DISSERTATION

DIAGNOSIS AND TREATMENT OF CRYPTOSPORIDIOSIS AND GIARDIASIS IN CATS AND DOGS IN THE UNITED STATES

Submitted by
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In partial fulfillment of the requirements
for the degree of Doctor in Philosophy
Colorado State University
Fort Collins, Colorado
Spring 2007

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANDREA VALERIA SCORZA ENTITLED DIAGNOSIS AND TREATMENT OF CRYPTOSPORIDIOSIS AND GIARDIASIS IN CATS AND DOGS IN THE UNITED STATES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DIAGNOSIS AND TREATMENT OF CRYPTOSPORIDIOSIS AND GIARDIASIS IN CATS AND DOGS IN THE UNITED STATES

The first two chapters of this dissertation review the organisms *Cryptosporidium* species and *Giardia* species, emphasizing the information most pertinent to dogs and cats. The research work described in chapters 3-9 focused primarily on the diagnosis and treatment of these 2 infectious agents in dogs or cats.

In Chapter 3, a monoclonal antibody-based immunofluorescence assay (IFA) was used to detect of *Cryptosporidium* species oocysts and *Giardia* species cysts in feces from cats and dogs. The *Cryptosporidium* IFA results were compared to those of a polymerase chain reaction assay (PCR), and a molecular and phylogenetic analysis on the 18S rDNA and heat shock protein-70 genes were performed on some of the *Cryptosporidium* positive samples. It was shown that the PCR assay was more sensitive than IFA for the detection of *Cryptosporidium* species, and that cats and dogs tested in this study were commonly positive for both organisms.

Genotype differentiation from the 18S rDNA fragment was not possible, so a PCR- Restriction Fragment Length Polymorphism (RFLP) that comprised a larger and polymorphic fragment of the 18S rDNA gene was performed and described in Chapter 4. Two hundred and ninety two samples were assayed, of which 179 were feline samples and 113 were canine samples. All the feline isolates with adequate DNA for study were *C. felis* and all the canine isolates were *C. canis.*

In Chapter 5, *G. duodenalis* isolates of dogs and cats were characterized at the *β-giardin* and at the *glutamate dehydrogenase* genes by PCR-RFLP and by sequencing analysis. Thirty six dog samples and 2 cat samples were analyzed. Among the dog isolates 32 corresponded with the restriction pattern of assemblage D and 4 with the assemblage C pattern by the *β-giardin* analysis. The two cat samples analyzed were typed as assemblage A.

In Chapter 6, the clinical management of four cats and a dog with chronic diarrhea and *Cryptosporidium* and *Giardia* co-infection is described. Different protocols utilizing metronidazole, fenbendazole, nitazoxanide, tylosin, azithromycin and paromomycin were tried in each individual patient with variable results. However, clinical disease ultimately resolved in each case. The study emphasizes the difficulties in management of these parasites in some patients.

In Chapter 7, three experiments are described that were designed to determine the optimal protocol for use of febantel, pyrantel, and praziquantel (FPP) for the treatment of giardiasis in experimentally-infected kittens. When, the United States formulation of FPP was administered at two small dog tablets/cat (approximately 56 mg/kg of febantel per cat), PO, for 5 days was administered to six cats, a significant decrease in cyst shedding and in the percentage of positive samples was observed in the treated group in comparison to the untreated group.

In Chapter 8, 26 laboratory cats were inoculated with a *Giardia* isolate from a cat with chronic diarrhea. The cats that became infected were then administered metronidazole benzoate (25 mg/kg, PO, q12hr for 7 days) to assess the effect on cysts shedding. All the cats became negative for *Giardia* by IFA and clinical signs of drug

toxicity were not detected during the study and so it was concluded that this protocol was safe and effective for the treatment of cats with giardiasis.

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ACKNOWLEDGMENTS

First, I would like to express my gratitude to my advisor Dr. Michael Lappin for giving me the opportunity of pursuing graduate studies when I first meet him in 1998. Since then I was able to complete my Masters degree, and then he encouraged me to pursue a Doctorate degree. I am also thankful for the various opportunities for learning, including traveling, which allowed me to interact with other scientists in my field. Apart from our work together, he also accompanied and supported me in other important moments in my personal life.

I would also like to thank my graduate committee members, Dr. Bachand, Dr. Jensen and Dr. Twedt for their scientific contribution and their help in improving the quality of my work.

The support from Dr. Lappin's laboratory staff was also very important, and included the handling and processing of numerous samples, as well as the handling of cats when necessary. My special thanks to Melissa Brewer and to Jennifer Hawley.

I am very thankful to Dr. Robert Burnett for his time and patience in teaching me the necessary molecular biology techniques, especially DNA sequencing and restriction length polymorphism.

I would also like to acknowledge Glenda Tatton Allen who has provided me with *Cryptosporidium* and *Giardia* fecal samples and with advices in parasitology. She is supportive and caring and has helped me to overcome my frustration.

I also want to thank Dr. Maria Margarita Alves who helped me with the interpretation of the *Cryptosporidium felis* genotyping, Dr. Simone Cacciò who provided me with *Giardia*

controls for the *Giardia* genotyping research, and Dr. William Black for his help in the construction of phylogenetic trees.

Furthermore, I would like to acknowledge Heska Corporation and Bayer Animal Health for their crucial support in the diagnostic and treatment studies.

Apart from the University, special thanks go to my family and friends. To my husband Mike, for his patience, understanding, and for being an example of integrity and perseverance. My parents who are a constant presence in my life, advising me, respecting me and for their constant search for knowledge. Also to my brother Mariano, Claudia, Valentina and Martin for their generosity and thoughtfulness. To Celia, Susana, Eliana, Sibel and Patricia Vigil for their invaluable company during this journey. Naturally, thanks also go to our cats Romito and Pulchinella.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

CRYPTOSPORIDIUM SPP.

1.1 TAXONOMY

Members of the genus *Cryptosporidium* are placed taxonomically within the kingdom Protozoa, phylum Apicomplexa, class Coccidea, order Cryptosporidiida (Marquardt, 2000).

Historical perspective

Edward Tyzzer was the first to identify the genus *Cryptosporidium* and to recognize its multispecies nature. Initially, he described *C. muris*, from the gastric glands of a laboratory mouse and then he reported a second species: *C. parvum* also isolated from the small intestine of a laboratory mouse (Xiao et al, 2004; Upton and Current, 1985). During the following fifty years after *Cryptosporidium* was first described, it was commonly confused with *Sarcocystis*. Once the differences with *Sarcocystis* were recognized, the erroneous concept of strict host specificity was applied to *Cryptosporidium* at the same time that numerous new species were identified (Xiao et al, 2004). Later on, cross-transmission studies demonstrated that *Cryptosporidium* isolated from different animals can be transmitted from one host species to another. For that reason, numerous *Cryptosporidium* that affected different mammals were named *C. parvum* (Xiao et al, 2004). Recently, molecular characterization of *Cryptosporidium* helped in the taxonomy and validation of multiple *Cryptosporidium* species (Xiao et al, 2004).

Species concept in Cryptosporidium

One of the main problems regarding *Cryptosporidium* taxonomy is the difficulty in fulfilling the definition of biological species. The definition of species as groups of interbreeding natural populations reproductively isolated from other groups is hard to apply to *Cryptosporidium* (Xiao et al, 2004). Although *Cryptosporidium* has a sexual stage and intraspecies sexual recombination has been demonstrated in *C. parvum* (Mallon et al, 2003), it is difficult to conduct genetic crossing studies with many *Cryptosporidium*. The vast reproductive potential of the parasite leads to a large number of genetically similar parasites co inhabiting restricted areas, which in turn results in mating between siblings. Thus *Cryptosporidium* tends to have a bias towards a clonal population structure (Awad-El-Kariem, 1999). Morphology, specifically oocyst structure, is one of the bases for apicomplexa taxonomy. However, oocysts of many *Cryptosporidium* species are virtually identical (Xiao et al, 2004). Therefore, other morphologic characteristics of the oocysts may be included in a taxonomy report. For example, *C. baileyi*, possessed a third type of merogonous stage not seen in *C. parvum* (Xiao et al, 2004). Infectivity is another criterion used for the characterization of the *Cryptosporidium* species. However, numerous variables including; infection dose, oocysts age, strain used, can affect the parasite development (Upton and Gillock, 1996; Xiao et al, 2004). Host specificity can also help to define a species. For example, cross transmission studies helped to distinguish *C. hominis* from *C. parvum* (Morgan-Ryan et al, 2002; Xiao et al, 2004). Biochemical differences, assessed by restriction fragment polymorphism analysis (RFLP) of genomic DNA, isozyme analysis and other methods can help to differentiate species (O'Donoghue, 1995; Xiao et al, 2004). These methods, however, are expensive and impractical because they require large numbers of parasites

to perform the assays and have many technical problems (Xiao et al, 2004). Recently, genetic differences at the species or genotype level became the key element in taxonomy because they correlate well with biological characteristics of the different *Cryptosporidium* species (Xiao et al, 2004).

At the 6th Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease at the Pasteur Institute in Paris, France, in a section related to *Cryptosporidium* taxonomy, it was suggested that four basic requirements should be fulfilled: 1: morphometric studies of oocysts; 2: genetic characterization; 3: demonstration of natural and whenever possible, at least some experimental host specificity; 4: compliance with the International Commission on Zoological Nomenclature (ICZN).

Cryptosporidium Species

Species that are currently considered valid are: *C. andersoni, C. baileyi, C. canis, C. felis, C. gailli, C. hominis, C. meleagridis, C. molnary, C. muris, C. parvum, C. wrairi, C. saurophilum and C. serpentis* (table 1.1).

Table 1.1
Valid *Cryptosporidium* species

Species	Major host	Minor host
C. muris	Rodents, bactrian camels	Humans, rock hyrax, mountain goats
C. andersoni	Cattle, bactrian camels	Sheep
C. parvum	Cattle, sheep, goat, humans	Deer mice, pigs
C. hominis	Humans, monkeys	Dugongs, sheep
C. wrairi	Guinea pigs	
C. felis	Cats	Humans, cattle
C. canis	Dogs	Humans
C. meleagridis	Turkeys, humans	Parrots
C. baileyi	Chicken, turkeys	Cocktiels, quails ostriches, ducks
C. galli	Finches, chicken, Capercalles, grosbeaks	
C. serpentis	Snakes, lizards	
C. saurophilum	Lizards	Snakes
C. molnari	Fish	

Xiao L, Fayer R, Ryan U, et al. *Cryptosporidium* Taxonomy: Recent Advances and Implications for Public Health. Clin. Microbiol. Rev. 2004;17(1):72-97.

Since cats and dogs are infected with host specific *C. felis* and *C. canis* respectively; the characteristics of only these *Cryptosporidium* will be described.

Cryptosporidium canis

The size of the oocysts of *C. canis* is 4.95 by 4.71 μm and had a length/width ratio of 1.05 (Fayer et al, 2001). These oocysts were indistinguishable from *C. parvum* oocysts and have common surface antigens. Oocysts obtained from a dog were infectious for a calf, but not infectious for neonatal BALB/c or for dexamethasone-treated and untreated C57BL6/N mice. Oocysts obtained from a human were also infective for a calf and sequence analysis of the small-subunit (SSU) rRNA and heat shock protein 70 (HSP-70) showed that these isolates were identical to the dog genotype previously identified (Morgan et al, 2000a; Sulaiman et al, 2000, Xiao et al, 1999a). *C. canis* infections had been also found in other dogs, coyotes, foxes and humans (Xiao et al, 2004).

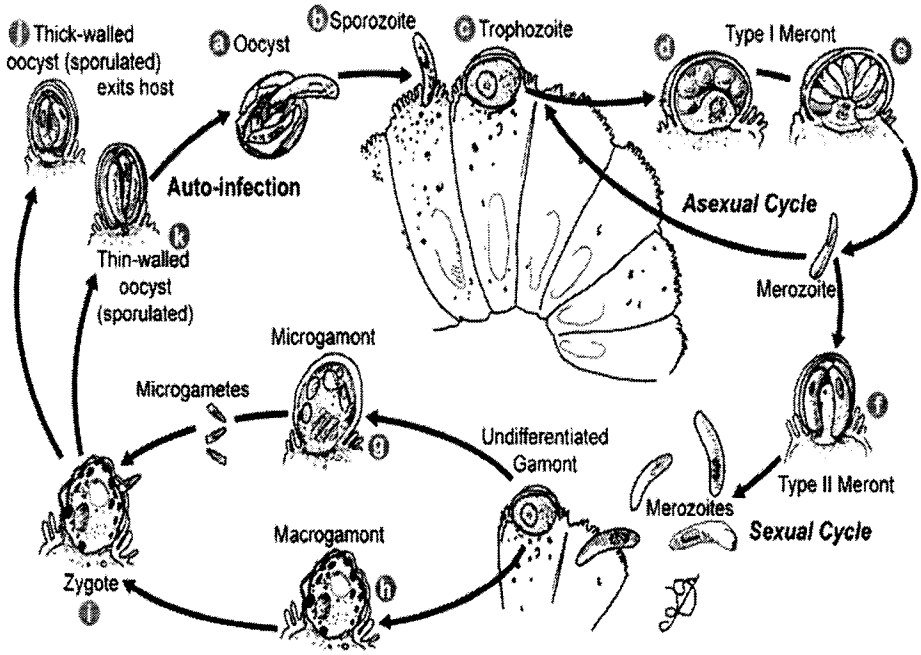
Cryptosporidium felis

C. felis oocysts are 5 by 4.5μm. Oocysts from a cat were fed to other cats, mice and guinea pigs but they were only infectious for the cats (Iseki, 1979). Prepatent and patent periods were 5 to 6 and 7 to 10 days post-inoculation, respectively. Other studies also reported oocysts of similar sizes and these isolates from cats were not infective to mice but they were infective to newborn lambs (Mtambo et al, 1996). The validity of *C. felis* was in doubt for several years, however, recent molecular characterization studies at the SSU rRNA, ITS-1, HSP-70, oocyst wall protein (COWP), and actin loci sustained the concept of *C. felis* as a valid species (Xiao et al, 2004). *C. felis* infections have been found in cats, humans and cattle.

1.2 LIFE CYCLE

The life cycle of cryptosporidians differs from most other coccidians (Marquardt, 2000). All stages of development (asexual and sexual) of cryptosporidia occur within one host. The cycle begins with the ingestion (and possibly other routes such as respiratory secretions) of sporulated oocysts by a host. Oocysts excyst in the gastrointestinal tract (or other tissues such as the respiratory tract), releasing infective sporozoites, which become enclosed as trophozoites within parasitiferous vacuoles of the microvillous surface of enterocytes. The trophozoites proliferate (asexually) by merogony to produce, sequentially, two types of meronts. Within 24 hours, type I meronts (containing eight merozoites) leave the parasitiferous vacuoles to invade other epithelial cells where they develop into more type I meronts or type II meronts (containing four merozoites). The type I meronts are thought to be capable of recycling indefinitely and, thus, the potential exists for new type I meronts to arise continuously.

The type II meronts do not undergo merogony but produce sexual reproductive stages (gamonts). The zygotes formed by sexual reproduction (gametogony between male microgamonts and female macrogamonts) form either thick-walled or thin-walled oocysts, each containing four sporozoites. Approximately 20% of the oocysts produced in the gut are "thin–walled" oocysts that failed to form an oocyst wall. Instead, a series of membranes surround the developing sporozoites (Fayer et al, 2000). Thus, *C. parvum* appears to have two autoinfective cycles: the first by continuous recycling of type I meronts and the second through sporozoites released from ruptured thin-walled oocysts. The thick-walled oocysts are passed in the feces and into the environment. Oocysts are infective upon excretion, thus, permitting direct fecal-oral transmission.



Life cycle of *C. parvum* (from: www.dpd.cdc.gov)

1.3 BIOLOGY OF CRYPTOSPORIDIUM

Cryptosporidium spp. are extremely infectious because of the following characteristics: the oocysts are resistant to most common disinfectants, they are ready for infection when passed in feces and a very low number of oocysts is enough to cause infection.

Resistant oocyst wall

The disulfide bond-rich wall provides a protective barrier for the sporozoites. *Cryptosporidium* spp. oocysts are bilayered, consisting of an inner and outer layer. The outer layer is comprised of acidic glycoproteins and it can be partially removed by treatment with sodium hypochlorite. The inner layer provides rigidity and elasticity to the oocysts wall due to the central glycolipid/lipoprotein layer and the thick inner filamentous layer composed of glycoproteins (Carey et al, 2004). Proteinase K was effective in disrupting the internal layer of the oocyst wall. Ultrasonication, chloroform treatment and phenol extraction did not disrupt the oocyst wall (Carey et al, 2004). Due to the consistency of the oocyst wall, *Cryptosporidium* oocysts can survive in the environment. In general, aged oocysts are more susceptible to disruption by environmental changes and disinfectants. A characteristic of *Cryptosporidium* oocysts is their resistance to chlorine as used in drinking water treatments and swimming pools. Table 1.2 showed some of the studies done evaluating the resistance of *Cryptosporidium* oocysts to different conditions.

Table 1.2
Effect of selected environmental factors on the viability and infectivity of *C. parvum* oocysts

Environmental parameter	Conditions	Oocyst viability/infectivity	Assessment method
Temperature	336h incubation from 10C to 30C in water	Infectious	BALB/c mouse infectivity
	1 min at >72C, 2 min or longer at 64C (water)	Non-infectious	BALB/c mouse infectivity
	Snap freezing	100% dead oocysts	DAPI/PI*
Desiccation	4h at room temperature	>99% dead oocysts	DAPI/PI
Tap Water	176 days in a flow-through system at room temperature	>96% dead oocysts	DAPI/PI
Sea Water	35 days at 4C under static lab conditions	38% dead oocysts	DAPI/PI
Cow feces	176 days submerged in solid feces at ambient temperature	66% dead oocysts	DAPI/PI
Human feces	178 days at 4C	78% dead oocysts	DAPI/PI

Adapted from:Carey CM, Lee H, Trevors JT. Biology, persistence and detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts. Water Res 2004;38:818-862.

*DAPI/PI: 4', 6'-diamidino-2-phenylindole and propidium idodide (vital fluorogenic dyes)

Since there is no effective treatment for cryptosporidiosis, hygienic measures are valid ways to control the infection. A study evaluated the effect of two products containing formaldehyde and hydrogen peroxide developed to be used as disinfectants in farms (Castro-Hermida et al, 2006). Viability assays showed a decreased in oocyst viability in association with an increase in exposure time, and when these oocysts were used to infect mice, the intensity of infection was significantly lower than in the control litters (Castro-Hermida et al, 2006). The authors concluded that both commercial disinfectants have anticryptosporidial activity. Formaldehyde and hydrogen peroxide were the main components; however, additional minority components may have some part in decreasing

oocyst infectivity (Castro-Hermida et al, 2006). UV disinfection technology is being used in the water industry because it was shown to be effective against other waterborne pathogens (Hijnen et al, 2006). Although *Cryptosporidium* oocysts are susceptible to UV radiation, the researchers suggested that for protozoan (oo)cysts, more investigation related to the calculation of the microbial inactivation time (MIC) of UV disinfection in water should be done (Hijnen et al, 2006).

Relatively small size

Due to it small size (4 to 6 μm), *C. parvum* oocysts pass through the most conventional filters, making *Cryptosporidium* a challenge to effective water treatment methods. Turbidity is use as an indicator of the concentration of small suspended particles including oocysts in water. However, drinking water that met the US safety standards, including those for turbidity, was responsible for the Milwaukee outbreak. Even after tightening the turbidity standards a *Cryptosporidium* outbreak occurred in Nevada where the turbidity of the water was extremely low (MacKenzie et al, 1994). Therefore, low turbidity water does not assure that drinking water will be free of *Cryptosporidium.*

Reverse-osmosis filters and 1um absolute filters have been shown to remove oocysts (Carey et al, 2004).

Low infectious dose

The median infective dose (ID50) of the Iowa strain of *C. parvum* in 29 healthy adult volunteers was 132 oocysts (DuPont et al, 1995). Using a mathematical model on the data from the Milwaukee outbreak, it was suggested that some individuals may have developed the infection after the ingestion of only one oocyst (Carey et al, 2004).

Another study were three different isolates were administered to healthy volunteers found that they differed in their duration of the infectivity and ID50 (Okhuysen et al, 1999) (table 1.3). In this study oocysts were measured by direct immunofluorescence (DFA), however, flow cytometry confirmed the presence of other light cryptosporidial infection that was not detected by DFA (Carey et al, 2004).

Table 1.3
ID 50 and duration of infectivity of three strains of *C. parvum*

C. parvum strain	number of infectivity volunteers tested	ID50 (oocysts)	duration of (hours)
Iowa (calf)	11	87	64.2
UPC (calf)	29	1,049	81.6
TAMU (horse)	14	9	94.5

Okhuysen PC, Chappell CL, Crabb JH, et al. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J Infect Dis 1999;180:1275-1281.

Fully infectious oocysts after shedding

Cryptosporidium oocysts sporulate inside the host and are ready for infection immediately when passed in feces. In addition it is difficult to control the extended period of shedding often after asymptomatic illness (Dillingham et al, 2002).

1.4 MEANS OF TRANSMISSION

Cryptosporidium enters to the environment through feces from human and non-human hosts. Proper treatment and disposal of human feces and animal manure are crucial to ensure a safe environment. It is estimated that humans produce a total of 232 million metric tons of feces per year (Fayer et al, 2004a). In developing countries, most of the feces are disposed with little or no treatment to the rivers or into the sea; in developed countries, most of the feces are aerobically digested before disposal into the river or coastal waters. The livestock manure production is approximately 5.45 billion

metric tons per year, most is deposited in the field with little or no treatment except in places with manure management programs, resulting in an accumulation of an incalculable number of pathogens (Fayer et al, 2004a).

Cryptosporidiosis can be transmitted in different ways:

Drinking water

Waterborne cryptosporidiosis is associated with the contamination of water with animal or human waste. Waterborne transmission is probably the most common way of transmission, whether by fully chlorinated drinking water or by sewage effluent. There have been outbreaks attributable to potable water, well water and surface water (Duffy and Moriarty, 2003). The ability of *Cryptosporidium* to tolerate the concentration of chloride applied during water treatment processes has been a contributing factor in many of the cases (Duffy and Moriarty, 2003). The first reported waterborne outbreak of cryptosporidiosis was in Braun Station, Texas in 1984. The biggest documented outbreak of *C. parvum* occurred in Milwaukee, Wisconsin in 1993 and was associated with a potable public water supply (MacKenzie et al, 1994). Worldwide, approximately 43 outbreaks related to drinking water have been reported since 1984 (Fayer et al, 2000). *C. parvum* oocysts have been detected in untreated wastewater and in treated drinking water (Fayer et al, 2000). Several studies documented the presence of oocysts in finished water samples from 3.8 to 33.3% of the time at concentrations from 10 to 484 oocysts per liter[3] when using conventional filtration methods in water treatment plants (Fayer et al, 2000). These levels represent daily exposure to persons using filter-purified tap water (Fayer et al, 2000). Between 20%-65% of the population in the United States is carrying antibodies against *Cryptosporidium* (Rossignol et al, 2006). However, the improvements

during the purification of water supplies, may increase the risk of acquiring cryptosporidiosis by reducing the protective immunity gained through the low exposure to *Cryptosporidium* oocysts (Rossignol et al, 2006).

Recreational water

A review article reported 31 outbreaks that affected more than 10,000 people associating cryptosporidiosis with exposure to recreational water (Fayer et al, 2000). Despite chlorination of the water, the infection occurred through fecal accidents by infants with diapers or incontinent individuals.

Cryptosporidium oocysts have been reported in marine waters off the coast of Hawaii and Puerto Rico (Fayer et al, 2004a).

Food borne transmission

The biology and the ecology of *Cryptosporidium* make its control in the food industry hard (Dawson, 2005). Only in the last 3-5 years, it was recognized that food may play a more significant role in the transmission of cryptosporidiosis than previously thought (Duffy and Moriarty, 2003). Approximately 10% of the *Cryptosporidium* and *Giardia* cases in the United States are food borne (Smith et al, 2006). Food can be contaminated from fertilizers made from animal or human feces, by contaminated water used for irrigation, or by infected food handlers. A recent study showed that 36% of the water used to irrigate vegetables in the United States and in various Central American countries tested positive for *Cryptosporidium* oocysts (Fayer et al, 2004). Oocysts of *C. parvum* have been reported in shellfish in the United States, Spain and Ireland but none of these findings correlates with food borne outbreaks of cryptosporidiosis (Fayer et al, 2000a). The food borne outbreaks reported occurred through unpasteurized milk and

apple juice, and the contamination of chicken salad by an infected person (Fayer et al, 2000a). *Cryptosporidium* oocysts do not survive the standard heating processes and their survival might be reduced by other processes during the preparation of the food (Dawson, 2005). However, a recent report showed that steaming contaminated mussels do not kill the infectivity of *C. parvum* (Gomez-Couso et al, 2006).

It is difficult to identify the source of infection due to the time delay in the consumption of food products and the onset of illness. Additionally, the lack of appropriated methods for routine detection of the parasite in food has also hindered epidemiological investigations (Duffy and Moriarty, 2003). A recent article emphasized the need of maximizing DNA extraction techniques and minimizing Taq polymerase inhibitors to have more sensitive techniques to detect pathogens in food (Smith et al, 2006).

Person-to-person transmission

In Brazil, 19% of the family members of children with cryptosporidiosis seroconverted or developed clinical signs of disease (Dillingham et al, 2002). During the Milwaukee outbreak, 5.4% of the household contacts developed cryptosporidiosis (MacKensey et al, 1995). When it is difficult to maintain an adequate sanitation, nosocomial and day care infections are likely to occur (Dillingham et al, 2002; Koch et al, 1985).

Animal-to-people transmission

The connection between cattle exposure and cryptosporidiosis, through direct or indirect contamination, clearly illustrate the zoonotic transmission. In one study, dairy farmers had a *Cryptosporidium* seroprevalence of 44% in comparison to 24% in other

individuals (Casemore et al, 1997). *C. felis* and *C. canis* have been reported in immunocompetent and immunosuppressed humans (Pedraza-Diaz et al, 2001; Xiao et al, 2001a; Pienizaek et al, 1999; Morgan et al, 2000b). This issue will be described in more detail in the zoonotic section.

Other environmental sources

It was shown that *C. parvum* oocysts ingested by Canadian geese (*Branta canadensis*) and Peking ducks (*Anas platyrhynchos*) passed through the gastrointestinal tract, were excreted in the feces for nearly one week, and were capable of infecting mice (Fayer et al, 2000). Later viable oocysts of *C. parvum* were recovered from feces of Canadian geese in fields where they rested along their migration route (Fayer et al, 2000). The fact that a wide variety of birds can be infected with *Cryptosporidium* shows that the oocysts can be spread over vast areas (Fayer et al, 2000).

The association of respiratory symptoms in immunocompromised patients with cryptosporidiosis has been reported (Fayer et al, 2000). Thus concept of airborne cryptosporidiosis has been theorized, but there have been no proven cases (Hojlyng et al, 1987).

Flies, cockroaches and other potential vectors

Some studies have experimentally shown *Cryptosporidium* spp. oocysts to be carried by beetles and flies (Fayer et al, 2000; Graczyk et al, 1999).

Seasonality and risk factors

In tropical and developing areas, cryptosporidiosis occurs more often in the warm or wet season, when increased rainfall result in greater spread of contaminated surface water used for drinking (Dillingham et al, 2002).

The groups implicated with the highest risk of infection are children and personnel in day care centers, farmers and animal handlers, and health care workers. Travelers are at risk when they travel from developed to developing countries with high prevalence of disease (Ramirez et al, 2004). Another risk factor seems to be weaning, especially at young age indicating that breast feeding provides some protection (Dillingham et al, 2002).

Cryptosporidium in wild mammals, marine mammals, birds, reptiles and fish

Cryptosporidium has been detected in most species of wild animals as well as birds, reptiles and fish. Unique genotypes have been reported in bears, marsupials, mountain gorillas and red deer among other animals (Xiao et al, 2003). *Cryptosporidium* has been also found in the dugong, in Australia, in California sea lions and in seals in artic Canada (Fayer et al, 2004b). Their zoonotic potential is unknown; nevertheless it is important to recognize that they contribute to the pool of *Cryptosporidium* oocysts identified in environmental samples (Xiao et al, 2003). In the future the studies on the *Cryptosporidium* isolates that infect marine and wildlife animals should be performed at a molecular level to establish the host range, transmission dynamics and zoonotic potential of these isolates (Applebee et al, 2005).

1.5 PATHOGENIC MECHANISM OF *C. PARVUM*

Excystation

Excystation is needed to liberate the sporozoite and its subsequent adherence to host cells. Several parasite proteolytic enzymes, including serine and cysteine endopeptidases and aminopeptidases have been reported. It is supposed that the role of these enzymes may participate in the degradation of the oocyst suture and assist in the release of the encased sporozoites (Chappell et al, 2003) (See table 1.4).

Host cell recognition and attachment

The sporozoites attach the epithelial cell by their anterior pole, followed by invagination of the host cell membrane (Deng et al, 2004). *Cryptosporidium* creates a niche for itself between the cell membrane and the cell cytoplasm; apparently, the chemotherapy resistance can be explained by its particular location. *Cryptosporidium* lies within a parasitophorus vacuolar membrane (PVM) which provides a protective function. As a difference with other coccidian, it has a unique structure called the feeder organelle membrane, which separates the cell cytoplasm from the parasite cytoplasm. The feeder organelle membrane is the site for nutrient uptake from the host cell. In other coccidians, the PVM acts as a nutrient uptake membrane. Additionally, since the PVM derives from the host cell membrane, it also has some of its absorptive properties (Tzipori and Ward, 2002). Sporozoite attachment and epithelial cell invasion are affected by sporozoite doses, pH, incubation time, divalent cations and the status of host cell differentiation (Deng et al, 2004).

These initial host-parasite interactions are complex processes that involve multiple parasite ligands and host receptors. During the zoite stages, the invasive apicomplexans have secretory organelles, named roptrias, micronemes and dense granules, and as a group are known as apical complex (Tzipori and Ward, 2002). These organelles secrete

proteins that facilitate the attachment, invasion and PVM formation. Recently it was demonstrated that *C. parvum* excrete the following proteins: GP900, galactose-N-acetylgalactosamine (Gal/GalNAc)-specific lectin, gp15/40, thrombospondin-realted anonymous protein (TRAPs), CP47 and CSL glycoprotein. In general, these specific proteins are involved in attachment and invasion of *C. parvum* (Deng et al, 2004) (table 1.4).

Table 1.4
Pathogenic mechanisms of *C. parvum*

Molecular component	Location	Importance in infection
CSL	Apical complex of sporozoites and merozoites	Attachment/invasion;neutralized by monoclonal antibody to CSL or CSL receptor
GP900	Sporozoite microneme	Attachment/invasion;neutralized by specific antibody
GP40/15complex	Sporozoite surface, apical complex	Attachment/invasion;neutralized by monoclonal antibody or specific lectins
TRAP-C1	Microneme (proposed)	Locomotion;cell attachment and invasion (proposed)
CP47	Sporozoite apical complex	Unknown
CPS500	Sporoite apical complex	Attachment/invasion;neutralized by monoclonal antibody
Hemolysin H4	Unknown	Membrane disruption
Cp ABC transporter	Feeding organelle	Ion transport (proposed)
Proteases	Sporozoite surface; secreted?	Excystation
HSP70, HSP-90	Cytoplasm?	Adaptation to host environment and/or stressors

Chappell CL, Okhuysen PC, Clinton White Jr, A. Cryptosporidium parvum:infectivity, pathogenesis and host-parasite relationship. in *Cryptosporidium: From Molecules to Disease.* RCA Thompson, A Armson and UM Ryan. 2003 first edition. Elsevier.

1.6 HOST RESPONSE TO THE INFECTION

Epithelial cell responses to C. parvum infection

Intestinal epithelial cells are a physical barrier between the host internal environment from the host external environment and they produce a variety of cytokines and chemokines in response to pathogens.

Cytoskeleton modulation

A notorious feature of *C. parvum* infection is that after attachment to the host cell, it rearranges the host cytoskeleton actin at the site of the infection and it starts the initiation of the parasitophorous vacuole (Deng et al, 2004).

Apoptosis

C. parvum induces and inhibits apoptosis in the host cell. *C. parvum* induces apoptosis by two mechanisms: via the Fas receptor and the Fas ligand protein and via caspaces, which are a group of intracellular proteases (Ojeius et al, 1999; Chen et al, 1999). Removal of the infected cells by apoptosis helps the host because it maintains the epithelial barrier's integrity. *C. parvum* also induces apoptosis in the uninfected cells nearby limiting the spread of the infection. The induction of moderate levels of apoptosis limits the host's inflammatory response that could be detrimental for the survival of the parasite. *C. parvum* can also inhibit apoptosis in infected epithelial cells by activating nuclear factor kB (NF-kB) early after infection when the parasite need the host cell for growth and development (Deng et al, 2004).

Regulation of proinflammatory cytokines and immune regulators

C. parvum infection is characterized by mucosal inflammation with neutrophils, macrophages and lymphocytes in the lamina propria underlying the epithelial cells and

intraepithelial neutrophils and T lymphocytes (Theodos, 1998). Interleukin 8 (IL-8), and other pro-inflammatory cytokines like Gro-α, TNF-α, and RANTES are upregulated during *C. parvum* infection (Deng et al, 2004). Transforming grow factor-β (TGF- β) is an anti-inflammatory cytokine that seems to play a protective role in limiting the epithelial damage produced by infection with *C. parvum* (Deng et al, 2004). *C. parvum* activates prostaglandin h synthetase 2 expression and prostaglandin E2 and F2a in human intestinal cells (Laurent et al, 1998).

Enteric Beta Defensin (EBD) belongs to a group of peptides that are part of the innate defenses present in the intestinal tract. After *C. parvum* infection, EBD expression was up-regulated by 5-10 folds in calves, suggesting that the innate immune system also contributes in the response against *C. parvum* (Deng et al, 2004).

Intestinal immune responses to C. parvum infection

Cellular immunity mediated by CD4+ and CD8+α/β T cells is an important component for the resolution of *C. parvum* infection. γ/δ T cells participate in host immune responses against pathogenic infections. Recent studies using bovine and murine models of cryptosporidiosis suggested that γ/δ T lymphocytes may play a role in protective immunity against *C. parvum.* The results of studies done using a bovine model of cryptosporidiosis suggested that γ/δ T cells have a role in initiating the host immune responses (Wyatt et al, 1997; Deng et al, 2004). Results of studies done in mice suggested that γ/δ lymphocytes are not necessary for induction of intestinal inflammation but their presence alter the morphology of the ensuing lesion (Deng et al, 2004). The differences between γ/δ lymphocytes in cows and mice remain to be clarified and the role of γ/δ lymphocytes in the human and other animal responses is unknown.

Heat shock proteins (HSP) are polypeptides produced by cells to preserve cellular functions under conditions of stress and they can activate γ/δ T cells (Deng et al, 2004). It was hypothesized that epithelial cell expression of HSPs and that heat shock response might be activate the rapid recruitment of γ/δ T cells to *C. parvum* infected tissues and then subsequent immune response (Deng et al, 2004). In addition, during *C. parvum* infection, HSPs can also inhibit host apoptosis. The balance between pro and anti-apoptotic events is not completely elucidated for *C. parvum* infection (Deng et al, 2004).

Interferon- γ (IFN- γ) also appears to have an important role in innate immunity and in cell-mediated responses against *C. parvum* (Deng et al, 2004).

The synthesis of nitric oxide (NO) is elevated in patients with diarrhea caused by infectious pathogens (Gookin et al, 2006). It was demonstrated that mucosal expression of inducible NO synthase (iNOS) is increased in infected piglets and that inhibition of iNOS in vitro has no short-term effect on barrier function (Gookin et al, 2004; Gookin et al, 2006). It was shown that in *Cryptosporidium* infected piglets, iNOS expression is mainly in the epithelium, it was NF-kappaB dependent and it was not restricted to enterocytes (Gookin et al, 2006). The authors concluded that induction of iNOS is a non specific response of the epithelium that mediates the enterocytes reaction against *C. parvum* (Gookin et al, 2006).

1.7 CRYPTOSPORIDIOSIS IN HUMANS

Cryptosporidiosis has been reported in over 50 countries. In developed countries, seroprevalence rates range between 25-35%; in developing countries, seroprevalence rates are higher, up to 64% in Latin America, 57.5% in Brazil and 42% in China (Fayer et

al, 2004a). Therefore, cryptosporidiosis is a major public health concern in both developed and developing countries. In developed countries, water borne outbreaks have an important economic impact. It was estimated that the total illness-associated cost of the Milwaukee outbreak in 1993, was $96.2 million. The average total cost for a person with mild, moderate and severe illness during the outbreak was $116, $475 and 7,808 respectively (Corso et al, 2003). The United Kingdom will spend an average of £23 million/year to meet legal requirements for removal of *Cryptosporidium* from drinking water (Xiao et al, 2004).

