CRYPTOSPORIDIUM PARVUM, MOLECULAR ENVIRONMENTAL DETECTION AND IMPLICATIONS

by

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A Dissertation Submitted to the Faculty of the
DEPARTMENT OF VETERINARY SCIENCE AND MICROBIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
MAJOR IN PATHOBIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

2003
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ACKNOWLEDGMENTS

I would like to thank the following individuals for their help in the preparation of this dissertation as many individuals contributed to this research in very special ways. Many thanks to Dr. Charles R. Sterling for being a patient and supportive advisor and mentor, for having foresight outlining a complete doctoral program, and for providing me with the opportunity to conduct this research in his laboratory. Dr. B. Helen Jost not only played a critical role in conducting this research but her advice-giving nature helped me become a more competent scientist. Drs. Rodney Adam, Michael Hammer and Lynn Joens served on my committee and provided helpful suggestions and critical review of outlined hypotheses. In addition, Marilyn Marshall deserves special acknowledgement due to her giving nature, research ideas, and daily encouragement for completing this research. I am grateful to Ana Luisa Child for being my partner and giving support and advice on a daily basis. Thanks also to Dr. Michael Riggs and Deborah Schaeffer for giving critical reviews of hypotheses as well as continued collaboration. Thanks to Dr. Stephen Billington for helpful conversations and suggestions. Finally, I would like to acknowledge Paul Hoover and Carrie Reed whose laboratory assistance was greatly appreciated.
DEDICATION

To

Ana Luisa Child

here is to three years apart,
we did it,
now let us see what life brings together

And
my parents,
Jerry and Betty,
whose support, encouragement and love
kept me going from start to finish
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ABSTRACT

Cryptosporidiosis is a major cause of diarrheal illness worldwide and characterized by several daily bowel movements, resulting in fluid loss and dehydration. Two species, Cryptosporidium hominis and C. parvum (previously designated C. parvum genotype I and genotype II, respectively) are the main causative agents in human infection; however, thirteen Cryptosporidium species are taxonomically recognized of which ten are documented to infective humans. Complicating matters for disinfection, epidemiological, and treatment studies, C. parvum isolates infect multiple mammalian species while C. hominis solely infects humans. Due to inherent characteristics of the oocyst, disease prevention relies heavily on detection and not disinfection although the current detection method lacks sensitivity and specificity and is unable to determine Cryptosporidium spp., genotype, or viability/infectivity status. Molecular detection techniques, while having the ability to speciate, genotype and/or assess viability status, have not been properly evaluated for sensitivity or specificity. The purpose of this dissertation was to: 1) develop a C. parvum PCR based detection method and discuss its limitations (i.e. the lower limit of detection in both laboratory and environmental samples); and (2) to extend current epidemiological and molecular data rationalizing the multiple C. parvum host specific infectivity patterns. To fulfill these two objectives, three separate experiments were designed and executed. The results from which are included in the appendix as peer reviewed published manuscripts and are the basis of this dissertation.
1.0 INTRODUCTION:

1.1 Preface:

Parasitic beings exist in many different forms and the associated lifecycles are as numerous as the diversity of parasites themselves. This diversity ranges from 'non-living' prions and viruses, to bacteria, fungi, ciliates, amebas, worms, and ecto-parasites such as lice and ticks. Each of these parasites can be classified into different groups of parasitism. In the most general sense, the parasite/host relationship, coined in 1879 by de Bary, is defined as a symbiosis or basically “intimately living together” (Roberts and Janovy, 2000). From this general core, three major classifications are described: mutualism, commensalism, and parasitism.

Parasitic relationships in which both host and parasite derive benefit are defined as mutualism. An example of such is bacterial nitrogen fixation with leguminous plant such as peas and beans. These plants lack the enzyme, nitrogenase, to fix nitrogen from the environment. Rhizobium bacteria contain the enzyme and are able to fix atmospheric nitrogen under anaerobic conditions. The mutualistic relationship arises when root nodules are formed on the plant. The root nodules maintain the required anaerobic environment (via oxygen scavenging molecules such as leghaemoglobin of the plant) for nitrogenase to fix nitrogen that is then available to the plant. In return the root nodule provides a niche for protection and a place for the bacteria to grow.

Commensalism is a relationship in which the parasitic being benefits from the association and the host is neither helped nor harmed. Transportation is a form of commensalism. Remora fish use their modified mouth for attachment to sharks, manta
rays, whales, turtles and boats and thereby accessing free locomotion. In addition, the remora, by virtue of vicinity, obtains food without having to acquire food for itself.

Finally, in parasitism, the host is either directly harmed or in some manner, the parasite lives at the expense of the host organism. Organisms that cause disease are classified in this group. The focus of this dissertation, *Cryptosporidium parvum*, is such an example. The existence of *C. parvum* is completely dependent on the host. The host provides the proper environment for parasite growth, acquisition of nutrients, and reproduction, while itself is physically harmed during the process even to the extent of death in certain cases.

**Why study *C. parvum***? Without doubt, *C. parvum* infection constitutes a major cause of persistent diarrhea worldwide. Among those who are immunocompromised, cryptosporidiosis can be devastating and is often fatal (Vakil *et al.*, 1996; Wuhib *et al.*, 1994). The self-perpetuating life-cycle aspects of *C. parvum* combined with the lack of decisive chemotherapy contribute to this distressing issue. Among immunocompetent adults, cryptosporidiosis is a self-limiting disease, however the illness has major effects in economic losses (medical and productivity) (Corso *et al.*, 2003; Havelaar *et al.*, 2000). Among children, especially in developing countries, cryptosporidiosis and the related diarrhea burden is responsible for nutrition shortfalls that then exacerbates into slowed growth and development. Physically, cryptosporidiosis in children is responsible for impaired weight gain (Checkley *et al.*, 1997), stunting of height (Checkley *et al.*, 1998), and general lowered physical fitness (Guerrant *et al.*, 1999). In addition, dehydration and malnourishment episodes, both consequences of *C. parvum* infection, occurring during
the first two years of life correlate with long-term cognitive deficits (Guerrant et al., 1999; Niehaus et al., 2002). These combined effects of cryptosporidiosis warrant the past, current and future research on this organism.

The purpose of this dissertation is two fold: (1) develop a C. parvum PCR based detection method and discuss limitations of such molecular bases detection methods (i.e. the lower limit of detection in both laboratory and environmental samples); and (2) extend current epidemiological and molecular data rationalizing the multiple C. parvum host specific infectivity patterns. It is hoped that this information will be of assistance to both water and research communities by providing basic knowledge that can be built upon in the future.

1.2 History

In 1907, Tyzzer characterized a sporozoan parasite reproducing in the stomachs, specifically the gastric glands, of mice (Tyzzer, 1907). He described the organism as having asexual and sexual stages, a specialized attachment organelle, and ‘spores’ (oocysts) excreted in the feces (Tyzzer, 1907). In 1910 he named the organism Cryptosporidium muris, ‘crypto’ for small/hidden and ‘spore’ for the excreted oocyst (Tyzzer, 1910). In 1912, Tyzzer described a second sporozoan with oocysts smaller than C. muris infecting only the small intestine (Tyzzer, 1912). He named this second species C. parvum. Later, in 1929, he published the first report describing the developmental stages of C. parvum (Tyzzer, 1929).
The genus *Cryptosporidium* was not studied for the next 48 years, as it was not deemed to be of economic, medical, or veterinary importance. Although *C. meleagridis* was determined to be the causative agent for a major turkey kill-off in 1955 (D. Slavin, 1955), it wasn’t until 1971 that *C. parvum* was perceived important due to the economic impact as a result of bovine diarrhea caused by this organism (R. Panciera *et al.*, 1971). The first reported human *C. parvum* cases occurred in 1976 (Meisel *et al.*, 1976; Nime *et al.*, 1976a) and it wasn’t until the advent of HIV/AIDS in 1982 that cryptosporidiosis was recognized with worldwide interest (Anonymous, 1982).

In the early to mid 1990’s, greater than 20 *Cryptosporidium* species were described in various animals, however the validity of these multiple species is questionable and has not been confirmed (O'Donoghue, 1995). As of 1997, eight *Cryptosporidium* species were recognized based on host specificity, infection locality, and oocyst size (Fayer *et al.*, 1997): *C. baileyi* (chicken), *C. felis* (domestic cat), *C. meleagridis* (turkey), *C. muris* (house mouse), *C. nasorum* (fish), *C. parvum* (mammals), *C. serpentinis* (snakes), and *C. wrairi* (guinea pig). At present, with the advent of molecular tools and more intensive epidemiological studies, the genus *Cryptosporidium*, and specifically the species *C. parvum*, has been redefined and differentiated. Appearing at the genus level, five new species have been classified including *C. andersoni* (Lindsay *et al.*, 2000), *C. canis* (Fayer *et al.*, 2001; Morgan *et al.*, 2000), and *C. hominis* (Morgan-Ryan *et al.*, 2002), *C. molnari* (Alvarez-Pellitero and Sitja-Bobadilla, 2002), *C. saurophilum*, previously *Cryptosporidium* desert monitor genotype (Koudela and Modry, 1998), making a total of 13 recognized *Cryptosporidium* species (Table 1). Within the
bounds of the *C. parvum* species, there are now 4 recognized genotypes including bovine, monkey, mouse, and rabbit (Xiao et al., 2002). There are also several unclassified *Cryptosporidium* parasite isolates including bear, bovine genotype B, deer, deer mouse, ferret, fox, goose, lizard, marsupial, muskrat, opossum I and II, pig, skunk, snake, and tortoise (Xiao et al., 2002).

As of 1995 *C. parvum* had been isolated from 79 species of mammals ranging from wildlife to livestock and humans (O'Donoghue, 1995) with the conundrum being: 'how does one species infect so many different hosts?' Original evidence of different *C. parvum* genotypes and possible division of *C. parvum* into different species appeared in 1991. Restriction fragment length polymorphism (RFLP) and Southern blot analysis of a repetitive DNA region were used to demonstrated that human *C. parvum* isolates differed from bovine isolates (Ortega et al., 1991). This study was expanded upon with the first *Cryptosporidium* spp. PCR/RFLP based detection system (Awad-El-Kariem et al., 1993). Molecular techniques and infectivity patterns were further used to separate *C. parvum* into genotype I, infecting only humans with exceptions of a dugong (*Dugong dugon*) (Morgan et al., 1998b) and a gnotobiotic piglet model (Widmer et al., 2000a), and genotype II being infectious for bovine and other mammals (Awad-el-Kariem, 1996; Bonnin et al., 1996; Morgan et al., 1995). Only recently it was proposed that *C. parvum* genotypes I and II are not distinct genotypes but rather distinct *Cryptosporidium* species, *C. parvum* (genotype bovis) and *C. hominis* (Morgan-Ryan et al., 2002). This distinction is based upon host infectivity patterns and multilocus genomic assessment. This splitting of *C. parvum* into *C. parvum* genotype bovis and *C. hominis* is generally accepted and
lends itself to explaining how the single *Cryptosporidium* species could infect multiple species; when in fact two and possibly more *Cryptosporidium* species were causing the observed cryptosporidiosis in the multiple mammalian hosts.

### 1.3 Taxonomy

Taxonomy places *C. parvum* in the phylum Apicomplexa and class Sporozoa (Levine, 1980, 1984). This group includes many medically important human and veterinary parasites such as *Cyclospora cayetanensis*, *Eimeria* spp., *Isospora* spp., *Plasmodium* spp., *Sarcocystis* spp., and *Toxoplasma gondii* (Levine, 1984; Morgan *et al*., 1999). Along with other shared morphological characteristics, the inclusion of an apical complex found in the sporozoites and merozoites within these organisms is a primary distinguishing feature that is common among this group. Based on 18S rRNA gene sequence, *C. parvum* is a member of the protozoan Infrakingdom Alveolata, which is monophyletic and contains three Phyla: Apicomplexa syn. Sporozoa (apicomplexans), Ciliophora (ciliates), and Dinozoa (dinoflagellates) (Cavalier-Smith, 1993; Cavalier-Smith, 1999; Gajadhar *et al*., 1991).

### 1.4 Life Cycle

The life cycle is presented in Figure 1, conveying both sexual and asexual reproduction stages of *C. parvum*. The life cycle begins when a host ingests an infectious (sporulated) oocyst, the only exogenous stage of the life cycle. The oocysts themselves are composed of a tough two-layered wall and contain four haploid (chromosome copy
number: 1N) sporozoites. Unlike other intestinal Apicomplexa members, Cryptosporidium spp. oocysts have two unique features. First, the sporozoites are not contained within sporocysts, although this is under debate (Beier et al., 2001; Beier, 2000), and second, the oocysts are excreted fully sporulated, thus allowing for immediate infection of the next unsuspecting host. After ingestion and exposure to elevated temperature, pancreatic enzymes and/or bile salts, the sporozoites excyst from the oocyst. Depending on the Cryptosporidium spp., the infection locality differs. Generally, C. parvum excysts and infects the jejunum and terminal ileum of the intestinal tract. However, the respiratory tract is also known as an opportunistic site of infection. Cryptosporidium species such as C. andersoni and C. muris prefer to infect the gastric glands, while C. canis, C. parvum, and C. wrairi prefer to infect the small intestine, specifically, the jejunum. The released sporozoites adhere to an epithelial cell luminal surface probably involving Gal/GalNac specific lectins (Chen and LaRusso, 2000). The attachment prompts the microvilli to evaginate the sporozoite using the host actin cytoskeletal components of filamentous actin and the actin-binding protein α-actinin, thus creating a parasitophorous vacuole (Elliott and Clark, 2000; Elliott et al., 2001). Again, unlike other Apicomplexa members, Cryptosporidium parasites are unique and develop within the host cell outer membrane but remain extracytoplasmic. Next, within the parasitophorous vacuole, the “feeder organelle” develops between the host cell cytoplasm and the developing sporozoite. The function of the feeder organelle is yet to be fully determined, however, freeze-fracture electron microscopy of the feeder organelle/host cytoplasm interface demonstrated visible membrane folds in the feeder organelle with
some of the folds being connected to small cytoplasmic vesicles, indicating that the
feeder organelle may play an important role for incorporation of nutrients from the host
cell (Yoshikawa and Iseki, 1992). The infection subsequently progresses through asexual
and sexual stages. Each of the sporozoites that successfully navigates to and initiates
infection in an epithelial cell develops into a spherical trophozoite. The trophozoite
subsequently matures and begins the process of schizogony, or asexual reproduction (also
called merogony). Schizogony is the multiple fission (multiple mitoses) of the nucleus
before cytokinesis. Following schizogony and the generation of six to eight nuclei, the
trophozoite develops into a first-generation schizont (structurally called a type I meront).
Each of the nuclei is incorporated into a single merozoite. Merozoites are structurally
similar to sporozoites and after maturing and exiting the type I meront, infect another
host epithelial cell. The infection caused by a type I merozoite develops into either
another type I meront, thus creating a repeating cycle of type I meront generation, or into
a type II meront. Type II meronts also undergo schizogony and produce four type II
merozoites. The sexual stages begin when the type II merozoites exit the type II meront
and enter new host cells and develop into microgamonts and macrogamonts (both are
haploid). Microgamonts become multinucleated, with each nuclei developing into a
microgamete, while macrogametes maintain in a uninucleate state. At this stage the
microgametes exit the microgamont and fertilize a macrogamete, thus creating diploid
zygotes (2N copy number). At this stage, fertilization followed by meiosis and crossing
over of the chromosomes can occur thus providing opportunity for reassortment of genes
and recombination. The fertilized macrogamonts develop into immature oocysts
followed by *in situ* sporulation and the final development of an oocyst with four sporozoites. Some of the mature oocysts develop a thin wall, which allows for autoinfection of the host, while the majority develops a thick double oocyst wall that is excreted from host and into the environment. Secreted oocysts vary in size depending on the different *Cryptosporidium* species (Table 2) with *C. parvum* being basically round with dimensions of 5μm by 4.5μm (Figure 2) (Fayer *et al.*, 1997).

Outcrossing and sexual recombination were shown to occur and but the degree of such was not measured. In the INF-gamma knockout mouse model, two *C. parvum* isolates, expressing distinct microsatellite loci, were inoculated on separate days (one isolate was demonstrated to require more time to establish infection). The resulting populations were passed again through INF-gamma knockout mice (at low oocysts numbers in order to propagate large oocyst numbers for microsatellite analysis). Evidence of recombination did exist as one oocyst population expressed both microsatellite markers while maintaining the parental multilocus pattern. One limitation of this study is that the analysis was performed on a population of oocysts instead of single oocysts. Measures were taken to account for this issue however these actions were not 100% effective (Feng *et al.*, 2002).

1.5 Disease and Pathogenesis

*Cryptosporidiosis* is characterized by intermittent and profuse watery diarrhea with other symptoms including anorexia, abdominal cramps, headache (neuralgia), fatigue, fever, vomiting, and general weight loss (Fayer *et al.*, 1997; Goodgame, 1996;
Leav et al., 2003). In the immunocompromised population, fluid loss within a single day has been documented to be between twelve and seventeen liters with as many as 71 bowel movements (Fayer et al., 1997). Within the immunocompetent population, the duration of clinical symptoms in one group of fifty patients ranged from two to twenty-six days with a mean of 12 days (Jokipii and Jokipii, 1986). This variability in clinical signs makes diagnosis of Cryptosporidium infection somewhat subjective and microscopic identification of oocysts in stool is the definitive diagnosis (Arrowood, 1997). Cryptosporidiosis is generally self-limiting and disease resolves itself after 21 days in immunocompetent hosts. However, individuals with a compromised immune system are at risk of continued diarrhea, dehydration and eventually death if rehydration therapy is not established. Clinical features are outlined in Table 3.

Cryptosporidium parvum infection begins with ingestion of the sporulated oocyst, followed by sporozoite excystation in the small intestine, particularly the jejunum and terminal ileum, and finally enterocyte (and other epithelial cells) attachment and sporozoite directed superficial invasion and formation of the parasitophorous vacuole (Current, 1988; Fayer et al., 1997; Leav et al., 2003). As noted earlier, other Cryptosporidium species, such as C. muris, infect the gastric glands of the stomach rather than the small intestine. Once infection has been established, various host cell lesions can be observed. These lesions generally consist of villous atrophy and/or fusion, microvilli blunting, and epithelium metaplasia into cuboidal shaped cells (Heine et al., 1984b). The intestinal cell crypts, on the other hand, elongate and become hyperplastic
(Laurent et al., 1999). The severity of these observed lesions correlates with the number of infecting organisms (Godwin, 1991).

Although advances have been made in characterizing cryptosporidiosis infection, virulence determinants are not well characterized and the actual cause for the observed abnormalities within the normal absorptive and secretory functions of the gut is poorly understood (Okhuysen and Chappell, 2002). One explanation for the large volume of diarrhea observed is prostaglandin (PG) production/release from inflamed tissue (Argenzio et al., 1990; Argenzio et al., 1993; Laurent et al., 1998). In the porcine model, increased levels of prostaglandins PGE$_2$ and PGI were observed at the mucosal surface (Argenzio et al., 1990). High levels of PGs have been shown to inhibit neutral NaCl absorption resulting in secretory diarrhea (Argenzio et al., 1990; Argenzio et al., 1993; Laurent et al., 1998). It is hypothesized that leukocytes, resident and recruited, maybe one source for the high levels of PGs (Argenzio et al., 1993). It was shown that \emph{C. parvum} infected intestinal cells had increased prostaglandin H synthase-2 expression resulting in PGE$_2$ and PGF$_{2\alpha}$ production (Laurent et al., 1998).

