Characterizing *Cystoisospora canis* as a Model of Apicomplexan Tissue Cyst Formation and Reactivation

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ABSTRACT

Cystoisospora canis is an Apicomplexan parasite of the small intestine of dogs. *C. canis* produces monozoic tissue cysts (MZT) that are similar to the polyzoic tissue cysts (PZT) of *Toxoplasma gondii*, a parasite of medical and veterinary importance, which can reactivate and cause toxoplasmic encephalitis. We hypothesized that *C. canis* is similar biologically and genetically enough to *T. gondii* to be a novel model for studying tissue cyst biology. We examined the pathogenesis of *C. canis* in beagles and quantified the oocysts shed. We found this isolate had similar infection patterns to other *C. canis* isolates studied. We were able to superinfect beagles that came with natural infections of *Cystoisospora ohioensis*-like oocysts indicating that little protection against *C. canis* infection occurred in these beagles. The *C. canis* oocysts collected were purified and used for future studies. We demonstrated in vitro that *C. canis* could infect 8 mammalian cell lines and produce MZT. The MZT were able to persist in cell culture for at least 60 days. We were able to induce reactivation of MZT treated with bile-trypsin solution. In molecular studies, we characterized *C. canis* genetically using ITS1 and CO1 to build phylogenetic trees and found *C. canis* was most similar to *C. ohioensis*-like with ITS1 and more similar to *T. gondii* than any other coccidia using ITS1 and CO1. We identified genes and proteins involved with virulence, cyst wall structure, and immune evasion of *T. gondii* and examined the DNA of *C. canis* for orthologs. *C. canis* had orthologs with 8 of 20 *T. gondii* genes examined. Monoclonal and polyclonal antibody and lectin studies demonstrated similar tissue cyst wall proteins on *C. canis* MZT and *T. gondii* PZT. Our findings in vitro and using genetic characterization of *C. canis*

indicated the presence of similar genes and proteins, and its close phylogenetic location with *T. gondii* demonstrate that *C. canis* may serve as a model to examine tissue cyst biology. The system we described provides a simple model to produce tissue cysts and to study host factors that cause reactivation of tissue cysts.

DEDICATION

This dissertation is dedicated to my family, friends and husband for their unwavering love and support and in memory of Samuel Wendler, J. Kenley Carr and Carmen Miles.

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---Alice Elizabeth Houk- Miles, MPH---

ATTRIBUTIONS

My doctoral projects would not have been possible without the guidance and assistance of my committee, collaborators and colleagues who contributed to the research and completion of the manuscripts making up this dissertation.

Chapters 2 through 5:

David S. Lindsay, PhD (Department of Biomedical Sciences and Pathobiology) is a Professor of Parasitology at VMCVM and is the corresponding author on these manuscripts. He aided in project development, writing, and editing of all of the manuscripts.

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Chapter 2:

Thomas O'Connor, PhD, is a research fellow at IDEXX Laboratories. He helped with project development, editing of manuscripts and funding of the project.

Solange Maria Gennari, DVM, MS, PhD and Hilda F. J. Pena, PhD (Department of Preventative Veterinary Medicine and Animal Health) are a Professor and Post-Doctoral Researcher, respectively, from the College of Veterinary Medicine and Animal Science at the University of Sao Paolo in Brazil and are responsible for collecting the isolate of *C. canis* used to infect the beagles, resulting in the oocysts used for the other projects. They also assisted with editing of the manuscripts.

Chapter 4:

Caitlin M. Cossaboom, MPH, PhD (Department of Biomedical Sciences and Pathobiology) is a co-author on this manuscript and is currently a veterinary student at VMCVM. She helped with sequence data analysis, GenBank submission, and editing of the manuscripts.

Arielle Arnold, BS (Department of Biological Sciences) served as an undergrad research assistant and assisted in sample collection and data processing.

Chapter 5:

Jeannine S. Strobl, PhD (Department of Electrical and Computer Engineering) is a member of the research faculty at Virginia Tech and is a co-author on the manuscript. She provided mentorship and helped with project development and editing of the manuscript.

Nammalwar Sriranganathan, BVSc, MVSc, PhD, Diplomate ACVM (Department of Biomedical Sciences and Pathobiology) is a Professor at VMRCVM and is a co-author on the manuscript. He provided mentorship and helped with project development and editing of the manuscript.

Louis M. Weiss, MD. MPH (Departments of Pathology and Medicine) is a professor at the Albert Einstein College of Medicine. He provided information on *Toxoplasma* tissue cysts as well as the antibodies used in the study.

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GENERAL INTRODUCTION

Phylum Apicomplexa is comprised of such human and animal parasites as those responsible for malaria, toxoplasmosis and coccidiosis (Labesse et al., 2009). *Cystoisospora* species can be a causative agent of intestinal coccidiosis—a disease in which the primary clinical sign is diarrhea—in canids, felids, swine and humans worldwide (Lindsay et al., 2011).

Cystoisospora canis infects young dogs and can be distinguished from the three other *Cystoisospora* species that infect dogs due to its relative large size (>33 µm) compared to (<30 µm) for the others (Lindsay et al., 1997a). *Cystoisospora canis* has a prevalence around 4.4% in the United States (Little et al., 2009) although canine coccidiosis prevalence has been found to be as high as 38% (Kirkpatrick and Dubey, 1987; Lindsay, 1990). Once infective oocysts are consumed, it takes 9-11 days for the parasite to reproduce in the small intestine before the dog starts to shed oocysts in the feces and the process can continue for 8-10 days (Lindsay, 1990; Mitchell et al., 2007). Once the oocysts are shed into the environment, it takes 1-2 days for them to become infective to other dogs, depending on temperature and humidity (Lepp and Todd, 1974). The oocysts are able to survive in the environment for months and are resistant to disinfection (Barutzki et al., 1981; Buehl et al., 2006).

Transmission is usually fecal-oral and dogs become infected when they consume infective oocysts in the environment. It has also been found that paratenic hosts like rodents can become infected by ingesting oocysts and the parasite will leave the gut and create monozoic tissue cysts (MZT) in tissues like the lymph nodes, spleen and liver (Dubey, 1975; Mitchell et al., 2009). It is possible for dogs to become infected by ingestion of the tissue cysts in these paratenic hosts (Dubey, 1982; Houk and Lindsay, 2013). If *C. canis* is similar to its human-infectious counterpart *C. belli*, tissue cysts that form in the dog may also be able to reactivate,

allowing the parasite to travel back to the gut and cause intestinal disease once again (Lindsay et al., 1997b; Velasquez et al., 2001).

C. canis is a member of the family Sarcocystidae, made up of protozoa able to form tissue cysts, and *C. canis* is biologically similar to *Toxoplasma gondii* (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Samarasinghe et al., 2008). Rather than the monozoic tissue cysts containing a single parasite that *C. canis* forms, *T. gondii* is able to form polyzoic tissue cysts (PZT) as *T. gondii* multiples asexually producing bradyzoites in the cell before encysting (Dubey et al., 2009). These PZT are able to remain dormant in the tissue for the lifespan of the host while the parasite is able to evade the host immune response by suppressing apoptosis and recruitment of pro-inflammatory cells (Butcher et al., 2011; Sauer et al., 2013; Tomita et al., 2013). *T. gondii* affects approximately 30% of the human population worldwide (Skariah et al., 2010) and PZT reactivation with egress of bradyzoites can cause severe complications including encephalitis and ocular and congenital diseases (Dubey et al., 2009). *T. gondii* tissue cyst biology has proven itself very difficult to study and not much is known about the mechanisms behind reactivation (Bhadra et al., 2011).

Due to the complex nature of the *T. gondii* PZT, we proposed *C. canis*'s MZT as a model. Before using it as a model however, more in depth characterization than was present in the literature had to occur. We began by obtaining an isolate of *C. canis* from colleagues in Brazil and then testing the isolate's pathogenicity in beagles, as it has been found not all isolates or inoculum sizes of *C. canis* are equally infectious (Houk et al., 2013; Lepp and Todd, 1974; Mitchell et al., 2007).

Previous studies have examined the behavior of *C. canis* in vitro but reports about whether or not *C. canis* makes a MZT in cell culture or underwent asexual division differed so

we set out to examine whether or not our Brazilian isolate of *C. canis* underwent asexual development or produced MZT in different host cell types from different animal species. We then needed to see how long the MZT could be cultured and if we could stimulate a reactivation event allowing us to study PZT reactivation at a later date (Houk and Lindsay, 2013).

Finally, there had been no characterization of the genes or proteins of *C. canis*. We first wanted to perform phylogenetic analysis to identify the position of *C. canis* within the *Cystoisospora* clade and then confirm that *C. canis* is indeed, similar to *T. gondii*. We then searched the literature for genes known to be used in parasite virulence and identified proteins involved with *T. gondii* tissue cysts. We used PCR and immunofluorescence assays to determine the presence of genes and proteins orthologous between *C. canis* and *T. gondii*.

C. canis is a common parasite of dogs (Reinemeyer, 2007) but has not been the focus of much research. The literature often conflicts so the goal of this work was not only to elucidate which findings are more likely to be correct, but also to gain better understanding of the behavior of *C. canis* and the genes and proteins that allow it to be effective at infecting cells and remaining unmolested in a MZT state. In addition to furthering our knowledge about *C. canis* and its MZT, this research is also important for what we apply to our understanding of other apicomplexan parasites, like *T. gondii*, and their tissue cysts.

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Chapter 1: *Cystoisospora canis* - Literature Review

BACKGROUND

General History of C. canis

Cystoisospora (synonym *Isospora*) is a genus of protozoan parasites within the Phylum Apicomplexa that are commonly found infecting the intestinal tract of their hosts. *Cystoisospora* species can be found in canids, felids, swine and humans worldwide and are able to cause diarrhea (Frenkel, 1977; Lindsay et al., 2011). Originally, this genus was known as *Isospora* after being discovered by Schneider in 1881 but J.K. Frenkel proposed a name change in 1977 due to the ability of these parasites to leave the intestine and make monozoic tissue cysts in paratenic hosts (Frenkel, 1977). These parasites were originally thought to be most similar to *Eimeria* spp. due to their homoxenous—or one host—life cycle (Levine and Ivens, 1981). Later, molecular studies confirmed Frenkel's designation by studying the 18S rRNA locus and finding *Cystoisospora* spp. to be more similar to members of the family Sarcocystidae—which includes tissue cyst-forming coccidians *Toxoplasma gondii* and *Neospora caninum*—rather than Eimeriidae (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Matsubayashi et al., 2011).

Cystoisospora canis was first described by Nemeséri in 1959 (Mitchell et al., 2007) and is one of four known *Cystoisospora* species to infect dogs. While structurally similar to the other three, *C. canis* is easily distinguishable by its large size (> 33 µm) compared to the smaller (< 30 µm) *C. ohioensis*, *C. burrowsi*, and *C. neorivolta* (Lindsay et al., 1997a). Some other well-known species of *Cystoisospora* include: *C. belli* which infects humans, *C. felis* and *C. rivolta*

infecting cats and *C. suis* infecting pigs. There are several other species that are of less significance that we do not know much about as yet.

Life Cycle

Dogs become infected when a sporulated oocyst is consumed. Mechanical and chemical disruption from digestion causes the four sporozoites within each of two sporocysts in the oocysts to excyst. In the posterior portion of the small intestine, the sporozoites penetrate the lamina propria and undergo asexual development. After three generations, the merozoites then undergo sexual development which leads to the formation of oocysts. It takes 9-11 days before oocysts begin to be shed in the feces—the pre-patent period—and they may continue to be shed for 8-10 days—the patent period (Houk et al., 2013; Lindsay, 1990; Mitchell et al., 2007).

Once the oocysts are passed into the environment they can sporulate in 1-2 days, depending on temperature and humidity, and then infect other dogs (Lepp and Todd, 1974). The oocysts are able to survive for months in the environment and are resistant to disinfection (Barutzki et al., 1981; Buehl et al., 2006). In addition to canine infections, paratenic hosts like small rodents can consume the sporulated oocysts and become infected (Dubey, 1975; Dubey and Mehlhorn, 1978). In the paratenic hosts, the sporozoites leave the intestine and journey to other organs like lymph nodes, spleen, and liver where they encyst as monozoic tissue cysts (MZT) (Mitchell et al., 2009). Ingestion of MZT may serve as another mode of transmission for dogs (Dubey, 1982; Houk and Lindsay, 2013). MZT in a dog may reactivate and recolonize the intestines causing recrudescence of disease and renewed oocyst shedding similar to *C. belli* (Lindsay et al., 1997b; Velasquez et al., 2001).

Monozoic Tissue Cysts

Instead of undergoing the normal developmental cycle in the intestinal tract, some sporozoites may leave the lamina propria, invade extra-intestinal sites, and produce MZT in the host. In humans, mesenteric lymph nodes are most often infected but other tissues such as the liver, spleen, and tracheobronchial and mediastinal lymph nodes can be infected (Velasquez et al., 2011). The MZT contain a single parasite structurally resembling a sporozoite (Roberts et al., 1972) which Frenkel and Dubey (1972) termed a "zoite".

There are very few studies on how *C. canis* sporozoites behave once inside the host cells and reports of their behavior vary (Fayer and Mahrt, 1972). Studies suggest that *C. canis* sporozoites undergo binary fission (endodyogeny) as Fayer and Mahrt (1972) observed paired zoites within bovine and canine cells in vitro. Studies in mice tissues (Frenkel and Smith, 2003) and in vitro found that *C. canis* sporozoites instead produced MZT in African green monkey (CV-1) and bovine turbinate (BT) cells (Houk and Lindsay, 2013; Mitchell et al., 2009). These MZT contain a single asexual stage surrounded by a tissue cyst wall that does not undergo asexual multiplication. *Cystoisospora belli* (synonym *Isospora belli*) of humans undergoes endodyogeny (binary fission) in cell culture and does not produce tissue cysts in vitro (Oliveira-Silva et al., 2006). However, MZT do occur in the tissues of humans infected with *C. belli* (Comin and Santucci, 1994; Frenkel et al., 2003; Lindsay et al., 1997b; Michiels et al., 1994; Restrepo et al., 1987), in the tissues of dogs and cats infected with *Cystoisospora* species (Dubey and Frenkel, 1972) as well as the tissue of experimentally infected mice (Frenkel and Smith, 2003).

Monozoic tissue cysts of *C. belli* in histological sections are thick-walled and measure 12-22 x 8-10 µm in size, each containing a single zoite. Ultrastructurally, a *Cystoisospora* tissue cyst consists of a single centrally located zoite surrounded by a granular tissue cyst wall within a

parasitophorous vacuole (PV). Zoites contain the cellular organelles characteristic of infective asexual stages (sporozoites, merozoites, tachyzoites, bradyzoites) of coccidial parasites (Lindsay et al., 1997b; Mitchell et al., 2009). The tissue cyst wall is next to the limiting membrane of the PV. Recurrent clinical disease is common in both immunocompetent and immunosuppressed patients and it is believed to be due to reactivation of the zoites present in the *Cystoisospora* tissue cysts and migration to the human intestinal tract (Lindsay et al., 1997b; Velasquez et al., 2001). It is common for immunosuppressed patients to relapse with clinical disease once *C. belli* treatment has been stopped (Boyles et al., 2012; Lindsay et al., 2011).

