidiosis in poultry (Furr and Kennedy, 2001). For these reasons, there were two central hypotheses for this dissertation. Firstly, that *Cystoisospora canis* is a primary pathogen causing canine coccidiosis and secondly, ponazuril is highly effective in treating a wide range of coccidial parasites.

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CHAPTER 2

LITERATURE REVIEW

Canine Coccidiosis (Cystoisospora species)

The phylum Apicomplexa consists of a wide range of pathogenic species including coccidia, a large group of intestinal parasites that is of veterinary and medical importance. *Cystoisospora canis* (*syn. Isospora canis*), *Neopspora caninum*, *Sarcocystis neurona*, *Eimeria bovis* and *Toxoplasma gondii* are examples of coccidia that cause disease in animals. *Cystoisospora canis* Nemeséri, 1959; *Cystoisospora ohioensis* Dubey, 1975; *Cystoisospora burrowsi* Trayser and Todd, 1978; and *Cystoisospora neorivolta* Dubey and Mahrt, 1978 are four species that can cause coccidiosis in canids. Identification of *C. canis* is based on oocysts size (> 33 μm) (Lindsay et al., 1997). However, specific identification of the other three *Cystoisospora* spp. requires detailed structural examination of oocysts and life cycle studies due to the similarity in their oocysts (<30 μm) . These 3 coccidial species are usually grouped together as members of the *C. ohioensis* complex and the oocyst referred to as *C. ohiohensis*-like (Dubey et al., 1978) since they cannot be identified based on unsporulated oocysts (Table 1). *Cystoisospora canis* is thought to be more pathogenic than members of the *C. ohioensis* complex.

Coccidial infections are common in both domestic and wild canids. Prevalence in the United States varies greatly depending on location. One study reported a prevalence range between 10.6% and 72% domestic dogs (Catcott EJ, 1975). More recently, surveys estimate a range between 3-38% (CAPC

guidlines, http://www.capcvet.org, 2007). In Spain, prevalence ranged from 5%-14% in both stray and cared for dogs (Martínez-Carrasco et al., 2007). Canine coccidiosis can become a problem for breeding facilities due to the severity of disease in puppies (Penzhorn et al., 1992).

Cystoisospora Life Cycle

Cystoisospora species have a direct life cycle (Figure 1) that begins when sporulated oocysts are ingested by a canine definitive host (fecal-oral transmission). *Cystoisospora* species can also use paratenic hosts. Sporulated oocysts contain two sporocysts each with four sporozoites (Figure 2). Once ingested, sporozoites will become activated in the small intestine and emerge from the sporocysts to invade intestinal cells. The location and site in the intestines and the number of asexual generations varies with each species of canine *Cystoisospora* (Table 1). For *C. canis*, asexual replication by endodyogeny to produce 1st-generation schizonts containing merozoites is completed by 5-7 days post-infection (dPI) (Lepp and Todd 1974). By 8 dPI, 2nd and 3rd generation schizonts are complete and sexual stages, microgamonts (male) and macrogamonts (female) start to appear. After maturation of sexual stages, many microgametes will exit cells to fertilize macrogametes, producing oocysts. Oocysts can be found as early as 9 dPI (Lepp and Todd 1974). Once fully developed, oocysts rupture out of host cells and are excreted into the environment where they will undergo sporulation and become infective for other canine hosts. The prepatent and patent period vary depending on the *Cystoisospora* species (Table 1).

Sporozoites have also been known to leave the intestinal tract and infect other tissues in the canine definitive host (Dubey, 1975). These extraintestinal stages are termed monozoic or unizoite cysts or hypnozoites and consist of a single centrally located zoite surrounded by a thick wall (Figure 3). This is commonly seen with the human coccidia, *Cystoisospora belli* (syn. *Isospora belli*) (Restrepo et al., 1987). Canine *Cystoisospora* species are able to make use of paratenic hosts, such as mice, rats and hamsters (Frenkel and Dubey, 1972; Dubey, 1975; Dubey and Melhorn, 1978). Paratenic hosts become infected by ingesting sporulated oocysts but asexual and sexual development can not occur. Sporozoites will enter extraintestinal tissues but do not cause disease in these hosts. Monozoic cysts are commonly found in mesenteric lymph nodes of both definitive and paratenic hosts. However, liver and spleen can also be infected (Dubey and Frenkel, 1972). Coccidiosis can occur if a canine ingest a paratenic host harboring monozoic cysts; however the prepatent period is shortened.

Pathogenesis and Disease

Pathogenicity of *Cystoisospora canis* and other species occurring in dogs is controversial. Previous laboratory attempts to induce infections in dogs have been unsuccessful at proving that *Cystoisospora* species can be a primary pathogen (Dubey, 1978; Dubey and Fayer, 1976; Lepp and Todd, 1974). Development of asexual and sexual stages occurs within enterocytes and cells in the lamina propria in the small intestine of canine definitive hosts (Lepp and Todd, 1974; Dubey, 1978; Dubey, 1979). Host cell lysis is caused by maturation

and emergence of these stages and host cell rupture is especially destructive when hundreds of mature oocysts are being released from intestinal cells.

Disease is more common in young dogs than adults, but infection can also be asymptomatic even in puppies shedding oocysts. Clinical signs associated with canine coccidiosis include diarrhea, anemia, dehydration, abdominal pain, vomiting, anorexia, weight loss, apathy, tremors and convulsions, paresis, pneumonia, respiratory rales and staggering (Oduye and Bobade, 1979; Correa et al., 1983; Olson, 1985). Death is also possible in severe untreated cases but this is rare. Immunosuppression or stress from concurrent disease can intensify clinical signs of coccidiosis.

Dogs that recover from clinical disease caused by *C. canis* appear to develop immunity from future clinical *C. canis* infections. It is unclear why clinical signs are not seen in older dogs. One explanation is age related resistance (Kirkpatrick and Dubey, 1987). It is also unclear if infection with one species of *Cystoisospora* will provide immunity to the other 3 species.

Diagnosis of Cystoisospora sp.

Diagnosis of canine coccidiosis should be based on clinical history, signs, and fecal examination. Fecal samples processed by flotation procedures are examined for the presence of oocysts. However, the coprophagic habits of dogs can cause spurious parasites to be present in stool samples and *Eimeria spp.* oocysts must be distinguished from those of *Cystoisospora spp.* Bacterial and viral pathogens and other parasites should also be ruled out as potential causes of presenting clinical signs. The presence of *Cystoisospora* oocysts in feces does

not equal a primary cause of disease unless other pathogens have been ruled out.

Treatment and Prevention of Cystoisospora sp.

Many dogs will spontaneously eliminate an infection, but for those that do not, several drugs have proven effective in shortening the patent period. Currently, the only anticoccidial agent approved for treating canine coccidiosis is sulfadimethoxine. Sulfadimethoxine suppresses oocyst excretion and limits diarrhea associated with *Cystoisospora* infections (Lindsay and Blagburn, 1995). Toltrazuril, a triazinetrione, has been used successfully in Europe to treat various types of animal coccidiosis, including feline and canine coccidiosis due to *Cystoisospora* species (Daugschies et al., 2000; Lloyd and Smith, 2001). Many other drugs have aided in decreasing oocyst shedding and relieving clinical signs associated with canine coccidiosis, such as amprolium and ormetroprim in combination with sulfadimethoxine (Kirkpatrick and Dubey, 1987; Lindsay and Blagburn, 1995).

Precautions should be taken to minimize the spread of coccidia infection to other dogs, especially in kennels, animal research facilities and veterinary clinics. Removing fresh feces from animal housing areas and washing these areas will help prevent the spread of infection. Most disinfectants have little or no effect on *Cystoisospora* oocysts but ammonium hydroxide has shown some effect on this stage (Fayer, 1980). Dogs should be restricted from ingesting small rodents which can harbor monozoic cysts that can lead to infection.

Toxoplasma gondii

Canine coccidiosis posses little threat to humans. Humans are not susceptible to canine coccidial infections and therefore it is not considered a zoonosis. *Toxoplasma gondii* is another coccidian similar to *Cystoisospora* canis that uses domestic cats and other felids as the definitive host and is zoonotic. *Toxoplasma gondii* is an important coccidian parasite that causes infection worldwide. This parasite infects both humans and animals with a worldwide prevalence. According to the National Health and Nutrition survey (NHANES: 1999-2000), 15.8% of people between the ages of 12 and 49 in the United States are infected with *T. gondii* (Jones et al, 2003). Seroprevalence is higher at 54% in Southern European countries (Welton and Ades, 2005) but the lowest prevalence is found in Asian countries at 10.2% (Hung et al., 2005). *Toxoplasma* encephalitis is an important form of *T. gondii* infection most common in immunosuppressed patients. Before the institution of prophylactic treatment, it is estimated that 10% of AIDS patients in the U.S. and 30% in Europe die from toxoplasmosis each year (Luft and Remington, 1992).

Most *T. gondii* infections in humans occur from ingesting undercooked meat. Seroprevalence of *T. gondii* in farm animals varies widely among species and farming practices. Large economic losses occur due to toxoplasmosis in sheep, goats and pigs, which are more likely to carry this parasite than cattle in the United States (Lunden et al., 2002). The most important effect in sheep and goats is fetal mortality. In the U.S., the prevalence of *T. gondii* in swine has been determined to have a wide range of infection between < 1-69% (Dubey, 1990;

Dubey et al. 1990) and sheep average 65.5% (Dubey and Kirkbride, 1986). Young pigs are more likely to die from a *T. gondii* infection than adult pigs. Cattle appear to be more resistant to *T. gondii* infections with a wide prevalence range in the U.S. between < 1-100% (Dubey, 1986a). However, cattle do not suffer from clinical disease caused by this parasite. Instead, *Neospora caninum*, another coccidian parasite that is very similar to *T. gondii*, causes abortion in cattle.

Dogs are thought to contribute to human *Toxoplasma* infection by mechanically transporting oocysts (Frenkel et al., 2003) and through ingestion of dog meat where acceptable. Seroprevalence in dogs varies greatly. One study conducted in dogs from Baltimore, Maryland found a prevalence of 32.2% (Childs and Seegar, 1986). Areas around the world found canines with a seroprevalence of 67.4% in dogs from Sri Lanka (Dubey et al., 2007), 50% in dogs from Pakistan (Ahmad et al., 2001), 76.4% in dogs from Brazil (Cañón-Franco et al., 2004) and 26% of dogs from Austria (Wanha et al., 2005). *Neospora caninum* is a closely related parasite to *T. gondii* that causes disease in dogs. Similarities in morphology and presenting disease led to frequent misdiagnosis of *Neospora* until it was identified as a separate organism in 1988 (Dubey et al., 1988).

Life Cycle and Parasite Morphology of T. gondii

Toxoplasma gondii has a complicated life cycle which wasn't completely known until 1970. Members of the family Felidae, including domestic cats, are the only known definitive hosts for *T. gondii*. A variety of warm-blooded

mammals and birds serve as intermediate hosts, including humans. Felines are both the definitive host and an intermediate host (Figure 4).

Oocysts

Within the feline definitive host, sexual replication in the intestines occurs and leads to the production of oocysts which will be shed in the feces upon defecating. During an acute infection cats can shed millions of unsporulated oocysts measuring around 12 x 10 μm. Once in the environment sporulation occurs between 1 and 5 days (Dubey et al., 1970). This stage is extremely resistant to environmental factors and disinfectants. Sporulated oocysts are similar in structure to *Cystosispora* species and contain two sporocysts each with four sporozoites that are infective when ingested by an intermediate or definitive host (Figure 4). Sporozoites give rise to tachyzoite stages after ingestion by the host.

Tachyzoites

Tachyzoites are a rapidly multiplying stage that can replicate in any nucleated host cell. *Toxoplasma gondii* tachyzoites are crescent shaped and measure 2 μm by 6 μm. This stage replicates by endodyogeny forming numerous clones within a single parasitophorous vacuole (Figure 5). Host cell death occurs when it can no longer support the growth of tachyzoites leading to rapid infection of neighboring cells (Dubey et al., 1998). Clinical disease occurs from the destruction of tissues and a strong inflammatory response caused by rupturing of tachyzoites out of a parasitophorous vacuole. Tachyzoites are spread through the bloodstream and will readily cross the blood-brain, placental and retina

barriers. Tachyzoites are associated with an acute infection of *T. gondii* and will convert to bradyzoites where they will remain in tissue cysts for the life of the host.

Bradyzoites and Tissue Cysts

Bradyzoites are similar in structure to tachyzoites but are functionally different. Hundreds of bradyzoites grow within a thin walled tissue cyst and are considered to be a dormant stage (Figure 6). The size of tissue cysts depends on age, host cell type and the infecting *T. gondii* strain. Bradyzoites also replicate by endodyogeny but at a much slower rate within the host cell cytoplasm than tachyzoites. Tissue cysts can develop in any organ but are more prevalent in brain and both skeletal and cardiac muscles. Recrudescence of infection occurs when bradyzoites exit tissue cysts and convert back to tachyzoites, which will disseminate the infection within or to other tissues causing more cysts to form (Dubey et al, 1998). This usually occurs in immunosuppressed patients. Tissue cysts containing bradyzoites are associated with the chronic/latent phase of infection and are considered immunoprivileged since the immune system of the host does not attack them. In addition, most anticoccidial treatments have little effect on this stage. Tissue cysts are a common infective stage for both intermediate and feline definitive hosts (Weiss and Kim, 2000).

Life Cycle in the Feline Definitive Host

The *T. gondii* life cycle can be divided up into two parts: the life cycle in feline definitive hosts and the life cycle in intermediate hosts, which include humans. Cats can become infected by ingesting any of the three stages

discussed. However, ingestion of tachyzoites is rare and cats are more likely to become infected through ingestion of cysts containing bradyzoites in the tissue of an intermediate host or through fecal-oral transmission by ingestion of sporulated oocysts. In cats, bradyzoite-induced infection is more efficient than oocyst-induced infections (Dubey and Frenkel, 1976). *Toxoplasma gondii* infections in the definitive host undergo both sexual and asexual development (Frenkel JK, 1970). After ingestion of a bradyzoites in cysts, five schizont generations develop in the enteroepithelial cells in the intestine of the cat which give rise to the gamont sexual stages. Oocysts are produced and excreted in the feces around 3-5 days after ingestion of tissue cysts. Some bradyzoites will leave the intestines, divide as tachyzoites and form new cysts in tissues. *T. gondii* infections in cats are usually asymptomatic. Little is known about oocyst and tachyzoite induced infection in cats. It is generally believed that sporozoites from oocyst induced infection will convert to tachyzoites and leave the intestinal tract to infect other tissues. Tissue cysts will also form but some bradyzoites will re-enter the intestinal cells to undergo a bradyzoite-induced cycle as described above (Freyre et al., 1989). The prepatent period is longer when sporulated oocysts are ingested leading to the excretion of oocysts around 18 days post infection.

Life Cycle in the Intermediate Host

Intermediate hosts become infected through ingesting tissue cysts in raw or undercooked meat or by ingesting sporulated oocysts in contaminated food, water or soil. Oocyst-induced infection of *T. gondii* is more pathogenic in

intermediate hosts than in definitive hosts. Most research on sporulated oocyst infections of intermediate hosts has been conducted in mice (Dubey et al. 1997; Speer and Dubey, 1998). Dubey et al., (1997) reported sporozoites had begun infecting enterocytes in the small intestine of mice 30 minutes after oral inoculation with sporulated oocysts. Most sporozoites converted to tachyzoites and began infecting extraintestinal tissues by 18 hours PI. Bradyzoite formation began by 6 dPI. Enteritis, pneumonia and encephalitis in mice were common clinical signs 4 wks PI (Dubey et al., 1997). Bradyzoite-induced infections appear to be less pathogenic and less infectious when orally ingested by intermediate hosts. Dubey, (1997) reported bradyzoites had converted to tachyzoites and dissemination of the parasite occurred by 18 hours post oral ingestion of bradyzoites in mice. Congenital infections also occur in intermediate hosts and are particularly important in humans, sheep and goats. Clinical signs and abortions can occur when an intermediate host, commonly humans and sheep, becomes infected with *T. gondii* for the first time during pregnancy. Circulating tachyzoites in the mother's blood will infect the fetus through transplacental transmission which can have severe effects throughout the life of the offspring.

T. gondii Transmission and Pathogenesis

Hosts can be infected by 3 different routes: a) fecal-oral, ingestion of sporulated oocysts in contaminated food, water, soil and cat litter boxes, b) ingestion of tissue cysts containing bradyzoites and c) congenital infections. Humans can also become infected through organ transplantation (Aubert et al.,

1996; Renoult et al., 1997; Hermanns et al., 2001; Sarchi et al., 2007). However, this route of infection is rare. In immunocompetent humans, acute infection is usually asymptomatic but some infections can cause non-specific symptoms such as fever, lymphadenopathy, and fatigue (McCabe et al., 1987). Ocular *T. gondii* infections can occur in adults who have acquired the infection postnatally but are more common with congenital transmission. Toxoplasmic retinochoroiditis postnatally acquired in adults can cause blurred vision, pain, light sensitivity, tearing and possible vision loss (Latkany, 2007). In immunocompromised patients, such as those with AIDS, clinical disease usually occurs as a result of reactivation of chronic stages (tissue cysts) within the central nervous system of the patient (Luft and Remington, 1992). Toxoplasmic encephalitis is life-threatening in these patients if not treated. Clinical signs in immunocompromised patients are typically neurological and include seizures, speech abnormalities, altered cranial nerve function, muscle weakness and partial paralysis (Luft and Remington, 1992). Behavioral and psychomotor abnormalities, such as dementia and psychosis have also been reported (Luft et al., 1993). Multiple organs are likely be affected by an acute acquired *Toxoplasma* infection in AIDS or transplant patients.

Congenital infections occur when the mother acquires a primary *T. gondii* infection during pregnancy. Severity of disease in the fetus varies depending on when infection was acquired during pregnancy. The rate of transmission increases with each trimester but severity of disease in the fetus decreases. Dunn et al., (1999) found congenital infections occurred at a rate of 10-25%

when primary infection was acquired during the first trimester, 30-50% when acquired during the second trimester and 60-70% when maternal acquisition occurred during the third trimester. Maternal acquisition of *T. gondii* in the first 26 weeks of gestation increases the severity of clinical signs. Common clinical manifestations of congenital infections acquired during this time are intracerebral calcification, hydrocephalus, retinochoroiditis, mental retardation and encephalitis (Remington et al., 1995). The severity of congenital toxoplasmosis when infected in the third trimester (29 - 40 weeks) is much less and newborns may be asymptomatic but will likely develop clinical signs later on in life. Mothers who acquired *T. gondii* infections prior to pregnancy are unlikely to infect their unborn fetus.

T. gondii Diagnosis

Biologic, serologic or histologic methods are used to diagnose *T. gondii* infections. Serologic testing is the main source for diagnosis. Many assays have been developed to detect various isotypes of antibodies to aid in detection of an acute (IgM) or chronic (IgG) *T. gondii* infection (Table 2). Most assays used to diagnose infection today look at IgG or IgM antibodies.

<u>Sabin-Feldman dye test</u>

The Sabin-Feldman dye test was developed in 1948 and is considered the "gold standard" diagnostic assay for *T. gondii* infections. This assay is a complement-lysis based test that measures serum immunoglobulin through the use of live tachyzoites (Sabin and Feldman, 1948). The dye test is highly specific

and sensitive but is limited to reference laboratories due to its use of live organisms.

Enzyme-linked immunosorbent assays (ELISA)

This assay can be used to detect various isotypes of antibodies (IgG, IgA, IgM and IgE). IgG detection can only confirm the host has been infected with *T. gondii* and cannot differentiate between acute and chronic infection unless multiple samples are collected. Chronic toxoplasmosis can be detected by obtaining multiple samples over a period of time and examining IgG levels. If a latent *T. gondii* infection is present, the IgG antibody level will not change over time (Remington et al., 1995). IgM antibody detection can be indicative of a recently acquired infection and appear before IgG antibodies post-infection. However, this antibody can persist for over a year after a primary infection causing false positives especially in immunocompromised patients and pregnant women (Liesenfeld et al., 1997). Assays detecting IgM should not be used as the sole diagnostic test for determining an acutely acquired *T. gondii* infection in these patients. Instead, two assays should be conducted, first a sensitive IgM test followed by an IgG aviditiy test. Diagnosis of congenital toxoplasmosis through detection of IgM antibodies in cord blood has proven useful since IgM antibodies are unable to cross the placenta (Remington et al., 1968). Assays for the detection of IgA are more sensitive than IgM assays for detection of congenital infections in fetuses and newborns, but add little to diagnosing acute infections in adults (Stepick-Biek et al., 1990). In immunocompromised patients, IgA ELISA's rarely diagnose antibodies in their serum and a test for IgG

antibodies should be conducted. ELISA's detecting IgE and the IgE immunosorbent agglutination assay (ISAGA) may also be helpful in detecting a recently acquired *T. gondii* infection. Seropositivity for IgE last for only a brief period of time making this antibody ideal for diagnosis of an acutely acquired infection in adults (Pinion et al., 1990). However, in newborns and fetuses, IgE detection alone is not as useful as IgA antibodies but when used in conjunction with other diagnostic tests can provide valuable information. Wong et al., (1993) found IgE antibodies were detected by ELISA in 33% of patients with toxoplasmic encephalitis whereas IgA and IgM antibodies were not detectable.

Indirect fluorescent antibody test (IFAT)

This test also measures antibodies in serum but is not as sensitive as the dye test. False-positives occur in serum samples with anti-nuclear antibodies. Indirect fluorescent antibody tests are widely used and are available commercially. However, one disadvantage is the requirement of a microscope with a fluorescent light source.

Direct agglutination test

Development of this assay first occurred in 1965 (Fulton, 1965) and was later modified by Dubey and Desmonts, (1987) at which time the name was changed to the modified agglutination test (MAT). This test is ideal because no enzyme conjugates or special equipment is required. IgG antibodies are detected using formalin-fixed tachyzoites and serum. IgM antibodies can cause false-positive results but this can be avoided by adding 2-mercaptoethanol to the antigen before screening.

PCR assays have proven useful in the diagnosis of toxoplasmosis especially in detection of *in utero* and congenital infections and ocular infections. A wide variety of biological specimens such as amniotic fluid, blood, aqueous humor, cerebrospinal fluid (CSF) and body tissues can be tested using PCR. This method is also useful in diagnosing infection in immunosuppressed patients when serological assays fail to detect a response. Many *T. gondii* gene targets have been identified for use in PCR. The B1 gene was used to first identify *T. gondii* DNA from a single tachyzoite by Burg et al. in 1989 and is still widely used in PCR assays today.

Treatment and Prevention of Toxoplasmosis

In humans

Treating *T. gondii* infections depend on multiple components such as the immune status of the patient and the location of infection. In women diagnosed with an acute infection, treatment will be based on whether she is pregnant. Treatment is usually not needed in immunocompetent individuals with acquired or latent *T. gondii* infections. In immunocompromised patients and congenital infections many studies focus on prevention of chronic toxoplasmosis but most therapies have little effects on tissue cysts. Many therapies are based on experimental *in vivo* and *in vitro* studies since few large clinical trials have been conducted to determine the most effective treatments for infected patients. Common treatments act by inhibiting tachyzoite growth and when the treatment is stopped, tachyzoite growth resumes.

No single therapeutic agent is completely effective in managing infection so combinations of drugs are the preferred treatment strategy. In humans the current standard treatment of patients infected with *T. gondii* is a combination of a sulfonamides and pyrimethamine (Remington et al., 1995). This combination is recommended for treating immunocompetent (if treatment is necessary), immunocompromised hosts, congenital toxoplasmosis and ocular toxoplasmosis. However, this combination often leads to severe side effects associated with high toxicity and discontinuation of the regimen. Spiramycin has been used in treating toxoplasmosis acquired during pregnancy due to its affect on apicoplast function in the parasite and does not cross the placenta. Spiramycin is used to prevent fetal transmission once a maternal diagnosis is made (Remington et al., 1995). A combination of pyrimethamine, sulfadiazine and folinic acid should be alternated with spiramycin if fetal toxoplasmosis has been diagnosed (Remington et al., 1995). Clindamycin also poses anti-*T. gondii* activity (McMaster et al., 1973; Araujo and Remington, 1974; Djurković-Djaković et al., 1999), especially when used in combination with the standard drugs. Clindamycin is often used in combinations when sulfonamides cannot be tolerated. *Toxoplasma* encephalitis has been prevented and treated with trimethomprim-sulfamethoxazole in AIDS patients (Bozzette et al., 1995; Torre et al., 1998). Atovaquone has shown some activity on the *T. gondii* cyst stage which other drugs do not treat (Huskinson-Mark et al., 1991). In mice suffering from acute and chronic toxoplasmosis, atovaquone yielded significant protection against death (Araujo et al. 1991; Romand et al., 1993).