A second reason for diarrhea is via increased permeability within the intercellular junction complexes between intestinal epithelial cells. \textit{In vitro} infection showed increased permeability and decreased resistance between \emph{C. parvum} infected epithelial Caco-2 cells allowing leakage of fluids and molecules $\leq 1,000$ Da (Griffiths et al., 1994). This study also reported the release of cytoplasmic lactate dehydrogenase, indicating cell damage and ultimately death (Griffiths et al., 1994).
Of the characterized virulence factors, research has centered on attachment factors and parasitophorous vacuole formation. The first adhesion/locomotion molecule described is CPS-500, a polar-glycolipid, was shown to be non-proteinaceous in nature, comprised of mannose and inositol residues (Riggs et al., 1989; Riggs et al., 1999). It is involved in sporozoite and merozoite locomotion as well as host cell attachment as neutralizing monoclonal antibodies (mAbs) to one or more sensitive epitopes were shown to inhibit infection \textit{in vitro} (Bjorneby et al., 1990; Riggs et al., 1989; Riggs et al., 1999). CPS-500 also has terminal b-D-mannopyranosyl residues with mannose and inositol being predominant (Okhuysen and Chappell, 2002; Riggs et al., 1999). Mammals do not have terminal b-D-mannopyranosyl residues thus making it a possible therapeutic site for treating cryptosporidiosis (Okhuysen and Chappell, 2002; Riggs et al., 1999).

p23, a protein involved in parasite motility and attachment, was found on the sporozoite surface as well as being deposited in trails on microscope slides (Arrowood et al., 1991; Perryman et al., 1996). This small protein contains two distinct neutralization sensitive epitopes identified by two mAbs (C6B6 and 7D10), the effects of which were to reduce \textit{C. parvum} infection \textit{in vivo} in a mouse model (Perryman et al., 1996). The presence of p23 has also been shown to elicit an immune response and murine IgA mAbs against p23 were shown to be protective (Enriquez and Riggs, 1998). In addition, vaccination of cows with a recombinant p23 molecule resulted in the production of protective colostral antibodies (Perryman et al., 1999).

GP900 is a large secreted glycoprotein composed of 5 domains; domains 1 and 3 are cysteine rich; domains 2 and 4 act as separators and are comprised of polythreonine
regions; domain 5 is the largest and has a degenerate repeat structure (Barnes et al., 1998; Petersen et al., 1992). It is proposed that domain 5, with its many N-glycosylation sites forms a transmembrane segment while domains 2 and 4 are rigid and consequently extend domains 1 and 3 away from the parasite and closer to the host epithelium (Barnes et al., 1998). The proposed role of GP900 is that of attachment and invasion (Barnes et al., 1998; Petersen et al., 1997). As with CSP-500, GP900 is found on the surface of and shed from both sporozoites and merozoites (Petersen et al., 1997), is maintained in micronemes and deposited during the infection process (Barnes et al., 1998; Petersen et al., 1992). Antibodies against domains 1 and 3 inhibited sporozoite attachment/invasion in MDCK cells as assessed by microscopic observation (Barnes et al., 1998; Gut et al., 1991). Pre-treatment of Caco 2A cells with native GP900 or recombinant proteins consisting of domains 1 and 3 significantly blocked invasion (Barnes et al., 1998). While the authors concluded that invasion was blocked, these experiments did not determine whether attachment/adhesion or subsequent steps in invasion were mediated by GP900.

The circumsporozoite-like (CSL) glycoprotein is approximately 1300kDa and is found in both the micronemes and the dense granules of sporozoites and merozoites (Langer and Riggs, 1999; Riggs et al., 1994; Riggs et al., 1997; Schaefer et al., 2000). Collectively, CSL is comprised of 15 different molecular species (based on isoelectric focusing; range pI 3 to 10) and a subset of 6 molecules (pI 4 to 6.5) was specifically recognized by mAb 3E2, an antibody that recognizes a carbohydrate epitope on CSL. The range of different molecules is thought to be a reflection of differences in glycosylation and/or sialylation state of a single glycoprotein. Functionally, sporozoite
infection was completely inhibited in Caco 2A cells after exposure with mAb 3E2 and this mAb also passively protected in a mouse model (Riggs et al., 1997). Further characterization of CSL identified an 85 kDa epithelial cell receptor and pre-exposure of sporozoites with this receptor inhibited Caco 2A cell infection (Riggs et al., 1997).

Another C. parvum surface protein, TRAP-C1 (Spano et al., 1998), has sequence and structural similarities to the well-characterized thrombospondin family of adhesion molecules such as the Plasmodium spp. TRAP molecules (Robson et al., 1988), the MIC 2 protein of T. gondii (Wan et al., 1997), and the E. tenella Etp 100 (Tomley et al., 1991). TRAP-C1 is a sporozoite apical protein stored in the micronemes (Spano et al., 1998) and while not directly proven, its relatedness to the other adhesion molecules gives strong grounds for its role in C. parvum attachment/adhesion.

The most recent surface protein identified is the proteolytically cleaved Cpgp 40/15 (Cevallos et al., 2000; Strong et al., 2000). These two proteins, gp40 and gp15, are presumed to be the cleaved product of the larger protein, gp60 (Cevallos et al., 2000; Strong et al., 2000). Gp40 has been shown to share an epitope with GP900 and is functional in cell attachment (Cevallos et al., 2000; Strong et al., 2000).

Taken together, C. parvum possess an array of adhesion and invasion proteins. While each of these proteins is unique and some have been shown to have unique host cell receptors, these proteins might work in succession using more than one ligand and thus increasing the chances of attachment followed by invasion during the infection process. As an example, it is possible that initial attachment to the host glycocalyx
mediated by TRAP-C1 is followed by more specific attachment of GP900 and/or CSL to host surface membrane receptors (Langer and Riggs, 1999).

Also, it appears that *C. parvum* is selective in causing cellular distress. Recent *in vitro* findings have reported that cells infected with *C. parvum* do not undergo apoptosis while neighboring, non-infected cells are promoted to undergo this programmed cell death (Chen *et al.*, 2001; Heussler *et al.*, 2001; McCole *et al.*, 2000; Ojcius *et al.*, 1999; Widmer *et al.*, 2000b). The ability of *C. parvum* to prevent apoptosis includes up-regulation of host cell heat shock proteins in conjunction with parasite-dependent activation of NF-kappaB (Chen *et al.*, 2001; Heussler *et al.*, 2001). *C. parvum*-regulation of host cell apoptosis provides this organism with two major advantages. First, prevention of apoptosis within infected cells enables complete parasite development to the next stage by remaining intracellular and avoiding the host immune system. Second, promotion of neighboring cell apoptosis possibly directs any immune reaction away from the infected cell itself. This tactic to prevent host cell apoptosis has proven successful for other intracellular protozoan parasites including *Leishmania* spp., the microsporidian *Nosema algerae*, *Theileria* spp., *Toxoplasma gondii*, and *Trypanosoma cruzi* (Heussler *et al.*, 2001).

### 1.6 Treatment

Unlike certain Coccidia members that respond favorably to chemotherapy, for example treatment with trimethoprim-sulfamethoxazole resolves cyclosporiasis in seven days (Verdier *et al.*, 2000), over 400 compounds have been tested *in vivo* and *in vitro* to
treat cryptosporidiosis, all of which have demonstrated limited success with the exception of nitazoxanide (see below) (Tzipori and Ward, 2002). The refractive nature of *C. parvum* is thought, in part, to be a result of the parasitophorous vacuole providing shelter (Clark, 1999).

Passive immunotherapy has shown some degree of efficacy, although disease resolution has been partial rather than complete (Crabb, 1998). Approaches include mAb or polyclonal antibodies (Arrowood *et al*., 1989; Riggs, 2002; Riggs *et al*., 2002), antibodies delivered in egg yolks produced by hyperimmune hens (Cama and Sterling, 1991), ingestion of heat killed oocysts (Harp and Goff, 1995), colostrum from vaccinated cows (Fayer *et al*., 1989; Jenkins *et al*., 1999; Tzipori *et al*., 1986; Ungar *et al*., 1990b), and recombinant *C. parvum* surface proteins (Jenkins, 2001; Perryman *et al*., 1996; Takashima *et al*., 2003). Recombinant protein therapy is based on the hypothesis that *C. parvum* surface proteins are critical for motility, attachment and invasion and thus are targets for blocking infection. A recombinant p23 protein, expressed by bovine herpesvirus-1, was administered to rabbits and induced neutralizing p23 IgG antibodies (Takashima *et al*., 2003). Serum from the inoculated rabbits was then shown to be protective in HCT-8 cells for sporozoite infection indicating that these IgG antibodies are neutralizing.

Recently, nitazoxanide, a FDA approved drug for treatment in children, is showing promise in the treatment of cryptosporidiosis. Studies located in Africa (Amadi *et al*., 2002; Doumbo *et al*., 1997), Egypt (Rossignol *et al*., 2001), and Mexico (Diaz *et al*., 2003; Rossignol *et al*., 1998), reported reduction in oocyst shedding and/or complete
resolution of disease. In a double-blinded, placebo-controlled study in immunocompetent
children and adults, a three-day treatment regimen resolved diarrhea within seven days in
39 (80%) of 49 patients compared with 20 (41%) of 49 patients in the placebo control
group (Rossignol et al., 2001). However, a separate study reported treatment failure in an
HIV-positive patient (Giacometti et al., 1999).

The best treatment approach for immunocompetent and immunocompromised
individuals who are HIV negative remains oral rehydration therapy to prevent
dehydration. This includes fluid and electrolyte replacement, nutritional support,
antidiarrheal drugs, and antimicrobial drugs. The best approach for
immunocompromised individuals with HIV is the combination of oral rehydration
therapy and HAART treatment (Highly Active Antiretroviral Therapy) (Maggi et al.,
2000; Maggi et al., 2001). By greater suppression of viral replication thus keeping the
immune system operable, cryptosporidiosis will be resolved in the normal course of the
disease.

1.7 Immune Response

Control of Cryptosporidium infection, in both humans and ruminants, appears to
be age dependent with the young being very susceptible while adults are generally
refractory to infection (Gookin et al., 2002; Ortega-Mora and Wright, 1994). Lack of in
vitro cultivation and an ideal animal model have impeded identification of a protective
antigen to a certain extent (Ungar et al., 1990a). Yet, it is recognized that both cellular
and, to a lesser extent, humoral immunity play a role in the control of the disease.
Specifically, critical mediators included T lymphocytes, the cytokine gamma interferon (IFN-γ), and receptor/ligand interaction of CD40 and CD154 (CD40 ligand) for intercellular communications (Gookin et al., 2002).

Innate Immunity: Some of the intestinal epithelium innate immune responses consists of production of pro-inflammatory molecules and defensins from activated enterocytes (Tarver et al., 1998) as well as the presence of macrophages and natural killer (NK) cells (Bancroft and Kelly, 1994). IFN-γ has been demonstrated to play a role in innate immunity as treatment with anti-IFN-γ antibodies increases the severity of infection in both BALB/c and adult SCID mice (Chen et al., 1993b; McDonald and Bancroft, 1994; Ungar et al., 1991). In addition, the presence of IFN-γ has direct effects on epithelial cells including increased expression of MHC II receptors (Ruemmele et al., 1998), increased paracellular permeability of intercellular tight junctions (specifically loss of zonal occluden-1 function) (Han et al., 2003; Youakim and Ahdieh, 1999), and up-regulation of transmembrane CD40 expression (Kooy et al., 1999). These changes in the epithelium may enhance interaction with cytotoxic effector cells of the innate as well as cellular and humoral responses. In addition, pretreatment of Caco 2 and HT29 cells with IFN-γ (1 – 1000 U/ml) inhibited parasite development (McDonald et al., 1999; Pollok et al., 2001). NK cells are a likely source of IFN-γ, however the depletion of NK cells with anti-ASGM-1 antibodies and stimulation of NK cells with IL-12 in severe combined immunodeficient (SCID) mice had no effect on the pattern of C. parvum infection (McDonald and Bancroft, 1994; Rohlman et al., 1993). IFN-γ also directly induces nitric oxide production (NO) (Linn et al., 1997).
Cellular Immunity: Termination of cryptosporidiosis has been largely deciphered with T lymphocytes, CD4+ and CD8+ (cytotoxic T cells), playing major and minor roles, respectively (Chen et al., 1993a). In 1984, neonatal nude (athymic) mice were shown to develop chronic and eventually fatal cryptosporidiosis while control mice developed self-limited infections (Heine et al., 1984a). Subsequent studies involving SCID mice confirmed the same pattern indicating that T-cells and not B-cells play a role in eliminating disease (Chen et al., 1993b; Kuhls et al., 1992; McDonald et al., 1992; Mead et al., 1991a; Mead et al., 1991b). It was further demonstrated that SCID mice reconstituted with spleen cells taken from immunocompetent mice were able to resolve disease (Chen et al., 1993a; McDonald et al., 1992; Perryman et al., 1994). Immunocompetent or reconstituted SCID mice treated with anti-CD4+ or anti-IFN-γ had reduced or abolished ability to resolve disease while mice receiving anti-CD8+ antibodies showed no marked effect (Chen et al., 1993a; McDonald et al., 1992; Ungar et al., 1990a; Ungar et al., 1991). In addition, reconstituted mice had C. parvum-specific antibodies in serum as well as anti-CD8+ antibody treated mice, whereas anti-CD4+ antibody treated mice had no detectable C. parvum antibodies (Chen et al., 1993a). Mice deficient in major histocompatibility complex (MHC) class I (therefore lacking functional CD4+ T-cells) were susceptible to C. parvum infection while mice deficient in MHC II (lacking CD8+ T-cells) were not (Aguirre et al., 1994). Further supporting evidence comes from the roles of T-cell receptor types αβ or γδ (Waters and Harp, 1996). CD4+ T-cells generally express αβ type receptors while CD8+ T-cells express the γδ type. Adult and neonate mutant mice, lacking either one of these receptors, were challenged with C.
parvum infection. αβ T-cell receptors were required for recovery in both neonate and adult mice while γδ receptors played only a minor role in neonate protection and not at all in the adults (Waters and Harp, 1996). Cryptosporidiosis in HIV-infected patients, where susceptibility and severity are higher, also provides evidence for the importance of an effective CD4⁺ T-cell population (Flanigan et al., 1992). Confounding these observations, and therefore re-emphasizing the importance of the appropriate animal model, are reports indicating that CD8⁺ T-cells are equally important in the control of C. parvum infection in calves (Abrahamsen et al., 1997; Fayer et al., 1998a; Wyatt et al., 1997; Wyatt et al., 1999).

Further investigations studied the CD4⁺ T-cell population subtypes, T-cell helper-1 (Th1) and Th2 cells. Classification of Th1 or Th2 is based on the secreted cytokine types. Th1 cells secrete cytokines IFN-γ and IL-2 that promote a cell-mediated response (Abbas et al., 1996). Th2 cells secrete IL-4, IL-5, and IL-10, which are associated with an allergic or antibody-mediated response (Abbas et al., 1996). MAb depletion of IFN-γ and IL-12 (a key regulatory cytokine that promotes the Th1 response) increase severity and duration of disease (Aguirre et al., 1998; Chen et al., 1993b; McDonald et al., 1992; Ungar et al., 1991). Increased IFN-γ production by CD4⁺ T-cells, mesenteric lymph node cells and mucosal T-cells has been documented during C. parvum infections (Culshaw et al., 1997; Harp et al., 1994; Tilley et al., 1995). In addition, during the course of infection, intraepithelial lymphocytes (IELs) producing IFN-γ increased in cell quantity (Culshaw et al., 1997) as well as enhanced expression of IFN-γ mRNA (Fayer et al., 1998a; Wyatt et al., 1997). Lastly, injection of IL-12 one day prior to C. parvum
exposure completely protected newborn mice from disease development while depletion of IL-12 with mAbs increased parasite reproduction (Urban et al., 1996a; Urban et al., 1996b). The presence of IL-2 may also be involved in disease control. Treatment with anti-IL-2 antibodies in neonatal mice increased susceptibility to *C. parvum* infection (Enriquez and Sterling, 1993; Ungar et al., 1991). Also, *in vivo* and *in vitro* infections with *C. muris* and *C. parvum* detected increased levels of IL-2 (Davami et al., 1997; Harp et al., 1994). These studies indicate that a Th1 response is involved in immunological control during the early stages of infection.

A Th2 response is proposed to invoke disease resolution during late stages of primary infection. With IFN-γ being a main cytokine involved in the Th1 response, BALB/c mice genetically lacking IFN-γ (GKO) mice were infected with *C. parvum* but eventually recovered (Mead and You, 1998). Furthermore, anti-IFN-γ antibodies administered at the onset of disease intensified the infection while antibodies administered late in the disease process had no effect suggesting that IFN-γ levels are lower during the late stages of recovery (Aguirre et al., 1998). Antibody-mediated depletion of IL-4 and IL-5 reduced the Th2 response, resulted in increased *C. parvum* oocyst production and lengthened the duration of illness (Aguirre et al., 1998; Enriquez and Sterling, 1993).

Humoral Immunity: Early studies indicated that the humoral immune response is very important for protection against cryptosporidiosis (Campbell and Current, 1983). Surface IgG, IgM, and IgA levels are all associated with resolution of diarrhea and oocyst shedding (Casemore, 1987). However, the pattern of *C. parvum* infection (onset, peak, or
duration) did not differ between control and B cell-deficient neonatal BALB/c mice (Taghi-Kilani et al., 1990). Also, primary infection with C. parvum does not alleviate infection after a second exposure to oocysts but rather reduces clinical illness (Current and Bick, 1989; Newman et al., 1994; Okhuysen et al., 1998). Preliminary data suggest that it may be possible to develop immunity with frequent exposure to oocysts (Madico et al., 1997). A second exposure study with human volunteers demonstrated that fewer individuals shed oocysts after the second exposure (3 of 19; 16%) than after the first exposure (12 of 19; 63%) (P < 0.005) and IgG and IgA seroconversion was higher after the second exposure (Okhuysen et al., 1998). Yet, this antibody response did not correlate with the presence or absence of infection (Okhuysen et al., 1998).

Nitric oxide (NO), while not essential, was demonstrated to have a role in cryptosporidiosis recovery. C. parvum infection in mice resulted with increased levels of plasma NO concentrations as well as inducible NO synthase enzyme (iNOS) synthesis by the infected epithelium (Leitch and He, 1999). Mice genetically lacking iNOS or treated with iNOS blocking agents were more susceptible to infection had increases in amount and duration of oocyst shedding, and epithelial colonization (Leitch and He, 1994, 1999), however, recovery still ensued (Hayward et al., 2000; Kuhls et al., 1994a).

Taken together, these results indicate that CD4+ and IFN-γ are involved in disease resolution, while CD8+ T-cells, NO, and antibodies specific for C. parvum are involved to a lesser extent.
1.8 Ecology

The ecology of *C. parvum* centers around six major aspects of the organism 1) the sexual/sexual life cycle within the vertebrate small intestine including a) auto-infective cycles, b) production immediately infective oocysts and c) the large mass of oocysts produced each day; 2) the small size of the oocyst; 3) low infectious dose; 4) resistance of the oocyst to environmental and disinfection conditions; 5) lack of therapy or a vaccine; and 6) zoonotic potential.

