CRYPTOSPORIDIUM PARVUM, MOLECULAR ENVIRONMENTAL DETECTION AND IMPLICATIONS

by

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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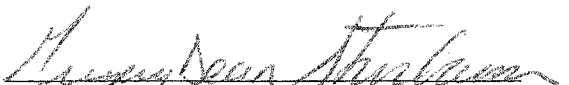
SIGNED: 
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DEDICATION

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. serpentis* (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 *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. *In vitro* infection showed increased permeability and decreased resistance between *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 *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 *C. parvum* infection *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 sialysation 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

(Gomez-Morales and Pozio, 2002). 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 occulden-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/asexual 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 clostrum 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 resistance, 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. meleagridis* (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 x 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 down stream 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 fluoroscein 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 show 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 inactive 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 difficultly 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 that 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.

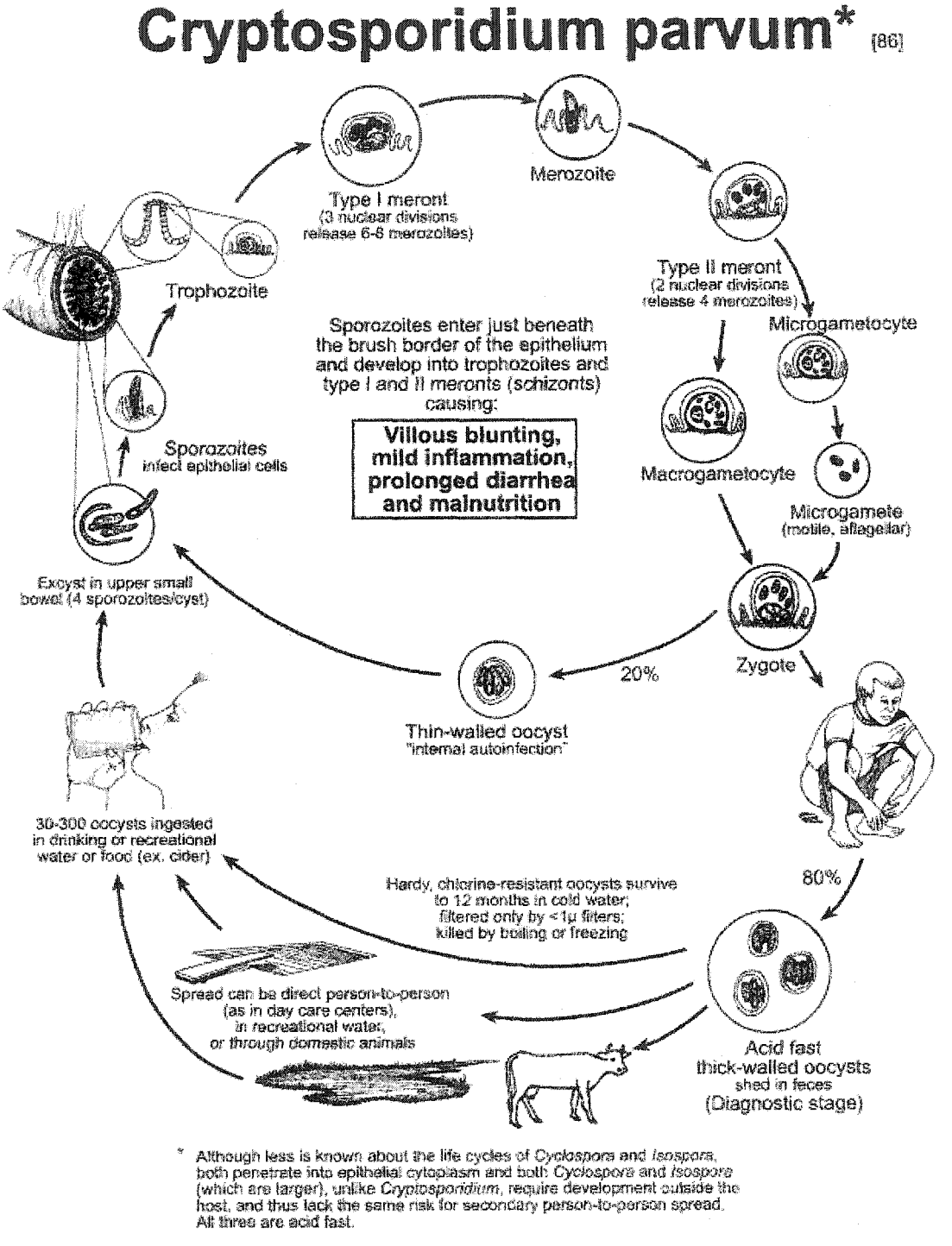

Figure 1. Life cycle of *Cryptosporidium parvum.* The life cycle illustration is from Dillingham *et al.*, 2002 and used with permission from the authors.

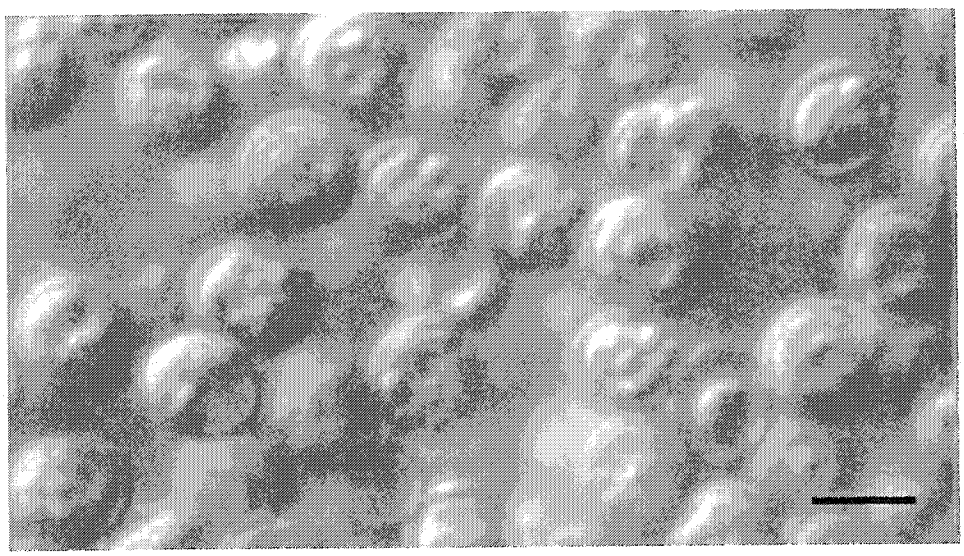

Figure 2. Nomarski differential contrast photomicrograph of *Cryptosporidium parvum* oocysts. Bar represents 5 μm.

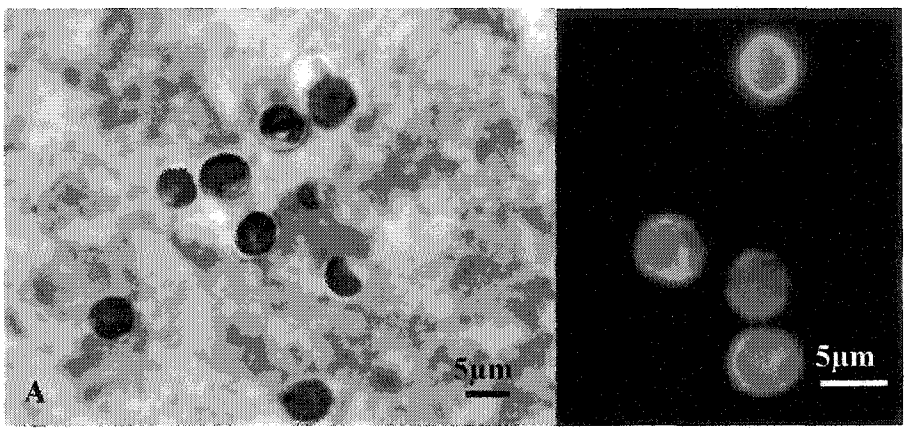

Figure 3. Differential staining techniques for identification of *Cryptosporidium parvum* oocysts in stool and environmental samples. Panel A: modified acid-fast stain; Panel B: Immunofluorescence Microscopy.

Table 1 Taxonomically recognized *Cryptosporidium* species

***Cryptosporidium* species**	**Genotype**	**Host**	**Reference**
C. andersoni		Mammal (*Bos taurus*)	(Lindsay *et al.*, 2000)
C. baileyi		Avian (*Gallus gallus*)	(Current *et al.*, 1986)
C. canis	Coyote, Fox, Dog	Mammal (*Canis familaris*)	(Fayer *et al.*, 2001; Morgan *et al.*, 2000)
C. felis		Mammal (*Felis catus*)	(Iseki, 1979)
C. hominis		Mammal (*Homo sapiens*)	(Morgan-Ryan *et al.*, 2002)
C. meleagridis		Avian (*Meleagris gallopavo*)	(Slavin, 1955)
C. molnari		Fish	(Alvarez-Pellitero and Sitja-Bobadilla, 2002)
C. muris		Mammal; House mouse	(Tyzzer, 1910)
C. nasorum		Fish (*Naso lituratus*)	(Hoover *et al.*, 1981)
C. parvum	bovis	Mammal (*Mus musculus*)	(Tyzzer, 1912)
C. saurophilum		Skink (*Eumeces schneideri*)	(Koudela and Modry, 1998)
C. serpentis		Reptile (multiple species)	(Levine, 1980)
C. wrairi		Mammal (*Cavia porcellus*)	(Vetterling *et al.*, 1971)

Table 2. Morphometric description of *Cryptosporidium* spp. oocysts

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

Table 3. Clinical Features of Cryptosporidiosis [A]

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

[A] Table adopted from Fayer *et al.*, 2000 for *Cryptosporidium parvum* and *C. hominis*
[B] AIDS: acquired immunodeficiency syndrome
[C] N/A: Not Applicable

Table 4. Cryptosporidiosis outbreaks due to contaminated drinking water [A]

Year	Location	Number of cases	Suspected cause	Reference
1984	Braun Station, TX	2,006	Contaminated well water	(D'Antonio *et al.*, 1985)
1987	Carrolton, GA	12,960	Treatment deficiencies	(Hayes *et al.*, 1989)
1989	Ayrshire, UK	27	Treatment deficiencies	(Smith *et al.*, 1989)
1990 - 1991	Isle of Thanet, UK	47	Treatment deficiencies	(Joseph *et al.*, 1991)
1991	Berks County, PA	551	Treatment deficiencies	(Moore *et al.*, 1993)
1992	York Shire, UK	125	Contaminated tap water	(Furtado *et al.*, 1998)
1992	Jackson County, OR	15,000	Treatment deficiencies	(Frost *et al.*, 1998; Moore *et al.*, 1993)
1993	Milwaukee, WI	403,000	Treatment deficiencies	(MacKenzie *et al.*, 1994)
1993	Waterloo, Canada	>1000	Contaminated tap water	(Rose *et al.*, 1997)
1993	Las Vegas, NV	103	Unknown	(Goldstein *et al.*, 1996)
1993	Wessex, UK	27	Contaminated tap water	(Furtado *et al.*, 1998)
1994	Kanagawa, Japan	461	Contaminated drinking water	(Kuroki *et al.*, 1996)
1994	Walla Walla, WA	104	Sewage contaminated well	(Dworkin *et al.*, 1996)
1995	Northern Italy	294	Contaminated water tanks	(Frost *et al.*, 2000; Pozio *et al.*, 1997)
1995	Gainesville, FL	77	Contaminated tap water	(Anonymous, 1996a)
1996	Ogose, Japan	>9000	Unfiltered ground water	(Yamazaki *et al.*, 1997)
1996	New York, NY	>30	Contaminated apple cider	(Anonymous, 1997a)
1997	Shoal Lake, Ontario	100	Unfiltered lake water	(Anonymous, 1997b)
1997				
1998	Guadarrama, Spain	21	Contaminated tap water	(Rodriguez-Salinas Perez *et al.*, 2000)
1998	Brushy Creek, TX	32	Sewage contaminated well	(Anonymous, 1998c)
1999	Northwest England, UK	360	Unfiltered surface water	(Anonymous, 1999)
2000	Belfast, Northern Ireland	129	Contaminated drinking water	(Glaberman *et al.*, 2002)
2000	Lancashire, England	58	Contaminated drinking water	(Howe *et al.*, 2002)
2001	Belfast, Northern Ireland	230	Contaminated drinking water	(Glaberman *et al.*, 2002)

[A] Table adopted from Fayer *et al.*, 2000.

Table 5. *Cryptosporidium parvum* detection methodologies and associated lower limits of detection (LLOD)

Detection	Oocyst Concentrations	Determined By	LLOD[1]	Reference
PCR/18S rRNA[2]	900, 90, 9, 1	Serial Dilution	90 oocysts	(Johnson *et al.*, 1995)
RT-PCR/*hsp70*[3]	1000, 100,50,25,12,1	Serial Dilution	1 oocyst	(Stinear *et al.*, 1996)
PCR/18S rRNA	5000, 1000,500, 50, 5	Serial Dilution	50 oocysts	(Rochelle *et al.*, 1997)
PCR/Unknown region	5000, 1000,500, 50, 5	Serial Dilution	50 oocysts	(Rochelle *et al.*, 1997)
PCR/Unknown region	5000, 1000,500, 50, 5	Serial Dilution	5 oocysts	(Rochelle *et al.*, 1997)
Oocysts/IMS[4]/IFA[5]	100	Flow Cytometry	100 oocysts	(Reynolds *et al.*, 1999)
PCR/Unknown region	100, 50, 10, 1	Serial Dilution[1]	1 oocyst[6]	(Wu *et al.*, 2000)
Oocysts/membrane dissolution/IFA	4 X 10^4, 100	Serial Dilution	<100 oocysts	(McCuin *et al.*, 2000)
Oocysts/PCR-18s rRNA	10, 7, 5, 4, 3, 2, 1	Micromanipulation	1 oocyst	(Sturbaum *et al.*, 2001b)
Oocysts/IMS/PCR-18s rRNA	1000, 500, 250, 100,	Serial Dilution[1]	10 oocysts	(Lowery *et al.*, 2000)
Oocysts/IMS/IFA	10, 5	Serial Dilution	5 oocysts	(McCuin *et al.*, 2001)
Oocysts/IMS/RT-PCR/*hsp70*	10000, 1000, 100, 8	Serial Dilution	8 oocysts	(Monis and Saint, 2001)
Oocysts/IMS/PCR-18s rRNA	15, 10, 5	Flow Cytometry	5 oocysts	(Sturbaum *et al.*, 2001a)
Hollow fiber ultrafilter/IFA	10000, 1000, 600	Serial Dilution	Not Determined	(Kuhn and Oshima,
Oocysts/Flocculation/IFA	1 X 10^6, 1000, 10, 5, 1	Serial Dilution	10 oocysts	(Karanis and Kimura,
Oocysts/IMS/Real-Time PCR	10000, 1000, 100, 10, 5	Serial Dilution	10 oocysts	(Hallier-Soulier and Guillot, 2003)
DNA/Real-Time PCR	1000, 100, 10, 1	Serial Dilution	1 oocyst[6]	(Guy *et al.*, 2003)
Oocysts/Real-Time PCR	775, 75	Serial Dilution	75 oocysts	(Fontaine and Guillot,

[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

4.0 References

Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature. **383**: 787-793.