Canine Coccidiosis and Host Immune Response to Coccidial Infections

C. canis infection behaves the way many coccidial infections do. Clinical signs of disease include watery or bloody diarrhea, weight loss, lethargy, vomiting, dehydration, anorexia and, rarely, fatality may occur. Disease in healthy animals is generally self-limiting but sulfadimethoxine, or other off-label drugs, and supportive care may be administered to help control the infection (Houk et al., 2013; Mitchell et al., 2007).

There is some controversy about the ability of *C. canis* to cause infection in dogs. Studies by Neméseri (1960) found that clinical coccidiosis could be achieved with an inoculum of 5-8 x 10^4 oocysts but Lepp and Todd (1974) could not produce disease with 1-1.5 x 10^5 oocysts of an isolate from Illinois. More recently, Mitchell et al. (2007) and Houk et al. (2013) were able to infect 6-8 week beagles with 1 x 10^5 oocysts using oocysts isolated from either a dog from Virginia or Brazil and achieve clinical disease. This supports the argument by Levine and Ivens (1981) that strain differences may result in different pathogenicity in dogs.

Young puppies are the most susceptible to infection with *C. canis*. It is possible to infect a day old pup if enough oocysts are used but susceptibility to infection increases with age until the pup is weaned. It is thought that this phenomenon has something to do with the physiological and biochemical properties of the intestine which change when the pup's diet changes from milk to solid food. Lepp and Todd (1974) found that dogs that were fed 1 x 10^6 oocysts at 6 weeks of age were immune from further *C. canis* infection after 2 months and immune after 1 month when given 2 x 10^6 oocysts at 10 weeks of age (Lepp and Todd, 1974; Levine and Ivens, 1981). While previous infection with *C. canis* was protective, prior *C. ohioensis*-like infection did not protect against *C. canis* (Mitchell et al., 2007).

C. canis is an intracellular parasite like *T. gondii* so it primarily generates a Th1 immune response from the host which relies on CD8+ and IFN-γ to protect from reinfection and keep the tissue cyst dormant (Sauer et al., 2013). Apicomplexans are able to interfere with host cell signaling to suppress apoptosis and upregulate the production of interleukin 12 (IL-12) which blocks the pro-inflammatory responses to recruit IFN-γ (Butcher et al., 2011). This means there is no cell-mediated killing of MZT and the parasites are able to remain throughout the lifespan of the host in a delicate balance.

Epidemiology, Transmission and Prevention

Young puppies are the primary ones infected with *C. canis* (Mitchell et al., 2007). The prevalence of *C. canis* in dogs in the United States averages around 4-5% (Blagburn et al., 1996; Little et al., 2009) although canine coccidiosis can reach as high as 38% (Kirkpatrick and Dubey, 1987; Lindsay, 1990). Patent infection is most often found in dogs from the southeastern portion of the U.S. because of temperature and humidity, physiologically stressed or stray animals who

hunt and feed on infected paratenic hosts, or in those who live in areas with poor sanitation where fecal-oral transmission is more likely (Blagburn et al., 1996; Lindsay, 1990).

MOLECULAR CHARACTERIZATION

As previously mentioned, phylogenetic analysis of the rRNA 18S gene has shown that *Cystoisospora* spp. are more closely related to *Toxoplasma* than *Eimeria*, indicating that *Cystoisospora* belongs in the family Sarcocystidae along with *T. gondii* (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Samarasinghe et al., 2008). Little has been done to molecularly characterize *C. canis* and there is limited sequence data in GenBank. Samarasinghe et al. (2008) made efforts to phylogenetically characterize other species of *Cystoisospora* and using partial 18S, ITS1 and 5.8S sequences for other Sarcocystidae and developed primers that amplify the internal transcribed spacer 1 (ITS1) rDNA of *Cystoisospora* spp. The ITS1 region has been used historically because there are several copies between DNA subunits encoding for ribosomes found in all organisms. Because of the availability and relative ease of amplifying the ITS1 locus, it has been widely used in phylogenetic and other molecular studies. From the ITS1, primers were designed to only amplify a *Cystoisospora*-specific region of that locus. They were also able to use restriction fragment length polymorphism (RFLP) to distinguish between species using the AluI restriction enzyme. This was especially thought to be important for the smaller and morphologically indistinguishable *C. ohioensis* complex that occurs in dogs (He et al., 2012b; Matsubayashi et al., 2011; Samarasinghe et al., 2008).

Another genetic marker used for phylogenetic study is the 5'-end of the cytochrome *c* oxidase subunit 1 (CO1) gene of the mitochondria. While 18S is the most readily available genetic marker for phylogenetic studies, CO1 has become more widely used in recent years and is a part of the Barcode of Life initiative, a global effort to catalog and identify new species by using short,

standardized DNA sequences (Ogedengbe et al., 2011; Teletchea, 2010). Mitochondrial CO1 is found in all eukaryotes since it is involved in oxidative phosphorylation during cellular respiration and is the most widely used genetic target for animal barcoding. Studies performed with other coccidia, found CO1 to be better than more traditionally used 18S in resolving species into monophyletic clades. When comparing the two markers, CO1 had a greater distance between different species and because it codes for a protein, it is easier to align sequences (Ogedengbe et al., 2011; Ogedengbe et al., 2013; Teletchea, 2010).

GENES AND PROTEINS INVOVLED WITH VIRULENCE AND PATHOGENESIS

Parasite Invasion into Host Cells

As *T. gondii* is an obligate intracellular parasite, it is necessary for its survival and reproduction to infect new host cells. Due to the conserved nature of invasion mechanisms, we can make hypotheses about other apicomplexans (Lebrun, 2007). When the parasites invade cells, there are three main organelles within the apical complex working together to penetrate the cell and establish the PV—micronemes, rhoptries and dense granules (Carruthers and Sibley, 1997; El Hajj et al., 2006). Micronemal proteins are associated with gliding and attachment to the host. Micronemes and rhoptries work together to form tight attachments between the parasite and the host cell before invading the host cell and situating itself inside the PV created by the dense granule proteins and rhoptries (Coppens et al., 1999; Labesse et al., 2009). After invasion, a change in host cell transcription patterns can be seen resulting in the up-regulation of pro-inflammatory cytokines, protection from apoptosis, changes in metabolism and cell growth and differentiation (Blader and Saeij, 2009).

Rhoptries are of particular interest because of the role of their proteins in parasite-host cell interactions. Six to 12 rhoptries are found in each cell and they are club-shaped with a bulbous base (bulb) and a duct (neck) that extends to the anterior pole of the parasite (Lebrun, 2007). There are two types of rhoptry proteins: rhoptry neck proteins (RONs) and proteins that originate from the bulb (ROPs). RONs interact with micronemes during invasion and help propel the parasite through the host cell's plasma membrane into the newly forming PV (Carruthers et al., 2000). ROPs are associated with the membrane of the PV that the RONs helped form and ROPs can interact with the host cell mitochondria and enter the nucleus to change host cell gene expression (Beckers et al., 1994; El Hajj et al., 2006; Labesse et al., 2009).

While many of the ROPs contribute to establishment and survival of the parasite in the host cell, the ROP2 family is especially interesting as they can anchor the host cell mitochondria to the parasitophorous vacuole membrane (PVM) control the proliferation of *T. gondii* and appear to be critical for rhoptry genesis (Bradley et al., 2005; El Hajj et al., 2007; Nakaar et al., 2003; Sinai and Joiner, 2001). There are at least 12 proteins known to be included in the ROP2 family: ROP2, 4, 5, 7, 8, 11, 16, 17, and 18 (Labesse et al., 2009). Some of the most intensely studied are ROP5, ROP16 and ROP18. ROP5 has been implicated as a major virulence factor that can reduce the amount IFN-γ-inducible-immunity related GTPases in the PV membrane so that the integrity of the membrane is not compromised and the parasites inside can evade cell-mediated killing by IFN-γ (Niedelman et al., 2012; Zheng et al., 2013). Upon invasion into the host cell, ROP16 is injected into the cytoplasm before traveling to the host cell nucleus where it has the ability to control host signaling pathways and suppresses the macrophage pro-inflammatory response (Butcher et al., 2011). ROP18 is a protein kinase which can control the proliferation of parasites within the cell and its interaction with the PV membrane suggests an

ability to modify other proteins—those from dense granules, micronemes or other rhoptries—and help control host cell function and/or signaling (El Hajj et al., 2007).

Due to the importance of the parasite's organelles in the apical complex in virulence, they have been targeted for vaccine research. Dense granules, micronemes, and rhoptries all have vaccine candidates with GRA4, MIC3, and ROP5 being some of the most researched. Some of the most promising studies for a vaccine against *T. gondii* have combined multiple proteins from each organelle with a surface antigen of tachyzoites (SAG1) (Ismael et al., 2003; Zheng et al., 2013). The ROP2 family appear to only have homologs in Sarcocystidae and since they are not found in other apicomplexans like *Eimeria* or *Plasmodium*, any vaccine using these may only be good for coccidia closely related to *T. gondii* (El Hajj et al., 2007).

Tissue Cyst Proteins

It is important to study the tissue cyst wall because research suggests that latent tissue cysts are able to evade the immune response so that they may last for the life of the host and serve as reservoirs for reactivated infection (Tomita et al., 2013). We know that *C. canis* has a relatively thick tissue cyst wall compared to *T. gondii*'s 0.25-0.75 µm (Dubey et al., 1998) but otherwise, there have been no studies on its composition. However, there have been studies on the *T. gondii*'s cyst wall and due to their biological similarities, inferences can be made. Researchers have used immunofluorescent studies to better understand the tissue cyst wall composition. One reagent used is *Dolichos biflorus* lectin (Zhang et al., 2001) which recognizes sugar residues at the CST1 glycoprotein in the tissue cyst wall of *T. gondii*. The CST1 glycoprotein has been indicated to be a key structural component of tissue cysts and Tomita et al. were able to find a monoclonal antibody that binds to CST1. They were also able to develop polyclonal antisera to BAG1/heat

shock protein 30 which is involved in differentiation *of T. gondii* from the acute to the latent tissue cyst stage (Tomita et al., 2013).

CONCLUSIONS

In summary, apicomplexans are important to research as they are responsible for diseases affecting both the human and animal populations worldwide. Studies have been conducted to determine the life cycle and pathology of *C. canis* infection but only preliminary research has been conducted to study the behavior of MZT in cell culture and there is no published molecular data. While simple measures can be taken to prevent parasitic infection—adequately washing hands, foods and work surfaces, staying away from unfiltered water, cooking meat to an appropriate temperature, picking up feces in a timely manner and adequately cleaning pet enclosures—is important to study these parasites so advances can be made in drug and vaccine discovery.

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Chapter 2: Experimentally Induced Clinical *Cystoisospora canis* Coccidiosis in Dogs with Prior Natural Patent *C. ohioensis*-like or *C. canis* Infections

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ABSTRACT

Diarrhea caused by intestinal coccidia (*Cystoisospora* species) is a common problem in pet dogs and in dogs in animal shelters. *Cystoisospora canis* has the largest oocysts of the 4 named species of coccidia infecting dogs. The present study examined an isolate of *C. canis* obtained from a dog from São, Paulo, SP, Brazil. Oocysts sporulated within 2 days at room temperature and 20 sporulated oocysts were 37.6 by 28.6 µm (range 35-42 by 26-31 µm). Most sporulated oocysts contained 2 sporocysts each with 4 sporozoites although a few (<1%) were *Caryospora*-like contained 1 sporocyst with 8 sporozoites. Two experiments using a total of 11 female 6-wk-old beagles were conducted to determine the pathogenicity of oral infection with 5 x 10^4 sporulated oocysts of this isolate of *C. canis*. Five of the 11 dogs had natural infections with *C. ohioensis*-like (N=4) or *C. canis* (N=1) prior to the predicted patent period of 9-10 days. Ten of the dogs developed diarrhea with occasional blood, and 3 dogs were affected to the extent that clinical treatment for coccidiosis using sulfadimethoxine was recommended. Dog CRU had a natural *C. canis* infection and did not develop clinical disease after oral infection with *C. canis* oocysts. This dog had a prepatent period of 9 days and a patent period of 3 days, corresponding to experimental infection with the new isolate of *C. canis*. It excreted fewer *C. canis* oocysts than did the other dogs. The 4 dogs with natural *C. ohioensis*–like infection all developed clinical disease, and 1 required treatment. The prepatent period was 9–10 days, and the patent period was 10–11 days in these dogs. All 6 dogs not naturally infected with *Cystoisospora* developed clinical disease, and 2 required treatment. The prepatent period was 9–10 days, and the patent period was 8–12 days. The present study confirms that *C. canis* is a primary pathogen for young dogs. It demonstrates that prior infection with *C. canis* but not *C. ohioensis*–like coccidia confers some resistance to clinical disease and a decrease in oocyst production in dogs challenged with *C. canis*.

INTRODUCTION

Coccidia of the genus *Cystoisospora* are common intestinal parasites of dogs, cats, and humans worldwide (Frenkel, 1977). Dogs are hosts for 4 named species of *Cystoisospora*, and oocysts of *C. canis* can be definitively identified based on their structure in fecal samples because of their large size (>33 µm) when compared to the oocysts of *Cystoisospora ohioensis*, *Cystoisospora neorivolta*, and *Cystoisospora burrowsi*, which are smaller and structurally similar (<30 µm) to each other (Lindsay et al., 1997a). 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 (Lindsay et al., 1997a). New methods using PCR and sequencing or RFLP are being developed and will help better define these *C. ohioensis*–like parasites from dogs in the future (He et al., 2012a; Matsubayashi et al., 2011; Samarasinghe et al., 2008).

Little is known about immunity or resistance to intestinal coccidial infection caused by *Cystoisospora* species in dogs. This is because it is difficult to obtain coccidia-free dogs from commercial suppliers and the logistics of keeping dogs coccidia free prior to experimental and challenge infections. In 1 study, solid immunity followed primary *C. canis* infection, and no *C. canis* oocysts were excreted after challenge (Becker, 1981). Eight of 22 dogs examined in a second study were excreting *C. ohioensis*-like oocysts in their feces prior to experimental infection with sporulated oocysts of *C. canis*. All 22 dogs developed clinical signs of coccidiosis and excreted *C. canis* oocysts in their feces (Mitchell et al., 2007).