1) All Apicomplexans are parasitic in nature and have a complex intracellular life cycle, which includes both sexual and asexual stages. Certain genera of the phylum infect only a single host (*Eimeria* spp.) while others can infect two or more separate hosts (*Toxoplasma*). The life cycle of *C. parvum* differs slightly from the general Apicomplexan life cycle in two major ways. First, *C. parvum* is different is that it has three routes for propagating the infection; two are autoinfection while the third involves transmission to other hosts. The first autoinfective route occurs during the asexual stages of reproduction. *Cryptosporidium* species differ from other Apicomplexa members, such as *Eimeria* spp., in that Type I merozoites are able to continually infect new epithelial cells in the absence of a healthy immune system (Current *et al.*, 1986). The second autoinfective route occurs with the production of a ‘thin walled’ oocyst. During the final stages of oocyst development, approximately 80% of the oocysts develop a ‘thick wall’ while the other 20% develop the ‘thin wall’ (Current, 1988). The thick walled oocysts are passed in feces and wait for exposure to the next host. While a portion of the ‘thin walled’ oocysts is also passed with the feces, some will excyst in the lumen of the
infected host’s intestine and these newly developed sporozoites are then able to establish infection in neighboring epithelial cells. Combined, the presence of thin-walled, autoinfective oocysts and the recycling of Type I meronts constitute a major health problem to those who lack an adequate immune system, due to malnourishment, drug therapy or immune deficiency. Second, *C. parvum* differs from other Apicomplexans in that the excreted ‘thick walled’ oocysts are immediately infectious to the next host (Current, 1988). Other oocysts, such as those of *Cyclospora*, are passed in an unsporulated state and need to proceed through the stage of sporogony to become infectious. This sporulation time can be days for *Toxoplasma gondii* (Hill and Dubey, 2002) to weeks for *Cyclospora cayetanensis* (Ortega *et al.*, 1993; Ortega *et al.*, 1994). *C. parvum* oocysts sporulate while still within the host and are passed as completely infectious entities for the next host. This trait provides a mechanism of direct fecal/oral transmission and thus can spread rapidly within a population. Finally, important to note is the number of progeny produced can be as great as $10^{10}$ oocysts in a single day. This high number of infectious oocysts increases the chances for immediate exposure to a second host as well as increasing the quantity of oocysts in source water (LeChevallier *et al.*, 1991).

2) The oocyst is approximately 4.5 X 5.5 micrometers in diameter (Table 2). Using water as a major transmission route, the oocysts are at the size limit of exclusion for conventional or direct filtration normally used in water treatment plants (WTP) (Clark, 1999). It should be noted that WTP are few and far between in developing countries and even if present, they are usually not operated to optimization for removal of
small particulates. Due to this inadequacy, it is easy to understand why transmission via water in developed and developing countries readily occurs.

3) The third ecological aspect is an experimentally determined low infectious dose. Three separate studies were performed with human volunteers and various C. parvum strains maintained by passage. In the first study, oocysts of the Iowa strain (bovine calf origin) were given to 29 human volunteers in single doses ranging from 30 to 1 million (DuPont et al., 1995). The median infective dose (ID$_{50}$) was 132 oocysts (DuPont et al., 1995). The follow up study tested three strains with the ID$_{50}$ being 87 for Iowa (bovine calf origin), 9 for Tamu (horse origin), and 1032 for UCP (bovine calf origin). Finally, the ID$_{50}$ of the Moredun strain (cervine origin) was determined to be 300 oocysts (Okhuysen et al., 2002). Also, in this final study, a single oocyst was able to infect GKO mice (Okhuysen et al., 2002).

4) Innate resistance of the oocyst plays a critical role in transmission of this disease. The ‘thick walled’ oocysts have been shown to be very hardy transmission packages both in natural environmental conditions and disinfection/removal processes (Korich et al., 1990; Smith and Rose, 1998). The oocyst has been demonstrated to be infectious for months either in environmental or in drinking water conditions (Fayer and Nerad, 1996; Fayer et al., 1996; Fayer et al., 1998c). Oocysts will remain infective if not disturbed from semisolid feces at ambient temperatures (Fayer et al., 1997), and oocysts maintained at 20°C for 6 months remained viable and infectious for suckling mice (Fayer et al., 1998c). Therefore, watershed management is of key interest due to potential run-off of fecal matter from livestock farms into rivers and reservoirs. Second, oocysts are
resistant to the chlorine concentrations used in water treatment facilities (2 ppm) and swimming pools/parks (Carpenter et al., 1999; Chauret et al., 1998; Korich et al., 1990; McAnulty et al., 1994). Exposure to these chlorine concentrations required greater than 2 days for oocyst inactivation (Korich et al., 1990). Oocysts aged in river waters and subsequently treated with chlorine and monochloramine were as infectious as control oocysts (Chauret et al., 1998). Unpurified oocysts introduced to a water matrix with 10 ppm of chlorine were fully infectious after 48 hours, indicating that fecal debris imparts a higher resistance to disinfection (Carpenter et al., 1999). In addition, over 25 chemical disinfectants, including household brands such as Lysol and Pine Sol, showed no marked reduction in oocyst viability as demonstrated via excystation (Fayer et al., 1997). Ammonia exposure at 1 and 5% concentrations for 30 minutes showed >60% and >94% reduction in sporozoite excystation (Fayer et al., 1997).

Other innate features that oocysts possess include limited resistance to freezing conditions while heating and desiccation are lethal (Anderson, 1985, 1986; Fayer, 1994). Oocysts frozen at −5°C remained infectious after two months and those frozen at −10°C were infectious for one week (Fayer and Nerad, 1996). Freezing at −20°C for 24 hours and snap freezing to −70°C killed oocysts (Fayer and Nerad, 1996).

5) The fifth aspect of C. parvum ecology is the refractory nature to chemotherapy and lack of a vaccine. As stated above, chemotherapy, even nitazoxanide, is not 100% effective and currently the best treatment remains supportive therapy. This refractive nature enables the infection to run its full course and in the process millions of oocysts are disseminated into the environment for potential future transmission. Vaccine
development has been limited and is directed towards passive immunotherapy (see treatment above) rather than active protective immunity (Jenkins, 2001). The approach of passive immunity is largely based on target populations most susceptible to infection, children from developing countries and neonatal ruminants (de Graaf et al., 1999). First, the most susceptible age (the young) corresponds with an immature immune system and therefore vaccine therapy would probably not be effective whereas passive immunity via colostrum is. Second, infection in immunocompetent adults is self-resolving and is rarely life threatening. Lastly, while cryptosporidiosis is endemic in many parts of the world, occurrence of the disease remains sporadic. Consequently, there is little perceived need for widespread vaccination (Jenkins, 2001).

6) With 152 mammalian hosts (Fayer et al., 2000) and multiple Cryptosporidium spp. and C. parvum genotypes able to infect humans, this large reservoir, both on livestock farms and in the wild, maintains a high zoonotic potential for cryptosporidiosis. However, as outlined in the transmission section, it appears that anthroponotic transmission occurs as much if not more than zoonotic transmission.

These ecological factors of C. parvum give rise to difficulty in controlling cryptosporidiosis. The highly resistant, small, fully sporulated and immediately infectious oocysts are an excellent means for transmission either directly or indirectly.

1.9 Epidemiology and Routes of Transmission

As a result of the different Cryptosporidium species that infect humans, cryptosporidiosis poses diverse public health challenges. During the time period from
initial recognition of human infection in 1976 (Meisel et al., 1976; Nime et al., 1976b) until the mid 1990’s, C. parvum was reported to infect over 79 different mammalian species (Fayer et al., 1997). Currently, there are 152 mammalian hosts of C. parvum and the unclassified C. parvum genotypes (Fayer et al., 2000). For years, researchers and medical personnel focused on C. parvum as being the only Cryptosporidium species causing infection in man, with a single reported human infection of C. meleagris (Ditrich et al., 1991). It is now documented that other Cryptosporidium species do cause human infection and these infections, while primarily documented in immunocompromised populations, are also observed in immunocompetent individuals (Meinhardt et al., 1996; Xiao et al., 2001a). Public health officials and researchers need not only to be informed about these new classifications but also, at the more basic level, determine risk factors (i.e. transmission patterns, survivability, disinfection resistance, etc.) of these other Cryptosporidium species and C. parvum genotypes.

Cryptosporidium infection has been documented on six continents and in over 40 countries (Kosek et al., 2001). In 1995, the incidence of C. parvum worldwide in immunocompetent individuals with diarrhea was reported to be 6.1% and 2.1% in developing and developed countries, respectively (Adal et al., 1995). In immunocompromised individuals with diarrhea, the incidence was 24% and 13.8% again in developing and developed countries, respectively (Adal et al., 1995). Similar incidence numbers were reported in a 1997 review of persons excreting oocysts (Guerrant, 1997). Two percent and 6.1% of immunocompetent and 14% and 24% of immunocompromised individuals were infected in developed and developing countries,
respectively (Guerrant, 1997). A serology study of infants, children and adolescents living in the United States reported that 20% had antibodies to *Cryptosporidium* (with a high of 58% in Oklahoma adolescents) (Kuhls *et al.*, 1994b). Serology in developing countries report that 65% of eight to ten year old children living in rural China are seropositive (Zu *et al.*, 1994) and 90% of infants living in a shantytown in Brazil are seropositive by their first birthday (Newman *et al.*, 1994). Lastly, based on positive stools, the cryptosporidiosis incidence rate in a cohort of Peruvian children less than two years old was 45% (Checkley *et al.*, 1997). These infection records indicate that evolved transmission routes of *Cryptosporidium* spp. are successful.

These transmission routes include 1) water-borne through drinking and recreational water, 2) food-borne, 3) person to person, both direct and indirect, 4) animal to person, 5) animal to animal, 6) possibly airborne, and 7) mechanical vectors.

1) Water-borne transmission. *Cryptosporidium* spp. oocysts have been found in surface water (LeChevallier *et al.*, 1991), ground water (Bridgman *et al.*, 1995; Hancock *et al.*, 1998; Moulton-Hancock *et al.*, 2000), treated drinking water (Lee *et al.*, 2002), untreated wastewater, filtered secondarily treated wastewater, activated sludge effluent (Chauret *et al.*, 1999; Madore *et al.*, 1987; Suwa and Suzuki, 2001, 2003), and combined sewer overflows and run-off events after rain fall (Kistemann *et al.*, 2002; Rose *et al.*, 1997). As stated above, the oocyst’s robustness and survivability in different conditions allows water transmission to be a high risk factor for cryptosporidiosis.

A partial list of documented water-borne outbreaks is presented in Table 4. Obviously, countless contaminated water sources in developing countries go unmonitored.
for the presence of *Cryptosporidium* let alone other fecal contaminant indicators. Discussed here are three examples of well-documented water-borne outbreaks.

In the summer of 1984, the first appropriately documented water-borne outbreak with confirmed oocysts in stool and serological tests occurred in a suburb of 5,900 persons outside of San Antonio, Texas (D'Antonio *et al.*, 1985). With diarrhea as the major symptom, 34 of 100 persons in a telephone survey reported being ill. Source of exposure was linked to potable, unfiltered artesian well water, which tested positive for fecal coliforms in all the homes in the community. Further investigation demonstrated that dye introduced to the sewage system later appeared in the well water (D'Antonio *et al.*, 1985).

An outbreak in Carroll Country, GA, 1987, illustrates the many possible failures that can occur to cause an outbreak (Hayes *et al.*, 1989). The outbreak was first recognized in college students reporting increased levels of gastroenteritis. In a telephone survey, 61% of household members exposed to the public water reported illness, while only 20% not exposed to the public water reported illness. It was finally determined that 12,960 of the 64,900 residents reported gastroenteritis. Oocysts were confirmed in 58 of 147 (39%) of afflicted persons and serology for *Cryptosporidium* was also positive. Oocysts were also confirmed via immunofluorescence in the treated water, in dead water mains, and in the raw water streams above the water treatment plant. Three possible entry points for oocyst contamination were documented. Cattle present in the watershed were positive with a low level *Cryptosporidium* infection. A blocked sewer line above the water treatment plant was overflowing and dye added to this overflow
reached the plant in six hours. Finally, the water treatment plant itself had failures in that: 1) the flocculation basins had had the mechanical agitators removed; 2) filter beds were not being sufficiently backwashed; and 3) general impaired filtration. It is most likely that a combination of all three failures were the cause of the outbreak.

The largest water-borne outbreak occurred in Milwaukee, WI, in 1993 (MacKenzie et al., 1994). Approximately 400,000 individuals (or nearly 25% of the metropolitan population) experienced gastrointestinal illness. Ice made before and during the outbreak was shown to contain oocysts thus implicating the public treated drinking supply. It is presumed that oocysts from Lake Michigan entered one of the two water treatment plants. Failed systems and possible routes of contamination include: 1) high turbidity was noted in the treated water possibly due to lowering the concentration of polyaluminum chloride or alum coagulant; 2) backwash water was recycled thus increasing the concentration of oocysts in the water to be treated; and 3) heavy rainfall increased potential of oocysts reaching Lake Michigan from a) cattle manure on the watershed area, b) abattoir waste, and c) sewage overflow. It was later determined that purified oocysts from four infected patients were 1) not infective in animals models and 2) genetically *C. parvum* genotype I (now *C. hominis*) (Xiao et al., 2001b). These results indicate that the main source of contamination was the sewage overflow of human waste. Incidentally, the Milwaukee wastewater treatment plant is also upstream from one of the two water treatment plants.

Finally, *Cryptosporidium* transmission in recreational waters (fountains, swimming pools, water-parks, and reservoirs) is well documented and has affected over
10,000 individuals (Anonymous, 1994, 2000, 2003; Fayer et al., 2000; MacKenzie et al., 1995a; Puech et al., 2001; Stafford et al., 2000). This successful transmission route is due to the combination of "accidents" (unexpected fecal contamination possibly from children) and oocyst chlorine resistance and low infectious dose.

2) Food-borne transmission. Unlike *Cyclospora cayetanensis* (Caceres, 1998; Fleming, 1998; Herwaldt and Ackers, 1997) and other food-borne agents, the role of *Cryptosporidium* spp. as food borne pathogens has not been well documented and is thought to be largely underestimated (Laberge and Griffiths, 1996). Documented cases include: a 1995 outbreak in Minnesota in which contaminated chicken salad was implicated (Anonymous, 1996b, c); a 1997 outbreak in Spokane, WA in which uncooked greens were implicated (Anonymous, 1998a, b); consumption of fresh pressed apple cider in Maine and New York (Fayer et al., 2000; Millard et al., 1994); consumption of milk, improperly or unpasteurized (Gelletlie et al., 1997; Harper et al., 2002; Romanova et al., 1992); and finally, in 1998, a cryptosporidiosis outbreak was linked with an infected food handler (Quiroz et al., 2000). In this final outbreak, molecular genotyping techniques matched the infected patient’s *C. parvum* genotype with that of the food handler; both were genotype I (Quiroz et al., 2000).

While examples of food borne cryptosporidiosis are limited, studies reporting the presence of oocysts in and on consumable foods are not. Oocysts have been found in filter feeding oysters, clams and mussels from the Chesapeake Bay (Fayer et al., 1999; Fayer et al., 2002; Graczyk et al., 1999c; Graczyk et al., 2000b), in oysters from Galicia, Spain (Freire-Santos et al., 2000), and mussels of the coast of Ireland (Chalmers et al.,
1997). Reported concentrations range from $4.4 \times 10^2$ (Graczyk et al., 2001) to >$10^3$ (Gomez-Bautista et al., 2000) oocysts per single mussel. It has been shown that oocysts can survive and remain infectious in oysters for at least four days, based on naturally occurring oocysts and mouse infectivity (Fayer et al., 1998b) and experimentally seeded oocysts and dye exclusion viability assays (Freire-Santos et al., 2002). A 2003 study, in which oocysts of *Eimeria acervulina* were used as a surrogate, demonstrated that oysters concentrated oocysts within six hours of exposure but were not detectable 96 hours later (Lee and Lee, 2003). After 48 hours, oocysts were detectable in the oyster feces indicating that the oocysts are being passed out of the oysters and not amassed (Lee and Lee, 2003). These results suggest that constant exposure of oocysts to oysters maybe required for consumption of oysters to be a public health risk for parasites. However, noting that numerous viral and bacterial outbreaks have been linked with consuming raw and under cooked shellfish (Lees, 2000; Lipp and Rose, 1997; Mackowiak et al., 1976), it remains pertinent that proper preparation should be taken to prevent any infections associated with shellfish consumption.

*C. parvum* oocysts have also been detected on a variety of raw vegetables including basil, cabbage, celery, carrots, cucumbers, cilantro leaves and roots, green onions, ground green chili, leeks, lettuce, parsley, radishes, and tomatoes (Monge and Arias, 1996; Monge et al., 1996; Ortega et al., 1997; Robertson and Gjerde, 2001a). Various routes of vegetable contamination include exposure to fertilization with human or animal feces, contaminated irrigation water, and infected farm/produce/food handlers.
Isolation techniques are less than optimal and parasite recovery rates remain as low as 1% (Bier, 1991; Robertson and Gjerde, 2001b).

3) Person to person. In Fortaleza, Brazil, family member transmission (from a child <3 years old to a secondary family member) was determined to be 19% based on infection or seroconversion (Current, 1994; Newman et al., 1994). A follow-up study to the Milwaukee waterborne incident determined the household transmission rate to be 5.4% and extending the outbreak an additional two months (MacKenzie et al., 1995b). The close personal contact occurring in hospitals and in elderly and child care centers affords high rates of nosocomial and care center transmission (Cordell and Addiss, 1994; Cordell, 2001; Koch et al., 1985; Neill et al., 1996).

4) Animal to person. Cryptosporidiosis was once thought to be mainly a zoonotic disease with the main transmission from calves to humans either directly or indirectly. Now, the prevalence of zoonotic transmission is being re-evaluated due to genotyping methodologies and the confirmation of C. hominis (genotype I), and thus anthroponotic transmission routes (Morgan-Ryan et al., 2002).

With the noted potential that multiple Cryptosporidium species infect both the immunocompromised and the immunocompetent, care should be taken when handling any species of animal. Working professionals in abattoirs, livestock farms (cattle, deer, goats, sheep, and pigs), veterinary clinics and schools, and research facilities are by default at a higher risk of exposure. Multiple reports have documented outbreaks in veterinary schools (Pohjola et al., 1986; Preiser et al., 2003), amongst farm workers (Stefanogiannis et al., 2001), and there is a correlation between increased incidence of
cryptosporidiosis and the birthing season of lambs during which bottle feeding is common (Casemore et al., 1986; Casemore, 1989).

Many review articles speculate and implicate companion pets as being a source of infection for not only Cryptosporidium spp. but other parasites as well (Robertson et al., 2000). While the zoonotic potential is present, in the single case study which a patient and the pet canine both had cryptosporidiosis at the same time, the human was determined to have the bovine genotype while the dog was infected with the a dog genotype, now C. canis (Abe et al., 2002).

5) Animal to animal. From 15 different Canadian farms, prevalence rates of 20%, 23%, 11% and 17% were documented in cattle, sheep, pigs and horses, respectively, with naturally occurring cryptosporidiosis (Olson et al., 1997b). A separate study reported cryptosporidiosis caused by C. parvum and C. muris in 59% and 2% of Holstein calves (386 total), respectively (Olson et al., 1997a). Both reports and numerous others indicated that high rates of transmission occur on livestock farms.

Sylvatic transmission maintains Cryptosporidium species in wildlife (Perz and Le Blancq, 2001). With 152 different mammals being potential C. parvum hosts as well as the non-parvum species and unclassified genotypes being detected in different animals, Cryptosporidium spp. transmission will be maintained. This abundance then poses a threat for water catchments that feed public water supplies and assessment and protection of the catchments will become more pertinent in the future.

6) Airborne. Although proposed in 1987 with incidents of pulmonary cryptosporidiosis in immunocompromised persons, airborne transmission has yet to be
officially documented as a route of infection (Hojlyng et al., 1987; Hojlyng and Jensen, 1988).