Abe, N., Kimata, I., and Iseki, M. (2002). Identification of genotypes of *Cryptosporidium parvum* isolates from a patient and a dog in Japan. J Vet Med Sci. **64**: 165-168.

Abrahamsen, M.S., Lancto, C.A., Walcheck, B., Layton, W., and Jutila, M.A. (1997). Localization of alpha/beta and gamma/delta T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves. Infect Immun. **65**: 2428-2433.

Adal, K.A., Sterling, C.R., and Guerrant, R.L. (1995) *Cryptosporidium* and related species. In: Infections of the Gastrointestinal Tract. M.J. Blaser, P.D.S., J.I. Ravdin, H.B. Greenberg, R.L. Guerrant (ed). Raven Press. New York. pp. 1107-1128.

Adham, S.S., Jacangelo, J.G., and Laine, J. (1994). Effect of membrane type on the removal of *Cryptosporidium parvum* and, *Giardia muris*, and MS2 virus. in: *Proceedings of the American Water Works Association's Annual Conference*. American Water Works Association. New York, NY. pg 477.

Aguirre, S.A., Mason, P.H., and Perryman, L.E. (1994). Susceptibility of major histocompatibility complex (MHC) class I- and MHC class II-deficient mice to *Cryptosporidium parvum* infection. Infect Immun. **62**: 697-699.

Aguirre, S.A., Perryman, L.E., Davis, W.C., and McGuire, T.C. (1998). IL-4 protects adult C57BL/6 mice from prolonged *Cryptosporidium parvum* infection: analysis of CD4+alpha beta+IFN-gamma+ and CD4+alpha beta+IL-4+ lymphocytes in gut-associated lymphoid tissue during resolution of infection. J Immunol. **161**: 1891-1900.

Aiello, A., Xiao, L., Limor, J., Liu, C., Abrahamsen, M., and Lal, A. (1999). Microsatellite analysis of the human and bovine genotypes of *Cryptosporidium parvum*. J Euk Microbiol. **46**: 46S-47S.

Alvarez-Pellitero, P., and Sitja-Bobadilla, A. (2002). *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata L.* and *Dicentrarchus labrax L.* Int J Parasitol. **32**: 1007-1021.

Amadi, B., Mwiya, M., Musuku, J., Watuka, A., Sianongo, S., Ayoub, A., and Kelly, P. (2002). Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. Lancet. **360**: 1375-1380.

Anderson, B.C. (1985). Moist heat inactivation of *Cryptosporidium* sp. Am J Public Health. **75**: 1433-1434.

Anderson, B.C. (1986). Effect of drying on the infectivity of cryptosporidia-laden calf feces for 3- to 7-day-old mice. Am J Vet Res. **47**: 2272-2273.

Anderson, W.L., Champlin, T.L., Clunie, W.F., Hendricks, D.W., Klein, D.A., Kugrens, P., and Sturbaum, G.D. (1996). Biological Particle Surrogates for Filtration Performance Evaluation. in: *Water Quality Technical Conference, 1996.* American Water Works Association. Toronto, Ontario. pg

Anonymous. (1994). *Cryptosporidium* infections associated with swimming pools--Dane County, Wisconsin, 1993. MMWR Morb Mortal Wkly Rep. **43**: 561-563.

Anonymous. (1996a). Outbreak of cryptosporidiosis at a day camp--Florida, July-August 1995. MMWR Morb Mortal Wkly Rep. **45**: 442-444.

Anonymous. (1996b). From the Centers for Disease Control and Prevention. Foodborne outbreak of diarrheal illness associated with *Cryptosporidium parvum*--Minnesota, 1995. JAMA. **276**: 1214.

Anonymous. (1996c). Foodborne outbreak of diarrheal illness associated with *Cryptosporidium parvum*--Minnesota, 1995. MMWR Morb Mortal Wkly Rep. **45**: 783-784.

Anonymous. (1997a). Outbreaks of Escherichia coli O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider--Connecticut and New York, October 1996. MMWR Morb Mortal Wkly Rep. **46**: 4-8.

Anonymous. (1997b). Outbreak at a first nation community on Shoal Lake, Ontario, Canada. Cryptosporidium Caps Newslett. **2**: 2-3.

Anonymous. (1998a). From the Centers for Disease Control and Prevention. Foodborne outbreak of Cryptosporidiosis--Spokane, Washington, 1997. JAMA. **280**: 595-596.

Anonymous. (1998b). Foodborne outbreak of cryptosporidiosis--Spokane, Washington, 1997. MMWR Morb Mortal Wkly Rep. **47**: 565-567.

Anonymous. (1998c). Outbreak in Texas - sewage leak suspected as source of groundwater contamination. Cryptosporidium Caps Newslett. **3**: 1-2.

Anonymous. (1999). Outbreak in NW England - water samples found positive. Cryptosporidium Caps Newslett. **4**: 1.

Anonymous. (2000). Outbreak of gastroenteritis associated with an interactive water fountain at a beachside park--Florida, 1999. MMWR Morb Mortal Wkly Rep. **49**: 565-568.

Anonymous. (2003). Surveillance data from swimming pool inspections--selected states and counties, United States, May--September 2002. MMWR Morb Mortal Wkly Rep. **52**: 513-516.

Anusz, K.Z., Mason, P.H., Riggs, M.W., and Perryman, L.E. (1990). Detection of Cryptosporidium parvum oocysts in bovine feces by monoclonal antibody capture enzyme-linked immunosorbent assay. J Clin Microbiol. **28**: 2770-2774.

Argenzio, R.A., Liacos, J.A., Levy, M.L., Meuten, D.J., Lecce, J.G., and Powell, D.W. (1990). Villous atrophy, crypt hyperplasia, cellular infiltration, and impaired glucose-Na absorption in enteric cryptosporidiosis of pigs. Gastroenterology. **98**: 1129-1140.

Argenzio, R.A., Lecce, J., and Powell, D.W. (1993). Prostanoids inhibit intestinal NaCl absorption in experimental porcine cryptosporidiosis. Gastroenterology. **104**: 440-447.

Arrowood, M., and Sterling, C. (1987). Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. Journal of Parasitology. **73**: 314-319.

Arrowood, M. (1997) Chapter 2: *Cryptosporidium* Diagnosis. In: *Cryptosporidium* and Cryptosporidiosis. Fayer, R. (ed). CRC Press, Inc. New York. pp. 43-64.

Arrowood, M.J., Mead, J.R., Mahrt, J.L., and Sterling, C.R. (1989). Effects of immune colostrum and orally administered antisporozoite monoclonal antibodies on the outcome of *Cryptosporidium parvum* infections in neonatal mice. Infect Immun. **57**: 2283-2288.

Arrowood, M.J., Sterling, C.R., and Healey, M.C. (1991). Immunofluorescent microscopical visualization of trails left by gliding *Cryptosporidium parvum* sporozoites. J Parasitol. **77**: 315-317.

Arrowood, M.J., and Donaldson, K. (1996). Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J Eukaryot Microbiol. **43**: 89S.

Atherholt, T., LeChevallier, M.W., Norton, W.D., and Rosen, J.S. (1998). Effect of rainfall on *Giardia* and *Cryptosporidium*. JAWWA. **90**: 66-80.

Australian-Drinking-Water-Guidelines. (1998). National Health and Medical Research Council. Agricultural and Resource Management Council of Australia and New Zealand.

Awad-El-Kariem, F.M., Warhurst, D.C., and McDonald, V. (1993). Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. Parasitology. **109**: 19-22.

Awad-el-Kariem, F.M. (1996). Significant parity of different phenotypic and genotypic markers between human and animal strains of *Cryptosporidium parvum*. J Eukaryot Microbiol. **43**: 70S.

Bancroft, G.J., and Kelly, J.P. (1994). Macrophage activation and innate resistance to infection in SCID mice. Immunobiology. **191**: 424-431.

Barnes, D.A., Bonnin, A., Huang, J.X., Gousset, L., Wu, J., Gut, J., Doyle, P., Dubremetz, J.F., Ward, H., and Petersen, C. (1998). A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Mol Biochem Parasitol. **96**: 93-110.

Baxby, D., Blundell, N., and Hart, C.A. (1984). The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in faeces. J Hyg (Lond). **93**: 317-323.

Beier, T.B., Svezhova, N.V., and Sidorenko, N.V. (2001). Oocyst structure and problem of coccidian taxonomy. Tsitologiia. **43**: 1005-1012.

Beier, T.V. (2000). Further comment on the coccidian nature of cryptosporidia (Sporozoa: Apicomplexa). Parazitologiia. **34**: 183-195.

Belosevic, M., Guy, R.A., Taghi-Kilani, R., Neumann, N.F., Gyurek, L.L., Liyanage, L.R., Millard, P.J., and Finch, G.R. (1997). Nucleic acid stains as indicators of *Cryptosporidium parvum* oocyst viability. Int J Parasitol. **27**: 787-798.

Belosevic, M., Craik, S.A., Stafford, J.L., Neumann, N.F., Kruithof, J., and Smith, D.W. (2001). Studies on the resistance/reactivation of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts exposed to medium-pressure ultraviolet radiation. FEMS Microbiol Lett. **204**: 197-203.

Bern, C., Hernandez, B., Lopez, M.B., Arrowood, M.J., De Merida, A.M., and Klein, R.E. (2000). The contrasting epidemiology of *Cyclospora* and *Cryptosporidium* among outpatients in Guatemala. Am J Trop Med Hyg. **63**: 231-235.

Bern, C., Ortega, Y., Checkley, W., Roberts, J.M., Lescano, A.G., Cabrera, L., Verastegui, M., Black, R.E., Sterling, C., and Gilman, R.H. (2002). Epidemiologic differences between cyclosporiasis and cryptosporidiosis in Peruvian children. Emerg Infect Dis. **8**: 581-585.

Bier, J.W. (1991). Isolation of parasites on fruits and vegetables. Southeast Asian J Trop Med Public Health. **22 Suppl**: 144-145.

Bjorneby, J.M., Riggs, M.W., and Perryman, L.E. (1990). *Cryptosporidium parvum* merozoites share neutralization-sensitive epitopes with sporozoites. J Immunol. **145**: 298-304.

Black, E.K., Finch, G.R., Taghi-Kilani, R., and Belosevic, M. (1996). Comparison of assays for *Cryptosporidium parvum* oocysts viability after chemical disinfection. FEMS Microbiol Lett. **135**: 187-189.

Bodley-Tickell, A.T., Kitchen, S.E., and Sturdee, A.P. (2002). Occurrence of *Cryptosporidium* in agricultural surface waters during an annual farming cycle in lowland UK. Water Res. **36**: 1880-1886.

Bonnin, A., Fourmaux, M.N., Dubremetz, J.F., Nelson, R.G., Gobet, P., Harly, G., Buisson, M., Puygauthier-Toubas, D., Gabriel-Pospisil, G., Naciri, M., and Camerlynck, P. (1996). Genotyping human and bovine isolates of *Cryptosporidium parvum* by polymerase chain reaction-restriction fragment length polymorphism analysis of a repetitive DNA sequence. FEMS Microbiol Lett. **137**: 207-211.

Borchardt, M.A., and Spencer, S.K. (2002). Concentration of *Cryptosporidium*, microsporidia and other water-borne pathogens by continuous separation channel centrifugation. J Appl Microbiol. **92**: 649-656.

Bridgman, S.A., Robertson, R.M., Syed, Q., Speed, N., Andrews, N., and Hunter, P.R. (1995). Outbreak of cryptosporidiosis associated with a disinfected groundwater supply. Epidemiol Infect. **115**: 555-566.

Buckley, R., and Warnken, W. (2003). *Giardia* and *Cryptosporidium* in pristine protected catchments in central eastern Australia. Ambio. **32**: 84-86.

Caccio, S., Homan, W., van Dijk, K., and Pozio, E. (1999). Genetic polymorphism at the beta-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. FEMS Microbiol Lett. **170**: 173-179.

Caccio, S., Homan, W., Camilli, R., Traldi, G., Kortbeek, T., and Pozio, E. (2000). A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. Parasitol. **120**: 237-244.

Caceres, V. (1998). A foodborne outbreak of cyclosporiasis caused by imported raspberries. J Fam Pract. **47**: 231-234.

Cama, V.A., and Sterling, C.R. (1991). Hyperimmune hens as a novel source of anti-*Cryptosporidium* antibodies suitable for passive immune transfer. J Protozool. **38**: 42S-43S.

Campbell, A.T., Robertson, L.J., and Smith, H.V. (1992). Viability of *Cryptosporidium parvum* oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. Appl Environ Microbiol. **58**: 3488-3493.

Campbell, P., and Current, W. (1983). Demonstration of serum antibodies to *Cryptosporidium* sp. in normal and immunodeficient humans with confirmed infections. J Clin Micro. **18**: 165.

Carpenter, C., Fayer, R., Trout, J., and Beach, M.J. (1999). Chlorine disinfection of recreational water for *Cryptosporidium parvum*. Emerg Infect Dis. **5**: 579-584.

Casemore, D. (1987). The antibody response to *Cryptosporidium*: development of a serological test and its use in a study of immunologically normal persons. Journal of Infection. **14**: 125.

Casemore, D.P., Jessop, E.G., Douce, D., and Jackson, F.B. (1986). *Cryptosporidium* plus *Campylobacter*: an outbreak in a semi-rural population. J Hyg (Lond). **96**: 95-105.

Casemore, D.P. (1989). Bottle-fed lambs as a source of human cryptosporidiosis. J Infect. **19**: 101-104.

Cavalier-Smith, T. (1993). Kingdom protozoa and its 18 phyla. Microbiol Rev. **57**: 953-994.