In animals

Prophylactic treatment for domestic farm animals consists of medicated feed. Decoquinate has proven useful in feed for sheep and goats (Millard and Spelman, 1989). However, the need for continual treatment poses a problem for free ranging animals. A live vaccine to prevent abortion in sheep is available in some countries (Buxton and Innes, 1995). As with humans, combination therapy with pyrimethamine and sulfadiazine is widely used in animals (Dubey, 1986, Buxton et al., 1993). Clindamycin is usually chosen to treat toxoplasmosis in dogs and cats (Lappin et al., 1989; Davidson, 2000). In the feline definitive host, toltrazuril has proven to be fully effective against both asexual (schizonts) and sexual (gamont) stages that develop in the small intestine and on tachyzoites (extraintestinal stages) (Rommel et al., 1987).

Precautions can be taken to limit exposure to *T. gondii* in humans and animals. Many human infections occur through ingestion of tissue cysts in improperly prepared meat. Current recommendations by regulatory agencies include that meat should be stored in a freezer for at least a 24 hour period or until needed and meat should be thoroughly cooked through the center (Center for Disease control and Prevention, www.cdc.gov/toxoplasmosis, 2008). Any surface that has come in contact with raw meat, poultry or seafood should be washed with hot, soapy water, including hands, knives and cutting boards. Fruits and vegetables should be washed and/or peeled prior to eating.

In order to prevent acquisition of *T. gondii* infections from the environment, it is recommended that gloves be worn while gardening or contacting soil and

hands should be washed immediately after handling soil or sand. Outdoor sand boxes should be kept covered when not in use. Cats should be kept indoors and only fed commercial foods or cooked table scraps. Litter boxes should be cleaned daily to prevent oocysts from becoming infective. Pregnant women and immunocompromised patients should wear gloves and a mask when cleaning the litter box and hands should be immediately washed post cleaning. Avoid handling stray or unknown cats, especially if pregnant (Center for Disease control and Prevention, www.cdc.gov/toxoplasmosis, 2008).

Model Apicomplexan

Toxoplasma gondii is the one of the more manageable parasites to control with in a laboratory setting. Both, acute and latent stages of this parasite can be grown and maintained *in vitro*. Many animal models, specifically mice, are well developed providing a basic understanding of host response. Multiple molecular genetic manipulation techniques have been validated and are easily accessible to laboratories. Recently, the *T. gondii* genome has been sequenced and can be accessed through an online database (www.toxodb.org)(Gajria et al., 2008). These advances in parasite molecular biology have increased the desire for drug target based screening in commercial programs. So far, *T. gondii* has proven effective in confirming specific drug targets for *Eimeria tenella* (Donald and Liberator, 2002) and *Cryptosporidium parvum* (Streipen et al., 2004). Because of these advantages, *T. gondii* has emerged as the major model for apicomplexan parasites.

New Chemotherapeutic Agent

Ponazuril, a major metabolite of toltrazuril has recently been approved for treating *Sarcocystis neurona,* the causative agent of equine protozoal myeloencephalitis (EPM). In horses, it is effective at a dose of 5 or 10 mg/kg when given for 28 days consecutively (Furr et al., 2001). To date, no one has looked at this new anticoccidial's effect on other apicomplexan parasites similar to *S. neurona*, such as *T. gondii* or *C. canis.* Ponazuril's mode of action is currently unknown but is thought to act on the apicoplast, a non-photosynthetic plastid.

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Life cycle of *Cystoisospora canis*
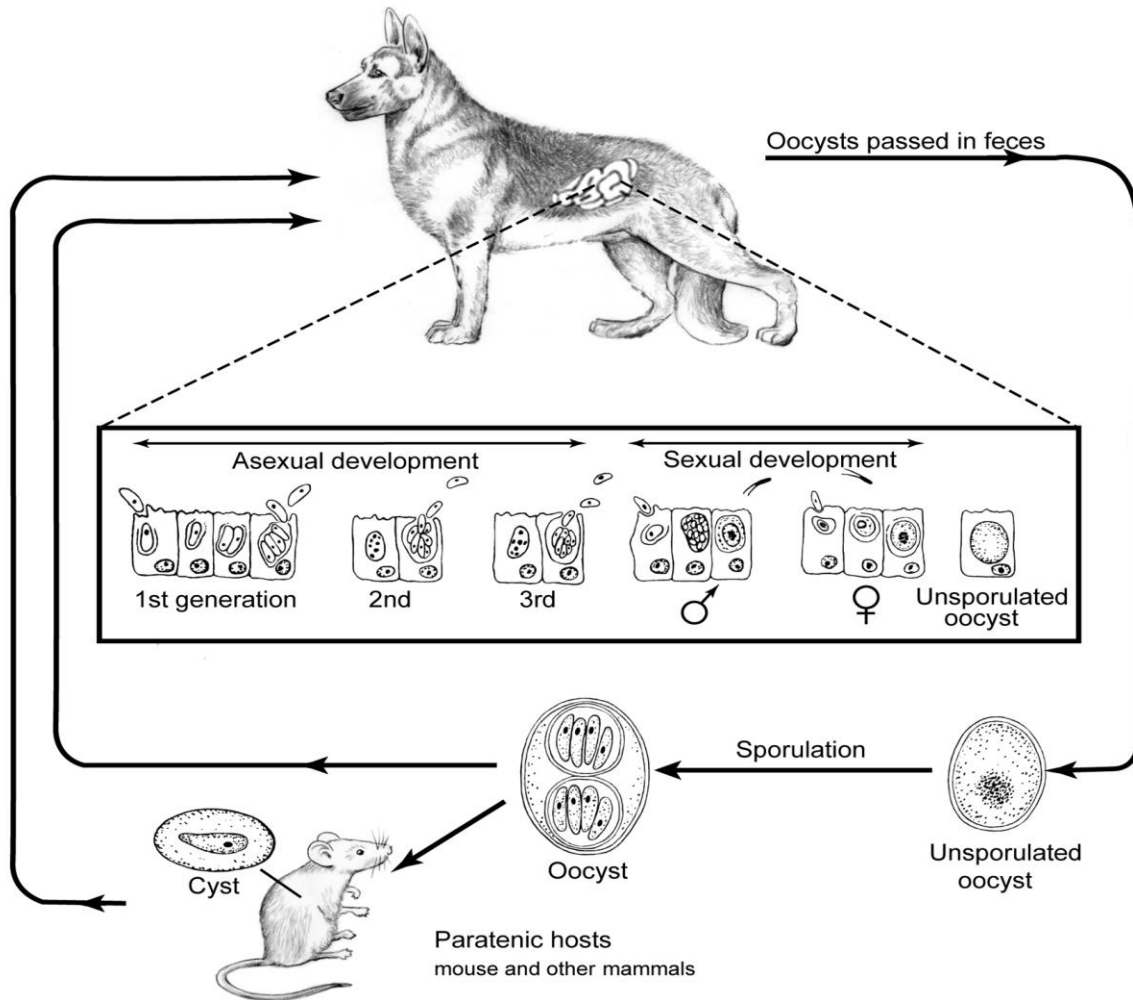
Figure 1. *Cystoisospora* species life cycle, specifically *C. canis.* (Adapted from Lindsay and Blagburn, 1994)
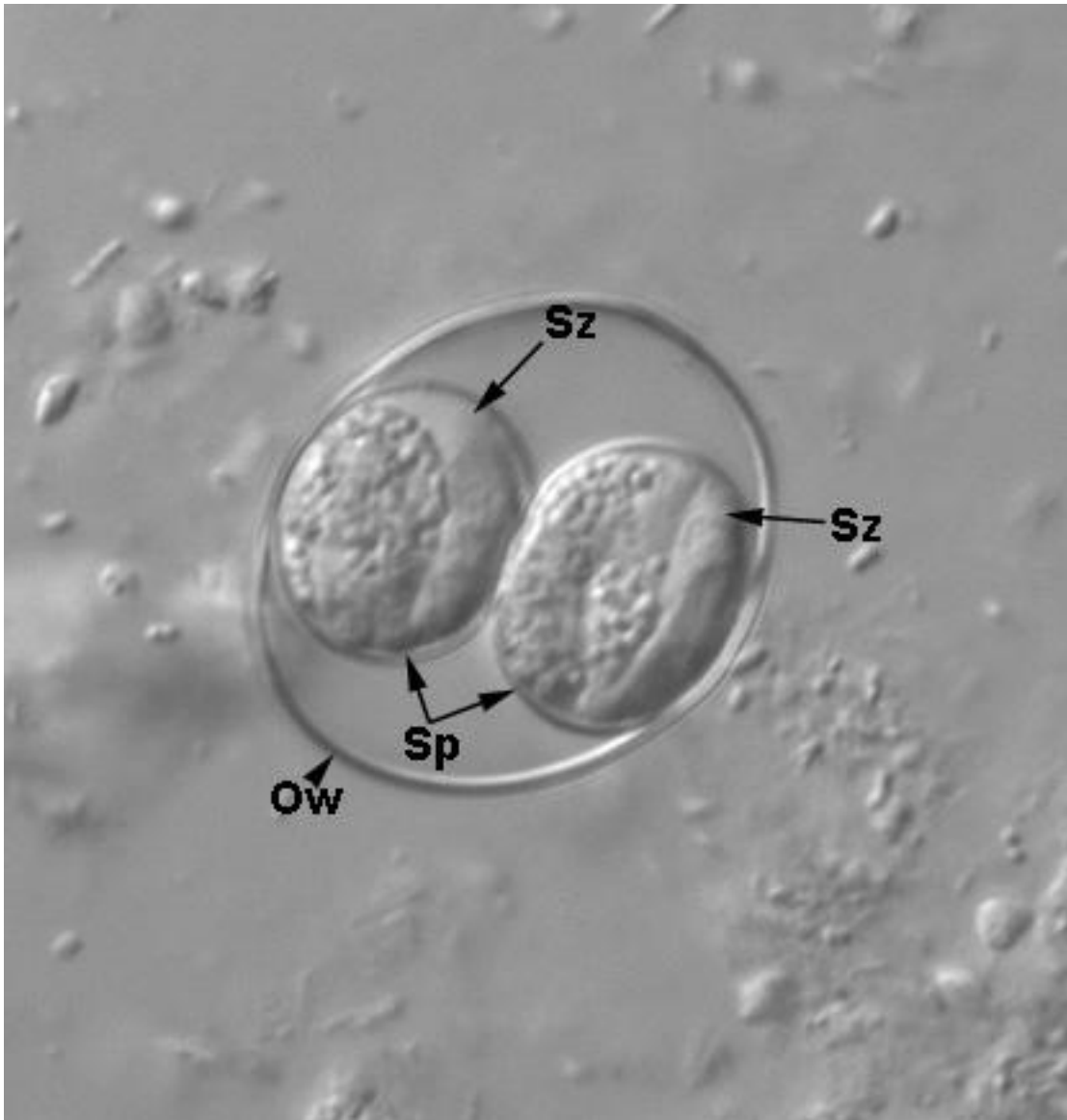
Figure 2. Sporulated *C. canis* oocyst (oil). Ow oocysts wall; Sp, sporocyst; Sz, sporozoite.
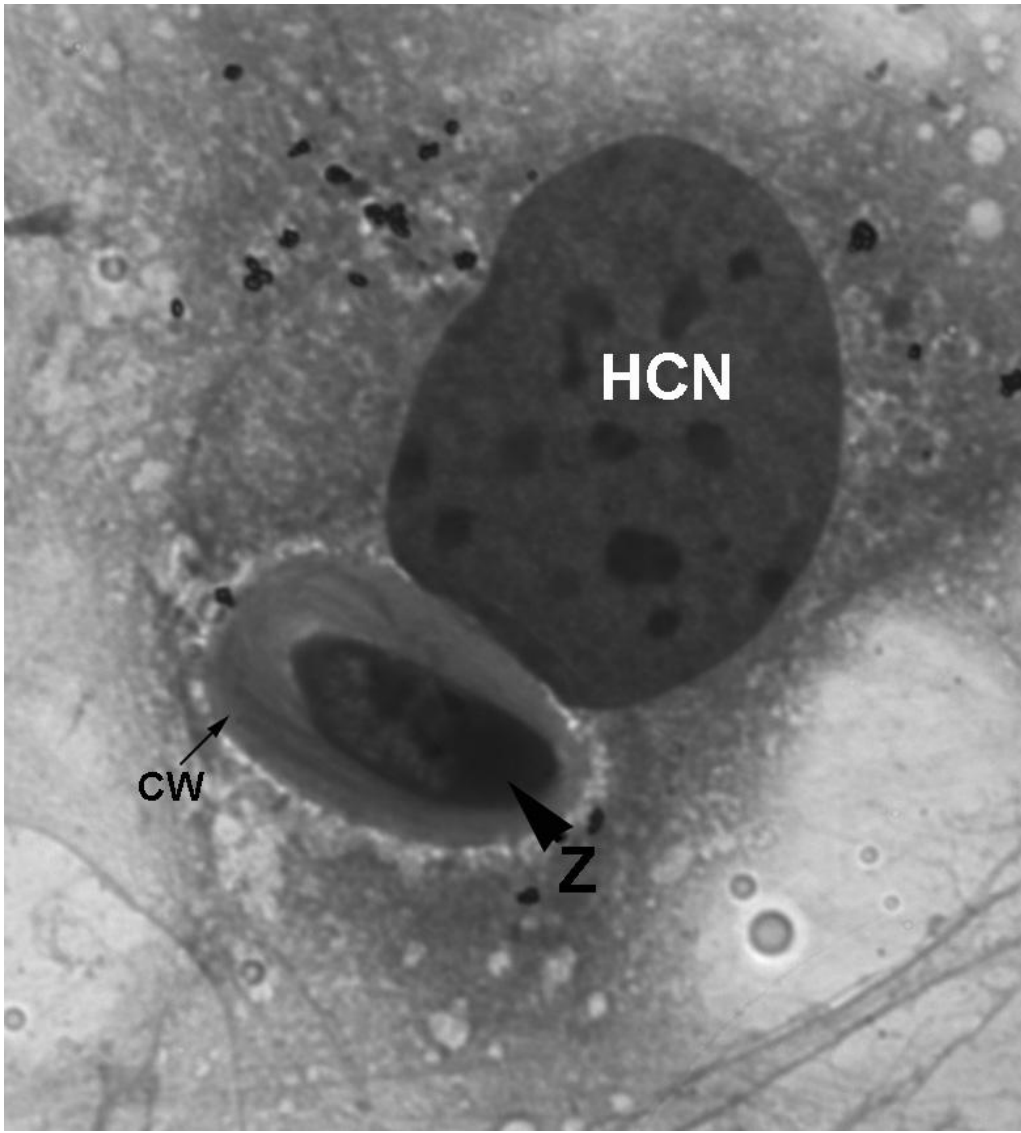
Figure 3. Monozoic cyst-like structure grown in cell culture (oil). A thick cyst wall

(Cw) that surrounds a single centrally located zoite (Z). HCN, host cell nucleus.
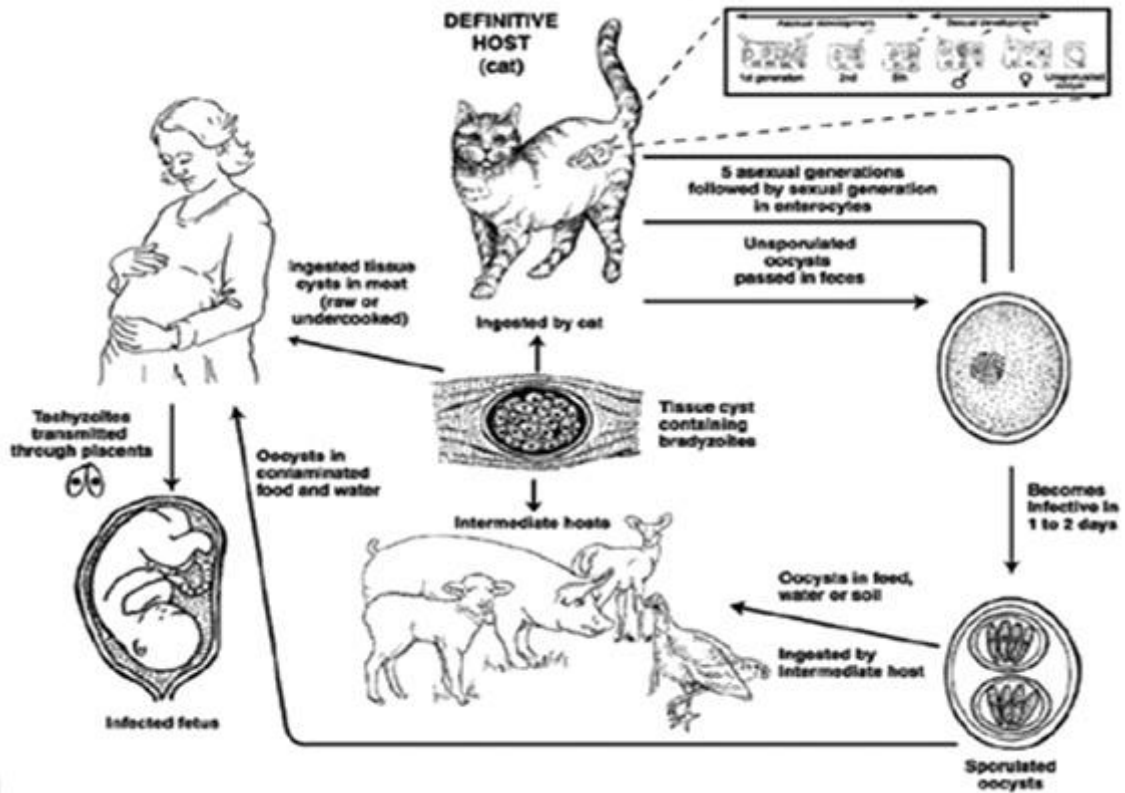
Figure 4. *Toxoplasma gondii* life cycle. *(*From Dubey JP and Lindsay DS. Biology of *Toxoplasma gondii* in cats and other animals. In World Class Parasites Vol. 9: Opportunistic Infections: *Toxoplasma, Sarcocystis*, and Microsporidia. Lindsay DS & Wiess LM (Eds). Kluwer Academic publishers. Boston, MA. Figure 1. pg   2. Copyright© 2004. Reprinted with kind permission of Springer Science and Business Media.)
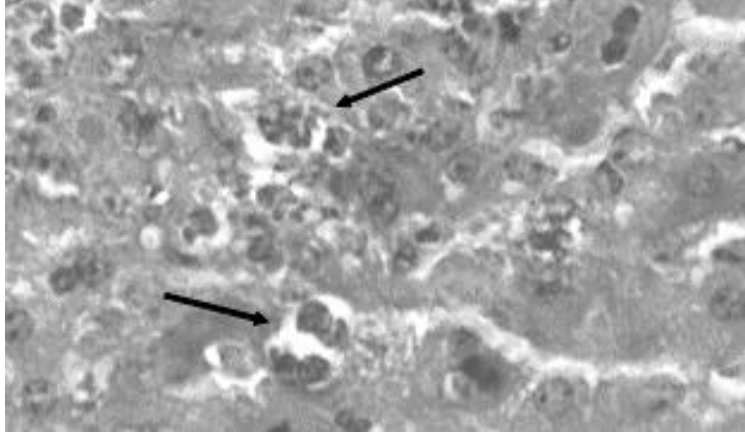
Figure 5: *T. gondii* tachyzoite in liver section of mouse.
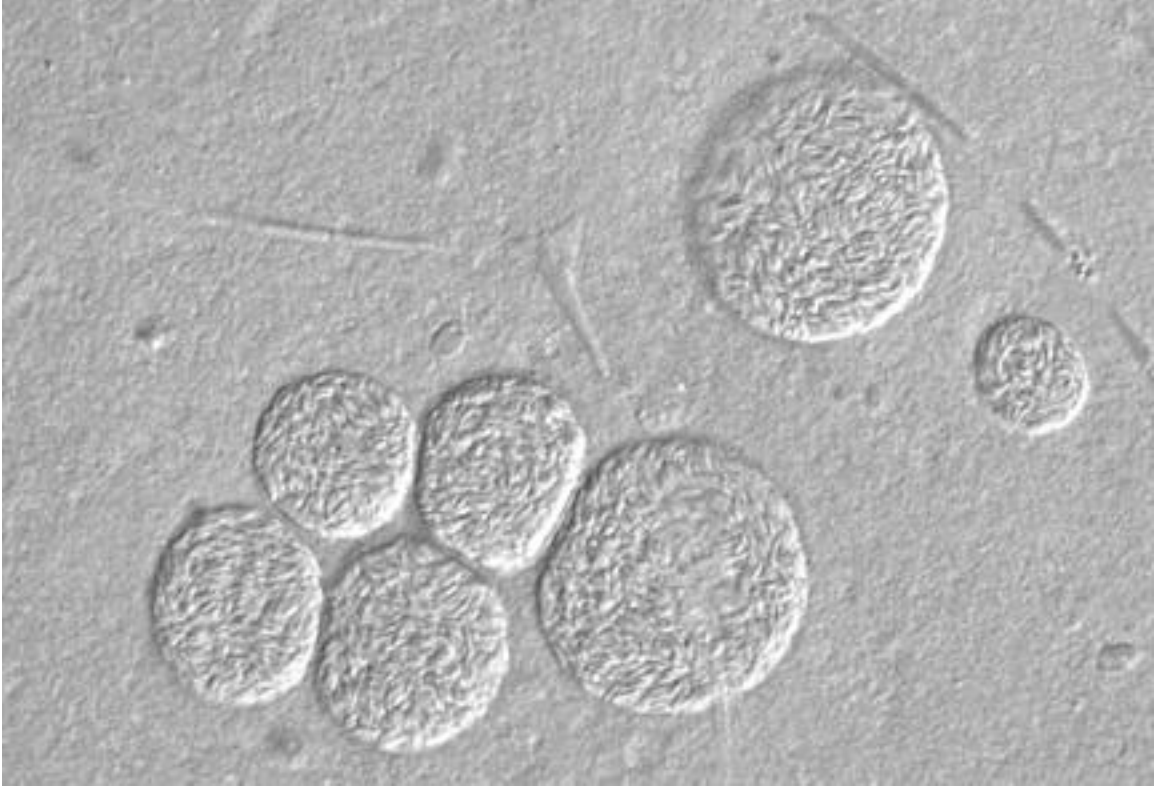
Figure 6: *T. gondii* tissue cyst containing bradyzoites.

Table 1. Species of *Cystoisospora* able to infect canines. *(Data from Lindsay DS and Blagburn BL. 1994. Biology of Mammalian Isospora. Parasitology Today. 10:214-220. and Levine ND and Ivens V. 1965. Isospora species in the dog. The Journal of Parasitology. 51:859-864.)*

	Oocysts LengthxWidth (μm)	Prepatent period (days)	Patent period (days)	Location in host[a]	Asexual stages
Cystoisospora canis	32-53 x 28-32	8-10	10-15	Epi, LP SI	3
Cystoisospora ohioensis	19-27 x 18-23	4-5	5-12	E SI[b] +LI	2
Cystoisopora burrowsi	17-22 x 16-19	5-7	9-15	E +LP SI	2
Cystoisospora neorivolta	20-27 x 15-24	6	13-23	LP SI	4

[a] Abbreviations: E, enterocytes: Epi, epithelium: SI, small intestine, mainly the posterior portion: LI, large intestine, LP, lamina propria

[b] *C. ohioensis* develops through out the small intestine.

Table 2. Serologic Assays used to Detect *T. gondii* Infections.