7) Mechanical vectors. Insects that frequent feces, including cockroaches, dung beetles, and houseflies, have been suggested as potential vectors (Graczyk et al., 1999a; Graczyk et al., 1999b; Graczyk et al., 2000a; Graczyk et al., 2003; Mathison and Ditrich, 1999; Zerpa and Huicho, 1994). Flies were experimentally verified to transmit infectious oocysts (Graczyk et al., 2000a). Flytraps were placed next to infected and non-infected calves over a period of seven to ten days. A single fly would transport between four and 131 oocysts and the total number of oocysts per collection trap varied from 56 to 4.5X10^3 (Graczyk et al., 2000a). These numbers are well above the experimentally determined infectious dose (DuPont et al., 1995). In addition, higher oocyst concentrations correlated with the calf being infected and actively shedding oocysts.

Two groups of microscopic water protozoans, ciliates and rotifers, as well as the nematode C. elegans (Dr. Ynes Ortega, personal communication) have been shown to ingest and concentrate C. parvum oocysts (Stott et al., 2001; Stott et al., 2003). The suggested role of oocyst ingestion by ciliates and rotifers is that of predation and food, however transport of oocysts in watersheds by these two protozoans would be possible. C. elegans would also be able to transport the ingested oocysts to new locations within soil and these findings corroborate oocysts movement in soil (Mawdsley et al., 1996).

It has also been postulated that birds are able to transport viable C. parvum oocysts over large areas. The first report found oocysts of an undetermined Cryptosporidium species in gulls (Smith et al., 1993). C. parvum oocysts ingested by
Canadian geese and Peking ducks were excreted after one week and remained infectious for mice (Graczyk et al., 1997). Feces from wild Canadian geese on their migratory route were determined to contain viable C. parvum oocysts (Graczyk et al., 1998). However, a recent study surveying gulls for the prevalence of Campylobacter spp. and Cryptosporidium spp. did not identify any oocysts in 205 fresh fecal specimens (Moore et al., 2002).

Along with these diverse routes of transmission, cryptosporidiosis, to a certain degree, follows a seasonal pattern. In developing countries, cryptosporidiosis occurrence increases during warm and/or wet seasons. It is hypothesized that increased rainfall leads to a greater spread of contaminated surface water used for drinking (Newman et al., 1994). An epidemiological study in Guatemala showed that while C. cayetanensis infection peaked during the warmer months, C. parvum infections were most common during the rainy season (Bern et al., 2000). A follow up study outside of Lima, Peru showed that both incidence of cryptosporidiosis and cyclosporiasis increased during the warm/wet season (Bern et al., 2002).

Application of molecular techniques has had a tremendous effect on epidemiological studies, as identification of the causative agent is no longer limited to culture-enriched protocols. Traditionally, biochemical typing, antibiotic resistance, isozymes, Western blotting, two-dimensional gel electrophoresis, and serology could characterize organisms that could be cultured in vitro. Now, characterization can be performed directly on a clinical or environmental sample without enriching or axenic culturing of the organism of interest. This facilitates typing of obligate intracellular
organisms for which in vitro cultivation is still lacking, inefficient, and/or is time consuming. Prior to molecular techniques, Cryptosporidium infections were attributed to the single Cryptosporidium species, *C. parvum*. Now, it is known that multiple Cryptosporidium species and genotypes infect all ages, with the propensity to infect the young and elderly, independent of immune status. This has implications with proper identification of routes of infection, source tracking, catchment protection and ultimately possible prevention of disease and outbreaks.

Currently, various genotyping protocols targeting different loci are available depending on the level of sensitivity needed for epidemiology. Protocols able to distinguish Cryptosporidium at the species and genotype levels target the 18S rRNA (Heitman et al., 2002; Perz and Le Blancq, 2001; Xiao et al., 2001b), beta-tubulin (Caccio et al., 1999; Rochelle et al., 1999b), COWP (Spano et al., 1997), and Hsp70 (Dalle et al., 2003; Gobet and Toze, 2001; Stinear et al., 1996). Protocols able to distinguish sub-genotypes target the surface protein Cgpg40/15 (Cevallos et al., 2000; Leav et al., 2002) and microsatellite markers (Aiello et al., 1999; Caccio et al., 2000; Feng et al., 2000). In addition, multiple species and genotype infections do occur which complicate epidemiology tracking.

1.10 Detection and Molecular Developments

The ubiquitous nature of Cryptosporidium spp and *C. parvum* specifically makes detection an essential aspect of prevention and spread of disease. Possible detection targets include life cycle stages such as the oocyst and sporozoites/merozoites, nucleic
acids, and metabolic products. Of these, due to stability, the oocyst has been and currently remains the selected target for detection both in clinical and environmental samples and this detection is largely based on microscopic identification. However, successful microscopic detection greatly depends on oocyst concentration and problematic for various reasons. First, due to size and shape, deciphering oocysts from other debris for correct identification is difficult and requires experienced microscopists to perform this not so glamorous duty. Second, low oocyst concentration increases the challenge of correct identification. In light of these two problems, microscopic detection of oocysts remains the gold standard to which all newly developed detection methodologies are compared against.

In clinical samples, due to profuse diarrhea and large oocyst production, detection is generally not a problem. However, cases of low-level infection do occur and combined with the tedious task of correct identification amongst the plethora of other fecal debris, misdiagnosis does ensue.

In environmental samples, a geometric mean of 2.7 oocysts/L (range: <0.007 to 484 oocysts/L, n=66) was documented in source waters (LeChevallier et al., 1991) and <1 oocysts/L (range: 0.001 to 0.48 oocysts/L, n=158) is reported in drinking waters (Rose et al., 1997). Again, these low oocyst concentrations combined with other debris, makes identification difficult.

Due to the low oocyst concentration, detection methodologies commonly employ a concentration step before microscopy. Techniques such as diethyl-ether centrifugation (Garcia et al., 1979; Garcia and Shimizu, 1981; Horen, 1983), Percoll density gradients
(Waldman et al., 1986), sequential Percoll/sucrose gradients (Arrowood and Sterling, 1987), Sheather’s sugar flotation (Current et al., 1983; Sheather, 1923), and sucrose/cesium chloride gradients (Arrowood and Donaldson, 1996) are used to purify and concentrate oocysts from fecal material. This semi-purified solution containing the oocysts is then used for down stream detection with differential stains and microscopy or other detection techniques.

Environmental concentration and purification techniques for oocyst recovery evolved from protocols originally developed for *Giardia*. An outbreak of giardiasis in Aspen, CO, 1965 (Craun, 1986) initiated the development of a microscopic based method that combined water filtration to concentrate *Giardia* cysts followed by zinc sulfate floatation for separation and iodine staining for identification (Quinones et al., 1988; Reference-Method, 1992). This basic detection flowchart including concentration, separation/isolation, and microscopic detection is generally followed for oocyst detection in water samples but with improvements. Filtration of the environmental sample is used to concentrate oocysts and, as noted before, samples sources include treated drinking water, ground water, surface waters (lakes/reservoirs, irrigation ditches, rivers, etc.), run-off events, sewage treatment effluent, and others. Various techniques have been used for large volume concentration. Adapted from the *Giardia* recovery methods, a 10 inch polypropylene cartridge filter with a 1 μm nominal porosity was the filter of choice of the EPA sponsored Information Collection Rule (ICR) (Musial et al., 1987; USEPA, 1996a). However, this filter was demonstrated to have low recovery efficiencies (LeChevallier et al., 1995; Shepherd and Wyn-Jones, 1996) and therefore other concentration techniques
were developed including membrane filtration (Ongerth and Stibbs, 1987), calcium carbonate flocculation (Vesey et al., 1993), continuous flow centrifugation (Borchardt and Spencer, 2002; Renoth et al., 1996; Zuckerman et al., 1999), flow cytometry (Vesey et al., 1994a; Vesey et al., 1994b), wound fiberglass cartridge filters (Kaucner and Stinear, 1998), foam filters (McCuin and Clancy, 2003), and capsule filters (Matheson et al., 1998). Once the water sample has been concentrated, oocyst separation is performed to remove sample debris for downstream applications. Each of these concentration techniques has positive and negative aspects. For example, recovery efficiencies for calcium carbonate flocculation (>70%) (Shepherd and Wyn-Jones, 1996) and continuous flow centrifugation (>90%) (Borchardt and Spencer, 2002) are good, however time restraints of >4 and >2 hours, respectively, restrict the use of these techniques for day-to-day applications. Currently, the EPA has approved two systems for oocyst recovery. First is a self-contained 1 µm absolute porosity pleated membrane capsule. The Envirochek™ HV Sampling Capsules (Pall Gelman Laboratory, Ann Arbor, MI) have a reported recovery rate of >70% (Matheson et al., 1998). The other approved filter is based on compression of foam to achieve the appropriate porosity to collect oocysts on the surface of the filter. The Filta-Max® (IDEXX Laboratories, Inc., Westbrook, Maine) recovery efficiency in tap water was 48.4% ± 11.8%, and in raw source waters, the range was 19.5 to 54.5% (McCuin and Clancy, 2003).

Examples of oocysts separation techniques used with environmental samples include Percoll/sucrose flotation (Arrowood and Sterling, 1987; LeChevallier et al.,
1995), Percoll/Percoll step gradient (Nieminski et al., 1995), flow cytometry (Vesey et al., 1994a), and immunomagnetic separation (IMS) (Johnson et al., 1995).

Microscopic based staining techniques (Figure 3) used with fecal samples include safranin methylene blue stain (Baxby et al., 1984), Ziehl-Neelsen modified acid-fast (Henricksen and Pohlenz, 1981), DMSO-carbol fuchsin (Pohjola et al., 1985), and immunofluorescent assays utilizing oocyst-reactive mAbs and polyclonal antibodies conjugated with fluorescein isothyocyanate (FITC). Other techniques include negative staining with nigrosin (Pohjola, 1984) and malachite green (Elliot et al., 1999) where the background is stained. Oocyst staining with these techniques is not 100% uniform and the discernment from fecal debris is difficult requiring experienced microscopists.

Oocysts in environmental samples are identified based on three general oocyst morphological characteristics. First, oocysts are detected via immunofluorescence labeling of the oocyst wall. Fluorescence intensity, oocyst wall thickness, characteristic folds in the wall are observed and assessed. Second, 4,6 diamidino-2'-phenylindole (DAPI) staining aids in identification, location, and number of sporozoite nuclei, if present. Finally, Nomarski microscopy facilitates oocyst internal morphology including identification of sporozoites. The triple combination of fluorescence, DAPI staining and internal structure assessment assists the microscopist to decide if the observed environmental object is an oocyst or not. However it is important to note that this methodology is not 100% specific and cross-reacting algae and other debris can interfere, thus making the identification process very subjective. In addition, with the exception of
C. muris, microscopic detection is not able to classify Cryptosporidium spp. or genotypes.

Non-microscopic based detection methods include immunological and molecular based techniques. Examples of immunological-based techniques include enzyme-linked immunosorbent assays (ELISA) (Anusz et al., 1990; Chapman et al., 1990; Knowles and Gorham, 1993; Ungar, 1990), reverse passive haemagglutination (Farrington et al., 1994), and solid-phase qualitative immunochromatographic assays (Garcia and Shimizu, 2000).

Molecular based protocols have been shown to be as sensitive as microscopic protocols and have the additional advantage of speciation and genotyping. For example, in combination with IMS, nested PCR was able to detect eight oocysts (Monis and Saint, 2001) seeded into treated waters. The different molecular platforms are as numerous as the different loci targeted for detection. PCR based protocols include and target the 18S rRNA (Johnson et al., 1995; Leng et al., 1996; Lowery et al., 2000; Sturbaum et al., 2002; Xiao et al., 1999), hsp70 gene (Gobet and Toze, 2001; Kaucner and Stinear, 1998; Monis and Saint, 2001), beta tubulin (Perz and Le Blancq, 2001), an unknown loci (Laxer et al., 1991) as well as multiple other protocols (Awad-El-Kariem et al., 1993; Gibbons and Awad-El-Kariem, 1999; Gile et al., 2002; Hallier-Soulier and Guillot, 2000; Morgan et al., 1998a; Scorza et al., 2003; Xiao et al., 2001b). Real-time PCR protocols are also demonstrating sensitivity and are able to distinguish different Cryptosporidium species (Fontaine and Guillot, 2002, 2003; Higgins et al., 2001; Limor et al., 2002; MacDonald et al., 2002; Tanriverdi et al., 2002). DNA array technology has been adapted to detect
and discriminate between different *Cryptosporidium* species and genotypes (Straub et al., 2002). Novel techniques such as fiber optics and biosensors (Snowden and Anslyn, 1999; Wang, 2000) are also showing promise.

Yet, none of these techniques has the ability to assess viability and infectivity. The need for establishing viability and/or infectivity is two fold. First, disinfection methodologies need to be accurately verified. Second, oocysts detected during routine monitoring or in an outbreak situation should be evaluated for viability/infectivity to assess the risk of exposure. The current gold standard is mouse infectivity, which is expensive, time consuming, and requires experienced personnel (Rochelle et al., 2002). In addition, mouse infectivity becomes a problem when dealing with *Cryptosporidium* spp. that do not infect mice (i.e., *C. hominis*). Therefore an alternative viability assessment technique is needed to reliably differentiate potentially infectious from non-infectious oocysts (Fricker and Crabb, 1998).

The vital dyes propidium iodide (PI, not membrane permeant) and DAPI (membrane permeant; affinity for nucleic acid) were originally touted as good indicators of viability (as measure by *in vitro* excystation) (Campbell et al., 1992; Jenkins et al., 1997), however they are now reported not to be accurate (Black et al., 1996).

Similarly, *in vitro* excystation was shown not to be a reliable measure of infectivity (Neumann et al., 2000a). Oocysts failing to excyst were found to be infectious in the mouse model and vice versa, and sporozoites that successfully excysted were not infectious (Belosevic et al., 1997; Neumann et al., 2000a; Neumann et al., 2000b).
Fluorescent in situ hybridization (FISH) (Vesey et al., 1998) and reverse transcriptase PCR (RT-PCR) (Jenkins et al., 2000) have both been shown to correlate with viability.

The single test that assesses both viability and infectivity is cell culture (CC) followed by RT-PCR (Di Giovanni et al., 1999; Rochelle et al., 1999a). However, the lower limit of detection (LLOD) has not been assessed for CC/RT-PCR and, therefore, application with environmental samples should be suspect.

Of these different types of concentration, separation and detection methodologies, one combination has been adopted by the EPA (USEPA, 1999). Method 1623 combines filtration (capsule or foam) followed by elution and oocyst separation via IMS. Detection and identification uses the triple combination of immunofluorescence, DAPI staining and Nomarski microscopy. Reported recovery efficiencies range from 36 to 75% (DiGiorgio et al., 2002; LeChevallier et al., 2003; McCuin and Clancy, 2003).
2.0 Present Studies

This dissertation addresses key issues concerning detection and proper identification of *C. parvum*. The first aim deals with the use of accurately quantified oocysts in order to determine the lower limit of detection (LLOD) of *C. parvum* oocysts in laboratory grade water. The second aim was to determine and compare the LLOD of Method 1623 with that of a nested-PCR detection approach in laboratory grade and environmental waters. The third aim stemmed from an observation of size difference in amplicons generated from the gene encoding the *C. parvum* surface protein GP900 between genotype I (*C. hominis*) and genotype II (*C. parvum bovis*). Therefore, the third aim was to amplify and sequence genes encoding known surface proteins from *C. parvum* and *C. hominis*. The gene sequences were compared to identify amino acid differences in the expressed proteins from these two organisms. Such differences may correspond to functional differences in host specificity, as seen with *C. hominis* preferentially infecting *Homo sapiens*.

The methods, results, and conclusions of these studies are presented in the manuscripts appended to this dissertation. The following is a summary of the most important findings in these manuscripts.

2.1 Present Study One (Appendix A)

This manuscript outlines for the first time in published literature, the use and validation of microscopic micromanipulation to isolate and deliver low numbers of *C. parvum* oocysts to a test vial of interest. In addition, a nested PCR primer set was
developed targeting the 18S rRNA and tested for sensitivity using micromanipulation and specificity using isolated DNA from multiple different species. Content within the manuscript discusses strengths and weaknesses of current techniques used for LLOD validation. It was determined that micromanipulation is an accurate technique able to deliver low numbers of oocysts to a test vial of interest. The nested PCR protocol had LLOD, in replicates of 50 and laboratory grade water, of 100% with ten oocysts and 38% with a single oocyst. Finally, it was concluded that micromanipulation, while accurate, is labor intense and does not lend itself to large-scale validation processes.

2.2 Present Study Two (Appendix B)

This study compared detection efficiencies of the EPA Method 1623 with the nested PCR protocol developed in Present Study One. Both methods had equal detection efficiencies giving positive detection at the five-oocyst level. In addition, non-specific PCR amplification results generated during the study revealed specificity issues that have implications effecting past, current and future molecular detection validation processes.

2.3 Present Study Three (Appendix C)

PCR amplicon size differences between the entire C. parvum and C. hominis attachment/invasion GP900 genes prompted further investigation of other surface proteins, including p23, Cpgp 40/15, and specific loci in GP900, domains 1, 3 and 5. Distinct demarcation was noted between C. parvum and C. hominis at all loci. These differences add support to the recent separation of C. parvum genotype I and II into the
recently classified *C. parvum* genotype bovis and *C. hominis* taxonomic titles. In addition, some of the gene sequence data correspond to differences in the deduced amino acid sequence. This information has implications explaining host-specificity differences observed among *Cryptosporidium* spp.
3.0 Research Contribution

Major drawbacks were noted when discussing current detection methodologies targeting Cryptosporidium oocysts in environmental samples, including but not limited to sensitivity and specificity. Microscopic protocols (i.e. Method 1623) are not sensitive (36 to 75%) (DiGiorgio et al., 2002; LeChevallier et al., 2003; McCuin and Clancy, 2003) and are unable to determine species, genotype, and viability/infectivity. The immunological techniques are potentially non-specific due to cross-reactivity with other microorganisms (Fayer et al., 2000). Molecular methodologies are still in their infancy and have not been fully evaluated. Obviously, detection protocols that are not sensitive or specific allow false positive and negative results to occur. These drawbacks have implications in disease prevention and epidemiology.

Prevention of cryptosporidiosis: The best prevention of communicable disease is education and as with cryptosporidiosis, especially in developing countries, boiling water for consumption and general sanitary practices has a large impact in the prevention of this disease. However, these precautions are not always followed in the developing world, and in the case of developed countries, the populace expects and demands that drinking and recreational water are free of possible pathogens and contaminants. With this high expectation, prevention currently relies on detection protocols and screening environmental water samples for potential contamination. Two stances have been taken concerning this screening process. First, screening is applied to unprocessed water and food samples assessing the risk of downstream potential contamination. Or second,
screening is performed on processed samples, specifically treated water, directly assessing the presence of contamination.

In the case of food, especially fruits and vegetables that are potentially exposed to contaminated irrigation or insecticide water, screening is not useful due to the extremely low recovery efficiency of current technology (Bier, 1991; Robertson and Gjerde, 2000, 2001b), and the sheer volume of food that would need to be tested on a day-to-day basis.

In raw water, oocyst screening establishes a baseline risk level. If oocysts are not present in the raw water, then there exists little risk of exposure. If oocysts are present in a raw water source, then the general risk to the public increases via consumption of potentially contaminated treated drinking water. Experimentally determined removal efficiencies of *C. parvum* oocysts for conventional and direct water treatments were two to three and one to two log reductions, respectively (Anderson *et al.*, 1996; Hashimoto *et al.*, 2001; Nieminski, 1994). Therefore, monitoring the level of oocyst concentrations in raw waters would indicate how effectively a water treatment plant would need to operate to remove any oocysts that might be present (i.e. greater than a four log reduction). The USEPA has taken this prevention approach requiring the monthly monitoring of raw waters to determine oocyst concentration. Once the oocyst concentration has been determined, then a numerically determined log reduction value is allocated. To meet this log reduction value, different treatment processes (i.e. membrane filtration, ultraviolet light disinfection) are required in a water treatment plant in order to lower the risk to the public. While this approach is preventive, the 'snap-shot' sampling once a month does not give the complete and accurate picture of oocyst levels and there is no guarantee that
the oocyst level determined on one day would be the same every day. Water catchments change day-to-day and year-to-year depending on water flow and human and animal influences.