Cavalier-Smith, T. (1999). Zooflagellate phylogeny and the systematics of protozoa. Biol Bull. **196**: 393-396.

Cevallos, A.M., Zhang, X., Waldor, M.K., Jaison, S., Zhou, X., Tzipori, S., Neutra, M.R., and Ward, H.D. (2000). Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. Infect Immun. **68**: 4108-4116.

Chalmers, R.M., Sturdee, A.P., Mellors, P., Nicholson, V., Lawlor, F., Kenny, F., and Timpson, P. (1997). *Cryptosporidium parvum* in environmental samples in the Sligo area, Republic of Ireland: a preliminary report. Lett Appl Microbiol. **25**: 380-384.

Chapman, P.A., Rush, B.A., and McLauchlin, J. (1990). An enzyme immunoassay for detecting *Cryptosporidium* in faecal and environmental samples. J Med Microbiol. **32**: 233-237.

Chauret, C., Nolan, K., Chen, P., Springthorpe, S., and Sattar, S. (1998). Aging of *Cryptosporidium parvum* oocysts in river water and their susceptibility to disinfection by chlorine and monochloramine. Can J Microbiol. **44**: 1154-1160.

Chauret, C., Springthorpe, S., and Sattar, S. (1999). Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. Can J Microbiol. **45**: 257-262.

Checkley, W., Gilman, R.H., Epstein, L.D., Suarez, M., Diaz, J.F., Cabrera, L., Black, R.E., and Sterling, C.R. (1997). Asymptomatic and symptomatic cryptosporidiosis: their acute effect on weight gain in Peruvian children. Am J Epidemiol. **145**: 156-163.

Checkley, W., Epstein, L.D., Gilman, R.H., Black, R.E., Cabrera, L., and Sterling, C.R. (1998). Effects of *Cryptosporidium parvum* infection in Peruvian children: growth faltering and subsequent catch-up growth. Am J Epidemiol. **148**: 497-506.

Chen, W., Harp, J.A., and Harmsen, A.G. (1993a). Requirements for CD4+ cells and gamma interferon in resolution of established *Cryptosporidium parvum* infection in mice. Infect Immun. **61**: 3928-3932.

Chen, W., Harp, J.A., Harmsen, A.G., and Havell, E.A. (1993b). Gamma interferon functions in resistance to *Cryptosporidium parvum* infection in severe combined immunodeficient mice. Infect Immun. **61**: 3548-3551.

Chen, X.M., and LaRusso, N.F. (2000). Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. Gastroenterology. **118**: 368-379.

Chen, X.M., Levine, S.A., Splinter, P.L., Tietz, P.S., Ganong, A.L., Jobin, C., Gores, G.J., Paya, C.V., and LaRusso, N.F. (2001). *Cryptosporidium parvum* activates nuclear factor kappaB in biliary epithelia preventing epithelial cell apoptosis. Gastroenterology. **120**: 1774-1783.

Clancy, J.L., Gollnitz, W.D., and Tabib, Z. (1994). Commercial labs: How accurate are they? JAWWA. **86**: 89.

Clark, D.P. (1999). New insights into human cryptosporidiosis. Clinical Microbiology Reviews. **12**: 554-563.

Cordell, R.L., and Addiss, D.G. (1994). Cryptosporidiosis in child care settings: a review of the literature and recommendations for prevention and control. Pediatr Infect Dis J. **13**: 310-317.

Cordell, R.L. (2001). The risk of infectious diseases among child care providers. J Am Med Womens Assoc. **56**: 109-112.

Corso, P.S., Kramer, M.H., Blair, K.A., Addiss, D.G., Davis, J.P., and Haddix, A.C. (2003). Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. Emerg Infect Dis. **9**: 426-431.

Crabb, J.H. (1998). Antibody-based immunotherapy of cryptosporidiosis. Adv Parasitol. **40**: 121-149.

Craik, S.A., Weldon, D., Finch, G.R., Bolton, J.R., and Belosevic, M. (2001). Inactivation of *Cryptosporidium parvum* oocysts using medium- and low-pressure ultraviolet radiation. Water Res. **35**: 1387-1398.

Craun, G.F. (1986). Waterborne giardiasis in the United States 1965-84. Lancet. **2**: 513-514.

Culshaw, R.J., Bancroft, G.J., and McDonald, V. (1997). Gut intraepithelial lymphocytes induce immunity against *Cryptosporidium* infection through a mechanism involving gamma interferon production. Infect Immun. **65**: 3074-3079.

Current, W. (1988). The biology of *Cryptosporidium*. ASM News. **54**: 605.

Current, W. (1994). *Cryptosporidium parvum*: household transmission. Ann Intern Med. **120**: 518.

Current, W.L., Reese, N.C., Ernst, J.V., Bailey, W.S., Heyman, M.B., and Weinstein, W.M. (1983). Human cryptosporidiosis in immunocompetent and

immunodeficient persons. Studies of an outbreak and experimental transmission. N Engl J Med. **308**: 1252-1257.

Current, W.L., Upton, S.J., and Haynes, T.B. (1986). The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. J Protozool. **33**: 289-296.

Current, W.L., and Bick, P.H. (1989). Immunobiology of *Cryptosporidium* spp. Pathol Immunopathol Res. **8**: 141-160.

Dai, X., and Hozalski, R.M. (2003). Evaluation of microspheres as surrogates for *Cryptosporidium parvum* oocysts in filtration experiments. Environ Sci Technol. **37**: 1037-1042.

Dalle, F., Roz, P., Dautin, G., Di-Palma, M., Kohli, E., Sire-Bidault, C., Fleischmann, M.G., Gallay, A., Carbonel, S., Bon, F., Tillier, C., Beaudeau, P., and Bonnin, A. (2003). Molecular characterization of isolates of waterborne *Cryptosporidium* spp. collected during an outbreak of gastroenteritis in South Burgundy, France. J Clin Microbiol. **41**: 2690-2693.

D'Antonio, R.G., Winn, R.E., Taylor, J.P., Gustafson, T.L., Current, W.L., Rhodes, M.M., Gary, G.W., Jr., and Zajac, R.A. (1985). A waterborne outbreak of cryptosporidiosis in normal hosts. Ann Intern Med. **103**: 886-888.

Davami, M.H., Bancroft, G.J., and McDonald, V. (1997). *Cryptosporidium* infection in major histocompatibility complex congeneic strains of mice: variation in susceptibility and the role of T-cell cytokine responses. Parasitol Res. **83**: 257-263.

de Graaf, D.C., Spano, F., Petry, F., Sagodira, S., and Bonnin, A. (1999). Speculation on whether a vaccine against cryptosporidiosis is a reality or fantasy. Int J Parasitol. **29**: 1289-1306.

Di Giovanni, G.D., Hashemi, F.H., Shaw, N.J., Abrams, F.A., LeChevallier, M.W., and Abbaszadegan, M. (1999). Detection of infectious *Cryptosporidium parvum* oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. Appl Environ Microbiol. **65**: 3427-3432.

Diaz, E., Mondragon, J., Ramirez, E., and Bernal, R. (2003). Epidemiology and control of intestinal parasites with nitazoxanide in children in Mexico. Am J Trop Med Hyg. **68**: 384-385.

DiGiorgio, C.L., Gonzalez, D.A., and Huitt, C.C. (2002). *Cryptosporidium* and *Giardia* recoveries in natural waters by using environmental protection agency method 1623. Appl Environ Microbiol. **68**: 5952-5955.

Ditrich, O., Palkovic, L., Sterba, J., Prokopic, J., Loudova, J., and Giboda, M. (1991). The first finding of *Cryptosporidium baileyi* in man. Parasitol Res. **77**: 44-47.

Doumbo, O., Rossignol, J.F., Pichard, E., Traore, H.A., Dembele, T.M., Diakite, M., Traore, F., and Diallo, D.A. (1997). Nitazoxanide in the treatment of cryptosporidial diarrhea and other intestinal parasitic infections associated with acquired immunodeficiency syndrome in tropical Africa. Am J Trop Med Hyg. **56**: 637-639.

Drescher, A.C., Greene, D.M., and Gadgil, A.J. (2001). *Cryptosporidium* inactivation by low-pressure UV in a water disinfection device. J Environ Health. **64**: 31-35.

DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., and Jakubowski, W. (1995). The infectivity of *Cryptosporidium parvum* in healthy volunteers. N Engl J Med. **332**: 855-859.

Dworkin, M.S., Goldman, D.P., Wells, T.G., Kobayashi, J.M., and Herwaldt, B.L. (1996). Cryptosporidiosis in Washington State: an outbreak associated with well water. J Infect Dis. **174**: 1372-1376.

Elliot, A., Morgan, U.M., and Thompson, R.C. (1999). Improved staining method for detecting *Cryptosporidium* oocysts in stools using malachite green. J Gen Appl Microbiol. **45**: 139-142.

Elliott, D.A., and Clark, D.P. (2000). *Cryptosporidium parvum* induces host cell actin accumulation at the host-parasite interface. Infect Immun. **68**: 2315-2322.

Elliott, D.A., Coleman, D.J., Lane, M.A., May, R.C., Machesky, L.M., and Clark, D.P. (2001). *Cryptosporidium parvum* infection requires host cell actin polymerization. Infect Immun. **69**: 5940-5942.

Enriquez, F.J., and Sterling, C.R. (1993). Role of CD4+ TH1- and TH2-cell-secreted cytokines in cryptosporidiosis. Folia Parasitol (Praha). **40**: 307-311.

Enriquez, F.J., and Riggs, M.W. (1998). Role of immunoglobulin A monoclonal antibodies against P23 in controlling murine *Cryptosporidium parvum* infection. Infect Immun. **66**: 4469-4473.

Fairley, C.K., Sinclair, M.I., and Rizak, S. (1999). Monitoring not the answer to *Cryptosporidium* in water. Lancet. **354**: 967-969.

Farrington, M., Winters, S., Walker, C., Miller, R., and Rubenstein, D. (1994). *Cryptosporidium* antigen detection in human feces by reverse passive hemagglutination assay. J Clin Microbiol. **32**: 2755-2759.

Fayer, R., Andrews, C., Ungar, B.L., and Blagburn, B. (1989). Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. J Parasitol. **75**: 393-397.

Fayer, R. (1994). Effect of high temperature on infectivity of *Cryptosporidium parvum* oocysts in water. Appl Environ Microbiol. **60**: 2732-2735.

Fayer, R., and Nerad, T. (1996). Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. **62**: 1431-1433.

Fayer, R., Trout, J., and Nerad, T. (1996). Effects of a wide range of temperatures on infectivity of *Cryptosporidium parvum* oocysts. J Eukaryot Microbiol. **43**: 64S.

Fayer, R., Speer, C.A., and Dubey, J.P. (1997) Chapter 1: The General biology of *Cryptosporidium*. In: *Cryptosporidium* and Cryptosporidiosis. Fayer, R. (ed). CRC Press, Inc. New York. pp. 1-41.

Fayer, R., Gasbarre, L., Pasquali, P., Canals, A., Almeria, S., and Zarlenga, D. (1998a). *Cryptosporidium parvum* infection in bovine neonates: dynamic clinical, parasitic and immunologic patterns. Int J Parasitol. **28**: 49-56.

Fayer, R., Graczyk, T.K., Lewis, E.J., Trout, J.M., and Farley, C.A. (1998b). Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol. **64**: 1070-1074.

Fayer, R., Trout, J.M., and Jenkins, M.C. (1998c). Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. J Parasitol. **84**: 1165-1169.

Fayer, R., Lewis, E.J., Trout, J.M., Graczyk, T.K., Jenkins, M.C., Higgins, J., Xiao, L., and Lal, A.A. (1999). *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg Infect Dis. **5**: 706-710.

Fayer, R., Morgan, U., and Upton, S.J. (2000). Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int J Parasitol. **30**: 1305-1322.

Fayer, R., Trout, J.M., Xiao, L., Morgan, U.M., Lai, A.A., and Dubey, J.P. (2001). *Cryptosporidium canis* n. sp. from domestic dogs. J Parasitol. **87**: 1415-1422.

Fayer, R., Trout, J.M., Lewis, E.J., Xiao, L., Lal, A., Jenkins, M.C., and Graczyk, T.K. (2002). Temporal variability of *Cryptosporidium* in the Chesapeake Bay. Parasitol Res. **88**: 998-1003.

Feng, X., Rich, S.M., Akiyoshi, D., Tumwine, J.K., Kekitiinwa, A., Nabukeera, N., Tzipori, S., and Widmer, G. (2000). Extensive polymorphism in *Cryptosporidium parvum* identified by multilocus microsatellite analysis. Applied and Environmental Microbiology. **66**: 3344-3349.

Feng, X., Rich, S.M., Tzipori, S., and Widmer, G. (2002). Experimental evidence for genetic recombination in the opportunistic pathogen *Cryptosporidium parvum*. Mol Biochem Parasitol. **119**: 55-62.

Flanigan, T., Whalen, C., Turner, J., Soave, R., Toerner, J., Havlir, D., and Kotler, D. (1992). *Cryptosporidium* infection and CD4 counts. Ann Intern Med. **116**: 840-842.

Fleming, C. (1998). A foodborne outbreak of *Cyclospora cayetanensis* at a wedding: clinical features and risk factors for illness. Arch Int Med. **158**: 1121-1125.

Fontaine, M., and Guillot, E. (2002). Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. FEMS Microbiol Lett. **214**: 13-17.

Fontaine, M., and Guillot, E. (2003). An immunomagnetic separation-real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. J Microbiol Methods. **54**: 29-36.

Freire-Santos, F., Oteiza-Lopez, A.M., Vergara-Castiblanco, C.A., Ares-Mazas, E., Alvarez-Suarez, E., and Garcia-Martin, O. (2000). Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J Parasitol. **86**: 853-854.

Freire-Santos, F., Gomez-Couso, H., Ortega-Inarrea, M.R., Castro-Hermida, J.A., Oteiza-Lopez, A.M., Garcia-Martin, O., and Ares-Mazas, M.E. (2002). Survival of *Cryptosporidium parvum* oocysts recovered from experimentally contaminated oysters (*Ostrea edulis*) and clams (*Tapes decussatus*). Parasitol Res. **88**: 130-133.

Fricker, C.R., and Crabb, J.H. (1998). Water-borne cryptosporidiosis: detection methods and treatment options. Adv Parasitol. **40**: 241-278.