In developing countries, cryptosporidiosis has its major impact on children, specifically on children younger than 5 years old. Peak occurrence of the infections occurs in infants younger than 2 years old (Newman et al, 1999; Bern et al, 2000). Children can have multiple episodes of cryptosporidiosis (Newman et al, 1999; Xiao et al, 2001a). In a follow up study of 185 children, those with cryptosporidiosis experienced several months of weight and height faltering, an effect that in young children persisted a year after infection (Checkley et al, 1998). Another study in children in Northeast Brazil showed a correlation between cryptosporidiosis during the first 2 years of life and reduced fitness among 4-6 years old (Guerrant et al, 1999). The association of early cryptosporidiosis with growth and cognitive deficits will consequently affect school performance. There is also a significant association between having diarrhea in the first two years of life and the delay with the start of schooling (Dillingham et al, 2002). The major brain development and synapse formation occurs during the first two years of life in humans. Early childhood illness can affect brain

development which may explain the lasting impact of childhood cryptosporidiosis (Dillingham et al, 2002).

Cryptosporidiosis occurs in immunocompetent and immunosuppressed individuals. In immunocompromised patients, such as those affected with the human immunodeficiency virus (HIV+), the severity of cryptosporidiosis increases as the CD4+ cells count falls, especially when the count falls below 200 cells/μl (Xiao et al, 2004).

Table 1.5. Some clinical features of cryptosporidiosis.

Characteristic	Immunocompromised host	Immunocompetent host
Susceptible population	Any age especially those with AIDS	Children (less 1 yrs)
Site of infection	Intestinal or extraintestinal	Usually intestinal
Enteric presentation	Asymptomatic, transient, chronic or fulminant	Asymptomatic, acute or persistent
Common clinical symptoms	Diarrhea, fever, jaundice, weight loss and vomiting	Diarrhea, fever, abdominal cramps, nausea and weight loss
Clinical duration	From 2 days to lifetime	Up to 2 weeks
Severity according to CD4+ count		
>200cells/mm3	Spontaneous resolution	Not available
<100cells/mm3	Chronic and extraintestinal	
<50 cells/mm3	Fulminant	
Likely outcome	Transient or asymptomatic (5-184 weeks) Chronic (3-127 weeks) Fulminant (2-28 weeks until death following infection)	High mortality in infants and young children in developing countries
Treatment	Highly active anti-retroviral therapy alone	No specific treatment indicated or with anti-parasitic agents

Carey et al, 2004. Biology, persistence and detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocyst. Water Res 38:818-862.

Cryptosporidium diarrhea is associated with impaired intestinal absorption and enhanced secretion (Carey et al, 2004). The mechanism of diarrhea is not exactly known

but it has been suggested that it can occur through the disruption of microvillous surface area, the presence of an enterotoxin, or adhesion factors affecting parasite attachment to host cells (Carey et al, 2004). When changes in the microvillous area and inflammatory response were evaluated in a porcine model, reduction in villous height and an expansion in crypts were found, yielding an approximate 1:1 villous-crypt ratio as compared to a 5:1 ratio seen in controls (Argenzio et al, 1990). An increase of inflammatory cells in the lamina propria from 46+/-116 cells in controls to 1,014+/-187 in infected villous was found (Argenzio et al, 1990). Histological findings revealed severe villous atrophy, crypt hyperplasia and inflammatory cells in the lamina propria. All these findings were associated with an altered glucose-stimulated Na+ and water absorption, as well as increase in Cl-secretion (Argenzio et al, 1990). It was suggested that diarrhea occurs from a combination of secretory diarrhea and sodium/glucose malabsorption due to villous atrophy and epithelia damage (Carey et al, 2004).

Much of the current research is devoted to elucidating the molecular interactions between parasite and host that lead to successful *Cryptosporidium* invasion and its development in the intestinal cells.

1.8 ZOONOTIC POTENTIAL

The interest in human cryptosporidiosis started approximately in 1980, at that time the infection was believed to be mainly zoonotic but the potential to person to person transmission was also considered (Hunter and Thompson, 2005). Cryptosporidiosis was known among veterinarians and farm workers, since numerous case reports documented the transmission of *Cryptosporidium* spp. from infected cattle to farm workers and veterinary students (Anderson et al, 1982; Levine et al, 1988). Since

1982, the number of cases dramatically increased as a result of the AIDS epidemic. Initially, most of the reported cases were attributed to immunosuppression, but with the development of new diagnostic techniques, outbreaks in immunocompetent people began to be recognized. By the late 1990s, genetic studies reported that *C. parvum* could be separated into two genotypes: type I which was only found in humans (now called *C. hominis*) and type II which infects humans and cattle (Peng et al, 1997; Mc Laughlin et al, 2000; Hunter and Thompson, 2005). Up till then, most the knowledge of this infection came from the scrutiny of outbreaks and little was know about the route of infection of isolated cases.

The risk factors of acquiring cryptosporidiosis for people in the developed countries are contact with other cases, travel abroad, and contact with cattle (Hunter and Thompson, 2005). However, contact with pets is either not significantly or negatively associated with risk (Robertson et al, 2002). There is also a negative association with the consumption of raw vegetables (Roy et al, 2004). In developing countries keeping pigs and dogs were found as significant risk factors for acquiring cryptosporidiosis (Molbak et al, 1994). However, none of these studies genotyped the isolates. Recently, it was reported that the majority of the human infections are caused by *C. parvum* cattle genotype and *C. hominis* (Thompson, 2003). All species of *Cryptosporidium* infect a limited range of animals (table 1.1) and when the host range includes humans, the parasite acquires public health significance (table 1.6). In the United States, human cryptosporidiosis is caused mainly by *C. hominis* (>75%), whereas in the United Kingdom, most cases are caused by *C. parvum* (Ramirez et al, 2004). A recent case control study in the UK, found two models of transmission of *Cryptosporidium* after

genotyping of the human isolates (Hunter et al, 2002). Humans infected with *C. hominis* are associated with foreign travel and with changing diapers and there is a negative association with eating fresh fruit. For *C. parvum*, the epidemiology is more complex, but there is a strong positive association with contact with cattle and a negative association with eating raw vegetables.

The host specific *C. felis* and *C. canis* have been reported in immunocompetent and immunosuppressed humans (Pedraza-Diaz et al, 2001; Xiao et al, 2001a; Pienizaek et al, 1999; Morgan et al, 2000b). However, there is not an apparent role for domestic pets as the source of infection for humans (Hunter and Thompson, 2005). Moreover, these negative associations can point to the protective effect of low levels of exposure leading to immunity. This was further demonstrated in a study were individuals with high anti-cryptosporidial antibodies titters were less likely to have diarrhea than those with low levels of antibodies (Frost et al, 2005).

Zoonosis in immunocompromised individuals

Immunocompromissed humans, particularly those living with AIDS have the highest risk. Although a study failed to find an association between pet ownership and cryptosporidiosis in HIV–infected individuals, in light of the small sample size of the study the authors believe that it would be prudent for immunocompromised pet owners to avoid direct contact with animal feces because the risk for them is probably higher (Glasser et al, 1998). According to the feline zoonoses guidelines from the American Association of Feline Practitioners it seems prudent to assume feces from all cats infected with *Cryptosporidium* spp. are a potential human health risk (Brown et al, 2003). Techniques for detecting *Cryptosporidium* spp. should be included in the diagnostic

evaluation of all cats with diarrhea and all cats in the homes of immunosuppressed individuals (Brown et al, 2003). The importance of cryptosporidiosis as a zoonotic disease became more apparent as a consequence of the AIDS pandemic, since cryptosporidiosis is considered an AIDS-defining illness. In the United States, there are several million people with compromised immune systems, of which approximately 1 million are infected with human immunodeficiency virus (HIV) (CDC, MMWR Morb Mortal Wkly Rep 1990). More than 60 % of households in the United States have pets; approximately 32% of the households have dogs and 27% having cats (Morrison, 2001). The level of pet ownership is constant among people infected by HIV (Glasser et al, 1998). Considering that pet ownership is beneficial to immunosuppressed individuals, more genotyping information regarding the *Cryptosporidium* that infects cats and dogs should be available. It is not know how companion animals acquire cryptosporidiosis. In recent studies, *Cryptosporidium baileyi* and *Cryptosporidium muris* oocysts had been recovered from cat feces (McGlade et al, 2003; Hajdušek et al, 2004). *Cryptosporidium muris* and *C. baileyi* are species found in rodents and in birds suggesting that cats can became infected through carnivorism. Additionally, cats and dogs with cryptosporidiosis can be subclinically infected carriers and act as a source of infection to other individuals or to the environment (Thompson et al, 2003). When a cat or dog became a carrier of an enteric zoonotic agent, the owner should be advised of the public health risk (Tuzio et al, 2005). If immunocompromised individuals or very young children could be exposed, the risk may be unacceptable. If the owners decide to keep the pet, the veterinarian should emphasize the importance of hygiene and sanitation (Tuzio et al, 2005).

Table 1.6.1:Occurrence of *Cryptosporidium* species in immunocompetent individuals

Country	Cases (numbers)	*C. parvum*	*C. hominis*	*C. meleagridis*	*C. felis*	*C. canis*		Cervine genotype		Monkey genotype	
Czech Rep.	Children (9)	9									
Czech Rep.	Not known (3)	2				1					
Denmark	Adult (44)	18	25	1							
France	Adult (11)	7	4								
Northern Ireland	Adult (39)	35	4								
Switzerland	Children (14)	3	11								
Switzerland	Not known (9)	9									
The Netherlands	Adult (29)	7	22								
England	Various (2414)	1354	1005	22		6	1				
Scotland	Various (137)	64	71							2	
Canada	Not known (150)	29	108					9			
Japan	Not known (1)	1									
Japan	All (22)	3	16	3							
Japan	Not known (3)	2	1								
Nepal	Not known (2)		2								
Kenya	All (9)		9								
Malawi	Children (9)	3	6								
Malawi	Children (43)	2	41								
Peru	Children (118)	20	76	10		4	9				
Brazil	Children (7)		7								
New Zealand	Not known (423)	223	198				2				
Total	3496 (51%)	1798 (51%)	1606 (46%)	36 (1%)		11(0.3%)	12 (0.3%)	9 (0.2%)		2	

Modified from Cacciò et al, 2005:Unravelling *Cryptosporidium* and *Giardia* epidemiology Trends Parasitol 2005;21(9):430-437.

Table 1.6.2:Occurrence of *Cryptosporidium* species in immunocompromised individuals

Country	Cases (numbers)	*C. parvum*	*C. hominis*	*C. meleagridis*	*C. felis*	*C. canis*	*C. muris*	*C. suis*
Vietnam	(3)		3					
Thailand	(29)		24	3	1		1	
Thailand	(34)	5	17	7	3	2		
Peru	(229)	34	204	38	10	12		1
Kenya	(6)	1	4	1				
Kenya	(24)	8	14	1			1	
Malawi	(2)	1	1					
Guatemala	(4)		4					
USA	(17)	2	15					
USA	(10)	1	5		3	1		
South Africa	(21)	5	16					
Italy	(9)	8			1			
France	(46)	22	14	3	6		1	
France	(13)	7	6					
Switzerland	(13)	7	2	1	3			
Portugal	(40)	22	11	3	4			
England	(27)	17	1	7	2			
Total	597	140 (23%)	341(57%)	64 (11%)	33 (6%)	15 (2%)	3	1

Modified from Cacciò et al, 2005:Unravelling *Cryptosporidium* and *Giardia* epidemiology Trends Parasitol 2005;21(9):430-437.

1.9 FELINE CRYPTOSPORIDIOSIS

Since Iseki reported the first case of feline cryptosporidiosis in 1979, prevalence studies and case reports of infected cats have been documented worldwide (table 1.7). The prevalence of *C. parvum* and *C. felis*, however is not completely known at this time and the results depend mainly on the study population and on the diagnostic test used.

Generally speaking, the prevalence in most of the studies tends to be higher in young animals and in shelter animals. Cats with cryptosporidiosis can be clinically or subclinically infected. The major determinants of the severity of disease caused by *Cryptosporidium* spp. are the immune status of the affected animal, the infective strain and the inoculation dose. As mentioned before, different *C. parvum* isolates demonstrated to have differences in infectivity in humans. It is unknown, however, if *C. parvum* or *C. felis* cause different degree of infection in cats.

Cryptosporidiosis in immunocompetent cats may not be associated with diarrhea or debilitation. Many cats that are shedding *Cryptosporidium* oocysts do not show clinical signs of disease (Iseki, 1979; Asahi, et al. 1991; Arai et al, 1990). Nevertheless, the histopathologic reports of these infected cats show loss of microvilli, degeneration of host epithelial cells and atrophy of the villi in heavy infections, which may predispose to malabsobtion or other gastrointestinal dysfunction (Poonacha and Pippin, 1982; Lappin et al, 1997a; Goodwin and Barsanti, 1990). Clinically infected cats can have diarrhea, anorexia and weight loss. Usually, clinical signs of disease occurred in cats with immunosuppression, preexisting disease in the intestinal tract, or coinfection with other infectious or non-infectious causes (Lappin et al, 1997a; Goodwin and Barsanti, 1990, Brizee-Buxton and Crystal, 1994). Immunosuppressed cats with clinical signs of

cryptosporidiosis include FeLV-infected cats, FIV-infected cats, or animals under other conditions of stress (Monticello et al, 1987; Goodwin and Barsanti, 1990; Mtambo et al, 1991; Bennet et al, 1985). Coinfection with infectious agents caused clinical signs of disease in some cats. *Cystoisospora* spp., and *Toxocara cati,* coronavirus and *Campylobacter* had been documented in cats with cryptosporidiosis and diarrhea (Monticello et al, 1987; Goodwin and Barsanti, 1990; Uga et al, 1989; Lent et al, 1993; Bennet et al, 1985). However, coinfection with *Capillaria spp., Spirometra erinacei, T. cati, Pharingostomum cordatum,* and *Cystoisospora felis* has been reported in healthy cats with cryptosporidiosis (Bennet et al, 1985). Coinfection with other protozoans including *Giardia* spp. and *Tritricomonas foetus* may aggravate clinical signs of cryptosporidiosis in cats (Keith et al, 2003, Perez-Trot et al, 2002; Gookin et al, 2001).

Non-infectious diseases associated with cryptosporidiosis in cats are lymphoma and inflammatory bowel disease (Lent et al, 1993; Brizee-Buxton and Crystal, 1994; Lappin et al, 1997a). In a case report of a cat with concurrent inflammatory bowel disease, the inflammatory changes resolved after the parasite was successfully treated with tylosin at a dose of 11 mg/kg PO twice daily for 28 days suggesting the inflammation was due to the parasitic infection (Lappin et al, 1997a).

Additionally, cats infected with *Cryptosporidium* spp. can be subclinically infected carriers. The administration of glucocorticoids to some chronically infected, oocyst-negative cats induced repeated oocyst shedding (Asahi et al, 1991; McReynolds et al, 2001).

Overall, infection in cats seems to be relatively common however, before PCR testing; cryptosporidiosis in cats was probably under diagnosed. This finding relates to

the relative insensitivity of some of the available tests and to the fact that cats with cryptosporidiosis shed very few oocysts. In a study of naturally-infected cats, the mean number of discharged oocysts for cats with and without diarrhea was 1,817 oocysts per gram of feces and 191 oocysts per gram of feces respectively (Uga et al, 1989).

Molecular analysis of the *Cryptosporidium* spp. in most of the studies mentioned previously was not performed and so the true prevalence of *C. felis* is unknown. Just recently, DNA amplification of 18S rDNA gene of *Cryptosporidium* isolates from cats showed that the cats in this study harbor *C. felis* (Fayer et al, 2006).

Table 1.7: Prevalence of *Cryptosporidium* spp. in Cats

Number Examined	Location	Test	Prevalence (%)	Study
600	United States	Serology	8.3	Mc Reynolds et al, 1999
258	Scotland	Serology (IgG)	74	Mtambo et al, 1995
205	Colorado	Microscopy	5.4	Hill et al, 2000
135	Czech Republic	Serology	57	Svobodovà et al, 1994
263	New York	Microscopy	3.8	Spain et al, 2000
173	North Carolina	Microscopy IFA	6.5	Nutter et al, 2004
418	Australia	Microscopy	0	Mc Glade et al, 2003
40	Australia	PCR	10	Mc Glade et al, 2003
102	Australia	Microscopy	1.2	Sargent et al, 1998
10	France	Microscopy	0	Chermett et al, 1989
300	Germany	Microscopy	1.3	Agustin-Bichl, 1984
13	Japan	Microscopy	38.5	Iseki, 1979
507	Japan	Microscopy	20	Uga et al, 1989
608	Japan	Microscopy	3.8	Arai et al, 1990
57	Scotland	Microscopy	12.3	Nash et al, 1995
235	Scotland	Microscopy	8.1	Mtambo et al, 1991
16	Egypt	Microscopy	6.2	El-Hohary et al, 1998

Modified from Lyndsay DS, Zajac AM. *Cryptosporidium* infections in cats and dogs. Comp Cont Ed Pract Vet 2004; 864-874

1.10 CANINE CRYPTOSPORIDIOSIS

Cryptosporidiosis in dogs is very similar to that in cats. As well as cats, dogs have a unique genotype: *C. canis* based on molecular analysis of their oocysts (Fayer, 2001). Cryptosporidiosis in dogs has been described in prevalence studies and in case reports (table 1.8).

As well as in other species, *Cryptosporidium* is more common in young animals. *Cryptosporidium* have been documented in pups with and without clinical findings of cryptosporidiosis (Wilson, 1983; Sisk et al, 1984). *C. canis* was isolated from a pup with gastric and intestinal cryptosporidiosis (Miller et al, 2003). Several case reports including severe diarrhea associated with co-infections of parvovirus, distemper or parasitism in pups have been reported (Turnwald et al, 1988; Fukushima and Helman, 1984; Willard and Bouley, 1999; Denholm et al, 2001, Aydin et al, 2004). Intestinal malabsorption was reported in an adult dog with cryptosporidiosis (Greene et al, 1990). In a recent study of *C. parvum* isolates, originally obtained from a patient and a pet dog, were found to have cattle and dog genotypes, respectively (Abe et al, 2002b).

The prevalence data for cryptosporidiosis in dogs and cats is highly variable, the studies mentioned previously had different study populations, different diagnostic techniques and most of them used assays that are not sensitive enough for the detection of feline and canine cryptosporidiosis. In addition, molecular analysis of the *Cryptosporidium* spp. in most of the studies mentioned previously was not performed and the prevalence of the specific *C. canis* and *C. felis* is unknown.

Table 1.8. Prevalence of *Cryptosporidium* spp. in dogs

Number Examined	Location	Test	Prevalence (%)	Study
130	Colorado	Microscopy	3.8	Hackett et al, 2003
200	California	Microscopy	2	el-Ahraf et al, 1991
49	Georgia	Microscopy	10.2	Jafri et al, 1993
100	Kentucky	Microscopy	17	Juett et al, 1996
421	Australia	Microscopy/PCR	0	Bugg et al, 1999
493	Australia	Microscopy	11	Johnston et al, 1993
195	Australia	Microscopy	7.1	Milstein et al, 1995
25	Egypt	Microscopy	12	El-Hohary et al, 1998
458	Check Republic	Microscopy	4.6	Svobodovà et al, 1994
57	Finland	Microscopy	0	Pohjola, 1984
29	France	Microscopy	44.8	Chermett et al, 1989
200	Germany	Microscopy	0	Agustin-Bichl,, 1984
213	Japan	Microscopy	1.4	Uga et al, 1989
140	Japan	PCR	9.3	Abe et al, 2002
257	Korea	Microscopy	9.7	Kim et al, 1998
81	Spain	Microscopy	7.4	Causape et al, 1996
101	Scotland	Microscopy	0	Simpson et al, 1988
100	Scotland	Microscopy	1	Grimason et al, 1993
450	Brazil	Microscopy	8.8	Lallo et al, 2006
450	Brazil	PCR	9.5	Lallo et al, 2006
2193	Argentina	Microscopy	2.2	Fontanarrosa et al, 2006
240	Italy	PCR	3.3	Giangaspero et al, 2006
166	Brazil	Microscopy	2.4	Huber et al, 2005

Modified from Lyndsay DS, Zajac AM. *Cryptosporidium* infections in cats and dogs. Comp Cont Ed Pract Vet 2004; 864-874

1.11 DIAGNOSIS OF CRYPTOSPORIDIOSIS

The diagnosis of cryptosporidiosis was initially done by microscopic techniques. Such conventional techniques, however, lack sensitivity, are laborious and require well trained microscopists for accurate identification of pathogens. Moreover, since *Cryptosporidium* oocyst shedding is sporadic, several samples are required for diagnosis. Additionally, a recent article demonstrated that morphology is not a reliable tool for designating species within *Cryptosporidium* (Fall et al, 2003).

Oocyst concentration procedures

To increase the recovery rate of *Cryptosporidium* spp. oocysts, stool concentration methods including Sheather's sucrose flotation, zinc sulfate flotation, saturated sodium chlorine methods, discontinuous sucrose, isopropynic or discontinuous Percoll, or cesium chloride gradient centrifugation, can be performed before any staining technique. In a review article where eight different concentration procedures were tested, NaCl flotation methods resulted in the highest recovery rates of 17% from bovine feces (Petry, 2000). The threshold of detection of *Cryptosporidium* oocysts was reported to be 10^6 oocysts/ml of feces, using unconcentrated fecal smears (Weber et al, 1991). Since cats generally only shed 10^3 oocyst/gram, oocyst concentration procedures are insensitive for use with cat feces.

Oocyst staining procedures.

The techniques more frequently used are safranin-methylene blue stain, Kinyoun, Ziehl-Neelsen and DMSO-carbol fuchsin. Ziehl-Neelson acid-fast is the most common used staining technique for *Cryptosporidium.* The oocysts can be stained with carbol-fuchsin and they retained the dye in the decolorizing step with the acid alcohol (Petry, 2000). The acid fastness of the oocysts allows differentiation from fecal material that is visualized by counter-staining with malaquite green or methylene blue. *Cryptosporidium* oocysts appear as pink to bright red spheres of approximately 5 μm in diameter (Petry, 2000).

In addition to acid fast staining, other staining techniques including, negative stains, such as light–green or mercuramine, and fluorescent stains, eg, phenol auramine

can be used. The advantage of these techniques is that they allow a fast screening of the specimen but they require a fluorescence microscope (Petry et al, 2000).

A study reported that the use of formalin-ethyl-acetate as a concentration method, prior to Ziehl-Neelsen stain, increases the threshold of detection to 10^5 oocysts per gram of watery stool in human samples (Weber et al, 1991). In acute symptomatic human patients, it is easy to detect oocysts in unconcentrated stools, but lower numbers in the stools of asymptomatic carriers may be missed using conventional techniques.

Monoclonal antibody based immunofluorescence staining of oocysts

Immunofluorescence detection procedures had been reported to be more sensitive and specific than acid fast staining methods. While the average detection limit using acid fast technique is 5 X 10^5 oocysts/gram of feces, the threshold of detection using IFA is 5 X 10^4 oocysts/gram of feces (Weber et al, 1991). Additionally, IFAs are easier to read for inexperienced laboratory personnel and screening of IFAs requires less time than other techniques (Weber et al, 1991). The main disadvantage of the technique is that IFA has a higher cost. Although overall a high sensitivity was reported in some studies, other authors have found no difference between IFA and acid-fast stain in stool samples from asymptomatic carriers (Petry, 2000). A recent study, reported that IFA had a lower sensitivity than modified Ziehl Neelsen technique when a single cat fecal sample was tested (Marks et al, 2004). The sensitivity of IFA equals the one of the MZN when 2 to 4 feline samples are tested (Marks et al, 2004). The same study found that oocyst fluorescence intensity varied from an intense apple-green to a weak green fluorescence. The variation in oocyst fluorescence maybe due to antigenic diversity within *Cryptosporidium* spp. or to infection with more than 1 *Cryptosporidium* spp. (Marks et al,

2004). When the samples were tested with a polyclonal antibody a higher sensitivity was achieved, which supports the previous hypothesis.

These assays had not been validated with *C. felis* or *C. canis,* therefore, when used for detection of *Cryptosporidium* spp. in feline and canine samples, false negative results may occur. The sensitivity of different IFAs ranges between 94% and 100% compared to acid fast and ELISA. Specificity rates of the IFA s were 100 % compared to acid fast stain and ELISA (Petry et al, 2000).

Detection of serum antibodies.

Humoral responses to cryptosporidial infections were first assayed by indirect immunofluorescence against infected histological tissue sections (Fayer et al, 1997). Microtiter enzyme immunoassays for the detection of antibody responses to crude cryptosporidial antigens have been developed in several laboratories employing antigens prepared from sonicated oocysts, whole frozen oocysts, zirconium bead disrupted and freeze-thawed oocysts, and oocysts prepared in an undefined manner (Fayer et al, 1997). Cross reactivity to cryptosporidial antigens by sera from individuals infected with other parasites is a potential drawback to all serologic assays. Detection of *Cryptosporidium* antibodies in serum has been used epidemiologically in the study of cryptosporidiosis in humans and other animals, including cats (Groves et al, 1994; Tzipori and Campbell, 1981; Svobodova et al, 1994; Lappin et al, 1997b). Serological evidence of *Cryptosporidium* spp. infection was detected in 50 of 600 (8.3%), 20 of 23 (87%) and 77 of 135 (57%) of the cats from the United States, Scotland and the Czech Republic respectively (McReynolds et al, 1999; Tzipori and Campbell, 1981; Svobodovà et al, 1994). In experimentally infected cats, *C. parvum* specific IgG was detected 4 weeks

after infection (McReynolds, 2001;Unpublished data. Master's Defense, Colorado State University). An ELISA for detection of feline *C. parvum* specific IgG was previously developed in our laboratory and was tested in naturally and experimentally infected cats (Lappin et al, 1997b). When the results of this ELISA were compared with the presence of oocysts in feces by zinc sulfate flotation and acid fast, the sensitivity, specificity, positive predictive value and negative predictive value were: 50%, 85.5%, 7.7% and 98.6%, respectively (Lappin et al, 1997b). In the same study, 26 of 170 cats (15.3%) were seropositive for *C. parvum* IgG but only 4 of 170 cats (2%) had oocysts in feces (Lappin et al, 1997b). Many cats with acute infection and diarrhea are likely to be seronegative, and then it is feasible that *Cryptosporidium* IgG titers will persist for months or years (McReynolds, 2001;Unpublished data. Master's Defense, Colorado State University). Thus, the presence of *C. parvum* specific IgG in cat serum suggests prior infection and did not correlate highly with oocyst shedding. However, the fecal tests used were very insensitive compared to PCR assay (Scorza et al, 2003). *Cryptosporidium* spp. infections often persist at low levels for years in some animals and so it is possible the antibody test results did not correlate to presence of oocysts because of false negative test results in the fecal assays. In dogs antibodies to *Cryptosporidium* were detected by indirect immunofluorescence in 16 of 20 (80%) samples (Tzipori and Campbell, 1981). However, to our knowledge, the use of *Cryptosporidium*–specific IgG ELISA in dogs was not reported. Overall, minimal data is available from dogs and cats to assess predictive values of antibody testing. Even though the presence of *Cryptosporidium* specific antibodies may not prove to not have diagnostic value in a

single individual, the serological test results will be valuable as an epidemiological marker of exposure in community–based prevalence studies (Farthing, 2000).

Fecal antibody detection

C. parvum induce mucosal infection, therefore IgA in feces would be expected to be the predominant antibody class stimulated. It was demonstrated that immunological tests on saline extracts of feces do not represent the true status of the gut humoral immune system (Hills et al, 1990; Petry, 2000). Nevertheless, several ELISAs for detection of *C. parvum* fecal antibodies have been reported.

Assays have been developed to evaluate if the presence of *C. parvum* antibodies in feces correlates better than serum antibodies with the presence of oocysts in feces (Peeters et al, 1992). Fecal and serum anti-*Cryptosporidium parvum* IgA, IgM, and IgG were monitored by an enzyme-linked immunosorbent assay after experimental and natural infection of calves with *C. parvum*. The analysis of fecal extracts established that IgA and IgM are the major immunoglobulins with activity against endogenous stages of *C. parvum* in the gut. The secretion of maximal IgA levels occurred during the same period of time in which oocysts levels in feces had began to drop noticeably. Therefore, a good temporal association was indicated between *C. parvum* expulsion and the amount of specific IgA in secretions (Peeters et al, 1992).

Fecal antigen detection by ELISA

Another alternative to increase the sensitivity of microscopic techniques in the diagnosis of cryptosporidiosis is the use of enzyme-linked immunosorbent assay (ELISA) for detection of free antigen in feces. These assays are specifically useful in

epidemiological studies in need of standardization, in laboratories without a fluorescence microscope, and in situations when processing batch specimens may be crucial.

A review article evaluated the sensitivity of several commercial kits using IFA as a reference test (Petry et al, 2000). The sensitivity of five of the kits was comparable to the sensitivity of IFA (range 94.5% -100%). Only two tests performed poorly, with sensitivities ranging from 29% (for one of the assays) and 66.3 to 93% (for the second assay). A recent article compared the sensitivity of three fecal ELISAs and IFA using MZN as gold standard in feline samples (Marks et al, 2004). One of the tests: ELISA 1, achieved the higher sensitivity (89%) on day 1, followed by another kit: ELISA 2 (80%) and the last kit: ELISA 3 performed poorly (15%). When samples of two consecutive days were examined, MZN, ELISA1 and ELISA 2 achieved a similar sensitivity (approximately 90%) (Marks et al, 2004). Another study also compared the sensitivity of a fecal ELISA kit and carbol fuchsin stain in the detection of *Cryptosporidium* in canine and feline samples (Cirak and Bauer, 2004). Twenty-six of 270 dog samples (9.5%) tested positive for *Cryptosporidium* by microscopy and 8 of 270 (2.95%) tested positive by the fecal ELISA (Cirak and Bauer, 2004). In the same study, none of the 100 cats tested positive for *Cryptosporidium* by microscopy, but 22 of 100 (22.4%) tested positive by the fecal ELISA. In general, ELISAs are likely to have lower specificity compared to IFA (90.3-100%) (Petry et al, 2000). Our laboratory, recently evaluated 2 lateral flow devices for the detection of *C. parvum* in human feces and found them to be inadequate for detection of *Cryptosporidium* spp. in feline feces (Bachman and Lappin, unpublished data, 2007). This may relate to antigenic differences between *C. felis* and *C. parvum*; currently

available point of care kits use monoclonal or polyclonal antibodies directed against *C. parvum*.

In microscopic techniques, there is a correlation between signal and morphology of the reactive oocyst. Such correlation is missing in ELISA and constitutes a major disadvantage. The read-out of the ELISA has to be taken for granted and false-positive reactions are impossible to differentiate from true positives.

Agglutination assays for detection of antigen in feces

An assay for detecting soluble antigen uses coagglutination of sensitized latex particles and protein A-rich *Staphylococcus aureus* cells sensitized with anti-*C. parvum* antiserum (Petry et al, 2000). The authors report this assay to be very sensitive, detecting antigen from a single oocyst per milliliter feces but the disadvantage of the assay lies in the time required to extract the antigen.

A reverse passive hemagglutination assay employs sheep erythrocytes sensitized with a monoclonal anti-*C. parvum* IgM antibody as an indicator system. This assay reported sensitivity similar to most ELISAs (Petry et al, 2000).

DNA –Based Techniques

A variety of molecular techniques have been developed for the detection and characterization of *Cryptosporidium*. Polymerase chain reaction (PCR) is extremely sensitive, easy to perform, can be used to analyze a large number of samples at one time, and can be adapted to speciate and strain type. Ideally, PCR amplification of a specific target DNA offers high sensitivity, specificity, reproducibility and ease of interpretation of the results. Since the first PCR for the detection of *Cryptosporidium* in fecal samples (Laxer et al, 1991) was developed, numerous PCR protocols have been described for the

detection of cryptosporidiosis in clinical and environmental samples. These initial assays were developed for the detection of *C. parvum* and did not differentiate among *Cryptosporidium* spp. It is unclear if these techniques cross react with other *Cryptosporidium* spp. or if they amplified other microorganisms. It was reported that the first technique, the Laxer PCR, also amplified DNA from *Eimeria* and *Giardia* (Xiao et al, 2003).

PCR has been reported to have increased sensitivity in comparison to microscopical and immunological-based techniques in clinical samples (daSilva et al, 1999; Webster et al, 1996). Some authors have reported threshold as low as a single oocyst in stool samples (Rochelle et al, 1997; Morgan et al, 1996; Webster et al, 1996). PCR has been used to detect *Cryptosporidium spp.* DNA in feline feces, and it was reported to be more sensitive than IFA (Sargent et al, 1998; Scorza et al, 2003). Although PCR is rapid, highly sensitive and accurate, it has several limitations. First, false positives can result from detection of naked nucleic acid non-viable organisms, and laboratory contamination. False negative results could be due to several factors including unequal distribution of oocysts through the sample, failure to adequately disrupt the oocysts and liberate DNA, absorption of DNA to stool constituents, degradation of DNA, and presence of PCR inhibitors, like bilirubin, humic acids and bile constituents.

Species differentiation

A limited number of PCR tools are available for the differentiation of *Cryptosporidium* species (table 1.9). Only a few genes, including small subunit (SSU) rRNA (or 18S rRNA), heat shock protein (HSP-70), oocyst wall protein (COWP) and actin genes for the most prevalent *Cryptosporidium* spp., had been characterized (Xiao et

al, 2003). Post- PCR analyses are based on direct sequencing of the amplicon or digestion with endonucleases followed by gel electrophoresis of the restriction fragments. SSU rRNA is the most common loci used for detection and differentiation of *Cryptosporidium* spp. The SSU rRNA is part of a gene complex in which each unit is composed by polymorphic regions interspread between conserved regions. The utility of this gene for molecular and phylogenetic analysis derives from the fact that different regions within the unit have different rates of evolution (Groth and Wetheral, 2000). For species identification, the analysis of highly or moderately conserved regions including 18S rRNAs and other structural and housekeeping genes is required (Cacciò et al, 2005). The most studied rRNA is the small subunit because it has the slowest rate of evolution (Hillis and Dixon, 1991). By designing PCR primers to be located in conserved regions that comprise polymorphic areas, we will be able to differentiate among *Cryptosporidium* spp. by sequencing analysis or by another differentiation technique. Nevertheless, care should be taken in selecting the primers because sequences conserved among several *Cryptosporidium*, could also be conserved among other eukaryotic organisms (Xiao et al, 2003).

The heat shock protein (HSP) gene belongs to a multigene family that is highly conserved in eukaryotes and prokaryotes. These proteins facilitate the folding of proteins in secretion and transport, and they are also involved in protection from host cell defenses and virulence (Khramtsov et al, 1995; Sulaiman et al, 2000). Under stress conditions, their expression is upregulated. HSP-70 of a *Cryptosporidium* bovine isolate was cloned and sequenced in 1991 (Khramtsov et al, 1995) and in the year 2000, sequence and phylogenetic analysis of various human and animal *Cryptosporidium* was performed.

HSP-70 demonstrated that it has higher heterogeneity than the SSU rRNA gene, and so it is a better target for genotyping. 18S rRNA restricts nucleotide changes to certain areas of the gene, whereas mutations in the HSP-70 are dispersed over the sequence. Therefore, phylogenetic analysis of the HSP-70 is more robust than the analysis of the 18S rRNA gene resulting in higher bootstrap values (Sulaiman et al, 2000).

Genotyping tools

The first PCR tool that differentiates the human and the bovine genotypes of *C. parvum* was developed in 1995 (Morgan et al, 1995). After that, many genotyping tools including PCR, random amplified polymorphic DNA PCR (RAPD-PCR), arbitrary primed PCR (AP-PCR), reverse transcription PCR (RT-PCR), followed by RFLP analysis, single-strand conformation polymorphism (SSCP) analysis, ELISA or DNA sequencing have been developed (Carraway et al, 1997; Xiao et al, 1999a; Sulaiman et al, 1998). Since these techniques can differentiate among the human and the bovine genotype of *C. parvum*, they help to understand the transmission of cryptosporidiosis in humans.

The published techniques that are able to identify species and genotypes are based on the rRNA or ITS, using PCR-RFLP (Morgan et al, 1999; Xiao et al, 1999b) or DNA sequencing (Morgan et al, 1998).

Subgenotyping tools

Subgenotyping is very useful in epidemiological settings; it allows the identification of clusters of cases, enabling a more accurate tracking of the infection/contamination source during an outbreak. The identification of subtypes requires highly discriminatory fingerprinting techniques which can identify individual

isolates or clonal or clonal lineages (Cacciò et al, 2005). The subgenotyping tools available are: DNA sequence analysis of microsatellites (Aiello et al, 1999; Cacciò et al, 2000), HSP-70 gene (Sulaiman et al, 2000), GP60 gene (Peng et al, 2001, Sulaiman et al, 2001) and a double stranded (ds) RNA (Xiao et al, 2001b) (table 1.9). Microsatellites and minisatellites are sequences that consist of tandem repeats of a simple motif of a variable length (10-100 bp for microsatellites and 1-5 bp for minisatellites). Microsatellites have long polymorphisms that occurred as a consequence of gaining or losing repeat units during DNA replication (Cacciò et al, 2005). Therefore, alleles of different sizes can be observed at the microsatellites loci allowing us the identification of genetic fingerprints (Cacciò et al, 2005). GP60-based assays supposed to be the ones with the higher resolution (Xiao et al, 2003). Results of sequencing analysis of this gene separate the *C. parvum* human and bovine genotype into at least eight allele families, each of which has various subgenotypes (Xiao et al, 2003).

Molecular tools for the analysis of environmental samples

The characterization of *Cryptosporidium* from environmental samples contributes to the understanding of the flow of the parasite in the environment. *Cryptosporidium* had been successfully isolated from seeded water samples (Xiao et al, 2003). PCR had been used in the detection of *C. parvum* oocysts seeded in milk or juice, with variable sensitivities (Xiao et al, 2003) and poor performance of PCR has been reported in the detection of *C. parvum* in soil samples (Xiao et al, 2003).