The United Kingdom has taken the opposite and second approach for prevention of cryptosporidiosis. This approach ignores oocyst levels in the raw water and only concerns itself with oocysts in the treated drinking water (UKDETR, 1998). In this case, monitoring for oocysts using Method 1623 is performed twenty-four hours a day and seven days a week. The maximal accepted oocyst concentration is ten per 100L and dispensation of water in excess of this concentration constitutes a criminal offense (Fairley et al., 1999). However, due to the lengthy time requirement of Method 1623 (a minimum of 8 to 12 hours per sample), this approach is not a preventive strategy. Ingestion of contaminated drinking water would occur hours before the contamination is even detected and therefore fails to immediately protect the public.

Unfortunately, while both approaches have good intentions of preventing disease and are good for public relations, both are established on a method that is not 100% sensitive nor has the ability to assess genotyping or viability issues. Because of these drawbacks, the Canadian Department of Health (Health-Canada, 1997, 2003) as well as Australia’s National Health and Medical Council (Australian-Drinking-Water-Guidelines, 1998) do not mandate monitoring of oocysts in raw or treated waters. Therefore the question arises: With the millions of dollars being spent on monitoring and assessing potential risk, would not it be more effective to invest in alternative prevention or risk assessment approaches?
Would sound judgment include upgrading all water treatment plants with current membrane filtration technology and ultraviolet light (UV) disinfection to ensure public safety? Membrane filtration is >90% effective at removing oocysts (Adham et al., 1994) and UV disinfection has been shown to effectively inactivate oocysts (Belosevic et al., 2001; Craik et al., 2001; Drescher et al., 2001; Linden et al., 2001). Other approaches, which are less expensive, incorporate water catchment protection and surrogate monitoring (Isaji, 2003).

Water catchments are the areas of land in which rain water and snowmelt are gathered as surface water that drain into creeks, ditches and eventually fill the lakes and reservoirs. Human activities occurring in catchments include agricultural practices with the manure fertilizer, abattoirs or livestock markets, and livestock farms with land drainage into water catchments. Wildlife such as deer is also found in catchments, as they also need a source of water. Consequently, catchments are vulnerable to pollution from human and environmental sources, which possibly lead to contamination of raw water sources and eventually might challenge a water treatment plant removal practice or infiltrate ground water. Studies have shown that Giardia and Cryptosporidium are present even in pristine catchments beyond the reach of urban or agriculture influence (Bodley-Tickell et al., 2002; Buckley and Warnken, 2003; Perz and Le Blancq, 2001). Rain-fall events were positively correlated with increased concentrations of oocysts (Atherholt et al., 1998) and regular monitoring of catchments was deemed inadequate and should include sampling during heavy run-off events (Kistemann et al., 2002).
Therefore, catchment protection plays an initial and critical role in preventing raw waters from being contaminated.

Surrogate monitoring including turbidity, particle counting, microscopic particulate analysis (MPA), latex microspheres, and bacteriophage and bacterial species such as *Clostridium perfringens* have had variable success correlating with oocyst removal after water treatment (Jakubowski *et al.*, 1996). For example, continuous turbidity monitoring of both raw and treated waters could give early indication and warning of a large incursion of unknown particulates challenging a treatment process or filter bed breakthrough in finished water, thus alerting water plant operators to an increased risk of oocysts in the treated water. Oocyst presence in raw waters and removal in treated waters is correlated with turbidity (Anderson *et al.*, 1996; Hsu and Yeh, 2003; LeChevallier *et al.*, 1991). Particle counters (a more sophisticated measure of turbidity) have established a correlation of water particulate removal with oocyst removal (Anderson *et al.*, 1996), but, are reported to be troublesome in day-to-day operations at water treatment plants. MPA is recommended for assessing water treatment performance of removing particles and the method has strong correlation with *Giardia* cyst removal and to a lesser extent *C. parvum* oocyst removal (Anderson *et al.*, 1996; Hancock *et al.*, 1996; USEPA, 1996b). The use of microspheres (five µm in diameter) correlated with oocyst removal but only with water treatment containing hydrophilic negatively charged filter media (Dai and Hozalski, 2003). Lastly, removal of *C. perfringens* and bacteriophages, to a lesser extent, correlated with oocyst removal (Payment and Franco, 1993; Venczel *et al.*, 1997). While individually these surrogates are not exact indicators
of Cryptosporidium presence or removal, the combined information of several of these surrogates may be very effective.

Would not a better approach include 1) water catchment protection – controlling and preventing major contamination events; 2) routine monitoring of raw water sources with a) turbidity, MPA, and other surrogates and b) direct detection of oocysts; 3) increased monitoring in raw water for oocysts if abnormalities are noted in surrogate levels (i.e. increased turbidity as a consequence of a rain event); and 4) incidental monitoring of treated water when there is: a) significant decrease in raw water quality (i.e. increased turbidity); b) treatment processes are disturbed (i.e. improper filter bed back-flush); c) or the report of a potentially outbreak. The need to monitor raw water for the presence of oocysts should be assessed individually for each source feeding a treatment plant, taking into account the characteristics of the catchment area and the nature of the water treatment process.

Epidemiology of cryptosporidiosis: Determining the source of contamination or exposure aids tremendously in the prevention of future outbreaks or isolated cases. Due to the multiple Cryptosporidium species and genotypes as well as the multiple transmission patterns (anthroponotic, sylvatic, and zoonotic) and routes (water, food, direct), this task is now reliant on the marriage of detection protocols and molecular techniques. Therefore, environmental epidemiology is dependent on the same detection protocols that have been shown not to be sensitive at recovering oocysts. For example, during an outbreak situation samples are collected from patients and possible environmental samples such as food or well water with the goal of linking patients with
the presumptive cause of contamination. The high concentration of oocysts in fecal material greatly aids in the success of detecting, extracting DNA and applying molecular techniques and thus giving a genotype and sub-genotype profile of those *Cryptosporidium* species causing disease during the outbreak. While the epidemiological link between patients can be strong based on exposure factors and genotyping characteristics, determining the source of contamination relies solely on the LLOD of any given molecular detection protocol. Again, knowing that the detection protocols are not sensitive greatly hinders the epidemiological process.

Notwithstanding any of the political or practical approaches of cryptosporidiosis control, the fact remains that prevention and epidemiology rely heavily on detection methods that have not been appropriately evaluated. This shortcoming is the thrust of this dissertation: Identification of and solutions to aspects that are lacking for proper evaluation of these detection methods.

The LLOD for the majority of these different protocols, both microscopic and molecular, has not been properly determined (Reynolds *et al.*, 1999). Taking into account the low infectious dose as well as low oocyst occurrence in raw and treated waters, the testing with low numbers of intact oocysts is needed to determine the LLOD of any given protocol. The use of previously extracted and serial diluted DNA does not include the difficulty of liberating DNA from oocysts and therefore is not a true measure sensitivity. If a nucleic acid based detection method is to be evaluated, then an extraction protocol must be included. Furthermore, confidence that the low intact oocyst test number is precise is of the utmost importance. Three oocyst-counting methods are used
to generate low oocyst numbers for testing. These include haemocytometer chamber counting followed by serial dilution, micromanipulation and flow cytometry/cell sorting. Serial dilution is not accurate due to the inherent standard error generated. Repeatedly, the coefficient of variation was determined to be greater than 10% in oocyst numbers counted between replicate aliquots of an oocyst suspension (Reynolds et al., 1999). In addition, oocyst numbers ranged from 80 to 120 organisms when enumerating from a 100 oocysts stock suspension (Reynolds et al., 1999). Micromanipulation, while being accurate, is labor intensive and does not lend itself to large-scale projects. Flow cytometry has been determined to be both accurate and precise for oocyst enumeration and delivery into to test vials and/or matrixes (Reynolds et al., 1999). In addition, flow cytometry is capable of generating large numbers of test vials at any desired oocyst concentration.

The majority of detection protocols are evaluated with a serial dilution of purified extracted DNA or intact oocysts via haemocytometer counting (Table 5) and thus, again, LLOD is not accurately assessed. Taking into consideration the time and financial constraints of more accurate enumerating systems, oocyst enumeration with serial dilution is appropriate during the initial validation of a newly developed detection protocol. However, in view of the >10% coefficient of variation and the documented range of counted oocysts at the 100 level, serial dilution should not be used with levels less than 100 oocysts to provide accurate oocyst numbers.

This identifies the first limitation of presently used detection techniques: Determining of the LLOD of any given detection protocol, whether microscopic or
molecular based, needs to be performed with accurately determined and delivered low numbers of oocysts. In an attempt to address this issue, the LLOD of a newly developed nested primer set was determined by testing with low numbers of oocysts isolated via micromanipulation (Appendix A). Validation of micromanipulation as an isolation and delivery tool consisted of isolating and dispensing 75 FITC-labeled individual oocysts from solution to microscope slides for immunofluorescence detection. Sixty-seven of the 75 single oocysts (89.3%) were detected and in no case were additional oocysts detected in a single transfer. With micromanipulation shown to be an accurate oocyst delivery tool, the LLOD was determined for a developed nested PCR protocol targeting the 18S rRNA. Oocysts concentrations of 10, 7, 5, 4, 3, 2, and a single oocyst were delivered directly into thin-walled PCR tubes in 1X PCR buffer and subjected to DNA liberation and nested-PCR detection. Detection efficiencies (%) were 100, 94, 92, 88, 76, 56, and 38 for 10, 7, 5, 4, 3, 2, and one oocyst, respectively. Therefore, the LLOD 100% of the time for this nested-PCR protocol was ten oocysts.

Taking into account that this molecular detection protocol and affiliated LLOD did not include factors that are inclusive to environmental detection protocols, such as raw water inhibitors, filtration and IMS, the LLOD of this nested-PCR protocol was next determined by seeding low oocyst numbers into raw waters with recovery/isolation by IMS (Appendix B). Oocyst seed doses of five, ten, and fifteen per milliliter were dispensed into Leighton tubes containing 10L equivalents from two raw water sources, a river and a reservoir. Tests were done in triplicate and the nested-PCR protocol was compared to microscopic IFA detection. At the five-oocyst level, microscopic
immunofluorescence detected four, three and two and three, six and four oocysts in the
two raw waters, indicating that IMS/IFA is able to recover and detect low numbers of
oocysts. The replicate with six oocysts detected (higher than the seed dose) is attributed
to naturally occurring oocysts in the river sample or extra oocysts being delivered during
the seeding process. Nested-PCR detection at the five-oocyst level gave positive
amplifications for all replicates in both raw waters, indicating that this molecular protocol
is as sensitive as microscopic detection with the added ability to speciate and genotype.

Interestingly, three of the replicates (one replicate at the three and two at the ten-
oocyst level) in the reservoir water gave a larger than expected PCR amplicon. Upon
investigation via DNA sequencing, it was determined that this amplicon did not originate
from *C. parvum* DNA but rather a dinoflagellate, a common free-living algal species.
Further testing involving DNA isolated from *Gymnodinium fuscum*, a dinoflagellate
species, indicated that this nested primer set was not specific for *C. parvum* as it
amplified the same size PCR amplicon as observed in replicates with the raw water
samples. This nested primer set was developed to amplify a segment in the 18S rRNA
gene. Alignment of the entire *C. parvum* 18S rRNA gene with that of *G. fuscum* revealed
86.5% pairwise identity, and therefore, false positive amplification was not a surprise.
During the process of designing the nested primer set, isolated DNA from various species
was tested to assess specificity. These species included *C. andersoni, C. baileyi, C.
muris, C. parvum, C. serpentis, Cyclospora cayetanensis, Eimeria neischulzi, Encephalitozoon (septata) intestinalis, Giardia duodenalis, Hammondia heydorni, Neospora caninum*, and bacterial species *Bacillus subtilis* and *Escherichia coli*. It was
determined that the nested primer set was specific for *Cryptosporidium* species and that RFLP was able to distinguish between different species and genotypes. Other protocols have included DNA from the following species for primer specificity: *Ascaris lumbricoides*, *Clonorchis* species, *Endolimax nana*, *Entamoeba coli*, *Hymenolepis nana*, *Taenia* species, *Trichuris trichiura*, and numerous bacterial species including *Campylobacter jejuni*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and multiple *Salmonella* spp (Johnson *et al.*, 1995; Stinear *et al.*, 1996). While common algal species were included during the evaluation of microscopic based immunofluorescent protocols (Clancy *et al.*, 1994; Rodgers *et al.*, 1995), none have been included during specificity validation with molecular protocols. In addition, the process of oocyst recovery and isolation from environmental samples does not result in purified oocysts and other organic and inorganic debris are present including other parasites, bacteria, algal species, and PCR inhibitors. With few exceptions (notably bead based systems), DNA extraction processes are non-selective and will capture DNA from all sources, including oocysts, algae, and naked DNA. As stated in the Taxonomy section, the *Cryptosporidium* genera is classified in the Alveolata group which consists of Sporozoa (apicomplexans), Ciliophora (ciliates), and Dinozoa (dinoflagellates). Therefore, because dinoflagellates and ciliates are related to the *Cryptosporidium* genera and are free living in many raw water matrixes, DNA from these organisms should be included during the molecular detection protocol validation process. A literature search revealed that not a single molecular detection method included DNA from algal species.
Thus, the second manuscript (Appendix B) demonstrated that IMS/nested-PCR is as sensitive as the current gold standard, IMS/IFA, and identified a major weakness in the specificity validation process with current PCR and DNA hybridization based protocols. It is recommended that PCR detection of a target organism from an environmental sample be based upon 1) highly specific and tested PCR primers with phylogenetically related species, 2) PCR amplicon size, 3) species-specific RFLP digestion, and/or 4) DNA sequencing for proper species identification.

The third manuscript (Appendix C) deviates from environmental detection validation, however the results have direct implications to specificity issues. During the process of investigating the gene encoding the C. _parvum_ attachment/invasion protein GP900, it was ascertained that the nucleotide sequence of genotype I (C. _hominis_) was approximately 500 bp less than that of C. _parvum_ genotype II (now C. _parvum_ bovis). Based on this observation, it was hypothesized that notable differences between C. _parvum_ bovis and C. _hominis_ would be present within the nucleotide and thus deduced amino acid sequence of GP900 as well as other attachment/invasion proteins. The significance of such differences ultimately may account for observed differences in host specificity. Previous reports had also speculated this distinction and differences between C. _hominis_ and C. _parvum_ bovis were noted in the gene coding the Cpgp40/15 surface protein (Cevallos _et al._, 2000; Leav _et al._, 2002; Strong _et al._, 2000).

To test this hypothesis, PCR primers amplifying Cpgp40/15, GP900 (domains 1, 3 and 5), and p23 were developed against C. _parvum_ bovis published sequences and used to amplify these loci from eight C. _hominis_ and five C. _parvum_ bovine isolates, Apple cider,
Glasgow, Iowa, Moredun, and Texas. All the targeted loci were successfully amplified and DNA sequenced with the exception of GP900 domain 3 from two of the C. hominis isolates.

DNA sequence alignment revealed numerous base pair differences with some of these alterations corresponding to differences in the predicted amino acid sequence. p23 was shown to be identical among each of the C. parvum bovine or C. hominis isolates, however, three amino acids differences were noted between the species. Interestingly, the third amino acid change (Asp to Glu) occurs in the final position of a six amino acid motif (Q-D-K-P-A-D) recognized by the neutralizing mAb C6B6 (Perryman et al., 1996). The Cpgp40/15 DNA sequence was conserved among C. parvum bovine isolates while highly variable within the C. hominis isolates as genotypic subtypes, Ia, Ib, Id, and Ie, were represented (Strong et al., 2000). Also, the deduced C. hominis Cpgp40/15 protein sequences varied in length from 276 to 306 amino acids. GP900 domain 1 contained four amino acid differences and GP900 domain 5 revealed seventeen between C. parvum bovis and C. hominis isolates. Surprisingly, alignment of the six C. hominis isolates that GP900 domain 3 was amplified and all five C. parvum bovine isolates did not reveal any differences in the nucleotide sequence.

These nucleotide and deduced amino acid differences within cpgp40/15, p23, and gp900 domains 1 and 5 lend support to the previously outlined hypothesis: variation within attachment/invasion proteins may account for the respective host infectivity patterns observed between C. parvum and C. hominis isolates. This information of amino acid changes between these Cryptosporidium species may be realized through future
studies concentrating on functionality and receptor/ligand interactions. In addition, this work has identified multiple differentiating nucleotide sites any of which may be used in genotyping or epidemiological studies and added to the number of potential loci that may act as a specific target of molecular detection.