We previously demonstrated that oocysts of *C. canis* isolated from pitbull puppies from Virginia are pathogenic for young beagle dogs (Mitchell et al., 2007) and that this isolate could be used to demonstrate drug efficacy in experimentally infected beagles (Reinemeyer, 2007).

Unfortunately, this line of *C. canis* was lost due to a normal decline in infectivity with oocyst age. The present study describes the oocysts of a new isolate of *C. canis* obtained from a dog from Brazil and their pathogenicity for beagle puppies. We demonstrate that this isolate is pathogenic for beagle puppies and that preexisting *C. ohioensis*-like infection does not prevent clinical disease due to *C. canis*. We also are able to present results from a puppy naturally infected with *C. canis* that demonstrate some protection from homologous infection.

MATERIALS AND METHODS

Oocysts larger than 33 µm in length were detected in the feces of a 7-mo-old female dog (Fig. 1) from the Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil, by 1 of us (H.F.J.P.) in April 2011. Some blood was present, but the feces were not diarrheic. Feces from this dog were collected and sent to the Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine (VAMDRCVM), Virginia Tech, Blacksburg, Virginia, for propagation and further study of canine coccidiosis. Oocysts were sporulated in 2% (v/v) sulfuric acid solution at room temperature for 4 days (Figs. 2, 3). Twenty oocysts were examined and measured using a calibrated ocular micrometer and an Olympus BX60 epifluorescent microscope equipped with differential contrast optics (Olympus America Inc., Center Valley, Pennsylvania). Concentrated oocysts were stored in 2% sulfuric acid solution at 4 C. Oocysts were sterilized using exposure to 100% commercial bleach solution for 10 min. The oocysts were washed free of bleach using sterile Hanks balanced salt solution without calcium or magnesium (HBSS). The numbers of oocysts present were determined by counting in a hemocytometer. Oocysts were 3 mo old when used for

experiment 1. Oocysts collected from dogs in experiment 1 were used to infect dogs in experiment 2, and the sporulated oocysts were 3 mo old.

Two experiments using a total of 11 female beagles were conducted (Table 1). Dogs were obtained at 6 wk of age (Covance, Cumberland, Virginia). The protocols used in the present report were approved by the Institutional Animal Care and Use Committee, Virginia Tech. All dogs were vaccinated and neutered and then adopted by members of the community following guidelines of Virginia Tech at the end of the study. Fecal samples were examined using centrifugal flotation in Sheathers' sugar solution and the McMaster egg counting technique. Fecal samples were examined daily or every other day until dogs were orally infected (if feces was available). Fecal oocyst numbers were determined by the McMaster method using 2 g of feces and mixing this sample in 28 ml of Sheathers' sugar solution. Both sides of a McMaster counting slide were loaded with the mixture. McMaster slides were allowed to sit for 5 min, and then all oocysts present were counted. The total number of oocysts counted was determined by multiplying the number counted by 50.

An inoculum dose of 5 x 10^4 sporulated *C. canis* oocysts in 0.25 ml of HBSS was used. Dogs were orally infected by mixing the *C. canis* oocysts and HBSS solution in an appropriate amount (25 g) of commercial dog food (Hill's Science Diet A\D, Topeka, Kansas) and placing the food in a bowl and allowing the puppies to consume the dog food. All dogs readily ate this mixture within 3–5 minutes, and none vomited the inoculum.

All puppies were examined daily, their health condition noted, and fecal samples were collected. Toys were provided to each pup, and they were housed so they could see, hear, and smell each other. Pups were allowed to exercise outside of their cage on the floor during the 15–

20 min it took to clean their cage and collect fecal samples. An attempt was made to collect large amounts of feces during the patent period to provide a source of oocysts for future studies.

Dogs that developed severe diarrhea (watery and/or bloody), inappetence >24 hr, discomfort, or dehydration were treated medically by the clinical veterinarian for coccidiosis with sulfadimethoxine (Albon 5% suspension; Pfizer Inc., New York, New York) dosage at 55 mg/kg orally on the first day, and then 27.5 mg/kg orally once daily for 3-5 days, and given supportive care (subcutaneous fluid boluses).

RESULTS

Oocysts survived shipping from Brazil and sporulated when placed in 2% sulfuric acid solution. Twenty oocysts were measured at 37.6 by 28.6 µm (range 35–42 by 26–31 µm) (Figs. 1–4). Oocysts in fresh samples contained a contracted sporont, and many had developed to the 2-sporoblast stage. Oocysts sporulated within 2 days at room temperature and contained 2 sporocysts each with 4 sporozoites (Fig. 2). *Caryospora*- like oocysts containing a single sporocyst enclosing 8 sporozoites (Fig. 3) were frequently present in samples sporulated for experimental studies. Autofluorescence of the oocyst and sporocysts was observed when samples were examined using UV light (Lindquist et al., 2003). In experiment 2, fully sporulated *C. canis* oocysts present from the infecting dose of bleach-treated *C. canis* oocysts were seen in the feces of a dog (CXR). This was 2 day post-inoculation (PI), and dog CXR was excreting unsporulated *C. ohioensis*–like oocysts from a preexisting natural infection (Fig. 4).

Diarrhea was not present in the 5 pups in experiment 1 when they were examined the day of arrival at VAMDRCVM. Three of 5 dogs in this group excreted *C. ohioensis*–like oocysts prior to infection with *C. canis* oocysts (Table 1; Fig. 5). The pre-patent period was 9 days, and

the patent period was 8–10 days in these dogs. One dog (VG) was treated for intestinal coccidiosis with sulfadimethoxine and fluids beginning day 12 post-infection. Clinical signs of diarrhea were first observed 9 days PI, and fecal samples became formed again on day 14 PI.

Diarrhea was not present in the 6 pups in experiment 2 when they were examined the day of arrival at VAMDRCVM. One of 6 dogs (CXR) in experiment 2 excreted *C. ohioensis*-like oocysts prior to infection with *C. canis* oocysts (Table I; Fig. 6). The prepatent period for *C. canis* was 10 days, and the patent period was 11 days in CXR. One of 6 dogs (CRU) excreted oocysts of *C. canis* oocysts prior to oral inoculation with *C. canis* oocysts (Table 1). Two dogs (CUA and CPX) were treated for intestinal coccidiosis with sulfadimethoxine day 9 PI.

DISCUSSION

Cystoisospora species oocysts are most prevalent in dogs under 1 yr of age (Barutzki and Schaper, 2011; Buehl et al., 2006; Fontanarrosa et al., 2006; Little et al., 2009). A survey of 1,199,293 canine fecal samples from the United States in 2006 demonstrated that 4.4% of the samples contained oocysts of *Cystoisospora* species (Little et al., 2009). This value was similar to the 2.6% to 4.8% prevalence previously found in surveys (n=7) from dogs from the United States (reviewed by Little et al., 2009). Beuhl et al. (2006) found that 8.7% of 3,590 diagnostic samples from Austrian dogs less than or equal to 2 years old contained *Cystoisospora* oocysts. They found that 78% of the positive dogs were 4 mo old or less. Barutzki and Schaper (2011) found *Cystoisospora* oocysts in 5.6% of 24,677 dogs from Germany, and Fontanarrosa et al. (2006) found that 12% of 1,293 samples from dogs contained *Cystoisospora* oocysts.

Diagnosis of the cause of diarrhea is often a challenge to veterinarians attending animal shelters because dogs enter the shelters with a variety of enteropathogens, many of which are pathogenic or zoonotic under defined circumstances (Tupler et al., 2012). The large size of *C. canis* oocysts makes them easily identified in fecal preparations from dogs with diarrhea. However, care should be taken to demonstrate other pathogenic microorganisms, physiological conditions, environmental conditions, age, and immune status of dogs prior to making a definitive diagnosis of cystoisosporiasis (Lindsay et al., 1997a). It is interesting to note that Daugschies et al. (2000) reported that natural *Cystoisospora* infections were regularly found in 3-4-wk-old pups in dog breeding facilities in Austria and that they were not always associated with diarrhea. The age of dogs used in our study was 6 wk old, and this would be at the end of or near the end of a patent *C. ohioensis*–like coccidial infection that was acquired at 4 wk old. No surveys of dogs in breeding facilities have been reported from the United States, but the finding that 6-wk-old pups came naturally infected indicates that dogs in breeding colonies in the United States most likely acquire primary infections at a young age. Severe diarrhea was not present in any of the puppies in our study naturally infected with *C. ohioensis*-like oocysts.

Caryospora-like oocysts have been observed in many studies on *Cystoisospora* species in mammals (Lindsay et al., 1997a). Little is known about factors that induce the development of *Caryospora*-like oocysts of what would normally be *Cystoisospora* species oocysts. Matsui et al. (1993) demonstrated that heat treatment of unsporulated *Cystoisospora rivolta* oocysts from cats at 50 C for 5 min caused an increase in the numbers of *Caryospora*-like oocysts that were produced after sporulation. Oocysts collected from cats inoculated with *Caryospora*-like oocysts of *C. rivolta* were *Cystoisospora*-like after sporulation, indicating that heat treatment did not induce a stable mutation. The biological importance of these *Caryospora*-like oocysts is

unknown. The heat stress treatment method of Matsui et al. (1993) may prove to be a useful method to examine gene expression associated with sporogony of *C. canis* oocysts.

Oral inoculation of 5 x 10^4 oocysts of our isolate of *C. canis* used in the present study from Brazil caused similar clinical signs and prepatent and patent periods in 6 week-old recipients, as did inoculation of 5 x 10^4 oocysts of an isolate of *C. canis* obtained from dogs from Virginia (Mitchell et al., 2007; Reinemeyer, 2007) in 6 wk-old pups. An isolate of *C. canis* from Illinois was not pathogenic for 6-wk-old pups inoculated with 1 x 10^5 sporulated oocysts (Lepp and Todd, 1974). Buehl et al. (2006) reported that 2 x 10^4 oocysts of *C. canis* obtained from dogs in Austria were pathogenic for 3 wk-old beagle puppies. Results of the present study support those of Buehl et al. (2006), Mitchell et al. (2007), and Reinemeyer et al. (2007) and confirm that *C. canis* is a primary pathogen in young dogs.

ACKNOWLEDGEMENTS

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FIGURES

Photomicrographs of fecal flotations from dogs experimentally infected with *Cystoisospora canis* obtained from a dog from Brazil.

Figure 1. Unsporulated oocyst of *C. canis* demonstrating contracted sporont (S). Bar = 10 µm.

Figure 2. Sporulated *C. canis* oocyst containing 2 sporocysts each with 4 sporozoites. Note the sporocyst residum (R). Bar = 10 µm.

Figure 3. *Caryospora*-type sporulated oocyst of *C. canis* containing 8 sporozoites and a single sporocyst. A sporocyst residum (R) is present. Bar = 10 µm.

Figure 4. Sample from dog CXR fed sporulated *C. canis* oocysts demonstrating the inoculated sporulated *C. canis* oocyst (arrow) from the inoculum and many smaller nonsporulated *C. ohioensis*-like oocysts (arrowheads) from a natural infection. Bar = 20 µm.

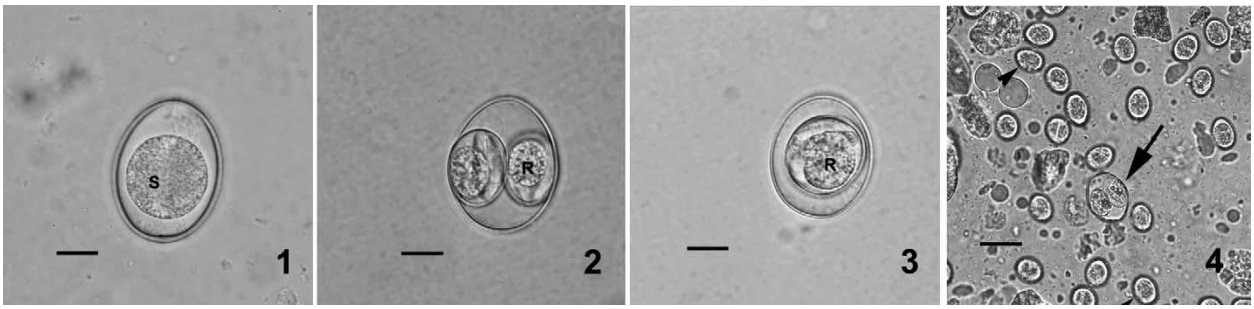


Figure 5. Results of *C. canis* oocyst counts from dogs in Experiment 1. Dogs VL and VI represented with solid line (no prior infection) and dogs VG, VH, and VJ represented with the dashed line (prior *C. ohioensis*-like infection).

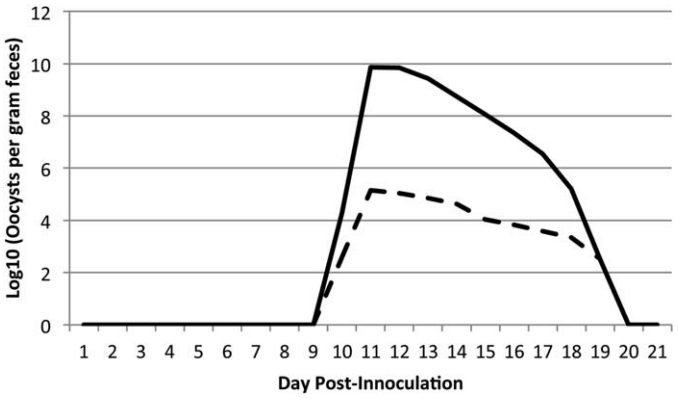
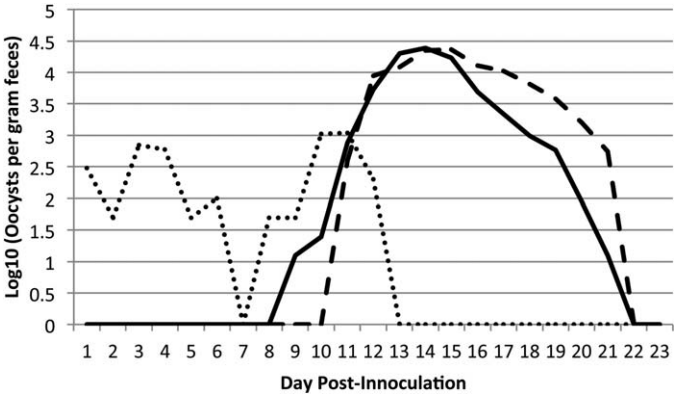


Figure 6. Results of *C. canis* oocyst counts from dogs in Experiment 2. Dogs CPX, CUA and CRW represented with the solid line group (no prior infection); dog CXR is the dashed line (prior *C. ohioensis*-like infection), and dog CRU is the dotted line (prior *C. canis* infection).



TABLES

Table 1. History of *Cystoisospora* oocyst excretion, results of clinical signs, and prepatent and patent periods following oral infection of dogs with 50,000 sporulated *C. canis* oocysts.