Oocysts infectivity and assessment of viability

The estimation of the viability of oocyst infectivity is important in assessing the survival in the environment, the success of the treatment processes and ultimately in quantifying

the public health risk. The following are the methods that can be used to test oocysts viability/infectivity.

Animal infectivity:

Animal models are the gold standard for the assessment of infectivity but animals may not represent the true risk of infection for humans (Duffy and Moriarty, 2003).

Cell lines: Approximately 20 cell lines showed to support *C. parvum* infection (Duffy and Moriarty, 2003). Recently, a study demonstrated a good correlation between infection in human ileocecal adenocarcinoma (HCT-8) cell lines and mouse infectivity. Therefore, cell lines can be an alternative to mouse infectivity (Duffy and Moriarty, 2003).

Exclusion/inclusion of vital fluorogenic dyes:

The most commonly used dyes are 4', 6'-diamidino-2-phenylindole and propidium idodide (DAPI/PI). This is a very fast method (<3 hours) and has the benefit of providing information on the relative percentages of viable and non-viable oocysts. However, it can overestimate oocysts viability, which will lead to overestimation of risk (Duffy and Moriarty, 2003).

Table 1.9: Targets, assays and main use of amplification-based techniques for *Cryptosporidium*

Target	Assay type	Identification
18S rDNA	PCR, nested PCR, sequencing, PCR-RFLP, Real-Time PCR, microarray	Species and genotype
Hsp 70	PCR, nested PCR, sequencing, Real-Time PCR, microarray	Species and genotype
COWP	PCR, nested PCR, sequencing, PCR-RFLP, microarray	Species and genotype
Actin	PCR, nested PCR, sequencing,	Species and genotype
B-Tubulin	PCR, nested PCR, sequencing, PCR-RFLP,	Species and genotype
GP60	PCR, nested PCR, sequencing,	Subgenotype
Microsatellites	PCR, nested PCR, sequencing, fragment typing	Subgenotype
Minisatellites	PCR, nested PCR, sequencing, fragment typing	Subgenotype
Extrachromosomal Double-stranded DNA	Reverse transcriptase, PCR, sequencing, heteroduplex mobility assays	Subgenotype

Modified from: Cacciò SM, Thompson RCA, McLaughlin J, et al. Unraveling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol 2005;21(9):430-437.

1.12 TREATMENT OF CRYPTOSPORIDIOSIS IN HUMANS

The treatment of cryptosporidiosis falls into three categories: antimicrobial therapy, symptomatic anti-diarrhecal treatment and immunotherapy.

Antimicrobial therapy

More than 100 compounds have been evaluated in laboratory animal models, but no treatment has successfully eliminated clinical and parasitological responses. In general, most of the agents studied received mixed success, many of the experiments include a small number of cases and when eradication of the parasite was achieved, it has frequently been difficult to reproduce (Smith and Corcoran, 2004). The resistance of *C. parvum* to chemotherapeutic agents may be associated with its unique location which is intracellular but extracytoplasmic. Recent genetic studies discovered differences between *Cryptosporidium* and other apicomplexans such as, the absence of an apicoplast and the presence of a mitochondrion which may account for many of the treatment failures (Striepen and Kissinger, 2004).

Spiramycin was considered to produce encouraging results in pilot studies, but consistent efficacy against cryptosporidiosis in HIV patients has not been confirmed in clinical trials (Smith and Corcoran, 2004). *Azithromycin,* has also been evaluated in animal models of infection and in human cryptosporidiosis with some encouraging preliminary results (Farthing, 2000). Azithromycin achieves high biliary concentrations, which might be helpful in eradicating biliary infections in HIV+ patients. However, when administered to 13 HIV+ patients, the majority continued to shed oocysts (Smith and Corcoran, 2004). Another study, reported good clinical and parasitologic responses in 13 children after three weeks of treatment (Allam and Shehab, 2002). However, other

studies have not reported a significant therapeutic response to azithromycin (Blanshard et al, 1997; Smith and Corcoran, 2004) An additional macrolide, *clarithromycin*, achieves good intracellular concentrations and has demonstrated to be a good prophylactic agent against cryptosporidiosis (Jordan, 1996). However, when clarithromycin and rifabutin were administered, it was rifabutin that was responsible for decreasing the relative risk of cryptosporidiosis (Fishenbaum et al, 2000).

Paromomycin is an aminoglycoside antibiotic that is poorly absorbed from the gastrointestinal tract. The doses used are generally 500 mg/kg four times a day, and sometimes it is recommended a maintenance therapy with 500 mg/kg be administered twice daily (Hewitt et al, 2000). The efficacy of the drug in AIDS patients with cryptosporidiosis is controversial. The limitations on clinical trials with AIDS patients are usually the small sample size of the studies and the presence of confounding factors, like the coinfection of the patients with other opportunistic infections. There have been some instances in which infection appears to have been eradicated, but in others, symptoms disappear but may re-occur during therapy. A possible explanation of the recurrence, may be that paromomycin does not penetrate biliary ducts (Hewitt et al, 2000). However, in clinical practice, paromomycin is commonly used to treat cryptosporidiosis in HIV-positive patients despite a paucity of data from randomized clinical trials (Hewitt et al, 2000). *Albendazole* was administered to HIV-patients with diarrhea of undifferentiated etiological agent. Although the number of patients with cryptosporidiosis was small, it appears that this drug was active in this group of patients (Farthling, 2000).

In recent years the most promising agent used for the treatment of cryptosporidiosis is *nitazoxanide*, a salicylanide derivative of nitrothiazole, which is effective against a broad spectrum of parasites and bacteria (Smith and Corcoran, 2004). Nitazoxanide was used to treat several gastrointestinal pathogens in Central and South America and it seems to be well tolerated, has a relative low incidence of adverse effects, and has no significant drug-to-drug interaction (Bobak, 2006). To date, nitazoxanide is the only drug approved by the US Food and Drug Administration (FDA) for the treatment of diarrhea caused by *Cryptosporidium* in children and in adults (Hemphill et al, 2006). The antiprotozoal mechanism of action is by interfering with the pyruvate ferredoxin oxidoreductase (PFOR)-mediated electron transfer reaction that is essential for anaerobic energy metabolism of the parasite (Gilles and Hoffman, 2002). It was demonstrated that the DNA derived PFOR protein sequence of *C. parvum* is similar to the one of *G. duodenalis* (Cohen, 2005). However, there is increasing evidence that other mechanisms could account for the activity of nitazoxanide (Hemphill et al, 2006). It is also possible that in *Giardia* and other protists, thiazolides also interfere in intracellular signaling pathways through their salicylic acid moiety (Hemphill et al, 2006). The interference in signaling pathways often leads to apoptosis (Hemphill et al, 2006). In the course of 31 clinical studies, 2,983 children were exposed to the drug and the studies demonstrated high efficacy rates (> 80%) (Cohen, 2005). Initially, a study in Mali indicated that nitazoxanide was effective in AIDS-related cryptosporidiosis and this observation was later was confirmed in a double-blind randomized placebo-controlled trial in Mexico (Duombo et al, 1997; Rossignol et al, 1998). More recent studies demonstrated clinical and parasitologic response rates of 80% and 70% respectively in immunocompetent

children, with lower response rates in immunocompromised individuals (Smith and Corcoran, 2004). In patients with HIV-related disease, success was variable depending on the degree of immunosuppression and the duration of the treatment (Amadi et al, 2002; Bailey and Erramouspe, 2004). The administration of 500 mg of nitazoxanide daily for three days was effective in treating cryptosporidial diarrhea in Egyptian immunocompetent children (Rossignol et al, 2006). Nitazoxanide also showed to be efficient in the treatment of biliary cryptosporidiosis in gerbils, so its use in humans with biliary cryptosporidiosis could be evaluated in the future (Hemphill et al, 2006). The most commonly report side effects in 613 treated children were abdominal pain (8%), diarrhea (2%), vomiting (1%) and headache (1%) (Cohen, 2005). However, these side effects were mild and transitory (Cohen, 2005). Furthermore, the broad spectrum of nitazoxanide against pathogens that cause persistent diarrhea and its safety profile, makes this drug suitable for the empiric therapy of diarrhea (Cohen, 2005).

Another modality of treatment is the use of a *combination of antimicrobials*. The administration of azithromycin and paromomycin was associated with significant reduction in oocyst excretion and some clinical improvement (Smith et al, 1998). In another study, the administration of paromomycin, azithromycin and nitazoxanide to an AIDS patient produced only partial benefit, yielding only slight improvement in clinical symptoms (Giacometti et al, 1999). A recent study, evaluated the efficacy of nitazoxanide (200 mg/kg/day) and paromomycin (100 mg/kg/day) for the treatment of biliary tract cryptosporidiosis in immunosuppressed gerbils (Baishanbo et al, 2006). The results of this study showed that oocyst shedding was partially suppressed in the treated

group and gall bladder infection was less frequent in nitazoxanide-treated gerbils than in controls (Baishanbo et al, 2006).

Mangiferin, a pure plant-derived principle inhibit in vitro growth of *C. parvum* (Tarantino et al, 2004). A recent study used mangiferin at two dosages (50mg/kg/day during 10 days and 100 mg/kg/day during 10 days) and compared its anti-*Cryptosporidium* effect with the one of paromomycin (100mg/kg/die during 10 days) in neonatal mice (Perrucci et al, 2006). Results of this study showed that although both paromomycin and mangiferin at 100mg/kg/day had anti-cryptosporidial activity, none of them were able to inhibit intestinal colonization of *C. parvum* in neonatal mice (Perrucci et al, 2006).

Isoflavone derivatives have antiparasitic activities in vitro (Stachulski et al, 2006). A series of isoflavone derivatives related to genistein showed excellent in vitro activity against *C. parvum* and also demonstrated to have high efficacy against *C. parvum* experimentally infected gerbils (Stachulski et al, 2006).

Anti-diarrhocal drugs

Supportive therapy includes fluid and electrolyte replacement, nutritional support and anti-diarrheal agents.

Immunotherapy

Passive immunotherapy

The use of bovine colostrums, colostral antibodies, monoclonal or polyclonal antibodies and lectins specific for certain glycoproteins on the surface of sporozoites, merozoites and oocysts, has been shown to reduce the severity of *Cryptosporidium* infection (Crabb, 1998; Griffiths, 1998). The administration of hyperimmune bovine

colostrums (HBC) improved symptoms and reduced oocysts shedding in experimentally infected calves (Riggs et al, 2002). Colostrom from cow's hyperimmunized with *C. parvum* oocysts achieved limited success in humans and non-human hosts (Smith and Corboban, 2004). There is an increasing focus on immunotherapy targeting apical complex and surface antigens involved in zoite motility (P23, CPS-500, GP15 and TRAP-C1) and invasion (GP900, CP47, G60/45/25, SCL and TRAP-C1) (Carey et al, 2004). A monoclonal antibody responsible for the CSL reaction, reduced but not eliminate, persistent infection in mice (Riggs et al, 2002). A study demonstrated that recombinant bovine herpesvirus-1 (BHV-1) expressing the P23 protein of *C. parvum* was able to neutralize antibodies in rabbits (Takashima et al, 2003). Some studies showed that polyclonal antibodies against whole *C. parvum* oocysts may provide passive immunity in mice (Arrowood et al, 1989; Carey et al, 2004).

Lectins are carbohydrate-binding plant proteins that mediate interactions between host and parasite. The identification of a sporozoite–specific lectin adherence factor as the cause of sporozoite attachment to the intestinal surface suggested that some of these glycoproteins may be a useful target for immunotherapy (Carey et al, 2004).

Probiotics

One study evaluated the effect of *Lactobacillus reuteri* and *Lactobacillus acidophilus* on the shedding of *C. parvum* oocysts in immunosuppressed mice (Alak et al, 1999). Results of the study showed that *L. acidophilus* was more effective than *L. reuteri* in reducing oocyst shedding. In a similar study, *L. reuteri* was found to effective in decreasing oocyst shedding in immunosuppressed mice (Alak et al, 1997). In a recent article, daily administration of *L. casei*-containing mixtures was showed to produce a

more rapid clearance of parasites in treated rats than in control rats (Guitard et al, 2006). However, the administration of this probiotic was unable to eradicate *Cryptosporidium* infection in rats (Guitard et al, 2006).

Immune reconstitution

The use of a protease inhibitor combined with one or two nucleoside analogues produces a marked reduction in viral load, an increased of CD4 T lymphocyte counts and a delay in the onset of HIV-related opportunistic infections. Several studies have confirmed that highly active antiretroviral therapy (HAART) improves HIV-related diarrhea, which is related to cryptosporidiosis and microsporidiosis and that there has been a decline in prevalence in intestinal cryptosporidiosis in HIV-infected patients following the wide spread use of protease inhibitors (Farthing, 2000). A recent study showed that protease inhibitors used in HAART reduce *C. parvum* sporozoite host cell invasion and parasite development in vitro, and inhibition is enhanced in combination with paromomycin. The anti-*Cryptosporidium* activity in HAART seems to be due to the aspartyl protease inhibitors of the human immunodeficiency virus included in HAART, which directly interfere with the life cycle of the parasite (Zardi et al, 2005). Additionally, protease inhibitors showed an indirect effect in the production of IL2 and IFN-γ, the second is considered the major inhibitor of *C. parvum* infection (Zardi et al, 2005). Unfortunately, HAART therapy is not easily accessible for HIV+ individuals in the developing world.

Further studies of the *Cryptosporidium* genome will contribute to the discovery of new genes and biochemical pathways that can be targeted in new treatments for cryptosporidiosis.

1.13 TREATMENT OF CRYPTOSPORIDIOSIS IN CATS AND DOGS

There are only a few studies about treatment for cryptosporidiosis in cats and dogs.

Clindamycin hydrochloride, at a dose of 25-mg/kg PO daily, was administered to a cat with chronic cryptosporidiosis and lymphocytic duodenitis (Lappin et al, 1997a). After 60 days of therapy there were no further improvement in stool consistency and oocysts were still detected. Therefore, clindamycin was discontinued on day 60 and tylosin was administered at 11mg/kg PO twice daily for 28 days. Stools were normal within 7 days of initiating therapy and the fecal samples assessed for oocysts during the treatment with tylosin were negative. Results from eight daily fecal examinations were negative for oocysts 6 months after completion of tylosin therapy. The inflammatory changes in the bowel resolved after treatment suggesting inflammation was from *C. parvum* infection. The cat remained normal for two years after treatment. We have treated many cats with presumed cryptosporidiosis with tylosin at a dosage of 10 to 15 mg/kg given orally twice daily 21 days, and diarrhea has resolved in about 50% of cases (Lappin MR, Scorza AV, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado: Unpublished data 2007). However, these observations are uncontrolled, and the signs in the affected cats may have resolved spontaneously. It is also possible the anti-inflammatory or antibacterial effects of tylosin played a role in clinical responses. In addition, tylosin is not tolerated by most cats because of its unpleasant taste.

Azithromycin, has also been evaluated in animal models of infection and in human cryptosporidiosis with some encouraging preliminary results (Farthing, 2000). In a

recent study, the administration of azithromycin to dairy cows infected with *Cryptosporidium*, significantly reduced oocyst shedding and improved clinical signs of diarrhea (Elitok et al, 2005). We currently recommend azithromycin at a dosage of 10 mg/kg given orally daily in cats that are intolerant or non-responsive to tylosin (Scorza and Lappin, 2006). The optimal duration of therapy is unknown but is usually several weeks. Other than potential for mild gastrointestinal side-effects, the drug appears very safe for use in cats.

Paromomycin was effective in clearing *Cryptosporidium* oocysts from the feces of a naturally infected cat with persistent diarrhea and in a group of experimentally inoculated cats (Barr et al, 1994; McReynolds et al, Unpublished data). However, the diagnostic tests used in the follow up period in both cases were not sensitive enough to detect animals in a carrier state. Care should be taken when this drugs is used in cats with bloody diarrhea. In one study of cats with *Tritrichomonas* infection treated with paromomycin, 4 out of 32 cats developed acute renal failure and 3 of the 4 cats became deaf (Gookin et al, 1999).

We have recently studied groups of presumably immunocompetent kittens and adult cats that were infected with both *Giardia* and *Cryptosporidium* species (Scorza AV, Lappin MR, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colo: Unpublished data, 2007). Cats with coinfections seem to be more difficult to treat than cats infected with either organism alone. Recently, we used paromomycin at a dosage of 150 mg/kg given orally daily to control diarrhea in two cats with resistant *Giardia* species and *C. felis* infection that had not responded to treatment with other drugs. However, treatment was

needed for more than 21 days to achieve maximal clinical response and stop fecal shedding. Paromomycin should be considered a rescue drug (to be used only in resistant infections) and should never be used in cats with bloody diarrhea, due to the risk of systemic absorption and induction of renal disease or deafness.

We are currently evaluating *nitazoxanide* to treat a number of small-animal parasites. Some of the dogs and cats with *Cryptosporidium* or *Giardia* species infections have had diarrhea resolve after the administration of nitazoxanide at a dosage of 25 mg/kg given orally every 12 hours for at least five days but it is too early to suggest an efficacy for this treatment (Scorza and Lappin, 2007).

Table 1.10. Drug Therapy for *Cryptosporidium* Infections in Cats and Dogs

Drug	Species	Dose (mg/kg)	Route	Interval (hr)	Duration (days)
Paromomycin	Dogs and Cats	125-165	PO	12	5
Azithromycin	Dogs	5-10	PO	12	5-7
	Cats	7-15	PO	12	5-7
Tylosin	Cats and Dogs	10-15	PO	8-12	21

Modified from: Barr SC. Giardiasis. In Infectious Diseases of the dog and cat, edition 3, 2005. Elsevier. Pag 736-742.,

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CHAPTER 2: INTRODUCTION AND LITERATURE REVIEW

GIARDIA SPP.

2.1 TAXONOMY

Giardia belongs to the family Hexamitidae, order Diplomonadida, Class Trepomonadea, Phylum Metamonada

Historical perspective

Antoine van Leeuwenhoek first observed *Giardia* in 1681, however, the first detailed description of the parasite was not provided until 1859 by Lambl (Thompson and Monis, 2004). The name *Giardia* was assigned by Kunstler for a flagellate found in the intestine of tadpoles of anuran amphibians. In 1888, Blanchard suggested that *Giardia* should be the generic name in commemoration of the parasite's first accurate description by Lambl (Thompson and Monis, 2004). In 1952, Filice classified *Giardia* species according to morphology and rejected the validity of host specificity as a criterion for taxonomy. Based on the shape of the characteristic internal microtubular structures known as median bodies and the overall shape of the trophozoites; Filice argued that *Giardia* could be divided into three morphologically different groups: *G. doudenalis*, *G. muris* and *G. agilis,* two of which can infect mammals (table 2.1).

Table 2.1
Recognized species in the genus *Giardia*

Species	Hosts	Morphological characteristics	Trophozoites dimensions length/width (μm)
G. duodenalis	Domestic and wild animals (humans)	Pear-shaped trophozoites	12-15/6-8
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies	20-30/4-5
G. muris	Rodents	Rounded trophozoites with small round median bodies	9-12/5-7
G. ardae	Birds	Rounded trophozoites with prominent notch in ventral disc and rudimentary caudal flagellum	~10/~6.5
G. psittaci	Birds	Pear-shaped trophozoites, with no ventro-lateral flange. Claw shaped median bodies	~14/~6

Thompson, RCA. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Vet Parasitol 2004;124:15-35.

Based on epidemiological observations and on the results of cross transmission studies, Filice concluded that within the three groups there were morphologically similar forms with different physiological characteristics. However, for an accurate taxonomy of all the different variants of the parasite, the scientific community would have to wait until a more discriminatory methodology was developed.

Genetic characterization

The subsequent development of axenic culture allowed the growth of large numbers of individual isolates for genetic characterization by alozyme electrophoresis. The results of these analyses produced highly valuable information about the genetic structure of *Giardia* and led to the finding of considerable levels of genetic diversity within the *G. duodenalis* group. However, the interpretation of the genetic information from a taxonomy perspective has been problematic. It was not possible to culture all the

Giardia isolates; for example, isolates from dogs are refractory to in vitro culture. In addition, much of the genetic information comes from a small pool of culture selected isolates and can not be consider representative of the genetic pool present in nature.

The application of PCR-based technology helps to overcome the limitations of in vitro culture and to obtain isolates from a variety of sources. PCR of the rDNA gene, glutamate dehydrogenase (GDH), elongation factor1-alpha (ef1-α) and triose isomerase (tpi) genes among other genes, help to clarify the genetic divisions among the *G. doudenalis* group (Hopkins et al, 1997; Monis et al, 1996; Monis et al, 1998; Monis et al, 1999) (table 2.2).

Table 2.2
Genotype and host range of isolates within the *Giardia doudenalis* morphological group

Genotype/Assemblage	Host range
Zoonotic/A	Humans, livestock, cats, dogs, beavers, guinea pigs, slow loris
Zoonotic/B	Humans, slow loris, chinchillas, dogs, beavers, rats, siamang
Dog/C, D	Dog
Livestock/E	Cattle, sheep, pigs
Cat/F	Cat
Rat/G	Domestic rats
Muskrats/Vole	Wild rodents

Thompson, RCA. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Vet Parasitol 2004;124:15-35.

The results of these experiments demonstrated that *G. duodenalis* is a species complex comprising a variety of genetically and phenotypically similar genotypes that exhibit differences in host specificity (Monis and Thompson, 2004; Thompson, 2004).

The *Giardia* species infecting humans are grouped in two major genotyping groups: Assemblage A and Assemblage B. There is plenty of genetic support indicating that these Assemblages should have separate species names, there is also genetic sub-

grouping within each Assemblage (Thompson, 2004). Assemblage A consists of two clusters AI and AII. The AI subgroup is a mixture of closely related human and animal isolates and most attention regarding the zoonotic potential has been focused on this subgroup. The AII subgroup consists entirely of human isolates. Assemblage B comprises two subgroups: BIII and BIV, of which the latter appears to be human specific (Thompson, 2004).

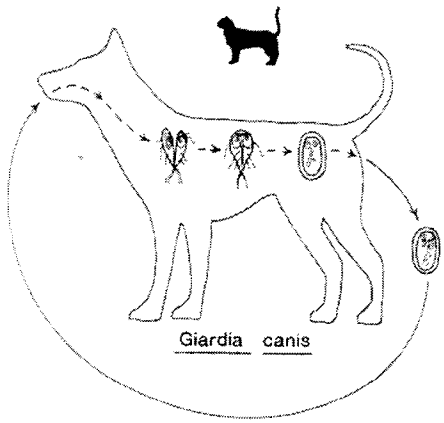
Alternatively, there are also host-specific groups confined to certain animal hosts, including dogs, cats, rats, voles/muskrats and hoofed animals (table 2.2). Therefore, maybe the dog genotype (Assemblage C/D) and the livestock genotype (Assemblage E) could be named *G. canis* and *G. bovis* as previously suggested (Thompson, 2002; Thompson, 2004).

2.2 LIFE CYCLE

Giardia occurs in two forms, the trophozoite and the cyst. The trophozoite is the active, motile form that is found in the intestinal tract. It is approximately 15 μm long and 8 μm wide and has a teardrop shape. The trophozoites can be identified under light microscopy by the smiling face appearance, which is formed by the two nuclei in the anterior third forming the eyes, the axonemes passing longitudinally between the nuclei, forming the nose, and the median bodies located transversely in the posterior third forming the mouth and four pair of flagella complete the shape (Barr and Bowman, 1994). The cyst is the resistant stage mainly responsible for transmission; it is approximately 12 μm long and 7 μm wide. The cyst contains two incompletely separated but form trophozoites, the axoneme, fragments of the ventral disks, and as many as four nuclei can be seen inside. The cyst is very resistant and can survive several months

outside the host in wet, cold conditions, but is susceptible to desiccation in dry and hot conditions.

The life cycle of *Giardia* is direct; a susceptible host became infected by ingestion of cysts, which excyst in the duodenum after exposure to gastric acid and pancreatic enzymes. The cyst contains two trophozoites that then separate, mature shortly after excystment and attach to the brush border of the villous epithelium. The distribution of *Giardia* in the intestinal tract varies according to the host and the diet. In dogs the organism was detected from the duodenum to the ileum and in cats trophozoites had been found through the intestinal tract. Trophozoites multiply by binary fission in the intestinal tract and then encyst by an unknown mechanism. Cysts are passed in the feces one or two weeks after the infection. Trophozoites can also passed in feces, especially in cats, but rarely survive for a significant period outside the host (Barr and Bowman, 1994).



Life cycle of *Giardia canis* (from: Veterinary Parasitology Reference Manual Fifth Edition W.J.Foreyt. Blackwell Publishing, 2001 pg34).

2.3 TRANSMISSION

Giardiasis is transmitted through the fecal-oral route. Similarly to *Cryptosporidium*, *Giardia* cysts are stable and can survive for weeks to months in the environment and a small number of cysts are enough to cause infection. Infected cattle can excrete approximately 10^7 *Giardia* cysts per gram of feces. Therefore, large numbers of *Giardia* cysts can be spread into the environment through manure resulting in the water or food contamination. The median infective dose is thought to be between 25-100 cysts, but human volunteers were infected with only 10 cysts (Fayer et al, 2004).

Waterborne transmission

Waterborne outbreaks of giardiasis are a major public health problem in many industrialized nations. Environmental contamination of the water systems result from human, agricultural and wildlife sources (Heitman, et al, 2002). The majority of the waterborne outbreaks occurred in unfiltered surface or groundwater systems impacted by surface run off or sewage discharges (Thompson, 2004). A study mentioned before, found that sewage effluent has the highest prevalence of *Giardia* (Heitman et al, 2002). In Canada and Italy, a high prevalence of *Giardia* cysts in raw sewage water was reported (Sulaiman et al, 2004). *Giardia* cysts have been found in > 2,350 samples of surface water from eight countries at nearly five cysts per liter, and in 21-100% of the samples examined (Fayer et al, 2004). In the United States, 18 waterborne outbreaks of *Giardia* occurred between 1984 and 1994 (Fayer et al, 2004). During 1998-2002, the total number of reported cases of giardiasis in the Unites States decreased; in the 2002, however, the number of cases increased. It is likely that this increase number of causes is because giardiasis became a national noticeable disease in 2002 (Hlavsa et al, 2005).

Foodborne infections

According to the Centers for Disease Control and Prevention, between 1988 and 1992, parasites contributed to a 2% of the food-borne outbreaks in the United States (Rose and Skilfo, 1999). Of the 17 total parasitic outbreaks during that time period, *Trichinella* was associated with 59% and *Giardia* cause the other 41% (Rose and Skilfo, 1999). Foodborne outbreaks, however, are not as well documented as waterborne outbreaks; and most of the foodborne outbreaks of *Giardia* were related to contamination through food handlers (Rose and Skilfo, 1999). Additionally, *Giardia* has been found in 60% of the water samples tested, used to irrigate vegetables in the United States and in some Central American countries (Thurston-Enriquez et al, 2002). *Giardia* had been recovered from coriander, mint, radishes and potatoes irrigated with wastewater (Lane and Lloyd, 2002). *Giardia*, as well as *Cryptosporidium* has been found in shellfish and in fresh, estuarine and coastal waters worldwide (Fayer et al, 2004).

Recreational water

Giardia cysts have been reported in marine waters off the coast of Hawaii and Puerto Rico (Fayer et al, 2004). *Giardia* was found in 41% and 64 % in Hong Kong water samples tested from beaches suitable and unsuitable for bathing respectively (Fayer et al, 2004).

Risk factors

Persons at increased risk of infection are:

1. Travelers to disease-endemic areas
2. Children in day-care centers
3. Close contact to infected persons

4. Persons who swallow/ingest contaminated water
5. Persons who are taking part in outdoor activities and do not practice hygienic behaviors.
6. Persons who have contact to infected animals

There is a marked seasonality in the onset of giardiasis, from early summer to early fall.

2.4 CYCLES OF TRANSMISSION

Giardia maintains its infection in mammals through the following four cycles. These cycles, however, may interact and the host range of some of the *Giardia* genotypes is unknown.

Humans

Human to human transmission of giardiasis can occur directly from places in poor hygienic conditions or indirectly through accidental ingestion of the cysts from contaminated water or food. Humans appeared to be infected most commonly with Assemblage B. In the UK, a study that examined 35 human samples reported that 64% were Assemblage B, 27% were Assemblage A and the rest of the samples were a combination of Assemblages A and B (Amar et al, 2002). In Australia, infections with Assemblage B were also more prevalent (70%) than infections with Assemblage A (Read et al, 2001). A recent study, in India reported similar percentages also, with 61% of the people infected with Assemblage B (Traub et al, 2004). Additionally, a study that examined isolates of *Giardia* from humans and dogs living in the same community found that all the human isolates were Assemblages A or B and the dog isolates were Assemblages C or D with the exception of only one isolate (Hopkins et al, 1997).

Cattle

Since beef and dairy cows have a high prevalence (sometimes 100%) of *Giardia* and calves can excrete between 10^5 and 10^6 cysts per gram of feces, there has been a heightened awareness of the role of cattle in the transmission of giardiasis (Thompson, 2004; O'Handley, 2002). Transmission occurs among infected calves and infected adults; among dairy cows the frequency of transmission is higher (Thompson, 2004). The grouping behavior of calves favors the transmission of *Giardia* cysts. A study in the United Kingdom found that contaminated soil is a reservoir for infection of newly introduced animals (Thompson and Monis, 2004). However, a study in Australia did not find *Giardia* cysts in the feces of adult heifers or in the soil (Thompson and Monis, 2004).

A study genotyping cattle in Canada reported that most of the samples tested were of the livestock genotype (Assemblage E), but a small part of the samples were infected with Assemblage A (Appelbee et al, 2002; Thompson, 2004). In Australia, a recent study, reported that 100% of the dairy calves were infected with the livestock genotype (Thompson, 2004).

Dogs and cats

Giardia is the most prevalent parasite of dogs and cats in Australia and has a high prevalence in pets worldwide (Thompson and Robertson, 2003). Molecular epidemiology studies demonstrated that dogs and cats may be infected with host-specific genotypes as well as with the zoonotic genotype (Thompson, 2004).

Wildlife

Giardia has been found in beavers, nutrias and deer, however, there is limited information on *Giardia* in wildlife (Thompson, 2004). Based on the information

available, it is possible that wildlife animals may harbor host-specific genotypes (Thompson, 2004). A study genotyping *Giardia* isolates from marsupials showed that they are infected with a novel genotype (Thompson, 2004). Nevertheless, recent studies demonstrated that beavers and the white-tailed deer can harbor the zoonotic Assemblages (Appelbee et al, 2002, Trout et al, 2003). *Giardia* cysts have been isolated from the feces of California sea lyons, from ringer seals in artic Canada and from harp, grey, and harbor seals in the Gulf of St Lawrence, Canada (Fayer et al, 2004).

Waterborne transmission

As previously stated, outbreaks of giardiasis are a major public health problem in many industrialized nations.

2.5 ZOONOTIC TRANSMISSION

While the WHO declared *Giardia* a zoonotic pathogen in 1979, no direct evidence of fecal-oral or waterborne transmission was established at that time (Thompson, 2004). The greatest zoonotic risk is for genotypes of *Giardia* in Assemblage A, specifically those in the subgroup AI, and to a lessen degree the genotypes in Assemblage B (Thompson, 2004).

Studies done in livestock suggested that the risk of acquiring giardiasis through cattle is probably minimal, at least in United States and in Australia, since most of the time cattle are infected with the specific genotype (Assemblage E) (Hoar et al, 2001; Thompson, 2004). Nevertheless, livestock are also infected with the zoonotic genotype, Assemblage A. Since calves with giardiasis shed between 10^5 and 10^6 oocysts per gram of feces, even a few cows infected with the zoonotic assemblage could be a public health risk (Xiao, 1994; Thompson, 2004). Recently, it was suggested that the zoonotic

genotype may be present only temporally in cattle under conditions in which the frequency of transmission with the livestock genotype, Assemblage E, is high and competition is likely (Thompson et al, 2004). However, another study reported that humans appeared to be the source of infection in a small number of dairy cows in a park in Uganda (Graczyk et al, 2002).

The association between beavers and waterborne outbreaks led the WHO to consider *Giardia* as a zoonotic agent (Thompson, 2004). Nevertheless, there is not much information about *Giardia* affecting wildlife, nor their zoonotic potential. At this point, *Giardia* of human origin appears to be the main source of water contamination. In the case of aquatic mammals found infected with *Giardia,* it is likely that these animals became infected from water contaminated with fecal material from infected humans (Thompson, 2004). The study that genotyped the *Giardia* isolate of a beaver origin, demonstrated that the source of *Giardia* infections in beavers was of human origin (Thompson, 2004). In addition, a study isolated *Giardia,* Assemblage A from clams, emphasizing the contamination with feces of mammalian origin (Graczyk et al, 1999).

Dogs and cats are infected with their host-specific genotypes Assemblages C, D and F and they can also harbor the zoonotic genotype Assemblage A. The clinical significance of giardiasis in cats and dogs appears to minimal, but there is a controversy regarding treatment in pets due to its zoonotic potential. Several studies genotyped *Giardia* isolates from dogs. A study done in Aboriginal communities in Australia, demonstrated that dogs were most commonly infected with the dog genotype (Hopkins et al, 1997). Another study, showed that in urban areas in Australia, the zoonotic genotype and the dog-specific genotype are equally common in dogs, suggesting the possibility of

zoonotic transmission (Thompson et al, 1999). This fact was also reported in another study where dogs from multi-dog households were more likely infected with the dog-specific genotype of *Giardia* than dogs in single-dog houses, stressing the possibility of human transmission (Bugg et al, 1999). A study in a growing tea communities in India where humans and dogs are infected with *Giardia,* found that 20% of the dogs were infected with the zoonotic genotype (Traub et al, 2002). A study in Japan found that all the dogs were infected with dog-specific genotype (Abe et al, 2003). In Mexico, two studies demonstrated that the dogs tested were infected with the zoonotic genotype A (Lalle et al, 2005; Eligio-Garcia et al, 2005). In Italy, two studies found that most of the dogs harbor the dog-specific genotypes but also some dogs harbor the zoonotic genotype (Lalle et al, 2005; Berrilli et al, 2004). The results of these studies can be explained according to the different habits of people and dogs in their habitats. In Aboriginal communities the dogs tend to be grouped together, therefore competitive interaction of the genotypes are likely to guarantee that the host-specific genotype predominate in respective host species. In situations where *Giardia* infections are common in humans and dogs, dogs would have equal contact with the zoonotic and the dog-specific genotypes (Thompson, 2004).

Up to this point the fact that similar genotypes are dispersed in different hosts is not certain evidence of zoonotic transmission. Samples from the study in India were characterized at three different loci and provided the first evidence of zoonotic transmission between a dog and a human in the same house (Traub et al, 2004).

2.6 PATHOGENIC MECHANISM OF *GIARDIA* SPP.

Giardia infection reduces the absorption in the small intestine leading to disaccharidase deficiencies, decreased absorption of electrolytes, nutrients and water, concluding in malabsorption, maldigestion and diarrhea.

Role of parasitic products

Giardia causes epithelial injury without invading the mucosa. Although the pathogenesis of *Giardia* is not clearly understood, it was reported that secretory-excretory products of the parasite are responsible for some of the epithelial abnormalities seen during the infection. Cytotoxic activity have been reported in HeLa and Vero cell monolayers that had been exposed to live trophozoites, but not to parasite free extracts or culture supernatants (Buret et al, 2002). It was also demonstrated that *Giardia* affect cytoskeleton proteins and that extracts from sonicated trophozoites may increase the rate of enterocytes apoptosis (Teoh et al, 2000; Chin et al, 2000).

Host immune factors

Host immune cells are implicated in the pathogenesis of giardiasis. One example is the transfer of activated T-cells that may cause villous atrophy in mice in the absence of *Giardia* infection (Farthing, 1993). It was also reported that T-lymphocytes mediate villous atrophy in other intestinal disorders (Buret et al, 2002). Infection of *G. muris* in immunocompetent mice produces a diffuse shortening of the microvilli and alter the disaccharidase activities, these changes, however, are not seen in T-cell deficient mice (Scott et al, 2000b). While these results clearly indicate the involvement of T cells in the pathogenesis of giardiasis; the basis of the mechanism involved is not completely clear.

Mast cells, the primary effectors of allergy, are also implicated in giardiasis. During *Giardia* infection, circulating and secretory anti-*Giardia* antibodies are secreted. Studies done in children with giardiasis reported an increased concentration of IgE associated with *Giardia* infection (Di Prisco et al, 1998). There is enough support to associate giardiasis with allergies and urticaria (Buret et al, 2002).

Effects on epithelial ultrastructure and function

The most common abnormalities associated with giardiasis are disaccharidase deficiencies (Buret et al, 2002). *Giardia* also impairs other digestive enzymes including trypsin, chemotropism, lipase and amylase (Buret et al, 2002). Enzyme deficiencies may or may be not be associated with villous atrophy. Several studies demonstrated that the reduced enzyme absorption is a consequence of the shortening of brush border microvilli at the sites where trophozoites are attached as well as in other areas (Buret et al, 1991; Buret et al, 1992). In conclusion, giardiasis produces a diffuse loss of brush border microvillus length, which causes epithelial maldigestion and malabsorption of electrolytes, nutrients and water resulting in diarrhea.