Serologic Assay	Antibody	Points of Interest	Reference
Sabin-Feldman dye test	IgG	• Considered "gold standard" • Not widely available b/c of the use of live organisms	Sabin and Feldman, 1948
Indirect fluorescent antibody test (IFAT)	IgG	• Safer than Dye test • Widely available • Special equipment needed • False-positives and False-negatives	Araujo et al., 1971
Direct agglutination test	IgG	• No special equipment needed • 2-β mercaptoethanol required • Excellent for screening animals	Dubey and Desmonts, 1987
Enzyme-linked immunosorbent assays (ELISA)	IgG	• Accurate • Confirms host is infected	Remington et al., 1995
	IgM	• Helps diagnose recently acquired infection • Use in conjunction with other assays • Diagnose congenital infection using cord blood	Remington et al, 1968
	IgA	• Diagnose infection in fetus or newborns • More sensitive than IgM for congenital diagnosis	Stepick-Biek et al., 1991
	IgE	• Positive for a shorter period of time than IgA or IgM • Diagnose acute infections • Toxoplasmic encephalitis patients are positive	Pinion et al, 1990

CHAPTER 3

CYSTOISOSPORA CANIS NEMESÉRI, 1959 (SYN. *ISOSPORA CANIS*), INFECTIONS IN DOGS: CLINICAL SIGNS, PATHOGENESIS, AND REPRODUCIBLE CLINICAL DISEASE IN BEAGLE DOGS FED OOCYSTS

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ABSTRACT

Canine intestinal coccidiosis is a cause of diarrhea in young dogs and dogs that are immunocompromised. Reports in the literature indicate that experimental reproduction of clinical coccidiosis with *Cystoisospora canis* (syn. *Isospora canis*) is difficult, and few studies have been done with *C. canis.* Experimental oral infections were attempted in 22, 6- to 8-wk-old female beagles with 5×10^4 (n = 2) or 1×10^5 (n = 20) sporulated *C. canis* oocysts. Diarrhea was observed in all inoculated dogs. Diarrhea began 2–3 days before oocyst excretion. Five of the 22 dogs were given an anticoccidial (sulfadimethoxine) because of their clinical signs. The mean prepatent period was 9.8 days (range, 9–11 days, n = 22 dogs), and the patent period was 8.9 days (range, 7–18 days, n = 20 dogs). Two dogs exhibiting clinical coccidiosis were examined at necropsy 10 days after infection. Developmental stages of *C. canis* were present in cells in the lamina propria throughout the entire small intestine in both dogs. Microscopic lesions observed in both of these dogs were villous atrophy, dilation of lacteals, and hyperplasia of lymph nodes in Peyer's patches. Results of bacterial and viral examinations of these 2 dogs were negative, indicating that intestinal coccidiosis was the cause of the diarrhea. Our study indicates that *C. canis* can be a primary cause of diarrhea in young dogs.

INTRODUCTION

Coccidia are common parasites of dogs worldwide. Dogs are hosts for *Cystoisospora canis* Nemeséri, 1959; *Cystoisospora ohioensis* Dubey, 1975; *Cystoisospora burrowsi* Trayser and Todd, 1978; and *Cystoisospora neorivolta* Dubey and Mahrt, 1978. In dogs, oocysts of *C. canis* can be definitively identified based on their structure in fecal samples because of their large size (>33 μm) when compared with the oocysts of *C. ohioensis*, *C. neorivolta*, and *C. burrowsi*, which are structurally similar (<30 μm) (Lindsay et al., 1997). The oocysts of these 3 similar-sized coccidial species are often grouped together and termed *C. ohioensis*–like oocysts because detailed structural examinations and life-cycle studies are needed before a definitive diagnosis can be made. The life cycle and transmission of *C. canis* has been examined by several groups of researchers (Neméseri, 1960; Lepp and Todd, 1974, 1976; Dubey, 1975b, 1982; Hilali et al., 1979; Becker et al., 1981). The life cycles and transmission of *C. ohioensis*, *C. neorivolta*, and *C. burrowsi*, have also been examined (Dubey, 1975a, 1978a, 1978b; Dubey and Mahrt, 1978; Dubey and Mehlhorn, 1978; Dubey et al., 1978; Trayser and Todd, 1978; Becker et al., 1981; Rommel and Zielasko, 1981.).

There is controversy over the pathogenicity of *C. canis* and other *Cystoisospora* species occurring in dogs. Severe clinical disease was not produced in 25, 6-wk-old or 6, 8-wk-old dogs inoculated with $1–1.5 \times 10^5$ *C. canis* oocysts of an Illinois isolate of the parasite (Lepp and Todd, 1974). Neméseri (1960) found that 5×10^3 oocysts of a Hungarian isolate of *C. canis* were not pathogenic for dogs, but an inoculum of 5 or 8×10^4 oocysts produced clinical

coccidiosis. The present study was done to evaluate the pathogenicity of an isolate of *C. canis* obtained from pit bull puppies. Additionally, the oocysts of *C. canis* are redescribed, and additional information on the life cycle of *C. canis* is presented.

MATERIALS AND METHODS

Source of oocysts

Oocysts consistent with the structure of *C. canis* were identified in the feces of 2 littermate pit bull puppies, housed at the Montgomery County animal shelter in Blacksburg, Virginia. The pups were 1–2 mo of age. Feces were collected from these puppies 1 or 3 times/wk from 26 February 2004 through 24 March 2004. These oocysts were mixed in 2% (v/v) sulfuric acid, filtered through 2 layers of cheesecloth, placed in a thin layer (4–6 mm) in 150-cm^2 tissue culture flasks with vented tops, and placed on a mechanical shaker for 4–6 days at room temperature. Oocysts were concentrated by flotation using Sheathers' sugar solution and stored at 4 C in 2% sulfuric acid until used. Oocysts were washed free of sulfuric acid in sterile Hanks balanced salt solution (HBSS) by centrifugation before use in experimental infections.

Dogs and fecal examinations

Five experiments using 22 female beagles were conducted (Table I). Dogs were obtained at 6–8 wk of age (Covance, Cumberland, Virginia). Weights were obtained upon the dogs' arrival at our facilities and at weekly intervals thereafter. Fecal samples were examined using centrifugal flotation in Sheathers' sugar solution. Fecal samples were examined daily until dogs were orally

infected (if feces were available). Samples were examined on days −1, 0, and 1–29 for coccidial oocysts. Quantitative fecal oocyst counts using the McMaster method were done when a dog became positive for the *C. canis* oocyst (Tables II, III). Briefly, the McMaster method was conducted by mixing 2 g of feces with 28 ml of Sheathers' sugar solution. Both sides of a McMaster counting slide were loaded with the mixture. Slides were allowed to sit for 5 min, and then all oocysts present were counted. The total numbers of oocysts counted was determined by multiplying the number counted by 50. Number 1 was used if the McMaster exam was negative, but the fecal float was positive.

Clinical signs

Clinical signs were recorded for each dog daily after clinical signs became apparent. Temperatures were obtained when dogs became clinically ill (Experiments 1–3) or at weekly intervals (Experiments 4–5). Fecal samples were scored daily (Table IV). Briefly, a score of 1 = normal-formed feces; 2 = mixture of loose and formed; 3 = completely loose but not liquid; and 4 = liquid. A note was made whether blood or mucus was present.

Hematocrit and total protein values were examined weekly in dogs from Experiments 4 and 5.

Experimental infections

Experiments 1–5 used an inoculum dose of 1×10^5 sporulated *C. canis* oocysts, whereas Experiment 3 used an inoculum dose of 5×10^4 sporulated *C. canis* oocysts in 2 of the 4 dogs in addition to the dose listed above in the remaining 2 dogs. Dogs were orally infected by mixing the appropriate amount of

sporulated oocysts in commercial dog food (Hills Science Diet A\D, Topeka, Kansas). All dogs readily ate this mixture within 3–5 min, and none vomited the inoculum.

One dog (BAS) in Experiment 1 was treated orally with 5 mg of prednisone daily for 3 days before infection and then daily on days 1–6 and 8–12 after infection (Table I). Results of Experiment 1 indicated that prednisone immunosuppression was not needed, and none of the other dogs was given this treatment. Dogs BAR, BAS, ALF, ASF, and AJY were treated with 25 mg/kg sulfadimethoxine (Pfizer Inc., Groton, Connecticut) for 2–3 days because of severe diarrhea (Table I).

Pathogenicity and development study (Experiment 5)

Experiment 5, using 8 dogs, was designed to determine the role of *C. canis* in the pathogenicity of diarrhea observed in the infected dogs and to rule out other causes, such as bacteria and viruses. The sporulated oocyst inoculum was treated with 50% v/v bleach solution for 5 min on an ice bath and then washed by centrifugation in cold sterile HBSS until the smell of bleach was no longer present. This inoculum was then streaked onto blood agar and TSA agar to detect bacteria that may have survived bleach treatment. This inoculum was used to infect 8 beagles.

Two dogs (AJV and AIZ-2) were killed 10 days postinoculation (PI). A board-certified pathologist (R.B.D) conducted the necropsy. Intestinal tissues were collected for bacteriological culture and histological examination. Additional tissues collected for histology only and fixed in 10% neutral buffered formalin

solution were mesenteric lymph nodes, liver, and spleen. Formalin-fixed tissues were embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin. Feces were collected for virology and examined by transmission electron microscopy (TEM) after negative staining at the Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas. Additionally, portions of ileum were fixed in 3% (v/v) glutaraldehyde in phosphate buffer (PBS, pH 7.4). Tissues were postfixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanols, passed through 2 changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, Pennsylvania). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10CA TEM operating at 60 kV. Digital images were captured using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, Massachusetts).

Thick sections of resin-embedded tissues were stained with methylene blue-Azure II-Basic fuchsin triple stain (Hayat, 1989) and mounted on glass slides for observation with light microscopy.

Immunohistochemistry

Immunohistochemistry was done to determine whether developmental stages of *C. canis* contained cross-reactive antigens to *Neospora caninum*, *Toxoplasma gondii*, or *Sarcocystis neurona*. Parasite-specific antisera were made in rabbits and used at dilutions of 1:500 and 1: 1,000. Paraffin-embedded tissue sections of *C. canis*–infected ileum were cut at 6 µm, mounted on glass slides, and used for immunohistochemical examinations using the avidin–biotin

immunoperoxidase complex (ABC) test, as previously described by Lindsay and Dubey (1989). Positive controls for parasite cross-reactivity were tissue sections containing developmental stages of *T. gondii*, *N. caninum*, or *S. neurona*.

Redescription of C. canis

Sporulated oocysts from pit bull puppies were examined using an Olympus BX60 microscope equipped with differential contrast optics and a digital camera. Measurements were obtained from 25 oocysts using oil emersion and a calibrated ocular micrometer.

RESULTS

Cystoisospora ohioensis–like oocysts were observed in the feces of dogs in Experiment 3 (4 of 4 dogs), Experiment 4 (2 of 4 dogs), and Experiment 5 (2 of 8 dogs) before infection with *C. canis* oocysts (Tables I–III). Clinical signs were not associated with the presence of these *C. ohioensis*–like oocysts. All dogs that excreted *C. ohioensis*–like oocysts were susceptible to clinical coccidiosis when fed *C. canis* oocysts orally (Table I). The 2 dogs (AJV and AIZ2) used in Experiment 5 for histology and pathology studies never excreted *C. ohioensis*–like oocysts, and that was a selection criterion for their use in the studies. The *C. canis* oocyst counts for the dogs in Experiments 1–5 are presented in Tables II and III.

Clinical signs

Clinical coccidiosis was induced in all dogs in Experiments 1–5 (Table I). Fecal scores are presented in Tables IV–V. Fecal scores of 3 or 4, indicating severe diarrhea, were usually seen 2–3 days before oocyst excretion. Clinical

signs were consistent with canine coccidiosis and included watery or bloody diarrhea, anorexia, weight loss, vomiting, and lethargy. Increased rectal temperatures were also noted in most dogs. Hematocrit and total protein values obtained from dogs in Experiments 4 and 5 were within normal ranges (37–55% hematocrit; 5.2–7.8 g/dl total protein) for dogs. Total weight gains for dogs ranged from 0.2 to 3.0 kg (Table VI).

All dogs excreted *C. canis* oocysts. The mean prepatent period was 9.8 days (range, 9–11 days, n = 22 dogs), and the patent period was 8.9 days (range, 7–18, n = 20 dogs).

Pathogenicity and development

Results of histopathological examination of small intestine documented asexual stages and sexual stages of *C. canis* within the subepithelial lamina propria of intestinal villi (Figs. 1–4). There was mild villous atrophy; moderate, diffuse villous epithelial cell attenuation; moderate crypt epithelial cell hyperplasia; occasional widely scattered, mildly dilated lacteals; and marked lymphoid hyperplasia of the Peyer's patches (Fig. 5). Occasional crypts contained a few eosinophils, polymorphonuclear leukocytes, and necrotic epithelial cells. Rare sexual stages of *C. canis* were present in the colon. Extraintestinal stages of *C. canis* were not detected in the mesenteric lymph nodes, but there was moderate-to-marked lymphoid hyperplasia, mild sinus histiocytosis, and occasional scattered foci of neutrophils and eosinophils.

No bacterial growth was observed on the blood agar or TSA agar plates after 3 days of incubation with sterilized oocysts mixture used to infect dogs. No

bacterial pathogens were isolated from the intestines of the 2 dogs killed and examined at necropsy. No viruses were detected by electron microscopy in the feces from these 2 dogs.

Schizonts, merozoites, macrogamonts, microgamonts, and oocysts were present in all sections of small intestines (Figs. 1–4) from both dogs. Developmental stages were located in a parasitophorous vacuole in host cells that were in the lamina propria. Different developmental stages appeared to be in the same host cell (Fig. 3). Immature schizonts and mature merozoites could also been seen in the same cell. Occasionally, macrogamonts and microgamonts were seen in the same host cell. Light microscopic observations on asexual stages occupying the same host cell were validated by examinations using TEM.

Immunohistochemistry

Developmental stages of *C. canis* did not react with antibodies to *T. gondii*, *N. caninum*, or *S. neurona*.

<center>**REDESCRIPTION**</center>

Cystoisospora canis.

Diagnosis.

Oocysts ovoid. Micropyle absent; oocyst residuum absent. Sporulated oocysts measure 37.2 ± 1.0 by 29.5 ± 1.2 µm (35–39 by 27–32 µm, n = 25); length to width ratio 1.3 ± 0.06 (1.16–1.38, n = 25). Two sporocysts present in each oocyst; sporocysts ellipsoidal, Stieda and substieda bodies absent, sporocyst residuum present, composed of a compact spherical mass or dispersed granules. Sporocysts measure 21.2 ± 0.9 by 16.3 ± 0.1 µm (19–23 by

15–18 µm, n = 25); length to width ratio 1.3 ± 0.08 (1.17–1.47, n = 25). Four sporozoites in each sporocyst.

Taxonomic summary.

Type host. Domestic dog, *Canis familiaris*.

Other hosts. Coyotes, *Canis latrans*, are experimental (Loveless and Anderson, 1975, Dubey, 1982; Dunbar and Foreyt, 1985) and natural hosts (Dubey, Fayer et al., 1978).

Paratenic hosts. Mice, cats, dogs, swine, sheep, water buffalos, and camels (Dubey, 1975b, Hilali et al., 1992, 1995; Zayed and El-Ghaysh, 1998). These studies are based on feeding tissues of naturally or experimentally infected animals and finding oocysts of *C. canis* in canine feces after feeding of host tissues.

Location in host. Inside of host cells, within the lamina propria of the duodenum, jejunum, and ileum of the small intestine and rarely the colon.

Prepatent period. From 9 to 11 days (Neméséri, 1960; Lepp and Todd, 1974; present study) if oocysts are used as inoculum. The prepatent period is 8–9 days in dogs fed *C. canis*–infected mice (Dubey, 1975b).

Patent period. Either 4 wk (Neméséri, 1960) or 7–15 days (present study).

Sporulation time. Sporulation is complete in 48 hr at 20 C and 16 hr at 30 or 35 C (Lepp and Todd, 1976).

Material deposited. A phototype (see Bandoni and Duszynski, 1988) of sporulated oocysts is deposited in the U.S. National Parasite Collection (USNPC), Beltsville, Maryland. USNPC no. 097291.00.

Remarks

Amorphous inclusions were present between the sporont and oocyst wall of many unsporulated *C. canis* oocysts. These inclusions have been observed in unsporulated *Cystoisospora suis* oocysts from pigs (Biester and Murray, 1934; Lindsay et al., 1980, 1982) and unsporulated *Cystoisospora rivolta* oocysts from cats (Dubey, 1979). This material is not present in fully sporulated oocysts of these *Cystoisospora* species.

DISCUSSION

Intestinal coccidial infections in naturally infected dogs have been examined in many countries (Dubey, Weisbrode et al., 1978; Boch et al., 1981; Correa et al., 1983; Kirkpatrick and Dubey, 1987; Penzhorn et al., 1992; Daugschies et al., 2000; Junker and Houwers, 2000). It is difficult to attribute intestinal disease to coccidia unless other pathogens are ruled out in a thorough search for disease-causing agents (Lindsay et al., 1997). Most studies rely only on clinical signs and do not examine tissues for lesions or other pathogenic agents. Penzhorn et al. (1992) studied a commercial German Shepherd breeding kennel in South Africa and found *Cystoisospora* sp. oocysts in the feces of dogs with diarrhea, some of which were also hemorrhaging. These authors were not able to demonstrate canine pathogenic bacteria or viruses in the feces of these dogs (Penzhorn et al., 1992). They were not able to link oocyst excretion by bitches to coccidial infections in their puppies. Daugschies et al. (2000) reported that natural *Cystoisospora* sp. infections were regularly found in 3- to 4-wk-old

pups in dog-breeding facilities and that they were not always associated with diarrhea.

Experimental studies on the pathogenicity of canine coccidia are few, and they often conflict each other. Dubey (1978b) found that 5×10^5 *C. ohioensis* oocysts (administered as 1×10^6 sporocysts in the original paper) caused diarrhea in experimentally infected 7-day-old pups but not weaned pups or young dogs. Microscopic changes associated with *C. ohioensis* infection included villous atrophy, necrosis of apical enterocytes, and cryptitis (Dubey, 1978b). Daugschies et al. (2000) reported puppies (age not given) experimentally infected with 4×10^4 oocysts of the *C. ohioensis* group developed catarrhal-to-hemorrhagic diarrhea. Little is known about the pathogenicity of *C. neorivolta* (Mahrt, 1967; Dubey and Mahrt, 1978) or *C. burrowsi* (Trayser and Todd, 1978; Rommel and Zielasko, 1981).

Levine and Ivens (1981) suggested that strain differences in pathogenicity of *C. canis* could be present in dogs. Neméseri (1960) found that 5×10^3 oocysts of a Hungarian isolate of *C. canis* were not pathogenic for dogs, but an inoculum of 5 or 8×10^4 oocysts produced clinical coccidiosis. In contrast, severe clinical disease was not produced in 25, 6-wk-old or 6, 8-wk-old pups inoculated with 1–1.5×10^5 *C. canis* oocysts (Lepp and Todd, 1974) isolated in dogs from Illinois. The pathogenicity of *C. canis* oocysts in the present study are more similar to what was reported by Neméseri (1960), rather than what was reported by Lepp and Todd (1974).

The present study demonstrated that *C. canis* is a primary pathogen in young dogs. Our histological studies demonstrated lesions (Fig. 5) in the small intestine, which were associated with the presence of developmental stages (Figs. 1–4) of *C. canis* and clinical signs of diarrhea. Bleach treatment of the inoculum rendered it free of bacteria, indicating that bacteria were not responsible for causing the clinical signs. Our attempts to demonstrate pathogenic bacteria and viruses in the 2 experimentally infected dogs examined at necropsy were negative, indicating that the coccidia were responsible for the clinical signs and microscopic lesions in these animals.

Solid immunity follows a primary *C. canis* infection, and no oocysts are discharged after challenge (Becker et al., 1981). We used young (6- to 8-wk-old) dogs in hopes of obtaining them before they developed a natural *C. canis* infection. Fortunately, none of our dogs came infected with *C. canis* because preinoculation fecal examinations for *C. canis* were negative and the timing of the prepatent period was consistent with the literature (Ne" />meséri, 1960; Lepp and Todd, 1974; Levine and Ivens, 1981). Prior infection is always a problem when working with coccidia in animals. Some of our dogs harbored *C. ohioensis*– like oocysts in their feces before infection (Tables I–III). However, this *C. ohioensis*– like infection did not prevent these dogs from being infected with *C. canis* nor did it preclude them from developing clinical signs. Neither of the 2 dogs used for microscopic lesion studies had prior infection with *C. ohioensis*– like coccidia. The reproducibility of clinical disease in this study suggests this is a good model

for canine coccidiosis. This canine coccidiosis model can be used to determine future efficacious anticoccidial agents.

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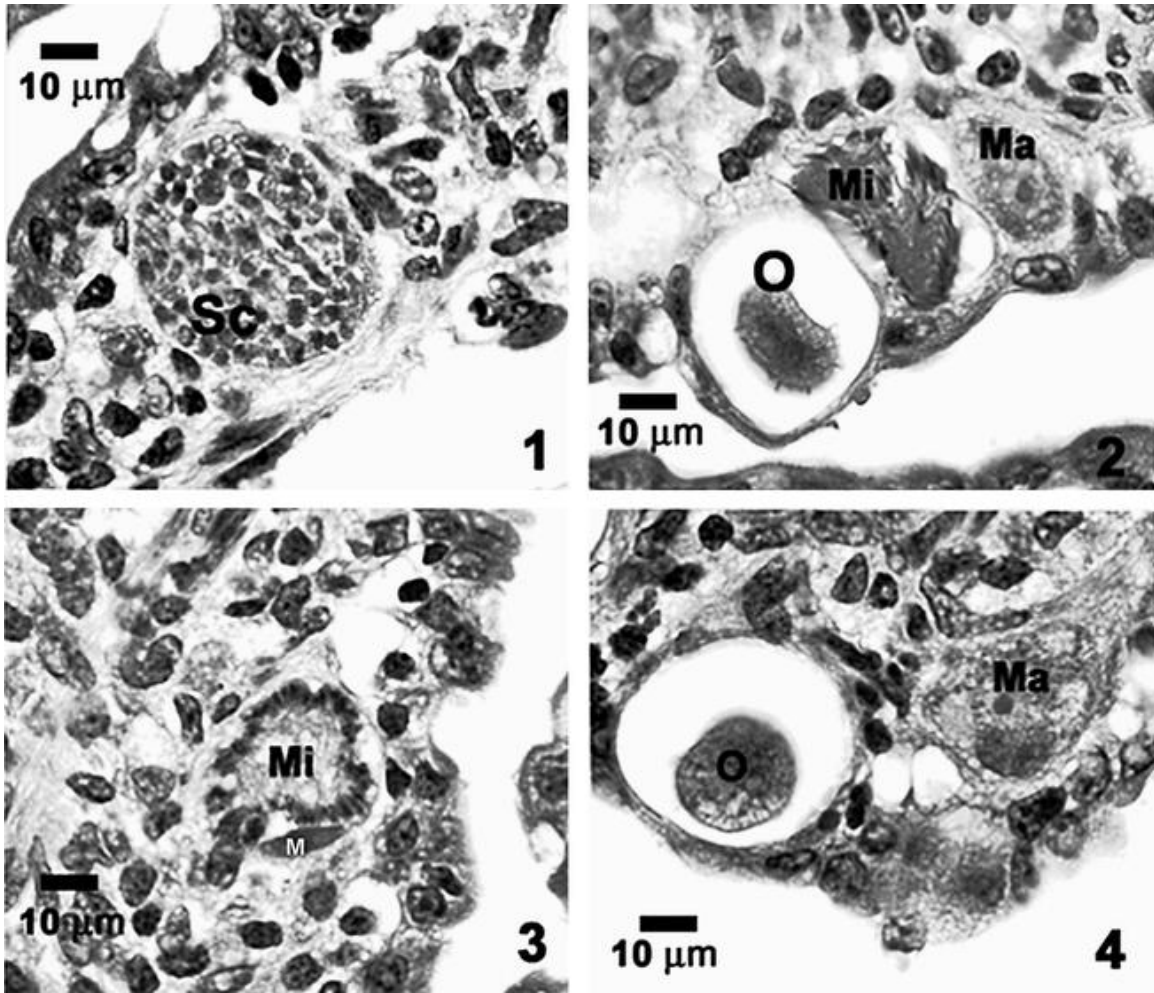
Figures 1–4. Hematoxylin and eosin stained histological sections of the ileum of dog AIZ infected with 1×10^5 *Cystoisospora canis* oocysts 10 days previously and demonstrating developmental stages in the intestinal lamina propria. (**1**) A mature schizont (Sc) containing numerous merozoites is located in a host cell in the lamina propria. (**2**) Several sexual stages including an oocyst (O), a mature microgamont (Mi) with microgametes, and a macrogamont (Ma) are present in this section. (**3**) An immature microgamont (Mi) that appears to be in the same host cell as a merozoite (M). The infected cell is in the lamina propria. (**4**) An oocyst with a contracted sporont (O) and a macrogamont (Ma) in the lamina propria.

Figure 5. Section of ileum from dog AIZ infected with 1×10^5 *Cystoisospora canis* oocysts 10 days previously. Note mild villous atrophy, dilated lacteals, and marked lymphoid hyperplasia of the Peyer's patches.

Table I. Experimental protocol for oral infection of dogs with sporulated oocysts of *Cystoisospora canis*, clinical signs, prepatent and patent periods in days.