On this last point, choosing the genetic loci for molecular detection is crucial as false positive reactions have been noted and proper specificity validation is rarely performed. Mainly due to sequence availability, the majority of the Cryptosporidium molecular detection protocols target conserved genes (i.e. 18S rRNA, beta tubulin, hsp70) (Sulaiman et al., 2000; Xiao et al., 1999). Conserved genes, as exemplified in Appendix B, can lead to false positive detection. Therefore, the alternative is to identify genes that are unique to Cryptosporidium. Loci hypothesized to be unique to C. parvum include microsatellites (Aiello et al., 1999; Caccio et al., 2000; Feng et al., 2000) and attachment/invasion proteins. Microsatellites are prone to frequent size changes in the number of nucleotide repeats. Thus, while very suitable for epidemiological studies, microsatellites are not appropriate to be used in detection protocols. Surface attachment/invasion proteins on the other hand are stable and constitute good markers for molecular detection. Currently, the use of Cryptosporidium surface protein loci in environmental detection protocols is limited, targeting COWP, Trap C-1 and Trap C-2 (Guy et al., 2003; Higgins et al., 2003; Lowery et al., 2001). It is anticipated that the differences identified within cpgp40/15, p23, and gp900 domains 1 and 5 will be incorporated into future detection protocols.
In conclusion, this dissertation identified and proposed solutions to drawbacks of past and current detection protocols, both microscopic and molecular. It is hoped that future detection methodologies will incorporate proper sensitivity and specificity validation practices. This dissertation also added information to *Cryptosporidium* attachment/invasion proteins. Hopefully the identification of nucleotide and deduced amino acid differences in these proteins between *C. parvum* and *C. hominis* isolates will lead to functional differences that can be inferred to host specificity issues.
Cryptosporidium parvum

Sporozoites enter just beneath the brush border of the epithelium and develop into trophozoites and type I and II meronts (schizonts) causing:

- Villous blunting,
- Mild inflammation,
- Prolonged diarrhea
and malnutrition

Figure 1. Life cycle of Cryptosporidium parvum. The life cycle illustration is from Dillingham et al., 2002 and used with permission from the authors.
**Figure 2.** Nomarski differential contrast photomicrograph of *Cryptosporidium parvum* oocysts. Bar represents 5 μm.
Figure 3. Differential staining techniques for identification of *Cryptosporidium parvum* oocysts in stool and environmental samples. Panel A: modified acid-fast stain; Panel B: Immunofluorescence Microscopy.
<table>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Genotype</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. andersoni</td>
<td></td>
<td>Mammal (Bos taurus)</td>
<td>(Lindsay et al., 2000)</td>
</tr>
<tr>
<td>C. baileyi</td>
<td></td>
<td>Avian (Gallus gallus)</td>
<td>(Current et al., 1986)</td>
</tr>
<tr>
<td>C. canis</td>
<td>Coyote, Fox, Dog</td>
<td>Mammal (Canis familiaris)</td>
<td>(Fayer et al., 2001; Morgan et al., 2000)</td>
</tr>
<tr>
<td>C. felis</td>
<td></td>
<td>Mammal (Felis catus)</td>
<td>(Iseki, 1979)</td>
</tr>
<tr>
<td>C. hominis</td>
<td></td>
<td>Mammal (Homo sapiens)</td>
<td>(Morgan-Ryan et al., 2002)</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td></td>
<td>Avian (Meleagris gallopavo)</td>
<td>(Slavin, 1955)</td>
</tr>
<tr>
<td>C. molnari</td>
<td></td>
<td>Fish</td>
<td>(Alvarez-Pellitero and Sitja-Bobadilla, 2002)</td>
</tr>
<tr>
<td>C. muris</td>
<td></td>
<td>Mammal; House mouse</td>
<td>(Tyzzer, 1910)</td>
</tr>
<tr>
<td>C. nasorum</td>
<td></td>
<td>Fish (Naso lituratus)</td>
<td>(Hoover et al., 1981)</td>
</tr>
<tr>
<td>C. parvum</td>
<td>bovis</td>
<td>Mammal (Mus musculus)</td>
<td>(Tyzzer, 1912)</td>
</tr>
<tr>
<td>C. saurophilum</td>
<td></td>
<td>Skink (Eumeces schneideri)</td>
<td>(Koudela and Modry, 1998)</td>
</tr>
<tr>
<td>C. serpentis</td>
<td></td>
<td>Reptile (multiple species)</td>
<td>(Levine, 1980)</td>
</tr>
<tr>
<td>C. wraithi</td>
<td></td>
<td>Mammal (Cavia porcellus)</td>
<td>(Vetterling et al., 1971)</td>
</tr>
</tbody>
</table>
Table 2. Morphometric description of Cryptosporidium spp. oocysts

<table>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Oocyst Length (μm)</th>
<th>Oocyst Width (μm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. andersoni</td>
<td>7.4 (6.0 – 8.1)</td>
<td>5.5 (5.0 – 6.5)</td>
<td>(Lindsay et al., 2000)</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>6.2 (5.6 – 6.3)</td>
<td>4.6 (4.5 – 4.8)</td>
<td>(Current et al., 1986)</td>
</tr>
<tr>
<td>C. canis</td>
<td>4.95 (3.68–5.88)</td>
<td>4.71 (3.68–5.88)</td>
<td>(Fayer et al., 2001)</td>
</tr>
<tr>
<td>C. felis</td>
<td>4.6 (3.2 – 5.2)</td>
<td>4.0 (3.0 – 4.0)</td>
<td>(Iseki, 1979)</td>
</tr>
<tr>
<td>C. hominis</td>
<td>5.2 (4.4 – 5.9)</td>
<td>4.9 (4.4 – 5.4)</td>
<td>(Morgan-Ryan et al., 2002)</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>5.2 (4.5 – 6.0)</td>
<td>4.6 (4.2 – 5.3)</td>
<td>(Lindsay et al., 1989)</td>
</tr>
<tr>
<td>C. molnari</td>
<td>3.2 – 5.5</td>
<td>3.0 – 5.0</td>
<td>(Alvarez-Pellitero and Sitja-Bobadilla, 2002)</td>
</tr>
<tr>
<td>C. muris</td>
<td>8.4 (6.6 – 7.9)</td>
<td>6.3 (5.3 – 6.5)</td>
<td>(Upton and Current, 1985)</td>
</tr>
<tr>
<td>C. nasorum</td>
<td>4.3 (3.5 – 4.7)</td>
<td>3.3 (2.5 – 4.0)</td>
<td>(Landsberg and Paperna, 1986)</td>
</tr>
<tr>
<td>C. parvum bovine genotype</td>
<td>5.0 (4.5 – 5.4)</td>
<td>4.5 (4.2 – 5.0)</td>
<td>(Upton and Current, 1985)</td>
</tr>
<tr>
<td>C. saurophilum</td>
<td>5.0 (4.4 – 5.6)</td>
<td>4.7 (4.2 – 5.2)</td>
<td>(Koudela and Modry, 1998)</td>
</tr>
<tr>
<td>C. serpentis</td>
<td>6.2 (5.6 – 6.6)</td>
<td>5.3 (4.8 – 5.6)</td>
<td>(Tilley et al., 1990)</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>5.4 (4.8 – 5.6)</td>
<td>4.6 (4.0 – 5.0)</td>
<td>(Vetterling et al., 1971)</td>
</tr>
</tbody>
</table>
Table 3. Clinical Features of Cryptosporidiosis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Immunocompetent Persons</th>
<th>Immunocompromised Persons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible Population</td>
<td>Adults of any age; Children, especially those under 1 year of age</td>
<td>Immunocompromised persons of any age especially those with AIDS&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Site of Infection</td>
<td>Intestinal</td>
<td>Usually intestinal but occurs extraintestinal including ocular and pulmonary sites</td>
</tr>
<tr>
<td>Enteric Presentation</td>
<td>Asymptomatic or acute</td>
<td>Asymptomatic, transient, chronic, or fulminant</td>
</tr>
<tr>
<td>Common Clinical Symptoms</td>
<td>Diarrhea, fever, abdominal cramps, vomiting, nausea, and weight loss</td>
<td>Severe diarrhea, fever, jaundice, weight loss and vomiting</td>
</tr>
<tr>
<td>Clinical Duration</td>
<td>Five to ten days</td>
<td>Two days to lifetime</td>
</tr>
<tr>
<td>Severity according to CD4+ Count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;200 cells/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>Spontaneous resolution</td>
</tr>
<tr>
<td>&lt;100 cells/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N/A&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Chromic and extraintestinal</td>
</tr>
<tr>
<td>&lt;50 cell/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>Fulminant</td>
</tr>
<tr>
<td>Estimated Outcome</td>
<td>Self-resolving in adults; High mortality in infants and young children in developing countries</td>
<td>High mortality</td>
</tr>
<tr>
<td>Treatment</td>
<td>Rehydration therapy</td>
<td>Active antiretroviral therapy and antiparasitic agents</td>
</tr>
</tbody>
</table>

<sup>A</sup> Table adopted from Fayer et al., 2000 for *Cryptosporidium parvum* and *C. hominis*

<sup>B</sup> AIDS: acquired immunodeficiency syndrome

<sup>C</sup> N/A: Not Applicable
<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Number of cases</th>
<th>Suspected cause</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>Braun Station, TX</td>
<td>2,006</td>
<td>Contaminated well water</td>
<td>(D'Antonio et al., 1985)</td>
</tr>
<tr>
<td>1987</td>
<td>Carrollton, GA</td>
<td>12,960</td>
<td>Treatment deficiencies</td>
<td>(Hayes et al., 1989)</td>
</tr>
<tr>
<td>1989</td>
<td>Ayrshire, UK</td>
<td>27</td>
<td>Treatment deficiencies</td>
<td>(Smith et al., 1989)</td>
</tr>
<tr>
<td>1990-1991</td>
<td>Isle of Thanet, UK</td>
<td>47</td>
<td>Treatment deficiencies</td>
<td>(Joseph et al., 1991)</td>
</tr>
<tr>
<td>1991</td>
<td>Berks County, PA</td>
<td>551</td>
<td>Treatment deficiencies</td>
<td>(Moore et al., 1993)</td>
</tr>
<tr>
<td>1992</td>
<td>York Shire, UK</td>
<td>125</td>
<td>Contaminated tap water</td>
<td>(Furtado et al., 1998)</td>
</tr>
<tr>
<td>1992</td>
<td>Jackson County, OR</td>
<td>15,000</td>
<td>Treatment deficiencies</td>
<td>(Frost et al., 1998; Moore et al., 1993)</td>
</tr>
<tr>
<td>1993</td>
<td>Milwaukee, WI</td>
<td>403,000</td>
<td>Treatment deficiencies</td>
<td>(MacKenzie et al., 1994)</td>
</tr>
<tr>
<td>1993</td>
<td>Waterloo, Canada</td>
<td>&gt;1000</td>
<td>Contaminated tap water</td>
<td>(Rose et al., 1997)</td>
</tr>
<tr>
<td>1993</td>
<td>Las Vegas, NV</td>
<td>103</td>
<td>Unknown</td>
<td>(Goldstein et al., 1996)</td>
</tr>
<tr>
<td>1993</td>
<td>Wessex, UK</td>
<td>27</td>
<td>Contaminated tap water</td>
<td>(Furtado et al., 1998)</td>
</tr>
<tr>
<td>1994</td>
<td>Kanagawa, Japan</td>
<td>461</td>
<td>Contaminated drinking water</td>
<td>(Kuroki et al., 1996)</td>
</tr>
<tr>
<td>1994</td>
<td>Walla Walla, WA</td>
<td>104</td>
<td>Sewage contaminated well</td>
<td>(Dworkin et al., 1996)</td>
</tr>
<tr>
<td>1995</td>
<td>Northern Italy</td>
<td>294</td>
<td>Contaminated water tanks</td>
<td>(Frost et al., 2000; Pozio et al., 1997)</td>
</tr>
<tr>
<td>1995</td>
<td>Gainesville, FL</td>
<td>77</td>
<td>Contaminated tap water</td>
<td>(Anonymous, 1996a)</td>
</tr>
<tr>
<td>1996</td>
<td>Ogose, Japan</td>
<td>&gt;9000</td>
<td>Unfiltered ground water</td>
<td>(Yamazaki et al., 1997)</td>
</tr>
<tr>
<td>1996</td>
<td>New York, NY</td>
<td>&gt;30</td>
<td>Contaminated apple cider</td>
<td>(Anonymous, 1997a)</td>
</tr>
<tr>
<td>1997</td>
<td>Shoal Lake, Ontario</td>
<td>100</td>
<td>Unfiltered lake water</td>
<td>(Anonymous, 1997b)</td>
</tr>
<tr>
<td>1998</td>
<td>Guadarrama, Spain</td>
<td>21</td>
<td>Contaminated tap water</td>
<td>(Rodriguez-Salinas Perez et al., 2000)</td>
</tr>
<tr>
<td>1998</td>
<td>Brushy Creek, TX</td>
<td>32</td>
<td>Sewage contaminated well</td>
<td>(Anonymous, 1998c)</td>
</tr>
<tr>
<td>2000</td>
<td>Belfast, Northern Ireland</td>
<td>129</td>
<td>Contaminated drinking water</td>
<td>(Glaberman et al., 2002)</td>
</tr>
<tr>
<td>2000</td>
<td>Lancashire, England</td>
<td>58</td>
<td>Contaminated drinking water</td>
<td>(Howe et al., 2002)</td>
</tr>
<tr>
<td>2001</td>
<td>Belfast, Northern Ireland</td>
<td>230</td>
<td>Contaminated drinking water</td>
<td>(Glaberman et al., 2002)</td>
</tr>
</tbody>
</table>

Table 4. Cryptosporidiosis outbreaks due to contaminated drinking water

A Table adopted from Fayer et al., 2000.
<table>
<thead>
<tr>
<th>Detection</th>
<th>Oocyst Concentrations</th>
<th>Determined By</th>
<th>LLOD¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR/18S rRNA²</td>
<td>900, 90, 9, 1</td>
<td>Serial Dilution</td>
<td>90 oocysts</td>
<td>(Johnson et al., 1995)</td>
</tr>
<tr>
<td>RT-PCR/hsp70³</td>
<td>1000, 100, 50, 25, 12, 1</td>
<td>Serial Dilution</td>
<td>1 oocyst</td>
<td>(Stinear et al., 1996)</td>
</tr>
<tr>
<td>PCR/18S rRNA</td>
<td>5000, 1000, 5000, 1000, 50, 5</td>
<td>Serial Dilution</td>
<td>50 oocysts</td>
<td>(Rochelle et al., 1997)</td>
</tr>
<tr>
<td>PCR/Unknown region</td>
<td>5000, 1000, 5000, 50, 5</td>
<td>Serial Dilution</td>
<td>5 oocysts</td>
<td>(Rochelle et al., 1997)</td>
</tr>
<tr>
<td>Oocysts/IMS⁴/IFA⁵</td>
<td>100</td>
<td>Flow Cytometry</td>
<td>100 oocysts</td>
<td>(Reynolds et al., 1999)</td>
</tr>
<tr>
<td>PCR/Unknown region</td>
<td>100, 50, 10, 1</td>
<td>Serial Dilution¹</td>
<td>1 oocyst⁶</td>
<td>(Wu et al., 2000)</td>
</tr>
<tr>
<td>Oocysts/membrane dissolution/IFA</td>
<td>4 × 10⁴, 100</td>
<td>Serial Dilution</td>
<td>&lt;100 oocysts</td>
<td>(McCuin et al., 2000)</td>
</tr>
<tr>
<td>Oocysts/PCR-18s rRNA</td>
<td>10, 7, 5, 4, 3, 2, 1</td>
<td>Micromanipulation</td>
<td>1 oocyst</td>
<td>(Sturbaum et al., 2001b)</td>
</tr>
<tr>
<td>Oocysts/IMS/PCR-18s rRNA</td>
<td>1000, 500, 250, 100, 10, 5</td>
<td>Serial Dilution¹</td>
<td>10 oocysts</td>
<td>(Lowery et al., 2000)</td>
</tr>
<tr>
<td>Oocysts/IMS/IFA</td>
<td>10, 5</td>
<td>Serial Dilution</td>
<td>5 oocysts</td>
<td>(McCuin et al., 2001)</td>
</tr>
<tr>
<td>Oocysts/IMS/RT-PCR/hsp70</td>
<td>10000, 1000, 100, 8</td>
<td>Serial Dilution</td>
<td>8 oocysts</td>
<td>(Monis and Saint, 2001)</td>
</tr>
<tr>
<td>Oocysts/IMS/PCR-18s rRNA</td>
<td>15, 10, 5</td>
<td>Flow Cytometry</td>
<td>5 oocysts</td>
<td>(Sturbaum et al., 2001a)</td>
</tr>
<tr>
<td>Hollow fiber ultrafilter/IFA</td>
<td>100000, 10000, 600</td>
<td>Serial Dilution</td>
<td>Not Determined</td>
<td>(Kuhn and Oshima, 2003)</td>
</tr>
<tr>
<td>Oocysts/Flocculation/IFA</td>
<td>1 × 10⁶, 1000, 100, 10, 5, 1</td>
<td>Serial Dilution</td>
<td>10 oocysts</td>
<td>(Karanis and Kimura, 2003)</td>
</tr>
<tr>
<td>Oocysts/IMS/Real-Time PCR</td>
<td>100000, 1000, 100, 10, 5</td>
<td>Serial Dilution</td>
<td>10 oocysts</td>
<td>(Hallier-Soulier and Guillot, 2003)</td>
</tr>
<tr>
<td>DNA/Real-Time PCR</td>
<td>1000, 100, 10, 5</td>
<td>Serial Dilution</td>
<td>1 oocyst⁶</td>
<td>(Guy et al., 2003)</td>
</tr>
<tr>
<td>Oocysts/Real-Time PCR</td>
<td>775, 75</td>
<td>Serial Dilution</td>
<td>75 oocysts</td>
<td>(Fontaine and Guillot, 2003)</td>
</tr>
</tbody>
</table>

¹ lower limits of detection (LLOD)
² 18S rRNA, Small Subunit ribosomal RNA
³ hsp70, 70 KDa heat shock protein
⁴ IMS, Immunomagnetic Separation
⁵ IFA, Immunofluorescent microscopy
⁶ as determined by serial dilution of C. parvum previously isolated DNA
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5.0 Appendices

5.1 Appendix A

Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts.

Sturbaum GD, Reed C, Hoover PJ, Jost BH, Marshall MM, Sterling CR.

To: Journals Dept.
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From: Gregory Dean Sturbaum
University of Arizona Ph.D. Candidate
CH Diagnostic & Consulting Service, Inc.
President and Secretary

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PMID: 11375178 [PubMed - indexed for MEDLINE]

Sturbaum GD, Klonicki PT, Marshall MM, Jost BH, Clay BL, Sterling CR.
Immunomagnetic separation (IMS)-fluorescent antibody detection and IMS-PCR detection of seeded Cryptosporidium parvum oocysts in natural waters and their limitations.
PMID: 12039759 [PubMed - indexed for MEDLINE]

Sincerely,

Gregory Sturbaum
Cryptosporidiosis is a self-limiting diarrheal infection in the immunocompetent individual, and with proper oral hydration therapy a full recovery is expected (5). The disease is caused by apicomplexan parasite Cryptosporidium parvum and to date is still without effective chemotherapy (19). The lack of drug therapy is of major concern in immunocompromised individuals in which the parasite's unique self-perpetuating life cycle can cause long-duration diarrhea resulting in major fluid loss (6).

The infectious stage of C. parvum, the oocyst, is shed in the feces of an infected individual. The oocysts have three major features that contribute greatly to the survival and spread of the organism. First, when shed in the feces, oocysts are immediately infectious to the next host (8). This readily leads to direct fecal/oral transmission. Second, oocysts are environmentally resistant and can remain infectious for 2 to 3 months or longer under proper conditions (17). Last, oocysts are small in size (4 to 6 μm in diameter) and resistant to the normal chlorine disinfection level used in water treatment plants and distribution systems, consequently allowing for the spread of C. parvum via drinking water (10, 17). This was illustrated in 1993, when an estimated 400,000 persons in Milwaukee, Wis., succumbed to a waterborne disease outbreak in which Cryptosporidium played a major contributing role (13).

The 1993 Milwaukee outbreak has led to continued monitoring of C. parvum in both untreated (lakes, reservoirs, rivers, and groundwater) and treated water supplies. Also, since 1993, detection methods have progressively improved. Initial techniques included the Information Collection Rule (EPA-600-R-95-178) and membrane filter dissolution (1) and calcium carbonate flocculation protocols (16). The latest oocyst recovery technique from water samples is immunomagnetic separation (IMS) as described by Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA (EPA-821-R-99-006). This protocol is becoming widely accepted, with recovery rates ranging from 62 to 100% (4, 9).

A limiting factor of all these detection techniques is that none is C. parvum specific. The protocols all follow the basic flow chart of concentrating a volume of water containing oocysts, followed by a purification step and detection with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies that lack C. parvum specificity. This is important, because not all species of Cryptosporidium are infectious for mammals but many do have the same size and shape of C. parvum oocysts. With the current detection methods lacking the ability to distinguish among species, some scientists have turned to molecular techniques to identify which species have been isolated from a water sample. These techniques include the PCR, restriction fragment length polymorphism (RFLP), and direct DNA sequencing (3, 11, 20). The need for improved oocyst isolation from water samples, followed by identification of the species of isolated Cryptosporidium oocysts, has led to the combination of IMS followed by PCR detection and species determination with RFLP (7, 12). This combination has been reported to detect small numbers of oocysts (12).

In addition, recent molecular studies have classified C. par-
the 18S rRNA gene. External primers ExCry1 (GCC ACT AGT CAT ATG CTT)
the difficulty of liberating DNA from the oocyst.

This report outlines a protocol that assesses the efficiency of low DNA template detection via PCR. By using micromanipulation techniques, low numbers of C. parvum oocysts, even a single oocyst, can be confidently and accurately delivered directly into PCR tubes for subsequent DNA liberation and PCR detection. Using this approach, the current techniques for detection and species determination of C. parvum can be evaluated.

MATERIALS AND METHODS

C. parvum oocyst stocks. The Iowa C. parvum genotype 2 isolate was propagated at the University of Arizona. A C. parvum genotype 1 isolate was collected by Atlantic Beeswax FRESMA field workers (Lima, Peru) engaged in long-term collaborative efforts and shipped to the University of Arizona. Oocysts were purified using discontinuous Percoll gradients and osmium chloride (2) and enumerated by hemocytoimeter, and aliquots of a single genotype were stored in antibiotic solution (0.01% Tween 20, 100 U/ml penicillin, 0.1 mg streptomycin/ml, and 0.1 mg gentamicin/ml) at 4°C. Genotype 2 oocysts used in this study were 2 months old. The genotype 1 oocysts used were less than 6 months old.