				Cystoisospora canis	
Experiment	Dog	Prior oocyst present	Clinical Signs	Prepatent period (days)	Patent period (days)
1	VL	No	Yes	9	8
1	VI	No	Yes	9	9
1	VG	*C. ohioensis*-like	Yes*	9	10
1	VH	*C. ohioensis*-like	Yes	9	10
1	VJ	*C. ohioensis*-like	Yes	9	10
2	CTZ	No	Yes	10	10
2	CPX	No	Yes*	9	12
2	CUA	No	Yes*	8	12
2	CRW	No	Yes	10	10
2	CXR	*C. ohioensis*-like	Yes	10	11
2	CRU	*C. canis*	No	9	3

* Treated with sulfadimethoxine for coccidiosis.

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Chapter 3: *Cystoisospora canis* (Apicomplexa: Sarcocystidae): Development of monozoic tissue cysts in human cells, demonstration of egress of zoites from tissue cysts, and demonstration of repeat monozoic tissue cyst formation by zoites

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ABSTRACT

Sporozoites of *Cystoisospora canis* penetrated and developed to monozoic tissue cysts in 4 human, 1 monkey, 1 bovine and 2 canine cell lines. No asexual division was documented although multiple infection of a single cell was observed. Examination of cultures using transmission electron microscopy demonstrated that they were monozoic tissue cysts and contained a single sporozoite. The appearance of monozoic tissue cysts in all cell lines was similar but the parasitophorous vacuole surrounding some sporozoites in DH82 dog macrophages was swollen. Monozoic tissue cysts were observed for up to 127 days in human pigmented retinal epithelial cells. Treatment of cell cultures containing monozoic tissue cysts with 0.75 sodium taurocholic acid and 0.25% trypsin stimulated egress of zoites (former sporozoites) from tissue cysts. Zoites collected from monozoic tissue cysts were able to penetrate and develop to monozoic tissue cysts in new host cells. Monozoic tissue cysts survived exposure to acid pepsin solution indicating that they would be orally infectious. The tissue cyst wall surrounding zoites did not autofluoresce as did oocyst and sporocyst walls exposed to UV light. We believe that *C. canis* can be used as a model system to study extra-intestinal monozoic tissue cysts stages of *C. belli* of humans.

Keywords: Cystoisospora canis, Isospora canis, *cell culture, tissue cyst*

INTRODUCTION

Coccidia of the genus *Cystoisospora* (Apicomplexa: Sarcocystidae) are common intestinal parasites of dogs, cats, and humans worldwide (Frenkel, 1977; Lindsay et al., 2011). Dogs are hosts for 4 named species of *Cystoisospora* and oocysts of *Cystoisospora canis* can be definitively identified based on their structure in fecal samples because of their large size (>33 µm) when compared to the oocysts of *Cystoisospora ohioensis*, *Cystoisospora neorivolta*, and *Cystoisospora burrowsi* which are smaller and structurally similar (<30 µm) to each other (Lindsay et al., 1997a). 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 (Lindsay et al., 1997a). New methods using PCR and sequencing or RFLP are being developed and will help better define these *C. ohioensis*-like parasites from dogs in the future (He et al., 2012a; Matsubayashi et al., 2011; Samarasinghe et al., 2008). Phylogenetic analysis of the rRNA 18S gene has shown that *Cystoisospora* spp. are more closely related to *Toxoplasma*, than *Eimeria*, indicating that *Cystoisospora* belongs in the family Sarcocystidae along with *T. gondii* (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Samarasinghe et al., 2008).

We have recently demonstrated that the dog coccidian *C. canis* will form monozoic tissue cysts in African green monkey kidney (CV-1) cells and in bovine turbinate (BT) cells (Mitchell et al., 2009). These monozoic tissue cysts contain a single asexual stage (zoite = intracellular sporozoite surrounded by a tissue cyst wall) that does not undergo asexual multiplication. *Cystoisospora belli* (synonym *Isospora belli*) of humans undergoes endodyogeny (binary fission) in cell culture and does not produce tissue cysts in vitro (Oliveira-Silva et al., 2006). However, monozoic tissue cysts do occur in the tissues of humans infected with *C. belli* (Comin and Santucci,

1994; Frenkel et al., 2003; Lindsay et al., 1997b; Michiels et al., 1994; Restrepo et al., 1987) and in the tissues of dogs and cats infected with *Cystoisospora* species (Dubey, 1975; Dubey and Frenkel, 1972). Instead of undergoing the normal developmental cycle in the intestinal tract, some sporozoites leave and invade extra-intestinal sites and produce monozoic tissue cysts in the host. In humans, mesenteric lymph nodes are most often infected but other tissues such as the liver, spleen, and tracheobronchial and mediastinal lymph nodes can be infected (Velasquez et al., 2011). Parasites are usually found as single stages structurally resembling sporozoites. Monozoic tissue cysts of *C. belli* in histological sections are thick walled and measure 12–22 µm × 8–10 µm in size, each containing a single zoite. Ultrastructurally, a *Cystoisospora* tissue cyst consists of a single centrally located zoite surrounded by a granular tissue cyst wall within a parasitophorous vacuole (PV). Zoites contain all the cellular organelles characteristic for infective asexual stages (sporozoite, merozoites, tachyzoite, and bradyzoites) of coccidial parasites (Lindsay et al., 1997b; Mitchell et al., 2009). The tissue cyst wall is next to the limiting membrane of the PV. Recurrent clinical disease is common in both immunocompetent and immunosuppressed patients infected with *C. belli* and it is believed to be due to reactivation of the zoites present in the tissue cysts and migration to the human intestinal tract (Lindsay et al., 1997b; Velasquez et al., 2001). It is common for immunosuppressed patients to relapse with clinical disease once *C. belli* treatment has been stopped (Boyles et al., 2012; Lindsay et al., 2011).

The present study was done to determine if sporozoites of an isolate of *C. canis* would produce monozoic tissue cysts in human cell lines, to determine if zoites could be passed in cell culture, and to develop a system to study the biology of *Cystoisospora* monozoic tissue cysts in vitro.

MATERIALS AND METHODS

Host cell lines and their maintenance

We examined human pigmented retinal epithelial (ATCC CRL-2302, HRE cells, epithelial morphology; Manassas, VA, USA); human ileocecal colorectal adenocarcinoma (ATCC CCL-244, HCT-8 cells, epithelial morphology, Manassas, VA, USA), human lung fibroblasts (ATCC CCL-171, MRC-5 cells, fibroblast morphology, Manassas, VA, USA), and human skin fibroblasts (ATCC CRL-1635, Hs68 cells, fibroblast morphology, Manassas, VA, USA) to determine if they would support the growth of *C. canis* (Table 1). We also examined bovine turbinate (ATCC 1390, BT cells, morphology epithelial, Manassas, VA, USA), Madin-Darby canine kidney (ATCC CCL-34, MDCK cells, epithelial morphology, Manassas, VA, USA), canine macrophages (DH82, mixed morphology, Dr. Michael J. Yabsley, University of Georgia, Athens, GA, USA), and African green monkey kidney (ATCC CCL-70, CV-1 cells, epithelial morphology, Manassas, VA, USA), to determine if they would support the growth of *C. canis* (Table 1). Host cells were grown in 25 cm^2 cell culture flasks or on 22 mm^2 glass coverslips in 6 welled cell culture plates in RPMI 1640 cell culture medium (Mediatech, Inc., Manassas, VA, USA) containing 100 IU penicillin/ml, 100 µg/ml streptomycin/ml and 10% (v/v) fetal bovine serum (FBS). Cells were maintained in the same medium except the concentration of FBS was 2%. Living cell cultures were examined using an inverted microscope equipped with phase-contrast optics (Zeiss Invertoskope).

C. canis ***oocysts and excystation of sporozoites from sporocysts***

Oocysts of *C. canis* were obtained from the feces of experimentally infected 6 week-old female beagles (Covance, Cumberland, VA, USA) fed 5×10^4 sporulated *C. canis* oocysts (Houk et al., 2013). The Institutional Animal Care and Use Committee, Virginia Tech, Blacksburg, VA, USA approved the protocols using dogs in the present study. All dogs were vaccinated and neutered then adopted by members of the community following guidelines of Virginia Tech at the end of the study.

Feces containing *C. canis* oocysts were sporulated in 2% (v/v) sulfuric acid for 4 days at room temperature. Sporulated oocysts were treated with 100% commercial bleach for 10 min to kill contaminating bacteria, viruses and yeast. Bleach was washed off of the oocysts by repeated centrifugation in sterile Hanks balanced salt solution magnesium and calcium (HBSS) (Mediatech, Inc., Manassas, VA, USA).

Sporulated *C. canis* oocysts in HBSS were ruptured with the use of a tissue grinder and then treated with excysting medium (ExM) containing at a final concentration of 0.75% (w/v) sodium taurocholic acid (Sigma Chemical Co., St. Louis, MO, USA) and 0.25% (w/v) trypsin in HBSS at 37°C (Mitchell et al., 2009). Excysted sporozoites were washed with HBSS, then 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 (maintenance medium).

Development of C. canis *monozoic tissue cysts*

Sporozoite numbers were determined using a hemocytometer. Host cells were grown on 22 mm^2 coverslips or 25 cm^2 cell culture flasks and inoculated with varying numbers of sporozoites. Coverslips were removed and processed for light microscopic examination after staining with Giemsa various days post-inoculation (PI). The 22-mm^2 coverslips were fixed in 10% (v/v) buffered formalin solution then post-fixed in 100% methanol, stained with Giemsa, and mounted on glass slides using PermountTM (Fisher Scientific Company, Fair Lawn, NJ, USA) for microscopic examination. Selected 22-mm^2 were stained with hematoxylin and then mounted on glass slides for microscopic examinations. Slides were observed and photographed using an Olympus BH60 microscope (Olympus America Inc., Center Valley, PA, USA) equipped with epifluorescent, UV, and differential interference contrast (DIC) optics.

Infected cell cultures in flasks or on 22 mm^2 coverslips were monitored daily or every other day to determine developmental progress. Select flasks were chosen for transmission electron microscopy or other manipulations based on observation of events viewed using the inverted microscope. Living preparations of cultures were examined by placing a drop of the test culture to be examined on a glass microscope slide and then placing a 22 mm^2 coverslip on the drop and viewing with bright field, UV or DIC optics.

Egress of sporozoites from cells containing tissue cysts

HRE or CV-1 cells in 25 cm^2 containing monozoic tis- sue cysts of *C. canis* were used to determine if exposure to ExM would induce parasite egress. The cell culture medium was removed and the monolayer washed in 37°C HBSS and then 37°C ExM was added. Cultures were incubated

for 5–10 min and examined with an inverted microscope. Once egress occurred, 2% maintenance medium was added and the contents placed in 15 sterile centrifuge tubes and the ExM removed by centrifugation. Organisms obtained from monozoic tissue cysts were termed zoites.

Repeated passage of* C. canis *zoites

The infectivity of zoites collected after egress form monozoic tissue cysts was examined in HRE or CV-1 cells grown on 22 mm^2 glass coverslips or in 25 cm^2 cell culture flasks. Zoites were collected as above and the ExM washed off by repeated centrifugation in HBSS. Zoites were suspended in appropriate medium and used as needed. Zoites were usually counted with a hemocytometer and used to infect host cells but occasionally zoites were not enumerated before use.

Passage of monozoic tissue cysts in infected host cell cultures

We examined if host cells containing monozoic tissue cysts could be maintained by standard trypsin–EDTA treatment used to maintain uninfected host cells. Infected HRE cell cultures containing monozoic tissue cysts in 25 cm^2 cell culture flasks were washed in HBSS and exposed to 0.05% (w/v) trypsin and 0.53 mM EDTA in HBSS (Mediatech, Inc., Manassas, VA, USA) for 5 min at 37°C. Host cells were disrupted from the plastic growth surface by gently tapping the flask with the palm of the hand then cells were suspended in 10% growth medium and plated into new 25 cm^2 cell culture flasks. This was conducted once a month for 4 consecutive months.

Exposure to acid pepsin solution

Exposure to acid pepsin solution was used to indicate if monozoic tissue cysts would survive exposure to conditions in the host's stomach and potentially be orally infectious. HRE cell cultures containing 20-day-old *C. canis* monozoic tissue cysts in 25 cm^2 cell culture flasks were washed in HBSS and exposed to acid–pepsin solution (0.5 g pepsin, 0.5 g NaCl, 98.6 ml distilled water, 1.4 ml HCl, pH 0.9) for 15 min. The acid–pepsin solution was removed by repeated centrifugation in growth medium. Collected zoites were used to infect additional HRE cells growing on 22 mm^2 coverslips.

Transmission electron microscopy

Twenty-five cm^2 flasks of HRE, CV-1, and DH82 cells infected with *C. canis* sporozoites were examined using transmission electron microscopy (TEM). The cell culture was fixed in 3% (v/v) gluterahldehyde in phosphate buffer (PBS, pH 7.4). Tissues were post-fixed 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, 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 AMT camera system (Advanced Microscopy Techniques Corp., Danvers, MA).

RESULTS

Development of monozoic tissue cysts

We found that sporozoites of *C. canis* penetrated and produced monozoic tissue cysts (Figs. 1–4) in all cell lines examined (Table 1). Motile sporozoites were observed in cultures after inoculation and they underwent gliding and flexing movements. Intracellular sporozoites were present within 1 h after inoculation. Intracellular sporozoites were never observed to undergo movement unless the host cells were treated to induce egress. Infected cell cultures were easily maintained by replacing the medium every 2–3 days for at least a month and 1 flask of HRE cells was visually monitored for up to 127 days and appeared to be viable. Only HCT-8 adenocarcinoma cells proved to be difficult to maintain long term. These HCT-8 cells became crowded due to lack of strong contact inhibition and monolayers deteriorated after 10 days or longer (Table 1). Sporozoites developed next to the host cell nucleus in all cell lines (Figure 1) Multiple penetrations occurred in all cell lines (Figs. 1 and 2).

The use of canine origin cells did not result in enhanced development. No multinucleate schizonts and no sexual stages were observed. Development of *C. canis* in DH82 canine macrophages revealed some interesting differences from the other cell lines. These cells appeared to actively phagocytize non-excysted and excysted oocysts, sporocysts, and walls of oocysts and sporocysts (Fig. 1). Occasionally sporozoites were seen in unusually large parasitophorous vacuoles present in DH82 cells. Multiple sporozoites were often present in DH82 cells (Fig. 1) but it was not possible to determine if sporozoites actively penetrated the DH82 cells or they were phagocytized.