Effects on epithelial cytoskeleton

The cytoskeleton of the enterocytes supports the cells structure, the brush border of the microvilli are anchored in the apical fibrillar terminal web of enterocytes where they associate with proteins such as myosin and foldrin. *Giardia* excretory-secretory products alter the cytoskeleton of the enterocytes, and these changes depend on intracellular rather than extracellular $Ca^{2}+$ (Buret et al, 2002). Within the microvilli, groups of filamentous actin are kept together by villin, frimbrin, and a 110-kDa protein-calmodulin complex (Buret et al 2002). A study demonstrated that rearrangement of F-

actin and focal loss of other structural proteins occurs during *Giardia* infection (Teoh et al, 2000).

Effects on epithelial permeability

The enterocytes cytoskeleton has an active role in the regulation of the intestinal permeability, which is altered after *Giardia* infection, resulting in impaired absorption. It was reported that loss of the epithelial barrier function may result from the disruption of the tight junctional proteins and/or from the contraction of the circumferential filament bands that contain actin, myosin, tropomyosin and α-actinin (Buret et al, 2002). The parasite load and the severity of the infection appear to be strain related (Buret et al, 2002). Despite strain differences, a study reported that in a *G. muris* infected mice, permeability to sugar probes was significantly increased (Scott et al, 2000a). The exact mechanism of how *Giardia* infection alters enterocytes permeability has yet to be elucidated. Furthermore, several proteins in the epithelial junctional complexes, including members of the zonula-occludens family (such as ZO-1), cingulin, and claudins, have been identified (Buret et al, 2002). Whether permeability alterations results from a direct effect on these protein is uncertain. It was also reported that *Giardia* induces changes in the enterocytes permeability and it is associated with enterocytes apoptosis (Chin et al, 2000).

2.7 HUMAN GIARDIASIS

Giardia, as well as *Cryptosporidium*, are found worldwide and are among the most frequently reported parasites worldwide. *Giardia* is the most common protozoan infection in humans and it occurs in industrialized and developing countries. In the developed world, there is an estimate of $2.8x10^8$ infections per year in humans (Lane and

Lloyd, 2002). A surveillance done by the Center for Disease and Control in the 1990s revealed the organism in every major region of the United States (Lebwohl et al, 2003).

In the developing countries the prevalence of giardiasis ranges between 10 and 20% of the general population, young children are at the highest risk (Lebwohl et al, 2003). Immunocompromissed groups are more susceptible to develop chronic giardiasis. Although is not a major cause of AIDS-associated diarrhea in the United States, prevalence of giardiasis and the chronicity of the infection is greater in the developing world (Lebwohl et al, 2003).

Infection can be directly via fecal-oral contact or indirectly through the ingestion of cysts through contaminated food or water. Once ingested, cysts passed into the stomach, where they are exposed to gastric acid. The acid pH in the stomach and the pancreatic proteases, promote rapid excystation within minutes of reaching the duodenum (Hawrelak, 2003). Trophozoites rapidly colonize the intestinal tract and start causing clinical signs. The symptoms of giardiasis vary from asymptomatic passage of cysts to chronic diarrhea, malabsobtion, and weight loss. Young individuals, immunosuppressed individuals and group-housed animals are at the highest risk. Acute small bowel watery diarrhea is most common; mixed bowel diarrhea, vomiting, and fever are less common. Feces are usually malodorous, pale and steatorrheic, weight loss or poor weight gain can occur. Other less common clinical signs are chills, urticaria and polyarthritis (Hawrelak, 2003). However, a great proportion of infected individuals are completely asymptomatic.

2.8 FELINE GIARDIASIS

Giardia spp. as well as *Cryptosporidium* was reported in prevalence studies, case reports and treatment studies. *Giardia* was first described in cats by two investigators in

1925 and subsequently, it was named *G. cati* by Deschiens and *G. felis* by Hegner (Kirkpatrick, 1986). However, no association with clinical disease was made at that time. After that *Giardia* was reported worldwide (table 2.3). Several studies documented that, younger cats appear to be more susceptible to infection and clinical disease (Kirkpatrick and Laczak, 1985; Kirkpatrick, 1986). *Giardia* transmission in cats can occur through direct contact with infected animals, particularly in catteries (Kirkpatrick and Laczak, 1985). Transmission can also occur through carnivorism if the organisms are present in the intestines of the prey species (Lappin, 2005). Infection may also be self-limited in 27-35 days in cats and dogs. The prepatent period of giardiasis on cats ranges from 5 to 16 days (mean of approximately 10 days). In cats, in contrast to dogs and humans, trophozoites are not found in the duodenum, but in the jejunum and ileum (Kirkpatrick and Farrell, 1984). Shedding of *Giardia* cysts by cats may fluctuate from undetectable to concentrations to > 1,000,000 cysts/gram of feces (Kirkpatrick and Farrell 1984). Peaks of oocysts shedding occur sporadically rather than cyclically and the duration between any two given peaks is generally from 2 to 7 days (Kirkpatrick and Farrell 1984).

Although most of the cats shedding *Giardia* do not show clinical signs of disease, *Giardia* in cats can induce illness in some cats. Clinical signs of feline giardiasis include chronic diarrhea and weight loss. The diarrhea is usually mucoid, pale, soft and has a strong odour, steatorrhoea may be present as well (Brightman and Slonka, 1976; Kirkpatrick and Farrell 1984, Nesvadba, 1979; Kirkpatrick, 1986). Commonly, infected cats are afebrile, do not vomit and exhibit normal total protein and hemogram values (Kirkpatrick, 1986). Co-infection of *Giardia* and other parasites have been reported in cats. Three studies documented the co-infection of *Giardia* and *Cryptosporidium* in cats

(Keith et al, 2003; Perez Trot et al, 2002; Scorza and Lappin, Unpublished). Another study reported an association between *Tritrichomonas foetus* and *Giardia* infection in cats (Gookin et al, 2004).

Table 2.3 Prevalence of *Giardia* spp. in Cats

Number Examined	Location	Test	Prevalence (%)	Study
206	Colorado	Microscopy	2.4	Hills et al, 2000
236	New York	Microscopy	7.3	Spain et al, 2001
1457	United States	Fecal ELISA	4.5	Zislin et al, 2002
3167	Germany	Microscopy	10	Barutzki et al, 2003
81	Servia	Microscopy	22	Nikolic et al, 2002
40	Australia	Microscopy	5	McGlade, 2003
40	Australia	PCR	80	McGlade, 2003
48	Italy	Fecal ELISA	4	Biancardi et al, 2004
39	Tasmania	Fecal ELISA	21	Mildstein and Goldmid, 1997
131	Brazil	Microscopy	6.1	Barrientos-Serra et al, 2003
135	Czech Republic	Microscopy	1	Svobodovà et al, 1994
135	Czech Republic	Serum	57	Svobodovà et al, 1994
97	Iran	Microscopy	7	Anwar, 1974
100	Japan	Microscopy	3	Iseki et al, 1976
600	Japan	Fecal ELISA	40	Itoh et al, 2005
305	The Netherlands	Microscopy	1	Roben et al, 2004

2.9 CANINE GIARDIASIS

Most of the studies on canine giardiasis were prevalence studies (table 2.4), however, several case reports have been published (Agresti, 1975). In Italy, *Giardia* was found in 11 of 300 dogs suffering from catarrhal enteritis (Barlough, 1979). Co-infection with other parasites was also common. Two of 20 dogs infected with *Giardia* were concurrently infected with coccidia (Barlough, 1979). Fourteen of 160 clinically infected dogs were infected with *Giardia* and other organisms (Barlough, 1979).

Some characteristics of canine giardiasis are the following. The study done by Sykes and Fox in London, found that young dogs shed an average of 2,000 cysts per gram of feces and the mean cyst count per gram of feces for all infected dogs was 705.8

(Sykes and Fox, 1989). The prepatent period of canine giardiasis ranges from 5 to 12 days (mean of approximately 8 days). In dogs, *Giardia* prefers the jejunum but has been found in the ileum and duodenum (Barr, 2005). Additionally, trophozoites had been in the upper intestinal tract in symptomatic dogs and in the lower intestinal tract in asymptomatic dogs (Barr, 2005).

A recent study reported that female sex appeared to be a predisposition factor for canine giardiasis (Biancardi et al, 2004). Shedding of *Giardia* cysts in lactating bitches was reported, however in this study the hormonal conditions of the dogs were unknown (Biancardi et al, 2004). In dogs, as well as in other species, a lower prevalence of infection was found when the weather was hot and dry (Biancardi et al, 2004).

Giardia was found more commonly in owned dogs (Biancardi et al, 2004; Itoh et al, 2001). However, many of the owned dogs in the Italian study, come from breeding kennels (Biancardi et al, 2004).

Another study also from Brazil, showed a higher prevalence in shelter animals than in house owned dogs (Huber at al, 2005). In the same study, most of the animals did not show clinical signs of disease (only non-diarrhea samples were collected) and the researchers did not find an association with sex (Huber et al, 2005). The presence of *Giardia* was associated with the presence of other gastrointestinal parasites, especially with *Cystoisospora* (Huber et al, 2005). In Canada, a recent study analyzed 102 samples from dogs that visited hospitalized people and found that 7% of them had *Giardia* cysts in their feces (Lefebvre et al, 2006). Unfortunately, these samples had been not been genotyped (Lefebvre, et al, 2006).

Table 2.4. Prevalence of *Giardia* spp. in Dogs

Number Examined	Location	Test	Prevalence (%)	Study
275	United Kingdom	Microscopy	14.5	Sykes and Fox, 1989
105	Italy	Fecal ELISA	19%	Biancardi et al, 2004
183	Italy	Fecal ELISA	55.2%	Papini et al, 2005
1281	Germany	Microscopy	2.3	Epe at al, 2004
55	Tasmania	Microscopy	14.5	Mildstein and Goldsmith, 1980
1035	Japan	Microcopy	14.6%	Itoh et al, 2001
5959	United States	Fecal ELISA	7.5	Zislin et al, 2002
49	United States	IFA	26.5	Jafri et al, 1993
1216	Canada	Fecal ELISA	7.2	Jacobs et al, 2002
102	Canada	Fecal ELISA	7	Lefebvre et al, 2006
101	India	PCR	20	Traub et al, 2004
458	Czech Republic	Microscopy	2, 5	Svobodovà et al, 1994
458	Czech Republic	Serum	36.5	Svobodovà et al, 1994
81	Servia	Microscopy	14.4	Nikolic et al, 2002
101	Scotland	Microscopy	13.9	Simpson etal, 1988
166	Brazil	Microscopy	31.33	Huber et al, 2005
106	Argentina	Microscopy	14.5	Taranto et al, 2000

2.10 DIAGNOSIS

Giardia represents a diagnostic dilemma: it is one of the most commonly misdiagnosed, underdiagnosed and overdiagnosed parasite (Dryden et al, 2006). The problems on the diagnosis of *Giardia* are the following: it can be easily mistaken by pseudoparasites, yeasts, the cysts are shed intermittently and repeated fecal analysis may be needed before recovering cysts, and cysts can easily be deteriorated in fecal flotation solutions (Dryden et al, 2006). The inability to recognize samples with few *Giardia* cysts by veterinarians and veterinary technicians is also another problem in the diagnosis (Dryden et al, 2006).

Since cats and dogs are commonly infected with *Giardia*, the American Association of Feline Practitioners recommended that all cats with diarrhea of 1 to 2 days in duration be evaluated by direct fecal examination for trophozoites of *Giardia* spp (Brown et al, 2003).

Direct smears

Giardia trophozoites can be seen in a very small amount of fresh diarrheic feces mixed with a drop of normal saline solution in a microscope slide and cover with a coverslip. At 100X trophozoites are recognized by their rapid motion, but their structural characteristics should be observed at 400X. Because trophozoites may be associated with mucus, the only visible motility may be the flagella (Leib and Zajac, 1999). While *Giardia* trophozoites can be confused with *Tritrichomonads* that are similar in size, they may be differentiated by an undulating membrane, a rolling form of motility, the lack of a concave surface and a single nucleus (Leib and Zajac, 1999). Since *Giardia* is detected in a direct smear by motility, examination should be performed on a fresh sample. A refrigerated sample or one examined several hours after collection probably contains no living organisms. The application of Lugol's solution, methylene blue, or acid methyl green to the wet mount helps in the visualization of the internal structures of the trophozoites (Lappin and Calpin, 1998). A study in dogs with the examination of fresh feces on three different days, detected 40% of the *Giardia* infected dogs (Zimmer and Burrington, 1986). Trophozoites are rarely found in solid feces.

Concentration techniques

If trophozoites are not seen in the direct smear, examination of cysts should be performed. Protozoa cysts have higher chances of detection after fecal concentration techniques, such as Sheather's sugar centrifugation and zinc sulfate centrifugation (Lappin and Calpin, 1998). Sugar solution is hypertonic and pulls of the cytoplasm of the cysts to one side which make it appear as a half or quarter moon. Zinc sulfate centrifugal flotation is the preferred technique to observe cysts. In addition to the identification of

Giardia cysts, eggs of other common parasites can be seen. Cysts can be confused with yeast, but *Giardia* should be easily recognized because of its distinct structure. Barium sulfate, several proprietary antidiarrheals and enemas administered before the collection of the feces may interfere with the detection of the cysts (Leib and Zajac, 1999). Due to the intermittent pattern of shedding, it is recommended that at least three samples should be examined over a period of about one week before ruling out the possibility of *Giardia* (Zimmer and Burrington, 1986; Dryden et al, 2006). In a study mentioned before, *Giardia* cysts were found in about 90% of the dogs when three fecal examinations were done (Zimmer and Burrington, 1986). A recent study recommends performing Zinc sulfate flotation and a fecal antigen kit on three fecal samples over a period of 7 days to ensure a correct diagnosis (Dryden et al, 2006).

If the samples cannot be examined immediately, they can be stored in the refrigerator for several days, but samples should not be frozen. Cysts are found in formed to pasty feces, trophozoites are rarely detected following fecal concentration methods. If the feces contain much fat, formalin-ethyl acetate sedimentation is the best technique for detecting cysts.

Duodenal aspirates

Duodenal aspirate, biopsy, brush border cytology, are methods of identifying *Giardia* trophozoites in the preferred milieu of the duodenum. Of these methods, duodenal biopsy appears to be the most sensitive. Recognition of trophozoites in biopsy specimens should be the standard for the diagnosis. However, the histological appearance of the mucosa was usually normal in humans or lesions characteristic of celiac disease have been seen in giardiasis (Lebwohl et al, 2003). Duodenal aspirate is less sensitive of duodenal

biopsy and results have been mixed when compared with stool examinations in humans. The different sensitivities of the duodenal aspirate may be related to the method of obtaining and transporting the aspirate, the mixing of duodenal contents with hypotonic solutions that can cause lyses of the trophozoites (Lebwohl et al, 2003). However, the use of PCR or EIA on duodenal aspirates has not been reported. The string test can obtain duodenal secretions for analysis without the need for duodenoscopy. The sensitivity of the string test and duodenal aspirates was shown to have similar sensitivities (Lebwohl et al, 2003). Another technique to obtain duodenal secretions is duodenal brush cytology.

Endoscopic or peroral string sampling of duodenal secretions and then examination of the sediment for trophozoites was once considered the best method for diagnosis of canine giardiasis (Leib and Zajac, 1999). In cats, due to the mid to distal small bowel location of the parasite it is unlikely to be positive. This test required endoscopy or exploratory laparotomy, and immediate examination of the fluid for trophozoites. In a study in which endoscopic exam was compared with zinc sulfate flotation, duodenal aspiration was positive in 89% of the cases while one Zinc sulfate flotation was positive in 39% of the cases (Pitts et al, 1983). Later studies contradicted these results; examination of a single Zinc sulfate examination detected 77% of the infected dogs while endoscopic exam detected only 67 % of the infected dogs (Leib and Zajac, 1999). Results of another study supported the later findings (Leib and Zajac, 1999), concluding that most of the animals with giardiasis were either identified by Zinc sulfate flotation or responded to treatment eliminating the need of endoscopic examination. In humans, however, duodenal aspirate and stool microscopy are complementary approaches to a diagnosis that can sometimes be elusive.

Monoclonal antibody based immunofluorescence staining of oocysts

A fluorescein-labeled monoclonal antibody that contains monoclonal antibodies is available and can simultaneously detect *Cryptosporidium* and *Giardia* (IFA; Merifluor *Cryptosporidium/Giardia*, Meridian Diagnostics, Cincinnati, Ohio). This test was originally developed to use in human samples, therefore it is possible that some feline and canine isolates may be missed (Lappin, 2005). Recently, IFA was reported more sensitive and specific than $ZnSO_4$ flotation, and was comparable to an antigen detection technique when used on refrigerated feline samples (Lappin et al, 2002). In addition, to read IFA slides a fluorescence microscope is needed, therefore, this assay can only be performed in diagnostic laboratories. Nevertheless, this IFA has been used in some prevalence studies in dogs and other animals (Xiao and Herd, 1994; Deng and Cliver, 1999). Recently when IFA was used *Giardia* was detected in 36.7 % case and control dogs) in contrast to Zinc sulfate flotation that only detected 3.5% of case dogs and 1.8% of control dogs) in a case control study in shelter dogs (Sokolow et al, 2005). The sensitivity and specificity the IFA kit has been reported to be 96-100% in human fecal samples (Ali and Hills, 2003).

Fecal antigen detection by ELISA

Over the last decade, several fecal ELISAs for the simultaneous detection of *Giardia* and *Cryptosporidium* have been marketed for use in people. The advantages of the fecal ELISAs are as follows: numerous samples can be screened at one time, tests can be read objectively in the case of the microplate assays, by a spectrophotometer, no trained microscopists are needed and in the case of the rapid assay the test can be done in 10 minutes. However, false positives and false negatives have been reported (Johnston et al, 2003). Numerous studies have been done in human and veterinary medicine to compare

the sensitivity, specificity and predictive values of the different tests with conventional microscopic detection. Two studies in which commercially available kits were compared agreed that Prospect *Giardia* Microplate assay was one of the best assays evaluated (Maraha and Buiting, 2000). In the second study the sensitivity of the test was 81% in comparison to microscopic examination (Maraha and Buiting, 2000). Although, most of these studies agreed that the Prospect Microplate assay is one of the best fecal antigen test, a study in the Netherlands found that the assay is more sensitive than microscopic examination when only one specimen is evaluated, but, when two samples are evaluated microscopic examination proves to be more sensitive (Mank et al, 1997). Recently, sensitivity, specificity , positive and negative predicted values for the new ImmunoCard STAT *Cryptosporidium/Giardia* rapid assay (Meridian Bioscience, Inc) were 93.5%, 100, 100, and 95.5% respectively when compared with conventional ova and parasite examination (Garcia et al, 2003). False negative results were obtained in samples with low parasites numbers (Garcia et al, 2003). In another study, the ImmunoCard STAT, and the ProSpecT EZ Microplate assay were compared to the Merifluor Direct fluoresce antibody, and it was found that the latter assay was the most sensitive test (Johnston et al, 2003). The sensitivities of the microplate assay and the rapid assay were 90.6% and 81.3% respectively and specificities for all the tests were ≥99% (Johnston et al, 2003).

One study reported a 98.7% agreement between the ProSpecT *Giardia/Cryptosporidium* Microplate Assay, Alexon-Trend, Inc, Ramsey, Minn) and the ColorPAC *Giardia/Cryptosporidium* Rapid Assay (Becton Dickinson, Sparks, Md) for the detection of *Giardia* (Katanik et al, 2001). In the same study the sensitivities and specificities were 100% and 98% respectively (Katanik, et al, 2001). The false positive

results found in the study were attributed to the presence of blood in the samples (Katanik et al, 2001).

As a concluding remark, fecal antigen kits can be useful in endemic areas of giardiasis or and in epidemiologic studies, as shown in a study in Egypt (Rashid et al, 2002). However, in not heavily infected samples or in low prevalence populations, microscopic examinations should also be performed.

In veterinary medicine, several studies compared the sensitivity of these tests with conventional microscopy. Since these tests were developed to be used in humans, some *Giardia* isolates from dogs and cats might be missed in the detection. Recently, a fecal antigen test for dogs and cats was released and it is currently been evaluated (Lappin, 2005). In one study done with dogs, the immunoassay used (ProSpecT *Giardia* Rapid Assay, Alexon Inc., Sunnyvale, California, USA) detected 31.6% of false negative results in comparison to the zinc sulfate results (Payne et al, 2002). This specific assay detected a specific *Giardia* antigen (GS65). The estimated quantity of antigens detected in positive samples ranges from 50 to 5000 ng/ml in human samples (Biancardi et al, 2004). This test, therefore, is sensitive when large amounts of antigens are shed, but, more sensitive techniques are required in cases of lighter infections (Payne et al, 2002; Biancardi et al, 2004). A recent study compared the $ZnCl_2$-NaCl flotation method and a commercially coproantigen ELISA (ProSpecT *Giardia* Microplate Assay, Alexon Inc., Sunnyvale, California, USA) for the detection of *Giardia* in shelter cats and dogs (Cirak and Bauer, 2004). In this study *Giardia* cysts were detected in 25 of 270 (9.5%) dogs and 0 of 100 cats by microscopic examination and in 79 of 270 (29.5%) dogs and in 22 of 100 (22%) of the cats by the fecal ELISA (Cirak and Bauer, 2004). In the microscopic examination of a

single sample one should expect a lower sensitivity due to the intermittent pattern of shedding of *Giardia.* However, a previous study found the same ELISA kit less sensitive than zinc sulfate flotation (Barr et al, 1992). The discrepancy of the results of this study with others done in dog samples might be attributed to the use of $ZnCl_2$-Nacl flotation, which could destroy the cysts, making the detection difficult, in comparison to the $ZnSO_4$ solution employed by other researchers (Cirak and Bauer, 2004). Another point of concern in this study was that the fecal ELISA test was more often positive in coproscopically *Giardia*-negative canine samples that contained *Isospora burrowsi/ohioensis* oocysts than in fecal samples without any parasite stage (Cirak and Bauer, 2004).

In a study recently completed in our laboratory, results of this ELISA (SNAP Giardia, IDEXX Laboratories, Inc. Westbrook, ME) and IFA were in agreement for 94.4% of the samples (Bachman D, Lappin MR, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colo: Unpublished data, 2006). If the results of either a wet mount examination or fecal flotation are positive, a fecal antigen test is not needed except as a confirmation test in questionable samples. It is our opinion that if *Giardia* species antigen testing is considered, the Snap *Giardia* assay is probably the most logical to use. However, the assay should be a supplemental test and should not replace fecal flotation and wet mount examination.

Serologic tests

The use of serologic tests has not proven to be useful in the clinical setting, and its results have been controversial in epidemiologic studies (Lebwohl et al, 2003). A study

compared the results of fecal examinations and serological findings in dogs and cats in the Czech Republic (Svobodovà et al, 1994). *Giardia* cysts were detected in 25 of the 458 (5.5%) dogs tested while at the same time *Giardia* specific antibodies were detected in 167 of 458 (35.6%) of the dogs tested (Svobodovà et al, 1994). In cats, *Giardia* cysts were detected in 1 of 135 (0.007%) cats and specific antibodies were found in 77 of the 135 (57%) of the cats tested.

Molecular Genetic Techniques

Molecular techniques are crucial in epidemiological studies where genotyping of *Giardia* strains is needed. In the clinical setting, molecular techniques are not routinely used. PCR based techniques can be more sensitive than conventional microscopic and immunologic techniques, therefore, they can be helpful in chronic cases that do not respond to conventional treatment.

Characterization using enzyme electrophoresis

Studies using isozyme or alozyme electrophoretic banding patterns reported extensive genetic diversity among the *G. duodenalis* group (Thompson and Monis, 2004). However, the majority of the studies had limited utility because they used too few characters, small number of samples or both (Thompson and Monis, 2004).

DNA Studies

Most of the studies of genetic polymorphisms have been done in *G. duodenalis*, it has been characterized by Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), M13 fingerprinting and nucleotide (nt) sequencing.

RFLP

RFLP of the whole genomic DNA of 15 isolates of *G. duodenalis* reveal two major groups in ethidium bromide staining (Nash et al, 1985). Ten axenic isolates were examined at the trophozoites variant-specific surface protein (VSP) gene, the RFLP were analyzed by Southern blot and two RFLP types that correspond to genetic groups I and II were identified (Andrews et al, 1989). A conserved region from the VSP gene family has been used to identify isolates of the groups I and II and the novel livestock genotype by PCR-RFLP (Ey et al, 1992). Other researchers used a PCR-RFLP of the glutamate dehydrogenase (gdh) gene to identify isolates belonging to Assemblages A and B and also demonstrating that the "Polish" and the "Belgium" genotypes were equivalent to Assemblages A and B (Monis et al, 1996). Another study also used a PCR-RFLP of the gdh gene to characterize isolates of *G. duodenalis* from different hosts, namely Assemblages AI, AII, BIII, BIV, C, D and E (Read et al, 2004).

RFLP analysis of the rRNA genes allowed for the differentiation of genotypes in a mixed infection. PCR-RFLP on cat and dogs samples will be described in detail in chapter 5.

DNA Fingerprinting

Minisatellites and random amplified polymorphic DNA (RAPD) were used for differentiation of *G. duodenalis* isolates. The first study using minisatellites used the bacteriphage M13 as a hybridization probe to detect minisatellite polymorphisms, and the authors were able to discriminate between clones that appear identical by RFLP of rRNA repeats (Upcroft et al, 1990). The same technique has been used to identify patients with mixed infections (Thompson and Monis, 2004). Despite the discriminatory power of this

approach, the long term stability of these markers should be established before using them in epidemiologic studies (Thompson and Monis, 2004).

Depending on the primers used, RAPD-PCR detected polymorphisms between different isolates and between cloned lines of the same isolates. The genetic analysis of RAPD banding patterns correlated well with the grouping of isozyme analysis (Morgan et al, 1993). Recently, a study used RAPDs to characterize 18 isolates of *G. duodenalis* from humans and grouped them into six clusters that have good correlation with clinical and epidemiological features (Pelayo et al, 2003). The stability of these markers, however, remains unknown.

Nucleotide sequencing

Nucleotide sequencing was used to establish evolutionary relationships between organisms and to identify sequences of diagnostic value. Different PCR of the rRNA, triose phosphate isomerase (tpi) and gdh genes were developed. Several PCRs have been done at the rRNA genes; one of the protocols was used to compare human and dog isolates from the same region (Hopkins et al, 1997). Of 35 samples four genotypes had been identified, two of them (Assemblage A and B) from humans and the other two from dogs (Hopkins et al, 1997).

Two studies used the tpi gene to characterize *Giardia* isolates: in one study 16 isolates were located in three genetic groups, then a RLFP was also described but it was found that this system misclassified some isolates (Baruch et al, 1996; Thompson and Monis, 2004). In the other recent study characterized 69 isolates from humans, dogs, and other mammals were characterized and the groups matched those of the major assemblages (Sulaiman et al, 2003; Thompson and Monis, 2004).

Another study used sequencing data of the gdh gene to characterize human *G. duodenalis* (Monis et al, 1998). The results were consistent with the ones observed by alozyme analysis; the samples were located in two groups corresponding to Assemblage A and B (Monis et al, 1998). The livestock samples were located in Assemblage A or in the new livestock group, and the dog derived isolates were placed in two lineages that correspond to the dog groups: Assemblages C and D (Monis et al, 1998). In addition, one group for cats (Assemblage A-E) and one comprising isolates from rats (Assemblage G) were first identified.

A PCR target on the *β-giardin* gene was developed in 1992, since giardins are considered to be unique for this parasite, the assay was highly specific and no cross-amplification with other protozoa or bacteria was amplified (Mahbuhani et al, 1992; Cacciò et al, 2002). Another tested *β-giardin* PCR was tested in 21 reference strains and 30 fecal samples. The sequencing analysis showed that on the basis of specific substitutions, each Assemblage (A, B, E, in this study) is different from the others (Cacciò et al, 2002). Furthermore, new genotypes in the patient samples were found (Cacciò, et al, 2002). The correlation of the results using the *β-giardin* assay was very good when compared to the SSU-RNA (Cacciò, et al, 2002). Additionally a RFLP-PCR was developed and different restriction patterns were observed for the three Assemblages. In a recent study the *β-giardin* PCR was used to characterize *Giardia* isolates from human, calves, dogs and a cat (Lalle et al, 2005). Humans and calves were infected with Assemblages A and B, dogs with Assemblages C, D and A and the cat was infected with Assemblage F (Lalle et al, 2005). In addition, sequence heterogeneity of the *β-giardin* gene allowed for the differentiation of a number of subgenotypes within the

Assemblages. Sequencing analysis of genes with a high degree of heterogeneity, such as the *β-giardin* or the tpi can provide a resolution as high as multilocus sequence typing (Lalle et al, 2005).

Table 2.9: Targets, type of assays and main use of amplification-based techniques for *Giardia*

Target	Assay type	Identification
16S rDNA	PCR, nested PCR, sequencing, microarray	Species and genotype
GDH	PCR, nested PCR, sequencing, PCR, RFLP	Species and genotype
TPI	PCR, nested PCR, sequencing, Real-Time PCR, microarray	Species and genotype
B-Giardin	PCR, nested PCR, sequencing, PCR-RFLP, Real-Time PCR, microarray	Genotype
EF-1 α	PCR, sequencing, PCR-RFLP	Genotype

Cacciò et al. Unraveling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol 2005; 21(9):430-437.

2.11 TREATMENT OF GIARDIASIS IN HUMANS

Recent studies suggested that, due to the increased risk of side effects and the potential emergence of antibiotic resistant organisms the treatment of giardiasis in humans should be based on a combination of nutritional intervention and phytotherapy as a first line approach. Conventional antibiotic treatment should be reserved for cases in which the nutritional approach has been ineffective.

Nutritional management

Nutritional management is based on foods and supplements that inhibit *Giardia* growth, replication or attachment to the enterocytes and that promote host immune defense against the parasite. Consuming a high-fiber, low simple-carbohydrate; low-fat diet will help reducing the acute symptoms of giardiasis. Adequate amounts of fiber in the diet will increase mucin production, sequester bile acids and help to sweep trophozoites out of the intestine. Consumption of a low-simple carbohydrate diet will

result in reducing the amounts of sugars in the intestinal lumen and in lessening the osmotic draw of water, therefore alleviating the diarrhea. The reduction of dietary fat leads to lower levels of bile acid secretion; this will inhibit the survival of *Giardia* trophozoites and reduce steatorrhoea. A study showed that surgical cholestasis or the use of bile-binding resins in the diet can significantly decrease the fecal excretion of *G. muris* cysts (Erlandsen, 2005).

Probiotics

The anti-*Giardia* mechanism of probiotics works in two ways: by competition for adhesion sites and nutrients that would be otherwise used by the parasite and by stimulation of the immune response (Hawrelak, 2003). It was reported that probiotics inhibit and induce immunological response against *Giardia*. *Lactobacillus johnsonii* strain La1, inhibit *Giardia* growth in vitro (Perez et al, 2001). Additionally, it has been shown that *L. johnsonii* La1, *L. acidophilus* strain LA5 and *L. rhamnosus* strain GG all enhance IgA immune responses, and that *L. rhamnosus* GG increases mucin production (Hawrelak, 2003). The actions and qualities of probiotics appear to be strain-specific (Lewis and Fredman, 1998). For a probiotic strain to have therapeutic effect, it should demonstrate gastric acid and bile tolerance, adherence to intestinal mucosa and temporary colonization of the intestinal tract (Dunne et al, 2001).

Dietary fiber

A study reported that animals consuming a low-fiber diet were more likely to have clinical signs of giardiasis when inoculated with *Giardia* cysts than animals on a high-fiber diet (Leitch et al, 1989). Fiber induces an increase in mucus secretion and, in combination with the movement of insoluble fiber, reduces trophozoites attachment to the

intestinal mucosa which decreases trophozoite colonization (Leitch et al, 1989). Insoluble fiber increases the number of goblet cells which in turn increases the levels of mucin. After ingestion, lignins and soluble fibers bind to bile salts, reducing the availability of these for *Giardia* trophozoites, which depend on these salts to survive.

Wheat germ

N-acetyl-D-glucosamine (NAG) residues are major structural components of *Giardia* cysts and trophozoites. Wheat germ contains a lectin (wheat germ agglutinin WGA) that binds to NAG residues (Hawrelak, 2003). It was demonstrated that exposure of *Giardia* trophozoites to WGA inhibited excystation by more than 90%. The wheat germ lectin inhibited excystation by interfering with proteolysis of the cysts wall proteins (Meng et al, 1996). Another study administered WGA to mice prior to *Giardia* inoculation and the treated mice had a 50% reduction in cysts excretion compared to control animals. The authors concluded that WGA did not kill the parasites, but prevented their growth, replication and attachment (Hawrelak, 2003).

Phytotherapy

Medicinal herbs have also been used to alleviate the symptoms of giardiasis and to clear up the infection.

Garlic (Allium sativa)

Garlic has traditionally been used as an antiparasitic and antimicrobial agent, its anti-*Giardial* activity has been measured in the following study. The IC_{50} of whole garlic extract and the allicin breakdown products diallyl disulfide, diallyl sulfide and allyl mercaptan are 0.3mg/ml, 0.1mg/ml, 1.3 mg/ml and 0.037mg/ml respectively (Harris et al,

2000). Incubation of *Giardia* trophozoites with whole garlic extract resulted in loss of flagellar movement and cell motility, internalization of flagella, and trophozoite swelling. The administration of garlic extract to children with giardiasis, reduced clinical and parasitological giardiasis within three days of beginning of the treatment (Soffar and Mokhtar, 1991). The anti-*Giardia* activity of garlic happens through several mechanisms. Allicin may inhibit the activity of *Giardia*'s cysteine protease, which are secretory products related to mucosal alterations (Hawrelak, 2003). Garlic may also stimulate mucosal production of nitric oxide synthase, increasing the release of nitric oxide by the enterocytes and therefore having direct anti-*Giardia* effects (Harris et al, 2000).

Berberine-containing herbs

Berberine is an isoquinoline alkaloid found in a number of medicinal plants and has been used in the treatment of gastrointestinal infections and intestinal parasites (Hawrelak, 2003). It has been shown that berberine and its derivatives have shown to induce morphological changes in the trophozoites and encourage the development of glycogen deposits (Hawrelak, 2003). When it was administered to humans with giardiasis a marked decline in gastrointestinal signs was observed with a 68% reduction in *Giardia* positive stools (Hawrelak, 2003).

Indian long pepper (Piper longum)

This herb has been known for its antihelminitc and carminative effects. Aqueous and alcohol extracts were demonstrated to have anti-*Giardia* properties (Hawrelak, 2003).

Pippali rasayana (PR)

PR is a combination of *Piper longum* and *butea monosperma.* Administration of PR significantly increased the macrophage migration index and phagocytic activity, suggesting that the anti-*Giardia* mechanism enhances the host immune response and host clearance mechanism (Hawrelak, 2003). The administration of PR to human patients with giardiasis resolved clinical signs, and parasitic response was seen in 92% of the patients (Hawrelak, 2003).

Flavonoid- containing herbs

Several plants containing flavonoids including, oregano, plantain leaves, mango leaves, also seems to have anti-*Giardia* activity (Hawrelak, 2003).

Curcumin

Curcumin is a dye derived from the rhizome of the plant *Curcuma longa* and has anti-cancer and antioxidant activity by inhibiting nuclear factor κ β (Perez-Arriaga et al, 2006). A recent article showed that curcumin also exhibit cytotoxic effect in *G. duodenalis* inhibiting the parasite growth and adherent capacity, it also induced morphological alterations and provoked apoptosis-like changes (Perez-Arriaga et al, 2006).

Propolis

Propolis was administered to children with giardiasis in Cuba. Cure rates, evaluated by duodenal aspiration, were 52% in the treated children (Hawrelak, 2003). A recent study, showed that propolis concentrations of 250 and 500 microg/ml were able to inhibit the growth of *Giardia* trophozoites by 60% (Freitas et al, 2006).

Anti-Giardia drugs

There have been multiple drugs used for the treatment of giardiasis in humans and other animals.

Metronidazole

Metronidazole has been used frequently for the treatment of giardiasis in animals and people; it was demonstrated to have an anti-*Giardia* effect in vivo and in vitro (Gardner and Hill, 2001). The protozoal toxicity of metronidazole is from short lived intermediates or free radicals that produce damage by interacting with DNA and possibly other molecules (Lindsay and Blagburn 2001). Side effects have been reported in 7% of the human patients (Harris et al, 2001). These include unpleasant metallic taste, gastrointestinal disturbances and central nervous toxicity among others (Harris et al, 2001). The failure of treatment has been attributed to poor intestinal absorption. Metronidazole has been associated with recurrence rates as high as 90% and the prevalence of metronidazole resistance may be as high as 20% (Upcroft and Upcroft, 2001).

Other nitroimidazoles

Benzoyl metronidazole, secnidazole, nimorazole, tinidazole and ornidazole have also been synthesized (Harris et al, 2001). These compounds have been synthesized to reduce the side effects of metronidazole; for instance, benzoyl metronidazole, is a tasteless form of metronidazole and it is often used in pediatric cases of giardiasis. Recently, tinidazole, was approved by the US Food and Drug Administration for the treatment of trichomoniasis, giardiasis, amebiasis, and amebic liver abscess in humans (Fung and Doan, 2005). In susceptible protozoal cells, tinidazole is reduced to cytotoxic intermediates that covalently bind to DNA, causing irreversible damage (Fung and Doan,

2005). In comparative trials, tinidazole was significantly more effective than metronidazole in the treatment of giardiasis ($P < 0.05$) (Fung and Doan, 2005).