FITC labeling of C. parvum oocysts. Purified oocysts were FITC labeled in solution using the indirect Hydrofluor Combo detection kit for Cryptosporidium and Giardia (Ensys Inc., Research Triangle Park, N.C.). Briefly, primary and secondary labeling reagents and bovine serum albumin (1:10 [vol/vol] each) were added to a 100-μl water matrix suspension containing approximately 10^7 oocysts (Iowa isolate). The labeling mixture was incubated in the dark for 1 h at room temperature with brief shaking every 5 min. FITC-labeled suspensions were then washed twice in 1× PCR buffer (50 mM Tris-Cl [pH 8.3], 50 mM KC1) (PE Applied Biosystems, Foster City, Calif.) and resuspended at 4°C. The suspensions were stored at 2°C to 8°C. By using the FITC-labeled oocysts described above, and transferred to 75 microscope slides. Single oocysts were dispensed into 3 μl of double-distilled H2O on a microscope slide followed by the addition of 3 μl of PCR master mixture with the exception of amplified DNA template. One micropipette (20-μm-inner-diameter tip opening) together with a micropump stabilized by a micromanipulator.

Microscopic isolation of single oocysts. An aliquot of approximately 200 FITC-labeled oocysts was dispensed onto a microscope slide and diluted further with 1× PCR buffer to achieve two to three oocysts per field of view at ×200. Single, intact, fluorescing, FITC-labeled oocysts were located using epifluorescence microscopy. For oocyst isolation, the single oocyst was observed with 100× phase microscopy and subsequently isolated using a manually pulled glass micropipette tip (20-μm-inner-diameter tip opening) together with an Ultramicro-Pump II (World Precision Instruments [WPI], Sarasota, Fla.) and Microtech controller (microprocessor-based controller) (WPI) (Fig. 1). A joystick micromanipulator (WPI) stabilized the microscopic anatomic manipulator. Single oocysts were then transferred and deposited to the test matrix of interest, a microscope slide, or 10 μl of 1× PCR buffer within a thin-walled PCR tube.

To validate the transfer of single oocysts, 75 single oocysts were isolated, as described above, and transferred to 75 microscope slides. Single oocysts were dispensed into 3 μl of double-distilled H2O on a microscope slide followed by the addition of 5 μl of smearing medium (Merck/Klutz Giardia and Cryptosporidium kit: Merck Diagnostics, Cincinnati, Ohio). The suspensions containing the oocyst were covered with a 15-mm-diameter coverslip and sealed with clear fingernail polish. The entire coverslip area was scanned using 200× fluorescence detection. Using this approach, the current techniques for de­

FIG. 1. Differential interference contrast microscopy of a single C. parvum oocyst during micromanipulation. Magnification, ×400. FITC- labeled oocysts were located using epifluorescence microscopy and isolated with 100× phase microscopy using a manually pulled glass micropipette (20-μm-inner-diameter tip opening) together with a micropump stabilized by a micromanipulator.
FITC-labeled oocysts were transferred to microscope slides for epifluorescence microscopy. Sixty-seven of the 75 (89.3%) single oocysts detected in a single transfer. These data indicate that isolation and delivery of single oocysts can be performed accurately and confidently.

The newly designed nested primer set described in this report was evaluated for C. parvum specificity and detection sensitivity with low numbers of oocysts. PCR conditions were optimized using purified DNA from C. parvum genotypes 1 and 2. As expected, the external primer set amplified 844- and 840-bp fragments from genotype 1 and genotype 2, respectively. Similarly, the nested primer set amplified 595- and 590-bp fragments from genotype 1 and genotype 2, respectively.

Isolated DNA from the xenodiagnosis of Cryptosporidium parvum species, p-1 and p-2, C. parvum genotypes 1 and 2, respectively. Lanes M, 50-bp molecular marker (Pharmacia, Piscataway, N.J.).

FIG. 2. Nested PCR amplification and restriction enzyme digests with Vgl and DraII of a segment within the 18S rRNA of Cryptosporidium species, p-1 and p-2, C. parvum genotypes 1 and 2, respectively.

PCR-RFLP DETECTION OF SINGLE C. PARVUM OOCYSTS 2667

RESULTS AND DISCUSSION

Technology for the detection of C. parvum has seen advances related to concentration of oocysts in filtered-water samples, and IMS has replaced differential centrifugation techniques to enhance oocyst isolation and concentration when screening environmental samples (4, 15). Subsequent detection with advanced molecular techniques has been used to detect and determine the species of low numbers of oocysts (12). Along with development of these sensitive techniques for isolation and detection needs to be proper evaluation with known numbers of oocysts. To recapitulate, the two main methods for evaluating PCR sensitivity for detecting C. parvum are (i) dilution of intact oocysts from a stock suspension enumerated with a hemocytometer and (ii) dilution of DNA extracted from a large population of oocysts.

Using a dilution series from an enumerated stock of oocysts intrinsically contains a standard deviation. Due to this standard deviation, the actual number of oocysts used to evaluate a detection limit is not known. Flow cytometry and other studies in which there are microscopically confirmed numbers of oocysts are beginning to be used to ensure that evaluation is being performed with known numbers of oocysts (22, 24). Dilution of DNA to the single-oocyst level does not take into account the difficulty of liberating genomic DNA from individual oocysts or the loss of DNA during purification procedures such as phenol-chloroform extraction or those associated with commercial kits to rid the DNA preparation of PCR inhibitors encountered in fecal and environmental samples.

In the view of the need to determine the true limit of PCR for detecting whole oocysts, the combination of an ultramicrocentrifugation attachment to a micromanipulator was used to deliver the desired number of oocysts into PCR tubes (Fig. 1). To validate oocyst isolation and delivery, 75 individual FITC-labeled oocysts were transferred to microscope slides and confirmation of single-oocyst transfer was performed by epifluorescence microscopy. Sixty-seven of the 75 (89.3%) single isolated oocysts were detected. In no case were additional oocysts detected in a single transfer. These data indicate that isolation and delivery of single oocysts can be performed accurately and confidently.

The newly designed nested primer set described in this report was evaluated for C. parvum specificity and detection sensitivity with low numbers of oocysts. PCR conditions were optimized using purified DNA from C. parvum genotypes 1 and 2. As expected, the external primer set amplified 844- and 840-bp fragments from genotype 1 and genotype 2, respectively. Similarly, the nested primer set amplified 595- and 590-bp fragments from genotype 1 and genotype 2, respectively.

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TABLE 1. Evaluation of nested PCR amplification and restriction enzyme digestion of a segment within the 18S rRNA of Cryptosporidium for species differentiation

<table>
<thead>
<tr>
<th>Test organism (strain, genotype)</th>
<th>PCR product (amplicon) obtained from</th>
<th>Results of RFLP digestion with:</th>
<th>Vp</th>
<th>Drall</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum (Peru, 1)</td>
<td>+ (844) + (593)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. parvum (Iowa, 2)</td>
<td>+ (840) + (590)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. parvum (KSU-1, 2)</td>
<td>+ (840) + (590)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. oocystis (KSU-2)</td>
<td>+ (836) + (583)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>C. andrewsi (KSU-3)</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>C. parvum (KSU-4, 2)</td>
<td>+ (840) + (590)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>+ (831) + (579)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>C. muris (187255)</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Cyclospora cayetanensis</td>
<td>+ (883)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Eimeria necatrix</td>
<td>+ (885)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Encephalitozoon intestinalis</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Giardia duodenalis</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Neospora caninum</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

* +, amplification or digestion; -, no amplification or digestion; ND, not done.

Multiple PCR fragments observed.

5 oocysts, 88% for tubes containing 4 oocysts, 76% for tubes containing 3 oocysts, 56% for tubes with 2 oocysts, and 38% for tubes containing a single oocyst.

To our knowledge, this is the first report evaluating C. parvum detection by PCR at the confirmed single-oocyst level. Under these conditions, single oocysts were detected by PCR with a 38% PCR amplification rate and false oocysts were required to achieve the 90% level. These results demonstrate the difficulty of PCR amplification with intact oocysts and are a reflection of the inherent problems when applying PCR detection to environmental samples.

REFERENCES

5.2 Appendix B

Immunomagnetic separation (IMS)-fluorescent antibody detection and IMS-PCR detection of seeded Cryptosporidium parvum oocysts in natural waters and their limitations.

Sturbaum GD, Klonicki PT, Marshall MM, Jost BH, Clay BL, Sterling CR.

To:     Journals Dept.  
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        N.W.  Washington, DC 20036-2904  
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Sincerely,  

Gregory Sturbaum

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Journals Department

Date 9-4-03
Immunomagnetic Separation (IMS)-Fluorescent Antibody Detection and IMS-PCR Detection of Seeded Cryptosporidium parvum Oocysts in Natural Waters and Their Limitations

Gregory D. Sturbaum,* Patricia T. Klonicki, Marilyn M. Marshall, B. Helen Jost, Brec L. Clay, and Charles R. Sterling

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Received 1 October 2001/Accepted 2 March 2002

Detection and enumeration of Cryptosporidium parvum in both treated and untreated waters are important to facilitate prevention of future cryptosporidiosis incidents. Immunomagnetic separation (IMS)-fluorescent antibody (FA) detection and IMS-PCR detection efficiencies were evaluated in two natural waters seeded with nominal oocyst seed doses of 5, 10, and 15 oocysts. IMS-FA detected oocysts at concentrations of at least 0.001 oocyst/liter (range, 0.001 to 0.48 oocyst/liter; geometric mean, 2.7 oocysts/liter; oocyst seed density as determined by dilution, 525 to 870) and in drinking waters (<1.7 to 56.6%; oocyst seed densities, 0.001 to 0.48 oocyst/liter; geometric mean, 2.7 oocysts/liter; oocyst seed density as determined by dilution, 525 to 870). In this study, yielded positive amplification for 17 of the 18 seeded water samples, and the amplicons were subjected to restriction fragment length polymorphism digestion and DNA sequencing for species identification. Interestingly, the two unseeded, natural water samples were also PCR positive; one amplicon was the same base pair size as the C. parvum amplicon, and the other amplicon was larger. These two amplified products were determined to be derived from DNA of Cryptosporidium muris and a dinoflagellate. These IMS-PCR results illustrate that (i) IMS-PCR is able to detect low oocyst numbers in natural waters, (ii) PCR amplification alone is not confirmatory for detection of target DNA when environmental samples are used, (iii) PCR primers, especially those designed against the rRNA gene region, need to be evaluated for specificity with organisms closely related to the target organism, and (iv) environmental amplicons should be subjected to appropriate species-specific confirmatory techniques.

Cryptosporidium parvum, an intestinal protozoan parasite, continues to be an important cause of waterborne gastrointestinal disease worldwide. Due to oocyst robustness consisting of environmental stability and resistance to normal water disinfection processes (14, 22), the low infectious dose (10), and the lack of chemotherapy (23), detection and enumeration of this organism in both treated and untreated waters have become a focus of the water industry in order to prevent future incidents of cryptosporidiosis.

The currently accepted technique for oocyst recovery from water samples is immunomagnetic separation (IMS)-fluorescent antibody (FA) detection, as described by United States Environmental Protection Agency (EPA) Method 1623 (25). The reported IMS-FA recovery rates for oocysts seeded into previously concentrated water pellets of various turbidities have been 62 to 100% (oocyst seed density as determined by dilution, 36 to 976) (20), 55.9 to 83.1% (oocyst seed density as determined by dilution, 89.1 to 98.7) (17), 68 to 83% (oocyst seed density as determined by dilution, 525 to 870) (3), and 84.3% (oocyst seed density as determined by flow cytometry, 100) (19). In an additional study, in which oocysts were seeded into 10-liter grab samples of various turbidities, the reported recoveries ranged from <1.7 to 56.6% (oocyst seed densities, 1,615 and 2,880) (8). However, the criteria for oocyst species identification and viability are not fulfilled by the current IMS-FA protocol (25).

Therefore, several protocols have been developed by using PCR coupled with restriction fragment length polymorphism (RFLP) for species identification (3, 7, 12, 13, 16, 18) and cell culturing for viability (6, 20). As determined by the combination IMS-PCR approach, the detection limits in treated water samples have been reported to be <5 oocysts (13), 8 oocysts (18), and 10 oocysts (16). In addition, the detection limits in raw waters were reported to be <5 oocysts (12) and between 1 and 100 oocysts (16).

Due to the reported low oocyst concentrations in source water supplies (range, <0.007 to 484 oocysts/liter; geometric mean, 27 oocysts/liter; n = 66) (15) and in drinking waters (range, 0.001 to 0.48 oocyst/liter; n = 158) (21), the need to evaluate the IMS-FA and IMS-PCR recovery efficiencies with low oocyst numbers was deemed imperative. In this study we undertook the task of evaluating IMS-FA detection and IMS-PCR detection with low numbers of C. parvum oocysts seeded into postconcentrated samples of natural waters.

MATERIALS AND METHODS

Cryptosporidium parvum. C. parvum oocysts (Iowa isolate) were propagated and purified at the University of Arizona (2). Oocysts from a single batch, which were less than 2 months old, were sent to the Wisconsin State Laboratory of Hygiene for flow cytometric enumeration and sorting with an Epics Elite flow cytometer ( Coulter Corporation, Miami, Fla.). Three oocyst preparations were flow counted and dispersed into 25 ml of 1% Tween 20 to obtain aliquots nominally contained 5, 10, or 15 oocysts. The enumerated and sorted oocysts were shipped to CH Diagnostic & Consulting Service, Inc. (Loveland, Colo.) for oocyst seeding and IMS isolation.
Natural waters. Twenty-liter grab samples were collected from two natural waters, Dowdy Lake and Cache la Poudre River, both located in the northern Rocky Mountains of Colorado. Previous work has determined the concentration of Cryptosporidium spp. in Cache la Poudre water to be 300 to 1,000 oocyst liter−1 (K. Gertig, personal communication). One-liter water concentrates were used to qualitatively identify algal species by bright-field microscopy at magnifications of ×100 and ×400.

The 20-liter grab samples were collected in carboys and concentrated by using Envirocheck sampling cups (Pall Gelman Laboratory, Ann Arbor, Mich.) at a flow rate of 0.5 g/min per m3. Particulate matter was eluted by using EPA Method 1623 (25). The final centrifuge pellet was measured and resuspended in 20 ml of double-distilled H2O. One-milliliter aliquots (each equivalent to 1 liter of sample water) were dispensed into 125-mm flat-sided Leighton tubes (Durall A.S., Oslo, Norway) for use in IMS.

Confirmation of oocyst seed dose. Each oocyst stock solution (5, 10, or 15 oocysts/ml) was vortexed for 2 min, which was followed by three 180° inversions just before the solutions were dispensed as 1-ml aliquots directly onto 1-mm-pore-size 25-mm-diameter black polycarbonate membrane filters on a filtered glass support. Three replicates were tested for each oocyst dose. The oocysts were labeled with fluorescent histagreement by using a Merifluor C/G detection kit for Cryptosporidium and Gondia (Meridian Diagnostics Inc., Cincinnati, Ohio). The membranes were scanned and enumerated at a magnification of ×200 by using fluorescent microscopy.

Oocyst seeding and IMS. For seeding oocysts, the oocyst stock solutions (5, 10, or 15 oocysts/ml) were vortexed for 2 min, which was followed by three 180° inversions just before the solutions were seeded as 1-ml aliquots into Leighton tubes containing 3-liter equivalents of either Dowdy Lake or Cache la Poudre River water. A total of six Leighton tubes for each nominal oocyst value were tested; three replicates were used for microscopic analysis, and three replicates were used for IMS/PCR analysis. In addition, one sample for each nominal oocyst value was spiked into deionized H2O, and one sample of each of the natural water 1-liter equivalents was used as a background control. All samples were subjected to IMS by using EPA Method 1623 (25) and two final digestion steps (50 μl each). Microscopic replicates for each nominal oocyst value were scanned at a magnification of ×200 by using fluorescent microscopy, and pre- and post-separation oocysts were confirmed at a magnification of ×1,000 with the vital dye 4′,6-diamidino-2-phenylindole (DAPI) and Nomarski differential interference contrast microscopy. For replicates that were subjected to PCR detection, the final digestion suspensions were transferred into 0.5-ml thin-wall PCR tubes, frozen at −4°C, and shipped to the University of Arizona.

PCR. The nested PCR primer pairs and conditions used in this study have been described previously (24), and they amplify a 590-bp region of the C. parvum 18S rRNA gene. The 100 μl digestion suspension were washed three times in 100 μl of 1 x PCR buffer (30 mM Tris-HCl [pH 8.0], 50 mM KCl) (PE Applied Biosystems, Branchburg, N.J.) by centrifugation (16,000 × g) and resuspension. The final pellet was reconstituted in a solution containing 25 μl of 1 x PCR buffer and 5 μl of Instinct-cmate (Bio-Rad, Hercules, Calif.). This 50-μl suspension was subjected to six freeze-thaw cycles (2 min in liquid nitrogen, followed by 2 min in a 98°C water bath), with a 36°C centrifugation (16,000 × g) performed between the third and fourth cycles. Lastly, the 30-μl suspension was centrifuged at 16,000 × g for 3 min. One 10-μl aliquot of the freeze-thaw supernatant was used directly in the PCR DNA template. PCRs were performed with a Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, N.Y.).

Automated DNA sequencing. Amplicons were purified by using a QIAquick PCR purification kit (QIAGEN Inc., Valencia, Calif.) and were subjected to DNA sequencing at the GATC Sequencing Facility (University of Arizona, Tucson). To identify possible matches, database searches were performed with the BLASTN algorithm (1).

**RESULTS AND DISCUSSION**

At the time of collection, the turbidity of Dowdy Lake was 8.9 nephelometric turbidity units yielding a final 0.9 ml centrifuge pellet, and the turbidity of Cache la Poudre River was 4.6 nephelometric turbidity units yielding a final 0.2 ml centrifuge pellet.

As stated previously, the oocyst concentrations in both treated and untreated water matrices are low (15, 21). Oocyst detection methods, therefore, need to be evaluated with low and accurately counted oocyst numbers. Flow cytometry was chosen to enumerate the oocyst stock solution instead of hemacytometer counting or micromanipulation since hemacytometer counting is prone to error due to the inherent standard deviation associated with dilutions and to potential oocyst clumping and micromanipulation, while accurate (89.3% delivery rate for a single oocyst) (24), is time-consuming. Using oocysts enumerated by flow cytometry ensures a standardized stock solution that can be readily dispensed as replicates samples. Confirmation of the number of oocysts seeded per milliliter as enumerated on black polycarbonate membranes is presented in Table 1. The oocyst concentrations were at or below the nominal seed dose in all replicates except those that received the 15 oocyst/ml dose. The variation in the oocyst seed dose can be accounted for by (i) the possibility that the levels of oocyst recovery with the membranes were not 100% and thus losses could have occurred or (ii) the possibility that by withdrawing 1-ml aliquots from the flow-enumerated stock solution a standard error was introduced and thus the oocyst seed dose was over- or underestimated. It has been reported that oocysts are unevenly distributed within stock suspensions (4, 9). To correct for this deficiency of the method in the future, it is recommended that oocyst seed doses be flow enumerated and sorted directly into the IMS Leighton tubes (19). Despite this, however, the seeded values used to evaluate IMS-FA and IMS-PCR recovery efficiencies in this study were at or below the nominal oocyst seed dose of interest.