Egress of sporozoites from monozoic tissue cysts

Exposure of monolayers containing *C. canis* monozoic tissue cysts to ExM resulted in activation of sporozoites and their eventual egress from the tissue cyst. The cell membranes of host cells underwent dissolution within 10 min and a mixture of host cell nuclei, tissue cysts containing zoites, empty tissue cysts, zoites, and host cell particulate matter was present (Fig. 3). Zoites within tissue cysts probed the anterior end of the tissue cyst using their apical end and flexed in the tissue cyst. Zoites eventually exited through the anterior end of the tissue cyst although an opening was not visible (Fig. 3).

Infectivity of egressed zoites

The infectivity of zoites collected from HRE, MDCK, DH82, and CV-1 cells were examined. Zoites were able to be collected from egress-induced cultures and they were infective for additional host cells. It was not possible for us to distinguish between monozoic tissue cysts that were produced by *C. canis* sporozoites from oocysts or monozoic tissue cysts produced by *C. canis* zoites collected from monozoic tissue cysts. We occasionally observed what appeared to be division in some cells inoculated with zoites. They contained 2–4 tightly packed organisms. These types of infected cells were always few in number and we were unable to further study their origin or track their fate.

Passage of infected host cells containing monozoic tissue cysts

We were able to maintain monozoic tissue cysts by using routine mammalian cell culture methods of passing host cells using trypsin-EDTA solution and re-plating of infected and non-infected host cells for 4 passages. Examination of host cells after exposure to trypsin-EDTA solution indicated that the infected cells rounded up but remained intact (Fig. 4) (normal uninfected cells were also rounded after trypsin-EDTA treatment). The study was ended after 4 months. Manipulation of host cells containing monozoic tissue cysts using these methods did not result in asexual division but did maintain infected host cells. No evidence of multiplication was observed in monozoic tissue cysts passaged this way.

Survival of monozoic tissue cysts in acid-pepsin solution

Monozoic tissue cysts of *C. canis* in HRE cells survived treatment with acid–pepsin solution for 15 min. Motile zoites but not tissue cysts were observed in preparations treated with acid–pepsin solution. This indicates the tissue cyst wall was digested away by the acid–pepsin solution. Zoites treated with acid–pepsin solution were infective for HRE cells and produced monozoic tissue cysts.

Transmission electron microscopy

Examination of infected host cells demonstrated conclusively that the infected cells contain monozoic tissue cysts (Fig. 5). The tissue cysts contained a single sporozoite surrounded by a

granular tissue cyst wall. Fully formed tis- sue cysts were present in HRE, MDCK, DH82 cells day 5 after infection the earliest time any culture was examined by TEM.

DISCUSSION

Development of C. canis *in different cell types*

Most *Cystoisospora* species develop by endodyogeny when sporozoites are used to infect cell cultures (Lindsay et al., 1997a). However, complete development of *Cystoisospora suis* in swine testicle (Lindsay et al., 1998) and porcine epithelial (Worliczek et al., 2013) cells has been reported. Most asexual development of *Eimeria* and *Cystoisospora* species usually occurs in primary cell lines from the definitive host (Doran, 1982). Fayer and Mahrt (1972) observed endodyogeny when sporozoites of *C. canis* were inoculated onto established cell cultures of embryonic bovine kidney (EBK), embryonic bovine trachea (EBT), and MDCK cells and primary cultures of embryonic canine kidney (PECK) and embryonic canine intestinal (PECI). They observed division by endodyogeny on day 3 PI in EBT, MDCK, and PECK and day 4 in EBK and PECI cells examined for up to 14 days PI. They did not find *C. canis* infected MDCK cells after day 5 PI. They did not mention monozoic cyst-like stages in any of the cell types they used on any examination date PI. Likewise, sexual stages of *C. canis* were not observed in any cell culture type. We observed monozoic tissue cysts in MDCK cells which had been infected for 2 months and did not see any indication that MDCK cells were somehow losing the parasites as suggested by Fayer and Mahrt (1972). Mitchell et al. (2009) reported that monozoic tissue cysts were present for at least 15 days in BT cells and 17 days in CV-1 cells. We were able to observe

monozoic tissue cysts in apparently viable HRE cells for up to 127 days of infection in the present study.

Oliveira-Silva et al. (2006) reported that *C. belli* from humans developed by endodyogeny in cell culture. They found that more development occurred in VERO cells (source Green monkey kidney) than in HCT-8 or epithelial lung carcinoma (ELC) cells from humans. Large parasitophorous vacuoles were seen frequently in MDCK cells but rarely in HCT-8 or ELC cells. In the current study, we observed large parasitophorous vacuoles in DH82 cells (Figure 1) but not CV-1 (monkey kidney similar to VERO) or other cell lines. Intracellular merozoites of *C. belli (Oliveira-Silva et al., 2006)* and *C. suis* (Lindsay and Blagburn, 1987) were motile in living cultures while intracellular stages of *C. canis* in our study were not motile unless treated with ExM.

Development of sporozoites of the present isolate of *C. canis* obtained originally from a dog from Brazil (Houk et al., 2013) in BT and CV-1 cell cultures is similar to what was reported for sporozoites of an isolate of *C. canis* from dogs from VA, USA (Mitchell et al., 2007). It is interesting to note that Fayer and Mahrt (1972) used 2 different sources of *C. canis* oocysts in their study. They did not report any differences in development due to *C. canis* oocyst source (Fayer and Mahrt, 1972).

Egress of C. canis *from monozoic tissue cysts*

Treatment of infected HRE or CV-1 cell cultures with ExM was effective in inducing activation of zoites resulting in egress from the tissue cyst. We examined ExM because it is known to cause activation and excystation of *Cystoisospora* species sporozoites (Lindsay et al., 1997a). The bile component of ExM has been shown induce motility of merozoite stages of mammalian

Eimeria in cell culture (Doran, 1982). The movement of zoites in monozoic tissue cysts was similar to what occurs in ExM activated sporozoites in sporocysts (Lindsay et al., 1997a).

Maintenance of C. canis *monozoic tissue cysts by serial passage*

In vitro studies are hindered by the need to collect sporozoites from sporulated oocysts. We examined the potential of *C. canis* to be maintained in continuous culture using passage of *C. canis* using activation with ExM and reinfection of cells and by passage of infected host cells using standard cell culture methods. Both methods were successful and additional studies are needed to improve and optimize the methods. Treatment of infected cell cultures with acid–pepsin solution was also a viable method to collect sporozoites from infected cells and they were infectious for new host cells. The tissue cyst wall was dissolved with this method and survival of stages suggests that oral ingestion of monozoic tissue cysts may be a viable method for parasite transmission. This has been demonstrated in mice infected with feline and canine *Cystoisospora* species and fed to appropriate definitive hosts (Dubey, 1975).

Observations on C. canis *monozoic tissue cyst structure*

Our observations using TEM confirmed that intracellular stages were monozoic tissue cysts based on structure (Dubey and Mehlhorn, 1978; Markus, 1983; Mehlhorn and Markus, 1976; Mitchell et al., 2009). The structure zoites was the same as described for *C. canis* sporozoites excysted from oocysts (Roberts et al., 1972). Additional studies are needed to compare monozoic

tissue cysts produced by egress or cell passage with monozoic tissue cysts produced from excysted sporozoites.

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FIGURES

Figure 1. Cystoisospora canis sporozoites 5 days after inoculation of DH82 canine macrophage cells. Many intracellular sporozoites (black arrows) are present and the tissue cyst wall is not always apparent. A large parasitophorous vacuole (black arrowhead) surrounding a sporozoite is visible. Two parasites (white arrowhead) have apparently infected the same cell. Oocyst and sporocyst walls can be seen (white arrows). The host cell nucleus (Hn) of some cells is visible. Bar = 20 µm.

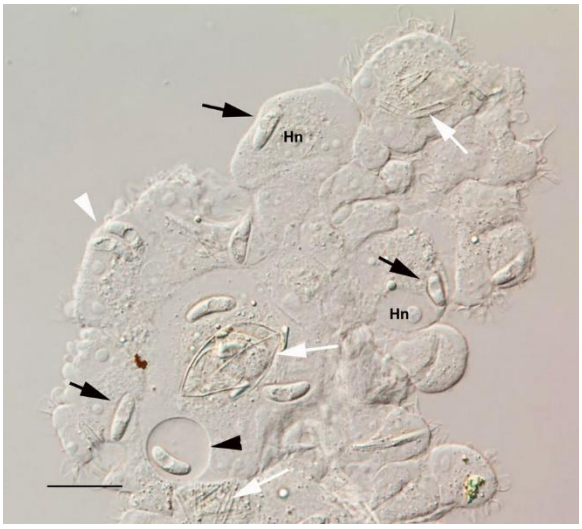


Figure 2. *Cystoisospora canis* sporozoites 10 days after inoculation of HRE cells. Many monozoic tissue cysts are present (arrows). One monozoic tissue cyst (arrowhead) has ruptured from its host cell during processing. Remaining oocyst and sporocyst walls (small arrowheads) are visible and are attached or have been ingested by HRE cells. Bar = 50 µm.

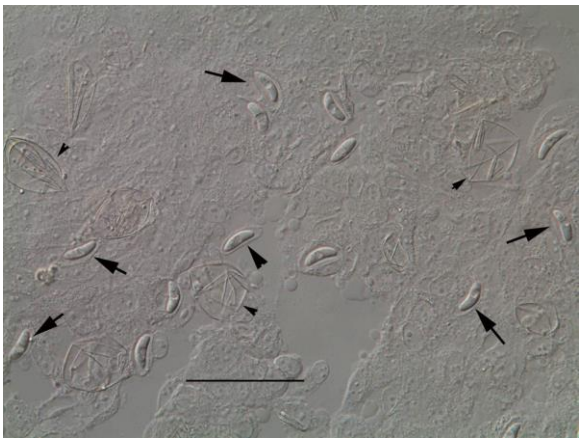


Figure 3. Egress of a zoite of *Cystoisospora canis* from a monozoic tissue cyst (MZT) exposed to excysting medium. (A) The zoite is exiting the MZT and its nucleus (N) is still within the MZT wall (arrow). (B) The zoite is continuing to exit MZT and its nucleus (N) is now on the outside of the MZT wall (arrow). (C) The zoite is completely outside the MZT (arrow). Bar = 10 µm.

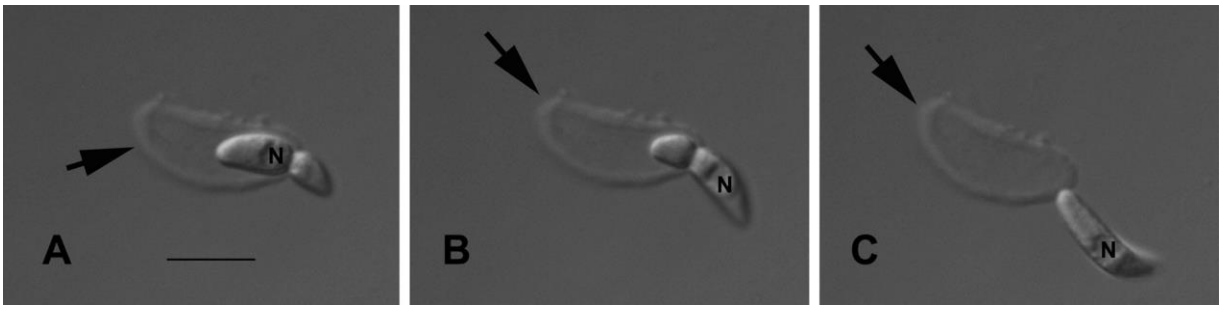
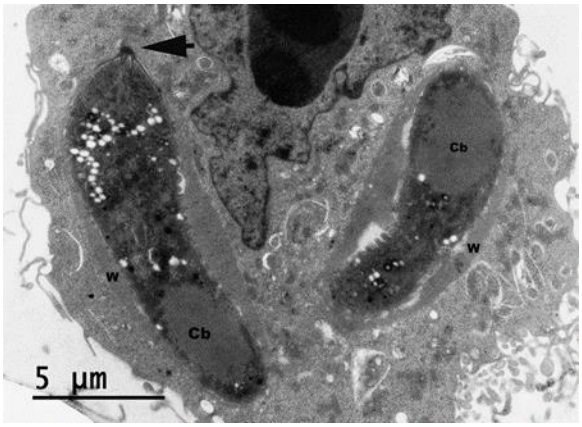


Figure 4. Three HRE cells after exposure to trypsin–EDTA solution for 5 min and used in passaging cell cultures. The intact tissue cyst wall (W) surrounds the zoite. Bar = 10 µm.

Figure 5. Transmission electron micrograph of a MDCK cell containing two monozoic tissue cysts of *Cystoisospora canis* five days after inoculation of sporozoites. The tissue cyst wall (W) and crystalloid bodies (Cb) of each zoite are visible. The arrow points to the apical complex that is visible in one zoite



TABLES

Table 1. Host cells used to grow monozoic tissue cysts of *Cystoisospora canis.*

Host / cell name	Cell type	Cell morphology	Results
Human / HRE	Pigmented retinal epithelial	Epithelial	Positive
Human / HCT-8	Adenocarcinoma	Epithelial	Positive[a]
Human / MRC-5	Lung	Fibroblast	Positive
Human / Hs68	Skin	Fibroblast	Positive
Monkey / CV-1	Kidney	Epithelial	Positive[b]
Dog / MDCK	Kidney	Epithelial	Positive
Dog / DH82	Macrophages	Variable	Positive[c]
Cow / BT	Turbinate	Epithelial	Positive[b]

[a] Cells not suitable for long-term maintenance of monozoic tissue cysts and cultured discarded 10 days after inoculation.

[b] Cells used in study by Mitchell et al. (2009) to produce monozoic tissue cysts.

[c] Unusually large parasitophorous vacuole sometimes present.

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Chapter 4: Phylogenetic Characterization of *Cystoisospora canis*

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ABSTRACT

Cystoisospora canis is an Apicomplexan parasite infecting young dogs. Very little has been done using molecular methods to characterize members of the genus *Cystoisospora*. The objective of this research was to phylogenetically characterize *C. canis* using both ITS1 rDNA locus and CO1 mitochondrial DNA markers. This project examines relationships within the *Cystoisospora* clade, the position of *C. canis* in this clade, and how similar its members are to other Sarcocystidae. PCR was performed using DNA isolated from oocysts using a modified phenol-chloroform DNA extraction protocol. PCR products were sequenced and phylogenetic trees were built using ClustalW2 and published sequences in GenBank. We found *C. canis* to be in agreement with the *Cystoisospora* sp. clade. Outside of *Cystoisospora* spp. *C. canis* is most similar to *T. gondii,* followed by *N. caninum,* which is in agreement with the literature. To our knowledge, this study was the first time *C. canis* has been analyzed phylogenetically.