Frost, F.J., Calderon, R.L., Muller, T.B., Curry, M., Rodman, J.S., Moss, D.M., and de la Cruz, A.A. (1998). A two-year follow-up survey of antibody to *Cryptosporidium* in Jackson County, Oregon following an outbreak of waterborne disease. Epidemiol Infect. **121**: 213-217.

Frost, F.J., Fea, E., Gilli, G., Biorci, F., Muller, T.M., Craun, G.F., and Calderon, R.L. (2000). Serological evidence of *Cryptosporidium* infections in southern Europe. Eur J Epidemiol. **16**: 385-390.

Furtado, C., Adak, G.K., Stuart, J.M., Wall, P.G., Evans, H.S., and Casemore, D.P. (1998). Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-5. Epidemiol Infect. **121**: 109-119.

Gajadhar, A.A., Marquardt, W.C., Hall, R., Gunderson, J., Ariztia-Carmona, E.V., and Sogin, M.L. (1991). Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. Mol Biochem Parasitol. **45**: 147-154.

Garcia, L.S., Brewer, T.C., and Bruckner, D.A. (1979). A comparison of the formalin-ether concentration and trichrome-stained smear methods for the recovery and identification of intestinal protozoa. Am J Med Technol. **45**: 932-935.

Garcia, L.S., and Shimizu, R. (1981). Comparison of clinical results for the use of ethyl acetate and diethyl ether in the formalin-ether sedimentation technique performed on polyvinyl alcohol-preserved specimens. J Clin Microbiol. **13**: 709-713.

Garcia, L.S., and Shimizu, R.Y. (2000). Detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens using the ColorPAC combination rapid solid-phase qualitative immunochromatographic assay. J Clin Microbiol. **38**: 1267-1268.

Gelletlie, R., Stuart, J., Soltanpoor, N., Armstrong, R., and Nichols, G. (1997). Cryptosporidiosis associated with school milk. Lancet. **350**: 1005-1006.

Giacometti, A., Burzacchini, F., Cirioni, O., Barchiesi, F., Dini, M., and Scalise, G. (1999). Efficacy of treatment with paromomycin, azithromycin, and nitazoxanide in a patient with disseminated cryptosporidiosis. Eur J Clin Microbiol Infect Dis. **18**: 885-889.

Gibbons, C., and Awad-El-Kariem, F. (1999). Nested PCR for the Detection of *Cryptosporidium parvum*. Parasitol Today. **15**: 345.

Gile, M., Warhurst, D.C., Webster, K.A., West, D.M., and Marshall, J.A. (2002). A multiplex allele specific polymerase chain reaction (MAS-PCR) on the dihydrofolate reductase gene for the detection of *Cryptosporidium parvum* genotypes 1 and 2. Parasitol. **125**: 35-44.

Glaberman, S., Moore, J.E., Lowery, C.J., Chalmers, R.M., Sulaiman, I., Elwin, K., Rooney, P.J., Millar, B.C., Dooley, J.S., Lal, A.A., and Xiao, L. (2002). Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. Emerg Infect Dis. **8**: 631-633.

Gobet, P., and Toze, S. (2001). Sensitive genotyping of *Cryptosporidium parvum* by PCR-RFLP analysis of the 70-kilodalton heat shock protein (HSP70) gene. FEMS Microbiol Lett. **200**: 37-41.

Godwin, T.A. (1991). Cryptosporidiosis in the acquired immunodeficiency syndrome: a study of 15 autopsy cases. Hum Pathol. **22**: 1215-1224.

Goldstein, S.T., Juranek, D.D., Ravenholt, O., Hightower, A.W., Martin, D.G., Mesnik, J.L., Griffiths, S.D., Bryant, A.J., Reich, R.R., and Herwaldt, B.L. (1996). Cryptosporidiosis: an outbreak associated with drinking water despite state-of-the-art water treatment. Ann Intern Med. **124**: 459-468.

Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Rodas, V., and Costas, E. (2000). Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl Environ Microbiol. **66**: 1866-1870.

Gomez-Morales, M.A., and Pozio, E. (2002). Humoral and cellular immunity against Cryptosporidium infection. Curr Drug Targets Immune Endocr Metabol Disord. **2**: 291-301.

Goodgame, R.W. (1996). Understanding intestinal spore-forming protozoa: cryptosporidia, microsporidia, isospora, and cyclospora. Annals Intern Med. **124**: 429-441.

Gookin, J.L., Nordone, S.K., and Argenzio, R.A. (2002). Host responses to *Cryptosporidium* infection. J Vet Intern Med. **16**: 12-21.

Graczyk, T.K., Cranfield, M.R., Fayer, R., Trout, J., and Goodale, H.J. (1997). Infectivity of *Cryptosporidium parvum* oocysts is retained upon intestinal passage through a migratory water-fowl species (Canada goose, *Branta canadensis*). Trop Med Int Health. **2**: 341-347.

Graczyk, T.K., Fayer, R., Trout, J.M., Lewis, E.J., Farley, C.A., Sulaiman, I., and Lal, A.A. (1998). *Giardia* sp. cysts and infectious *Cryptosporidium parvum* oocysts in the feces of migratory Canada geese (*Branta canadensis*). Appl Environ Microbiol. **64**: 2736-2738.

Graczyk, T.K., Cranfield, M.R., Fayer, R., and Bixler, H. (1999a). House flies (*Musca domestica*) as transport hosts of *Cryptosporidium parvum*. Am J Trop Med Hyg. **61**: 500-504.

Graczyk, T.K., Fayer, R., Cranfield, M.R., Mhangami-Ruwende, B., Knight, R., Trout, J.M., and Bixler, H. (1999b). Filth flies are transport hosts of *Cryptosporidium parvum*. Emerg Infect Dis. **5**: 726-727.

Graczyk, T.K., Fayer, R., Lewis, E.J., Trout, J.M., and Farley, C.A. (1999c). *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol Res. **85**: 518-521.

Graczyk, T.K., Fayer, R., Knight, R., Mhangami-Ruwende, B., Trout, J.M., Da Silva, A.J., and Pieniazek, N.J. (2000a). Mechanical transport and transmission of *Cryptosporidium parvum* oocysts by wild filth flies. Am J Trop Med Hyg. **63**: 178-183.

Graczyk, T.K., Fayer, R., Trout, J.M., Jenkins, M.C., Higgins, J., Lewis, E.J., and Farley, C.A. (2000b). Susceptibility of the Chesapeake Bay to environmental contamination with *Cryptosporidium parvum*. Environ Res. **82**: 106-112.

Graczyk, T.K., Marcogliese, D.J., de Lafontaine, Y., Da Silva, A.J., Mhangami-Ruwende, B., and Pieniazek, N.J. (2001). *Cryptosporidium parvum* oocysts in zebra mussels (*Dreissena polymorpha*): evidence from the St. Lawrence River. Parasitol Res. **87**: 231-234.

Graczyk, T.K., Grimes, B.H., Knight, R., Da Silva, A.J., Pieniazek, N.J., and Veal, D.A. (2003). Detection of *Cryptosporidium parvum* and *Giardia lamblia* carried by synanthropic flies by combined fluorescent in situ hybridization and a monoclonal antibody. Am J Trop Med Hyg. **68**: 228-232.

Griffiths, J.K., Moore, R., Dooley, S., Keusch, G.T., and Tzipori, S. (1994). *Cryptosporidium parvum* infection of Caco-2 cell monolayers induces an apical monolayer defect, selectively increases transmonolayer permeability, and causes epithelial cell death. Infect Immun. **62**: 4506-4514.

Guerrant, D.I., Moore, S.R., Lima, A.A., Patrick, P.D., Schorling, J.B., and Guerrant, R.L. (1999). Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg. **61**: 707-713.

Guerrant, R.L. (1997). Cryptosporidiosis: an emerging, highly infectious threat. Emerg Infect Dis. **3**: 51-57.

Gut, J., Petersen, C., Nelson, R., and Leech, J. (1991). *Cryptosporidium parvum*: *in vitro* cultivation in Madin-Darby canine kidney cells. J Protozool. **38**: 72S-73S.

Guy, R.A., Payment, P., Krull, U.J., and Horgen, P.A. (2003). Real-time PCR for quantification of giardia and cryptosporidium in environmental water samples and sewage. Appl Environ Microbiol. **69**: 5178-5185.

Hallier-Soulier, S., and Guillot, E. (2000). Detection of cryptosporidia and *Cryptosporidium parvum* oocysts in environmental water samples by immunomagnetic separation-polymerase chain reaction. Journal of Applied Microbiology. **89**: 5-10.

Hallier-Soulier, S., and Guillot, E. (2003). An immunomagnetic separation-reverse transcription polymerase chain reaction (IMS-RT-PCR) test for sensitive and rapid detection of viable waterborne *Cryptosporidium parvum*. Environ Microbiol. **5**: 592-598.

Han, X., Fink, M.P., and Delude, R.L. (2003). Proinflammatory cytokines cause NO*-dependent and -independent changes in expression and localization of tight junction proteins in intestinal epithelial cells. Shock. **19**: 229-237.

Hancock, C.M., Ward, J.V., Hancock, K.W., Klonicki, P.T., and Sturbaum, G.D. (1996). Assessing Water Treatment Plant Performance Using Microscopic Particulate Analysis (MPA). JAWWA. **88**: 24-34.

Hancock, C.M., Rose, J.B., and Callahan, M. (1998). The Prevalence of *Cryptosporidium* and *Giardia* in U.S. Groundwaters. JAWWA. **90**: 58-61.

Harp, J.A., Whitmire, W.M., and Sacco, R. (1994). In vitro proliferation and production of gamma interferon by murine CD4+ cells in response to *Cryptosporidium parvum* antigen. J Parasitol. **80**: 67-72.

Harp, J.A., and Goff, J.P. (1995). Protection of calves with a vaccine against *Cryptosporidium parvum*. J Parasitol. **81**: 54-57.

Harper, C.M., Cowell, N.A., Adams, B.C., Langley, A.J., and Wohlsen, T.D. (2002). Outbreak of *Cryptosporidium* linked to drinking unpasteurised milk. Commun Dis Intell. **26**: 449-450.

Hashimoto, A., Hirata, T., and Kunikane, S. (2001). Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in a conventional water purification plant. Water Sci Technol. **43**: 89-92.

Havelaar, A.H., De Hollander, A.E., Teunis, P.F., Evers, E.G., Van Kranen, H.J., Versteegh, J.F., Van Koten, J.E., and Slob, W. (2000). Balancing the risks and benefits of drinking water disinfection: disability adjusted life-years on the scale. Environ Health Perspect. **108**: 315-321.

Hayes, E.B., Matte, T.D., O'Brien, T.R., McKinley, T.W., Logsdon, G.S., Rose, J.B., Ungar, B.L., Word, D.M., Pinsky, P.F., Cummings, M.L., and et al. (1989). Large community outbreak of cryptosporidiosis due to contamination of a filtered public water supply. N Engl J Med. **320**: 1372-1376.

Hayward, A.R., Chmura, K., and Cosyns, M. (2000). Interferon-gamma is required for innate immunity to *Cryptosporidium parvum* in mice. J Infect Dis. **182**: 1001-1004.

Health-Canada. (1997). Federal Provincial Subcommittee on Drinking Water. Protozoa in drinking water: document for public comment. in: Canada Health, Canada.

Health-Canada. (2003). Summary of Guidelines for Canadian Drinking Water Quality. Federal-Provencial-Territorial Committee on Drinking Water. Canada.

Heine, J., Moon, H.W., and Woodmansee, D.B. (1984a). Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. Infect Immun. **43**: 856-859.

Heine, J., Pohlenz, J.F., Moon, H.W., and Woode, G.N. (1984b). Enteric lesions and diarrhea in gnotobiotic calves monoinfected with *Cryptosporidium* species. J Infect Dis. **150**: 768-775.

Heitman, T.L., Frederick, L.M., Viste, J.R., Guselle, N.J., Morgan, U.M., Thompson, R.C., and Olson, M.E. (2002). Prevalence of *Giardia* and *Cryptosporidium* and characterization of *Cryptosporidium* spp. isolated from wildlife, human, and agricultural sources in the North Saskatchewan River Basin in Alberta, Canada. Can J Microbiol. **48**: 530-541.

Henricksen, S.A., and Pohlenz, J.F. (1981). Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acat Vet Scand. **22**: 594-596.

Herwaldt, B.L., and Ackers, M.L. (1997). An outbreak in 1996 of cyclosporiasis associated with imported raspberries. The *Cyclospora* working group. N Engl J Med. **336**: 1548-1556.

Heussler, V.T., Kuenzi, P., and Rottenberg, S. (2001). Inhibition of apoptosis by intracellular protozoan parasites. Int J Parasitol. **31**: 1166-1176.

Higgins, J.A., Fayer, R., Trout, J.M., Xiao, L., Lal, A.A., Kerby, S., and Jenkins, M.C. (2001). Real-time PCR for the detection of *Cryptosporidium parvum*. J Microbiol Methods. **47**: 323-337.

Higgins, J.A., Trout, J.M., Fayer, R., Shelton, D., and Jenkins, M.C. (2003). Recovery and detection of Cryptosporidium parvum oocysts from water samples using continuous flow centrifugation. Water Res. **37**: 3551-3560.

Hill, D., and Dubey, J.P. (2002). *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin Microbiol Infect. **8**: 634-640.

Hojlyng, N., Holten-Andersen, W., and Jepsen, S. (1987). Cryptosporidiosis: a case of airborne transmission. Lancet. **2**: 271-272.

Hojlyng, N., and Jensen, B.N. (1988). Respiratory cryptosporidiosis in HIV-positive patients. Lancet. **1**: 590-591.

Hoover, D.M., Hoerr, F.J., Carlton, W.W., Hinsman, E.J., and Ferguson, H.W. (1981). Enteric cryptosporidiosis in a naso tang, *Naso lituratus*. J Fish Dis. **4**: 25.

Horen, W.P. (1983). Detection of *Cryptosporidium* in human fecal specimens. J Parasitol. **69**: 622-624.

Howe, A.D., Forster, S., Morton, S., Marshall, R., Osborn, K.S., Wright, P., and Hunter, P.R. (2002). *Cryptosporidium* oocysts in a water supply associated with a cryptosporidiosis outbreak. Emerg Infect Dis. **8**: 619-624.

Hsu, B.M., and Yeh, H.H. (2003). Removal of *Giardia* and *Cryptosporidium* in drinking water treatment: a pilot-scale study. Water Res. **37**: 1111-1117.