Expt.[*]	Dog	Dose of Oocysts	Clinical signs	Prepatent period	Patent period
1	BAR[†]	1×10^5	yes	11	10
1	BAS[†‡]	1×10^5	yes	9	10
2	AHC	1×10^5	yes	10	5
2	ALF[†]	1×10^5	yes	10	8
2	AHP	1×10^5	yes	10	8
2	AIZ1	1×10^5	yes	10	8
3	BAG[§]	5×10^4	yes	9	9
3	AXY[§]	5×10^4	yes	10	7
3	BBH[§]	1×10^5	yes	10	18
3	AYF[§]	1×10^5	yes	10	7
4	ASF[†§]	1×10^5	yes	10	10
4	ASH	1×10^5	yes	10	11

4	ASI	1×10^5	yes	10	8
4	ASG[§]	1×10^5	yes	10	8
5	AIY[§]	1×10^5	yes	10	9
5	AJU	1×10^5	yes	10	8
5	AKA	1×10^5	yes	10	7
5	AJY[†]	1×10^5	yes	10	7
5	AJA	1×10^5	yes	10	7
5	AJZ[§]	1×10^5	yes	10	8
5	AJV	1×10^5	yes	9	NA
5	AIZ2	1×10^5	yes	9	NA

*Experiment number.

[†] Dog treated with sulfadimethoxine because of clinical coccidiosis.

[‡]This dog was treated orally with 5 mg prednisone daily 3 days before infection and then daily on days 1-6 and daily on days 8-12 after infection.

[§] *Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

Table II. Daily McMaster's oocysts counts per gram of feces per dog (days 8-17).

ID	8	9	10	11	12	13	14	15	16	17
BAR[*]	0	0	0	30500	22000	118400	82750	33200	204600	134200
BAS[*†]	0	12300	0	0	43450	77250	77900	59700	39150	3450
AHC	0	0	1	28800	0	23300	4300	0	0	0
ALF[*]	0	0	1	31450	29150	30700	28050	8900	3400	100
AHP	0	0	1	140650	66250	51800	33800	20700	11100	800
AIZ	0	0	1	137950	58350	83500	54750	18800	4800	1250
BAG[‡]	0	250	81650	200250	215650	29050	7000	2800	150	50
BBH[‡]	0	0	14600	137300	1370300	98400	118900	47250	27950	24500
AXY[‡]	0	0	5450	123050	64250	28100	6450	1700	100	0
AYF[‡]	0	0	36700	125000	113350	64750	38050	6700	50	0
ASF[*‡]	0	0	100	75600	20700	2900	10000	5600	2100	200
ASH	0	0	1900	22750	96000	49800	8600	5600	2000	1950

ASI	0	0	500	9150	120400	23100	3000	3100	3300	900
ASG‡	0	0	11450	114400	55800	24500	5900	400	1050	150
AIY‡	0	1	4400	14400	32900	28200	13300	12500	3600	850
AJU	0	1	176500	36100	83900	40300	24200	6500	550	50
AKA	0	0	8350	106800	101600	38100	92500	5200	5450	0
AJY*	0	0	22400	122600	125100	92500	124400	22000	1100	0
AJA	0	0	38700	47500	122500	59900	16000	20400	6050	0
AJZ‡	0	0	40000	109300	167800	138800	53600	11800	5050	300
AJV	0	1	5900	0	0	0	0	0	0	0
AIZ-2	0	1	17550	0	0	0	0	0	0	0

*Dog treated with sulfadimethoxine because of clinical coccidiosis.

[†]This dog was treated orally with 5 mg prednisone daily 3 days before infection and then daily on days 1-6 and daily on days 8-12 after infection.

[‡]*Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

Table III. Daily McMaster's oocysts counts per gram of feces per dog (days 18-27).

ID	18	19	20	21	22	23	24	25	26	27
BAR[*]	82400	4500	100	0	0	0	0	0	0	0
BAS[*†]	350	0	0	0	0	0	0	0	0	0
AHC	0	0	0	0	0	0	0	0	0	0
ALF[*]	0	0	0	0	0	0	0	650	1250	600
AHP	0	0	0	0	0	0	0	0	0	0
AIZ	0	0	0	0	0	0	50	0	0	0
BAG[‡]	0	0	0	0	0	0	0	0	0	0
BBH[‡]	3550	700	0	0	0	3250	1500	1	1	1
AXY[‡]	0	0	0	0	0	0	0	0	0	0
AYF[‡]	0	0	0	0	0	0	0	0	0	0
ASF[*‡]	850	250	0	0	0	0	0	0	0	0
ASH	1150200	200	50	0	0	0	0	0	0	0
ASI	0	0	0	0	0	0	0	0	0	0

ASG‡	0	0	0	0	0	0	0	0	0	0
AIY‡	100	0	0	0	0	0	0	0	0	0
AJU	0	0	0	0	0	0	0	0	0	0
AKA	0	0	0	0	0	0	0	0	0	0
AJY*	0	0	0	0	0	0	0	0	0	0
AJA	0	0	0	0	0	0	0	0	0	0
AJZ‡	0	0	0	0	0	0	0	0	0	0
AJV	0	0	0	0	0	0	0	0	0	0
AIZ-2	0	0	0	0	0	0	0	0	0	0

* Dog treated with sulfadimethoxine because of clinical coccidiosis.

†This dog was treated orally with 5 mg prednisone daily 3 days before infection and then daily on days 1-6 and daily on days 8-12 after infection.

‡*Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

Table IV. Daily fecal scores[*] post-inoculation (P.I.) (dogs BAR-AYF).

Days P.I.	BAR[†]	BAS[†‡]	AHC	ALF[†]	AHP	AIZ	BAG[§]	BBH[§]	AXY[§]	AYF[§]
-1	1	1	2	1	1	1	1	1	1	1
0	2	1	2	1	1	1	1	1	1	1
1	2	1	2	2	1	1	1	1	1	2
2	2	1	2	2	1	1	1	1	1	1
3	1	1	1	2	1	1	1	1	1	1
4	1	1	2	2	2	2	1	1	1	1
5	2	1	2	2	2	2	1	2	1	1
6	2	3	1	1	2	2	1	2	1	2
7	1	2	1	1	2	1	1	2	1	2
8	1	1	4	4	2	1	2	2	2	2
9	4	3	4	4	3	3	3	3	2	1
10	4	4	4	4	4	4	4	2	2	3
11	4	4	4	4	4	3	3	2	2	3
12	4	2	4	4	4	2	4	1	2	3
13	4	2	3	4	4	3	4	3	3	3
14	4	2	3	4	4	2	2	2	1	2
15	3	4	4	4	4	3	2	2	1	2
16	4	4	3	3	3	3	2	2	1	2
17	4	4	3	3	3	3	2	2	1	1
18	3	3	2	2	2	2	1	1	1	1

19	3	2	1	1	2	1	1	1	1	1
20	2	2	2	2	2	1	1	1	1	1
21	2	2	2	2	2	1	1	1	1	1
22	2	2	1	2	1	1	1	1	1	1
23	3	2	1	2	1	1	1	1	1	1
24	2	1	1	2	2	1	1	1	1	1

[*]A score of 1 = normal formed feces; 2 = mixture of loose and formed; 3 = completely loose but not liquid; and 4 = liquid.

[†] Dog treated with sulfadimethoxine because of clinical coccidiosis.

[‡] This dog was treated orally with 5 mg prednisone daily 3 days before infection and then daily on days 1-6 and daily on

days 8-12 after infection.

[§]*Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

Table V. Daily fecal scores[*] post-inoculation (P.I.) (dogs ASF-AIZ-2).

Days P.I.	ASF[†‡]	ASH	ASI	ASG[‡]	AIY[‡]	AJU	AKA	AJY[†]	AJA	AJZ[‡]	AJV	AIZ-2
-1	1	1	1	1	3	2	3	2	2	2	2	2
0	1	1	1	1	2	1	1	1	1	1	1	1
1	1	1	1	1	3	1	1	1	1	1	1	1
2	1	1	1	1	3	2	2	3	3	3	3	2
3	1	1	1	1	3	3	1	3	1	3	3	3
4	1	1	1	1	3	2	2	2	2	3	3	2
5	1	1	1	1	3	3	2	2	2	3	3	2
6	1	1	1	1	3	3	2	2	2	3	3	2
7	1	1	1	2	4	2	1	1	1	2	2	1
8	1	1	2	2	NA[§]	4	2	2	1	3	3	3
9	3	2	1	4	4	4	4	4	4	4	4	4
10	4	4	4	4	4	4	4	4	4	4	4	4
11	4	4	4	4	4	4	4	4	4	4	NA	NA
12	4	4	4	4	4	4	2	4	4	4	NA	NA
13	NA	4	3	4	3	4	4	4	4	4	NA	NA
14	3	3	3	3	3	3	2	4	3	2	NA	NA
15	3	3	3	3	3	3	1	3	3	3	NA	NA
16	3	3	3	3	3	3	2	3	3	3	NA	NA
17	1	1	3	3	3	3	1	3	3	3	NA	NA
18	1	1	1	1	2	1	1	3	3	2	NA	NA

19	1	1	1	NA	3	3	2	3	3	3	NA	NA
20	1	1	2	1	2	3	1	1	2	1	NA	NA
21	1	1	1	1	1	1	2	1	2	1	NA	NA
22	2	1	1	2	1	1	1	1	2	1	NA	NA
23	1	1	1	1	3	1	1	1	2	2	NA	NA
24	1	1	1	1	1	2	1	1	1	1	NA	NA

[*] A score of 1 = normal formed feces; 2 = mixture of loose and formed; 3 = completely loose but not liquid; and 4 = liquid.

[†] Dog treated with sulfadimethoxine because of clinical coccidiosis.

[‡] *Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

[§] Not applicable because no sample was obtained that day post inoculation.

Table VI. Beginning and ending weights of dogs in kilograms.

Expt.[*]	Dog	Oocysts	Beginning	Ending	Total gain
1	BAR[†]	1×10^5	2.5	3.8	1.3
1	BAS[†‡]	1×10^5	2.8	4.3	1.5
2	AHC	1×10^5	2.6	2.8	0.2
2	ALF[†]	1×10^5	2.0	3.1	1.1
2	AHP	1×10^5	2.8	3.8	1.0
2	AIZ	1×10^5	1.8	2.7	0.9
3	BAG[§]	5×10^4	1.6	4.3	2.7
3	BBH[§]	5×10^4	1.5	4.5	3.0
3	AXY[§]	1×10^5	1.9	4.7	2.8
3	AYF[§]	1×10^5	1.5	4.5	3.0
4	ASF[†§]	1×10^5	1.0	2.5	1.5
4	ASH	1×10^5	1.1	2.8	1.7
4	ASI	1×10^5	1.2	3.0	1.8
4	ASG[§]	1×10^5	1.0	2.5	1.5
5	AIY[§]	1×10^5	1.5	3.0	1.5
5	AJU	1×10^5	1.7	2.7	1.0
5	AKA	1×10^5	2.0	3.2	1.2
5	AJY[†]	1×10^5	1.7	2.7	1.0
5	AJA	1×10^5	2.0	3.0	1.0

5	AJZ[§]	1 x 10^5	1.8	3.2	1.4
5	AJV[‖]	1 x 10^5	2.2	2.4	0.2
5	AIZ-2[‖]	1 x 10^5	1.7	2.0	0.3

[*]Experiment number.

[†] Dog treated with sulfadimethoxine because of clinical coccidiosis.

[‡]This dog was treated orally with 5 mg prednisone daily 3 days before infection and then daily on days 1-6 and daily on days 8-12 after infection.

[§]*Cystoisospora ohioensis*-like oocysts observed in the feces of dog prior to experimental oral infection with *C. canis oocysts*

[‖] Dog was killed and examined at necropsy.

CHAPTER 4

DEVELOPMENT AND ULTRASTRUCTURE OF *CYSTOISOSPORA CANIS*

NEMESÉRI, 1959 (SYN. *ISOSPORA CANIS*) MONOZOITC CYSTS IN TWO

NON-CANINE CELL LINES

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ABSTRACT

Cystoisospora canis is a coccidial parasite of the intestinal tract that can cause severe disease in dogs. Clinical signs include watery diarrhea, vomiting, fever, and weight loss. Extraintestinal stages of *Cystoisospora* spp. have been demonstrated in the mesenteric lymph nodes of paratenic hosts. Information on the biology of extraintestinal stages of canine *Cystoisospora species* is limited. The current study examined the development of *C. canis* in 2 noncanine cell lines and the ultrastructure of the monozoic cysts that formed. Monolayers of bovine turbinate cells and African green monkey kidney cells were grown on coverslips and inoculated with excysted *C. canis* sporozoites. Coverslips were collected on various days and fixed and stained for light microscopy (LM) or transmission electron microscopy (TEM). A single, centrally located, slightly crescent- shaped sporozoite surrounded by a thick cyst wall within a parasitophorous vacuole was observed with the use of LM and TEM. No division and no multinucleated stages were observed with either LM or TEM. With TEM, typical organelles of sporozoites were observed, such as rhoptries, dense granules, a crystalloid body, polysaccharide granules, and a conoid. The structure and ultrastructure of *C. canis* monozoic cysts produced in vitro are similar to extraintestinal cysts of other *Cystoisospora* species in experimentally infected animals and those of *Cystoisospora belli* observed in immunocompromised humans. This is the first study that fully demonstrates in vitro the development of what structurally resemble extraintestinal cysts of a *Cystoisospora* spp.

INTRODUCTION

Cystoisospora canis Neméséri, 1959 (syn. *Isospora canis*), *C. ohioensis* Dubey 1975, *C. burrowsi* Trayser and Todd, 1978 and *C. neorivolta* Dubey and Mahrt, 1978 are four canine coccidial parasites. *Cystoisospora ohioensis*, *C. burrowsi* and *C. neorivolta* oocysts are structurally similar and are usually grouped together and termed *C. ohioensis*-like until further diagnosis can be made. Oocysts of *C. canis* are much larger (>33μm) and are easily identified in fecal samples (Lindsay et al., 1997). In the United States, canine coccidiosis infection rates range between 0.6% and 72% (Catcott, 1979) and are commonly seen in breeding facilities.

Life cycle and transmission studies for *C. canis in vivo* have been extensively examined (Neméséri, 1960; Lepp and Todd, 1974, 1976; Dubey, 1975, 1982; Hilali et al., 1979; Becker et al., 1981; Mitchell et al., 2007). Recently, *C. canis* has been shown to be the primary cause of severe diarrhea in 8-wk-old female beagle pups, indicating *C. canis* can be highly pathogenic (Mitchell et al., 2007). These puppies were infected orally with sporulated *C. canis* oocysts mixed in their food (Mitchell et al., 2007). Transmission is usually fecal-oral, however ingestion of a paratenic host containing extraintestinal monozoic cysts will also cause a patent infection. *Cystoisospora spp.* can form monozoite cysts in extraintestinal tissues in both the canine definitive host and paratenic hosts. Tissues most commonly infected with monozoic cysts are the mesenteric lymph nodes, spleen and liver. Patent infections occur when a canine definitive host ingests paratenic hosts, such as a small rodent infected with these

extraintestinal cyst stages (Dubey and Mehlhorn, 1978). However, the prepatent period is shorter and clinical signs associated with cystoisosporosis are not as severe when compared to ingestion of sporulated oocysts (Dubey, 1975).

Few studies have examined the development of *C. canis* in cell culture. The greatest development of *Cystoisospora species* in vitro usually occurs in primary cell lines from the host of the parasite (Doran, 1982). The current study examines *C. canis* development in African green monkey kidney cells (CV-1) and bovine turbinate cells (BT). This study also describes ultrastructure of monozoic cysts grown in cell culture.

MATERIALS AND METHODS

Inoculum

Cystoisospora canis oocysts were identified based on structure in the feces of 2 littermate pitbull puppies that were estimated to be 1-2 mo of age (Mitchell et al. 2007). Oocysts used in this study were collected from the feces of experimentally infected 8-wk-old beagles (Mitchell et al., 2007) Oocysts were collected and sporulated as previously described (Mitchell et al., 2007).

Sporulated *C. canis oocysts* were ruptured using a tissue grinder and then treated with an excysting medium containing 1.5% taurocholic acid (w/v) and 0.5% trypsin (w/v) in Hanks balanced salt solution (HBSS) at 37 C. Excystation media was washed off with HBSS. Excysted sporozoites were concentrated by centrifugation and resuspended in 2% fetal bovine serum in RPMI 1640 media supplemented with 100 U penicillin/ml and 100 mg streptomycin/ml.

Cell Culture

Bovine turbinate cells (BT, ATTC CRL-1390 American Type Culture Collection, Manassas Virginia) and African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1, ATTC CCL-70, American Type Culture Collection, Manassas Virginia) were grown to confluence on 22-mm^2 glass coverslips in 6-well cell culture plates in growth media that consisted of 10% fetal bovine serum in RPMI 1640 medium, supplemented with 100 U penicillin/ml and 100 mg streptomycin/ml. Coverslips were incubated at 37 C in a humidified incubator containing 5% CO_2 and 95% air.

Cell monolayers were inoculated with 1×10^5 excysted *C. canis* sporozoites. Twenty-four hours post inoculation the medium was removed; the monolayer was rinsed with HBSS, and replaced with maintenance media. Cover slips were removed and fixed in 10% buffered formalin on days 2, 6, 8, 10, 13, 15 post-infection (PI) for BT cells and days 2, 7, 10, 13, 16, 17 PI for CV-1 cells. Cover slips were stained with Diff-Quik® (Dade Berhing Inc., Newark, DE) and mounted on slides. The lengths and widths of 30 zoites and cyst walls and/or parasitophorous vacuoles were determined using a calibrated ocular micrometer under oil immersion on days 2, 10, 15 or 16 PI for BT and CV-1 cells, respectively.

Transmission Electron Microscopy

African green monkey kidney cells were grown to confluence in flasks and infected with 1×10^3 excysted *C. canis* sporozoites. On days 2, 6, 9, 11, and 15 PI, monolayers were removed by scrapping with a cell scrapper and suspensions

were pelleted by centrifugation. Pellets were fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4). Cell pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanol, passed through two changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10CA TEM operating at 60 kV. Digital images were captured using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, MA).

RESULTS

Cell culture findings

Excysted *C. canis* zoites infected both cell types and formed monozoic cysts. In both cell types, monozoite cysts had a single centrally located sporozoite within a parasitophorous vacuole. A thick cyst wall surrounded each zoite by the final collection day (Fig. 1). Most zoites were slightly crescent or oblong in shape. Multinucleated stages, sexual stages and oocysts were not observed in either CV-1 or BT cells. Replication of zoites was not observed with in the cyst wall at any time point. The surface of zoites appeared smooth with no projections or visible grooves. An area of pale blue staining was observed towards the center and/or at the posterior end in the zoites cytoplasm (Fig 1). In both cell types, sporozoites were positioned next to the host cell nucleus and in some cases were causing an indentation in the host cell nucleus. Two days post infection, zoites in BT cells were surrounded by a parasitophorous vacuole with no visible cyst wall (Fig 2a). In BT cells at 10 dPI, a cyst wall appeared to

originate from the surface of the zoites to begin filling in the parasitophorous vacuole (Fig 2b) and by 15 dPI most of the parasitophorous vacuole was filled with a thick cyst wall (Fig 2c). However, in CV-1 cells at day 2 post infection, thick cyst walls had already begun filling in parasitophorous vacuoles around some zoites and at day 10 PI many zoites were still surrounded by a parasitophorous vacuole with little to no cyst wall (Fig 3a and b). By 16 dPI, many zoites were surrounded by thick cysts walls but a few zoites remained in parasitophorous vacuole with no wall present (Fig 3c). Two sporozoites zoites were observed in the same host cell at 15 d PI in BT cells (Fig 4). This was not seen at any other time point in BT cells or in zoites grown in CV-1 cells.

Mean measurements of 30 *C. canis* zoites and the surrounding cyst walls and/or parasitophorous vacuole grown in BT and CV-1 cells and collected on various days PI is presented in Table 1. The measurement range of 30 *C. canis* monozoic cysts grown in BT cell and collected at 2 dPI is 11.0-25.0 μm x 1.5-9.0 μm (length x width), collected at 10 d PI is 13.0-24.0 μm x 7.5-12.0 μm and collected on 15 d PI is 10.0-23.0 μm x 7.0-13.0 μm. The measurement range of 30 *C. canis* monozoic cysts grown in CV-1 cells collected at 2 d PI is 13.0-23.0 μm x 6.0-13.0 μm, collected at 10 d PI is15.0-23.0 μm x 6.0-11.0 μm and collect at 16 d PI is 16.0-24.0 μm x 7.0-14.0 μm. In both BT and CV-1 cells the parasites did not increase in size from 2 d PI to 15 or 16 d PI, respectively.

Ultrastructural findings

Monozoic cysts were viewed in CV-1 cell samples collected for TEM. Findings were similar to light microscopy in that only one zoite was seen in each

cyst. Division of zoites within cysts was not seen at any time point. A cyst consisted of a single centrally located zoite surrounded by a thick granular/fibrous cyst wall within a PV (Fig 5). Only one zoite was found in a single host cell on viewing the micrographs. Some zoites had a fibrillar material between its surface and the inner surface of the thick granular wall. It was noted that the particulate material of the cyst wall was not as thick in immature cysts and were in a large PV compared to a more mature cyst where the PV was smaller. Tubular structures were noted at the interface of the tissue cyst wall and fibrillar material surrounding the zoite (Fig 6). The fibrillar area appears to originate from the surface of the zoite. A typical coccidian three-layered pellicle confined each zoite. The outer unit membrane surrounded the whole parasite and the inner membrane complex was interrupted at the anterior polar ring (Fig 7). Micropores were not seen. Organelles typical of coccidial sporozoites were present, such as a large crystalloid body, dense granules, micronemes and granules similar to polysaccharides and lipids were located in the cytoplasm of each zoite. The crystalloid body was composed of numerous, small, electron dense granules and was seen at the posterior end of the parasite. The crystalloid body was circular shaped and was not bound by a membrane but was surrounded by many dense bodies (Fig 8). Amylopectin-like granules were seen anterior to the crystalloid body suggesting that this is the posterior crystalloid body. The posterior crystalloid body was in close proximity to the zoites nucleus (not shown). At the apical end of the zoite, conoid and polar rings typical of coccidian parasites were seen (Fig 7). The ducts of at least 2 rhoptries can also

be seen going through the center of the conoid. The number of electron dense rhoptries could not be determined. Micronemes were located through out the zoites cytoplasm but were more numerous in the anterior region of the zoite. Dense bodies were located anteriorly and posteriorly to the zoites nucleus. Lipid-like and polysaccharide-like granules were present through out the zoites cytoplasm and did not appear to be concentrated at any particular end of the zoite. The mean length and width of zoites at 15 d PI was $8.40 \pm 1.53 \times 4.30 \pm 0.18$ µm.

DISSCUSSION

Cystoisospora species of dogs and cats are known to have extraintestinal stages (Dubey and Frenkel, 1972; Dubey, 1975, 1978, 1979; Dubey and Mehlhorn, 1978). Most of the information known about these stages comes from tissue feeding studies due to the rare observance of them in tissue sections. These dormant stages are thought to be the cause of intermittent oocysts shedding through out the life of canine and feline definitive hosts. The present study shows that *C. canis* was able to enter 2 non-canine cell types and develop into monozoic cysts. These monozoic cysts grown in cell culture resemble extraintestinal cyst stages of other mammalian *Cystoisopora* species. This is the first study to describe monozoic cysts of any *Cystoisospora* sp. grown in cell culture.

Our study suggests that the zoite with in these cyst stages are actually sporozoites based on zoite ultrastructure and the lack of division of zoites with in the PV at any time point. Malarial hypnozoites also originate from sporozoites

and are the cause of relapse of infection (Krotoski, 1989). *Cystoisospora* merozoite formation occurs through endodyogeny and through schizogony for *Eimeria* species. Neither of these merozoite forming processes were observed. However, Fayer and Mahrt, (1972) observed reproduction, most likely by endodyogeny, of *C. canis* sporozoites grown in 5 different cell culture types, 2 of which were primary canine cell lines. The zoite pairs were attached at their posterior ends. In all 5 cell lines, sporozoites entered the host cells close to the nucleus and were surrounded by a PV; encysted stages or sexual stags were not mentioned (Fayer and Mahrt, 1972).

We found only 1 zoite in each tissue cyst in BT and CV-1 cells. This is in agreement with reports for human, feline and other canine *Cystoisospora* species with in host cells (Dubey and Mehlhorn, 1978; Dubey, 1979; Lindsay et al., 1997). In a few instances, it appears that 2 sporozoites were able to enter a single BT host cell simultaneously. Lindsay et al., (1997) reported that more than one tissue cyst, lacking a developed cyst wall was able to occupy the same host cell. Our study shows that 2 monozoic cysts are able to develop tissue cyst walls within the same host cell. It is unclear whether the tissue cysts share a PV or are in separate vacuoles but it does not appear reproduction has taken place based on tissue cyst wall formation.