Benzimidazoles

Mebendazole has been use for the treatment of giardiasis and trichomoniasis in humans (Harris et al, 2001). A large-scale human study administering albendazole reported an average efficacy between 62-95% compared with 97% for metronidazole (Wright et al, 2003). Higher levels of efficacy using albendazole require multiple doses. Benzimidazoles may have anti-*Giardia* effect by interacting with the colchicines site in tubulin in the microtubules, resulting in the disruption of assembly and disassembly (Harris et al, 2001). Selective toxicity is achieved because the drug is minimally absorbed from the host intestine (Harris et al, 2001).

Quinacrine

Quinacrine was shown to be effective against giardiasis since 1941 (Harris et al, 2001). It is effective in vivo and in vitro against *Giardia* and it has the advantage over nitroimidazoles in that it can also kill *Giardia* cysts (Harris et al, 2001). The exact mechanism of action is unknown but it is believed to interfere with flavin components of the enzymes such as NADH oxidase, decreasing oxygen consumption (Harris et al, 2001). Side effects such as neurotoxicity and gastrointestinal disturbance were reported, and because it crosses the placenta, it is contraindicated in pregnancy.

Furazolidone

Furazolidone was shown to have anti-*Giardia* activity both in vivo and in vitro (Wright et al, 2003). Selective toxicity occurs as furazolidone is only minimally absorbed from the intestine and is only activated by the parasite (Wright et al, 2003). Its

mechanism of action is unknown, but it may act as an electron acceptor, as in the case of metronidazole (Harris et al, 2001). Side effects are seen in 10% of the human patients (Harris et al, 2001).

Paromomycin

Paromomycin is a nonabsorbable aminoglycoside, therefore it is recommended in pregnant patients with giardiasis. Anti-*Giardia* activity was reported in vitro and in clinical trials (Harris et al, 2001). The proposed mechanism of action relies on the binding of the drug to the giardial SSU-RNA, in this way inhibiting protein synthesis (Wright et al, 2003).

Nitaxozanide

Nitaxozanide has a broad antiparasitic and antibacterial spectrum. It was first described in 1975 by Rossignol and was initially developed as a veterinary antihelminthic (Fox and Saravolatz, 2005). The antiprotozoal mechanism works by interfering with the pyruvate ferredoxin oxidoreductase-mediated electron transfer reaction that is essential for parasite's anaerobic energy metabolism (Gilles and Hoffman, 2002). However, this might not be the only pathway by which nitazoxanide exhibits antiprotozoal activity (Fox and Saravolatz, 2005). The nitro group in nitazoxanide, would play a crucial role, similar to the one postulated for the related drug metronidazole (Hemphill et al, 2006). Nitazoxanide was approved by the US Food and Drug Administration (FDA) for the treatment of diarrhea caused by *Giardia* in children and in adults (Fox and Saravolatz, 2005, Hemphill et al, 2006). Contrary to metronidazole, nitazoxanide was demonstrated to be largely non-mutagenic, so resistance to this drug will be delayed (Hemphill et al, 2006). Nitazoxanide may also have a function in treating metronidazole-resistant

giardiasis (Abboud, et al, 2001). In vitro, nitazoxanide and its metabolite tizoxanide were reported to be eight times more effective than metronidazole against *Giardia* metronidazole-susceptible strains (Adagu et al, 2002). It was administered at a dose of 100 mg bid for 3 days to children aged 2-3 years and it was effective in resolving the infection with a five day treatment regimen (Ortiz et al, 2001). Studies treating children infected with mixed parasitic infections showed eradication rates of *G. duodenalis* of 71% to 94% (Abaza et al, 1998; Romero et al, 1997). The drug has a few side effects and requires a short course of treatment. Since more than 99% of tizoxanide is bound to plasma proteins, care should be taken to administer nitazoxanide with other highly plasma protein bound drugs (Hemphill et al, 2006). However, no drug-drug interaction studies in vivo and studies in patients with compromised renal or hepatic function had been done (Fox and Saravolatz, 2005).

Due to the resistance to antiprotozoal drugs and no reliable treatment exist, efforts to find new therapeutic agents must continue. At this time, the interest is focus on the encystment mechanisms of *Giardia.* Since *Giardia* cysts walls contained unique proteins and polysaccharides that differed from their hosts, making them potential chemotherapeutic targets (Jarroll and Şener, 2003). Recently, a study incubated *Giardia* cysts with metronidazole or furazolidone and then analyzed the effects of these drugs in the encystations process (Hausen et al, 2006). Furazolidone, showed to be more effective than metronidazole to reduced in vitro cysts production (Hausen et al, 2006). The encystation process as an experimental model for treatment of giardiasis was not widely used and should be considered according to the authors (Hausen et al, 2006).

2.12 TREATMENT OF GIARDIASIS IN CATS AND DOGS

In veterinary medicine, there are few studies that utilized dose titrations and evaluation of drugs in experimentally infected animals. In most studies, fecal samples were only assessed for short periods of time after treatment and immune suppression was not induced to evaluate whether infection was eliminated or merely suppressed. Infection with *Giardia* does not appear to cause permanent immunity and so re-infection can occur, this hampers the assessment of treatment studies.

Because of the potential for zoonotic transmission, the treatment of *Giardia*-infected cats may be advocated whether or not they are clinically ill. In chronic cases, the possibility of underlying disorders such as inflammatory bowel diseases, bacterial overgrowth, exocrine pancreatic insufficiency, and immunodeficiency should also be considered.

As described previously, in human medicine, a combination of nutritional intervention and phytotherapy is the first line of approach for treating. However, the efficacy of these approaches for treating giardiasis in cats is unknown. In clinical practice, when vomiting and small bowel diarrhea are the main clinical signs, highly digestible bland diets are indicated. If large bowel diarrhea is the principal clinical sign, high fiber diets are used. Thus, while dietary manipulation is often used, most veterinary clinicians also prescribe drugs. Treatment options currently available or used historically include the following.

Metronidazole

Metronidazole was effective in eliminating *Giardia* cyst shedding in naturally and experimentally infected cats (Nesvadba 1979; Shatto 1981; Zimmer 1987; Scorza and

Lappin, 2004). Metronidazole may also help correct secondary bacterial overgrowth. Thus, if fecal cytology is consistent with concurrent bacterial disease, it is generally prescribe at the maximal dosage of 25 mg/kg given every 12 hours for seven days (Scorza and Lappin, 2004). Signs of toxicity, including gastrointestinal and central nervous system toxicity have been associated with the administration of metronidazole in some dogs (Dow et al, 1989) and kittens (Caylor and Cassimatis, 2001). Neurological toxicity may develop following either chronic therapy or acute high doses (Caylor and Cassimatis, 2001).

There are two formulations of metronidazole available for oral administration. Products containing metronidazole benzoate are commercially available in some countries and the drug is available for formulation in the United States. Metronidazole USP induces salivation and inappetance when administered orally to some cats (Groman, 2000).

Ipronidazole has been reported effective in treating giardiasis in two dogs (Barr, 2005). *Tinidazole* appears to have an anti-*Giardia* activity similar to metronidazole but it is not available in the United States (Barr, 2005).

Quinacrine was reported to be 100% effective in treating canine giardiasis but it is associated with 50% rate of side effects (Barr, 2005). In addition it has not been demonstrated effective in cats and it can not be administered to pregnant animals (Barr, 2005).

Benzimidazoles

In cats, *fenbendazole* was shown to be safe when administered to healthy, adult, non-pregnant cats at a dosage five times higher than the approved dosage (Schwartz et al, 2000). The suggested dose for fenbendazole for the treatment of giardiasis is 50mg/kg, PO q 24hs for 3 -5 days (Plumb's Veterinary Drug Handbook). However, when fenbendazole was administered to cats concurrently infected with *Giardia* and *Cryptosporidium parvum,* only four out of eight cats stopped shedding *Giardia* cysts (Keith et al, 2003).

Albendazole has been used successfully in dog studies, but can cause bone marrow suppression in cats and so we do not currently prescribe it to cats or dogs (Stokol et al, 1997).

Oxbendazole, another benzimidazole used in Europe, was 100% effective against *Giardia* in kennel dogs when used with other control methods (Barr, 2005).

Furazolidone

In cats, the administration of furazolidone causes inappetance and vomiting. Additionally, efficacy data for the treatment of giardiasis in cats is minimal (Brightman and Slonka 1976; Kirkpatrick 1985).

Paromomycin and *Nitaxozanide*

In our laboratory we have prescribed paromomycin or nitazoxanide in some cats with naturally occurring, resistant giardiasis by using the protocols described for cryptosporidiosis. However, none of the data are controlled, so whether these drugs are reasonable first choice drugs is debatable (Scorza and Lappin, 2006). Additionally, renal

failure and deafness occurred in some cats after treatment with paromomycin (Gookin et al, 1999).

Other therapeutic agents used in veterinary medicine

The administration of a combination of febantel, pyrantel, and praziquantel (FPP; Drontal Plus, Bayer Animal Health, Shawnee Mission, KS), has been evaluated for the treatment of giardiasis in dogs (Barr et al, 1998; Barutzki et al, 2001; Payne et al, 2002). In a recent study, three experiments were performed to evaluate the efficacy of Drontal® Plus for the treatment of giardiasis in experimentally infected kittens (Scorza et al, 2005). The American and the European formulation of Drontal® Plus had been evaluated different dosages. When, the United States formulation of Drontal® Plus at two small dog tablets/cat, PO, for 5 days was administered to six cats, a significant decrease in cysts shedding was observed in the treated group in comparison to the untreated. A significant decrease in the percentage of positive samples in the treated group was also observed. In addition, four of the six treated cats remained positive after two doses of immunosuppression (Scorza et al, 2005).

Immunotherapy

When a *Giardia* vaccine was used as a preventative in kittens challenged with a *Giardia* isolate from a lamb, the vaccine lessened the severity of the clinical signs and decreased the cysts shedding (Olson et al, 1996). This vaccine contained 150ug of protein that was obtained by disrupting axenically cultured *G. duodenalis* trophozoites isolated from a sheep. This vaccine induced a strong serum and mucosal humoral immune response. In a recent study, the use of a commercial *Giardia* vaccine for the treatment of kittens experimentally infected with a human strain of *Giardia* did not completely

eliminate the organism (Stein et al, 2003). It is currently unknown whether the vaccines protect against the challenge of feline specific *Giardia* isolates, and since the vaccines have only been available for a short period of time, vaccine side-effects are also unknown. Since the products are adjuvanted, vaccine-associated sarcomas may eventually be documented in cats. Infected puppies that received the *Giardia* vaccine showed less clinical signs and less shedding of oocysts than non-vaccinated puppies (Olson et al, 2000). The same vaccine was used successfully in *Giardia*-infected dogs that did not respond to conventional treatment (Olson et al, 2001). However, none of these studies had a control group. Another group of researchers, found no significance differences among vaccinated and unvaccinated asymptomatic dogs during the 6 months trial (Anderson et al, 2004). A researcher witnessed that numerous asymptomatic infected dogs developed diarrhea after receiving the vaccine (Barr, 2005).

Recently, *silymarin*, a mixture of a flavoniod and phenylpropanoid isolated from the plant *Silybum marianum* was evaluated for the treatment of canine giardiasis (Chon and Kim, 2006). Silymarin, has antioxidant and anti-inflammatory properties and lately, it was demonstrated to be hepatoprotective in infections with other protozoa (Chon and Kim, 2006). Results from this study showed no difference between the efficacy of metronidazole alone and silymarin plus metronidazole. However, the antioxidant activity of silymarin helped to compensate the weight loss and that metronidazole cause and also increased the levels of serum total proteins and albumin caused by metronidazole (Chon and Kim, 2006).

Treatment failures are common in humans and other animals with giardiasis. It is likely that no drug will be universally effective for the treatment of giardiasis. Therefore,

in clinical practice, the drug and protocol used should be varied according to each individual patient and all other options for treatment should be considered.

Prevention

It is suggested that in control environments four main approaches should be sued to control *Giardia*: 1) decontamination of the environment, 2) drugs, 3) cleaning cysts from coats and 4) preventing reintroduction of infection (Barr, 2006). Animals can be bathed with regular pet shampoos and through rinsed. Then, they should be bathed again (perianal area) with an ammonium quaternary compound. Amonium quaternary compounds can be irritants to the skin and mucous membranes but when applied for 3-5 minutes it does not seem to cause irritation (Barr, 2006). The use of *Giardia* vaccines as preventatives should be based on both the prevalence of *Giardia* in the population of animals to be vaccinated and the risk of zoonosis. There are pros and cons for *Giardia* vaccination that should be considered for each animal. Since domestic pets play a minimal role in human giardiasis, vaccination may not be necessary in most situations. Nevertheless, in crowded environments like catteries, or in the pets of immuno-suppressed individuals, vaccination might be appropriated.

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CHAPTER 3

Detection of *Cryptosporidium* species DNA in Feces of Cats and Dogs in the United States

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For submission to the Journal of the American Veterinary Medical Association

Detection of *Cryptosporidium* species DNA in Feces of Cats and Dogs in the United States

Key words: *Cryptosporidium*, PCR, IFA, cats, dogs

3.1 Structured Abstract

Objective: To compare a polymerase chain reaction assay (PCR) and a monoclonal antibody-based immunofluorescence assay (IFA) for detection of *Cryptosporidium* species in cats and dogs in the United States. Additionally, to perform a molecular and phylogenetic analysis on the 18S rDNA and heat shock protein loci (hsp-70) on some of the *Cryptosporidium* positive samples.

Design: Prospective

Sample population: Fecal samples of cats and dogs with diarrhea that did not respond to conventional treatment were sent to the authors for evaluation for *Cryptosporidium* species infection.

Procedures: IFA and PCR (18S rDNA and hsp-70)

Results: 292 samples were assayed, of which 71 (24.3%) tested positive for *Cryptosporidium* by PCR and 8 (2.7%) tested positive for *Cryptosporidium* by IFA and PCR. Of the 179 feline samples assayed, 52 (29%) tested positive for *Cryptosporidium* by PCR and 6 (3.3%) tested positive for *Cryptosporidium* by IFA and by PCR. Of the 113 canine samples assayed, 19 (16.8%) tested positive for *Cryptosporidium* by PCR and 2 (1.7%) tested positive by both IFA and PCR. The overall percent of agreement between PCR and IFA test results were 78% for all samples from cats and dogs, 74% for feline samples and 85% for canine samples.

Conclusions and Clinical Relevance: PCR was more sensitive than IFA for the detection of naturally infected cats and dogs, and the cats and dogs tested in this study were infected with *C. felis* and *C. canis* respectively.

3.2 Introduction.

Cryptosporidium is a coccidian parasite that infects a wide variety of vertebrates including cats and dogs. The species implicated in the majority of mammalian cryptosporidiosis is *C. parvum.* Within *C. parvum,* there are several unique genotypes that have been associated with specific hosts including cats, and dogs, namely *C. felis* and *C. canis,* respectively. In the United States, the prevalence of *Cryptosporidium* spp. in cats ranges from 3.8% to 8.2% (Table 1).[1-5] In dogs the prevalence *Cryptosporidium* ranges from 2% to 17% (Table 1).[4-9] Cryptosporidiosis can cause a wide variety of symptoms, ranging from the subclinical passage of oocysts in feces, to diarrhea, malabsorption, and other clinical signs of gastrointestinal disease.[10-15]

While previous work has shown the prevalence of cryptosporidiosis in companion animals to be highly variable (Table 1), the results cannot be directly compared between publications because of differences in study populations and diagnostic techniques. Many of the previously published studies used insensitive techniques and are likely to underestimate the prevalence rates of *Cryptosporidium* species infections. In addition, molecular analysis of the *Cryptosporidium* species isolates was not performed in most of the studies and so the prevalence of the specific *C. canis* and *C. felis* is not completely known.

Polymerase chain reaction assays (PCR) have been used to amplify *Cryptosporidium* species DNA from feces of cats and dogs and is thought to be the most

sensitive of the currently available techniques.[16-19] In addition PCR can be used in combination with other molecular techniques to genotype *Cryptosporidium* isolates.[16, 20-22] Molecular analysis of multiple loci, including 18S rDNA and hsp-70, had previously identified *C. felis* and *C. canis* as valid species.[16, 21-22]

The objectives of this study were: to use a *C. parvum* PCR and a commercially available immunofluorescent antibody assay (IFA) to detect *Cryptosporidium* species in fecal samples from cats and dogs with diarrhea in the United States; to compare the sensitivity of IFA vs. PCR for the detection of *Cryptosporidium* species; and to determine the *Cryptosporidium* species by phylogenetic analysis to have a broader understanding of the *Cryptosporidium* species that infect domestic cats and dogs in the United States.

3.3 Materials and Methods

3.3.1 Source of isolates

Fecal samples of cats and dogs with diarrhea that did not respond to conventional treatment were sent to the laboratory of two of the authors (MRL, AVS) for evaluation for *Cryptosporidium* species infection. The study was announced by word of mouth and one of two authors (MRL, AVS) spoke to each of the veterinarians about the cases. Samples were mailed on cold packs and either processed on arrival or stored at -20°C until processed. Samples were collected from 2000 to 2004.

3.3.2 Direct Immunofluorescence Assay

Each fecal sample was diluted 1:4 in 0.01 M phosphate buffered saline solution (pH = 7.2) and a thin fecal smear made on microscope slides supplied in an in vitro direct immunofluorescence assay (IFA) capable of simultaneous detection of *Giardia* cysts and

Cryptosporidium oocysts.[a] The assay was then performed and interpreted following the manufacturer's instructions.

3.3.3 DNA Isolation

DNA was extracted from feces using an adaptation of a previously published protocol.[23] The only differences were the use of a Mini-BeadBeater [b] for the disruption of the oocysts and use of the FastDNA kit.[c] for DNA extraction

3.3.4 18S rDNA gene amplification and sequencing

PCR amplification was done following a published protocol.[19] The primers used (awaF-995=5'-TAGAGATTGGAGGTTGTTCCT-3' and awaR-1206=5' CTCCACCAACTAAGAACGGCC-3') amplify a 256 base pair product.[24]

DNA sequences were analyzed both forward and reverse direction using an ABI3100 Genetic Analyzer.[d]

3.3.5 Hsp-70 gene amplification and sequencing

PCR amplification was performed as previously described.[25] A 311-bp region of the heat shock gene hsp-70 was amplified using the forward primer HSPF1 (5'-ATG TCT GAA GGT CCA GCT ATT GGT ATT GA-3') and the reverse primer HSPR1 (5' GAT TGG CTT RTC CTT YGG RCC 3'). DNA sequences were analyzed in both forward and reverse direction as described

3.3.6 Comparison of IFA and PCR

Results of PCR and IFA were compared by calculating the overall percentage of agreement and the kappa statistic.[26]

3.3.7 Phylogenetic analysis

Phylogenetic alignments were performed in Clustal W and its default settings.[e] The alignments were confirmed visually and adjustments were made as needed. Distance based, parsimony, and maximum likelihood analyses were performed by PAUP (Version 4.0b10) [f] and trees were constructed using PHYLIP (version3.5) [g]. Bootstrap analyses were as performed on 1000 replicates.

3.4 Results

3.4.1 Detection of Cryptosporidium spp. *from diarrheic samples from cats and dogs by PCR and IFA.*

A total of 292 samples were assayed, of which 71 (24.3%) tested positive for *Cryptosporidium* by PCR and 8 (2.7%) tested positive for *Cryptosporidium* by IFA and PCR (Table 2). Of the 179 feline samples assayed, 52 (29%) tested positive for *Cryptosporidium* by PCR and 6 (3.3%) tested positive for *Cryptosporidium* by IFA and by PCR (Table 2). Of the 113 canine samples assayed, 19 (16.8%) tested positive for *Cryptosporidium* by PCR and 2 (1.7%) tested positive by both IFA and PCR (Table 2). No samples tested positive for *Cryptosporidium* spp. by IFA exclusively.

3.4.2.Comparison of IFA and PCR. The percent of agreement between the two tests was calculated first, however, this comparison does not consider the agreement due to chance alone. Cohen's kappa is calculated to adjust for chance agreement but depends on the degree of symmetry in the classification agreements (IFA+PCR+ and IFA-PCR-) as well as the degree of symmetry in the classification disagreements (IFA+PCR- and IFA-PCR+). For a given percentage of agreement, K depends on the distribution of agreements and disagreements. In this study, most classification agreements were negative results (IFA-PCR-) and all classification disagreements were PCR+IFA-

resulting in considerable asymmetry in both classification agreements and disagreements. This lead to an artificially low kappa values which were not informative, and therefore were not reported. [27] If the distribution of agreements had been symmetrical, all kappa values would have increased two fold. However, there were no IFA+/PCR- results and the disagreements could never be symmetrical. The overall percent of agreement between PCR and IFA test results were 78% for all samples from cats and dogs, 74% for feline samples and 85% for canine samples (Table 2). Compared to results from PCR, very few samples tested positive by IFA and all the samples that tested positive by IFA tested positive by PCR as well. Conversely, all the samples that tested negative by PCR also tested negative by IFA.

3.4.3 Sequencing and phylogenetic analysis of 18S rDNA gene. Partial sequences of the *Cryptosporidium* 18S rDNA gene were obtained from 15 domestic cats and 3 dogs (Table 3). Nucleotide alignments of these isolates revealed that both dog-derived *Cryptosporidium* and cat-derived *Cryptosporidium* were identical to each other (Figure 1). Partial sequences of the 18S rDNA gene from all 15 domestic cats and 3 dog matched *C. parvum* when blasted in Genebank. Consistent results were obtained in the 3 phylogenetic analyses of the rRNA gene. All the cat and dog isolates were in different clusters due to nucleotide differences (Figure 1) but were grouped together (Figure 2). The groping support was 83% bootstrap by parsimonium analysis. Reference isolates from Genebank grouped together with groping support of 57% Bootstrap by parsimonium analysis.

3.4.4 Sequencing and phylogenetic analysis of Hsp-70 gene. Partial sequences of the *Cryptosporidium* hsp-70 gene were obtained from 21 domestic cats and 4 dogs. Overall

the percentage of homology among the isolates ranges between 96 and 98%. The canine isolate was slightly different from the feline isolates; the percentage of homology between itself and the feline isolates was between 85 and 87%. Partial sequences of the hsp-70 gene from the 4 positive dogs and 21 positive cats matched *C. canis* and *C. felis*, respectively when blasted in Genebank (Table 3). For the phylogenetic analysis we could only use 10 *Cryptosporidium* positive samples from cats. The phylogenetic analysis of the hsp-70 gene was consistent with the 18S rDNA analysis and the isolates grouped in one cluster (Figure 3). The groping support was 98% bootstrap by parsimony analysis. Reference isolates from Genebank grouped together with groping support 100% Bootstrap by parsimonius analysis

3.5 Discussion

Cryptosporidium species infection in dogs and cats was detected by PCR approximately ten times more than by IFA in this study. Positive and negative control samples were included on every PCR run and there is no reason to believe that the PCR results were falsely positive. These results were similar to those of other recent publications[17-18] and in cats experimentally infected with *C. parvum.*[19] It is likely that previous studies might have underestimated the prevalence of *Cryptosporidium* in companion animals due to the lack of sensitivity of the techniques used. The overall percentage of agreement between PCR and IFA test results in the study described herein was between 74% and 85%. The results suggest that PCR is more sensitive and specific than IFA for detecting infection in naturally-exposed animals.

In this study, samples were only available from animals with diarrhea and so the diagnostic sensitivity and specificity and predictive values could not be calculated.

Twenty-four percent (71/292) of the samples having chronic diarrhea that did not respond to symptomatic treatment tested positive for *Cryptosporidium* by PCR. However, most of the *Cryptosporidium* infected cats and dogs do not have clinical signs.[4] Nevertheless, the histopathologic reports of these infected cats show loss of microvilli, degeneration of host epithelial cells and atrophy of the villi in heavy infections, which may predispose to malabsorbtion or other gastrointestinal dysfunction.[13-15] Usually, clinical signs of disease occurred in patients with immunosuppression, preexisting disease in the intestinal tract, or coinfection with other infectious or non-infectious causes.[13,15,28] Overall infection in cats seems to be relatively common however, before PCR testing; cryptosporidiosis in cats was probably under diagnosed. This finding relates to the relative insensitivity of most of the available tests and to the fact that cats with cryptosporidiosis shed very few oocysts.[29] Additionally, in our study, the effect of previous treatment can veiled the results.

The primers that we used in the 18S rDNA PCR detected *C. parvum*, and some of the *C. felis* and *C. canis* strains reported in the Genebank. Amplification of the 18S rDNA and the hsp-70 loci by PCR in combination with other molecular techniques had been widely used for molecular characterization of *Cryptosporidium* parasites in different species.[21-22, 30] 18S rDNA is the most common loci used for detection and differentiation of *Cryptosporidium* spp. The 18S rDNA is part of a gene complex in which each unit is composed by polymorphic intergenic regions interspread between conserved regions. By designing PCR primers to be located in conserved regions that comprise polymorphic areas, we will be able to differentiate among *Cryptosporidium* spp. by sequencing analysis or by another differentiation technique. The 18S rDNA PCR assay used in this

study was primary used to be more sensitive that microscopic techniques. Unfortunately, the primers that we used in this study were located in a small conserved region of approximately 256 base pairs for *Cryptosporidium* spp. and did not span an intergenic region. Therefore, differentiation at a species level was not achieved with the 18S rDNA data.

In contrast, the hsp gene belongs to a multigene family that is highly conserved in eukaryotes and prokaryotes. Hsp-70 demonstrated that it has higher heterogeneity than the conserved region of the 18S rDNA gene that was used, and therefore it is a better target for genotyping. 18S rDNA restricts nucleotide changes to intergenic regions of the gene, whereas mutations in the hsp-70 are dispersed over the sequence. Therefore, phylogenetic analysis of the hsp-70 is more robust than the analysis of the conserved regions of the 18S rDNA gene resulting in higher bootstrap values.[21]

The hsp-70 primers that we used in this study had been also previously used for phylogenetic analysis in dogs.[25] The phylogenetic analysis of this gene allows us to differentiate *Cryptosporidium* at a species level showing that isolates from cats group together, separately from isolates from dogs that in turn form their own groups. Additionally, according to sequencing analysis the cats and dogs tested in this study were infected with *C. felis* and *C. canis* respectively.

Although the risk of acquiring *Cryptosporidium* from domestic pets appears to be minimal, *C. felis* and *C. canis* have been detected in humans.[31-34] More studies genotyping oocysts from cats and dogs should be performed in order to confirm the likelihood of zoonotic infection. Furthermore, cats and dogs can be subclinically infected

carriers acting as a source of infection to other individuals or to the environment. This has been a particular concern with the pets of immunosuppressed human patients.

Acknowledgments: This study was partially founded by Heska Corporation, Fort Collins, Colorado.

3.6 Tables

Table 1. Prevalence of *Cryptosporidium* spp. in Cats in the United States

Number	Species	Location	Assay	Prevalence	Ref #
600	Feline	USA	Serology	8.3%	1
205	Feline	Colorado	Acid fast stain	5.4%	2
263	Feline	New York	ELISA	3.8%	3
173	Feline	North Carolina	IFA	6.5%	5
130	Canine	Colorado	IFA	3.8%	6
200	Canine	California	Acid fast stain	2.0%	7
49	Canine	Georgia	Acid fast stain	10.2%	8
100	Canine	Kentucky	Acid fast stain	17.0%	9

Modified from reference 4.

Table 2. Distribution of *Cryptosporidium* species IFA and PCR results using feces from naturally infected cats and dogs in the United States.

	Sample set		
Result	Total	Feline	Canine
IFA positive, PCR positive	8	6	2
IFA positive, PCR negative	0	0	0
IFA negative, PCR positive	63	46	17
IFA negative, PCR negative	221	127	94
Percent agreement	78%	74%	85%

Table 3. *Cryptosporidium* isolates and sequence analysis results

Lab ID	Species	18S rDNA amplification	HSP-70amplification
98	feline	*C. parvum*	*C. felis*
104	feline	*C. parvum*	*C. felis*
127	feline	*C. parvum*	-
133	canine	*C. parvum*	-
137	canine	*C. parvum*	*C. canis*
161	feline	*C. parvum*	*C. felis*
162	feline	*C. parvum*	*C. felis*
163	feline	-	*C. felis*
164	feline	-	*C. felis*
178	feline	*C. parvum*	-
192	feline	-	*C. felis*
205	canine	*C. parvum*	-
219	canine	-	*C. canis*
226	feline	-	*C. felis*
227	feline	*C. parvum*	*C. felis*
230	feline	*C. parvum*	*C. felis*
232	feline	*C. parvum*	*C. felis*
233	feline	*C. parvum*	*C. felis*
235	feline	-	*C. felis*
238	feline	*C. parvum*	*C. felis*
239	feline	*C. parvum*	-
240	canine	-	*C. canis*
244	feline	*C. parvum*	*C. felis*
254	feline	-	*C. felis*
323	feline	-	*C. felis*
324	feline	*C. parvum*	*C. felis*
401	feline	-	*C. felis*
404	canine	-	*C. canis*
439	feline	-	*C. felis*
446	feline	-	*C. felis*
454	feline	*C. parvum*	-

Ref:-:not available

3.7 Figures

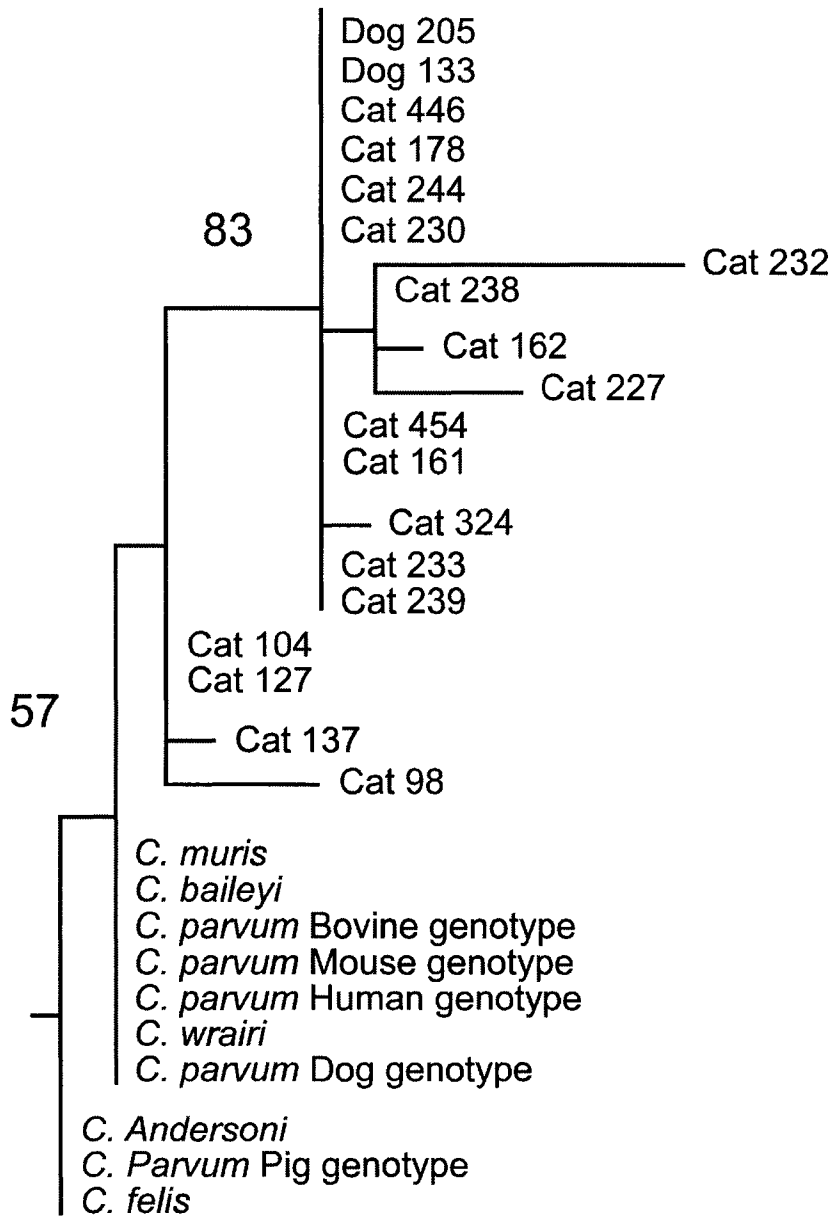
Figure 1: *Cryptosporidium* 18S rDNA consensus sequence

TTAGAGATTGGAGGTTGTTCCTTACTCCTTCAGCACCTTATGAGAAATCAAAG
TCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTG
ACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACG
GGAAAACTCACCAGGTCCAGACATAGGAAGGATTGACAGATTGATAGCTCTT
TCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAAGA

Alignments of 18S rDNA sequence data from the divergent isolates

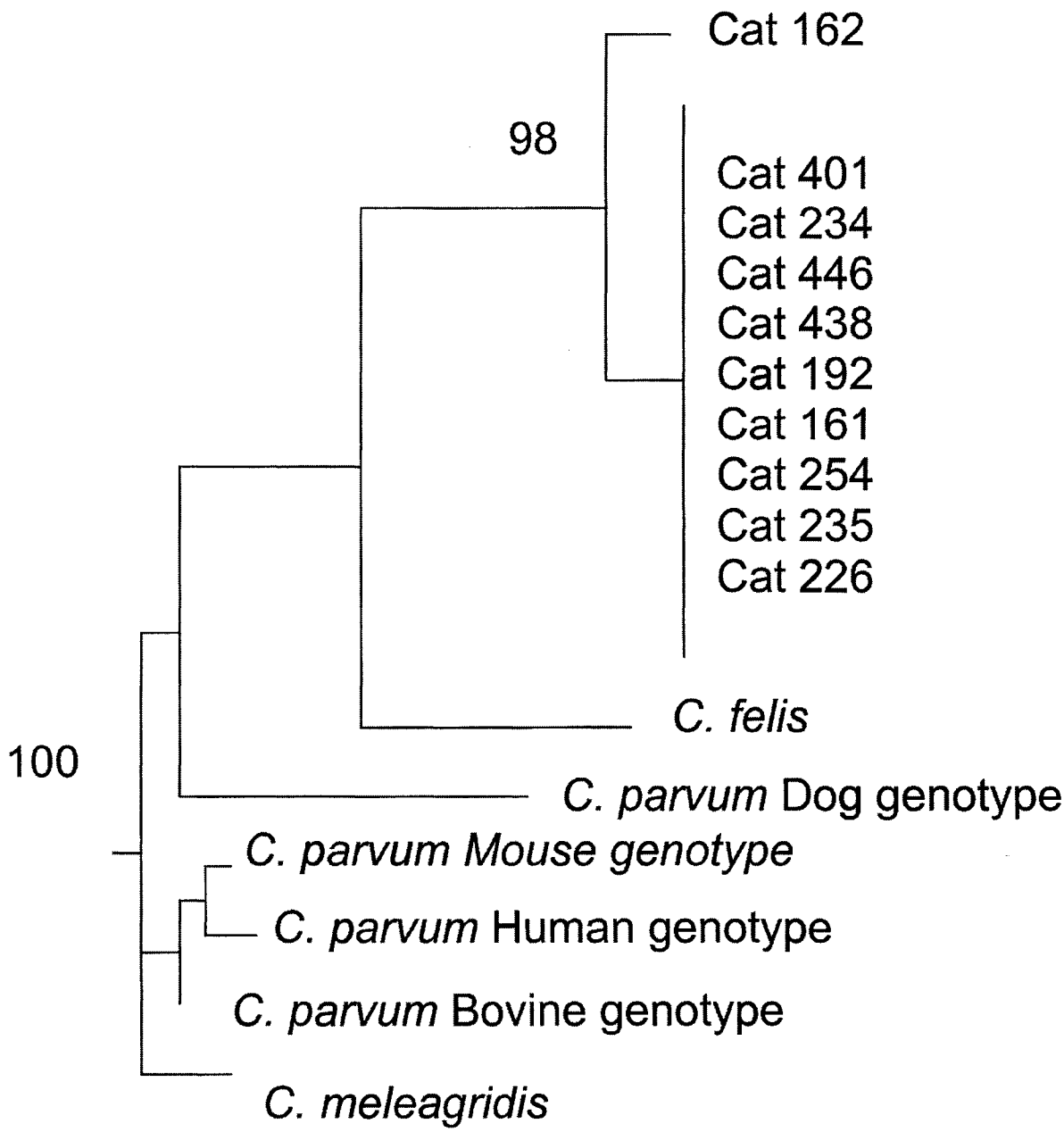
```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
232             TTAGAGATTG GAAGTTGTTC CTCTACTCCT TCAGCACC-T TAT-GAGAAA
238             TTAGAGATTG GAAGGTTGTT CCTTACTCCT TCAGCACC-T TAT-GAGAAA
162             TTAGAGATTG GAAGGTTGTT CCTTACTCCT TCAGCACC-T TAT-GAGAAT
227             TTAGAGATTG GAAGGTTGTT CCTTACTCCT TCAGCACC-T TAT-GAGAAA
CONSENSUS       TTAGAGATTG GA-GGTTGTT CCTTACTCCT TCAGCACC-T TAT-GAGAAA
                          *
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
104             AGGAA-TTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCTTAATTT
127             AGGAA-TTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCTTAATTT
137             AGGAA-TTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCTTAATTT
98              AGGAA-TTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCTTAATTT
CONSENSUS       AGGAA-TTGA CGGAAGGGCA CCACCAGGAG TGGAGCCTGC GGCT-AATTT
                                                                   *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
104             GACTCAACAC GGGAAAACTC ACCAGGTCCA GACATAGGAA GGATTGACAG
127             GACTCAACAC GGGAAAACTC ACCAGGTCCA GACATAGGAA GGATTGACAG
137             GACTCAACAC GGGAAAACTC ACCAGGTCCA GACATAGGAA GGATTGACAG
98              GACTCAACAC GGGAAAACTC ACCAGGTCCA GACATAGGAA GGATTGACAG
CONSENSUS       GACTCAACAC GGGAAAACTC ACCAGGTCCA GACATAGGAA GGAT-GACAG
                                                                 *
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
104             ATTGATAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCCGTTCTTA
127             ATTGATAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCCGTTCTTA
137             ATTGATAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCCGTTCT?G
98              ATTGATAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCCGTTCTA?
CONSENSUS       ATTGATAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCC-TTCTTA
                                                                   *

* Asterisks indicate different bases
```

Figure 2



Parsimonius tree based on the *Cryptosporidium* 18S rDNA sequences

Figure 3



Parsimonius tree based on the *Cryptosporidium Hsp-70* sequences

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3.9 Footnotes

a. Merifluor Crypto/Giardia IFA kit, Meridian Diagnostic Corporation, Cincinnati, OH.

b. Biospec Products, Bartlesville, OK

c. Qbiogene, Irvine, CA

d. Applied Biosystems, Foster City, CA

e. Lasergene, DNASTAR, Madison, WI

f. Sinauer Associates, Sunderland, MA

g.http://evolution.genetics.washington.edu/phylip.html-

CHAPTER 4

Genotyping of *Cryptosporidium* spp. isolates from dogs and cats in the United States

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For submission to the International Journal for Parasitology

4.1 Abstract

Cryptosporidium spp. are coccidian parasites that infect a wide variety of vertebrates including humans, cats and dogs. The species implicated in mammalian cryptosporidiosis is *C. parvum.* Within *C. parvum,* there are several unique genotypes that have been associated with specific hosts including cats, and dogs, namely *C. felis* and *C. canis* respectively. In the United States, the prevalence of *Cryptosporidium* spp. in cats and dogs ranges from 2% to 29.4% (Scorza et al, 2007). However, because molecular analysis of *Cryptosporidium* spp. has only been performed on some isolates and the techniques used for genotyping have varied among studies, the prevalence of the specific *C. felis* and *C. canis* is not completely known. The objective of this study is to genotype *Cryptosporidium* isolates of cats and dogs from the United States using Polymerase Chain Reaction –Restriction Fragment Length Polymorphism (PCR-RFLP) assay to have a broader knowledge of the species that infect these animals. All the fourteen isolates tested with this technique were *C. felis* and all four canine isolates tested were *C. canis.*

4.2 Introduction

Cryptosporidium spp. are protozoan parasites that infect a wide variety of vertebrates. The species implicated in the majority of mammalian cryptosporidiosis is *C. parvum.* Recently, molecular typing techniques determined that cats and dogs are infected with specie-specific *C. felis* and *C. canis* respectively (Morgan et al, 2000a; Sulaiman et al, 2000, Xiao et al, 1999a; Xiao et al, 2004). Even so, *C. felis* and *C. canis* have been found in immunocompetent and immunosuppressed humans and, when the host range includes humans, the parasite acquires public health significance (Pieniasek et al, 1999; Alves et al, 2000; Alves, 2006; Guyot et al, 2001; Xiao et al, 2001; Cama et al, 2003; Matos et al, 2004).