Isaji, C. (2003). Integrated water quality management for drinking water of good quality. Water Sci Technol. **47**: 15-23.

Iseki, M. (1979). *Cryptosporidium felis* sp. n. (protozoa: Eimeriorina) from the domestic cat. Jpn J Parasitol. **28**: 285.

Jakubowski, W., Boutros, S., Faber, W., Fayer, R., Rose, J.B., Schaub, S., Singh, A., and Stewart, M.H. (1996). Environmental Methods for *Cryptosporidium*. JAWWA.

Jenkins, M.B., Anguish, L.J., Bowman, D.D., Walker, M.J., and Ghiorse, W.C. (1997). Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. **63**: 3844-3850.

Jenkins, M.C., O'Brien, C., Trout, J., Guidry, A., and Fayer, R. (1999). Hyperimmune bovine colostrum specific for recombinant *Cryptosporidium parvum* antigen confers partial protection against cryptosporidiosis in immunosuppressed adult mice. Vaccine. **17**: 2453-2460.

Jenkins, M.C., Trout, J., Abrahamsen, M.S., Lancto, C.A., Higgins, J., and Fayer, R. (2000). Estimating viability of *Cryptosporidium parvum* oocysts using reverse transcriptase-polymerase chain reaction (RT-PCR) directed at mRNA encoding amyloglucosidase. J Microbiol Methods. **43**: 97-106.

Jenkins, M.C. (2001). Advances and prospects for subunit vaccines against protozoa of veterinary importance. Vet Parasitol. **101**: 291-310.

Johnson, D.W., Pieniazek, N.J., Griffin, D.W., Misener, L., and Rose, J.B. (1995). Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl Environ Microbiol. **61**: 3849-3855.

Jokipii, L., and Jokipii, A.M. (1986). Timing of symptoms and oocyst excretion in human cryptosporidiosis. N Engl J Med. **315**: 1643-1647.

Joseph, C., Hamilton, G., O'Connor, M., Nicholas, S., Marshall, R., Stanwell-Smith, R., Sims, R., Ndawula, E., Casemore, D., Gallagher, P., and et al. (1991). Cryptosporidiosis in the Isle of Thanet; an outbreak associated with local drinking water. Epidemiol Infect. **107**: 509-519.

Karanis, P., and Kimura, A. (2002). Evaluation of three flocculation methods for the purification of *Cryptosporidium parvum* oocysts from water samples. Lett Appl Microbiol. **34**: 444-449.

Kaucner, C., and Stinear, T. (1998). Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. Appl Environ Microbiol. **64**: 1743-1749.

Kistemann, T., Classen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V., and Exner, M. (2002). Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Appl Environ Microbiol. **68**: 2188-2197.

Knowles, D.P., Jr., and Gorham, J.R. (1993). Advances in the diagnosis of some parasitic diseases by monoclonal antibody-based enzyme-linked immunosorbent assays. Rev Sci Tech. **12**: 425-433.

Koch, K.L., Phillips, D.J., Aber, R.C., and Current, W.L. (1985). Cryptosporidiosis in hospital personnel. Evidence for person-to-person transmission. Ann Intern Med. **102**: 593-596.

Kooy, A.J., Prens, E.P., Van Heukelum, A., Vuzevski, V.D., Van Joost, T., and Tank, B. (1999). Interferon-gamma-induced ICAM-1 and CD40 expression, complete lack of HLA-DR and CD80 (B7.1), and inconsistent HLA-ABC expression in basal cell carcinoma: a possible role for interleukin-10? J Pathol. **187**: 351-357.

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., and Sterling, C.R. (1990). Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol. **56**: 1423-1428.

Kosek, M., Alcantara, C., Lima, A.A., and Guerrant, R.L. (2001). Cryptosporidiosis: an update. Lancet Infect Dis. **1**: 262-269.

Koudela, B., and Modry, D. (1998). New species of *Cryptosporidium* (Apicomplexa, Cryptosporidiidae) from lizards. Folia Parasitol. **45**: 93–100.

Kuhls, T.L., Greenfield, R.A., Mosier, D.A., Crawford, D.L., and Joyce, W.A. (1992). Cryptosporidiosis in adult and neonatal mice with severe combined immunodeficiency. J Comp Pathol. **106**: 399-410.

Kuhls, T.L., Mosier, D.A., Abrams, V.L., Crawford, D.L., and Greenfield, R.A. (1994a). Inability of interferon-gamma and aminoguanidine to alter *Cryptosporidium parvum* infection in mice with severe combined immunodeficiency. J Parasitol. **80**: 480-485.

Kuhls, T.L., Mosier, D.A., Crawford, D.L., and Griffis, J. (1994b). Seroprevalence of cryptosporidial antibodies during infancy, childhood, and adolescence. Clin Infect Dis. **18**: 731-735.

Kuhn, R.C., and Oshima, K.H. (2001). Evaluation and optimization of a reusable hollow fiber ultrafilter as a first step in concentrating *Cryptosporidium parvum* oocysts from water. Water Res. **35**: 2779-2783.

Kuroki, T., Watanabe, Y., Asai, Y., Yamai, S., Endo, T., Uni, S., Kimata, I., and Iseki, M. (1996). An outbreak of waterborne Cryptosporidiosis in Kanagawa, Japan. Kansenshogaku Zasshi. **70**: 132-140.

Laberge, I., and Griffiths, M.W. (1996). Prevalence, detection and control of *Cryptosporidium parvum* in food. Int J Food Microbiol. **32**: 1-26.

Landsberg, J.H., and Paperna, I. (1986). Ultrastructural study of the coccidian *Cryptosporidium* sp. from stomachs of juvenile cichlid fish. Dis Aquat Org. **71**: 625.

Langer, R.C., and Riggs, M.W. (1999). *Cryptosporidium parvum* apical complex glycoprotein CSL contains a sporozoite ligand for intestinal epithelial cells. Infect Immun. **67**: 5282-5291.

Laurent, F., Kagnoff, M.F., Savidge, T.C., Naciri, M., and Eckmann, L. (1998). Human intestinal epithelial cells respond to *Cryptosporidium parvum* infection with increased prostaglandin H synthase 2 expression and prostaglandin E2 and F2alpha production. Infect Immun. **66**: 1787-1790.

Laurent, F., McCole, D., Eckmann, L., and Kagnoff, M.F. (1999). Pathogenesis of *Cryptosporidium parvum* infection. Microbes Infect. **1**: 141-148.

Laxer, M.A., Timblin, B.K., and Patel, R.J. (1991). DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. American Journal of Tropical Medicine and Hygiene. **45**: 688-694.

Leav, B.A., Mackay, M.R., Anyanwu, A., RM, O.C., Cevallos, A.M., Kindra, G., Rollins, N.C., Bennish, M.L., Nelson, R.G., and Ward, H.D. (2002). Analysis of sequence diversity at the highly polymorphic Cpgp40/15 locus among *Cryptosporidium* isolates from human immunodeficiency virus-infected children in South Africa. Infect Immun. **70**: 3881-3890.

Leav, B.A., Mackay, M., and Ward, H.D. (2003). *Cryptosporidium* species: new insights and old challenges. Clin Infect Dis. **36**: 903-908.

LeChevallier, M., Norton, W., and Lee, R. (1991). Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl Environ Microbiol. **57**: 2610-2616.

LeChevallier, M.W., Norton, W.D., Siegel, J.E., and Abbaszadegan, M. (1995). Evaluation of the immunofluorescence procedure for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl Environ Microbiol. **61**: 690-697.

LeChevallier, M.W., Di Giovanni, G.D., Clancy, J.L., Bukhari, Z., Bukhari, S., Rosen, J.S., Sobrinho, J., and Frey, M.M. (2003). Comparison of method 1623 and cell culture-PCR for detection of *Cryptosporidium* spp. in source waters. Appl Environ Microbiol. **69**: 971-979.

Lee, M.B., and Lee, E.H. (2003). Passage of a coccidial parasite (*Eimeria acervulina*) through the Eastern oyster (*Crassostrea virginica*). J Food Prot. **66**: 679-681.

Lee, S.H., Levy, D.A., Craun, G.F., Beach, M.J., and Calderon, R.L. (2002). Surveillance for waterborne-disease outbreaks--United States, 1999-2000. MMWR Surveill Summ. **51**: 1-47.

Lees, D. (2000). Viruses and bivalve shellfish. Int J Food Microbiol. **59**: 81-116.

Leitch, G.J., and He, Q. (1994). Arginine-derived nitric oxide reduces fecal oocyst shedding in nude mice infected with *Cryptosporidium parvum*. Infect Immun. **62**: 5173-5176.

Leitch, G.J., and He, Q. (1999). Reactive nitrogen and oxygen species ameliorate experimental cryptosporidiosis in the neonatal BALB/c mouse model. Infect Immun. **67**: 5885-5891.

Leng, X., Mosier, D.A., and Oberst, R.D. (1996). Differentiation of *Cryptosporidium parvum*, *C. muris*, and *C. baileyi* by PCR-RFLP analysis of the 18S rRNA gene. Vet Parasitol. **62**: 1-7.

Levine, N.D. (1980). Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. J Parasitol. **66**: 830-834.

Levine, N.D. (1984). Taxonomy and review of the coccidian genus *Cryptosporidium* (protozoa, apicomplexa). J Protozool. **31**: 94-98.

Limor, J.R., Lal, A.A., and Xiao, L. (2002). Detection and differentiation of *Cryptosporidium* parasites that are pathogenic for humans by real-time PCR. J Clin Microbiol. **40**: 2335-2338.

Linden, K.G., Shin, G., and Sobsey, M.D. (2001). Comparative effectiveness of UV wavelengths for the inactivation of *Cryptosporidium parvum* oocysts in water. Water Sci Technol. **43**: 171-174.

Lindsay, D.S., Blagburn, B.L., and Sundermann, C.A. (1989). Morphometric comparison of the oocysts of *Cryptosporidium meleagridis* and *Cryptosporidium baileyi* from birds. Proc Helminthol Soc Wash. **56**: 91.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., and Blagburn, B.L. (2000). *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos taurus*. J Eukaryot Microbiol. **47**: 91-95.

Linn, S.C., Morelli, P.J., Edry, I., Cottongim, S.E., Szabo, C., and Salzman, A.L. (1997). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. Am J Physiol. **272**: G1499-1508.

Lipp, E.K., and Rose, J.B. (1997). The role of seafood in foodborne diseases in the United States of America. Rev Sci Tech. **16**: 620-640.

Lowery, C.J., Moore, J.E., Millar, B.C., Burke, D.P., McCorry, K.A., Crothers, E., and Dooley, J.S. (2000). Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. J Med Microbiol. **49**: 779-785.

Lowery, C.J., Moore, J.E., Millar, B.C., McCorry, K.A., Xu, J., Rooney, P.J., and Dooley, J.S. (2001). Occurrence and molecular genotyping of *Cryptosporidium* spp. in surface waters in Northern Ireland. J Appl Microbiol. **91**: 774-779.

MacDonald, L.M., Sargent, K., Armson, A., Thompson, R.C., and Reynoldson, J.A. (2002). The development of a real-time quantitative-PCR method for characterisation of a *Cryptosporidium parvum* in vitro culturing system and assessment of drug efficacy. Mol Biochem Parasitol. **121**: 279-282.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addis, D.G., Fox, K.R., Rose, J.B., and Davis, J.P. (1994). A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. New England Journal of Medicine. **331**: 161-167.

MacKenzie, W.R., Kazmierczak, J.J., and Davis, J.P. (1995a). An outbreak of cryptosporidiosis associated with a resort swimming pool. Epidemiol Infect. **115**: 545-553.

MacKenzie, W.R., Schell, W.L., Blair, K.A., Addiss, D.G., Peterson, D.E., Hoxie, N.J., Kazmierczak, J.J., and Davis, J.P. (1995b). Massive outbreak of waterborne *Cryptosporidium* infection in Milwaukee, Wisconsin: recurrence of illness and risk of secondary transmission. Clin Infect Dis. **21**: 57-62.

Mackowiak, P.A., Caraway, C.T., and Portnoy, B.L. (1976). Oyster-associated hepatitis: lessons from the Louisiana experience. Am J Epidemiol. **103**: 181-191.

Madico, G., McDonald, J., Gilman, R.H., Cabrera, L., and Sterling, C.R. (1997). Epidemiology and treatment of *Cyclospora cayetanensis* infection in Peruvian children. Clinical Infectious Disease. **24**: 977-981.

Madore, M.S., Rose, J.B., Gerba, C.P., Arrowood, M.J., and Sterling, C.R. (1987). Occurrence of *Cryptosporidium* oocysts in sewage effluents and selected surface waters. J Parasitol. **73**: 702-705.

Maggi, P., Larocca, A.M., Quarto, M., Serio, G., Brandonisio, O., Angarano, G., and Pastore, G. (2000). Effect of antiretroviral therapy on cryptosporidiosis and microsporidiosis in patients infected with human immunodeficiency virus type 1. Eur J Clin Microbiol Infect Dis. **19**: 213-217.

Maggi, P., Larocca, A.M., Ladisa, N., Carbonara, S., Brandonisio, O., Angarano, G., and Pastore, G. (2001). Opportunistic parasitic infections of the intestinal tract in the era of highly active antiretroviral therapy: is the CD4(+) count so important? Clin Infect Dis. **33**: 1609-1611.

Matheson, Z., Hargy, T.M., McCuin, R.M., Clancy, J.L., and Fricker, C.R. (1998). An evaluation of the Gelman Envirochek capsule for the simultaneous concentration of *Cryptosporidium* and *Giardia* from water. J Appl Microbiol. **85**: 755-761.

Mathison, B.A., and Ditrich, O. (1999). The fate of *Cryptosporidium parvum* oocysts ingested by dung beetles and their possible role in the dissemination of cryptosporidiosis. J Parasitol. **85**: 678-681.

Mawdsley, J.L., Brooks, A.E., and Merry, R.J. (1996). Movement of the protozoan pathogen *Cryptosporidium parvum* through three contrasting soil types. Biol Fertil Soils. **21**: 30-36.

McAnulty, J.M., Fleming, D.W., and Gonzalez, A.H. (1994). A community-wide outbreak of cryptosporidiosis associated with swimming at a wave pool. Jama. **272**: 1597-1600.

McCole, D.F., Eckmann, L., Laurent, F., and Kagnoff, M.F. (2000). Intestinal epithelial cell apoptosis following *Cryptosporidium parvum* infection. Infect Immun. **68**: 1710-1713.