Key words: Cystoisospora canis*, phylogenetic analysis, internal transcribed spacer region, cytochrome oxidase C, RFLP*

INTRODUCTION

Intestinal infections caused by *Cystoisospora* spp. coccidia are common in humans and animals (Lindsay et al., 1997a). *Cystoisospora canis* is an Apicomplexan parasite and is one of four known *Cystoisospora* to infect dogs. It can be differentiated morphologically based on its large size (>33 µm) in comparison to *C. burrowsi*, *C. neorivolta* and *C. ohioensis* (<30 µm) which are morphologically indistinguishable and grouped together as *C. ohioensis*-like when referenced (Houk and Lindsay, 2013; Lindsay et al., 1997a; Mitchell et al., 2007). Young puppies, elderly and immune-compromised dog are most likely infected with *C. canis*. As with most protozoa, infection with *Cystoisospora* spp. is associated with young age, confinement and poor sanitary conditions such as those seen in animal shelters and large-scale breeding facilities

Dogs become infected when a sporulated oocyst is consumed in contaminated food or water. *C. canis* travels to the small intestine where the oocysts undergo excystation and replicates asexually and sexually. After 8-10 days environmentally-hardy oocysts are produced which are shed into the environment where they sporulate in 1-2 days and then are infectious for other dogs (Lepp and Todd, 1974). In addition to canine infections, paratenic hosts like small rodents can consume the sporulated oocysts and become infected. In the paratenic hosts, the sporozoites leave the intestine and journey to other organs like lymph nodes, spleen, and liver where they encyst as monozoic tissue cysts (MZT) (Mitchell et al., 2009) which may serve as another route of infection for dogs (Dubey, 1982; Houk and Lindsay, 2013). It may be possible that *C. canis* behaves as *C. belli*, in that extra-intestinal MZT are able to reactivate during the life of the host to recolonize the gut and produce oocysts once more (Lindsay et al., 1997b; Velasquez et al., 2001).

Cystoisospora spp. were originally a part of the *Isospora* genus, described in 1881 by

Schneider, and thought to be more closely related to *Eimeria* spp. based on their shared one-host life cycle. However, in 1977, Frenkel proposed the genus *Cystoisospora* after discovering *I. felis* and *I. rivolta* create tissue cysts (Frenkel, 1977). Phylogenetic studies using the rRNA 18s gene, indicated that *Cystoisospora* was a member of the Sarcocystidae family rather than Eimeriidae because *Cystoisospora* was found to be more similar to *Toxoplasma gondii* and *Neospora caninum* than *Eimeria* spp. (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Matsubayashi et al., 2011). Samarasinghe et al. (2008) were able to develop a primer set that only amplified a *Cystoisospora*-specific portion of the ITS1 locus and they performed phylogenetic analysis with *C. belli, C. felis, C. ohioensis*-like, *C. rivolta* and *C. suis* and were able to describe their relationship to each other, as well as to other apicomplexans like *T. gondii*, *N. caninum, Sarcocystis* spp., *Eimeria* spp. It was further confirmed that *Cystoisospora* is most similar to *T. gondii* and *N. caninum* and firmly entrenched in the Sarcocystidae family rather than Eimeriidae (Samarasinghe et al., 2008). *C. canis* was not included in their studies and so its relationship to other *Cystoisospora* spp. is currently unknown and there has been no published sequence data for *C. canis*.

Our hypothesis was that after genetic characterization *C. canis* and other *Cystoisospora* species of mammals would fall into clades that reflect their host species. The ITS1 locus is part of the 18S rDNA region, the most commonly used locus used for genetic studies with these coccidia and the CO1 mitochondrial DNA is part of the DNA barcoding project and believed to be more accurate (Ogedengbe et al., 2011). The present study used common phylogenetic markers—internal transcribed spacer 1 (ITS1) locus and cytochrome *c* oxidase subunit 1 (CO1)—to discover the relationship of *C. canis* to other *Cystoisospora* spp. as well as other Sarcocystidae.

MATERIALS AND METHODS

Collection and purification of oocysts

Oocysts were obtained from the feces of experimentally infected beagle puppies (Houk et al., 2013). Oocysts were sporulated, concentrated, sterilized and excysted (when needed to collect sporozoites) as previously described (Houk and Lindsay, 2014). Purified oocysts were stored at 4ºC in Hanks balanced salt solution free of calcium and magnesium until used for the present study.

Prior to use for DNA extraction, oocysts were pelleted in a 2 ml centrifuge tube and 1 ml sterile phosphate buffered saline (PBS) was added. The 2 ml tube was re-centrifuged, the supernatant poured off, and the pellet was resuspended in 1 ml of deoxynucleotide (dNTP) -free H_2O and stored at -20ºC until use.

Genomic DNA Isolation

Genomic DNA was isolated from the frozen oocyst pellets by adding 1 mL lysis buffer AL, 25 µL proteinaseK, and RNase A (0.4µg/mL) (QIAGEN Group, Valencia, CA) to the pellet and incubating at 56°C overnight. The next day dithiothreitol (Acros Organics, Thermo Fisher Scientific Inc., Pittsburgh, PA) was added to a final concentration of 0.01 M and incubated at 37°C for 1 hour to stabilize the DNA. A phenol-chloroform-isoamyl alcohol (25:24:1 extraction was performed followed by two chloroform (Fisher BioReagents, Thermo Fisher Scientific Inc., Pittsburgh, PA) extractions to get DNA in aqueous phase. DNA was precipitated using 0.2 volumes of 10M ammonium acetate (Fisher Chemical, Thermo Fisher Scientific Inc., Pittsburgh, PA) and 2 volumes of 95% ethanol overnight at 4°C. Precipitated DNA was pelleted at 16,000 x g for 20 minutes, the ethanol-containing supernatant removed, and remaining ethanol was

allowed to evaporate for 10 minutes before the DNA was suspended in Tris-EDTA (Fisher BioReagents, Thermo Fisher Scientific Inc., Pittsburgh, PA) (pH 8.0) and kept at 4°C for future use.

Polymerase chain reaction and sequencing **Cystoisospora canis**

Platinum® PCR SuperMix High Fidelity (Invitrogen™ Life Technologies) was used for all PCR reactions with 10ng/ µL DNA used per 50 µL reaction. The internal transcribed spacer 1 (ITS-1) rDNA locus was amplified with a nested protocol. ITSF and EMR7 amplify the 3' end of the 18S rRNA region and the 5' end of the 5.8S region respectively. The PCR product was then used to amplify an approximately 450 bp portion of the ITS-1 rDNA locus of *Cystoisospora* species using ITSGF and ITSR2 primers (Samarasinghe et al., 2008). Both rounds used the following protocol: Initial denaturation of 94ºC for 5 minutes followed by 45 cycles (denaturation at 94ºC for 30 seconds, annealing at 60ºC for 20 seconds, and extension at 68ºC for 35 seconds) with a final extension at 68ºC for 7 minutes.

Toxo_CO1 forward and reverse primers used to sequence the CO1 region of *C. felis* (JN227478) submitted to GenBank by Ogedengbe and Barta (2012) were used to amplify the approximately 750 bp cytochrome *c* oxidase subunit 1 mitochondrial DNA region. The protocol had an initial denaturation at 94ºC for 5 minutes followed by 40 cycles (denaturation at 94ºC for 20 seconds, annealing at 45ºC for 30 seconds extension at 68ºC for 90 seconds) followed by a final extension at 68ºC for 10 minutes.

The PCR products were run on a 1% agarose gel with 7 µL ethidium bromide and a 100 bp ladder (Invitrogen Life Technologies, Grand Island, NY). The gel was run at 70V for 120 minutes using and then the PCR product was visualized with a Kodak Gel Logic 200 Imaging

System (Kodak, Rochester, NY) and Carestream Molecular Imaging Software (Carestream Health, Inc., New Haven, CT). The DNA was excised and purified form the gel using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and the concentration and purity of the genomic DNA was determined spectrophotometrically using the NanoDrop 2000 (NanoDrop, Wilmington, DE). The amplified products were submitted to the Genomics Research Laboratory at the Virginia Bioinformatics Institute, Blacksburg, VA for sequencing.

Restriction Fragment Length Polymorphism

RFLP was performed with the *Cystoisospora*-specific ITS1 PCR product using the Alu1 restriction enzyme. The protocol listed in the information pack was followed, the restriction digest was incubated at 37ºC for three hours. The product was run at 70V for 120 minutes on a 2% agarose gel with 7 µL ethidium bromide and a 100 bp ladder. In addition, to confirm laboratory results, restriction digest was simulated with NEB Cutter (Version 2.0, New England BioLabs, Inc.) (http://nc2.neb.com/NEBcutter2/) using *Cystoisospora* species ITS1 sequences in GenBank with the *C. canis* sequencing results from VBI.

Sequencing

Sanger sequencing was performed by Virginia Bioinformatics Institute (Blacksburg, VA) in triplicate in order to generate a good quality consensus sequence. The consensus sequence was constructed using BioEdit (version 7.2.5, Ibis Biosciences) and the sequence was analyzed using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Restriction digest of Alu1 was checked with NEB cutter (http://tools.neb.com/NEBcutter2/index.php) and the ITS1 locus was analyzed for other sites cut with commercial restriction enzymes. After the sequence

was submitted to GenBank, multiple parasite sequence alignment and phylogenetic analysis was performed using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Phylogenetic Analysis

ClustalOmega was used to align the sequences and ClustalW2 was used for phylogenetic analysis of neighbor-joining relationships and percent identity matrixes. *T. gondii* and *N. caninum* were included as they have been found to be the closest parasites phylogenetically in the literature.

To make the ITS1 phylogenetic tree, the following parasites were included: *Cystoisospora canis* (KP411387), *C. ohioensis*-like (EU124688), *C. felis* (KP411388), *C. rivolta* (EU124686), *C. belli* (EU124687), *C. suis* (EU124685), *Toxoplasma gondii* (L49390), and *Neospora caninum* (KC710332).

For the CO1 phylogenetic tree, the following published sequences (followed by GenBank accession numbers if published) were utilized: *C. canis*, *C. felis* (JN227478.1), *T. gondii* strain ME49 (HM771690), and *N. caninum* strain NC-1 (HM771688).

RESULTS

Phylogenetic analysis with Internal Transcribed Spacer 1

The *Cystoisospora*-specific ITS1 product was 411 bp (351 bp after alignment, clean-up and creation of a consensus sequence). This similar to other *Cystoisospora* sequences in GenBank. The *C. canis* ITS1 sequence was found to be in 99.7% agreement with the

Cystoisospora ohioensis-like ITS1 sequence and distantly related to the *C. felis* sequence (85%) (Table 1).

RFLP using ITS1 sequence and AluI restriction enzyme

On a 2% gel using AluI as a restriction enzyme, the 411 bp *C. canis* ITS1 sequence was cut once—yielding two bands—and using the NEB Cutter software, we found the fragment sizes to be 294 and 117 bp. The two RFLPs most similar, *C. ohioensis*-like and *C. rivolta*, also had two bands.

Phylogenetic analysis with Cytochrome c *Oxidase subunit 1*

The CO1 primers yielded a PCR product of approximately 750 bp product. *Cystoisospora felis* was the only *Cystoisospora* spp. in GenBank and *C. canis* had a 99% agreement with *C. felis*. *T. gondii* and *N. caninum* were the most similar outside of the *Cystoisospora* clade with identities of approximately 79% and 77% respectively (Table 1).

DISCUSSION

There has been no work done on characterizing *C. canis* genetically. In this study, we analyzed ITS1 rDNA locus and CO1 mitochondrial DNA and compared its sequence to other species of *Cystoisospora*. We found that *C. canis* was most similar to *C. ohioensis*-like using ITS1 and the RFLP analysis shows that *C. canis* and *C. ohioensis*-like have the most similar fragment sizes and patterns confirming that *C. canis* is closest to *C. ohioensis*-like when using the ITS1 locus. CO1 finds *C. canis* to be most similar to *C. felis—C. canis* had approximately a

85% similarity to *C. felis* with ITS1—but these results cannot be fully compared due to the lack of other *Cystoisospora*, most importantly *C. ohioensis*-like, CO1 sequences available in GenBank.

Previous studies have found that *Cystoisospora* spp. are more similar to *T. gondii* and *N. caninum* than any other coccidia (Samarasinghe et al., 2008). Using the ITS1 locus and CO1 we found *C. canis* to be closer to *T. gondii* than *N. caninum*.

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We thank Dr. Boris Striepen and his group at the University of Georgia for their helpful comments on DNA isolation. We are grateful to Dr. John Barta and his group at the University of Guelph for the cytochrome *c* oxidase subunit 1 primers. Supported in part by a grant from the National Center for Veterinary Parasitology (Oklahoma State University).

FIGURES

Figure 1. A phylogenetic tree using ITS1 locus. Relationships with *Cystoisospora* spp., *T. gondii*, and *N. caninum* examined using neighbor-joining.

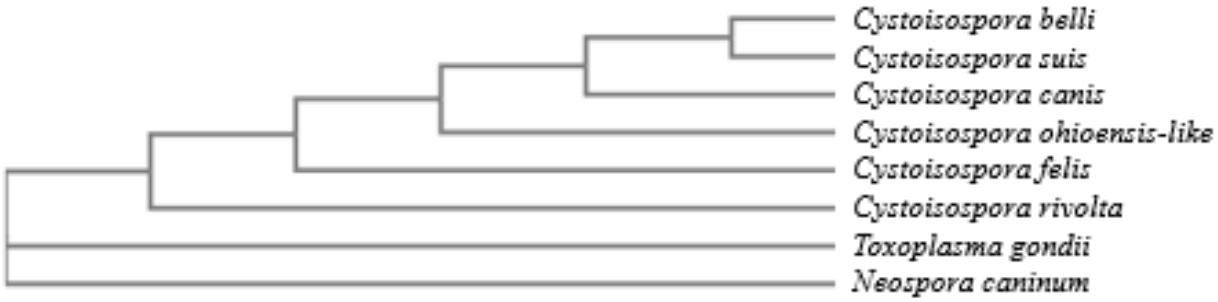


Figure 2. Phylogenetic tree using CO-1 built using neighbor-joining relationships. *T. gondii*, *N. caninum*, and *Eimeria tenella* were included.