In the United States, the prevalence of *Cryptosporidium* spp. in cats and dogs ranges from 2 to 29.4% (McReynolds et al, 1999; Hill et al, 2000; Spain et al, 2001; Lyndsay and Zajac, 2004; Nutter et al, 2004; Scorza et al, 2007). In most of the studies only microscopic examination was used for diagnosis and therefore the prevalence of the specific *C. canis* and *C. felis* is not completely known. Only recently, researchers started using PCR for the detection of *Cryptosporidium* in prevalence studies (Table 1). PCR has been used to detect cryptosporidiosis in cats (Sargent et al, 1998; Abe et al, 2002, Scorza et al, 2003). Additionally, PCR has been used worldwide for the detection of canine cryptosporidiosis (Table 1). A recent study from Italy reported a prevalence of 3.3% (8/240) in dogs by PCR, the sequence analysis showed that seven dogs harbored *C. parvum* cattle genotype and only one dog was infected with *C. canis* (Giangaspero et al, 2006).

While the results of these studies showed that presence of *Cryptosporidium* species DNA in feces of dogs and cats is common, few isolates have been genotyped.

However, based on the limited number of samples assessed to date, it appears dogs and cats are exposed to a number of different species other than *C. parvum*, *C. felis*, and *C. canis*.

In recent studies *C. baileyi* and *C. muris* oocysts have been recovered from cat feces suggesting that cats can be infected through carnivorism (Hajdušek et al, 2004; Thompson, 2003). Because cats and dogs with cryptosporidiosis can be subclinically infected carriers of *Cryptosporidium* species, they could act as a source of infection for other individuals or for the environment (Tuzio et al, 2005). When a cat becomes a carrier of an enteric zoonotic agent, the owner should be advised of the public health risk (Tuzio et al, 2005). Since the zoonotic risk of *Cryptosporidium* appears to vary by the species, more genotyping information is needed.

Cryptosporidium genotyping techniques have been based on the PCR amplification of different genes and posterior sequencing of the amplicon or digestion with endonucleases followed by gel electrophoresis of the restriction fragments (Spano et al, 1997; Gibbons et al, 1998; Morgan et al, 1998; Spano et al, 1998 a, b; Sulaiman et al, 1998; Cacciò et al, 1999; Xiao et al, 2001). The amplification targets were the small subunit (SSU) rRNA (or 18S rRNA), heat shock protein (HSP-70), oocyst wall protein (COWP), β-tubulin and actin genes (Cacciò et al, 2005). SSU rRNA is the most common loci used for detection and differentiation of *Cryptosporidium* spp. The SSU rRNA is part of a gene complex in which each unit is composed by polymorphic regions interspread between conserved regions. In a previous study, we performed a molecular and phylogenetic analysis on *Cryptosporidium* isolates from cats and dogs, in a small and conserved area of the SSU rRNA gene (Scorza et al, 2007). Therefore, is was not

possible to differentiate *C. felis* from *C. canis* from the fragment amplified (Scorza et al, 2007). In this study, we used a PCR-RFLP that comprised a larger and polymorphic fragment of the SSU rRNA gene (Xiao et al, 2000). Our results demonstrated that cats and dogs tested in our previous study were infected with *C. felis* and *C. canis* respectively (Scorza et al, 2007). However, up to now only a small number of *Cryptosporidium* isolates from cats and dogs have been genotyped (Xiao et al, 1999b; Morgan et al, 2000a; Alves, 2006; Fayer et al, 2001; Scorza et al, 2006).

Our objective was to genotype *Cryptosporidium* isolates from cats and dogs from the United States to have a broader knowledge of the species that infect these animals. We have used a PCR-RFLP that is widely used in the genotyping of *Cryptosporidium* isolates and that previously demonstrated to amplify *C. felis* and *C. canis* (Xiao et al, 2000; Jiang and Xiao, 2003).

4.3 Materials and Methods

4.3.1 Source of isolates

Fecal samples from cats and dogs with chronic diarrhea that did not respond to conventional treatment were submitted to our laboratory for assessment for *Cryptosporidium* species infection by used of previous published PCR assays and a commercially available IFA (Scorza et al, 2007). In that study, *Cryptosporidium* species results were determined for some isolates by sequencing the hsp-70 gene. Of the positive samples detected in that study, adequate *Cryptosporidium* DNA for further study was available for 14 cats and 4 dogs. The samples were store at -20C until analyzed in this study.

4.3.2 DNA Extraction

DNA was extracted following a previously published protocol (Scorza et al, 2003).

4.3.3 PCR-RFLP of the SSU-rRNA gene

The amplification of the SSU-rRNA gene was performed following a previously published protocol (Xiao et al, 1999a). For the first amplification, 2µl of DNA were amplified using the primers SSU1 (5'-TTC TAG AGC TAA TAC ATG CG-3') and SSU2 (5'-CCC ATT TCC TTC GAA ACA GGA -3'), in 25µl of PCR water and 25µl of HotStart Quiagen Master Mix (Quiagen, Valencia, California). Two µl of the first amplification product were amplified using the primers SSU3 (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG -3') and SSU4 (5'-AAG GAG TAA GGA ACA ACC TCC A-3') (Xiao et al, 1999a; 2000). The amplification was performed in a thermocycler (Perkin Elmer 9600 Thermocycler, Wellesley, Massachusetts) using the following protocol: 95C for 10 minutes, followed by 35 cycles of 95C for 45 seconds, 55C for 45seconds and at 72C for 1 minute followed by 10 minutes at 72C.

Twenty µl of the SSU-rRNA PCR products were digested using 2 µl of *VspI* or 4 µl of *SspI* (New England Biolabs INC., Beverly MA), 5µl of each corresponding buffer and the water necessary to reach a final volume of 50 µl (Xiao et al, 1999a).

Following the restriction enzyme digestion, 7 µl of each sample were analyzed in 2% agarose gels in TBE buffer, stained with ethidium bromide (40 µg/µl) and viewed by ultraviolet transilluminator.

4.4 Results

Restriction digestion of the *SspI* products yielded the two bands pattern (390bp and 426bp) for *C. felis* and the three bands pattern for *C. canis* (109-112, 254, 417-418bp). These patterns had been previously reported by other researchers (Tables 2-4, Figure 1, Xiao et al, 1999b; Alves, 2006).

The patterns observed after *VspI* restriction digestion were also the expected ones (Table 5). For *C. felis* most of the cat isolates had two types of products; one showing a two band pattern (104, 658bp)which represents the copy "A" of the SSU-rRNA gene and one with a three band-pattern (104,182,476bp)which represents the copy "B" of the SSU-rRNA gene. The former copy is the predominant product (Table 5). *For C. canis* the

anticipated two band pattern (102, 633bp) was observed. As for *SspI*, these patterns had been previously reported (Xiao et al, 1999; Alves, 2006; Gatei et al, 2002; Tables 2-4). Since we did not have any more DNA of these isolates, sequence analysis could not be done. We attempt to sequence the 100bp and 600 bp products of feline isolate 178 (after *VspI* digestion); the sequence of the 600bp matches other *C. felis* isolates reported in the Gene bank (100% homology).

4.5 Discussion

Several studies genotyped *Cryptosporidium* parasites from different hosts (Spano et al, 1997; Morgan et al, 1998; Spano et al, 1998a, 1998b; Sulaiman et al, 1998; Cacciò et al, 1999; Pieniasek et al, 1999; Alves et al, 2000; Guyot et al, 2001; Xiao et al, 2001; Cama et al, 2003; Matos et al, 2004; Sulaiman et al, 2000). Among the techniques used PCR-RFLP and sequence analysis of the COWP, DHFR, TRAP-C1 and SSU-rRNA are the most prevalent (Spano et al, 1997; Spano et al, 1998a, 1998b; Gibbons et al, 1998; Xiao et al, 1999a, 2000). We previously genotyped *Cryptosporidium* isolates from dogs and cats from the United States by PCR followed by sequencing analysis of the SSU-rRNA and hsp-70 genes (Scorza et al, 2007). Sequence analysis of the SSU-rRNA data did not allow us to genotype the *Cryptosporidium* isolates due to the small and conserved region amplified (Scorza et al, 2007). Therefore, we decide to genotype the isolates previously mentioned by the PCR-RFLP technique used in this study. A study in which these techniques were compared showed that the PCR-RFLP, which we used in our study, was the only one capable of amplifying C. *felis* and *C. canis* (Jiang and Xiao, 2003). Additionally, to provide more robust information at least two loci should be included on the genotyping analysis and at least one locus should be SSU-rRNA (Cacciò

et al, 2005). Therefore, we decided to include the hsp-70 sequencing results from a previous *Cryptosporidium* genotyping study (Scorza et al, 2007).

The results obtained after restriction endonucleases analysis of the SSU rRNA gene were the expected ones for *C. felis* and *C. canis*. The restriction digestion of the *C. felis* isolates by *VspI* showed that they have the presence of both copies A and B that codified the SSU-rRNA gene. This can be seen by the presence of four bands in the agarose gel. In most of our isolates, the copy A is predominant over the B. This can be observed by an increase of intensity in the bands corresponding to the A copy in comparison to the fragments corresponding to the B copy. Le Blancq found that in the *Cryptosporidium* genome there are five copies of the rDNA gene (Le Blancq et al, 1997). In the five copies of rDNA there are two different nucleotide sequences that they called A and B; four of the five copies have the sequence A and the other one has the sequence B (Le Blancq et al, 1997). These differences, however, do not alter the RFLP pattern obtained by digestion of *C. parvum* with *SspI* and *VspI* (Alves, 2006). In contrast to *C. parvum*, in the isolates of *C. felis* the proportion of 4 copies of A and one copy of B was not observed and this altered the RFLP pattern of *VspI* (Alves, 2006). In a previous study the *VspI* digestion of uncloned PCR products produced four visible bands instead of the usual two to three bands seen in other *Cryptosporidium* spp. (Xiao et al, 1999b). When the cloned products were digested, it was shown that this was the result of the presence of two types of products: one with the two-band pattern and the other with a three-band pattern (Xiao et al, 1999b). Sequence analysis of these cloned PCR products yielded two types (A and B) of SSU-rRNA sequence of each isolate with minor differences between the two sequences (Xiao et al, 1999b). Heterogeneous copies of the SSU rRNA gene are

found in all the *Cryptosporidium* species examined (Xiao et al, 1999b). In *C. felis*, when the sequence analysis of the B copy was compared with the A sequence, an insertion, a deletion and a substitution of one nucleotide was observed, resulting in an additional restriction pattern for *VspI* (Xiao et al, 1999b). Our results also correlate with two other studies (Gatei et al, 2002; Alves, 2006; Table 5). In Thailand, two *C. felis* isolates were found in HIV patients, one of them has the pattern of the A copy and the other one has the pattern of the B copy (Gatei et al, 2002). In Portugal seven *C. felis* isolates were characterized, two of them were cat isolates and the other five were human isolates (Alves, 2006, Figures 3, 4). The cat isolates presented both patterns with a preponderance of the A copy, and among the human isolates five of them have a mixed pattern; in three of them the pattern of A copy was predominant and in the other two, the predominant pattern of the B copy (Alves et al, 2006, Figure 3, 4).

The difference between the heterogeneous copies is small, therefore the impact of these differences on phylogenetic analysis is limited (Xiao et al, 1999b). Additionally, *C. felis* can be differentiated from other species by *SspI* digestion, which shows no differences between products from A and B units of the SSU rRNA gene (Xiao et al, 1999b). The hsp-70 gene has a higher heterogeneity than the SSU rRNA gene (Sulaiman et al, 2000). Therefore, sequencing analysis of the 311 bp fragment was enough for differentiate among the *C. canis* and *C. felis* and for supporting the results obtained by PCR-RFLP of the SSU rRNA gene.

In conclusion, this PCR-RFLP tool was useful in discriminating *Cryptosporidium* isolates from cats and dogs. Since we only used a small number of isolates and we did not have sequence information from the SSU-rRNA of these isolates, we are collecting

more canine and feline *Cryptosporidium* isolates to perform a larger molecular and phylogenetic analysis.

4.6 Tables

Table 1. Prevalence of *Cryptosporidium* spp. by PCR in cats and dogs

Number Examined	Location	Test	Prevalence (%)	Study
140dogs	Japan	PCR	9.3	Abe et al, 2002
450dogs	Brazil	PCR	9.5	Lallo and Fernandez-Bondan, 2006
240dogs	Italy	PCR	3.3	Giangaspero et al, 2006
40 cats	Australia	PCR	10	Mc Glade et al, 2003
421 dogs	Japan	PCR	0.3	Satoh et al, 2006
179 cats	USA	PCR	29.4	Scorza et al, 2006
113 dogs	USA	PCR	16.7	Scorza et al, 2006

Modified from Lyndsay and Zajac, 2004

Table 2: RFLP (in bp) in the SSU-rRNA gene of *C. parvum*, *C. felis* and *C. canis* genotypes

Species	Source	*Ssp*I digestion	*Vsp*I digestion
C. felis	cat	15, 33, **390, 426**	102, **104, 182, 476**
C. canis	dog	20, 33, **105, 254, 417**	94, **102, 633**
C. parvum	Bov A	11, 12, **108, 254, 449**	102, **104, 628**
C. parvum	Bov B	9, **119, 254, 449**	102, **104, 625**

Numbers in bold are the sizes of bands visible on the electrophoresis gel
From Xiao et al, 1999a

Table 3: Summary of the genotype results of the *Cryptosporidium* isolates identify in this study

Sample ID	Species	HSP-70	SSU-rRNA	
		Seq. Result	*SspI*	*VspI*
98	feline	*C. felis*	*C. felis*	*C. felis* A+
178	feline	-	*C. felis*	*C. felis* A+
240	canine	*C. canis*	*C. canis*	*C. canis*
192	feline	*C. felis*	*C. felis*	*C. felis* A+
227	feline	*C. felis.*	*C. felis*	*C. felis* A+
238	feline	*C. felis*	*C. felis*	*C. felis* A+
133	canine	-	*C .canis*	*C. canis*
230	feline	*C. felis*	*C. felis*	*C. felis* A+
401	feline	*C. felis*	*C. felis*	*C. felis* B+
163	feline	*n.a.*	*C. felis*	*C. felis* A+
446	feline	*C. felis*	*C. felis*	*C. felis* A+
205	canine	*C. canis*	*C. canis*	*C. canis*
243	feline	-	*C .felis*	*C.felis* A+
325	feline	-	*C. felis*	*C. felis* A+
233	feline	*C. felis*	*C. felis*	*C. felis* A+
232	feline	*C. felis.*	*C. felis*	*C. felis* A+
404	canine	*C. canis*	*C .canis*	*C. canis*
244	feline	-	*C. felis*	*C. felis* A+

References: A+: copies A and B present, with predominance of the A copy;
B+: copies A and B present, with predominance of the B copy
-: non available

Table 4: Occurrence of *C. felis* and *C. canis* in immunocompetent individuals

Country	Cases (numbers)	*C. felis*	*C. canis*	*Genotyping technique*	*Notes* (RFLP fragment size)	*Refs*
Czech Rep.	Not known (3)	1		SS rRNA/Seq		
				HSP-70		Ryan et al, 2003
England	Various (2414)	6	1	SS rRNA/Seq		Cacciò et al, 2005
Peru	Children (118)	4	9	SS rRNA/RFLP		Xiao et al, 2001
New Zealand	(423)		2	SS rRNA/RFLP		Learmont et al, 2004

Table 5: Occurrence of *C. felis* and *C. canis* in immunocompromised individuals

Country	Cases (numbers)	*C. felis*	*C. canis*	*Genotyping technique*	*Notes* (RFLP fragment size)	*Refs*
Thailand	(29)	1		SSU rRNA/Seq		Tiangptip and Jongwutiwes,2002
Thailand	(34)	2	2	SSU rRNA/RLFP	A: 658, 104, 102(human)	
				and Seq	B: 477, 181, 104, 102(human)	Gatei et al, 2002
Peru	(229)	10	12	SSU rRNA/RFLP		Cama et al, 2003
USA	(10)	3	1	SSU rRNA/Seq		Pieniazek et al, 1999
Italy	(9)	1		SSU rRNA/Seq		
				COWP/RFLP		Cacciò et al, 2002
France	(46)	6		SSU rRNA/Seq		Guyot et al, 2001
Switzerland	(13)	3		SSU rRNA		
				HSP-70		
				Acetyl-Coa		Morgan et al, 2000b
Portugal	(40)	4		SSU rRNA/RFLP		Matos et al, 2004
Portugal		7		SSU rRNA/RFLP	A+: 658, 104, 102 (2 cats)	
					A+: 658, 104, 102 (3 humans)	
					B+: 476, 182, 104, 102 (2 humans)	Alves, 2006
England	(27)	2		SSU rRNA		
				COWP		Cacciò et al, 2005

Modified from Cacciò et al, 2005: Unraveling *Cryptosporidium* and *Giardia* epidemiology Trends Parasitol 2005;21(9):430-437
References: A+: copies A and B present, with predominance of the A copy; B+: copies A and B present, with predominance of the B copy

4.7 Figures

Figure 1: Electrophoretic separation of the SSU rDNA nested products (834bp) after restriction digestion with the endonuclease *SspI*. Lane m1: 100 bp marker, lane m2: 50 bp marker, lanes C: *C. canis*, lanes F: *C. felis*

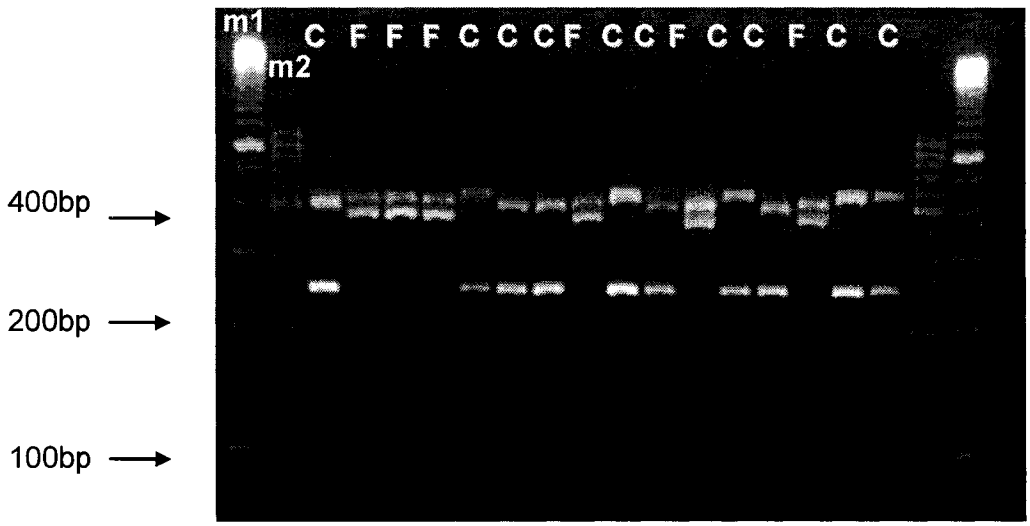


Figure 2: Electrophoretic separation of the SSU rDNA nested products (834bp) after restriction digestion with the endonuclease *VspI*. Lane m1: 100 bp marker, lane m2: 50 bp marker; lanes C: *C. canis*, lanes F: *C. felis* A+: *C. felis* isolates with copies of A and B, with predominance of the A copy.

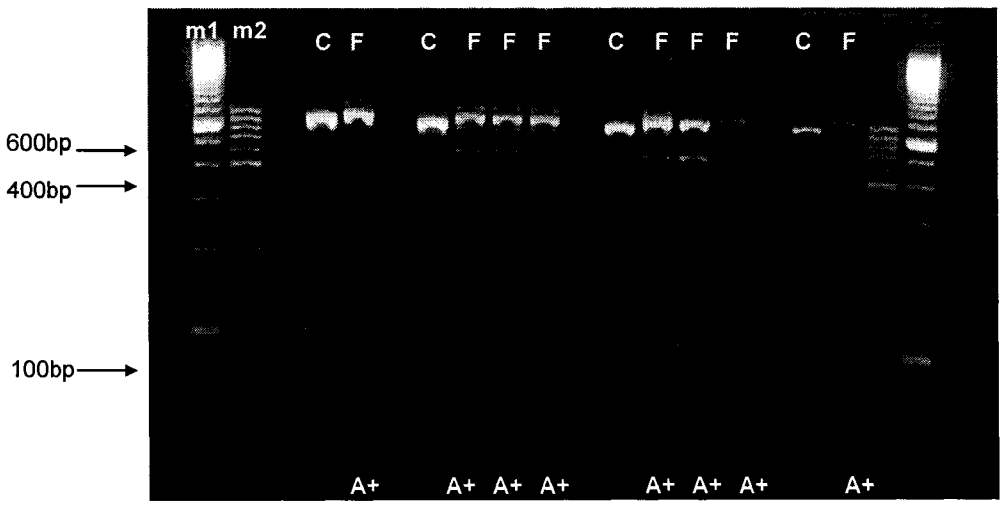


Figure 3: Electrophoretic separation of the SSU rDNA nested products (834bp) after restriction digestion with the endonuclease *VspI*. These are *C. felis* isolates from Portuguese humans included as a comparison of our *C. felis* isolates. Lane m: 100bp marker, lanes Cf A: *C. felis* isolates with copies of A and B, with predominance of the A copy, lanes Cf B: *C. felis* isolates with copies of A and B, with predominance of the B copy. From Alves, 2006.

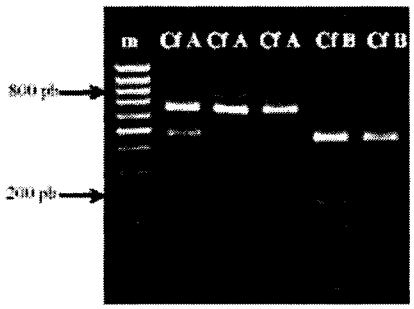
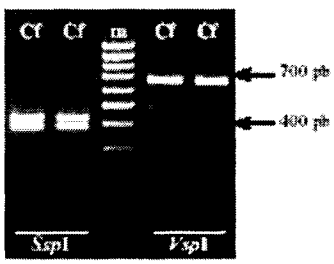


Figure 4: Electrophoretic separation of the SSU rDNA nested products (834bp) after restriction digestion with the endonucleases *SspI* and VspI . These are *C. felis* isolates from Portuguese cats included as a comparison of our *C. felis* isolates. Lane m: 100bp ladder, lanes Cf A: *C. felis* isolates with copies of A and B, with predominance of the A copy, lanes Cf B: *C. felis* isolates with copies of A and B, with predominance of the B copy. From Alves, 2006.



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CHAPTER 5

Genotyping of *Giardia duodenalis* isolates from dogs and cats in the United States

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For submission to the Journal of Parasitology

5.1 ABSTRACT: Cats and dogs are infected with morphologically indistinguishable strains of *G. duodenalis*. In the United States, the prevalence of giardiasis in dogs and cats ranges from 7 to 26%, however in most of the studies genotyping of the isolates was not performed. This study genetically characterized *G. duodenalis* isolates of dogs from the United States at the *β-giardin* and at the *glutamate dehydrogenase* (gdh) genes by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and by sequence analysis. Thirty six dog samples and two cat samples were analyzed. Based on the PCR-RFLP and sequence analysis at the *β-giardin* gene, 32 of the dog isolates were assemblage D and 4 were assemblage C. The two cat samples analyzed were assemblage A. These results were supported by the gdh assay with the exception of two discordant results. Our study showed that all the *G. duodenalis* isolates present in 36 dogs of the United States are dog-specific genotypes and that the cats were infected with the zoonotic genotype. To determine *G. duodenalis* zoonotic potential from companion animals it is crucial to genetically characterized isolates from cats and dogs.

5.2 INTRODUCION

Giardia spp. are one of the most common parasites in domestic animals. Dogs and cats are infected with morphologically indistinguishable strains of *G. duodenalis*. Molecular genetic studies, however, demonstrated that *G. duodenalis* is a species complex comprising at least seven major assemblages (Monis and Thompson, 2003). Assemblage A and B are found in humans and in other mammals including livestock and pet animals (Monis and Thompson, 2003). The other genetic assemblages within the *G. duodenalis* group are confined to specific animal hosts, like cats (assemblage F) and dogs (assemblages C and D) (Monis and Thompson, 2003). The World Health Organization

declared *Giardia* a zoonotic pathogen in 1979. The role of companion animals in zoonotic transmission of giardiasis is beginning to be unraveled as a result of the molecular characterization of their isolates. Several studies genotyped *Giardia* isolates from dogs and cats world wide using different techniques and genetic markers (Hopkins et al, 1997, Monis et al, 1999, Traub et al, 2002, van Keulen et al, 2002, Abe et al, 2003, Sulaiman et al, 2003, Read et al, 2004, Itagaki et al, 2005, Vasilopulos et al, 2004) (Table1). The first evidence of zoonotic transmission from pets was reported recently between a dog and a human living in the same house (Traub et al, 2004). In the United States, the prevalence of giardiasis in dogs and cats ranges anywhere from 2.4 to 26%, however in most of these studies genotyping of the isolates was not performed, so the zoonotic risk is unknown (Jafri et al, 1993, Hills et al, 2000, Spain et al, 2001, Zislin et al, 2002).

The objective of this study was to genetically characterize *G. duodenalis* isolates of dogs and cats from the United States at the β-giardin and gdh genes by PCR-RFLP and by sequence analysis.

5.3 MATERIALS AND METHODS

5.3.1 Source of isolates: Fecal samples from dogs and cats positive for *Giardia* spp. by IFA, ELISA or, microscopic examination after Zn SO_4 flotation, were used in this study. The majority of the samples were identified at a parasitology laboratory (Antech Diagnostics, New York). The rest of the samples were submitted for eggs and ova examination to the Colorado State University Diagnostic laboratory and some of the cat isolates were donated by Dr. Lora Ballweber from the Mississippi Veterinary Research & Diagnostic Laboratory. The samples were processed on arrival.

5.3.2 DNA Isolation: Giardia spp. cysts were concentrated following a standard sucrose centrifugal- flotation protocol before DNA extraction. An aliquot (300 µl) of the supernatant was homogenized using the Mini-BeadBeater (Biospec Products, Bartlesville, Oklahoma) for the disruption of the cysts and DNA was extracted using the FastDNA kit (Qbiogene, Irvine, California).

5.3.3 PCR-RFLP of the β-giardin locus and sequencing analysis: Giardia cati (cat #: 50163, ATCC, Manassas, Virginia) DNA and DNA from assemblages A1, A2, B and E donated by Dr. Simone Cacciò were included for comparison. The amplification of a fragment of 511-bp of the *β-giardin* was performed following a previously published protocol (Lalle et al, 2005 a). For the first amplification, 2µl of DNA were amplified using the primers BGF116 (5'-AGC AAC ATG AAC CAG CGC GTC AG-3') and BGR725 (5'-GCC TTC TCG CGG TCG ATC TCC TC-3') designed by Dr. Cacciò, in 25µl of PCR water and 25µl of HotStart Quiagen Master Mix (Quiagen, Valencia, California). Two µl of the first amplification product were amplified using the primers BGF115 (5'-GAA CGA GAT CGA GGT CCG-3') and BGR 648 (5'-CTC GAC GAG CTT CGT GTT-3') (Lalle et al, 2005 a). DNA amplification was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, California). The thermal protocol was: 95C for 15 minutes, followed by 35 cycles of 95°C for 30 seconds, 55C for 30seconds and at 72°C for 1 minute followed by 7 minutes at 72 C. Ten µl of the *β-giardin* PCR products were digested using *Hae III* (New England Biolabs INC., Beverly MA, USA) as previously published (Cacciò et al, 2002).

Following the restriction enzyme digestion, 7 µl of each sample were analyzed using Methaphor ® Agarose (Cambrex, Rockland, Maine) gels in TBE buffer, stained with

ethidium bromide (40 μg/μl) and viewed by ultraviolet transilluminator. DNA sequences were analyzed both forward and reverse direction using an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, California). The sequenced b-giardin fragments were compared with those in the Genebank database by BLAST analysis.

5.3.4 PCR-RFLP of the gdh locus: The amplification of a 432 fragment of the gdh locus was performed following a previously published protocol (Read et al, 2004). For the first amplification, 2μl of DNA were amplified using the primers GDHef (5'-TCA ACG TYA AYC GYG GYT TCC GT-3') and GDHiR (5'-GTT RTC CTT GCA CAT CTC C-3') in 25μl of PCR water and 25μl of HotStart Quiagen Master Mix (Quiagen, Valencia, California). Two μl of the first amplification product were amplified using the primers GDHif (5'-CAG TAC AAC TCY GCT CTC GG-3') and GDHiR (Read et al, 2004) in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, California). The thermal protocol is 95C for 15 minutes, followed by 35 cycles of 95°C for 30 seconds, 55C for 30seconds and at 72°C for 1 minute followed by 7 minutes at 72 C. PCR products were gel extracted using the PCR gel extraction or a purification kit (Quiagen, Valencia, California). DNA sequences were analyzed both forward and reverse direction using an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Five of the gdh PCR products were digested using *Nla IV*(New England Biolabs INC., Beverly MA, USA) (Read et al, 2004).

Following the restriction enzyme digestion, 7 μl of each sample were analyzed on 2% agarose gels in TBE buffer, stained with ethidium bromide (40μg/μl) and viewed by ultraviolet transilluminator. DNA sequences were analyzed both forward and reverse direction using an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City,

California). The sequenced b-giardin fragments were compared with those in the Genebank database by BLAST analysis.

5.4 RESULTS

5.4.1 PCR-RFLP of the β-giardin locus and sequencing analysis: Amplification of the nested b-giardin produced the expected 511bp product (data not shown). A total of 38 samples were processed, 36 were canine samples and two were feline samples (Table1). Of the 36 canine samples, 32 belonged to assemblage D and four to assemblage C. The two feline samples were assemblage A.

Restriction digestion using *Hae III* yielded the expected patterns for assemblage A (fragments of 186, 150, 110, and 50bp), for assemblage C (fragments of 194, 150, 102, 50 bp) and for assemblage D (fragments of 200, 194, 117 bp). These patterns were identified by gel electrophoresis. Each sample was amplified and cut by restriction enzymes at least twice. The results are displayed in Table 2 and Figure 1.

5.4.2 PCR of the GDH locus and sequencing analysis: Amplification of the nested gdh gene produced the expected 432 bp product (data not shown). A total of 33 samples were processed, 32 were canine samples and only one feline sample (Table1). Of the 32 canine samples, 30 belonged to assemblage D and two to assemblage C. The feline sample was assemblage A. The restriction enzyme cuts yielded the expected patterns for *Nla IV*, for assemblage A1 (150,120, 90 bp) and for assemblage C (190, 120, 70 bp) bands and for assemblage C. Among assemblage D, different patterns were observed including (250, 120 bp as reported) (Read et al, 2004), (250,100bp) and (250, 120, 100 bp). The results are displayed in Table 2 and Figure 2.

The sequences were compared with those in the Genebank database by BLAST analysis and the results matched the RFLP patterns.

5.5 DISCUSSION

Giardiasis is a very prevalent disease in pets worldwide (Thompson and Robertson, 2003). To determine *G. duodenalis* zoonotic potential from companion animals it is crucial to genetically characterized isolates from cats and dogs. Most of the genetic characterization studies used PCR-RFLP, Random Amplified Polymorphism DNA (RAPD), M13 fingerprinting and nucleotide sequencing. The assays used in this study had been previously used on cat and dog samples and were demonstrated to be highly specific and to have a very good correlation when compared with other genetic markers (Cacciò et al, 2002, Lalle et al, 2005a, 2005b). The use of the β - giardin gene has several advantages including high specificity, since giardin genes are unique to *Giardia* species, high sequence heterogeneity which provide the same resolution than when using a multilocus typing system, and a very good correlation when compared with other genetic markers (Cacciò et al, 2002; Lalle et al, 2005a). Gdh, as well as β-giardin, also has been widely used in a variety of publications (Abe et al, 2003; Monis et al, 1996; 1998; Homan et al, 1998; Rimhanen-Finne et al, 2002; Read et al, 2004). Additionally, gdh has also a very high discriminatory power (Read et al, 2004). The objective of the majority of the studies previously mentioned was the genetic characterization of *G. duodenalis* isolates. However, the relationship among isolates and the biological significance of this genetic heterogeneity is still not clear (Thompson and Monis, 2004).

The dogs in this study were infected with host specific genotypes and the two cats were infected with the zoonotic genotype. The genotyping results of all the isolates with

the exception of isolates NY4 and NY33 correlates by the β – giardin and gdh markers. Other researchers also occasionally found discordant genotyping results (Read et al, 2004; Traub et al, 2002). In one study, *Giardia* isolates from various animals were compared at the gdh gene and at the 18S rDNA genes (Read et al, 2004). Of 21 cats, two were assemblage D by the 18S rDNA PCR assay and assemblage B by the gdh PCR assay. Two other isolates were assemblage D by the 18S rDNA PCR assay and assemblage C by the gdh PCR assay (Read et al, 2004). Among nine dogs, one was assemblage D by the 18S rDNA PCR assay and assemblage B by the gdh PCR assay, another one was assemblage C by the 18S rDNA PCR assay and assemblage B by the gdh PCR assay, and two dogs were assemblage D by the 18S rDNA PCR assay and assemblage C by the gdh PCR assay (Read et al, 2004). Eleven out of 29 human isolates also showed discordant results; three isolates were assemblage D by 18S rDNA PCR assay and assemblage B or A by the elongation factor-1 (ef1-α) and by the triosephosphate isomerase (tpi) PCR assays (Traub et al, 2004). Seven samples were assemblage C by the 18S rDNA PCR assay and assemblage B or A by ef1-α and tpi PCR assays (Traub et al, 2004).

Our two discordant results might be attributed to introgression and retention of ancestral polymorphisms (Anderson, 2001). These events can result in divergent alleles segregating in the same population and identical alleles occurring in genetically distinct population or species (Anderson, 2001). Additionally, these two samples justify the need to perform multilocus analysis (Anderson, 2001). Mixed infections in which one assemblage predominates over the other can also explain discordant results. However, no evidence of mixed infection was found in the chromatograms.