The results of IMS-FA microscopic detection and confirmation of oocysts seeded into deionized H2O and naturally occurring waters are summarized in Table 1. In all but one case,
In order to reduce naturally occurring PCR inhibitors. During the washing process, oocysts could have been inadvertently lost. Alternatively, it is possible that the limits of detection with this nested primer set were not met. However, this is unlikely since the detection limits were determined to be 100, 94, 92, 88, 76, 56, and 38% for 10, 7, 5, 4, 3, 2, and 1 oocysts, respectively (24).

Interestingly, the two natural water background control samples, while negative as determined by the IMS-FA technique, were both positive as determined by the IMS-PCR (Table 2), and the amplicon from Dowdy Lake was visibly larger (approximately 625 bp) than the expected 590-bp amplicon of C. parvum. The PCR amplicon (approximately 625 bp) than the amplified C. parvum 18S rRNA sequences of C. muris (GenBank accession no. AF093496) with a pairwise identity value of 98.8% and the 624-bp Dowdy Lake amplicon as a dinoflagellate amplicon (Gymnodinium spp.; GenBank accession no. AF274260) with a pairwise identity value of 90.5% (Table 2).

### Table 2. Seeded oocyst detection by IMS-PCR and confirmation of identity by RFLP analysis and DNA sequencing

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of oocysts/ml</th>
<th>Replicate</th>
<th>PCR detection</th>
<th>RFLP digest</th>
<th>Identity determined by sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized H₂O</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>ND¹</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Dowdy Lake</td>
<td>0</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>Gymnodinium spp.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>C. parvum</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>Gymnodinium spp.</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>C. parvum</td>
</tr>
<tr>
<td>Cache la Poudre River</td>
<td>0</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>C. parvum</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>C. parvum</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>C. parvum</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>Undetermined</td>
</tr>
</tbody>
</table>

¹, +, RFLP-positive digestion; -, RFLP-negative digestion.
² Identity as determined by nucleotide sequencing and BlastN search.
³ ND, not done.
⁴ The PCR amplicon (approximately 625 bp) was larger than the expected 590-bp amplicon of C. parvum.

Recognizing the possibility that all the oocyst-seeded water samples could be PCR positive due to background DNA and not seeded C. parvum oocysts, we performed RFLP analysis and DNA sequencing of all samples. DraII digestion of the Cache la Poudre background amplicon resulted in two fragments of 298 and 286 bp, while the positive control C. parvum amplicon was undigested (Fig. 1G), a result that would be expected if the amplicon was derived from C. muris. Next, the 18S rRNA sequences of Gymnodinium spp. and C. parvum were examined for a restriction enzyme able to distinguish the signature as determined by nucleotide sequencing and BlastN search.
FIG. 1. Nested PCR (A) and RFLP digestion with DraI (B) or DraII (C) of Cryptosporidium spp. and two natural water matrices with or without seeded C. parvum oocysts following IMS. Lanes 1 to 4, amplicons from known isolates; lanes 5 to 9, amplicons from water matrices; lane 1, C. parvum Iowa isolate; lane 2, C. andersoni; lane 3, C. muris; lane 4, G. fetus; lane 5, Dowdy Lake background sample; lane 6, Cache la Poudre background sample; lane 7, Dowdy-5-3; lane 8, Dowdy-10-1; lane 9, Poudre-15-1; lanes M, 50-bp molecular weight marker (Gibco BRL, Grand Island, N.Y.).

two organisms. DraI was identified, and subsequent digestion of the Dowdy Lake background sample produced predicted 352- and 272-bp fragments, while the positive control C. parvum amplicon was undigested (Fig. 1B).

All seeded PCR-positive water samples were then subjected to digestion with DraI, DraII, and VspI. Restriction digestion with VspI was performed to confirm that PCR amplicons were from C. parvum genotype 2 and not genotype 1, and subsequent VspI digestions of all background and seeded water samples were negative (Table 2). However, three samples were digested with either DraI or DraII: replicate 3 of Dowdy Lake water seeded with 5 oocysts (Dowdy-5-3) was digested with DraI, replicate 1 of Dowdy Lake water seeded with 10 oocysts (Dowdy-10-1) was digested with DraI, and replicate 1 of Cache la Poudre River water seeded with 15 oocysts (Poudre-15-1) was digested with DraII (Fig. 1B and C). In addition, the PCR amplicons of samples Dowdy-5-3 and Dowdy-10-1 were both visually larger than the positive control C. parvum amplicon (Fig. 1A), as was the Dowdy Lake background amplicon. Therefore, preliminary data suggested that DNA from a dinoflagellate was amplified in samples Dowdy-5-3 and Dowdy-10-1 and DNA from C. muris was amplified in sample Poudre-15-1. DNA sequencing and BlastN (1) searches identified both Dowdy-5-3 and Dowdy-10-1 amplicons as a dinoflagellate amplicon, possibly Gymnodinium spp., like the amplicon in the Dowdy Lake background sample. The pairwise levels of identity between the Dowdy-5-3 and Dowdy-10-1 samples and Gymnodinium spp. were 91.3 and 90.1%, respectively.
The pairwise level of identity between samples Dowdy-5-3 and Dowdy-10-1 was 95.3%. Unfortunately, there was not sufficient Poudre-15-1 amplicon to successfully sequence it (Table 2). Two additional attempts were made to PCR amplify a nested amplicon from the Poudre-15-1 external master mixture, followed by Drall digestion and DNA sequencing. Both attempts resulted in undigested amplicons, and sequence data identified the new amplicons as C. parvum amplicons. These results suggest that Poudre-15-1 contained DNA of both C. muris and C. parvum. This finding has important implications. Since multiple Cryptosporidium spp. can be present in both environmental and treated water samples, the PCR methods used for detecting C. parvum in water (i) need to be confirmed with C. parvum-specific primers, (ii) should incorporate species-specific RFLP analysis, and/or (iii) should include DNA sequencing for proper identification.

The positive PCRs for the environmental background samples prompted continued evaluation of the nested primer set. DNA from Cryptosporidium andersoni KSU-3 and C. muris 108735 (both donated by Steve Upton, Kansas State University), as well as Gymnodinium fuscum CCMP1677 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton McKown Point, West Boothbay Harbor, Maine), were extracted by using a QiAamp DNA stool mini kit (Qiagen Inc.) as recommended by the manufacturer. The PCR conditions were as previously described (24), and restriction enzyme digestion with Dral or Drall followed amplification. Both C. andersoni and C. muris were PCR positive, and digestion with Dral was able to discriminate these organisms from the C. parvum positive control (Fig. 1A and C). Isolated DNA from G. fuscum also was digested with Dral and produced the predicted bands, as seen in the Dowdy Lake background sample (Fig. 1A and B). Finally, DNA sequencing of G. fuscum revealed pairwise levels of identity with the Dowdy Lake background amplicon, Dowdy-5-3 amplicon, and Dowdy-10-1 amplicon of 90.3, 89.7, and 88.9%, respectively.

The finding that DNA from an unknown Dowdy Lake dinoflagellate and isolated DNA from cultured G. fuscum both were amplified by the nested primer set protocol emphasizes the need to further evaluate primer sets designed specifically to detect C. parvum in environmental samples. Gymnodinium spp. and Cryptosporidium spp. are members of the protozoan infrakingdom Alveolata sensu Cavalier-Smith (6). Alveolata is a robust, monophyletic taxon, as confirmed by rRNA phylogenies (5, 11), and contains three phyla: Apicomplexa (synonym, Apicomplexa; apicomplexans), Ciliophora (ciliates), and Dinocozoa (dinoflagellates). Furthermore, it is recognized that the levels of ribosomal DNA homology are high among species, genera, and even families, and similar levels of homology can be observed among rapidly diverging lineages, as is the case for the three phyla constituting the Alveolata. In fact, relevant to this study, alignment of complete 18S rRNA gene sequences of C. parvum (GenBank accession no. AF108864) and Gymnodinium spp. revealed 86.5-89.5% pairwise identity. Because C. parvum oocysts, dinoflagellates, and ciliates are common in aquatic habitats and because of the close phylogenetic relationship among these phyla, it is not entirely surprising to observe that a given 18S rRNA primer set amplifies DNA derived from multiple alveolate members present together in an environmental sample. Therefore, we believe that other defined 18S rRNA primer sets employed for environmental screening and detection of C. parvum should also be tested with DNA templates derived from common aquatic microorganisms (e.g., Gymnodinium spp.) that are closely related to C. parvum.

In summary, this study demonstrated that IMS-FA and IMS-PCR both were able to detect low numbers of oocysts (i.e., 5 oocysts) seeded into natural waters with low turbidities. We recommend that ongoing studies evaluating C. parvum recovery and detection methods incorporate low oocyst seed doses. Furthermore, to obviate introduction of a standard deviation, we strongly believe that oocysts should be enumerated by flow cytometry and sorted directly into designated experimental vessels for use in a selected method. Lastly, the recommendations stated above concerning proper identification of a Cryptosporidium sp. in a water sample also applies to the entire water matrix. Water samples contain diverse assemblages of organisms, and DNA liberation and recovery techniques do not discriminate between the different organisms; thus, DNA isolated from a water sample is representative of all the organisms present in the sample. Therefore, we recommend that PCR detection of a target organism from an environmental sample be based upon (i) highly specific and tested PCR primers, (ii) PCR amplicon size, (iii) species-specific RFLP digestion, and/or (iv) DNA sequencing for proper species identification.

ACKNOWLEDGMENTS

This research was funded by CH Diagnostic & Consulting Service, Inc., and by the Sterling Parasitology Laboratory. We thank Leigh Jacobs and Walter Narro for propagating and purifying the C. parvum oocysts. We also thank Shawn Sliango, Ali Alyassir, and the CH Diagnostic & Consulting Service working group for concentrating the water samples and performing the IMS technique.

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5.3 Appendix C

Nucleotide changes within three *Cryptosporidium parvum* surface protein encoding genes differentiate genotype I from genotype II isolates.

Sturbaum GD, Jost BH, Sterling CR.

*Molecular and Biochemical Parasitology.* 2003 Apr 25;128(1):87-90.
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Nucleotide changes within three *Cryptosporidium parvum* surface protein encoding genes differentiate genotype I from genotype II isolates

Gregory D. Sturbaum, B. Helen Jost, Charles R. Sterling

C. parvum is an enteric protozoan parasite that infects intestinal epithelial cells of a diverse group of mammals, causing diarrhea and a myriad of associated symptoms [1,2]. The disease is self-limiting in immunocompetent individuals, but can become chronic and lead to wasting and possibly death in immunocompromised hosts, due to the organism’s self-perpetuating lifecycle [3–5]. *C. parvum* is now partitioned into two genotypes based upon host infection specificity [6] and DNA sequence data [7–15]. Type I predominantly infects humans, with exceptions [6,16], and type II infects both humans and numerous other mammals, primarily ruminants [12,17].

A number of *C. parvum* surface proteins have been identified and characterized on the basis of inhibiting/blocking sporozoite/merozoite gliding, attachment, membrane fusion, and parasitophorous vacuole formation. These proteins include the circumsporozoite-like glycoprotein exoantigen [18], GP900 [19–21], p23 [22], Cpgp40/15 [23,24], and the 47-kDa membrane associated protein [25]. The research characterizing these proteins, both genetic and functional, has been performed almost exclusively on type II isolates, with the exception of Cpgp40/15 [26]. Cpgp40/15 is reported to be highly polymorphic among type I isolates and shows differences between type I and type II isolates [23,24,27,28]. This observation implies that polymorphisms in this gene could account for differences in host receptor specificity [26]. Extending this observation further, one could hypothesize that genetic differences within other attachment and invasion proteins could collectively explain why *C. parvum* type I preferentially infects humans, while being completely absent from natural ruminant populations.

In a preliminary endeavor, we analyzed the genes encoding three *C. parvum* surface proteins, Cpgp40/15, GP900 and p23, for nucleotide sequence differences between type I and type II isolates.

The *C. parvum* isolates in this study were typed at two genetic loci [13,29] and classified as genotype I or II. In total, DNA isolated from 8 type I isolates, Y049, 278, 339, 830, 1487, 1531 (donated by Dr. Robert Gillman, Johns Hopkins University and Asociacion Benefica PRISMA, Lima, Peru), HFL-6 and HMS-C (donated by Dr. Michael Arrowood, Centers of Disease Control and Prevention, Atlanta, GA) and 5 type II isolates, Apple Cider, Glasgow, Iowa, More­dun, and Texas (propagated at the University of Arizona) was subjected to PCR amplification and automated DNA sequencing of the targeted genes.

Oligonucleotide primers p23–45 ATATTTTTACCTTC­CTTCACCT– and p23–569 AACCTTAATAAAAAA­CACTCTATTG were used to amplify a 549 bp fragment of the p23 gene. Nucleotide sequencing of the p23 PCR products revealed that all type I isolates were identical, as were all type II isolates (GenBankAY129677–AY129689). However, ten nucleotide differences were present between type I and II isolates and consisted of seven base transitions and three base transversions. Of these changes, three resulted in amino acid changes in the p23 protein. Ser68 and Pro72 in type I isolates are changed to Pro and Ala in type II isolates, respectively. At amino acid 90, there is a conservative substitution of Asp in type II isolates, to Glu in type I isolates. Interestingly, this third amino acid change (Asp to Glu) occurs in the final position of a six amino acid motif (Q-D-K-P-A-D) recognized by the neutralizing mAb C6B6.
The putative N-linked glycosylation site (N-X-S/T) at amino acids 15–17 [22] is conserved between both genotypes. In addition, there is a base pair deletion (AGA at sequence nucleotide positions 434–436) 35 bases downstream of the end of the p23 open reading frame in all type I when compared with type II isolates (data not shown).

The primers set Cpgp40/15–24 ATTACTCTCCGTCCTATAGCTCCTC and Cpgp40/15–936 GATTGCAGAAACGGAGGAAGCC amplifies a fragment of the Cpgp40/15 gene and sequence data from type I isolates varied in length from 831 to 937 base pairs (AF528755–AF528762), in agreement with previous reports stating that cpgp40/15 is highly variable in this genotype [23,26–28]. The deduced genotype I protein sequences varied in length from 276 to 306 amino acids (data not shown). Based on alignment with previously published cpgp40/15 sequences, Peruvian isolates Y049 and 830 were classified as subtype la [23], isolates 278 and 339 were subtype Id [23], and isolates 1487 and 1531 were subtype le [28]. In addition, subtype le isolates differ from the other subtype I subgenotypes by containing Asp instead of Glu in the second position of the putative Gp40/Gpl5 cleavage site (E-E) [23,24], US isolates Y049 and 830 did not contain a putative glycosylation site. Translated into the 857 amino acid sequence revealed 17 differences between the type I and II isolates. Prior studies analyzing the differences between the two Cryptosporidium genotypes have examined many genetic loci including the following genes: β tubulin [8,30,31], the Cryptosporidium oocyst wall protein [15], 18S SSU rRNA [7,32–34], HSP70 [13,14], the rRNA internal transcribed spacer region-1 [35], microsatellite regions [7,9], and the thrombospondin-related adhesive protein of gp900 domain 3 was troublesome to amplify therefore separate primer sets were developed. Primer set gp900D3-1562 AACTGAGATGTAGTTAATAACCTG and gp900D3-2031 TTGCAGTATGCTGTTTACCG amplified a 489 bp fragment from isolates 278, 339, 1487, 1531, and all type II isolates, of which 476 bp were sequenced (GenBank AF527865–AF527875).

Primers gp900D3-1581 ACCTGAGATGTGTTGTTTCCG and gp900D3-2080 GTGTAGATGTGTTGTTGTC were developed and used to amplify a 522 bp fragment from isolates HFL-6 and HM5-C. NCBI Y049 and 830 did not amplify with any combination of the above primers. Nucleotide alignments revealed three amino acid substitutions between the type I and II isolates. Alignment of the amino acid sequences with the two published domain 3 sequences, AF068065 and U83169, revealed similar amino acid substitutions within AF068065. These differences are Arg for Gly at position 591, Asp for Gly at position 626, and Ser for Cys at position 649 [20,21]. Also, the reported consensus glycosaminoglycan attachment sequence (S-G-X-G) is conserved amongst all the isolates [21].

Lastly, primers gp900D5-2490 ACCAATTCGAGGTTCT-CAGCACGAGCACAAATAGC and gp900D5-5129 ATTTTCGTTCTGCTCAGCTCAACTGACTTCCCG were used to amplify a 2671 bp fragment of gp900 domain 5. The Long PCR procedure utilized the PCR SuperMix High Fidelity kit (Invitrogen, Carlsbad, CA) and an extension time of 3 min. Walking sequencing generated 2572 bp from all the isolates (GenBank AF528754–AF528764), with the exceptions of HFL-6 and HM5-C, using primers gp900D5-2490 ACCAATTCGAGGTTCTCAGCACGAGCACAATAGC, gp900D5-2987 AGCAACTCCATCTCC-CCC, gp900D5-3343 ATGGATCTCCATTTGTCCGG, gp900D5-3843 TGTTACATTATACCC, gp900D5-4400 CACAAAGTGAAATGTCAGG, and gp900D5-4711 GTAAGCTCCGACTTGGAG. The nucleotide alignment revealed 50 base transitions and eight base transversions spaced throughout domain 5, differentiating type I and type II isolates. Translation into the 857 amino acid sequence revealed 17 differences between the type I and II isolates. The Texas isolate has a unique amino acid substitution of Pro for Ala at position 983, which was compared with other isolates. There were no differences between the genotypes in any of the predicted N-linked glycosylation sites [21]. Of the numerous amino acid repeats reported by Barnes et al. [21], only one substitution, Ser for Thr at position 1280, was noted between the type I and II isolates. This study systematically compared the gene sequences of major surface attachment/invasion proteins of C. parvum type I and II isolates. Prior studies analyzing the differences between the two Cryptosporidium genotypes have examined many genetic loci including the following genes: β tubulin [8,30,31], the Cryptosporidium oocyst wall protein [15], 18S SSU rRNA [7,32–34], HSP70 [13,14], the rRNA internal transcribed spacer region-1 [35], microsatellite regions [7,9], and the thrombospondin-related adhesive protein of
Cryptosporidium-1 [10,11]. The genetic characterization of these loci collectively provide undeniable evidence that C. parvum has delineated into two separate molecular, host-specific, and epidemiologically transmission patterns. The data provided in this work provides further strong evidence for the demarcation between the genotype I and genotype II images of C. parvum, as well as adding information on numerous restriction enzyme cleavage sites for use in future genotyping protocols. The nucleotide and deduced amino acid differences within the genes encoding cgpg40/15, p23, and gp900 domains 1 and 5 between the two C. parvum genotypes lend support to the previously outlined hypothesis: variation within C. parvum surface proteins may account for the observed difference in infection patterns of genotype I versus genotype II, and that these dissimilarities may be realized through receptor/ligand interactions. This hypothesis is further indirectly supported with the recent findings of differences between type I and II surface glycoproteins, specifically Cgpg40/15 [36]. Distinct blotting patterns within surface proteins gp15 and p23 were demonstrated between type I and II isolates by Western blot analysis using convalescent and immune serum from gnotobiotic piglets [36]. It was hypothesized that the low level of genetic similarity within cgpg40/15 (i.e., high level of phenotypic variability) accounts for observed Western blot differences between type I and II isolates [36]. The observations gleaned from this study extend our knowledge of already noted differences between C. parvum genotypes I and II. These observations also pave the way for ongoing studies designed to bridge the gap between genetic and functional differences in these two genotypes.

Acknowledgements

The authors thank Dr. Michael Riggs, Deborah Schaefer, Dr. Stephen Billington and Marilyn M. Marshall for scientific discussions and critical review of the manuscript. We also thank Walter Narro and Leigh Jacobs for propagating and purifying the isolates used in this study.

References