Few studies have grown *Cystoisospora* species in cell culture, so comparison of the cyst measurements grown in cell culture had to be made against monozoic cysts found in host tissues, unless noted otherwise. In our study, length and width of zoites within the monozoic cysts did not increase or

decrease with time in cultured cells. The cyst wall around the single zoite grew larger with time to fill in the PV. Dubey and Mehlhorn, (1978) measured single zoites within tissue cysts found in lymph nodes of mice fed *C. ohioensis* oocysts and found the zoites increased in size starting at 1 d PI with a mean measurement of 5.8 x 2.1 μm and ending at day 39 post infection with a mean measurement of 12.8 x 6.4 μm. At 14 d PI, the mean length and width of the zoites was 12.5 x 6.2 μm, which is no different than the zoites measured on day 36 PI (Dubey and Mehlhorn, 1978). The average length and width of paired *C. canis* zoites grown in cultured embryonic canine kidney cells was 12.2 x 3.8 μm when measured 3 d PI (Fayer and Mahrt, 1972). Again, suggesting that host and site specificity of the cell type may have a role in development of these cysts in cell culture. Our study found the mean length and width of *C. canis* zoites grown in BT and CV-1 cells are similar to extraintestinal tissue cysts found in definitive and transport hosts of other *Cystoisospora* species.

Ultrastructure of *C.canis* monozoic cysts grown in CV-1 cells are similar to cysts of other species found in tissues. The appearance of the centrally located zoite was that of typical coccidian sporozoites (Roberts et al., 1970; Roberts et al., 1972; Lindsay et al., 1997). The thick granular material that makes up the cyst wall has been observed in tissue cyst stages of many *Cystoisospora* species (Dubey and Frenkel, 1972; Dubey and Mehlhorn, 1978; Lindsay et al., 1997) and is similar to the fibrous covering of caryocysts of *Caryospora bigenetica* (Sundermann and Lindsay, 1989). In this study, the crystalloid body was made up of small, electron dense granules (Roberts et al., 1972) and was located

posterior to the nucleus. Crystalloid bodies have been described in sporozoites of most *Cystoisospora* species (Dubey and Frenkel, 1972; Mehlhorn and Markus, 1976; Dubey and Mehlhorn, 1978; Lindsay et al., 1997) and have a similar appearance to beta-glycogen particles. Roberts et al., (1972) noted an anterior and posterior crystalloid body in freshly excysted *C. canis* sporozoites. They noted amylopectin-like granules along the periphery of the posterior crystalloid body which is a typical association of refractile bodies in *Eimeria* species. Crystalloid bodies are thought to be analogous to refractile bodies found in sporozoites and merozoites of *Eimeria* species (Roberts and Hammond, 1970; Hammond et al., 1970) and are thought to be associated with the transfer of stored food but their true function remains unknown (Garnham et al., 1969; Desser, 1970).

Cystoisospora belli (syn. *Isospora belli*) is a coccidial parasite of humans that can cause serious disease in an immunocompromised host. Extraintestinal stages of *C. belli* have been reported as a probable cause of relapse for isosporiasis in AIDS patients (Restrepo et al., 1987; Michiels et al., 1994). In these patients numerous monozioc cysts are present in extraintestinal tissue. It is thought that these extraintestinal stages represent merozoites that have left the intestinal tract (Lindsay et al., 1997). As previously mentioned, we believe our cell culture derived cysts represent sporozoites. Recently, Siripanth et al., (2004) were able to grow *C. belli* from early schizogony through to sexual stages in human ileocecal adeno carcinoma cells (HCT-8), which are considered to be both host and site-specific in humans. They also observed early stage asexual

development of *C. belli* in a host specific but not a site-specific cell line (human larynx carcinoma, Hep-2) (Siripanth et al., 2004). In our study we did not use host or site-specific canine cell types as in the studies mentioned above. This could explain the lack of reproduction in our study compared to other studies which observed division of zoites within the PV. *C. belli* monozoic cysts in portions of spleen from a patient with AIDS averaged 12.2 x 2.5 µm and tissue cyst walls averaged 2.1 µm thick (Lindsay et al., 1997). Average measurements of monozoic cysts from our study are similar to this measurement. Division of zoites or projections from the zoites surface was not observed in any of the micrographs in this study. Lindsay et al., (1997) noted grooves and projections in the pellicles of *C. belli* zoites suggesting these projections could be mistaken for a second zoite within the same tissue cyst using light microscopy. The surface of the zoite appears to be directly associated with the fibrillar material surrounding it. This was also observed in *C. belli* monozoic cysts of found in mesenteric lymph nodes of an immunocompromised patient (Lindsay et al., 1997). However, the origin of the fibrillar material surrounding the zoite is currently unknown.

Extraintestinal tissue cysts of *Cystoisospora* species are often compared biologically to tissue cyst containing bradyzoites in patients with toxoplasmic encephalitis. Both are dormant tissue stages and are not susceptible to anticoccidial treatments. The cell culture derived monozoic cysts of *C. canis* would be a convenient model for examining chemotherapeutic agents or other treatment options for extraintestinal tissue cyst.

ACKNOWLEDGEMENTS

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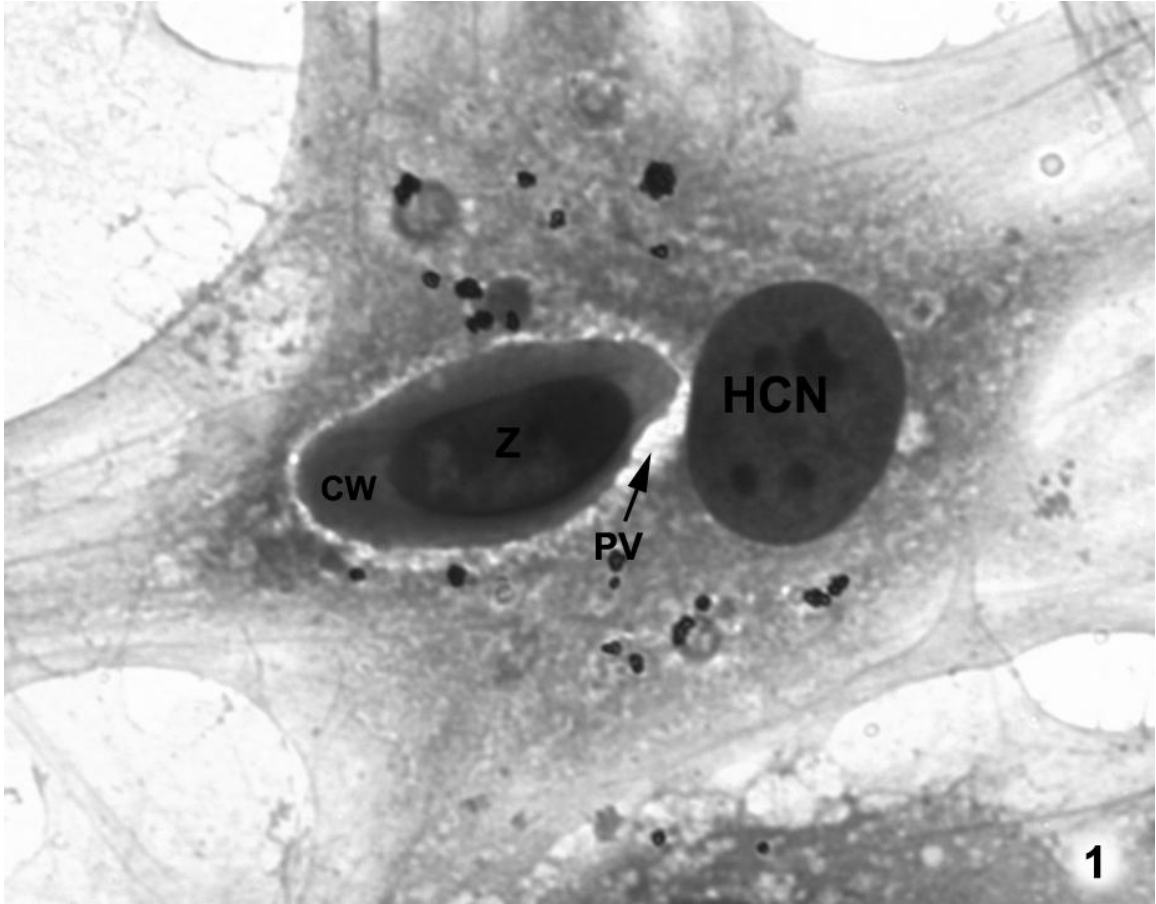
FIGURE 1. Light microscope appearance of monozoic cyst developed in bovine turbinate cells (oil). A single zoite (Z) was located in the center the parasitophorous vacuole (PV) and was surrounded by a thick cyst wall (CW). The cell culture derived monozoic cysts was located close to the host cell's nucleus (HCN).
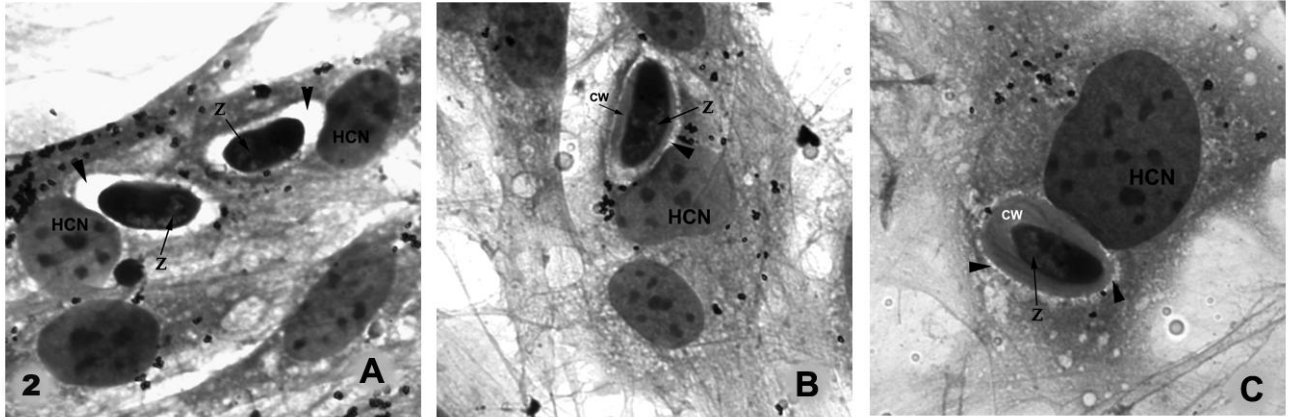
FIGURE 2. Cell culture derived monozoic cysts in BT cells observed by light microscopy. Notice all zoites are located close to the host cell's nucleus (HCN). (**A**) At 2 days post infection zoites (Z) were surrounded by a parasitophorous vacuole (arrow head) with no visible cyst wall (40x). (**B**) By 10 d PI a cyst wall (CW) has began to fill in the parasitophorous vacuole (arrow head) around the zoite (Z) (40x). (**C**) At 15 d PI the parasitophorous vacuole (arrow head) around the zoite (Z) is completely filled with the cyst wall (CW) (oil).
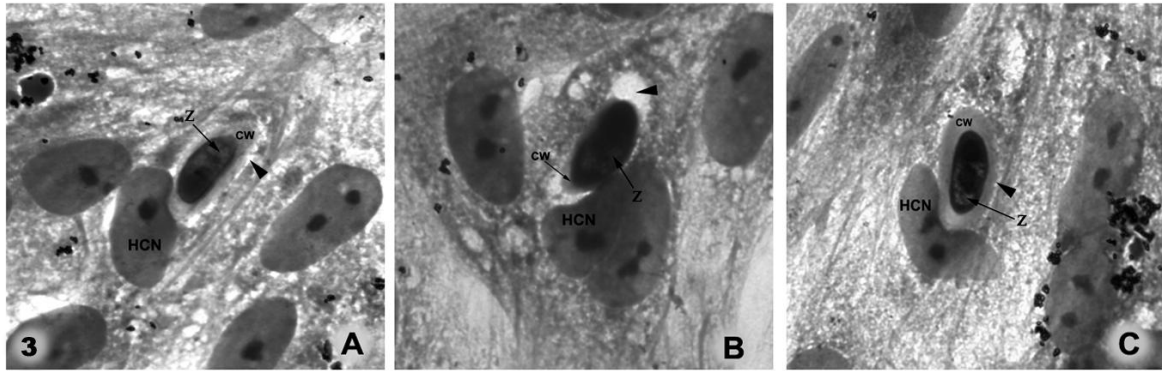
FIGURE 3. Cell culture derived monozoic cysts in CV-1 cells observed by light microscopy (40x). Notice all zoites are located close to the host cell's nucleus (HCN). (A) At 2 dPI a thick cyst wall (CW) surrounding the zoite (Z) and at (B) 10 dPI some zoites were surrounded by a parasitophorous vacuole (arrow head). (C) At 16 dPI most zoites (Z) were surrounded by a thick cyst wall (CW) that has filled in the parasitophorous vacuole (arrow head).
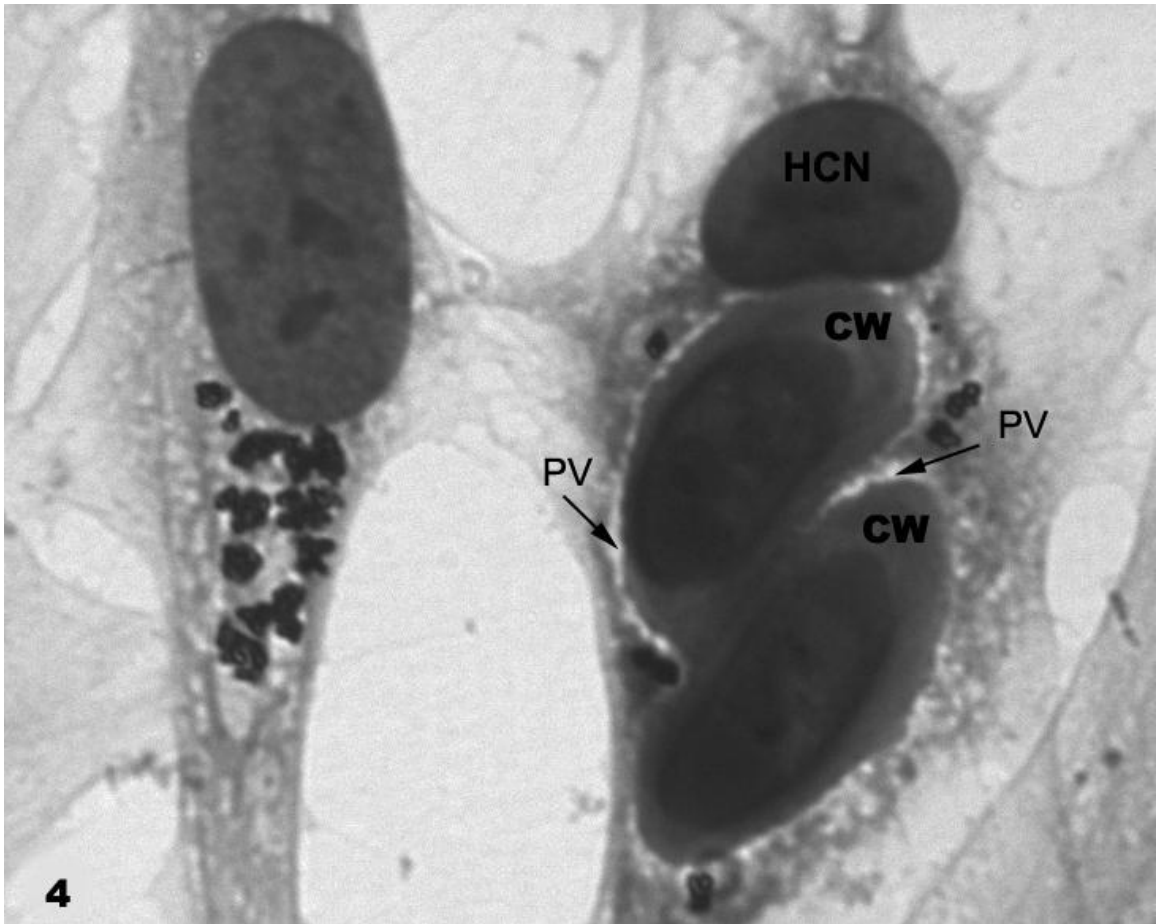
FIGURE 4. Two monozoic cysts develop in the same BT cell at 15 dPI (40x). Both zoites appear to be in the same parasitophorous vacuole (PV) but the cyst wall (CW) around each zoite appears to have developed individually. Development occurred near the host cell nucleus (HCN).

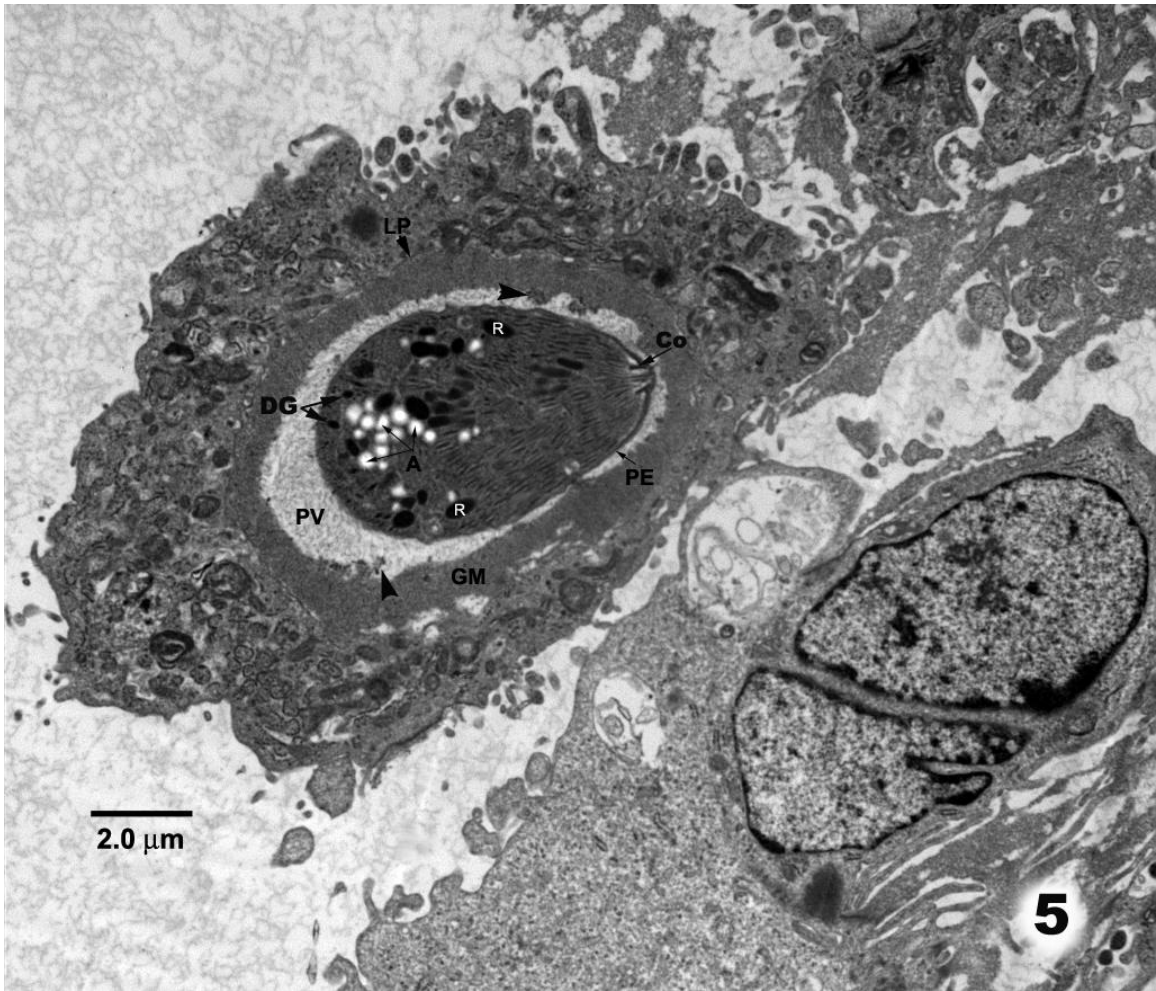FIGURE 5. Transmission electron micrograph of a monozoic cyst in CV-1 cells 15 dPI. Note the thick granular material (GM) that makes up the cyst wall which is beneath the limiting membrane (LP) of the parasitophorous vacuole (PV). The zoite has a three layerd pellicle (PE) and contains typical sporozoite organelles; Amylopectin granules (A), rhoptries (R), dense granules (DG) and a conoid (Co).
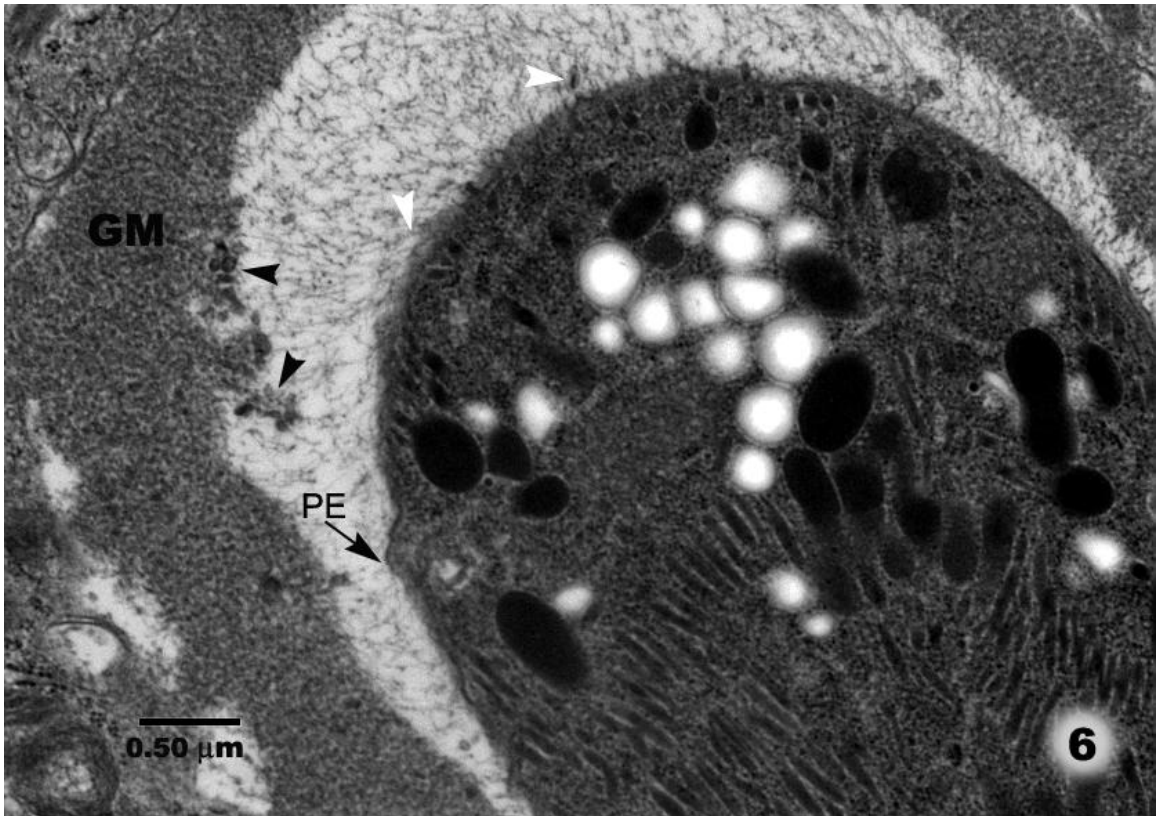
FIGURE 6. Transmission electron micrograph of a monozoic cyst in CV-1 cells 15 dPI. Tubular structures (black arrow head) at the interface of the granular material (GM) of the tissue cyst wall and fibrillar material (white arrow heads) surrounding the zoite. Note the fibrillar area appears to originate from the surface of the zoite (PE).
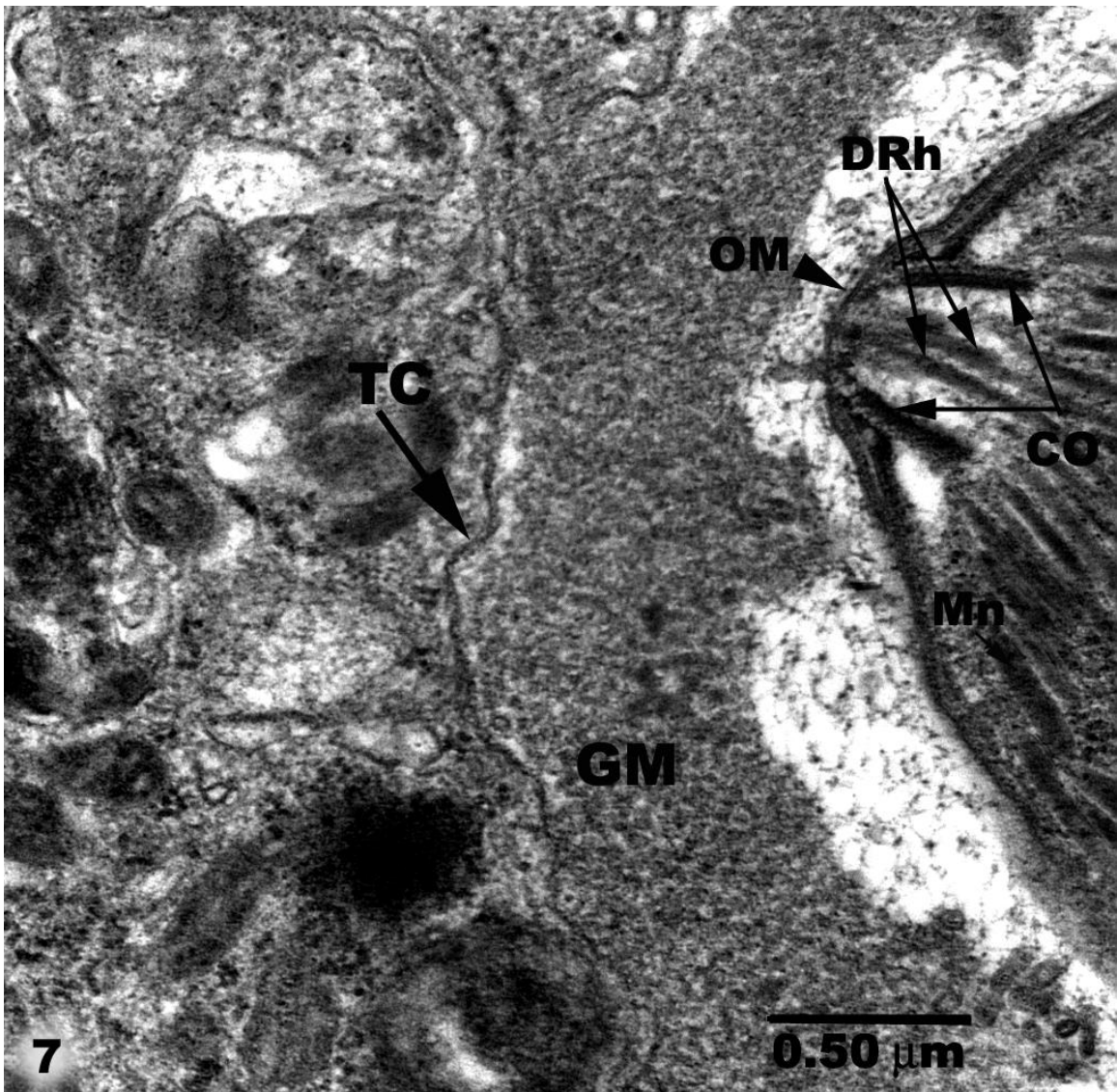
FIGURE 7. Transmission electron micrograph of the apical end of a cultured cyst 15 dPI. The outer membrane unit (OM) of the three layered pellicle surrounds the whole zoite. A tissue cyst limiting membrane (TC) is contacting the outer surface of the granular material (GM) which has filled in the parasitophorous vacuole. Note a typical coccidian conoid (Co) with two rhoptry ducts (DRh) running through the center. Micronemes (MN) can be seen throughout the zoite.
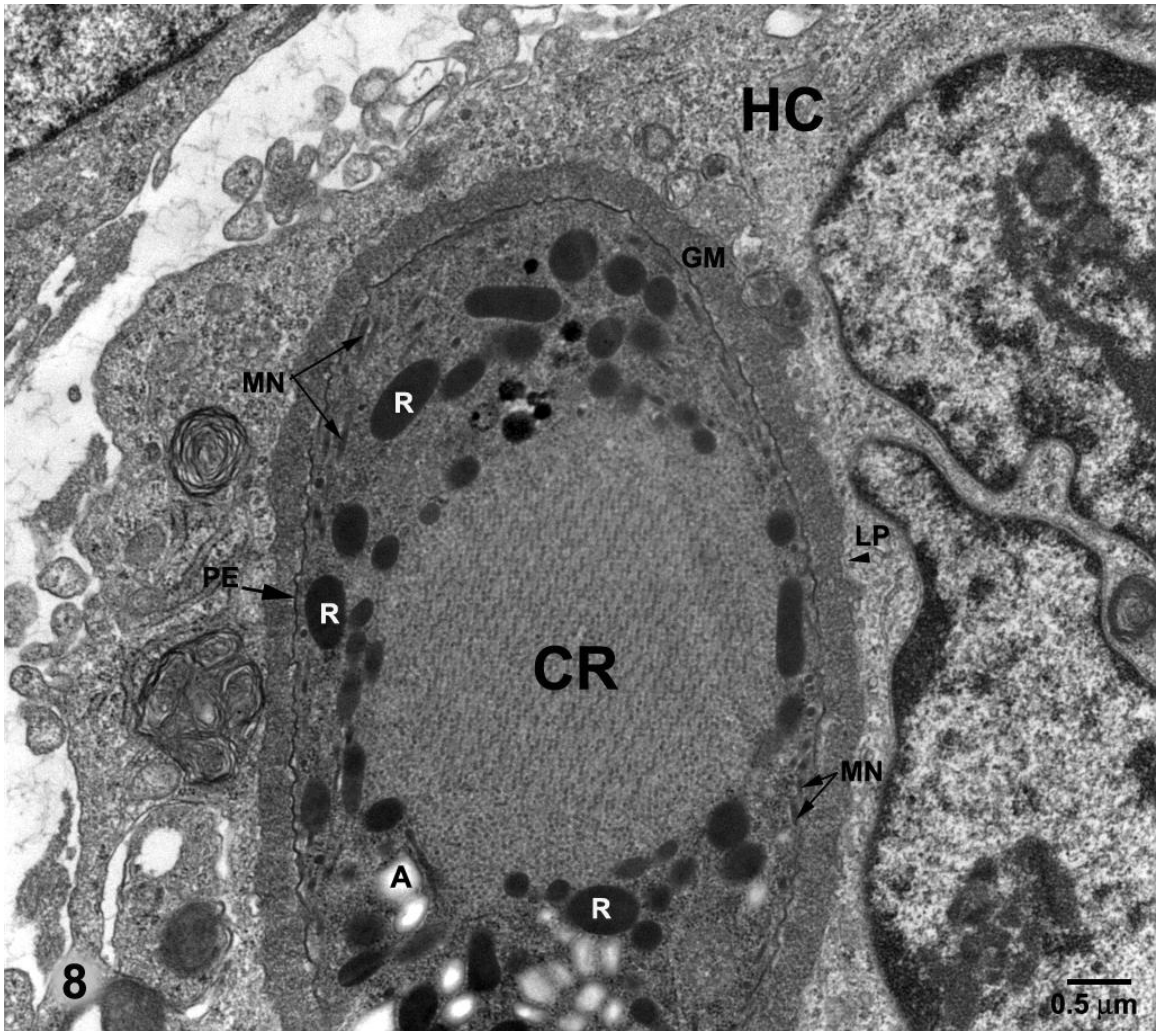
FIGURE 8. Transmission electron micrograph of cell cultured monozoic cyst collected 9 dPI. A zoite that demonstrates a large posterior crystalloid body (CR), rhoptries (R), micronemes (MN), amylopecitn-like granules (A), a three layered pellicle (PE) and a cyst wall of granular material (GM) within the limiting membrane (LP) of the parasitophorous vacuole. Host cell (HC).