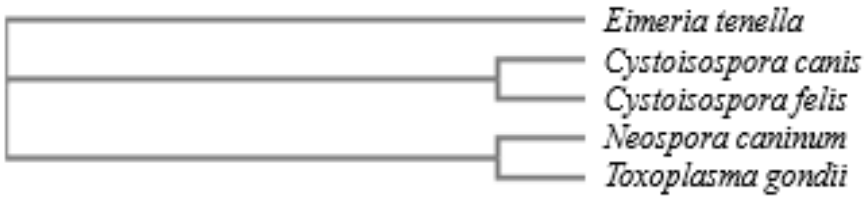
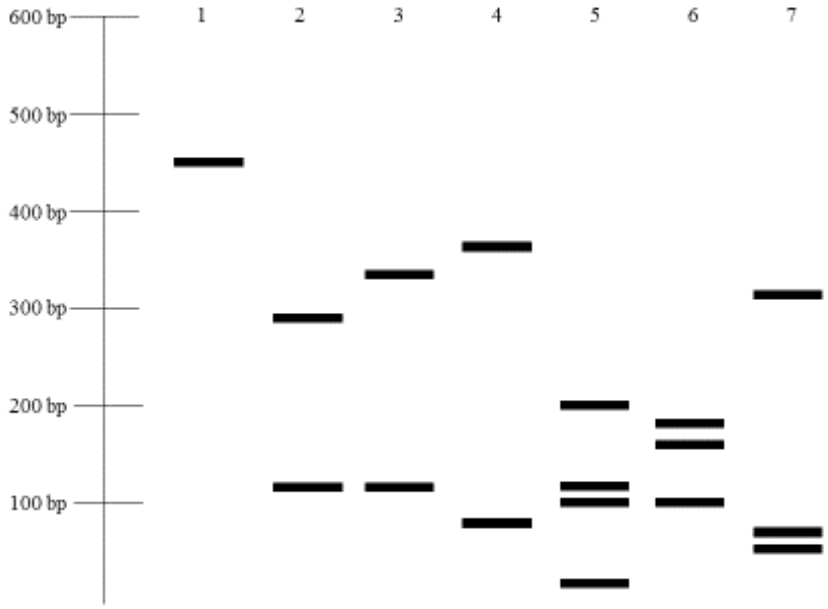


Figure 3. Schematic representation of RFLP patterns of *Cystoisospora* spp.-specific ITS1 locus PCR products cut with AluI restriction enzyme. 1. Control-uncut *C. canis* ITS1, 2. *C. canis*, 3. *C. ohioensis*-like, 4. *C. rivolta*, 5. *C. belli*, 6. *C. suis*, 7. *C. felis*.



TABLES

Table 1. Results from the percent identity matrixes generated by phylogenetic analysis of *C. canis* to other coccidia using ITS1* and CO1 locus**.

	***C. ohioensis*-like**	***C. felis***	***C. rivolta***	***C. belli***	***C. suis***	***T. gondii***	***N. caninum***
***C. canis* ITS1**	99.72%	85.38%	85.13%	83.78%	77.26%	61.10%	55.81%
***C. canis* CO1**	-	96.85%	-	-	-	78.85%	77.36%

**C. canis* (KP411387), *C. ohioensis*-like (EU124688), *C. felis* (KP411388), *C. rivolta* (EU124686), *C. belli* (EU124687), *C. suis* (EU124685), *Toxoplasma gondii* (L49390)

**** C. canis* (no accession number), *C. felis* (JN227478.1), *T. gondii* strain ME49 (HM771690), *N. caninum* strain NC-1 (HM771688)

- No sequence found in GenBank to use for phylogenetic comparison.

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Chapter 5: *Cystoisospora canis* has proteins orthologous to *Toxoplasma gondii* important for virulence and pathogenesis

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ABSTRACT

Cystoisospora canis is a protozoan parasite that causes gastrointestinal disease in puppies. It is a member of family Sarcocystidae and closely related to *Toxoplasma gondii*, a parasite able to infect virtually any warm-blooded vertebrate and responsible for a significant health and financial burden each year. Both parasites are able to produce tissue cysts in host cells. *C. canis* produces tissue cysts that contain one infective organism (zoite) and are monozoic tissue cysts (MZT). *T. gondii* produces tissue cysts that contain several infective organisms (bradyzoites) and are polyzoic tissue cysts (PZT). It is believed that tissue cysts may last for the lifespan of the host. If the tissue cyst reactivates, due to immunosuppression then clinical infection can occur once more. Significant damage may occur if the tissue cyst is in a delicate area like the brain or eyes (*T. gondii*) or intestinal infection resulting in oocyst production can occur (*Cystoisospora*). Tissue cyst biology of MZT and PZT is not well understood. We hypothesize that *C. canis* has orthologous genes and tissue cyst wall proteins with *T. gondii*. We chose 20 virulence genes of *T. gondii* and *N. caninum* and conducted PCR on DNA of *C. canis* for orthologs. Additionally, we used immunofluorescence assays to examine the MZT of *C. canis* for similar tissue cyst proteins known to be present on PZT of *T. gondii*. We found *C. canis* to have 8 of the 20 virulence genes in *T. gondii* and *N. caninum.* In immunofluorescence assays using polyclonal (BAG5) and monoclonal (SalmonE) antibodies to the PZT of *T. gondii* demonstrated similar binding with the MZT of *C. canis*. *Dolichos bilflorus* lectin which reacts with PZT of *T. gondii* also bound MZT of *C. canis*.

Keywords: Cystoisospora canis, Toxoplasma gondii, *orthologous genes of interest, rhoptries*

INTRODUCTION

Phylum Apicomplexa contains parasites that infect a great number of humans and animals worldwide and are the causative agent of such diseases as coccidiosis, malaria, cryptosporidiosis and toxoplasmosis (Labesse et al., 2009). *Cystoisospora canis* is an apicomplexan parasite that is found in the small intestine of dogs worldwide. Most members of the genus *Cystoisospora* can produce tissue cysts that contain a single organism (Frenkel, 1977) and are termed monozoic tissue cysts (MZT) to reflect that only 1 organism is present (Lindsay et al., 2014). *Cystoisospora* spp. are biologically and molecularly related to the tissue cyst forming coccidia *Toxoplasma gondii* and *Neospora caninum* (Barta et al., 2005; Carreno et al., 1998; Franzen et al., 2000; Samarasinghe et al., 2008) which are important protozoal parasites of mammals. These coccidia can cause debilitating disease, especially in young and immune-compromised patients. They produce polyzoic tissue cysts (PZT) filled with a great number of latent parasite stages (bradyzoites) in the tissues of their hosts which may last for the host's lifespan. *Cystoisospora* form MZT rather than PZT and they can reactivate causing renewed shedding of oocysts, lesions and recrudescence of clinical signs in the definitive host (Lindsay et al., 1997b; Velasquez et al., 2001).

The events that lead to this reactivation are not well understood but reactivation is associated with immune suppression (*Cystoisospora* spp., *T. gondii*) or pregnancy (*N. caninum*). Reactivation of tissue cysts can produce severe effects such as encephalitis, retinochoroiditis, hepatitis, abortion, and diarrhea (Dubey et al., 2009). Hosts can also begin to re-shed oocysts of *Cystoisospora* (but not *T. gondii*) in their feces and serve as a source of infection for others (Lindsay et al., 1997b; Velasquez et al., 2001). Several studies have examined the mechanisms leading to tissue cyst formation of *T. gondii* and *N. caninum*. Little has been done to determine

the events that lead to reactivation of latent tissue cysts of these species. Very little is known about the factors inducing tissue cyst formation of *Cystoisospora* in vitro and little is known on factors inducing reactivation.

Invasion into the cell and establishment of a parasitophorous vacuole (PV) involves multiple organelles and proteins. There are three main organelles of interest—dense granules, micronemes and rhoptries (Carruthers and Sibley, 1997; El Hajj et al., 2006). All three are located in the apical complex of the parasite and secrete proteins that assist with invasion and formation of the PV. Rhoptries in particular are of interest because of the functions of the proteins they secrete. There are two types: RONs are rhoptry proteins found in the neck region which work with the micronemes to propel the parasite within the host cell plasma membrane into the newly forming PV (Carruthers et al., 2000) and ROPs are proteins from the bulb part of the rhoptry which become associated with the PV membrane and are secreted into the host cell (Beckers et al., 1994; El Hajj et al., 2006; Labesse et al., 2009). The proteins secreted into the host cell may perform functions such as interacting with the host cell mitochondria or in changing gene expression so the recruitment of pro-inflammatory cells is suppressed (Butcher et al., 2011) .

This project had two aims 1) to survey genomic *C. canis* DNA to find genes orthologous to *T. gondii*; 2) to determine if *C. canis* MZT have similar tissue cyst wall proteins to *T. gondii* using two antibodies and a lectin known to bind to sugar residues on the tissue cyst wall of *T. gondii*. Our hypothesis was that *C. canis* has similar genes and proteins to *T. gondii* important for virulence and pathogenesis of the parasite. Using immunofluorescent assays in conjunction with molecular studies we were able to compare *T. gondii* with *C. canis* and our results support this hypothesis.

MATERIALS AND METHODS

Genomic DNA Isolation

Genomic DNA was isolated from the frozen oocyst pellets by adding 1mL lysis buffer AL, 25 µL proteinaseK, and RNase A (0.4µg/mL) (QIAGEN Group, Valencia, CA) to the pellet and incubating at 56°C overnight. The next day dithiothreitol (Acros Organics, Thermo Fisher Scientific Inc., Pittsburgh, PA) was added to a final concentration of 0.01 M and incubated at 37°C for 1 hour. A phenol-chloroform-isoamyl alcohol (25:24:1) extraction was performed followed by two chloroform (Fisher BioReagents, Thermo Fisher Scientific Inc., Pittsburgh, PA) extractions to get DNA in aqueous phase. DNA was precipitated using 0.2 volumes of 10M ammonium acetate (Fisher Chemical, Thermo Fisher Scientific Inc., Pittsburgh, PA) and 2 volumes of 95% ethanol. DNA was allowed to precipitate at 4°C overnight before being centrifuged for 20 minutes at 16,000 x g and at 4°C. Liquid was then removed from the pellet and the pelleted DNA was allowed to air-dry for 10 minutes before being suspended in Tris-EDTA (Fisher BioReagents, Thermo Fisher Scientific Inc., Pittsburgh, PA) (pH 8.0) and incubated at 40°C until DNA dissolved. Isolated DNA was stored at 4°C for future use.

Genes of interest

We used oligoprimer sequences described in the literature and the *Toxoplasma* Genomics Database (http://toxodb.org/toxo/) to design primers for other genes coding for proteins of interest. Using these primers, we examined genomic DNA of *C. canis* for orthologous genes found in *T. gondii*, *N. caninum*, *Sarcocystis neurona* and *Eimeria spp*. Genes coding for actin

(ACT1), cyst wall protein (CST1), rhoptries (RON1, RON2, RON3, RON4, ROP5, ROP11, ROP12, ROP13, ROP15, ROP16, ROP18, Rab11 and toxofilin), ApiTranscriptase 2 (AP2Ib), micronemes (MIC26 and MIC3) and dense granule proteins (GRA12 and GRA22) were targeted using PCR (see Table 1). Actin was used as a control as it can be found in all eukaryotic cells and it is necessary for structure and motility of the parasite (Poupel et al., 2000). ApiTranscriptase 2 and ROP18 are found to be important in stage differentiation and proliferation (El Hajj et al., 2007; Reid et al., 2012). Dense granules and micronemes are found in all apicomplexans but the genes that were chosen were those specific only to *T. gondii* or *N. caninum* (Reid et al., 2012) to help us determine to which *C. canis* is more closely linked. And finally, literature has shown that the rhoptries are very important to virulence and pathogenesis of the parasite so we decided to look for a broad range of rhoptry genes, some with known importance and others thought to be important but with the exact function unknown are unknown (Bradley et al., 2005; Reid et al., 2012; Zheng et al., 2013).

Polymerase Chain Reaction

GoTaq® Green Mastermix (Promega, Madison, WI)) was used for each reaction. A ME49 isolate of *T. gondii* was used as the positive control and nuclease-free water was used as the negative control to ensure lack of contamination. *N. caninum* (NC-1) was also used as a comparison as some genes are found in *T. gondii* but not *N. caninum* and vice versa. This was useful in determining whether or not *C. canis* was more biologically similar to *T. gondii* or *N. caninum*.

The PCR protocol was the following: initial denaturation of 94ºC for 5 minutes followed by 45 cycles (denaturation at 94ºC for 30 seconds, annealing 49-63ºC—depending on the T_m of the specific primer—for 20 seconds, and extension at 72ºC for 35 seconds) with a final extension at 72ºC for 7 minutes for all of the primers.

PCR product analysis and sequencing

PCR products were loaded into a 1% agarose gel with a 100 bp ladder (Invitrogen Life Technologies, Grand Island, NY). The gel was run at 70V for 120 minutes before being visualized with a Kodak Gel Logic 200 Imaging System (Kodak, Rochester, NY) and Carestream Molecular Imaging Software (Carestream Health, Inc., New Haven, CT). To confirm the identity of the PCR products, the bands of DNA were excised and purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and the concentration and purity of the genomic DNA was determined spectrophotometrically using the NanoDrop 2000 (NanoDrop, Wilmington, DE). The DNA was submitted to the Genomics Research Laboratory at the Virginia Bioinformatics Institute (VBI), Blacksburg, VA for Illumina sequencing. The sequence data was analyzed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Collection of Parasites for Immunofluorescence Assay Test

Monozoic tissue cysts were collected from human pigmented retinal epithelial cells (ATCC CRL-2302, HRE cells, epithelial morphology; Manassas, VA, USA) infected with sporozoites 36 days previously, washed with phosphate buffered saline (PBS, pH 7.2), and plated

on to 3 –welled Teflon-coated IFAT slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Antigen-containing slides were left to dry at room temperature for 4 to 12 hours and stored at -20ºC until used. An IFAT slide containing tissue cysts of the ME49 isolate of *T. gondii* was made using similar methods and served as a positive control. *T. gondii* (RH) tachyzoites were used as the negative control.

Examination of the tissue cyst wall with BAG5 and SalmonE

Fifty microliters each of BAG5 polyclonal antisera, SalmonE monoclonal antibody, and TgIDE4 monoclonal antibody—the former two reagents recognize *T. gondii* tissue cysts and TgIDE4 only recognizes the tachyzoite stage—were placed on respective wells of *C. canis* and *T. gondii* ME49 and RH slides. They were incubated at room temperature for 1 hour and then washed twice with PBS for 5 minutes each. Next, 50µL fluoroscein-labeled goat-anti mouse secondary antibody (1:50 dilution in PBS) was incubated on the slides for 30 minutes and then washed with PBS as before. The secondary antibody was also added a second time to a well containing only a PBS primary treatment to test for self-antibody binding. FluoromountG (SouthernBiotech, Birmingham, AL) was used to mount the slides. The slides were examined with an Olympus BX60 epifluorescent microscope.

Examination of the tissue cyst wall with* Dolichos bilflorus *lectin

Dolichos biflorus lectin (DBL) labeled with fluorescein isothiocyanate (E. Y. Laboratories, San Mateo, California USA) was diluted to 1mg/ml with PBS and incubated with

monozoic tissue cyst-containing slides and *T. gondii* slides for 30 minutes at room temperature in a humidified box. Following 2 consecutive washes in PBS, slides were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA), and assessed using an Olympus BX60 epifluorescent microscope equipped with DIC optics.