McCuin, R.M., Bukhari, Z., and Clancy, J.L. (2000). Recovery and viability of *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts using the membrane dissolution procedure. Can J Microbiol. **46**: 700-707.

McCuin, R.M., Bukhari, Z., Sobrinho, J., and Clancy, J.L. (2001). Recovery of *Cryptosporidium* oocysts and *Giardia* cysts from source water concentrates using immunomagnetic separation. J Microbiol Methods. **45**: 69-76.

McCuin, R.M., and Clancy, J.L. (2003). Modifications to United States Environmental Protection Agency methods 1622 and 1623 for detection of *Cryptosporidium* oocysts and *Giardia* cysts in water. Appl Environ Microbiol. **69**: 267-274.

McDonald, A.C., Pollok, R.C., and Farthing, M.J. (1999). Interferon-gamma inhibits development of *Cryptosporidium parvum* in human enterocytes. in: *: Society of*

Protozoologist 52nd Annual Meeting and 6th International Workshop of Opportunistic Pathogens. North Carolina State University. pg 34, W37.

McDonald, V., Deer, R., Uni, S., Iseki, M., and Bancroft, G.J. (1992). Immune responses to *Cryptosporidium muris* and *Cryptosporidium parvum* in adult immunocompetent or immunocompromised (nude and SCID) mice. Infect Immun. **60**: 3325-3331.

McDonald, V., and Bancroft, G.J. (1994). Mechanisms of innate and acquired resistance to *Cryptosporidium parvum* infection in SCID mice. Parasite Immunol. **16**: 315-320.

Mead, J.R., Arrowood, M.J., Healey, M.C., and Sidwell, R.W. (1991a). Cryptosporidial infections in SCID mice reconstituted with human or murine lymphocytes. J Protozool. **38**: 59S-61S.

Mead, J.R., Arrowood, M.J., Sidwell, R.W., and Healey, M.C. (1991b). Chronic *Cryptosporidium parvum* infections in congenitally immunodeficient SCID and nude mice. J Infect Dis. **163**: 1297-1304.

Mead, J.R., and You, X. (1998). Susceptibility differences to *Cryptosporidium parvum* infection in two strains of gamma interferon knockout mice. J Parasitol. **84**: 1045-1048.

Meinhardt, P.L., Casemore, D.P., and Miller, K.B. (1996). Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. Epidemiol Rev. **18**: 118-136.

Meisel, J.L., Perera, D.R., Meligro, C., and Rubin, C.E. (1976). Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology. **70**: 1156-1160.

Millard, P.S., Gensheimer, K.F., Addiss, D.G., Sosin, D.M., Beckett, G.A., Houck-Jankoski, A., and Hudson, A. (1994). An outbreak of cryptosporidiosis from fresh-pressed apple cider. Jama. **272**: 1592-1596.

Monge, R., and Arias, M.L. (1996). Presence of various pathogenic microorganisms in fresh vegetables in Costa Rica. Arch Latinoam Nutr. **46**: 292-294.

Monge, R., Chinchilla, M., and Reyes, L. (1996). Seasonality of parasites and intestinal bacteria in vegetables that are consumed raw in Costa Rica. Rev Biol Trop. **44**: 369-375.

Monis, P.T., and Saint, C.P. (2001). Development of a nested-PCR assay for the detection of *Cryptosporidium parvum* in finished water. Water Res. **35**: 1641-1648.

Moore, A.C., Herwaldt, B.L., Craun, G.F., Calderon, R.L., Highsmith, A.K., and Juranek, D.D. (1993). Surveillance for waterborne disease outbreaks--United States, 1991-1992. MMWR CDC Surveill Summ. **42**: 1-22.

Moore, J.E., Gilpin, D., Crothers, E., Canney, A., Kaneko, A., and Matsuda, M. (2002). Occurrence of *Campylobacter* spp. and *Cryptosporidium* spp. in seagulls (*Larus* spp.). Vector Borne Zoonotic Dis. **2**: 111-114.

Morgan, U.M., Constantine, C.C., O'Donoghue, P., Meloni, B.P., O'Brien, P.A., and Thompson, R.C. (1995). Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified polymorphic DNA analysis. Am J Trop Med Hyg. **52**: 559-564.

Morgan, U.M., Pallant, L., Dwyer, B.W., Forbes, D.A., Rich, G., and Thompson, R.C. (1998a). Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. J Clin Microbiol. **36**: 995-998.

Morgan, U.M., Xiao, L., Hill, B.D., O'Donoghue, P., Limor, J., Lal, A.A., and Thompson, R.C. (1998b). Detection of the *Cryptosporidium parvum* "human" genotype in a dugong (*Dugong dugon*). J Parasitol. **86**: 1352-1354.

Morgan, U.M., Xiao, L., Fayer, R., Lal, A.A., and Thompson, R.C. (1999). Variation in *Cryptosporidium*: towards a taxonomic revision of the genus. Int J Parasitol. **29**: 1733-1751.

Morgan, U.M., Xiao, L., Monis, P., Fall, A., Irwin, P.J., Fayer, R., Denholm, K.M., Limor, J., Lal, A., and Thompson, R.C. (2000). *Cryptosporidium* spp. in domestic dogs: the "dog" genotype. Appl Environ Microbiol. **66**: 2220-2223.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C., Olson, M., Lal, A., and Xiao, L. (2002). *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. J Eukaryot Microbiol. **49**: 433-440.

Moulton-Hancock, C., Rose, J.B., Vasconcelos, G.J., Harris, S.I., Klonicki, P.T., and Sturbaum, G.D. (2000). *Giardia* and *Cryptosporidium* Occurrence in Groundwater. JAWWA. **92**: 117-123.

Musial, C.E., Arrowood, M.J., Sterling, C.R., and Gerba, C.P. (1987). Detection of *Cryptosporidium* in water by using polypropylene cartridge filters. Appl Environ Microbiol. **53**: 687-692.

Neill, M.A., Rice, S.K., Ahmad, N.V., and Flanigan, T.P. (1996). Cryptosporidiosis: an unrecognized cause of diarrhea in elderly hospitalized patients. Clin Infect Dis. **22**: 168-170.

Neumann, N.F., Gyurek, L.L., Finch, G.R., and Belosevic, M. (2000a). Intact *Cryptosporidium parvum* oocysts isolated after in vitro excystation are infectious to neonatal mice. FEMS Microbiol Lett. **183**: 331-336.

Neumann, N.F., Gyurek, L.L., Gammie, L., Finch, G.R., and Belosevic, M. (2000b). Comparison of animal infectivity and nucleic acid staining for assessment of *Cryptosporidium parvum* viability in water. Appl Environ Microbiol. **66**: 406-412.

Newman, R.D., Zu, S.X., Wuhib, T., Lima, A.A., Guerrant, R.L., and Sears, C.L. (1994). Household epidemiology of *Cryptosporidium parvum* infection in an urban community in northeast Brazil. Ann Intern Med. **120**: 500-505.

Niehaus, M.D., Moore, S.R., Patrick, P.D., Derr, L.L., Lorntz, B., Lima, A.A., and Guerrant, R.L. (2002). Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian shantytown. Am J Trop Med Hyg. **66**: 590-593.

Nieminski, E.C. (1994). *Giardia* and *Cryptosporidium* removal through direct filtration and conventional treatment performance. in: *Proceedings of the American Water Works Association Annual Conference*. American Water Works Association. New York. pg 462.

Nieminski, E.C., Schaefer, F.W., 3rd, and Ongerth, J.E. (1995). Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl Environ Microbiol. **61**: 1714-1719.

Nime, F., Burek, J., Page, D., Holscher, M., and Yardley, J. (1976a). Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology. **70**: 592.

Nime, F.A., Burek, J.D., Page, D.L., Holscher, M.A., and Yardley, J.H. (1976b). Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology. **70**: 592-598.

O'Donoghue, P.J. (1995). *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parsitol. **25**: 139-195.

Ojcius, D.M., Perfettini, J.L., Bonnin, A., and Laurent, F. (1999). Caspase-dependent apoptosis during infection with *Cryptosporidium parvum*. Microbes Infect. **1**: 1163-1168.

Okhuysen, P.C., Chappell, C.L., Sterling, C.R., Jakubowski, W., and DuPont, H.L. (1998). Susceptibility and serologic response of healthy adults to reinfection with *Cryptosporidium parvum*. Infect Immun. **66**: 441-443.

Okhuysen, P.C., and Chappell, C.L. (2002). *Cryptosporidium* virulence determinants - are we there yet? Int J Parasitol. **32**: 517-525.

Okhuysen, P.C., Rich, S.M., Chappell, C.L., Grimes, K.A., Widmer, G., Feng, X., and Tzipori, S. (2002). Infectivity of a *Cryptosporidium parvum* isolate of cervine origin for healthy adults and interferon-gamma knockout mice. J Infect Dis. **185**: 1320-1325.

Olson, M.E., Guselle, N.J., O'Handley, R.M., Swift, M.L., McAllister, T.A., Jelinski, M.D., and Morck, D.W. (1997a). *Giardia* and *Cryptosporidium* in dairy calves in British Columbia. Can Vet J. **38**: 703-706.

Olson, M.E., Thorlakson, C.L., Deselliers, L., Morck, D.W., and McAllister, T.A. (1997b). *Giardia* and *Cryptosporidium* in Canadian farm animals. Vet Parasitol. **68**: 375-381.

Ongerth, J.E., and Stibbs, H.H. (1987). Identification of *Cryptosporidium* oocysts in river water. Appl Environ Microbiol. **53**: 672-676.

Ortega, Y.R., Sheehy, R.R., Cama, V.A., Oishi, K.K., and Sterling, C.R. (1991). Restriction fragment length polymorphism analysis of *Cryptosporidium parvum* isolates of bovine and human origin. J Protozool. **38**: 40S-41S.

Ortega, Y.R., Sterling, C., Gilman, R.H., Cama, V.A., and Diaz, F. (1993). *Cyclospora* species-a new protozoan pathogen of humans. N Engl J Med. **328**: 1308-1312.

Ortega, Y.R., Sterling, C.R., and Gilman, R.H. (1994). A new coccidian parasite (Apicomplexa: Eimeriidae) from humans. J Parasitol. **80**: 625-629.

Ortega, Y.R., Roxas, C.R., Gilman, R.H., Miller, N.J., Cabrera, L., Taquiri, C., and Sterling, C.R. (1997). Isolation of *Cryptosporidium parvum* and *Cyclospora cayetanensis* from vegetables collected in markets of an endemic region in Peru. Am J Trop Med Hyg. **57**: 683-686.

Ortega-Mora, L.M., and Wright, S.E. (1994). Age-related resistance in ovine cryptosporidiosis: patterns of infection and humoral immune response. Infect Immun. **62**: 5003-5009.

Payment, P., and Franco, E. (1993). *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl Environ Microbiol. **59**: 2418-2424.

Perryman, L.E., Mason, P.H., and Chrisp, C.E. (1994). Effect of spleen cell populations on resolution of *Cryptosporidium parvum* infection in SCID mice. Infect Immun. **62**: 1474-1477.

Perryman, L.E., Jasmer, D.P., Riggs, M.W., Bohnet, S.G., McGuire, T.C., and Arrowood, M.J. (1996). A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Mol Biochem Parasitol. **80**: 137-147.

Perryman, L.E., Kapil, S.J., Jones, M.L., and Hunt, E.L. (1999). Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. Vaccine. **17**: 2142-2149.

Perz, J.F., and Le Blancq, S.M. (2001). *Cryptosporidium parvum* infection involving novel genotypes in wildlife from lower New York State. Appl Environ Microbiol. **67**: 1154-1162.

Petersen, C., Gut, J., Doyle, P.S., Crabb, J.H., Nelson, R.G., and Leech, J.H. (1992). Characterization of a > 900,000-M(r) *Cryptosporidium parvum* sporozoite glycoprotein recognized by protective hyperimmune bovine colostral immunoglobulin. Infect Immun. **60**: 5132-5138.

Petersen, C., Barnes, D.A., and Gousset, L. (1997). *Cryptosporidium parvum* GP900, a unique invasion protein. J Eukaryot Microbiol. **44**: 89S-90S.

Pohjola, S. (1984). Negative staining method with nigrosin for the detection of cryptosporidial oocysts: a comparative study. Res Vet Sci. **36**: 217-219.

Pohjola, S., Jokipii, L., and Jokipii, A.M. (1985). Dimethylsulphoxide-Ziehl-Neelsen staining technique for detection of cryptosporidial oocysts. Vet Rec. **116**: 442-443.

Pohjola, S., Oksanen, H., Jokipii, L., and Jokipii, A.M. (1986). Outbreak of cryptosporidiosis among veterinary students. Scand J Infect Dis. **18**: 173-178.

Pollok, R.C., Farthing, M.J., Bajaj-Elliott, M., Sanderson, I.R., and McDonald, V. (2001). Interferon gamma induces enterocyte resistance against infection by the intracellular pathogen *Cryptosporidium parvum*. Gastroenterology. **120**: 99-107.

Pozio, E., Rezza, G., Boschini, A., Pezzotti, P., Tamburrini, A., Rossi, P., Di Fine, M., Smacchia, C., Schiesari, A., Gattei, E., Zucconi, R., and Ballarini, P. (1997). Clinical cryptosporidiosis and human immunodeficiency virus (HIV)-induced immunosuppression: findings from a longitudinal study of HIV-positive and HIV-negative former injection drug users. J Infect Dis. **176**: 969-975.

Preiser, G., Preiser, L., and Madeo, L. (2003). An outbreak of cryptosporidiosis among veterinary science students who work with calves. J Am Coll Health. **51**: 213-215.

Puech, M.C., McAnulty, J.M., Lesjak, M., Shaw, N., Heron, L., and Watson, J.M. (2001). A statewide outbreak of cryptosporidiosis in New South Wales associated with swimming at public pools. Epidemiol Infect. **126**: 389-396.

Quinones, B.E., Hibler, C.P., and Hancock, C.M. (1988) Comparison of the Modified Reference Method and Indirect Fluorescent Antibody Technique for Detection of *Giardia* Cysts in Water. In: Advances in Giardia Research. Wallis, P.M. and Hammond, B.R. (ed). University of Calgary Press. Calgary, Canada. pp. 215 - 217.

Quiroz, E.S., Bern, C., MacArthur, J.R., Xiao, L., Fletcher, M., Arrowood, M.J., Shay, D.K., Levy, M.E., Glass, R.I., and Lal, A. (2000). An outbreak of cryptosporidiosis linked to a foodhandler. J Infect Dis. **181**: 695-700.