Table I. Light Microscopy measurements of 30 monozoic cyst grown in cell culture (Mean ± SD).

Day PI	BT Cells		CV-1 Cells	
	Zoite	PV and/or Cyst wall	Zoite	PV and/or Cyst wall
2	12.0 ± 1.4 x 5.1 ± 0.8 µm	2.6 ± 0.9 x 1.4 ± 0.7µm	13.6 ± 1.4 x 5.8 ± 0.6 µm	2.9 ± 1.2 x 1.5 ± 0.9 µm
10	13.9 ± 1.0 x 5.8 ± 0.6 µm	3.4 ± 1.1 x 2.2 ± 0.8 µm	13.8 ± 1.1 x 6.2 ± 0.6 µm	3.1 ± 1.3 x 1.3 ± 0.8 µm
15	13.6 ± 0.9 x 6.0 ± 0.6 µm	4.2 ± 0.8 x 4.0 ± 0.8 µm	NA	NA
16	NA	NA	13.8 ± 1.0 x 5.9 ± 0.8 µm	3.8 ± 1.1 x 2.4 ± 1.0 µm

CHAPTER 5

MODE OF ACTION OF PONAZURIL AGAINST *TOXOPLASMA GONDII* TACHYZOITES IN CELL CULTURE

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ABSTRACT

Toxoplasma gondii is an important apicomplexan parasite of humans and other warm-blooded animals. Ponazuril is a triazine anticoccidial recently approved for use in horses in the United States. We investigated the mode of action of ponazuril against developing RH strain of *T. gondii* tachyzoites in African green monkey kidney cells. Host cells were infected with 2.0×10^5 tachyzoites and treated with 5 µg/ml ponazuril. Cultures were fixed and examined by transmission electron microscopy 3 days after treatment. Ponazuril interfered with normal parasite division. This led to the presence of multinucleate schizonts stages. Up to six tachyzoites were observed partially budded from the surface of these schizonts. Large vacuoles developed in these schizonts and they eventually degenerated.

INTRODUCTION

Toxoplasma gondii is an important parasite of humans and other warm-blooded animals. There are about 1,500,000 cases of toxoplasmosis in the United States each year and about 15% of those infected have clinical signs (7,11). Congenital toxoplasmosis has long been recognized because of the devastating results it can have on the infected fetus (6). These include hydrocephalus, blindness, and mental retardation. Congenitally infected children that are less severely infected may suffer from a variety of neurological related aliments throughout their lives (12). In the United States it is estimated that 85% of women of child-bearing age are at risk for toxoplasmosis (7) and that up to 4,000 cases of congenital toxoplasmosis occur each year (6). Toxoplasmic encephalitis (TE) became recognized as an AIDS defining illness in the early 1980's and TE is still the most important neurological component of AIDS (10). Toxoplasmosis is also a frequent and fatal complication in patients that receive organ transplantation (13). The annual economic impact of toxoplasmosis in the human population in the United States is about $7.7 billion (1).

Ponazuril is the major metabolite of toltrazuril, a triazine anticoccidial used in the poultry industry. Ponazuril has been shown to be active against *Sarcocystis neurona* in vitro (8) and in vivo (3) and against *Neospora caninum* in vivo (4). The present study was conducted to determine the in vitro activity of ponazuril against the RH strain of *T. gondii*.

MATERIALS AND METHODS

African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection) were grown to confluence in 25 cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin G/ml and 100 mg streptomycin/ml. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air.

Ponazuril (lot PFA101; Bayer HealthCare Animal Health) was used in the present study. Ponazuril was dissolved in DMSO and to make a stock solution of 1 mg/ml. Cell monolayers were inoculated with 2.0×10^5 RH strain *T. gondii* tachyzoites. Two hours after inoculation, the medium was removed and replaced with maintenance medium containing ponazuril at a concentration of 5.0 µg/ml. Control flasks received maintenance medium without ponazuril. Three days after infection the infected monolayers were removed from the plastic growth surface by scrapping with a cell scrapper. The suspensions were pelleted by centrifugation and processed for transmission electron microscopy (TEM). The pellet was fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4). Cell pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanol, passed through two changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc.). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10CA TEM operating at 60 kV. Digital images were captured using an ATM camera system (Advanced Microscopy Techniques Corp.).

RESULTS AND DISCUSSION

The non-treated tachyzoites developed by endodyogeny (2). Ponazuril treatment interfered with normal tachyzoite division. Tachyzoites that had not undergone nuclear division appeared normal. Alterations were seen in dividing organisms. The most prominent alteration was the presence of up to 6 nuclei in some stages. Tachyzoites were observed partially budded from the surface of these schizonts (Fig. 1). Many schizonts contained various size vacuoles ranging from 2 to 15 in number and up to 3.4 µm in diameter. Some of the larger vacuoles contained membranous inclusions. The golgi of some schizonts appeared slightly swollen. Some schizonts were degenerated. Our findings are similar to those reported by Lindsay et al. (9) for diclazuril against *T. gondii*. The apicoplast is a reported target for the triazine anticoccidials (5). We are currently investigating the molecular mode of action of ponazuril against *T. gondii*.

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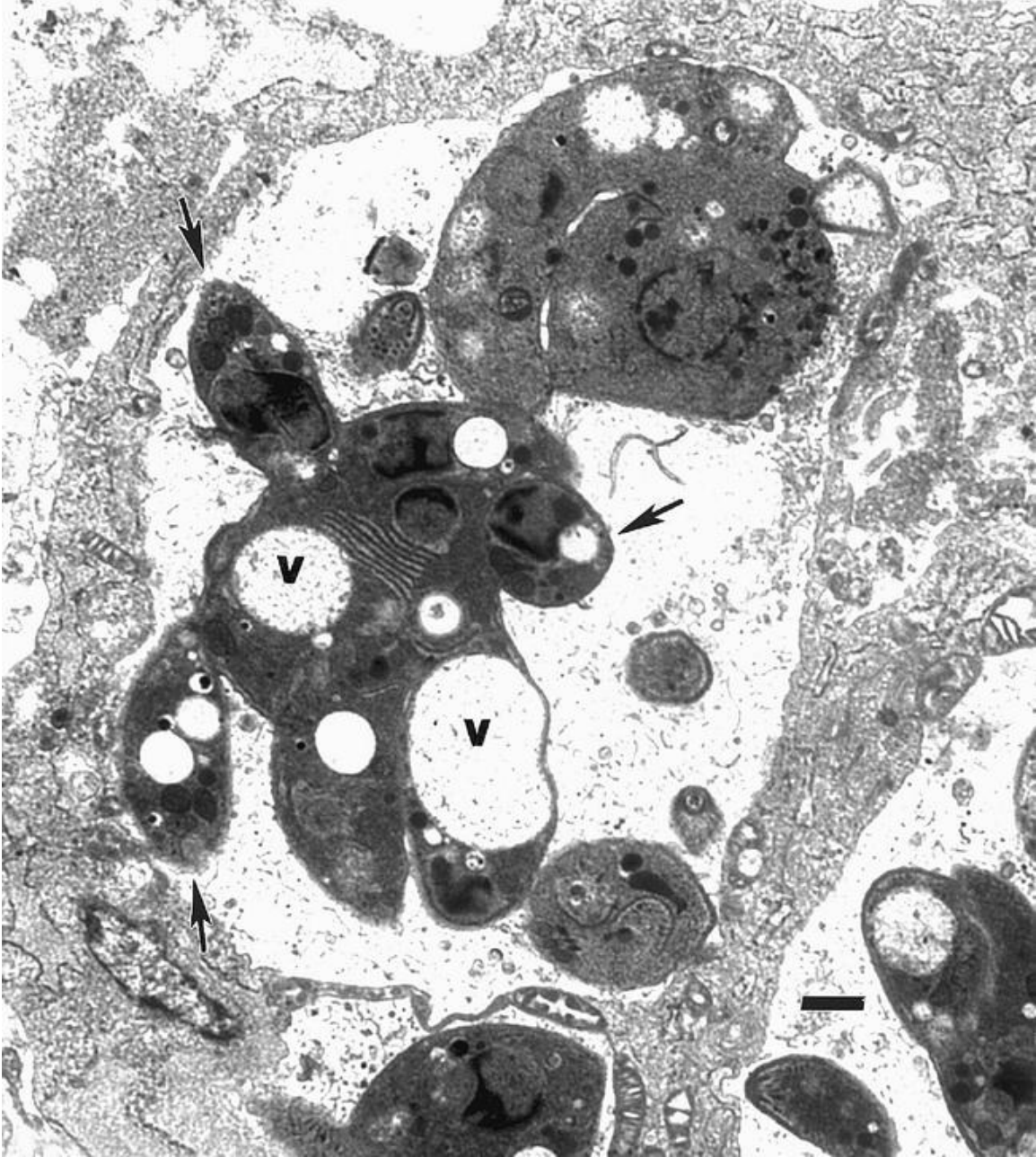
Fig. 1. Transmission electron micrograph of *Toxoplasma gondii* treated with ponazuril. Note the large vacuoles (V) in the parasite and the tachyzoites (arrows) partially budded from the surface. Bar = 1 µm.

CHAPTER 6

THE EFFECTS OF PONAZURIL ON DEVELOPMENT OF APICOMPLEXANS

IN VITRO

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ABSTRACT

We examined the effects of 5 µg/ml ponazuril treatments on developing tachyzoites of *Neospora caninum* and merozoites of *Sarcocystis neurona* to better determine the mode of action of this anticoccidial drug. Both parasites develop asexually by endogenesis. *Neospora caninum* was selected for study because it develops by endodyogeny, which results in two tachyzoites being produced internally, and *S. neurona* was selected because it develops by endopolygeny which results in many merozoites being produced internally. Ponazuril inhibited development of *N. caninum* after approximately 48 h post-exposure. Treated tachyzoites of *N. caninum* developed vacuoles and underwent degeneration. Ponazuril also inhibited development of merozoites of *S. neurona*. Treated merozoites and maturing schizonts of *S. neurona* developed vacuoles and underwent degeneration. The ability of *S. neurona* schizonts to undergo cytokinesis was inhibited. Our results are discussed in relation to previous ultrastructural research on endogenesis of tachyzoites of *Toxoplasma gondii* undergoing endodyogeny which indicated that ponazuril induced multinucleate stage formation and inhibited cytokinesis. Ponazuril is believed to act on the apicoplast and our study demonstrates that this agent may express its inhibitory effects in different phenotypic manners on different apicomplexan parasites. The enzyme/enzyme systems that are the inhibitory target of ponazuril may be different in these apicomplexans, or the results of inhibition may affect different pathways downstream of its initial site of action in these parasites.

INTRODUCTION

The tissue cyst-forming coccidia *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis neurona* are economically important apicomplexan parasites. Congenital toxoplasmosis in humans has long been recognized by its devastating results, including the triad of hydrocephalus, blindness, and mental retardation in severely infected infants (Jones et al. 2001b). Additionally, congenitally infected children who are less severely infected may suffer from a variety of neurological-related ailments throughout their lives (Roberts and Frenkel 1990). In the United States it is estimated that 85% of women of child-bearing age are at risk for toxoplasmosis (Jones et al. 2001a) and that up to 4,000 cases of congenital toxoplasmosis occur each year (Jones et al. 2001b). Toxoplasmosis is also a frequent and fatal complication in patients that receive organ transplantation (Soave 2001) and toxoplasmic encephalitis is still an important neurological component of AIDS (Luft and Chua 2000). The annual economic impact of toxoplasmosis on the human population in the U.S. is about $7.7 billion (Buzby and Roberts 1996).

Neospora caninum was first recognized as a cause of neonatal paralysis in dogs but was soon found to be a major cause of bovine abortions worldwide (Dubey and Lindsay 1996). It is structurally and biologically similar to *T. gondii* and was confused with that parasite for almost a century (Dubey and Lindsay 1996). Dogs are the definitive host of *N. caninum* (Lindsay, Dubey, and Duncan 1999a; McAllister et al. 1998).

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Equine protozoal myeloencephalitis (EPM) is caused by *S. neurona*. This disease, known since the early 1960's, is a major neurological syndrome of horses in the Americas (Dubey et al. 2001). It was not named until 1991, when it was isolated and grown in cell culture (Dubey et al. 1991). Horses are accidental hosts whose central nervous system is invaded by the schizonts and merozoites of *S. neurona*. The sarcocyst stages of *S. neurona* have not been found in any tissues of the horse. The Virginia opossum, *Didelphis virginiana*, is the only known definitive host in North America (Dubey and Lindsay 1998).

Apicomplexans divide asexually by two basic mechanisms: endogenesis and exogenesis (Chobotar and Scholtyseck 1982). We are interested in endogenesis because it represents the most common mode of asexual replication of the pathogenic stages in the tissue cyst-forming coccidia of mammals. In endogenesis, merozoites are produced internally in association with nuclei, centrioles, and centrocones (Chobotar and Scholtyseck 1982; Dubey, Lindsay, and Speer 1998; Speer and Dubey 2001, 2005). The membranes of the future merozoites (tachyzoites) and apical complex develop internally. In contrast, in exogenesis merozoite formation is initiated by a thickening of the inner membrane complex with the merozoites developing in association with this complex. They are eventually extruded from the surface of the schizont (Chobotar and Scholtyseck 1982). Development by endogenesis can further be subdivided into endodyogeny and endopolygeny. Endodyogeny is production of two organisms internally by endogenesis. Endopolygeny is production of many organisms internally by endogenesis.

Ponazuril is a triazine anticoccidial that is used to treat EPM, and it is a major metabolite of toltrazuril. Toltrazuril is an anticoccidial drug that is used to prevent coccidiosis in poultry in many parts of the world. In vitro (Darius, Mehlhorn, and Heydorn 2004a; Lindsay, Dubey, and Kennedy 2000) and in vivo (Darius, Mehlhorn, and Heydorn 2004b; Franklin et al. 2003; Gottstein et al. 2001) studies indicate that ponazuril is active against *N. caninum* and *S. neurona*. We have previously demonstrated that ponazuril is highly active against *T. gondii* in vitro and in vivo (Mitchell et al. 2004) and that 5 μg/ml ponazuril affects the ability of *T. gondii* tachyzoites to undergo endodyogeny (Mitchell et al. 2003). Multinucleate schizont-like stages are induced in *T. gondii* treated with ponazuril, indicating an effect on parasite cytokinesis (Mitchell et al. 2003).

The present study was done to better understand the mode of action of ponazuril against tissue cyst-forming coccidia. Our first hypothesis was that since *N. caninum* and *T. gondii* both divide by endodyogeny (Dubey, Lindsay, and Speer 1998; Lindsay et al. 1993), the effects of ponazuril treatment would be similar if not identical for these apicomplexans. Our second hypothesis was that since *S. neurona* divides by endogenesis, although it is characterized as endopolygeny (Speer and Dubey 2001), the effects of ponazuril treatment would be similar to those observed for other apicomplexans that divide by endogenesis, even if by endodyogeny.

MATERIALS AND METHODS

Light microscopy studies of ponazuril inhibition

Light microscopy studies were undertaken to determine when ponazuril exerted its inhibitory effects on developing *Neospora caninum* and *Sarcocystis neurona*. Monolayers of African green monkey (*Cercopithecus aethiops*, American Type Culture Collection, CCL-70) kidney (CV-1) cells were grown in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (Mitchell et al. 2003) on 22-mm^2 cover slips, placed on the bottom of two 6-well plates. The CV-1 cells were infected with 2 × 10^5 *N. caninum* (NC-1 isolate) tachyzoites. After a 3-h incubation period at 37°C to allow the tachyzoites to enter host cells, the infected media were removed and replaced with a maintenance medium (RPMI 1640 medium containing 2% fetal calf serum and antibiotics) without ponazuril or with a maintenance medium containing 5 μg/ml ponazuril. Ponazuril (lot PFA101; Bayer HealthCare Animal Health, Shawnee Mission, KS) was dissolved in DMSO and then made up to a stock solution of 1 mg/ml. Plates were incubated at 37°C in a humidified incubator containing 5% CO_2 and 95% air. One cover slip was removed from both the control plate and the ponazuril-treated plate at 13, 24, 48, 72, and 94 h post-treatment and placed in 10% (v/v) buffered formalin for 1 h at room temperature. Cover slips were then placed in 100% methanol until staining.

Cover slips were stained using Diff-Quick stain (Dade Behring Inc., Newark, DE) and allowed to dry before mounting onto slides with Permount. The number of parasites in 100 host cells was determined at each examination. The number of divisions that had occurred by endodyogeny was calculated as follows: if 1 tachyzoite was present, it was recorded as 0 divisions; if 2 tachyzoites were present, it was recorded as 1 division; if 3 or 4 tachyzoites were

present, it was recorded as 2 divisions; if 5–8 tachyzoites were present, it was recorded as 3 divisions; if 9–16 tachyzoites were present, it was recorded as 4 divisions; and if 17 or more tachyzoites were present, it was recorded as >5 divisions. Similar studies were conducted using Hs68 cells (human foreskin fibroblast, CRL-1635, American Type Culture Collection, Manassas, VA).

Similar methods were used to examine the effects of ponazuril treatment on development of *S. neurona*. The CV-1 cells were grown on 22-mm^2 cover slips placed on the bottom of 6-well plates. Cover slips were stained and the numbers of immature and mature schizonts (=schizonts with fully formed merozoites) present were recorded for the first 100 infected cells. *Sarcocystis neurona* does not grow in Hs68 cells so comparative studies were not done in this cell type.

Transmission electron microscopy

Host CV-1 cells were grown to confluence in eleven 25-cm^2 plastic cell culture flasks. Host cells were infected with 2×10^6 tachyzoites of *N. caninum* or merozoites of *S. neurona* for 3 h at 37°C, after which the infected media were removed and replaced with control media or maintenance media containing 5 µg/ml ponazuril (as above). Flasks were incubated at above conditions.