RESULTS

Orthologous genes found in C. canis

Eight of 20 *T. gondii* genes—ACT1, CST1, GRA12, GRA22, RON3, ROP11, ROP13, ROP16—surveyed had an orthologous gene in *C. canis* that we were able to amplify. The PCR products were purified and sequenced at VBI. We were able to create consensus sequences for 6 of the 8 genes that we plan on submitting to GenBank at a future date.

Immunofluorescence Assays

T. gondii ME49 PZT and *C. canis* MZT fluoresced with BAG5 polyclonal antisera, SalmonE monoclonal antibody and *Dolichos bilflorus* lectin. The RH negative control did not demonstrate fluorescence (Table 3 and Figure 1) with the former 3 reagents but the tachyzoites did fluoresce with TgIDE4 monoclonal antibody. The *C. canis* and *T. gondii* tissue cysts initially treated only with PBS and then secondary antibody did not fluoresce and sequential addition of secondary antibody did not indicate self-antibody binding.

DISCUSSION

Although there was a multitude of genes and proteins to choose from, after examining the literature, we chose to test. *C. canis* for 20 genes of interest in this pilot study. We chose a genes conserved within eukaryotes (ACT1), involved in stage differentiation and parasite replication (AP2Ib and ROP18), and some that interact with proteins in the cell and help with trafficking across the membrane (Rab11 and Toxofilin). The main organelles involved with virulence and pathogenesis are the rhoptries, micronemes and dense granules so we chose to amplify some of their gene products although we mostly concentrated on proteins found in the rhoptry bulb (ROP) and rhoptry neck (RON) as we found them to be the important and diverse. GRA12 and MIC26 were used to help elucidate whether *C. canis* is more similar to *T. gondii*, a very non-host and tissue specific parasite, or *N. caninum*, a parasite that has a much more limited host range and uses dogs as the definitive host. GRA12 is specific to *T. gondii* and MIC26 is specific to *N. caninum* so being able to amplify a PCR product with GRA12 but not MIC26 suggests that *C. canis* is more like *T. gondii* than *N. caninum*, even though they both share a canine definitive host.

Orthologous gene products were amplified in 8 of 20 genes tested in *C. canis* (see Table 2). The first, ACT1 was included because actin is found in all eukaryotes and we wanted a primer set to serve as a control. Next, CST1 is a tissue cyst wall protein found to be very important for the structural integrity of tissue cysts and ability to evade killing by the immune system (Tomita et al., 2013). GRA12 and GRA22 are dense granule proteins found in *T. gondii.* GRA12 is found in *T. gondii* but not but not *N. caninum* and GRA22 is involved in parasite egress from the parasitophorous vacuole (Okada et al., 2013; Reid et al., 2012). *C. canis* appears to have several orthologous rhoptry proteins. RON3 is involved in the formation of the

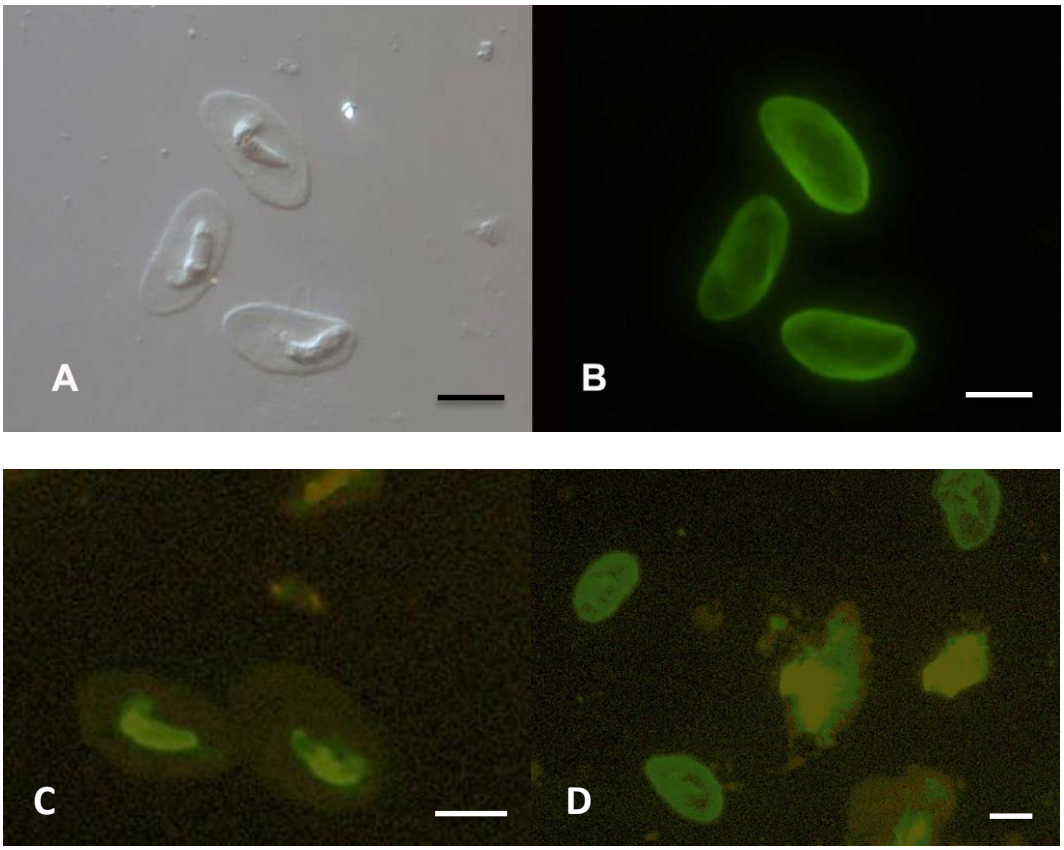
parasitophorous vacuole which surrounds the parasite in the cell. ROP11 and ROP13 play a part in invasion into the host cell and ROP16 is able to enter the host cell nucleus upon invasion and regulate gene expression to suppress the pro-inflammatory reaction and prevent the cells from being attacked by the host's Th1 immune response. Importantly, Hajj et al. (2006) found that *T. gondii* ROPs from the ROP2 family like ROP16 only have homologs with *N. caninum* rather than the more distantly related apicomplexans like *Plasmodium* spp. (El Hajj et al., 2006).

The mechanisms behind tissue cyst reactivation are relatively unknown. Previous studies have found the CST-1 glycoprotein to be an important part of tissue cyst stability and immune evasion and the BAG5 heat shock protein 30 has been associated with stage differentiation in *T. gondii* (Zhang et al., 2001). Fluorescence of *C. canis* tissue cysts with the immunofluorescence assay indicates that *C. canis* MZT have homologous proteins to those found on *T. gondii* PZT. Cross-reactivity a possibility but use of positive and negative controls should account for such an issue. To counteract any cross-reactivity of the lectin, the use of inhibitors is an option.

In conclusion, our hypothesis that *C. canis* has genes and proteins orthologous to *T. gondii* was supported. These genes code for proteins involved in cell entry, immunomodulation allowing persistence within the cell and structural integrity of the tissue cyst. This knowledge serve as an entry point for further studies of the tissue cyst and what causes reactivation and subsequent pathogenesis and potential mortality in animals and humans infected with apicomplexans.

FIGURES

Figure 1. A) *C. canis* tissue cysts observed with DIC. *C. canis* tissue cysts fluoresced under UV light B) DBL C) BAG5 polyclonal antisera D) SalmonE monoclonal antibody. Bar = 10µm.



TABLES

Table 1. Targeted gene products and primer information.

Gene Product of Interest	Importance	Primer Designed or Literature Source
Apicomplexan transcription factor (AP2Ib)	Transcription factor; implicated and control of lifecycle and development of parasite stages	5'-CAGACTGTGGAGGAAGCACA-3' 5'-CTCCATCTCGACCTCTTTGC-3'
Actin (ACT1)	Conserved gene in eukaryotes; used for structure and motility	5'- TCCCGTTTCTGCGTTAGACT -3' 5'- CTGACCTCGCTGAAAACCTC -3'
CST1 cyst wall protein (CST1)	Cyst wall protein involved in PZT structural integrity and immune evasion	5'- TCCCAGTGTGCCTTCCTTAC -3' 5'- TACCACAGCTCCGTCAACAG -3'
Dense granule protein 12 (GRA12)	*T. gondi*i-specific; involved in formation and function of parasitophorous vacuole	5'- TTTCACCATCCAAGCCTCTC -3' 5'- GTTTCTACCCTGCCAGATGC -3'
Dense granule protein 22 (GRA22)	Involved in parasite egress from the parasitophorous vacuole	5'- TGTGATGCGTATGTGTGCAG -3' 5'- TTCTCGGTCAGCTTTCTGGT -3'
Microneme protein 3 (MIC3)	*T. gondii* vaccine target; involved in attachment and invasion of the parasite	5'-GAGTCTTGCTCCGTCTTTCG-3' 5'-GTCCAAGTTTTCGACGCATT-3'
Microneme protein 26 (MIC26)	*N. caninum*-specific; involved in attachment and invasion of parasite	5'- AAACAGACCAGTCCGTCCAC -3' 5'- GGAGGTCTTGTGAGGGTTGA -3'
Ras-related protein (Rab11)	Regulates trafficking proteins and lipids to the rhoptries	Bradley et al., 2005
Rhoptry neck proteins 1-4 (RON1-4)	Conserved in apicomplexans; host cell invasion and formation of parasitophorous vacuole membrane	Bradley et al., 2005

(table continued)

Table 1(*continued)*

Gene Product of Interest	**Importance**	**Primer Designed or Literature Source**
Rhoptry protein 5 (ROP5)	Key virulence factor—helps maintain PV membrane and evade cell-mediated killing by immune system; *T. gondii* vaccine target	Bradley et al., 2005
Rhoptry proteins 11, 12, 13, and 15 (ROP11, 12, 13, 15)	*T. gondii* specific; host cell invasion	Bradley et al., 2005
Rhoptry protein 16 (ROP16)	ROP2 family; Interferes with host signaling pathways to modulate pro-inflammatory host cytokines	5'- GAGAAATGGTGGTTGCGACT -3' 5'- GGCGGAGTTGGTAAAGACAA -3'
Rhoptry protein 18 (ROP18)	ROP 2 family; Important for virulence; protects the parasitophorous vacuole from host immune system	Reid et al., 2012
Toxofilin	Interacts with parasite actin and protein phosphatase	Bradley et al., 2005

Table 2. Presence of genes of interest in *T. gondii* (positive control) and *C. canis*.

Gene	Present in *T. gondii*	Present in *C. canis*
ACT1	+	+
Ap2bI	+	-
CST1	+	+
GRA12	+	+
GRA22	+	+
MIC3	+	-
MIC26	-	-
Rab11	+	-
RON1	+	-
RON2	+	-
RON3	+	+
RON4	+	-
ROP5	+	-
ROP11	+	+
ROP12	+	-
ROP13	+	+
ROP15	+	-
ROP16	+	+
ROP18	+	-
Toxofilin	+	-

Table 3. Results of antibody and lectin studies.

Reagent	***T. gondii* (ME49)**	***C. canis***	***T. gondii* (RH)**
PBS	-	-	-
SalmonE monoclonal antibody	+	+	-
BAG5 polyclonal antisera	+	+	-
Dolichos biflorus lectin	+	+	-
*Tg*IDE4 monoclonal antibody	-	-	+

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Chapter 6: General Conclusions

This dissertation examined the suitability of *Cystoisospora canis* MZT formation as a model for apicomplexan tissue cyst reaction and was the first to analyze molecular data about the *C. canis*. These studies together support the use of the *C. canis* MZT as a model for *T. gondii* PZT and other Sarcocystidae.

We were able to contribute to knowledge about the pathogenesis of *C. canis* with heterologous isolates (Brazil vs USA) and re-infect dogs previously naturally infected with *C. canis* or *C. ohioensis*-like oocysts. Previous studies found dogs previously infected with *C. canis* had immunity to challenge but we found challenge with a different isolate is able to result in patent infection. Infection with an American isolate of *C. canis* did not protect against super-infection with a Brazilian isolate of *C. canis* although the duration of severity of clinical signs was reduced. There was also no evidence of cross-species protection as dogs naturally infected with *C. ohioensis*-like oocysts were able to be infected with *C. canis* and demonstrate normal pre-patent and patent periods.

We also studied the behavior of *C. canis* in mammalian cell cultures. Research in the 1970's indicated that *C. canis* was able to replicate by binary fission in cell culture but our studies indicate this is not the case. We infected a variety of cell lines from multiple species and different cell types and found tissue cysts with only a single zoite that did not replicate. We were also pleased to find that MZT are durable and able to be maintained in cell culture for long periods of time allowing them to be maintained until needed for research. And finally, we found we could induce reactivation of the MZT with bile-trypsin solution which simulates the digestive process found in hosts. This work demonstrates that *C. canis* can be easily manipulated and maintained in cell culture for future studies.

There are a limited number of *Cystoisospora* sequences in GenBank and there have been no published *C. canis* sequences. We were able to use ITS1 and CO1 genetic markers, commonly used in phylogenetic studies, to analyze *C. canis* and assign it a position in the *Cystoisospora* clade and a place among the Sarcocystidae family. Using the ITS1 marker we found a 99% identity to *C. ohioensis*-like complex of *Cystoisospora* which also infect dogs. We used CO1 as well but as there are no sequences in GenBank for *C. ohioensis*-like we could not get a confirmation of our analysis. Also, previous studies have found *Cystoisospora* spp. to be most similar to *T. gondii* and *N. caninum* and this work further examined those relationships and found *C. canis* to be more similar to *T. gondii* than other members of the Sarcocystidae. One would think that due to its canine host, *C. canis* would be more similar to *N. caninum* but this does not appear to be the case.

Finally, we studied the literature to find genes and proteins of interest and used PCR and immunofluorescent assays to determine if *C. canis* has orthologous genes and proteins to those found in *T. gondii*. We surveyed 20 genes with products involved in various important functions from motility, to penetration into the cell, to formation of the parasitophorous vacuole, to changes in host cell gene expression and immune modulation. *C. canis* does appear to possess genes and proteins important for virulence and protection from the immune system although not to the extent that *T. gondii* does as only 8 of the 20 *T. gondii* genes had orthologs in *C. canis*. When taking into account the behavior of *C. canis* however, the lack of some of these genes and their products makes sense because *C. canis* does not appear to have all the same life cycle stages as *T. gondii*. Surveying *C. canis* for genes of interest is the first step with the next being *de novo* sequencing of the genome to get a more in-depth look at genes of interest as well as those unique to *C. canis*.

There is very little known about *C. canis*. These studies were conducted to discover more about this parasite and help make inferences about its behavior in the host. The more we know

about parasites like *C. canis*, the more we can work on not only improving immunotherapeutic strategies but, more importantly, prevention of infection.