Reference-Method (1992) Pathogenic Protozoa: *Giardia lamblia*. In: Standard Methods for the Examination of Water and Wastewater 18th Edition. Greenberg, A., Clesceri, L.S., and Eaton A.D. (ed). American Public Health Association and American Water Works Association. Hanover, MD. pp. 9.125.

Renoth, S., Karanis, P., Schoenen, D., and Seitz, H.M. (1996). Recovery efficiency of *Cryptosporidium* from water with a crossflow system and continuous flow centrifugation: a comparison study. Zentralbl Bakteriol. **283**: 522-528.

Reynolds, D.T., Slade, R.B., Sykes, N.J., Jonas, A., and Fricker, C.R. (1999). Detection of *Cryptosporidium* oocysts in water: techniques for generating precise recovery data. J Appl Microbiol. **87**: 804-813.

Riggs, M.W., McGuire, T.C., Mason, P.H., and Perryman, L.E. (1989). Neutralization-sensitive epitopes are exposed on the surface of infectious *Cryptosporidium parvum* sporozoites. J Immunol. **143**: 1340-1345.

Riggs, M.W., Cama, V.A., Leary, H.L., Jr., and Sterling, C.R. (1994). Bovine antibody against *Cryptosporidium parvum* elicits a circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistent cryptosporidiosis in SCID mice. Infect Immun. **62**: 1927-1939.

Riggs, M.W., Stone, A.L., Yount, P.A., Langer, R.C., Arrowood, M.J., and Bentley, D.L. (1997). Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J Immunol. **158**: 1787-1795.

Riggs, M.W., McNeil, M.R., Perryman, L.E., Stone, A.L., Scherman, M.S., and O'Connor, R.M. (1999). *Cryptosporidium parvum* sporozoite pellicle antigen recognized by a neutralizing monoclonal antibody is a beta-mannosylated glycolipid. Infect Immun. **67**: 1317-1322.

Riggs, M.W. (2002). Recent advances in cryptosporidiosis: the immune response. Microbes Infect. **4**: 1067-1080.

Riggs, M.W., Schaefer, D.A., Kapil, S.J., Barley-Maloney, L., and Perryman, L.E. (2002). Efficacy of monoclonal antibodies against defined antigens for passive immunotherapy of chronic gastrointestinal cryptosporidiosis. Antimicrob Agents Chemother. **46**: 275-282.

Roberts, L., and Janovy, J. (2000) Foundations of Parasitology. Boston McGraw-Hill, Inc.

Robertson, I.D., Irwin, P.J., Lymbery, A.J., and Thompson, R.C. (2000). The role of companion animals in the emergence of parasitic zoonoses. Int J Parasitol. **30**: 1369-1377.

Robertson, L.J., and Gjerde, B. (2000). Isolation and enumeration of *Giardia* cysts, *Cryptosporidium* oocysts, and *Ascaris* eggs from fruits and vegetables. J Food Prot. **63**: 775-778.

Robertson, L.J., and Gjerde, B. (2001a). Occurrence of parasites on fruits and vegetables in Norway. J Food Prot. **64**: 1793-1798.

Robertson, L.J., and Gjerde, B. (2001b). Factors affecting recovery efficiency in isolation of *Cryptosporidium* oocysts and *Giardia* cysts from vegetables for standard method development. J Food Prot. **64**: 1799-1805.

Robson, K.J., Hall, J.R., Jennings, M.W., Harris, T.J., Marsh, K., Newbold, C.I., Tate, V.E., and Weatherall, D.J. (1988). A highly conserved amino-acid sequence in

thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature. **335**: 79-82.

Rochelle, P.A., De Leon, R., Stewart, M.H., and Wolfe, R.L. (1997). Comparison of primers and optimization of PCR conditions for detection of Cryptosporidium parvum and Giardia lamblia in water. Appl Environ Microbiol. **63**: 106-114.

Rochelle, P.A., De Leon, R., Johnson, A., Stewart, M.H., and Wolfe, R.L. (1999a). Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. Appl Environ Microbiol. **65**.

Rochelle, P.A., Jutras, E.M., Atwill, E.R., De Leon, R., and Stewart, M.H. (1999b). Polymorphisms in the beta-tubulin gene of *Cryptosporidium parvum* differentiate between isolates based on animal host but not geographic origin. J Parasitol. **85**: 986-989.

Rochelle, P.A., Marshall, M.M., Mead, J.R., Johnson, A.M., Korich, D.G., Rosen, J.S., and De Leon, R. (2002). Comparison of in vitro cell culture and a mouse assay for measuring infectivity of *Cryptosporidium parvum*. Appl Environ Microbiol. **68**: 3809-3817.

Rodgers, M.R., Flanigan, D.J., and Jakubowski, W. (1995). Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Appl Environ Microbiol. **61**: 3759-3763.

Rodriguez-Salinas Perez, E., Aragon Pena, A.J., Allue Tango, M., Lopaz Perez, M.A., Jimenez Maldonado, M., and Dominguez Rodriguez, M.J. (2000). Outbreak of cryptosporidiosis in Guadarrama (Autonomous Community of Madrid). Rev Esp Salud Publica. **74**: 527-536.

Rohlman, V.C., Kuhls, T.L., Mosier, D.A., Crawford, D.L., and Greenfield, R.A. (1993). *Cryptosporidium parvum* infection after abrogation of natural killer cell activity in normal and severe combined immunodeficiency mice. J Parasitol. **79**: 295-297.

Romanova, T.V., Shkarin, V.V., and Khazenson, L.B. (1992). Group cryptosporidiosis morbidity in children. Med Parazitol (Mosk). 50-52.

Rose, J., Lisle, J., and LeChevallier, M. (1997) Ch. 4: Waterborne Cryptosporidiosis: Incidence, Outbreaks, and Treatment Strategies. In: *Cryptosporidium* and Cryptosporidiosis. Fayer, R. (ed). CRC Press, Inc. New York. pp. 93 - 109.

Rossignol, J.F., Hidalgo, H., Feregrino, M., Higuera, F., Gomez, W.H., Romero, J.L., Padierna, J., Geyne, A., and Ayers, M.S. (1998). A double-'blind' placebo-

controlled study of nitazoxanide in the treatment of cryptosporidial diarrhoea in AIDS patients in Mexico. Trans R Soc Trop Med Hyg. **92**: 663-666.

Rossignol, J.F., Ayoub, A., and Ayers, M.S. (2001). Treatment of diarrhea caused by *Cryptosporidium parvum*: a prospective randomized, double-blind, placebo-controlled study of Nitazoxanide. J Infect Dis. **184**: 103-106.

Ruemmele, F.M., Gurbindo, C., Mansour, A.M., Marchand, R., Levy, E., and Seidman, E.G. (1998). Effects of interferon gamma on growth, apoptosis, and MHC class II expression of immature rat intestinal crypt (IEC-6) cells. J Cell Physiol. **176**: 120-126.

Schaefer, D.A., Auerbach-Dixon, B.A., and Riggs, M.W. (2000). Characterization and formulation of multiple epitope-specific neutralizing monoclonal antibodies for passive immunization against cryptosporidiosis. Infect Immun. **68**: 2608-2616.

Scorza, A.V., Brewer, M.M., and Lappin, M.R. (2003). Polymerase chain reaction for the detection of *Cryptosporidium* spp. in cat feces. J Parasitol. **89**: 423-426.

Sheather, A.L. (1923). The detection of intestinal protozoa and mange parasites by floatation technique. J Comp Pathol Ther. **36**: 260-275.

Shepherd, K.M., and Wyn-Jones, A.P. (1996). An evaluation of methods for the simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts from water. Appl Environ Microbiol. **62**: 1317-1322.

Slavin, D. (1955). *Cryptosporidium meleagridis* (sp. nov.). J Comp Pathol. **65**: 262.

Smith, H.V., Patterson, W.J., Hardie, R., Greene, L.A., Benton, C., Tulloch, W., Gilmour, R.A., Girdwood, R.W., Sharp, J.C., and Forbes, G.I. (1989). An outbreak of waterborne cryptosporidiosis caused by post-treatment contamination. Epidemiol Infect. **103**: 703-715.

Smith, H.V., Brown, J., Coulson, J.C., Morris, G.P., and Girdwood, R.W. (1993). Occurrence of oocysts of *Cryptosporidium* sp. in *Larus* spp. gulls. Epidemiol Infect. **110**: 135-143.

Smith, H.V., and Rose, J.B. (1998). Waterborne cryptosporidiosis: current status. Parasitol Today. **14**: 14-22.

Snowden, T.S., and Anslyn, E.V. (1999). Anion recognition: synthetic receptors for anions and their application in sensors. Curr Opin Chem Biol. **3**: 740-746.

Spano, F., Putignani, L., McLauchlin, J., Casemore, D.P., and Crisanti, A. (1997). PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol Lett. **150**: 209-217.

Spano, F., Putignani, L., Naitza, S., Puri, C., Wright, S., and Crisanti, A. (1998). Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Mol Biochem Parasitol. **92**: 147-162.

Stafford, R., Neville, G., Towner, C., and McCall, B. (2000). A community outbreak of *Cryptosporidium* infection associated with a swimming pool complex. Commun Dis Intell. **24**: 236-239.

Stefanogiannis, N., McLean, M., and Van Mil, H. (2001). Outbreak of cryptosporidiosis linked with a farm event. N Z Med J. **114**: 519-521.

Stinear, T., Matusan, A., Hines, K., and Sandery, M. (1996). Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by reverse transcription-PCR. Appl Environ Microbiol. **62**: 3385-3390.

Stott, R., May, E., Matsushita, E., and Warren, A. (2001). Protozoan predation as a mechanism for the removal of *Cryptosporidium* oocysts from wastewaters in constructed wetlands. Water Sci Technol. **44**: 191-198.

Stott, R., May, E., Ramirez, E., and Warren, A. (2003). Predation of *Cryptosporidium* oocysts by protozoa and rotifers: implications for water quality and public health. Water Sci Technol. **47**: 77-83.

Straub, T.M., Daly, D.S., Wunshel, S., Rochelle, P.A., DeLeon, R., and Chandler, D.P. (2002). Genotyping *Cryptosporidium parvum* with an hsp70 single-nucleotide polymorphism microarray. Appl Environ Microbiol. **68**: 1817-1826.

Strong, W.B., Gut, J., and Nelson, R.G. (2000). Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect Immun. **68**: 4117-4134.

Sturbaum, G.D., Marshall, M.M., Klonicki, P., Jost, B.H., Clay, B.L., and Sterling, C.R. (2001a). Evaluation of nested-PCR detection rates with known low numbers of *Cryptosporidium parvum* oocysts. in: *Water Quality Technology Conference*. A. W. W. A. Nashville, TN. pg

Sturbaum, G.D., Reed, C., Hoover, P.J., Jost, B.H., Marshall, M.M., and Sterling, C.R. (2001b). Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. **67**: 2665-2668.

Sturbaum, G.D., Klonicki, P.T., Marshall, M.M., Jost, B.H., Clay, B.L., and Sterling, C.R. (2002). Immunomagnetic separation (IMS)-fluorescent antibody detection and IMS-PCR detection of seeded *Cryptosporidium parvum* oocysts in natural waters and their limitations. Appl Environ Microbiol. **68**: 2991-2996.

Sulaiman, I.M., Morgan, U.M., Thompson, R.C., Lal, A.A., and Xiao, L. (2000). Phylogenetic relationships of Cryptosporidium parasites based on the 70-kilodalton heat shock protein (HSP70) gene. Appl Environ Microbiol. **66**: 2385-2391.

Suwa, M., and Suzuki, Y. (2001). Occurrence of *Cryptosporidium* in Japan and counter-measures in wastewater treatment plants. Water Sci Technol. **43**: 183-186.

Suwa, M., and Suzuki, Y. (2003). Control of *Cryptosporidium* with wastewater treatment to prevent its proliferation in the water cycle. Water Sci Technol. **47**: 45-49.

Taghi-Kilani, R., Sekla, L., and Hayglass, K.T. (1990). The role of humoral immunity in *Cryptosporidium* spp. infection. Studies with B cell-depleted mice. J Immunol. **145**: 1571-1576.

Takashima, Y., Xuan, X., Kimata, I., Iseki, M., Kodama, Y., Nagane, N., Nagasawa, H., Matsumoto, Y., Mikami, T., and Otsuka, H. (2003). Recombinant bovine herpesvirus-1 expressing p23 protein of *Cryptosporidium parvum* induces neutralizing antibodies in rabbits. J Parasitol. **89**: 276-282.

Tanriverdi, S., Tanyeli, A., Baslamisli, F., Koksal, F., Kilinc, Y., Feng, X., Batzer, G., Tzipori, S., and Widmer, G. (2002). Detection and genotyping of oocysts of *Cryptosporidium parvum* by real-time PCR and melting curve analysis. J Clin Microbiol. **40**: 3237-3244.

Tarver, A.P., Clark, D.P., Diamond, G., Russell, J.P., Erdjument-Bromage, H., Tempst, P., Cohen, K.S., Jones, D.E., Sweeney, R.W., Wines, M., Hwang, S., and Bevins, C.L. (1998). Enteric beta-defensin: molecular cloning and characterization of a gene with inducible intestinal epithelial cell expression associated with *Cryptosporidium parvum* infection. Infect Immun. **66**: 1045-1056.

Tilley, M., Upton, S.J., and Freed, P.S. (1990). A comparative study on the biology of *Cryptosporidium serpentis* and *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae). J Zoo Wildlife Med. **21**: 463.

Tilley, M., McDonald, V., and Bancroft, G.J. (1995). Resolution of cryptosporidial infection in mice correlates with parasite-specific lymphocyte proliferation associated with both Th1 and Th2 cytokine secretion. Parasite Immunol. **17**: 459-464.

Tomley, F.M., Clarke, L.E., Kawazoe, U., Dijkema, R., and Kok, J.J. (1991). Sequence of the gene encoding an immunodominant microneme protein of *Eimeria tenella.* Mol Biochem Parasitol. **49**: 277-288.

Tyzzer, E. (1907). A sporozoan found in the peptic glands of the common mouse. Proc Soc Exp Biol Med. **5**: 12.

Tyzzer, E. (1910). An extracellular coccidium, *Cryptosporidium muris* (gen. et sp. nov.) of the gastric glands of the common mouse. Journal of Medical Research. **23**: 487.