Previous genotyping studies showed different results according to the different habits of people and dogs in their habitats. When dogs tend to be grouped together, competitive interaction of the genotypes are likely to guarantee that the host-specific genotype predominate in respective host species (Bugg et al, 1999; Hopkins et al, 1997). In Aboriginal communities in Australia dogs were most commonly infected with the dog genotype (Hopkins et al, 1997). Dogs from multi-dog house holds were more likely infected with the dog-specific genotype of *Giardia* than dogs in single-dog houses (Bugg et al, 1999).

When *Giardia* infections are common in humans and dogs, dogs would have equal contact with the zoonotic and the dog-specific genotypes (Traub et al, 2002). In urban areas in Australia, the zoonotic genotype and the dog-specific genotype are equally common in dogs (Thompson et al, 1999). In a community in India where humans and dogs were commonly infected with *Giardia,* 20% of the dogs were infected with the zoonotic genotype (Traub et al, 2002). In Japan, dogs harbor both the dog specific and the zoonotic genotype while cats were infected with the cat-specific genotype (Abe et al, 2003; Itagaki et al, 2005). In Italy, two studies found that most of the dogs harbor the dog-specific genotypes but also some dogs harbor the zoonotic genotype (Lalle et al, 2005a; Berrilli et al, 2004). In Mexico, two studies demonstrated that the dogs tested were infected with the zoonotic genotype (Lalle et al, 2005a; Eligio-Garcia et al, 2005). In Colombia, six cats were infected with the cat specific genotype (Santin et al, 2006).

Our results described the *Giardia* genotypes found in dogs and cats in the USA, without making any inference regarding their zoonotic potential. Unfortunately, we did not know if the samples examined come from household dogs, or shelter dogs or which

region in particular. We also did not have information regarding the health status of the pet owners or whether they live in close contact with their dogs. So, our data just report the genotypes of the dogs evaluated without making any inference regarding their zoonotic potential.

The fact that similar genotypes are dispersed in different hosts is not formal evidence of zoonotic transmission. Samples from a study in India were characterized at three loci and provided the first evidence of zoonotic transmission between a dog and a human in the same house (Traub et al, 2004). To profoundly establish the role of companion animals in the transmission of giardiasis, more studies that concurrently genotype isolates from animals and humans living in close contact should be performed

Acknowledgments: This study was founded by a grant provided by Bayer Animal Health. The authors also thank Antech Diagnostic Laboratories for supplying some of the isolates analyzed in the study.

5.6 TABLES

Table 1: Genotypes of *G. duodenalis* isolates from dogs and cats

Country	Reference	Genetic Marker	Species	Assemblage
Australia	Hopkins et al, 1997	SSU-rRNA (Seq)	Dogs n=5	4 dog genotype 1=dog+human
	Monis et al, 1998	GDH (Seq)	Dogs n=11	10 C 1 D
	Monis et al, 1999	Multilocus	Dogs n=3	2 C 1 D
	Read et al, 2004	GDH (RFLP, Seq) SSU-rRNA (Seq)	Dogs n=9	1 A1 2 BIV 4 C 2 D
			Cats n=21	9 AI 2 BIV 2 C 7 D 1 E
	Mc.Glade et al, 2003	SSU-rRNA (Seq)	Cats n=14	group4 (~dog genotype)
India	Traub et al, 2002	EF1-α (RFLP) SSU rRNA (RFLP) TPI (RFLP)	Dogs n=20	(1 AI, 4 AII, 6 B,3 AII/B) (4B, 10A) (3 AII, 2 AI, 2 BIII/IV)
Japan	Abe et al, 2003; Itagaki et al, 2005	GDH (RFLP, Seq) GDH (Seq)	Dogs n=4 Dogs n=24	C, D 14 A 3 A/D 1 C 6 D
			Cats n=3	F
Mexico	Lalle et al, 2005; Eligio-Garcia et al, 2005	β-Giardin (RFLP, Seq) SSU-rRNA (Seq)	Dogs n=5 Dogs n=11	AI A
Italy	Lalle et al, 2005;	β-Giardin (RFLP, Seq)	Dogs n=21	6 A 1 C 13 D 1 A/D
			Cats n=1	F
	Berrilli et al, 2004	SSU-rRNA (Seq)	Dogs n=17	11 C 2 A 1D 2 A/C 1 C/D
			Cats n=1	A
Colombia	Santin et al, 2006	SSU-rRNA (Seq)	Cats n=6	F
The Netherlands	van der Giessen et al, 2006	SSU-rRNA (Seq) GDH (Seq)	Dogs n=2	D
USA	van Keulen et al, 2002	SSU-rRNA (Seq)	Dogs n=15	8A 7B
			Cats n=9	6A 3B
	Sulaiman et al, 2003	TPI (RFLP-Seq)	Dogs n=15	C
	Vasilopulos et al, 2004	GDH(Seq)	Cats n=6	4A 2F

Ref : RFLP: PCR-RFLP, Seq: sequencing analysis

Table 2: Genotypes of the *G. duodenalis* isolates used in this study

Sample ID	Species	β-giardin RFLP	β-giardin Sequence	GDH RFLP	GDH Sequence
Feline 7.14.05	fel	A	-	-	
NY1	can	D	D	D	D
NY4	can	C	C	D	D
NY5	fel	A	A	A	A1
NY6	can	D	D	D	D
NY13	can	D	D	D	D
NY14	can	D	D	-	-
NY15	can	C	C	C	C
NY16	can	D	D	D	D
NY21	can	D	D	D	D
NY22	can	D	D	D	D
NY23	can	D	D	D	D
NY24	can	D	D	D	D
NY26	can	D	D	D	D
NY27	can	D	D	D	D
NY29	can	D	D	D	D
NY32	can	D	D	D	D
NY33	can	C	C	D	D
NY34	can	D	D	D	D
NY35	can	D	D	D	D
NY36	can	D	D	D	D
NY37	can	D	D	-	-
NY39	can	D	D	-	-
NY40	can	D	D	D	D
NY41	can	D	D	D	D
NY43	can	D	D	D	-
NY44	can	D	D	D	D
NY45	can	C	C	C	C
NY50	can	D	D	D	D
NY52	can	D	D	D	D
NY53	can	D	D	D	D
Giardia1 (2.22.06)	can	D	D	D	-
Giardia2 (2.22.06)	can	D	D	D	-
Giardia3 (2.22.06)	can	D	D	D	-
Giardia4 (2.22.06)	can	D	D	D	-
67	can	D	D	D	D
68	can	D	D	D	D
Giardia 6.3.06	can	D	D	-	-

Ref: -:not available

5.7 FIGURES

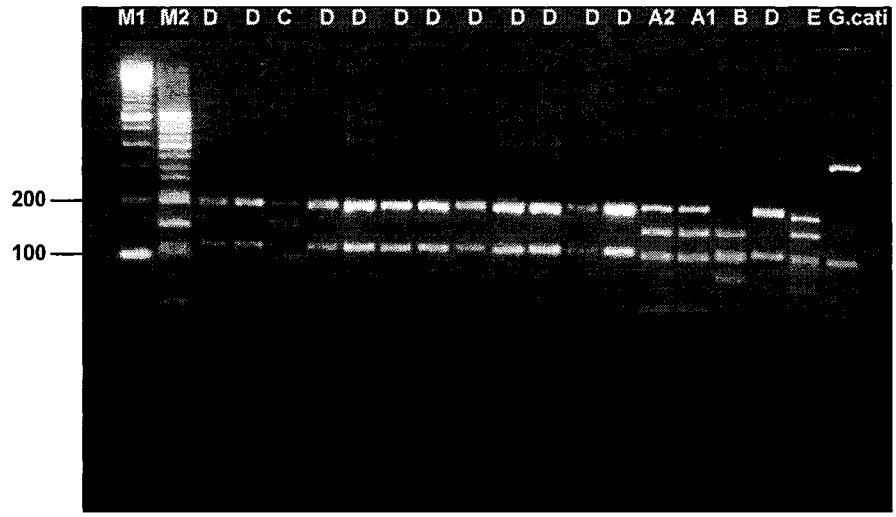

FIGURE 1: Electrophoretic separation of the b-giardin nested products (511bp) after restriction digestion with the endonucleases *Hae III.* Lanes M1: 100 bp marker, M2:50 bp marker, A1: assemblage A1, A2: assemblage A2, B: assemblage B, C: assemblage C, D: assemblage D, E: assemblage E, G. Cat isolate 50163, ATCC, Manassas, Virginia

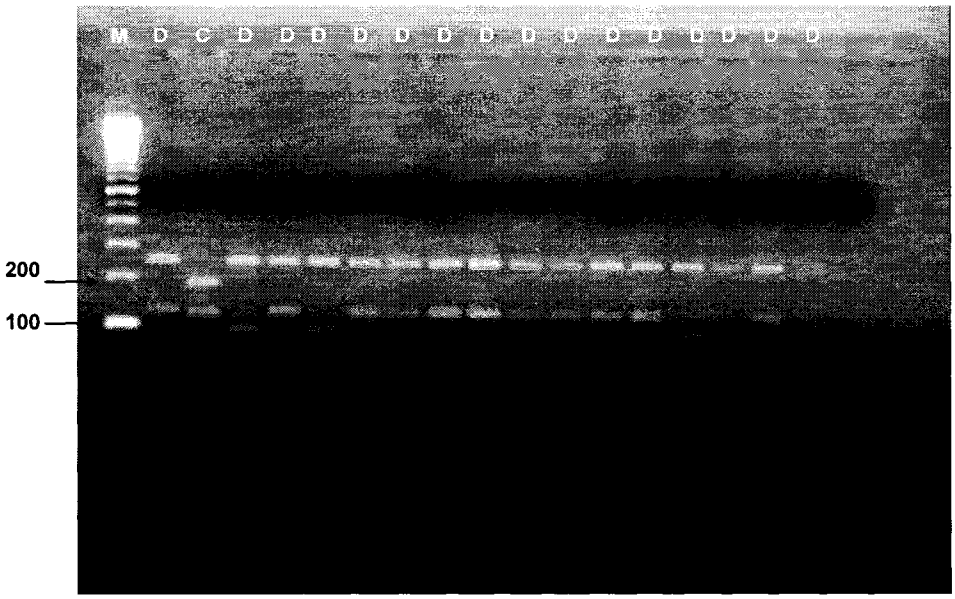

FIGURE 2: Electrophoretic separation of the gdh nested PCR products (432 bp) after restriction digestion with the endonuclease *Nla IV*. Lanes: 100 bp ladder, C: assemblage C, D: assemblage D.

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Cryptosporidium spp. and *Giardia* spp. coinfections in four naturally-infected cats and a dog

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For submission to the Journal of the Veterinary Medical Association

Structured Abstract:

Case Description: Clinical management of four cats and a dog co-infected with *Cryptosporidium* spp. and *Giardia* spp..

Clinical Findings: The four cats had a history of approximately 5 months of chronic mixed bowel diarrhea that failed to respond to food trials and multiple conventional treatment protocols. The dog had bloody diarrhea, anemia and interstitial pneumonia.

Treatment and Outcome: Attempted treatments varied by the case but drugs used included azithromycin, fenbendazole, metronidazole, nitazoxanide, and tylosin. Based on results of an in vitro direct immunofluorescence assay[a] (IFA), all five animals became negative for *Giardia* spp. and *Cryptosporidium* spp., However, the two cats evaluated by polymerase chain reaction (PCR) assay remained positive for *Cryptosporidium* spp. and one cat was IFA positive for *Giardia* spp. and *Cryptosporidium* spp. 5 days after discontinuing therapy.

Clinical Relevance: Dogs and cats with chronic diarrhea that do not respond to conventional therapeutic trials should be screened for *Cryptosporidium* spp. and *Giardia* spp. infection. Clinical signs in animals infected with both parasites can be difficult to control. Administration of drugs with presumed activity against *Cryptosporidium* spp. and *Giardia* spp. may not eliminate infection and reinfections may be common since these parasites do not cause permanent immunity.

This report describes the clinical management of four cats and a dog suspected to have diarrhea from *Cryptosporidium* spp. and *Giardia* spp. coinfection. These cases were managed between 2002 and 2005.

Cats 1 (1 year old, domestic longhair, male neutered) and 2 (3.5 month old domestic longhair, male neutered) lived in the same home in north-central Colorado and were suspected to have chronic diarrhea from dual infection with *Cryptosporidium* spp. and *Giardia* spp. infection, documented by IFA.[a] At the time of the submission of this dissertation, the source of the cats was unknown. Both cats had been administered several conventional treatments (drugs and doses unavailable) but the diarrhea did not resolve and so the cats were entered into an azithromycin treatment study at Colorado State University. Physical examinations were normal in both cats. Because of the dual infection and chronic diarrhea, the cats were evaluated for evidence of immunodeficiency by performing a complete blood cell count, serum biochemical panel, urinalysis, and CD4+ and CD8+ lymphocyte counts. The only abnormalities detected were an elevated neutrophil count (12,500/μl; N = 2,000-12,000/μl) and a slight increase in ALP activity (75 IU/L; N = 7-65 IU/L) in Cat 1. Prior to administration of azithromycin[b] (10 mg/kg PO daily for 14 days), three fecal samples per cat were collected (pre-treatment) for evaluation for *Cryptosporidium* spp. DNA in feces by PCR assay,[1] *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts by IFA,[a] and trophozoites of *Giardia* spp. and *Tritrichomonas foetus* by wet mount examination (Table 1). Samples were then collected daily during the treatment period (intra-treatment), and then three times per week during the week after treatment (post-treatment). Diarrhea did not resolve in either cat, both cats remained positive for *Cryptosporidium* DNA by PCR assay, and for *Giardia* spp. cysts

by IFA. Paromomycin[c] was then administered at 500 mg/cat PO daily for five days. After treatment, the consistency of the feces of both cats was normal and both cats were negative for *Giardia* spp cysts by IFA testing, but both cats remained positive for *Cryptosporidium* spp. DNA by PCR assay. In an attempt to resolve *Cryptosporidium* spp. oocyst shedding, paromomycin was administered again one month later. After administration of paromomycin, follow up fecal tests were performed over 4 weeks (7 samples) and one week (2 samples) for the first and second treatment periods, respectively (Table 1). After treating the cats with both azithromycin and paromomycin, both cats became negative for *Giardia* spp. and *Cryptosporidium* spp. by IFA but remained PCR assay positive for *Cryptosporidium* spp. DNA in feces (Table 1). Although parasitologic clearance was not achieved, the feces of both cats became harder. After the administration of the last treatment, (approximately 4 years) the owner reported that cats 1 and 2 are healthy and they did not have any other episodes of diarrhea.

Cat 3 (3 months old, domestic medium hair male) and Cat 4 (6 months old, domestic longhair male neutered) also lived in the same house in north-central Colorado after adoption from a local animal shelter. The two kittens had a history of approximately 5 months of chronic mixed bowel diarrhea and that had not resolved after diet changes or administration of metronidazole, amoxicillin-clavulanate,[d] fenbendazole [e] or sulfadimethoxine.[f] Over a 5 month period before consultation with the authors, the referring veterinarian had shown both cats to be negative for FeLV antigen,[g] FIV antibodies,[g] *Salmonella* spp. and *Campylobacter* spp. on fecal culture, and gastrointestinal parasites by fecal flotation. However, *Giardia*-like organisms were noted on a fecal direct, infrequent *Clostridium* spores were seen on fecal cytology, feces was

positive for antigen of *Cryptosporidium* spp.[h] and *Giardia* spp.[h] by ELISA, and feces was positive for *C. perfringens* enterotoxin.[h] One of the authors (MRL) was then consulted and recommended treating both cats with tylosin[i] at 15 mg/kg, PO, q12hr, for 7 days. However, no significant improvement in clinical signs was seen and it was difficult to administer this drug to the cats. A few days after stopping tylosin, fecal samples were collected and sent to our laboratory at Colorado State University for performance of *Cryptosporidium* and *Giardia* IFA;[a] both cats were positive for both organisms (Table 2) The fecal samples were stored at -20°C and PCR assay for *Cryptosporidium* spp. was performed 6 months later; both cats were positive.[1] After performance of the IFA test, azithromycin [b] administered at 10 mg/kg PO daily for 10 days was prescribed but was discontinued after three days because the diarrhea worsened. Because Cat 3 was having bloody diarrhea, metronidazole was administered by the referring veterinarian again to both cats. After two weeks of metronidazole therapy, the consistency of the feces improved in both cats but the diarrhea returned when the metronidazole was discontinued. The authors had performed experiment work (unpublished data) with nitazoxanide, a drug with anti-*Giardia* and anti-*Cryptosporidium* activity in people. [2, j] One of the authors (MRL) supplied an experimental formulation of the compound and it was administered at 50mg/kg PO daily for 5 days. If the diarrhea did not resolve by day 14, the dose of the nitazoxanide was to be increased to 100 mg/kg, PO daily for another 5 days. However, the diarrhea become worse in both cats and the drug was also difficult to administer, therefore nitazoxanide was discontinued. Based on IFA testing at that time, cat 3 was positive for *Giardia* spp. but negative for *Cryptosporidium* spp. and cat 4 was positive for both *Cryptosporidium* spp. and *Giardia* spp. One week later, paromomycin [c]

was administered at 150 mg/kg PO daily for 5 days. The fecal consistency immediately after treatment was thicker and samples collected on days 5 and 10 post-treatment after paromomycin showed that both cats were still positive by *Giardia* spp. by IFA on day 5 post-treatment and then they became negative for *Cryptosporidium* spp. and *Giardia* spp. by IFA on day 10 post-treatment. Because of the partial improvement in stool consistency, it was recommended that paromomycin be continued for another 10 days with fecal samples assayed on days 15, 20, 25, 30 and 36 after the first administration of paromomycin. After ten days of paromomycin administration, both cats were negative for *Giardia* and *Cryptosporidium* by IFA (Table 2). After 20 days of administration, paromomycin was discontinued in cat 3 because blood was again detected in the stool and in cat 4 because no further improvement had been noted. At that time a semi-moist diet was begun, *Tritrichomonas foetus* culture [3] was submitted, and orbifloxacin [k] was administered at 22.7 mg/cat, daily for 10 days for possible *T. foetus* coinfection.[4] The *T. foetus* culture was negative in both cats but the feces of both cats were thicker after 7 days of orbifloxacin therapy and so the medication was discontinued. On day 31 post-treatment with paromomycin, cat 3 was still positive for *Giardia* spp. by IFA and on day 35 post-treatment cat 4 was positive for *Cryptosporidium* spp. and *Giardia* spp. by IFA. The consistency of the feces was from soft to normal in cats 3 and 4 (Table2). We are currently attempting to contact the owner to have information about the health status of cats 3 and 4.

Case 5 was a 3 month old female Papillon dog. On arrival to the referring veterinarian's clinic, the dog was depressed and lethargic, had bloody diarrhea, and was positive for *Giardia* spp. cysts on fecal examination. The dog was administered 0.45%

NaCl and 2.5% dextrose, sucralfate [l] (¼ tablet PO), famotidine (0.4mg) IV and cefazolin (20mg) IV. The initial hemogram showed anemia and chest radiographs showed possible interstitial pneumonia and mild distention of the cervical esophagus. The next day the dog was improved and so was discharged with instructions to administer metronidazole at 25 mg/kg, PO, q12hrs for 7 days for the *Giardia* spp. infection, amoxicillin-clavulanate[d] at 20.6 mg/kg, PO, q12hrs for 7 days for the suspected interstitial pneumonia, sucralfate for the bloody diarrhea, and a vitamin supplement. After 10 days, the diarrhea was still present and grossly appeared to contain blood and mucous. The dog was active but hardly eating and drinking; metronidazole was administered for another week. After two weeks of metronidazole administration, the dog was still positive for *Giardia* spp. on fecal flotation and one *Strongyloides* larvae were also noted. After administration of metronidazole for another 5 days, 222 mg of fenbendazole[m] per day for 5 days was added to the food. Chest radiographs made at that time showed that the pneumonia was resolving. Clavamox was administered for 2 more weeks and orbifloxacin (2.8mg/kg PO daily for 28 days) and nebulizations with saline and amikacin were added to the treatment regimen. The next fecal flotation was still positive for *Giardia* spp. and so fenbendazole was prescribed for an additional 14 days. The referring veterinarian also added psyllium[n] to the food and recommended cleaning the environment with a quaternary ammonium compound. Two weeks later the fecal flotation remained positive for *Giardia* spp. cysts and so the dose of metronidazole was increased to 62.5 mg, PO q12hrs for 5 days while continuing the administration of fenbendazole. A CBC and a chemistry panel were performed in order to discard any underlying immunodeficiency. Abnormalities noted included a lymphocytosis (5,311/μl; N = 690-4500 /μl) and hypoproteinemia (Total

protein 4.7 g/dl: N = 5-7.4 g/dl). Amoxicillin-clavulanate (for an additional 14 days), orbifloxacin (for an additional 19 days), metronidazole (for an additional 5 days), and fenbendazole (for an additional 14 days) therapies were continued. Meantime, the constituency of the feces was still soft. One month later, a *Giardia* vaccine[o] was administered and fenbendazole prescribed at 222 mg daily for 90 days was begun. However, the stool consistency remained soft and the next fecal float remained positive for *Giardia* spp. At that time one of the authors (MRL) was consulted and it was recommended that the dog be evaluated for *Cryptosporidium* spp. coinfection. An IFA confirmed that the dog was coinfected with *Giardia* spp. and *Cryptosporidium* spp. and tylosin was administered at 11 mg/kg, PO, q12hr for 28 days. By day 9 of the treatment, stools were normal and the dog was negative for both parasites by IFA. The medication was discontinued and a week later the dog appeared to be healthy.

Cryptosporidium spp. and *Giardia* spp. are common parasites found around the world. In studies of dogs and cats performed in the United States, the prevalence rate of *Cryptosporidium* spp. is between 2 and 17% [5-12] and the prevalence of *Giardia* spp. is between 2.4% and 26.5% for dogs.[6,7,11,13] However, while infection by either organism is common, dual infection seems to be less common. To our knowledge only 5 studies have reported *Cryptosporidium* spp. and *Giardia* spp. co-infections in cats or dogs. [14,15-18] In the previous work performed on dogs and cats with chronic diarrhea in our laboratory (See Chapter 3 of this dissertation), only 7 of 292 animals were positive for both *Cryptosporidium* spp. and *Giardia* spp. infection[14] In addition, there have been few case reports of co-infection of *Giardia* spp. and *Cryptosporidium* spp. in humans,[19-20] calves,[21] and horses.[22] However, both organisms are transmitted by the fecal-oral route and are

common environmental contaminants and so presumably many animals are exposed to both agents. For example, in serological studies of people, anti-*Giardia* and anti-*Cryptosporidium* spp. antibodies are commonly detected. Thus, failure to detect dual infection may relate to organism clearance for the majority of the animals. However, *Cryptosporidium* spp. are very small and easy to miss on fecal flotation and so failure to detect dual infection in previous studies may also relate to poor sensitivity of previously utilized tests.

While both *Giardia* spp. and *Cryptosporidium* spp. have been associated with diarrhea, both organisms can be detected in feces of normal animals.[23-24] It is unknown why some animals are colonized and others develop infection and disease. In general, giardiasis and cryptosporidiosis are more common in young and shelter animals. Disease may also be more likely in immunosuppressed animals. In the animals described herein, 4 were young and the dog had evidence of pneumonia as well as the diarrhea which may have suggested the presence of an underlying immunodeficiency. Thus, disease associated with multiple parasitism may relate to poor host immune responses. However, the presence of multiple parasites may also potentiate development of disease; it was proposed that in people, infection with *Giardia* spp. or *Cryptosporidium* spp. might predispose one infection to the other.[25] This has also been proposed in animals. For example, it has been previously reported that coinfection with other protozoans including *Giardia* spp.[17] and *T. foetus* [3] may aggravate clinical signs of cryptosporidiosis in cats. In other work, co-infection with *Cystoisospora* spp., *Toxocara cati,* coronavirus and *Campylobacter* spp. had been reported in cats with clinical signs of cryptosporidiosis.[26,27]

Although co-infections with *Cryptosporidium* spp. and *Giardia* spp. are uncommon, in cases of chronic diarrhea that do not respond to conventional treatments, it recommended that veterinarians screen for both parasites. At this time, we believe that fecal flotation plus IFA testing are the most practical initial diagnostic tests; *Giardia* spp. antigen testing and *Cryptosporidium* spp. PCR assay may be needed in difficult cases. Repeated testing maybe required to detect infection in some animals. [28]

There have been numerous drugs assessed for the treatment of giardiasis and cryptosporidiosis in animals including man. For *Cryptosporidium* spp., more than 100 compounds have been evaluated in laboratory animal models or naturally infected animals, but no treatment has uniformly resulted in resolution of clinical signs and led to parasitological cure. Most treatments including azithromycin and paromomycin, were used in humans infected with cryptosporidiosis and had mixed success.[29] Azithromycin achieves high biliary concentrations and has been used in humans and cows infected with cryptosporidiosis.[29-30] In cats, azithromycin has been administered for the treatment of other infections, but there are no reports of its use in treating feline cryptosporidiosis.

To our knowledge, there is only one published report in which a cat coinfected with *Cryptosporidium* spp. and *Giardia* spp. was treated successfully for *Cryptosporidium* spp. with spiramycin at a dose of 70 mg/kg for 7 days.[16] There are only a few studies reporting treatment for cryptosporidiosis in cats and dogs. Tylosin at 11 mg/kg PO twice daily for 28 days was administered to a cat with chronic cryptosporidiosis and lymphocytic duodenitis.[31] Stools were normal within 7 days of initiating therapy and the fecal samples were negative for oocysts 6 months after completion of tylosin therapy. At the authors' laboratory many cats with presumed

cryptosporidiosis were treated with tylosin at a dosage of 10 to 15 mg/kg given orally twice daily 21 days, and diarrhea has resolved in about 50% of cases (Lappin MR, Scorza AV, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado: Unpublished data 2007). However, these observations are uncontrolled, and the signs in the affected cats may have resolved spontaneously. It is also possible the anti-inflammatory or antibacterial effects of tylosin played a role in clinical responses. However, tylosin has an unpleasant taste and is not tolerated by most cats. We believe tylosin had a beneficial clinical affect in the dog described herein as all other previously prescribed drugs were apparently ineffective. While tylosin has no known activity against *Giardia* spp., the cysts of the organism were not found on the final IFA. It is likely the organism numbers had decreased to below detectable limits of the assay but the dog was still infected.

Paromomycin was effective in clearing *Cryptosporidium* spp. oocysts from the feces of a naturally infected cat with persistent diarrhea[32] and in a group of experimentally inoculated cats.[33] However, in these studies, the parasitologic response was measured by microscopic techniques that are not very sensitive for the detection of feline and canine cryptosporidiosis. In this study IFA and PCR were used and they are the most sensitive techniques for detection of *Cryptosporidium* spp.[34] The drug also is thought to have anti-*Giardia* effects which is why it was chosen for administration to Cats 1-4. In addition, paromomycin should never be used in cats with bloody diarrhea, due to the risk of systemic absorption and induction of renal disease or deafness.[35]

To date, nitazoxanide is the only drug approved by the FDA to treat diarrhea caused by *Cryptosporidium* and *Giardia* species in children.[36-37] Toxicity studies have

shown nitazoxanide to be safe in cats.[38] In some *Cryptosporidium* spp. or *Giardia* spp. infected dogs and cats, diarrhea resolves after the administration of nitazoxanide at a dosage of 25 mg/kg given orally every 12 hours for at least five days but it is too early to suggest an efficacy for this treatment.[2] Because the drug was not tolerated by the Cats 3 and 4 in this report, we cannot assess its efficacy. Recently, it was reported that *Cryptosporidium* parasites lack of an organelle called apicoplast which is target by macrolides in other apicomplexa parasites. This could potentially explain the parasite resistance to these antibiotics.[39]

The resistance of *Cryptosporidium* spp. to chemotherapeutic agents may also be associated with its unique location which is intracellular but extracytoplasmic. Analysis of the *C. parvum* and *C. hominis* genomes reveled that certain biochemical pathways are lost or simplified in *Cryptosporidium* spp. parasites in comparison to other apicomplexa.[39] Conversely, *Cryptosporidium* have atypical biochemical pathways and genes that can be targets for new chemotherapeutics. [39] Additionally, the production of thin-wall oocyst that excyst endogenously and the resistant of oocysts to the most common disinfectants explains persistent infections.

Treatment failures are also common in *Giardia* infected patients. Reinfection, inadequate drug levels, inmunosuppression, drug resistance, and sequestration of the organism in the gall-bladder or pancreatic ducts are all proposed reasons.[40] The most commonly used drugs to treat giardiasis in cats and dogs are metronidazole and fenbendazole. Metronidazole was effective in producing parasitological and clinical response in naturally and experimentally infected cats.[41-44] Fenbendazole at was shown to be safe when administered to healthy, adult, non-pregnant cats at a dosage five times

higher than the approved dosage. [45] Nevertheless, when fenbendazole was administered to cats co-infected with *Giardia* and *Cryptosporidium parvum,* only four out of eight cats stopped shedding *Giardia* cysts. [17]

Febantel is a benzimidazole found in a combination product containing febantel, pyrantel, and praziquantel.[p] The administration of two small dog tablets/cat (approximately 50 mg/kg of febantel), PO, for 5 days, to experimentally infected cats decreased cyst shedding. [46] Additionally, 4 of the 6 treated cats remained negative for cysts even after administration of glucocorticoids in an attempt to induce immunosuppression. Febantel is metabolized in part to fenbendazole which is likely to explain its benefit for giardiasis. The *Giardia* vaccine used as a preventative in dogs was apparently successfully in eliminating cyst shedding and diarrhea in a group of naturally infected dogs.[47] However, the administration of 3 doses of a commercially available feline *Giardia* vaccine to 8 experimentally infected kittens did not stop cyst shedding[48] Consequently, whether the vaccine is an effective immunotherapy in naturally infected cats is unknown.

It appears that animals with co-infections are more difficult to treat successfully than animals infected with either organism alone.[3, 17] The cases presented here have common findings (table 1). The animals became negative for either parasite a few days after being treated with any of the drugs. However, if we extend the follow up period they became positive for either parasite. All the cats became temporarily negative for both parasites after the administration of paromomycin. Cats 1 and 2 became negative for *Giardia* by IFA after treatment with azithromycin and paromomycin, however, they remained positive for *Cryptosporidium* spp. As for cat 4 we did not obtain samples from

days 30 and 35 post-treatment with paromomycin. However, if cat 3 become positive it is also possible that cat 4 remained infected with *Giardia* and *Cryptosporidium* as well. The dog became negative for both parasites after the administration of tylosin but follow up testing after the last treatment was short. It might be possible that the dog resolved the diarrhea but remained being a carrier for any or both parasites. While azithromycin and paromomycin are not the first choice drugs for giardiasis, both have anti-*Giardia* activity and might explained why cats 1 and 2 clear up the infection. The therapeutic efficacy of paromomycin ranges from 55% to 88% and azithromycin has in vitro anti *Giardia* activity compared to that of metronidazole.[49]

Paromomycin is an aminoglycoside antibiotic that is poorly absorbed from the gastrointestinal tract. The administration of paromomycin helped in improving the diarrhea in the four cats of this study. Two of the cats (cats 3 and 4) were also receiving orbifloxacin [k] at the same time as paromomycin [c] which is a fluoroquinolone antibiotic indicated for the treatment of gram-negative and aerobic gram-positive bacilli and cocci. [50] Synergism of orbifloxacin with aminoglycosides has been reported. [50] So, it might be possible that the clinical signs in cats 3 and 4 improved due to synergism between paromomycin and orbifloxacin. Additionally, orbifloxacin might have helped controlling bacterial overgrowth and also in the treatment of a false negative *Pentatrichomonas* infection.[4] The dog also improved the diarrhea while receiving tylosin and orbifloxacin.

Treatment failures are common in animals with giardiasis and cryptosporidiosis. It is possible that no drug will be commonly effective for the treatment of this parasitic infection. Some treatments can help improve the clinical signs but infection may be unlikely to clear. Consequently, the drug and protocol used should be varied according to

each individual patient. In chronic cases, the possibility of underlying disorders should also be considered. Additionally, infection with *Giardia* and *Cryptosporidium* does not appear to cause permanent immunity and so re-infections are likely to occur.

Cats and dogs are infected with the host-specific *Cryptosporidium* and *Giardia* spp. genotypes; when they are infected and have formed stools they shed low number of (oo)cysts.[51-52] The frequency of finding these genotypes in immunocompetent humans is very low, so the chances for a healthy person to contact cryptosporidiosis from a cat or dog with formed stools is likely to be small.

Footnotes

a. Merifluor Crypto/Giardia IFA kit, Meridian Diagnostic Corporation, Cincinnati, OH.

b. Azithromycin, Pfizer Co, NY, NY.

c. Paromomycin Humatin, Pfizer, NY, NY.

d. Clavamox: Glaxo Smith Kline, Research Triangle Park, NC.

e. Panacur: Intervet, Millsboro, DE.

f. Albon: Pfizer Animal Health, Exon, PA.

g. SNAP Combo: IDEXX Laboratories, Portland, ME.

h. IDEXX Veterinary Services

i. Tylan: Elanco, Greenfield, IN.

j. Nitazoxanide: IDEXX Pharmaceuticals Greensboro, NC.

k. Orbax: Schering Plough, Omaha, NE.

l. Carafate: Aventis Pharmaceuticals Inc, Kansas City, MO.

m. Panacur granules 1gr (222 mg/gr): Intervet, Millsboro, DE

n. Metamucil: Dist. By Procter & Gamble, Cincinnati, OH.

o. Giardia vaccine: Giardia lamblia vaccine, Fort Dodge, Fort Dodge, IA.

p. Drontal® Plus, Bayer Animal Health, Merriam, KS.

Table 1: Effect of treatment on *Cryptosporidium* spp. and *Giardia* spp. infection in coinfected cats 1 and 2.

Azithromycin 10 mg/kg daily PO during 14 days			
Pre-treatment	IFA (#pos/#total) G C	PCR (#pos/#total) C	Consistency
Cat 1	3/3 1/3	2/3	diarrhea
Cat 2	1/3	2/3	diarrhea
Intra-treatment	IFA (#pos/#total) G C	PCR (#pos/#total) C	Consistency
Cat 1	10/11 3/11	11/11	diarrhea
Cat 2	5/11 2/11	11/11	diarrhea
Post-treatment	IFA (#pos/#total) G C	PCR (#pos/#total) C	Consistency
Cat 1	4/4	4/4	diarrhea
Cat 2	1/4	4/4	diarrhea
Paromomycin 500mg/kg daily PO during 5 days			
Post-treatment	IFA (#pos/#total) G C	PCR (#pos/#total) C	Consistency
Cat 1	1/7	2/7	normal
Cat 2	1/7	2/7	normal
Paromomycin 500mg/kg twice daily during 5 days			
Post-treatment	IFA (#pos/#total) G C	PCR (#pos/#total) C	Consistency
Cat 1	0/2	2/2	normal
Cat 2	0/2	1/2	soft-normal

References: *G: Giardia*; *C: Cryptosporidium*; #pos: number of positive samples; # total: number of total samples collected during that period.

Table 2: Effect of treatment on *Cryptosporidium* spp. and *Giardia* spp infection in coinfected cats 3 and 4.

Tylosin				
Pre-treatment	IFA (#pos/#total)		PCR (#pos/#total)	Consistency
	G	C	C	
Cat 3	1/1	1/1	1/1	diarrhea
Cat 4	1/1	1/1	1/1	diarrhea
Post-treatment	IFA (#pos/#total)		PCR (#pos/#total)	Consistency
	G	C	C	
Cat 1	1/1	1/1	0/1	diarrhea
Cat 2	1/1	1/1	0/1	diarrhea
Nitazoxanide				
Pre-treatment	IFA (#pos/#total)		PCR (#pos/#total)	Consistency
	G	C	C	
Cat 3	1/1	1/1	0/1	diarrhea
Cat 4	1/1	0/1	0/1	diarrhea
Paromomycin				
Pre-treatment	IFA (#pos/#total)		PCR (#pos/#total)	Consistency
	G	C	C	
Cat 3	3/6	1/6	0/1	normal
Cat 4	4/7	2/7	0/1	soft-normal

References: *G: Giardia*; *C: Cryptosporidium*; #pos: number of positive samples; # total: number of total samples collected during that period.

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CHAPTER 7

Efficacy of a combination of febantel, pyrantel, and praziquantel for the treatment of kittens experimentally infected with *Giardia*

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Previously published in the Journal of Feline Medicine and Surgery (J Feline Med Surg. 2006;8(1):7-13.)

Summary

This study evaluated the effect of two combination products containing febantel, pyrantel, and praziquantel (FPP) for the treatment of *Giardia* in experimentally infected kittens. In Experiment 1, five kittens were administered the United States formulation of FPP at doses of 37.8 mg/kg, 7.56 mg/kg, and 7.56 mg/kg, respectively, PO, q24h, for 5 days and four kittens remained as controls. In experiment 2, five kittens were administered the European formulation of FPP at the doses of 12.5 mg/kg, 12 mg/kg, and 4.16 mg/kg, respectively, PO, q24h, for 5 days and four kittens remained as controls. In experiment 3, six kittens were administered the United States formulation of FPP at 56.5 mg/kg, 11.3 mg/kg, 11.3 mg/kg, respectively, PO, q24h, for 5 days and five kittens remained as controls. Thirteen days post-treatment, kittens testing negative for *Giardia* cysts were administered 20 mg/kg methylprednisolone acetate, IM, weekly for a maximum of 2 injections. Feces were analyzed for *Giardia* cysts using a direct immunofluorescence test. After experiment 3, four of the six treated kittens, but no control kittens, remained negative for *Giardia* after the administration of methylprednisolone acetate.