Flasks were scraped on days 5, 6, 7, 8, and 9 post-treatment using a cell scraper to remove CV-1 cell monolayers infected with *N. caninum* or *S. neurona*. Control cells infected with *N. caninum* and *S. neurona* were collected 5- and 6- d post-treatment, respectively. Experiments were repeated using NC-1 *N. caninum* tachyzoites and Hs68 cells. The scraped media were removed and pelleted by

centrifugation. The pellets were fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4) for transmission electron microscopy (TEM). Pellets were then fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium phosphate buffer and rinsed twice with this buffer. The cell pellets were dehydrated in an ethanol series and were cleared by being passed through two changes of propylene oxide. Pellets were embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA) and thin sections were stained with uranyl acetate and lead citrate. Samples were examined with a Zeiss 10CA TEM operating at 60 kV and digital images were taken using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, MA).

RESULTS

Effects of ponazuril on apicomplexans

Multiplication rates of tachyzoites of *N. caninuim* were similar for the first 24 h (Table 1). Between 24 and 48 h, ponazuril began affecting tachyzoite development. At 72 h, the distinction between individual tachyzoites was obscured in most ponazuril-treated cells, making quantitative counts difficult. The numbers of host cells containing more than 5 divisional cycles remained >25% in ponazuril-treated cells but was never >10% in infected controls (Table 1), suggesting that host cell lysis was not occurring as readily in ponazuril-treated cells due to the presence of non-viable tachyzoites. Similar results were obtained in Hs68 cells (data not shown).

There was little visible difference between the development of ponazuril-treated and control schizonts of *S. neurona* using light microscopy of cell

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cultures. Four separate experiments were conducted, and no conclusive results were obtained (data not presented).

Transmission electron microscopy

For *N. caninum*, results are based on examination of 14 micrographs of control parasites in CV-1 cells and 40 micrographs of ponazuril-treated stages in CV-1 cells. Control NC-1 tachyzoites grown in maintenance medium were crescent-shaped or ovoid, and contained a conoid, rhoptries, dense granules, micronemes, and other organelles typical of apicomplexan tachyzoites. Some contained lipid or glycogen-like vacuoles. Ponazuril treatment caused the degeneration of tachyzoites. Multiple large vacuoles were present in the cytoplasm of degenerating tachyzoites (Fig. 1). These vacuoles may have originated from the apicoplast, the mitochondrion or from the fusion of lipid or glycogen-like vacuoles or combinations of these occurrences. The nuclear membrane often appeared to be swollen. Some tachyzoites viewed 7- and 8-d post-treatment maintained their natural shape and appeared normal. Ponazuril did interfere with normal tachyzoite division in a few parasites causing the presence of multiple nuclei, but this was not a frequent occurrence. Similar results were obtained for *N. caninum* that had infected ponazuril-treated Hs68 cells (data not shown).

For *S. neurona*, results are based on examination of 12 micrographs of control parasites in infected CV-1 cells and 52 micrographs of ponazuril-treated stages. Control infected host cells examined 5–9 d post-treatment appeared normal, and various stages of endopolygeny were observed. Ponazuril-treated

and infected CV-1 cells either appeared normal or contained degenerating schizonts or groups of degenerating merozoites. Vacuoles were present in the schizonts and in developing merozoites (Fig. 2). Merozoites were often in the process of budding from the surface of these degenerating schizonts. Occasionally, apparently viable merozoites could be seen in the same host cell as degenerating schizonts with budding merozoites. This suggests that some merozoites may have completed development before the complete effects of ponazuril were expressed on the schizont.

DISCUSSION

Light microscopic studies.

Ponazuril has minimal effect on *N. caninum* endogenesis up to 48 h, approximately the time needed for four divisional cycles. This contrasts with the findings of Mitchell et al. (2003) who found that ponazuril inhibited endogenesis of *T. gondii* after the second division. These delayed effects of inhibitory action have been found for many different classes of chemical agents that inhibit endogenesis of *T. gondii* (Beckers et al. 1995; Lindsay and Blagburn 1994). These findings might be due to the differences in divisional cell cycles between the 2 parasites. The cell cycle of *T. gondii* is 8–10 h, while that of *N. caninum* is 14–15 h (Sundermann and Estridge 1999). The rounded appearance of ponazuril-treated tachyzoites of *N. caninum* was similar to the description of Darius et al. (2004a) who observed ponazuril-treated tachyzoites of *N. caninum* using light microscopy.

Our light microscopic studies with ponazuril and *S. neurona* were inconclusive. The cell cycle of *S. neurona* takes 3 d (Lindsay et al. 1999b) and division is by endopolygeny. Since we examined ponazuril-treated *S. neurona* infected CV-1 cells up to 11-d post-treatment, this should have allowed for a minimum of three divisional cycles of *S. neurona*, sufficient time for ponazuril to exert its antiparasitic effect. Lindsay et al. (2000) used a merozoite production assay conducted at 10-d post-treatment to determine that ponazuril inhibited merozoite production of *S. neurona*. Lindsay et al. (2000) also determined that ponazuril did not cause mortality of *S. neurona* in cell cultures, but it did inhibit the growth rate as measured by merozoite production. Higher doses may be completely lethal, but we were unable to examine doses higher than 5 µg/ml ponazuril because ponazuril induced changes in the CV-1 host cells at doses >5 µg/ml (data not presented).

Transmission electron microscopy.

The present study determined that ponazuril affects endogenesis of *N. caninum* differently than endogenesis of *T. gondii*, despite the fact that both develop by endodyogeny. The development of both species was inhibited at 5 µg/ml ponazuril. However, the drug had a distinct effect on the cytoplasmic divisional process of *T. gondii* (Mitchell et al. 2003), preventing cytoplasmic division and inducing the formation of multinucleate schizont-like stages. Only one instance of a multinucleate tachyzoite of *N. caninum* was observed in the present study. Ponazuril had a more direct effect on tachyzoites of *N. caninum*, causing their degeneration. Darius et al. (2004a) examined the effects of

30 µg/ml ponazuril on developing *N. caninum* tachyzoites, and their findings are similar to those we observed with 5 µg/ml ponazuril. They attributed the action of ponazuril to adverse effects on the apicoplast and the tubular mitochondrion, and noted that ponazuril caused a swelling of these important organelles, which eventually caused tachyzoite death. The vacuoles that we observed in ponazuril-treated tachyzoites might possibly have been in the apicoplast and mitochondrion, but we could not conclusively demonstrate that the ponazuril-induced lesions were always confined to these two organelles. Darius et al. (2004a) did not report the presence of multinucleate stages of *N. caninum*, and we observed only one such stage, suggesting that this is not the usual effect of ponazuril treatment on *N. caninum*.

Using TEM, we were able to determine that ponazuril affected developing schizonts of *S. neurona*. The schizonts developed vacuoles that eventually led to their degeneration. Inhibition of schizont cytokinesis was also observed, similar to that observed in ponazuril-treated *T. gondii* (Mitchell et al. 2003). The presence of some normal-appearing schizonts in ponazuril-treated infected CV-1 cells using TEM further supported the findings of Lindsay et al. (2000), who showed that ponazuril was not completely cidal (kills) for all stages, but that it was static (inhibits) at this dose. The distinction between cidal and static is often dose dependent (Lindsay and Blagburn 2001). Additionally, agents that are static in vitro may be cidal in vivo because of assistance from the host's immune system (Mitchell et al. 2004).

Ponazuril may act on the apicoplast of coccidial parasites (Darius et al. 2004a; Hackstein et al. 1995). The apicoplast is an exciting new drug target for apicomplexan parasites, and its metabolic functions are many (Gornicki 2003; Seeber 2003). Our study has demonstrated that ponazuril may exert its inhibitory effect in phenotypically distinct manners on closely related apicomplexan parasites. The molecular reasons for these phenotypic differences await further study.

In conclusion, dramatic differences were observed for ponazuril treatment of the two apicomplexans that develop by endodyogeny—tachyzoites of *N. caninum* versus tachyzoites of *T. gondii*. Thus, we must reject our first hypothesis that endodyogenous apicomplexans are similarly affected by ponazuril. When we compared two apicomplexans that develop by endogenesis, but by endodyogeny as in *T. gondii* and by endopolygeny as in *S. neurona*, the effects of ponazuril treatment were very different for these two parasites. Thus, we must reject our second hypothesis that all endogenetic apicomplexans are similarly affected by ponazuril.

ACKNOWLEDGEMENTS

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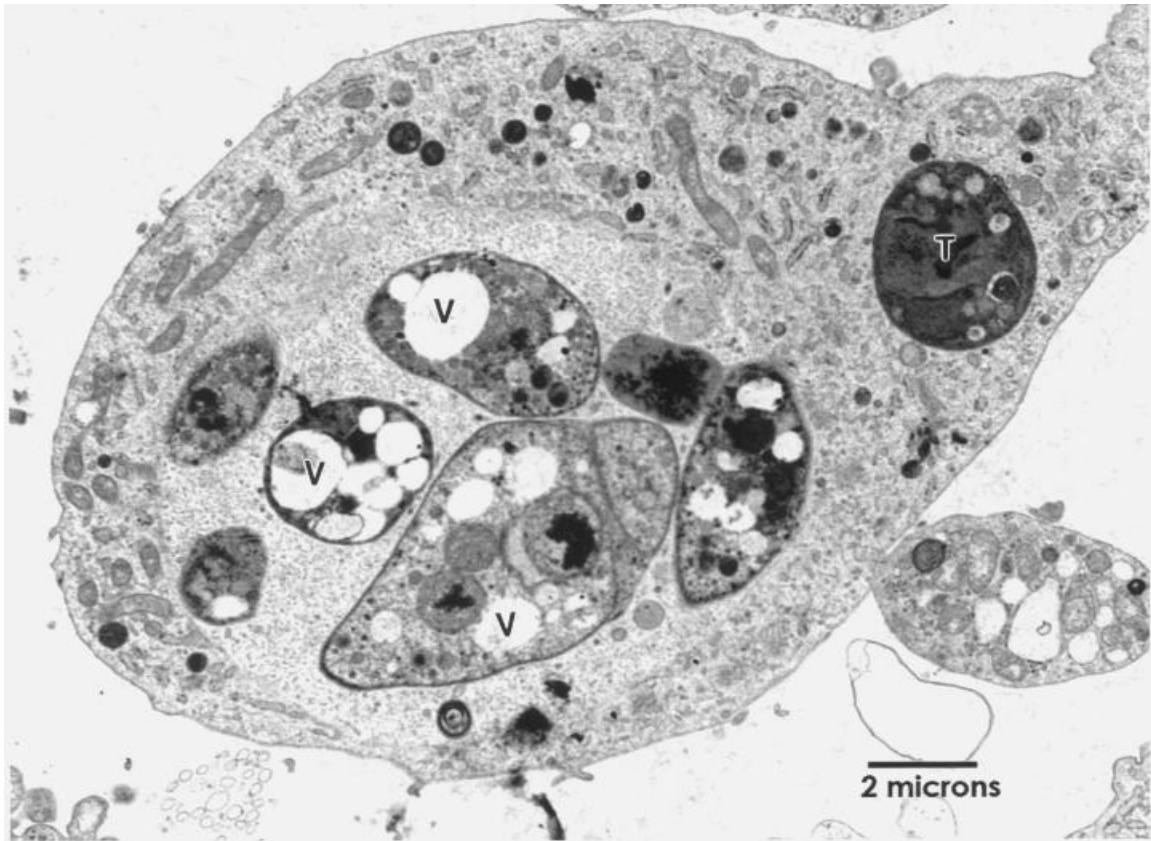
Fig. 1. Transmission electron micrograph of a group of degenerating tachyzoites of Neospora caninum in a CV-1 cell treated with ponazuril. Note the vacuoles (V) in individual tachyzoites. A tachyzoite (T) that contains few vacuoles is also present.

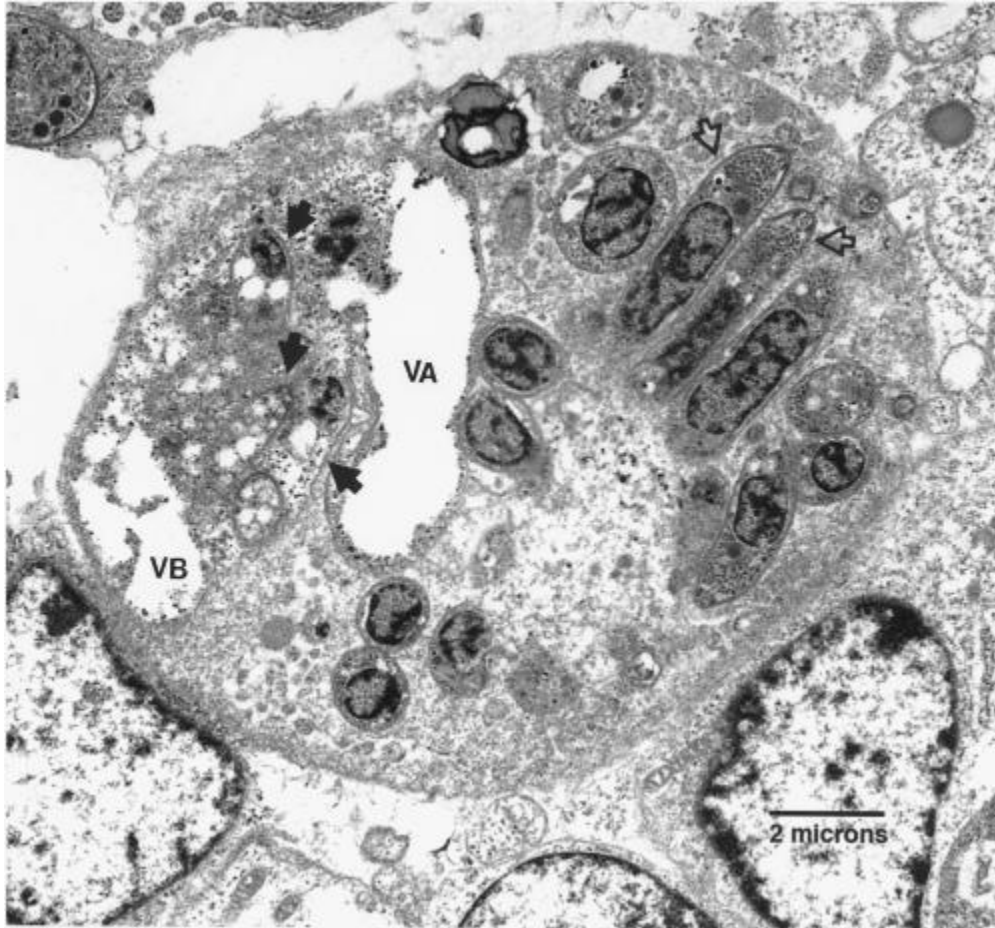Fig. 2. Transmission electron micrograph of a group of two degenerating schizonts of Sarcocystis neurona in a CV-1 cell treated with ponazuril. Large vacuoles (VA) (VB) are present in central portions of the schizonts. The anterior portions of some merozoites appear normal (open arrows), while others (arrows) appear to be degenerating due to increased vacuolization.

Table 1. Effect of 5 mg/ml ponazuril treatment on multiplication of *Neospora caninum* tachyzoites in 100 CV-1 cells in culture.

Examination time (h)	Treatment	Number of divisions observed per infected cell					
		0	1	2	3	4	>5
13	Control	20	31	46	3	0	0
13	Ponazuril	21	43	34	2	0	0
24	Control	6	14	34	32	8	6
24	Ponazuril	18	19	31	20	8	4
48	Control	30	4	6	7	5	8
48	Ponazuril	24	22	6	9	14	25
72	Control	18	41	15	10	10	6
72	Ponazuril	9	14	11	10	5	51
94	Control	15	21	25	21	10	8
94	Ponazuril	13	11	9	1	0	66

CHAPTER 7

EFFICACY OF PONAZURIL *IN VITRO* AND IN PREVENTING AND TREATING *TOXOPLASMA GONDII* INFECTIONS IN MICE

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ABSTRACT

Toxoplasma gondii is an important apicomplexan parasite of humans and other warm-blooded animals. Ponazuril is a triazine anticoccidial recently approved for use in horses in the United States. We determined that ponazuril significantly inhibited *T. gondii* tachyzoite production ($P < 0.05$) at 5.0, 1.0, or 0.1 μg/ml in African green monkey kidney cells. We used outbred female CD-1 mice to determine the efficacy of ponazuril in preventing and treating acute toxoplasmosis. Each mouse was subcutaneously infected with 1,000 tachyzoites of the RH strain of *T. gondii*. Mice were weighed daily, and ponazuril was administered orally in a suspension. Mice given 10 or 20 mg/kg body weight ponazuril 1 day before infection and then daily for 10 days were completely protected against acute toxoplasmosis. Relapse did not occur after prophylactic treatments were stopped. *Toxoplasma gondii* DNA could not be detected in the brains of these mice using polymerase chain reaction (PCR). One hundred percent of mice treated with 10 or 20 mg/kg ponazuril at 3 days after infection and then daily for 10 days were protected from fatal toxoplasmosis. Sixty percent of mice treated with 10 mg/kg ponazuril at 6 days after infection and 100% of mice treated with 20 mg/kg or 50 mg ponazuril 6 days after infection and then daily for 10 days were protected from fatal toxoplasmosis. Relapse did not occur after treatments were stopped. *Toxoplasma gondii* DNA was detected in the brains of some, but not all, of these mice using PCR. The results demonstrate that ponazuril is effective in preventing and treating toxoplasmosis in mice. It

should be further investigated as a safe and effective treatment for this disease in animals.

INTRODUCTION

Toxoplasma gondii is an important parasite of humans and other warm-blooded animals. About 1,500,000 human cases of toxoplasmosis are reported in the United States each year, and about 15% of those infected have clinical signs (Mead et al., 1999; Jones, Kruszon-Moran et al., 2001). Congenital toxoplasmosis has long been recognized because of the devastating effects it can have on the infected fetus (Jones, Lopez et al., 2001). These include hydrocephalus, blindness, and mental retardation. Congenitally infected children who are less severely infected may suffer from a variety of neurological-related aliments throughout their lives (Roberts and Frenkel, 1990). In the United States, it is estimated that 85% of women of child-bearing age are at risk for toxoplasmosis (Jones, Kruszon-Moran et al., 2001) and that up to 4,000 cases of congenital toxoplasmosis occur each year (Jones, Lopez et al., 2001). Toxoplasmic encephalitis (TE) became recognized as an acquired immunodeficiency syndrome (AIDS)–defining illness in the early 1980s, and TE is still the most important neurological component of AIDS (Luft and Chua, 2000). Toxoplasmosis is also a frequent and fatal complication in patients who receive organ transplantation (Soave, 2001). The annual economic impact of toxoplasmosis in the human population in the United States is about $7.7 billion (Buzby and Roberts, 1996).

Ponazuril is the major metabolite of toltrazuril, a triazine anticoccidial used in the poultry industry. Ponazuril has been shown to be active against *Sarcocystis neurona* in vitro (Lindsay and Dubey, 2000) and in vivo (Franklin et

al., 2003) and against *Neospora caninum* in vivo (Gottstein et al., 2001). The present study was carried out to determine the in vitro and in vivo activity of ponazuril against the RH strain of *T. gondii*.

MATERIALS AND METHODS

Cell culture

African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Manassas, Virginia) were grown to confluence in 25-cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum in Roswell Park Memorial Institute 1640 medium, supplemented with 100 U penicillin/ml and 100 mg streptomycin/ml. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air.

Ponazuril and in vitro efficacy

The activity of ponazuril (lot PFA101; Bayer HealthCare Animal Health, Shawnee, Kansas) was determined in a tachyzoite production (TP) assay (Lindsay and Blagburn, 1994). Ponazuril was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 1 mg/ml. Dilutions were made from this stock solution, and the highest concentration of DMSO in any solution was 0.01% (v/v). Cell monolayers were inoculated with 2.5 × 10^5 RH strain *T. gondii* tachyzoites. Two hours after inoculation, the medium was removed and replaced with maintenance medium containing ponazuril at concentrations of 0.1, 1.0, or 5.0 μg/ml (Fig. 1). Control flasks received maintenance medium without ponazuril. Four flasks were used per ponazuril treatment dose. The TP assay was

conducted after 4 days of treatment. The numbers of tachyzoites (mean of 16 counts/treatment [4 counts/flask]) present was determined by counting in a hemacytometer.

To determine when ponazuril acted on *T. gondii*, CV-1 cells were grown to monolayers on 22-mm^2 glass coverslips in 6-well cell culture plates. The CV-1 cells were inoculated with 1×10^5 tachyzoites, and 2 hr later the media were removed and replaced with media containing 5 µg/ml ponazuril. Replicate plates were treated with media containing 0.1% DMSO but no ponazuril. Coverslips were removed and examined 4, 9, 20, 24, and 48 hr after the addition of ponazuril-containing medium or control medium. The number of parasites in 100 host cells was determined at each observation time.

The following procedure was used to determine whether ponazuril treatments killed *T. gondii*. After the medium was collected for the TP assay, the cell monolayer was rinsed twice with maintenance medium to wash off any residual ponazuril, and 5 ml of maintenance medium was added to the flask. The flasks were then examined for 30 days for renewed growth of parasites, monolayer destruction, or both.

Statistical analysis

Mean tachyzoite counts were log transformed to stabilize variances before analysis and then back transformed for presentation. The MIXED procedure of SAS (SAS ver. 6.12, SAS Institute Inc., Cary, North Carolina) was used to perform analysis of variance. Tukey's honest significant difference ($P = 0.05$) was used to compare means.

Mice and examination for *Toxoplasma gondii*

For in vivo studies, a suspension of 50 mg ponazuril per milliliter (lot 2161AA) was obtained from Bayer HealthCare Animal Health. This suspension was diluted in distilled water and used for in vivo testing. Groups of 5 female CD-1 mice were used to determine the effects of treatment with ponazuril in the prevention and treatment of toxoplasmosis (Table I). All mice were inoculated subcutaneously in the dorsal scapular region with 1×10^3 tachyzoites. During the study, impression smears were made from the livers or lungs of any mice that died and were examined unstained by light microscopy for tachyzoites. At 8 wk postinoculation (PI), all surviving mice were bled from the retroorbital plexus. The serum was collected and examined for antibodies to *T. gondii* in a modified direct agglutination assay (MAT) (Dubey and Desmonts, 1987).

***Toxoplasma gondii* polymerase chain reaction**

Brains were examined for *T. gondii* DNA using the primers described by Jauregui et al. (2001). The DNA was extracted from 0.5 g of brain tissue from mice in groups 3–9 (Table I) using a commercial DNA extraction kit (DNA Maxi Kit, Qiagen, Valencia, California). The purified DNA was diluted 1:100, and a 20-µl aliquot was taken and mixed with 200 µl of InstaGene Matrix (Bio-Rad, Hercules, California). The samples were then incubated in a 56 C water bath for 30 min. The samples were vortexed and then placed in boiling water for 8 min. The samples were vortexed and centrifuged in a microfuge for 2–3 min. A 20-µl aliquot of the supernatant was used per 50 µl polymerase chain reaction (PCR). The remaining supernatant was stored at −20 C. PCR was performed on each

sample using Ready To Go PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) and a Hybaid OmniGene thermocycler. The detection primers were based on the *T. gondii* ITS1 sense primer 5′-GATTTGCATTCAAGAAGCGTGATAGTAT-3′ and antisense primer 5′-AGTTTAGGAAGCAATCTGAAAGCACATC-3′. Mouse β-actin was used as a positive control for DNA isolation and PCR (sense primer 5′-TCACCCACACTGTGCCCATCTACGA-3′ and antisense primer 5′-CAGCGGAACCGCTCATTGCCAATGG-3′). Standard PCR reaction conditions were used with the following amplification parameters: 94 C for 5 min, 35 cycles at 94 C for 1 min, at 62 C for 1 min, at 72 C for 1 min, and at 72 C for 10 min. The PCR products were run on a 1% agarose gel.

RESULTS

Effects on tachyzoite production

There was a significant effect of ponazuril treatment ($P < 0.05$) on tachyzoite production. Tukey's test indicated that the 1.0 µg/ml treatment was not significantly different ($P > 0.05$) from the 5.0 µg/ml treatment, but all other pairwise comparisons were significant ($P < 0.05$) (Fig. 1).

Host CV-1 cells treated with 5 µg/ml ponazuril contained only 4 parasites at observation times of 20 hr or greater. The CV-1 cells that contained *T. gondii* and that were not treated had 8 or more tachyzoites at these observation times. Results of timed observations indicated that ponazuril inhibits *T. gondii* replication after the second division by endodyogeny approximately 20 hr after treatment.

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Prevention of toxoplasmosis.

All nontreated mice developed acute toxoplasmosis and died or were killed 9–11 days PI (x = 10 PI) (Table I). No mouse in group 3 or 4 given 10 or 20 mg/kg ponazuril 1 day before infection and then daily for 10 days died. None of the mouse developed acute toxoplasmosis after prophylactic treatments were stopped. Three of 5 mice in group 3 tested serologically positive for *T. gondii* using the MAT, and 1 of 5 mice in this group was positive by PCR on brain tissue (Fig. 2). All 5 mice tested serologically negative in the MAT in group 4, and *T. gondii* DNA was not detected in the brains of these mice by PCR (Table I).

Treatment of acute toxoplasmosis

All nontreated mice developed acute toxoplasmosis and died or were killed 9–11 days PI (x = 10 PI). Five of 5 mice (100%) in group 5 and 5 of 5 mice (100%) in group 6 were protected from fatal toxoplasmosis (Table I). All mice were serologically positive for *T. gondii* in groups 5 and 6 on the MAT. PCR was done on the brains of 4 mice in group 5, and 1 was positive, whereas PCR was done on the brains of all mice in group 6, and they were all positive.

Three of 5 mice (60%) in group 7 and 5 of 5 mice (100%) in group 8 were protected from fatal toxoplasmosis. Deaths occurred on days 9 and 12 PI in group 7. All 3 mice in group 7 and all 5 mice in group 8 tested serologically positive in the MAT. All group 7 and 8 mice tested positive for *T. gondii* by PCR. One of 5 mice in group 9 died 11 days PI. This mouse had aspiration pneumonia, and its death was probably not due to toxoplasmosis. The 4 other mice in group 9 survived until the end of the study. All surviving mice in group 9 were positive

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by the MAT. The brains of 4 mice in group 9 were examined by PCR, and 1 was positive. Relapse did not occur after treatments were stopped.

DISCUSSION

The present study demonstrates that ponazuril is effective in preventing and treating toxoplasmosis in mice. The lack of mortality and detection of *T. gondii* DNA in the brain of only 1 mouse treated prophylactically with 10 mg/kg and no mouse treated prophylactically with 20 mg/kg indicates that ponazuril is highly effective in the prevention of toxoplasmosis. Ponazuril at 10 or 20 mg/kg was also 100% effective in preventing mortality in mice with 3-day-old, established *T. gondii* infections but did not prevent the parasite from eventually reaching the brain in these mice as determined by PCR on brain tissue. Treatment of clinical toxoplasmosis at 6 days after infection was less effective with 10 mg/kg (60% survival) than with 20 or 50 mg/ kg (100% survival; excluding 1 mouse in 50 mg/kg group that died of aspiration pneumonia).

Pyrimethamine alone or combined with sulfadiazine is the most commonly used treatment for human toxoplasmosis, whereas clindamycin and atovaquone are also frequently used (Luft and Chua, 2000). Ponazuril appears to be superior to clindamycin or atovaquone for the treatment of murine toxoplasmosis. Nikolic et al. (1999) found that treatment with 50 or 400 mg/kg clindamycin hydrochloride in the feed daily for 3 wk prevented mortality from the RH strain of *T. gondii*. Atovaquone given orally in the feed at 100 mg/kg for 14 days prevented death in 13% of the mice infected with the RH strain of *T. gondii* and examined by Djurkovic-Djakovic et al. (1999).

Diclazuril is a triazine anticoccidial related to ponazuril that has been evaluated against toxoplasmosis. Lindsay and Blagburn (1994) demonstrated that diclazuril prevented deaths from toxoplasmosis in 80 and 100% of mice treated 1 day before infection with 1 or 10 mg/kg diclazuril and then daily for 10 days after infection with RH strain of *T. gondii*. Lindsay et al. (1995) found that oral diclazuril at 10 mg/kg was 100 and 90% effective in preventing deaths in mice when given at 3 or 6 days, respectively, after infection with RH strain *T. gondii*. This activity is similar to that seen for ponazuril at 20 mg/kg in the present study.

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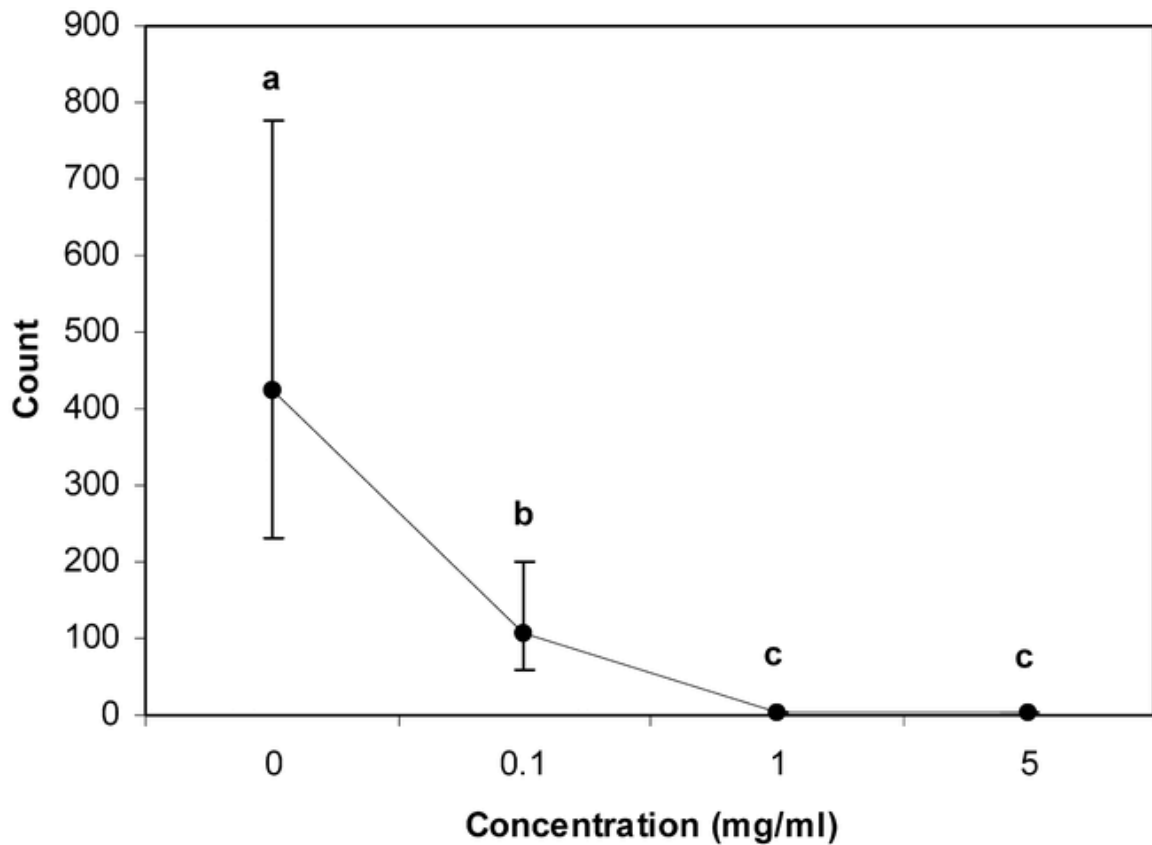
Figure 1. Activity of ponazuril against RH strain *Toxoplasma gondii* in CV-1 cell culture. Bars = 95% confidence intervals. Points with different letter are significantly different ($P < 0.05$) from each other.

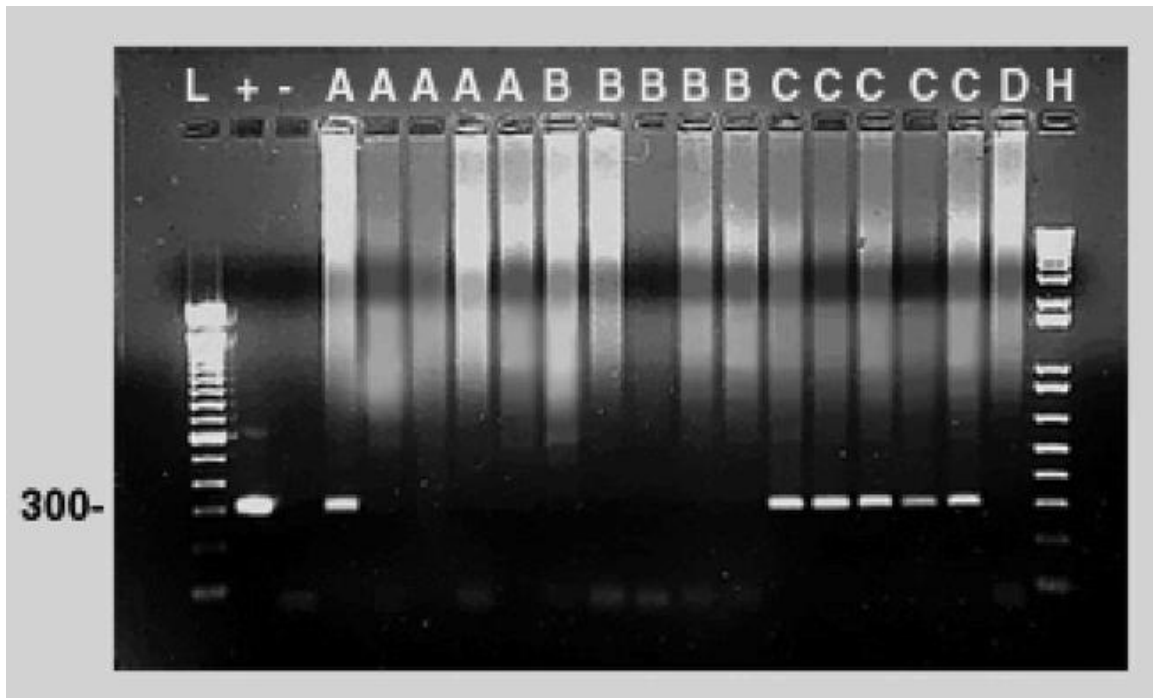Figure 2. Results of *Toxoplasma gondii* ITS1 PCR on DNA from brains of mice infected with the RH strain of *T. gondii* and treated with ponazuril. (L) 100-bp ladder, (+) positive control *T. gondii* DNA, (−) negative control no DNA, (A) DNA from individual mice from group 3, (B) DNA from individual mice from group 4, (C) DNA from individual mice from group 6, (D) DNA from a mouse from group 9 and (H) 1kb+ ladder.

TABLE I. Protocol for evaluating the effects of ponazuril against *Toxoplasma gondii* in mice.

Group	Treatment*	No. mice/no. survived[†]	PCR[‡]
1	Distilled water 3 days after infection	5/0	ND[§]
2	Distilled water 1 day before infection	5/0	ND
3	10 mg/kg ponazuril 1 day before infection	5/5	5/1
4	20 mg/kg ponazuril 1 day before infection	5/5	5/0
5	10 mg/kg ponazuril 3 days after infection	5/5	4/1
6	20 mg/kg ponazuril 3 days after infection	5/5	5/5
7	10 mg/kg ponazuril 6 days after infection	5/3	3/3
8	20 mg/kg ponazuril 6 days after infection	5/5	5/5
9	50 mg/kg ponazuril 6 days after infection	5/4[¶]	4/1

* Mice in groups 1 and 2 never received ponazuril. Mice in groups 3 and 4 received ponazuril 1 day before and on the day of infection and then daily for 10 days. Mice in groups 5–9 were treated daily for 10 days with ponazuril at the indicated day after infection.

[†] Number of mice inoculated/number of mice surviving infection.

[‡] Results of PCR; number tested by PCR on brain/number positive by PCR on brain.

[§] ND, not determined.

[¶] One mouse died in this group due to aspiration pneumonia.

CHAPTER 8

PREVENTION OF RECRUDESCENT TOXOPLASMIC ENCEPHALITIS USING PONAZURIL IN AN IMMUNODEFICIENT MOUSE MODEL

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ABSTRACT

Encephalitis due to *Toxoplasma gondii* occurs in animals and in humans. The present study examined the efficacy of ponazuril, the major metabolite of toltrazuril, against recrudescent toxoplasmosis in an interferon gamma gene knockout mouse model. Groups of mice were orally infected with 10 *T. gondii* tissue cysts (Type III, chicken isolate) and then placed on sodium sulfadiazine (SD, 1 mg/ml in drinking water)) to prevent death from acute infection and allow for the development of chronic infection. Group 1 was infected and treated with SD for the remainder of the study. Group 2 was infected and treated with SD followed by ponazuril treatment for recrudescent toxoplasmosis. Group 3 was infected and treated with SD followed by water treatment (= no treatment) for recrudescent toxoplasmosis. Group 4 was not infected and used as non-infected, non-medicated controls. Immunohistochemistry for *T. gondii* tissue cysts using BAG1 antisera (tissue cyst/bradyzoite specific) and rabbit polyclonal sera (usually weak reactions on tissue cysts but strong reactions with tachyzoites) were done on duplicate sections to compare parasite populations in the brains. All mice in group 3 developed recrudescent toxoplasmosis and died or were killed. None of the other mice developed toxoplasmosis and none died. Mice in group 1 which were treated with only SD had minimal lesions (mean = 2) in their brains. Lesion scores in mice in group 2 were variable (mean = 2.5). Mice in group 3 had extensive lesions (mean = 5). The control mice in group 4 were lesion (mean = 1) free. Lesion scores obtained with BAG1 anti-sera were identical to lesion scores obtained with polyclonal anti-*T. gondii* sera. This study

demonstrates that ponazuril is active against recrudescent toxoplasmic encephalitis.

INTRODUCTION

Toxoplasmic encephalitis (TE) is a well-recognized problem in humans and domestic and companion animals (Dubey 1993). Pyrimethamine and sulfadiazine (SD) are the mainstay treatments for TE in humans and animals (Lindsay and Dubey 1999) but toxic side effects can occur with the use of this combination of agents (Lindsay and Blagburn 2000). Additional non-toxic chemotherapeutic agents are needed to combat TE. Ponazuril is the major metabolite of toltrazuril, a triazine anticoccidial used in the poultry industry in Europe and South America. Ponazuril has been shown to be active against *Sarcocystis neurona* in vitro (Lindsay, Dubey, and Kennedy 2000; Mitchell et al. 2005) and in vivo (Franklin et al. 2003) and is approved for use in horses in the United States to treat equine protozoal myeloencephalitis. Ponazuril is also active against the related parasite *Neospora caninum* when tested in vitro (Darius, Mehlhorn, and Heydorn 2004a; Mitchell et al. 2005) and in vivo (Darius, Mehlhorn, and Heydorn 2004b; Gottstein et al. 2001) and against *Toxoplasma gondii* when tested in vitro (Mitchell et al. 2003, 2005) and in vivo (Mitchell et al. 2004). The present study was done to determine the in vivo activity of ponazuril against TE in an immunodeficient mouse model.

MATERIALS AND METHODS

A 50 mg/ml ponazuril (lot.2161AA) suspension was obtained from Bayer HealthCare Animal Health. This suspension was diluted in distilled water and used for in vivo testing. This is the same suspension used by Mitchell et al. (2004) in their studies on prevention and treatment of acute toxoplasmosis in

mice. Ponazuril was used at a dosage of 20 mg/kg to treat mice. Mice were treated orally once daily with ponazuril.

Tissue cysts of a type III strain of *T. gondii* isolated from chickens (Dubey et al. 2003) were obtained from the brains of experimentally infected mice. Groups of interferon γ gene knockout (INFG-KO) mice were each orally fed 10 tissue cysts. Group 1 was infected and treated with SD for the remainder of the study. Group 2 was infected and treated with SD followed by ponazuril treatment for recrudescent toxoplasmosis. Group 3 was infected and treated with SD followed by water (i.e. no treatment) for recrudescent toxoplasmosis. Group 4 was not infected and used as non-infected non-medicated controls (Table 1). Infected mice were treated with 1 mg/ml SD in the drinking water beginning 4 days post-inoculation (PI) to prevent death from toxoplasmosis and to allow the development of chronic infections. Mice were treated with SD for 77 days at which time groups 2 and 3 were taken off of SD for 7 days to allow for recrudescence of infection (Table 1). Mice in group 2 were then treated for 10 days with 20 mg/kg ponazuril (Table 1) while the mice in group 3 were not treated.

During the study, impression smears were made from the livers or lungs of any mice that died, and were examined unstained by light microscopy for tachyzoites. Their brains were removed and fixed in 10% neutral buffered formalin solution. All surviving mice in all four groups were killed 10 days after ponazuril treatment was initiated. Their brains were removed and fixed in 10% neutral buffered formalin solution and processed for staining with hematoxylin

and eosin. Additional brain sections from each mouse were processed for immunohistochemical staining with BAG1 antibodies to demonstrate tissue cysts (BAG1 is specific for bradyzoites) and separate brain sections were processed for immunohistochemical staining with rabbit polyclonal anti-*T. gondii* serum to demonstrate tachyzoites and other reactive stages of *T. gondii* (Dubey et al. 2001; Lindsay and Dubey 1989; McAllister et al. 1996).

After staining, slides were examined using light microscopy and a lesion score assigned. The entire section stained with BAG1 was examined and the following scoring system was used: 0=0 tissue cysts; 1=1–3 tissue cysts; 2=4–6 tissue cysts; 3=7–10 tissue cysts; 5=≥11 tissue cysts. The brain sections stained with the rabbit polyclonal anti-*T. gondii* serum were scored by counting groups of tachyzoites in the entire section: 0=0 groups; 1=1–12 groups; 2=13–20 groups; 3=21–32 groups; 4>32 groups.

RESULTS and DISCUSSION

Mice in group 1 which were treated with only SD during the study had no mortality and minimal lesions (mean=2) in their brains (Table 1). None of the mice in group 2 treated with SD and then ponazuril died. Lesion scores were variable (mean=2.5). All mice in group 3 taken off of sodium sulfadiazine and treated with water died within 10 days. These mice had extensive lesions (mean=5) in their brains. The control mice in group 4 were lesion-free (mean=1). Lesion scores obtained with BAG1 anti-sera were identical to lesion scores obtained with polyclonal anti-*T. gondii* sera. Tissue cysts and tachyzoites were observed in mice from all groups except those in group 4.

Ponazuril is highly active in this animal model of recrudescent TE. It also has excellent activity in preventing and treating acute toxoplasmosis in out-bred mice when used at 20 mg/kg for 10 days (Mitchell et al. 2004). The apicoplast is probably the site of activity of ponazuril and other triazine anti-coccidials (Hackstein et al. 1995). The apicoplast is a vestigial, non-photosynthetic plastid that has been described in apicomplexan parasites (Köhler et al. 1997). It probably was acquired by secondary endosymbiosis of a green alga. The function of the plastid is not known but it is essential for parasite survival. In plants, the plastids are the site for many biochemical pathways including the biosynthesis of folate, amino acids, ubiquinone, heme, nucleotides, lipids, and starch (Roberts et al. 1998). Because they are not present in vertebrate cells, the apicoplast is an attractive drug target for chemotherapy specifically against apicomplexan parasites (Fichera and Roos 1997).

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Table 1. Protocol and immunohistochemistry scores of mice with recrudescent toxoplasmosis treated with 20 mg/kg ponazuril

Group/mouse #	Sex	Treatment[a]	BAG 1[b] score	Polyclonal[c] score
1/1	M	T.g.+SD+SD	1	1
1/2	M	T.g.+SD+SD	ND	ND
2/1	F	T.g.+SD+pon	4	4
2/2	F	T.g.+SD+pon	0	0
2/3	F	T.g.+SD+pon	0	0
2/4	F	T.g.+SD+pon	1	1
2/5	M	T.g.+SD+pon	3	3
2/6	M	T.g.+SD+pon	1	1
2/7	M	T.g.+SD+pon	1	1
3/1	F	T.g.+SD+water	4	4
3/2	F	T.g.+SD+water	4	4
3/3	F	T.g.+SD+water	4	4
4/1	M	Water only	0	0
4/2	F	Water only	0	0

[a]Infected mice (groups 1–3) were treated with 1 mg/ml SD in the drinking water beginning 4 days post-inoculation (PI) to prevent death from toxoplasmosis and to allow the development of chronic infections. Mice were treated with SD for 77 days at which time groups 2 and 3 were taken off of SD for 7 days to allow for recrudescence of infection. Mice in group 2 were then treated for 10 days with 20 mg/kg ponazuril while the mice in group 3 were not treated.

[b]BAG1 is a tissue cyst-specific antiserum that stains bradyzoites.

[c]Polyclonal anti-*T. gondii* serum recognizes tachyzoites and other stages.

T.g., mice infected with *Toxoplasma gondii*; SD, sulfadiazine treatment given; pon, ponazuril treatment given; M, male; F, female; nd, not determined.

CHAPTER 9

GENERAL CONCLUSIONS

This dissertation confirmed that *C. canis* is a primary pathogen of young canines and the sole cause of clinical disease, as described in Chapter 3. Puppies were obtained at a young age to limit the possibility of a natural *C. canis* infection. Bacterial pathogens were ruled out by treating the oocyst inoculum with bleach, rinsing with sterile HBSS and streaking blood agar and TSA plates prior to inoculation. No bacterial growth occurred on the streaked plates after allowing ample time for bacteria growth, concluding our inoculum was bacteria free. Intestinal sections from 2 dogs were collected for bacteriological culture and histology. Bacterial pathogens were not isolated from intestinal tissues. Viruses were ruled out by examining fecal samples by TEM. Examination of histopathological intestinal tissues confirmed a *C. canis* infection was present. By infecting small groups of pups over a 2 year period of time, we were able to reproduce severe clinical disease in puppies within a laboratory environment. From this experimental design we conclude that sporulated *C. canis* oocysts given at a dose of 1×10^5 in young dogs is sufficient to cause canine coccidiosis and is considered a good model to be used for anticoccidials studies. We did not look at ponazuril's efficacy on *C. canis* infections in dogs. However, Reinemeyer et al., 2007 modified our canine coccidiosis model and evaluated the efficacy of ponazuril on *C. canis* infections. A single dose of ponazuril at 20 to 50 mg/kg significantly reduced oocysts counts by >94%.

Chapter 4 examined the growth of *C. canis* in cell culture. Interestingly, sporozoites released into cultures of 2 non-primary canine cell lines, developed into unizoites. These zoites maintained the typical characteristics of extraintestinal cysts found in paratenic and definitive host tissues. A centrally located zoite with a thick cyst wall developed within a parasitophorous vacuole. No replication occurred in either cell type. Examination using TEM, confirmed these stages are unizoic cysts based on morphology. Future studies should examine if unizoic cysts grown in cell culture can cause canine coccidiosis.

The need for new therapeutic agents to treat coccidia remains a priority in human and animal health. The study described in chapter 5 looked at a possible mode of action of ponazuril against *T. gondii*. Transmission electron microscopy determined ponazuril interferes with tachyzoite division. Tachyzoites normally divide by endodyogeny. Ponazuril treated *T. gondii* tachyzoites had multiple disfigured nuclei and were not able to separate from parent tachyzoites creating schizont-like stages. Large vacuoles, swollen Golgi and membranous inclusions in treated parasites were also present. We did not examine if the disfigured, ponazuril treated tachyzoites maintained their infectivity in vitro or the ability to cause disease in vivo. The apicoplast, a non-photosynthetic plastid and vital organelle, is thought to be the target for ponazuril and other triazine agents. Currently, the function(s) of the apicoplast are not clearly defined but it has been determined that is essential to the parasites survival (Marechal and Cesbron-Delauw., 2001). To determine at what concentration ponazuril is effective *in vitro* against *T. gondii*, a tachyzoite production assay was described in chapter 7.

Results showed tachyzoites were inhibited at a concentration of 1.0 μg/ml and this was not statistically different from a treatment dose of 5.0 μg/ml. Ponazuril proved to be coccidial static once treated media was removed and replaced with maintenance media.

We also determined that ponazuril affects the second round of tachyzoite endogenous replication (chapter 7). However, *N. caninum* was not affected until the 4th replication suggesting the differences in the divisional life cycles between these two parasites may affect how drugs act on the tachyzoites for each species (chapter 6). TEM of ponazuril treated *N. caninum* found dead tachyzoites with in a parasitophorous vacuole. Multinucleated schizont-like stages were not observed. Ponazuril's effect on *S. neurona* was also evaluated but our studies were inconclusive (chapter 6).

In chapter 7 and 8, we determined ponazuril is highly active in preventing and treating acute and chronic *T. gondii* infections in mice. Acute toxoplasmosis was prevented in mice pre- treated at 20 mg/kg ponazuril one day prior to infection according to PCR and serology results. Ponazuril was effective in preventing fatal toxoplasmosis when given 3 or 6 dPI at a dose of 10 or 20 mg/kg but all mice tested PCR positive for *T. gondii* confirming infection was present. Tissue cysts associated with chronic toxoplasmosis were reduced in brains from immunodeficient mice treated with 20 mg/kg ponazuril for 10 days.

LITERATURE CITED

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