7.2 Introduction

Giardia lamblia is a flagellate parasite that can cause significant gastrointestinal disease in a wide variety of mammals including kittens and humans. *G. lamblia* is a species complex comprising at least eight major assemblages which can be host specific, or not. To date, only the genetic group I from Assemblage A has been demonstrated to infect both humans and animals, thus proving zoonotic transmission (Monis and Thompson, 2003). In the United States, *Giardia* spp. infection was detected in 5 of 206 (2.4%) adult kittens tested in Colorado (Hills et al., 2000) and in 19 of 263 (7.2 %) kittens tested in New York State (Spain et al., 2001). In Europe, *Giardia* spp. infection was detected in 228 of 2234 (10.2%) kittens tested in Germany (Barutzki, 2001) and 18 of 81 (22.2%) kittens tested in Serbia (Nikolic et al., 2002). In Australia, 2 of 40 (5%) and 32 of 40 (80%) kittens tested positive for *Giardia* by microscopy and by Polymerase Chain Reaction (PCR) respectively; suggesting than highly sensitive techniques are required for the diagnosis of giardiasis in kittens (McGlade et al., 2003). Thus, it is possible that even larger numbers of kittens are infected by *Giardia* than previously estimated.

Albendazole, metronidazole and fenbendazole have been used most commonly for the treatment of feline and canine giardiasis. Albendazole was effective in the treatment of canine giardiasis, but was ineffective in one study of *Giardia*-infected kittens (Barr, 1993) and has been associated with bone marrow suppression in kittens (Stokol et al., 1997). Metronidazole has been showed to be effective in eliminating *Giardia* cyst shedding in some naturally and experimentally-infected kittens (Nesvadba, 1979; Shatto, 1981; Zimmer, 1987; Scorza and Lappin, 2004). However, gastrointestinal

and central nervous system toxicity has been associated with the administration of metronidazole in some dogs (Dow et al., 1989) and kittens (Caylor and Cassimatis, 2001). Administration of fenbendazole at a dosage five times greater than the approved dosage was well tolerated by healthy, adult, non-pregnant kittens (Schwartz et al., 2000). When fenbendazole was administered to eight kittens concurrently infected with *Giardia* spp. and *Cryptosporidium parvum,* only four stopped shedding *Giardia* cysts (Keith et al., 2003). A vaccine containing inactivated *Giardia* trophozoites, originally developed for the prevention of infection, has proven beneficial in the treatment of some naturally infected dogs (Olson et al., 2001). However, the administration of three *Giardia* vaccinations did not lessen or eliminate cyst shedding in treated kittens when compared to untreated control kittens (Stein et al., 2003).

The administration of a combination of febantel, pyrantel, and praziquantel (FPP),[a,b] has been evaluated for the treatment of giardiasis in dogs (Barr et al., 1998; Barutzki et al., 2001; Payne et al., 2002). The United States (US) and the European formulations of FPP are available. When FPP was developed for the United States, efficacy data on roundworms, hookworms and whipworms was submitted with a dose of at least 25 mg/kg to achieve highly effective whipworm control (Personal communication, Bayer Animal Health, 2005). For Europe, studies have shown that a lower dosage of febantel was sufficient for gastrointestinal nematode control including whipworm and thus, a different formulation was licensed (Personal communication, Bayer Animal Health, 2005).

To our knowledge, this drug combination has not been evaluated for the treatment of giardiasis in kittens.

The purpose of this study was to evaluate the United States and European formulations of FPP for the treatment of *Giardia* spp. infections of experimentally infected kittens.

7.3 Materials and Methods

7.3.1 Animals. Twelve week old, mixed sex kittens (n = 26) were purchased from a commercial vendor. The kittens were vaccinated subcutaneously (SQ) with a feline panleukopenia virus, herpesvirus 1, and calicivirus vaccine on 2 occasions 4 weeks apart and administered pyrantel pamoate PO twice before shipping to Colorado State University. On arrival, kittens were shown to be FeLV antigen and FIV antibody negative (SNAP Combo, IDEXX Laboratories, Portland, ME) and normal on complete blood cell count, serum biochemical panel, and urinalysis. The kittens were housed separately, fed a commercial feline diet at libitum, and were observed daily for attitude, stool consistency, and signs of drug toxicity including anorexia, vomiting and diarrhea.

7.3.2 Fecal assays. Prior to inoculation with *Giardia*, three fecal samples from each cat collected within a 10 day period, and assessed for the presence of enteric parasites by microscopic examination of feces after zinc sulfate centrifugation, following standard procedure for Colorado State University. In addition, each fecal sample was diluted 1:4 in 0.01 M phosphate buffered saline solution (pH = 7.2) and a thin fecal smear made on microscope slides supplied in an in vitro direct immunofluorescence assay (IFA) capable of simultaneous detection of *Giardia* cysts and *Cryptosporidium* oocysts (Merifluor Crypto/Giardia IFA kit, Meridian Diagnostic Corporation, Cincinnati, OH). After slides

were stained as instructed by the manufacturer, the number of cysts per slide were counted by use of a fluorescence microscope. A cyst score was then assigned from 0 to 4 based on the following: 0 = 0 cysts per slide; 1 = 1 to 250 cysts per slide; 2 = 250 to500 cysts per slide; 3 = greater than 500 cysts per slide; 4 = TNTC ("too numerous to count", defined as >100 cysts per 10x objective field).

7.3.3 Experimental design. Each of the three fecal samples collected from the 26 kittens were shown to be negative for enteric parasites prior to infection.

Experimental inoculation with Giardia cysts. *Giardia* cysts from a naturally infected cat, and identified by IFA, were administered to two of the kittens to produce enough *Giardia* cysts to infect the 24 kittens to be used in the treatment study. After withholding food for 12hr, each cat was given 2 x 10^4 *Giardia* cysts in water by stomach tube while sedated with ketamine (5-10 mg/kg, IV) and acepromazine (0.002 mg/kg, IV).

Our original study design included the infection of 24 kittens that would be randomized into three treatment groups and one control group, with treatment beginning on day 10 post-infection. However, only 17 of 26 kittens shed *Giardia* cysts in consistently detectable numbers and the prepatent period varied between kittens. Therefore, the following three experiments were performed sequentially.

Experiment 1. On day 28 post-inoculation (PI), there were nine kittens shedding *Giardia* cysts. The US formulation of FPP (Drontal Plus, Bayer Animal Health, Shawnee Mission, KS) at the approximate dosages of 37.8 mg/kg, 7.56 mg/kg, and 7.56 mg/kg, respectively, was administered to five kittens, PO, q24h, for five days. The four remaining kittens served as the untreated control group.

Experiment 2. On day 42 PI, an additional five, previously untreated kittens were persistently shedding *Giardia* cysts. The European formulation of FPP (Drontal Plus, Bayer Vital GmbH, Leverkusen) at the approximate dosages of 12.5 mg/kg, 12.0 mg/kg, and 4.16 mg/kg, respectively, was administered to these five kittens PO, q24h, for five days. The untreated kittens from experiment 1 had continued to shed large numbers of *Giardia* cysts and were used as the control group.

Experiment 3. After completion of experiments 1 and 2, 11 kittens were persistently shedding *Giardia* cysts. The US formulation of FPP at the approximate dosages of 56.5 mg/kg, 11.3 mg/kg, and 37.8 mg/kg, respectively, was administered to six kittens, PO, q24h, for five days. The other five kittens served as the untreated control group. Three of the six treated kittens had been control kittens in the previous experiments and three had previously been administered FPP but were still persistently infected. Three of the five control kittens had never been treated and two had previously been administered FPP but were still persistently infected.

Thirteen days after each treatment period, all kittens that were negative for *Giardia* cysts were administered 20 mg/kg methylprednisolone acetate, IM weekly for a maximum of 2 injections. Kittens shedding cysts after the first injection of methylprednisolone were considered *Giardia*-infected and did not receive the second injection. Kittens that remained negative for cysts on the last day of each experiment (Day 32 after beginning treatment) were considered not to have *Giardia* infection.

Clinical monitoring. Each cat was examined daily throughout the study. Fecal samples were collected daily and analyzed by IFA. Consistency of feces was classified daily as: normal = 1; soft = 2; watery = 3). Appetite was assessed as: normal = 1; less than normal =

2; and anorexia = 3. Complete blood cell count, serum biochemical panel and urinalysis were performed 5 and 19 days after completion of the FPP treatment period in experiments 1 and 2.

7.3.4 Statistical Analysis. For each cat group, the mean *Giardia* cyst score, percentage of *Giardia*-positive fecal samples, and percentage of soft stools were calculated within time periods; pre-treatment period (7 days [experiments 1 and 2] or 14 days [experiment 3] prior to drug administration), intra-treatment period (5 days), post-treatment period 1 (7 days post-treatment) and post-treatment period 2 (from days 8-14 post-treatment). Differences between treated and control cat groups within each period were evaluated using ANOVA appropriate for a repeated measure experiment (The MIXED procedure of SAS, SAS Institute, Cary, NC).

Treatment, period (time), and the treatment by time interaction were considered fixed effects. For the percentage of *Giardia*-positive fecal samples and percentage of soft stool samples, an arcsine transformation was used in the statistical analysis. In addition, Wilcoxon's rank sum test was also used to assess for mean *Giardia* cyst score differences between groups during the intra-treatment and post-treatment periods. Statistical significance was defined as $P < 0.05$.

7.4 Results

7.4 1 Experiment 1. Over the course of the experiment, a total of 159 fecal samples were assessed by IFA. Overall, positive results were detected in 56 of 86 samples (65%) in the treatment group and in 72 of 73 samples in the control group (98%). When percentages of positive samples were compared between the treatment and control groups (Table 1),

statistical significance was detected for the treatment by time interaction (P=0.0006). In the pre-treatment period, there were no differences between the groups for percentage positive fecal samples or cyst scores (Tables 1 and 2). Compared to the untreated kittens, kittens treated with FPP had significantly fewer positive fecal samples in post-treatment periods 1 and 2 and significantly lower cyst scores in post-treatment period 1. One of the 5 treated kittens was still cyst negative on day 13 post-treatment but resumed cyst shedding after one dose of methylprednisolone acetate. Overall, diarrhea was detected in 6 of 86 samples (6.9%) in the treatment group and 4 of 73 samples (5.5%) in the control group; differences between treated and control cat groups were not detected.

7.4.2 Experiment 2. Over the course of the experiment, a total of 135 samples were assessed by IFA. Overall, positive results were detected in 61 of 76 samples (80%) in the treatment group and in 58 of 59 samples in the control group (98%). When the percent of positive samples were compared between the treatment and control groups (Table 1), statistical significance was detected for the treatment by time interaction (P=0.0004). In the pre-treatment period, there were no differences between the groups for percent positive fecal samples or cyst scores (Tables 1 and 2). Compared to untreated kittens, treated kittens had significantly fewer positive fecal samples in the intra-treatment period and post-treatment period 1 and significantly lower cyst scores in post-treatment period 1. None of the kittens became cyst negative in post-treatment periods 1 or 2. Overall, diarrhea was detected in 5 of 76 samples (6.5%) in the treatment group and 5 of 59 samples (8.7%) in the control group; differences between treated and control cat groups were not detected.

7.4.3 Experiment 3. Over the course of the experiment, a total of 170 samples were assessed by IFA. Overall, positive results were detected in 52 of 109 samples (47%) in the treatment group and in 56 of 61 samples in the control group (92%). When percentages of positive samples were compared between the treatment and control groups (Table 1), statistical significance was detected for the treatment by time interaction (P = 0.0012). In the pre-treatment period, there were no differences between the groups for the percent positive fecal samples or cyst scores (Tables 1 and 2). Compared to untreated kittens, treated kittens had significantly fewer positive fecal samples in the intra-treatment period and post-treatment periods 1 and 2 and significantly lower mean cyst scores in post-treatment periods 1 and 2. On day 13 post-treatment, five of the six treated kittens remained negative for *Giardia* by IFA, therefore were administered methylprednisolone acetate as described. Four kittens remained *Giardia* cyst negative for the duration of the study. Overall, diarrhea was detected in 7 of 109 samples (5.5%) in the treatment group and 0 of 61 samples (0%) in the control group; differences between treated and control cat groups were not detected.

Other than salivation by some kittens, signs of toxicity were not recognized and CBC and serum biochemical panel results were normal after the three experiments.

7.5 Discussion

In this study, it would have been optimal for all kittens to have become infected with *Giardia*, to have had the same prepatent period and have shown clinical signs of giardiasis. Nevertheless, in the kittens that developed detectable infections, chronic, persistent shedding did occur. During the 7-14 day pre-treatment time periods in each of the three experiments, there was no difference in *Giardia* cyst shedding between the

treated and control kittens. In addition, the untreated control kittens continued to consistently shed *Giardia* cysts through each of the intra-treatment and post-treatment periods of each experiment. Thus, we believe that the statistical differences detected between treated and control kittens are most likely a treatment effect rather than due to chance. Because of the variable prepatent period and the sequential design of the 3 experiments, we believe the results should be interpreted cautiously. However, we also believe that the results should be reported and suggest that further experiments assessing the use of FPP for treatment of feline giardiasis be pursued using clinically affected cases.

Treated kittens in experiment 3 were administered higher doses of FPP than treated kittens in experiments 1 and 2. These kittens also had the best intra-treatment and post-treatment results, including failure to detect cyst shedding in four of six treated kittens even after administration of glucocorticoids. While *Giardia* infection can spontaneously resolve (Barr, 1998), we believe it is more likely the FPP played a role in organism clearance at this dose because each of the control kittens was persistently infected. However, it is possible that the four kittens that became *Giardia*-negative after treatment were still infected but at a level below the sensitivity limit of the IFA and that the glucocorticoid protocol we used was not high enough to induce shedding. We believe that if FPP is studied further for the treatment of feline giardiasis, minimum doses of 56.5 mg/kg, 11.3 mg/kg, and 37.8 mg/kg, respectively, should be used. This dose is similar to that reportedly effective for the treatment of giardiasis in dogs (Barr et al., 1998; Barutzki et al., 2001). In addition, when fenbendazole was administered to kittens with *Giardia* and *Cryptosporidium* spp. co-infections at 50 mg/kg, PO q24h, for 5 days, *Giardia*

infection was eliminated in four of eight kittens (Keith et al., 2003). The anti-*Giardia* activity of FPP is thought to be from the febantel component, which is metabolized into fenbendazole, oxfendazole and other compounds after oral administration (Barr et al., 1998). Administrations of the two formulations of FPP were well tolerated by the kittens; the only side-effect that was observed in these kittens was transient salivation after the administration of the tablets. Nevertheless, when FPP was first launched in Australia, there were reports of side effects (transient neurotoxic signs characterized by uncoordinated movements) and it was decided not to continue with FPP, but to launch Drontal (Pyrantel/Praziquantel) for cats. At that time it was believed that the side effects were caused by febantel, while pyrantel is generally not absorbed. However, after launching Drontal there were some neurotoxic signs in cats still recognized, suggesting the toxicity was not related to febantel. Today, after many years of monitoring, there are no correlations to either cats' breed or age. Thus, the reasons for the first findings remain unclear (Personal communication, Bayer Animal Health, 2005). Diarrhea was uncommon in the kittens in this study; therefore we could not determine whether the administration of the FPP lessened the clinical signs of giardiasis.

The failure of all 24 inoculated kittens to develop detectable patent infections could be from an insufficient *Giardia* cyst dose, the administration of a minimally virulent strain, innate resistant of some kittens, specific immunity in some kittens, or failure to detect *Giardia* cysts because of low level infection. While there is no standardized *Giardia* infection protocol reported for kittens; the infective dose in humans is thought to range from 10 to 1000 cysts (Flaubert, 2000). Thus, we assumed, 2×10^4 cysts of a feline isolate should be sufficient to cause infection in kittens. The *Giardia*

cysts were concentrated following the instructions of a published protocol (O' Handley et al., 2000), stored at 4°C for a maximum of 10 days, and were inoculated into all kittens at the same time. Thus, each kitten should have received a similar number of viable *Giardia* cysts. We infected the kittens with *Giardia* cysts derived from a naturally infected cat with clinical signs of giardiasis. However, it may possible that the strain that we administered was not virulent enough to produce infection and clinical signs of giardiasis in all the kittens. Kittens as well as children are thought to be more susceptible to *Giardia* infection than adults (Barr, 1998). However, protection against giardiasis can be acquired passively by neonates from immune mothers (Farthing, 1990). It is possible that maternal antibodies against *Giardia* were still present in some kittens at the time of inoculation, blocking infection. *Giardia* cyst shedding by kittens may fluctuate from undetectable levels to concentrations of > 1,000,000 cysts/gram of feces and the duration between any two given peaks of cysts excretion in kittens ranged from 2 to 7 days in one study (Kirkpatrick and Farrell, 1984). Thus, it is also possible that some of the kittens were previously infected by *Giardia* at undetectable levels and so were immune to infection. However, we believe that this hypothesis is unlikely because results were consistently negative in uninfected kittens through the study and the IFA used here is one of the most sensitive tests for the diagnosis of giardiasis. When used with cervine feces, the detection limit of this IFA is 500 cysts/ml of feces (Deng and Cliver, 1999). In addition, we also tested one sample from each kitten by a commercially available antigen test (Prospect Giardia Microplate Assay, Alexon Trend, Ramsey, MN) and all the samples tested were negative.

7.6 Acknowledgments:

The authors would like to thank Jason Eberhardt, Melissa Brewer, and Jennifer Hawley for their help in collecting samples and with animal care. This project was supported by a grant from Bayer Animal Health.

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7.8 Tables

Table 1. Percentage of fecal samples positive for *Giardia* cysts in untreated kittens and kittens treated with a combination product containing febantel, praziquantel, and pyrantel.

	Pre-treatment	**Intra-treatment**	**Post-treatment Period 1**	**Post-treatment Period 2**
Experiment 1				
Control kittens	100	100	95	100
Treatment kittens n=5	83	100	13	70
P value	0.1370	1.	<0.0001	<0.0001
Experiment 2				
Control kittens	95	100	100	100
Treatment kittens n=5	100	72.89	60	90
P value	0.4721	0.0184	0.0002	0.3512
Experiment 3				
Control kittens	100	100	91	77
Treatment kittens n=6	100	61	3	13
P value	1	<0.0058	< 0.0001	0.0001

*P-values represent back transformed results from arsine analysis

Table 2. Cyst score for *Giardia* cysts in untreated kittens and kittens treated with a combination product containing febantel, praziquantel, and pyrantel.

	Pre-treatment	**Intra-treatment**	**Post-treatment Period 1**	**Post-treatment Period 2**
Experiment 1				
Control kittens	2.8	1	2.5	2.05
Treatment kittens n=5	2.4	1	0.14	0.87
P value	1	0.3711	0.0131	0.2109
Experiment 2				
Control kittens	2.48	2.11	1.87	2.06
Treatment kittens n=5	1.68	1.1	0.57	2.4
P value	0.135	0.1248	0.0243	0.4606
Experiment 3				
Control kittens	1.22	1.5	1.4	1.6
Treatment kittens N=6	1.2	0.7	0.17	0.29
P value	0.8518	0.1611	0.004	0.004

*P-values from Wilcoxon Sum Rank test

CHAPTER 8

Metronidazole for the treatment of feline giardiasis

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Previously published in the Journal of Feline Medicine and Surgery (J Feline Med Surg. 2004:6(3):157-60)

8.1 Summary:

There are several drugs available for the treatment of giardiasis in cats, including metronidazole. The purpose of this study was to determine whether metronidazole benzoate administered at a dose of 25 mg/kg, PO, twice a day for seven days lessens or eliminates *Giardia* cyst shedding in cats with chronic infection. Twenty six, adult, laboratory-reared cats were used in this study. Sixteen cats had been inoculated orally with cysts of a human *Giardia* isolate and had completed a *Giardia* vaccine study in one animal holding room. The other ten cats were infected with the same *Giardia* presumably by contamination from the adjacent room where the *Giardia* vaccine study cats were located. From each cat, a fecal sample was collected within one week of the start of treatment and then every 2 to 4 days for 15 days after treatment was completed. Fecal samples were analyzed for the presence of *Giardia* cysts using a commercially available direct immunofluorescence test (IFA). Clinical signs of drug toxicity were not detected during the study.

8.2 Introduction

Giardia is a flagellate protozoan with worldwide distribution that causes significant gastrointestinal disease in a wide variety of vertebrates, including cats and people (Barr 1998). Mammalian isolates are all currently classified as *G. lamblia*. Recently, two or three genotypes of *Giardia* in people, two genotypes in dogs (Hopkins et al 1997), and a genotype that may be unique to cats have been identified (Homan et al 1998, Lu et al 1998). *Giardia* infection can occur in healthy cats as well as cats with acute or chronic small bowel diarrhea with or without weight loss. Infection is common; in recent studies, *Giardia* spp. were detected in 5 of 206 (2.4%) of the adult cats tested in Colorado (Hill et al 2000), 19 of 263 (7.2 %) of the kittens tested in New York State (Spain et al 2001), and 228 of 2234 (10.2%) of the cats tested in Germany (Barutzki 2001).

Because some *Giardia* spp. are zoonotic, treatment is frequently considered for some healthy carriers as well as clinically ill cats. In the United States, there are several protocols that have been assessed for the treatment of giardiasis in cats. Administration of the drugs albendazole, fenbendazole, furazolidone and metronidazole as well as use of a *Giardia* vaccine as immunotherapy have all been assessed in small numbers of cats. Albendazole was ineffective in one group of cats (Barr et al 1993) and was associated with bone marrow suppression (Stokol et al 1997). Fenbendazole was shown to be safe when administered to healthy, adult, nonpregnant cats at a dosage five times higher than the approved dosage (Schwartz et al 2000). However, when fenbendazole was administered to cats concurrently infected with *Giardia* and *Cryptosporidium parvum*, only four out of eight cats stopped shedding *Giardia* cysts (Keith et al 2003).

Furazolidone causes inappetance and vomiting in many cats and efficacy data for the treatment of giardiasis in cats is minimal (Brightman and Slonka 1976, Kirkpatrick 1985). A vaccine containing inactivated *Giardia* trophozoites developed for vaccination of kittens (Olson et al 1996) was recently assessed as an immunotherapy in experimentally inoculated cats but there were no differences in cyst shedding between treated cats and control cats (Stein et al 2003).

Metronidazole has been used frequently for the treatment of giardiasis in cats and people. However, the studies that have shown metronidazole to be effective in eliminating *Giardia* cyst shedding in naturally and experimentally infected cats only included small numbers of cats (Nesvadba 1979, Shatto 1981, Zimmer 1987). In addition, gastrointestinal and central nervous system toxicity has been associated with metronidazole administration to cats (Caylor and Cassimatis 2001).

The objectives of this study were to determine whether administration of metronidazole benzoate using one protocol lessens *Giardia* cyst shedding in cats and to clinically assess for toxicity.

8.3 Materials and Methods

Animals. Twenty-six laboratory-reared, mixed sex cats between one and two years of age were purchased from a commercial breeder. Sixteen cats were housed in one animal holding room (Stein et al 2003) and the other 10 cats (Scorza AV et al 2003) were located in an adjacent room. The cats were housed in individual cages, were fed a commercial feline diet at libitum, and were observed daily for attitude, stool consistency, and signs of drug toxicity including neurological disorders, lethargy, anorexia, vomiting, diarrhea and

hepatoxicity. The protocol used in this study was reviewed and approved by the Colorado State University Animal Care and Use Committee in accordance with federal regulations. The cats were adopted to private homes after the completion of the study.

Experimental design. Each of the 26 cats treated in this study were known to be chronically infected with a human *Giardia* isolate. Sixteen cats were housed in one animal holding room and had been inoculated orally with 10^6 cysts 10 months before being treated in this study. They had been persistently infected since that time, and had been either control cats or had failed treatment with *Giardia* vaccine given as immunotherapy (Stein et al 2003).

The other 10 cats were infected with *C. parvum* 16 months previously (Scorza AV et al 2003). By retrospective evaluation of stored feces, it was determined that the *C. parvum*-infected cats had been infected with *Giardia* for approximately five months prior to use in this study. These 10 cats were presumed to have been accidentally exposed to *Giardia* from the adjacent room where the other 16 cats were housed. The two groups of cats were infected by the same *Giardia* isolate as determined by comparison of a sequence analysis of the GDH locus (Homan et al 1998). These 10 cats had either not been treated for *Giardia* infection or had failed treatment with fenbendazole, administered at 50 mg/kg, PO, daily for 5 days (Keith et al 2003).

Metronidazole benzoate (PCCA, Houston, Texas) was administered to all 26 cats at 25 mg/kg, PO, q12hr for 7 days. A liquid formulation was used with a final concentration of 25 mg/ml of base which is equivalent to 40 mg of benzoate. From each cat, a fecal sample was collected within one week of the start of treatment and then every 2 to 4 days for 15 days after treatment was completed. The fecal samples were analyzed

for the presence of *Giardia* cysts by use of a commercially available direct immunofluorescence assay (IFA; Merifluor *Cryptosporidium/Giardia*, Meridian Diagnostics, Cincinnati, Ohio).

8.4 Results

In the 3 month period prior to treatment in this study, all of the cats were positive for *Giardia* cysts by IFA in at least two of four fecal samples tested; the majority of the cats were positive in the last four fecal samples. All the cats in the study were positive for *Giardia* cysts within one week of the administration of metronidazole benzoate. Clinical signs of drug toxicity, including neurological disorders, lethargy, anorexia, vomiting and diarrhea were not detected in any of the cats during or after treatment. All 26 cats were negative for *Giardia* cysts by IFA in the three fecal samples collected within the 15-day post-treatment period.

8.5 Discussion

Because all 26 cats treated here had been shedding *Giardia* cysts for five to 10 months (Keith et al 2003, Stein et al 2003), including the fecal sample collected within one week prior to treatment, we believed that the cats can be considered to be chronically infected. Thus, it is likely that the failure to document a single cyst in any sample from any of 26 cats within 15 days of treatment with metronidazole benzoate indicates a treatment response, not spontaneous resolution of cyst shedding. It would have been optimal to have included an untreated control group in this study, but because each cat had persistently shed *Giardia* cysts for months, to lessen the need for more infected cats, and to attempt to clear the infection so the cats could be adopted, all were treated. It also

may have been optimal to monitor for cyst shedding for longer periods of time. However, because the prepatent period for *Giardia* cyst shedding in dogs and cats is approximately 2 weeks and the organism is extremely infectious, it is impossible to determine whether positive samples detected after that time are a repeat infection or a failure to eliminate infection. Thus, most *Giardia* treatment studies are discontinued within one week of completing the treatment period (Barr et al 1994, Barr et al 1998).

Our results correlate with previous reports that showed metronidazole to be effective in lessening or eliminating *Giardia* cyst shedding when administered to individual cats; in some studies duration of parasitological follow up was not stated (Nesvadba 1979, Shatto 1981, Zimmer 1987). In another study, metronidazole eliminated *Giardia* spp. cyst shedding by two naturally infected and two experimentally infected cats for four or five four weeks post-treatment (Kirkpatrick and Farrell 1984). In that study, a modified ZnSO4 flotation technique was used to assess for presence of *Giardia* cysts in feces. Administration of metronidazole at the dose of 22 mg/kg twice a day for five weeks, eliminated cyst shedding as detected by ZnSO4 flotation in seven naturally infected cats; the diarrhea either ceased or was markedly diminished (Zimmer 1987). In the study described here, we could not determine whether the administration of metronidazole lessened the clinical signs of giardiasis, since none of the cats had diarrhea.

Shedding of *Giardia* cysts by cats may fluctuate from undetectable to concentrations to > 1,000,000 cysts/gram of feces (Kirkpatrick and Farrell 1984). The IFA used in the present study is thought to be one of the most sensitive tests for the diagnosis of giardiasis (Deng and Cliver 1999); in preliminary studies in our laboratory,

the threshold of detection in spiked feline fecal samples ranges between 60 and 110 *Giardia* cysts per gram of feces. In another study of cats infected with the human *Giardia* strain used in this study, the IFA was more sensitive and specific than ZnSO4 flotation and comparable to an antigen detection technique when used on refrigerated samples (Lappin et al 2002). However, it cannot be determined with absolute certainty whether administration of metronidazole benzoate eliminated infection or just inhibited cyst shedding to undetectable limits. We purposely chose not to euthanatize the cats to confirm or deny the presence of the organism in the intestinal tract. Recently it was reported that polymerase chain reaction (PCR) was more sensitive when compared with microscopy and ELISA in cat feces infected with giardiasis (McGlade et al 2003). PCR should be considered for use in future *Giardia* treatment studies.

There are two formulations of metronidazole available for oral administration; metronidazole benzoate was used here. Products containing metronidazole benzoate are commercially available in some countries and the drug is available for formulation in the United States. Metronidazole USP induces salivation and inappetance when administered orally to some cats (Groman 2000). In contrast, metronidazole benzoate is very well tolerated by cats, as seen in this report. Whether the two formulations have equivalent activity against *Giardia* spp. in cats has not been determined. The protozoal toxicity of metronidazole is from short lived intermediates or free radicals that produce damage by interacting with DNA and possibly other molecules (Lindsay and Blagburn 2001). The recommended dose of metronidazole in cats is between 10 and 60 mg/kg body weight per day (Lindsay and Blagburn 2001). Vomiting, inappetance, hepatotoxicity and rarely, central nervous toxicity can occur with metronidazole therapy

(Caylor and Cassimatis 2001). Neurological toxicity may develop following either chronic therapy or acute high doses (Caylor and Cassimatis 2001). Even though central nervous toxicity is rare, clinicians should be aware of the potential complications associated with the use of metronidazole. The results of this study suggest that administration of metronidazole to cats at 25 mg/kg, PO, q12hr for 7 days is unlikely to cause toxicity.

Many different *Giardia* strains had been identified; cats in this study were infected with a human isolate. To our knowledge, there have not been studies assessing sensitivity of different *Giardia* strains to different drugs in cats. It is possible that the strain that we used in this study was very sensitive to metronidazole. Treatment failures are common in humans and other animals with giardiasis; reinfection, inadequate drug levels, inmunosuppression, drug resistance, and sequestration of the organism in the gall-bladder or pancreatic ducts are all proposed reasons (Nash et al 2001). To further assess the effect of different *Giardia* strains on treatment results, case control treatment studies of naturally infected and experimentally infected cats will be needed. It is likely that no drug will be universally effective for the treatment of giardiasis. Therefore, in clinical practice, the drug and protocol used here should be adjusted for each individual patient having considered all options for treatment.

8.6 Acknowledgments:

The authors would like to thank the following people for their help in collecting samples and animal care; Dr. Carey Keith, Melissa Brewer, and Jennifer Hawley.

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CHAPTER 9: CONCLUSIONS

9.1 CONCLUSIONS

The experimental work described in chapters 3, 4 and 5 of this dissertation described how the incorporation of molecular techniques has had an unprecedented impact on our understanding of *Cryptosporidium* and *Giardia*. For example, in Chapter 3, the increased sensitivity of these techniques was used to demonstrate that *Cryptosporidium* parasites are more prevalent among cats and dogs than previously thought. In this chapter, we amplified DNA of a *Cryptosporidium* species from 24.3% of the chronic diarrhea samples collected from dogs and cats. Therefore, we believe that clinicians can now include cryptosporidiosis in the differential diagnosis of diarrhea. However, it should be emphasized that many times cats and dogs are asymptomatic carriers of the organism and the detection of DNA in feces does not prove disease causation. In Chapter 3 we also began to explore use of molecular techniques to allow genotypic differentiation among morphological indistinguishable organisms. The information of the amplification and posterior sequence analysis of the hsp-70 gene demonstrated that the cats and dogs in the United States are infected with the host-specific *C. felis* and *C. canis* genotypes.

In chapter 4, we continued genotyping *Cryptosporidium* isolates from cats and dogs. Since we were not able to obtain genotyping information from the 18S rDNA sequencing data in the chapter 3, we used a very reliable PCR-RFLP at the 18S rDNA gene that allowed us to further differentiate among the *Cryptosporidium* that infect cats and dogs in the United States. This technique also confirmed that the isolates that we assessed corresponded with the *C. felis* and *C. canis* host specific genotypes. The

combination of results from Chapters 3 and 4 confirm the results of others that most dogs and cats are infected with host adapted *Cryptosporidium* spp. and so hopefully are of minimal danger to human health.

In chapter 5, we also confirmed by the PCR- RFLP and sequencing analysis of the β-giardin and gdh genes that the dogs in our study were infected with the *G. duodenalis* host-specific genotype and that the two cats tested were infected with the zoonotic genotype. This work also confirms that of others suggesting that not all animals infected with *Giardia* are carrying zoonotic genotypes. However, we believe that collection and assessment of more isolates from multiple areas of the world are needed to further assess prevalence rates and relative risk to humans.

The second part of this dissertation was designed to assess different treatments for cryptosporidiosis and giardiasis. The work described in Chapter 6 is one of the first reports of *Cryptosporidium-Giardia* co-infection in cats and dogs and emphasized the difficulties in the management of these cases when diarrhea occurs. We conclude that multiple combinations of anti-parasitic drugs may need to be adjusted to individual naturally-infected animals with different parasitologic and clinical responses.

In Chapter 7, we showed laboratory infected cats resolve *Giardia* infection after treatment with metronidazole benzoate with minimal side-effects. We concluded that this was significant in that cats are generally intolerant of metronidazole USP.

In Chapter 8, we evaluated different doses and formulation of a combination of febantel, pyrantel, and praziquantel (FPP) for the treatment of giardiasis in experimentally infected kittens. One of the protocols administered significant decreased cysts shedding and the percentage of positive samples in the treated group in comparison

to the untreated group. This work allowed for an additional treatment protocol for veterinarians to consider when treating cats for clinical giardiasis.

9.2 REMARKS AND FUTURE DIRECTIONS

In 2004, *Giardia* and *Cryptosporidium* have been included in the "Neglected Diseases Initiative" which consists of a group of parasitic, bacterial and viral diseases with focal distribution and heterogeneous overlapping. They occur mostly in developing countries where climate, poverty and lack of access to services influence outcomes. When considered together, these diseases exhibit a considerable and increasing global burden, and impair the ability of those infected to achieve their full potential, both developmentally and socio-economically in missed opportunities. The major expectation was that molecular tools would allow us to obtain more information about genotyping, epidemiology, biology, and host- parasite interaction.

The results of the diagnosis chapters 3, 4, and 5 showed that molecular techniques allow us to genotype *Cryptosporidium* and *Giardia* from dog and cat samples from the United States. PCR followed by restriction analysis with endonucleases or sequencing analysis is widely used however; still we need to overcome some inconvenients and standardization between laboratories to interpret results. Although (oo) cysts DNA extraction methods have been commonly used in research labs, still is a problem to isolate DNA from feces from these organisms. In the isolates used on our studies, a decreased sensitivity was found when samples were not processed upon arrival or after shipping and prolonged storage at 4°C. Also the PCR step and the restriction analysis should be optimized from the published protocols. In terms of molecular typing for both

Cryptosporidium and *Giardia* isolates a multilocus approach is essential and some studies only count with a single gene analysis. The genotypic information of different *Cryptosporidium* genes is generally in agreement.

For *Giardia* isolates, genotypic analysis of different genes showed that results from one gene are not always in agreement with results of another gene. Regardless more genotyping analysis is needed in order to clarify this issue. Genotyping analysis should be come from isolated clinical cases, case-control studies or outbreaks. Each of the different study types will allow us to the impact of different factors like traveling contact with livestock or recreational water.

Additionally, in future studies it would be important to obtain information about clinical signs, immune status and nutrition to see if we can found association of clinical signs and particular assemblages/genotypes. In veterinary medicine, no information about the relationship between severity of clinical signs and assemblage/genotype is available.

Additionally to clarify the zoonotic transmission of cats and dogs isolates to humans more studies genotyping isolates from animals and their owners should be performed in order to clearly address this long standing question. The existence of different patterns in the RFLP gels of some of the assemblage D isolates in our study can be evidence of particular subtypes. Other techniques including real time PCR with melting curve analysis and subgenotyping of cats and dogs isolates should be perform.

Numerous drugs had been unsuccessfully used for the treatment of cryptosporidiosis. Recently nitaxozanide had been approved to the treatment of cryptosporidiosis and giardiasis in children. So far the only treatment that showed an

outstanding impact against cryptosporidiosis was the use of highly active antiretroviral therapy (HAART) in HIV positive patients. Protease inhibitors included in HAART have an anti-cryptosporidial activity along with the effect in restoring the immune function. However, most individuals living in developing countries do not have access to HAART. In veterinary medicine, we still have to evaluate protocols on the promising agent nitaxozanide.

The complete sequence of *C. parvum* and *C. hominis* will assist in the discovery of new genes and new biochemical pathways to develop novel therapies for cryptosporidiosis. Regrettably the advances in our knowledge were not paralleled by improvements in drug therapies.

In the attempts to treat giardiasis, treatment failure and resistance are also well documented. In most cases of treatment failure in humans, cure is achieved by prolonged treatment with high doses of drugs. Once resistance among microbes develops, the organisms can remain resistant long after the drug is no longer prescribed. Moreover, compensatory mutations can allow resistant parasites to develop wild type fitness and persist in the field. When treating giardiasis in cats and dogs all options of treatments should be considered. However, care should be taken when administering drugs to safeguard the effective drugs for as long as possible. It is essential to identify the cause of the diarrhea, the mechanisms of resistance, and evaluate the option of not to treat in absence of symptoms and ensuring total compliance with the drug protocol administered.