Tyzzer, E. (1912). *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. Arch Protistenkd. **26**: 394.

Tyzzer, E. (1929). Coccidiosis in gallinaceous birds. American Journal of Hygiene. **10**: 269.

Tzipori, S., Roberton, D., and Chapman, C. (1986). Remission of diarrhoea due to cryptosporidiosis in an immunodeficient child treated with hyperimmune bovine colostrum. Br Med J (Clin Res Ed). **293**: 1276-1277.

Tzipori, S., and Ward, H. (2002). Cryptosporidiosis: biology, pathogenesis and disease. Microbes Infect. **4**: 1047-1058.

UKDETR. (1998). Department of the Environment, Transportation, and the Regions. Water Supply (Water Quality) Regulations 1998. UK.

Ungar, B.L. (1990). Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. J Clin Microbiol. **28**: 2491-2495.

Ungar, B.L., Burris, J.A., Quinn, C.A., and Finkelman, F.D. (1990a). New mouse models for chronic *Cryptosporidium* infection in immunodeficient hosts. Infect Immun. **58**: 961-969.

Ungar, B.L., Ward, D.J., Fayer, R., and Quinn, C.A. (1990b). Cessation of *Cryptosporidium*-associated diarrhea in an acquired immunodeficiency syndrome patient after treatment with hyperimmune bovine colostrum. Gastroenterology. **98**: 486-489.

Ungar, B.L., Kao, T.C., Burris, J.A., and Finkelman, F.D. (1991). *Cryptosporidium* infection in an adult mouse model. Independent roles for IFN-gamma and CD4+ T lymphocytes in protective immunity. J Immunol. **147**: 1014-1022.

Upton, S.J., and Current, W.L. (1985). The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. J Parasitol. **71**: 625-629.

Urban, J.F., Jr., Fayer, R., Chen, S.J., Gause, W.C., Gately, M.K., and Finkelman, F.D. (1996a). IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with *Cryptosporidium parvum*. J Immunol. **156**: 263-268.

Urban, J.F., Jr., Fayer, R., Sullivan, C., Goldhill, J., Shea-Donohue, T., Madden, K., Morris, S.C., Katona, I., Gause, W., Ruff, M., Mansfield, L.S., and Finkelman, F.D. (1996b). Local TH1 and TH2 responses to parasitic infection in the intestine: regulation by IFN-gamma and IL-4. Vet Immunol Immunopathol. **54**: 337-344.

USEPA. (1996a). National primary drinking regulation: monitoring requirements for public drinking water suppliers: *Cryptosporidium*, *Giardia*, viruses, disinfection byproducts, water treatment plant data and other information requirements. Federal Register. **61**: 24354-24388.

USEPA (1996b) Microscopic Particulate Analysis for Filtration Plant Optimization. EPA No. 910-R-96-001. United States Environmental Protection Agency.

USEPA (1999) Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Washington, D.C. United States Environmental Protection Agency.

Vakil, N.B., Schwartz, S.M., Buggy, B.P., Brummitt, C.F., Kherellah, M., Letzer, D.M., Gilson, I.H., and Jones, P.G. (1996). Biliary cryptosporidiosis in HIV-infected people after the waterborne outbreak of cryptosporidiosis in Milwaukee. N Engl J Med. **334**: 19-23.

Venczel, L.V., Arrowood, M., Hurd, M., and Sobsey, M.D. (1997). Inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores by a mixed-oxidant disinfectant and by free chlorine. Appl Environ Microbiol. **63**: 1598-1601.

Verdier, R.I., Fitzgerald, D.W., Johnson, W.D., Jr., and Pape, J.W. (2000). Trimethoprim-sulfamethoxazole compared with ciprofloxacin for treatment and prophylaxis of *Isospora belli* and *Cyclospora cayetanensis* infection in HIV-infected patients. A randomized, controlled trial. Ann Intern Med. **132**: 885-888.

Vesey, G., Slade, J.S., Byrne, M., Shepherd, K., and Fricker, C.R. (1993). A new method for the concentration of *Cryptosporidium* oocysts from water. Journal of Applied Bacteriology. **75**: 82-86.

Vesey, G., Hutton, P., Champion, A., Ashbolt, N., Williams, K.L., Warton, A., and Veal, D. (1994a). Application of flow cytometric methods for the routine detection of *Cryptosporidium* and *Giardia* in water. Cytometry. **16**: 1-6.

Vesey, G., Narai, J., Ashbolt, N., Williams, K., and Veal, D. (1994b). Detection of specific microorganisms in environmental samples using flow cytometry. Methods Cell Biol. **42 Pt B**: 489-522.

Vesey, G., Ashbolt, N., Fricker, E.J., Deere, D., Williams, K.L., Veal, D.A., and Dorsch, M. (1998). The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. J Appl Microbiol. **85**: 429-440.

Vetterling, J.M., Jervis, H.R., Merrill, T.G., and Sprinz, H. (1971). *Cryptosporidium wrairi* sp. n. from the guinea pig *Cavia porcellus*, with an emendation of the genus. J Protozool. **18**: 243-247.

Waldman, E., Tzipori, S., and Forsyth, J.R. (1986). Separation of *Cryptosporidium* species oocysts from feces by using a percoll discontinuous density gradient. J Clin Microbiol. **23**: 199-200.

Wan, K.L., Carruthers, V.B., Sibley, L.D., and Ajioka, J.W. (1997). Molecular characterisation of an expressed sequence tag locus of *Toxoplasma gondii* encoding the micronemal protein MIC2. Mol Biochem Parasitol. **84**: 203-214.

Wang, J. (2000). From DNA biosensors to gene chips. Nucleic Acids Res. **28**: 3011-3016.

Waters, W.R., and Harp, J.A. (1996). *Cryptosporidium parvum* infection in T-cell receptor (TCR)-alpha- and TCR-delta-deficient mice. Infect Immun. **64**: 1854-1857.

Widmer, G., Akiyoshi, D., Buckholt, M.A., Feng, X., Rich, S.M., Deary, K.M., Bowman, C.A., Xu, P., Wang, Y., Wang, X., Buck, G.A., and Tzipori, S. (2000a). Animal propagation and genomic survey of a genotype 1 isolate of *Cryptosporidium parvum*. Mol Biochem Parasitol. **108**: 187-197.

Widmer, G., Corey, E.A., Stein, B., Griffiths, J.K., and Tzipori, S. (2000b). Host cell apoptosis impairs *Cryptosporidium parvum* development in vitro. J Parasitol. **86**: 922-928.

Wu, Z., Nagano, I., Matsuo, A., Uga, S., Kimata, I., Iseki, M., and Takahashi, Y. (2000). Specific PCR primers for *Cryptosporidium parvum* with extra high sensitivity. Molecular and Cellular Probes. **14**: 33-39.

Wuhib, T., Silva, T.M., Newman, R.D., Garcia, L.S., Pereira, M.L., Chaves, C.S., Wahlquist, S.P., Bryan, R.T., Guerrant, R.L., Sousa Ade, Q., and et al. (1994). Cryptosporidial and microsporidial infections in human immunodeficiency virus-infected patients in northeastern Brazil. J Infect Dis. **170**: 494-497.

Wyatt, C.R., Brackett, E.J., Perryman, L.E., Rice-Ficht, A.C., Brown, W.C., and O'Rourke, K.I. (1997). Activation of intestinal intraepithelial T lymphocytes in calves infected with *Cryptosporidium parvum*. Infect Immun. **65**: 185-190.

Wyatt, C.R., Brackett, E.J., and Barrett, W.J. (1999). Accumulation of mucosal T lymphocytes around epithelial cells after in vitro infection with *Cryptosporidium parvum*. J Parasitol. **85**: 765-768.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., and Lal, A.A. (1999). Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol. **65**: 1578-1583.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H., and Lal, A.A. (2001a). Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J Infect Dis. **3**: 492-497.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., and Lal, A. (2001b). Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Applied and Environmental Microbiology. **67**: 1097-1101.

Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R., and Lal, A.A. (2002). Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. Int J Parasitol. **32**: 1773-1785.

Yamazaki, T., Sasaki, N., Takahashi, S., Satomi, A., Hashikita, G., Oki, F., Itabashi, A., Hirayama, K., and Hori, E. (1997). Clinical features of Japanese children infected with *Cryptosporidium parvum* during a massive outbreak caused by contaminated water supply. Kansenshogaku Zasshi. **71**: 1031-1036.

Yoshikawa, H., and Iseki, M. (1992). Freeze-fracture study of the site of attachment of *Cryptosporidium muris* in gastric glands. J Protozool. **39**: 539-544.

Youakim, A., and Ahdieh, M. (1999). Interferon-gamma decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. Am J Physiol. **276**: G1279-1288.

Zerpa, R., and Huicho, L. (1994). Childhood cryptosporidial diarrhea associated with identification of *Cryptosporidium* sp. in the cockroach *Periplaneta americana*. Pediatr Infect Dis J. **13**: 546-548.

Zu, S.X., Li, J.F., Barrett, L.J., Fayer, R., Shu, S.Y., McAuliffe, J.F., Roche, J.K., and Guerrant, R.L. (1994). Seroepidemiologic study of *Cryptosporidium* infection in children from rural communities of Anhui, China and Fortaleza, Brazil. Am J Trop Med Hyg. **51**: 1-10.

Zuckerman, U., Armon, R., Tzipori, S., and Gold, D. (1999). Evaluation of a portable differential continuous flow centrifuge for concentration of *Cryptosporidium* oocysts and *Giardia* cysts from water. J Appl Microbiol. **86**: 955-961.

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.

Applied and Environmental Microbiology. 2001 Jun;67(6):2665-8.

To: Journals Dept.
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University of Arizona Ph.D. Candidate
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Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts.
Appl Environ Microbiol. 2001 Jun;67(6):2665-8.
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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.
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Sincerely,

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Species-Specific, Nested PCR-Restriction Fragment Length Polymorphism Detection of Single *Cryptosporidium parvum* Oocysts

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Concurrent with recent advances seen with *Cryptosporidium parvum* detection in both treated and untreated water is the need to properly evaluate these advances. A micromanipulation method by which known numbers of *C. parvum* oocysts, even a single oocyst, can be delivered to a test matrix for detection sensitivity is presented. Using newly developed nested PCR-restriction fragment length polymorphism primers, PCR sensitivity was evaluated with 1, 2, 3, 4, 5, 7, or 10 oocysts. PCR detection rates (50 samples for each number of oocysts) ranged from 38% for single oocysts to 92% for 5 oocysts, while 10 oocysts were needed to achieve 100% detection. The nested PCR conditions amplified products from *C. parvum*, *Cryptosporidium baileyi*, and *Cryptosporidium serpentis* but no other *Cryptosporidium* sp. or protozoan tested. Restriction enzyme digestion with *VspI* distinguished between *C. parvum* genotypes 1 and 2. Restriction enzyme digestion with *DraII* distinguished *C. parvum* from *C. baileyi* and *C. serpentis*. Use of known numbers of whole oocysts encompasses the difficulty of liberating DNA from the oocyst and eliminates the standard deviation inherent within a dilution series. To our knowledge this is the first report in which singly isolated *C. parvum* oocysts were used to evaluate PCR sensitivity. This achievement illustrates that PCR amplification of a single oocyst is feasible, yet sensitivity remains an issue, thereby illustrating the difficulty of dealing with low oocyst numbers when working with environmental water samples.

Cryptosporidiosis is a self-limiting diarrheal infection in the immunocompetent individual, and with proper oral hydration therapy a full recovery is expected (6). 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-*

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vum into genotypes 1 and 2 (5, 18, 21). Genotype 1 is found in humans, while genotype 2 can be found in both infected humans and animals. These findings are of major concern in understanding the epidemiology of *C. parvum* infections and have resulted in the hypothesis of two separate transmission cycles, one anthroponotic and one zoonotic (14).

These current methods for detecting low numbers of oocysts utilize the dilution of either oocysts or *C. parvum* DNA to achieve the nominal value of a single oocyst. Using the dilution method with oocysts results in a number plus or minus the standard deviation, and therefore exact numbers of oocysts are not known. Using the dilution method with *C. parvum* DNA does not take into account 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 Asociacion Benefica PRISMA field workers (Lima, Peru) engaged in long-term collaborative efforts and shipped to the University of Arizona. Oocysts were purified using discontinuous sucrose gradients and cesium chloride (2) and enumerated by hemocytometer, and aliquots of a single genotype were stored in antibiotic solution (0.01% Tween 20, 100 U of penicillin, 0.1 mg of streptomycin/ml, and 0.1 mg of 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 10^5 oocysts (Iowa isolate). The labeling mixture was incubated in the dark for 1 h at room temperature with brief vortexing every 10 min. FITC-labeled suspensions were then washed three times in 1× PCR buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl) (PE Applied Biosystems, Branchburg, N.J.). Washing the oocysts in 1× PCR buffer consisted of centrifugation at 14,000 × *g* for 3 min followed by resuspension of the packed pellet in 500 μl of 1× PCR buffer. After the final centrifuge step, the pellet was resuspended in 100 μl of 1× PCR buffer.

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 (≈20-μm-inner-diameter tip opening) together with an UltraMicroPump II (World Precision Instruments [WPI], Sarasota, Fla.) and Micro4 controller (microprocessor-based controller) (WPI) (Fig. 1). A joystick micromanipulator (WPI) stabilized the micropipette ultramicropump. Single oocysts were then transferred and dispensed 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 H_2O on a microscope slide followed by the addition of 3 μl of mounting medium (Merifluor *Giardia* and *Cryptosporidium* kit; Meridian Diagnostics, Cincinnati, Ohio). The suspension containing the oocyst was covered with a 15-mm-diameter coverslip and sealed with clear fingernail polish. The entire coverslip area was scanned using 200× fluorescence microscopy to confirm the presence of singly isolated oocysts.

PCR. The nested PCR primers designed for this study amplify a region within the 18S rRNA gene. External primers ExCry1 (GCC AGT AGT CAT ATG CTT GTC TC) (bp 16 to 38) and ExCry2 (ACT GTT AAA TAG AAA TGC CCC C) (bp 838 to 859) amplify an 844-bp fragment from genotype 1 and an 840-bp fragment from genotype 2. Nested primers NesCry3 (GCG AAA AAA CTC GAC TTT ATG GAA GGG) (bp 173 to 199) and NesCry4 (GGA GTA TTC AAG GCA TAT GCC TGC) (bp 739 to 765) amplify a 593-bp fragment from genotype 1 and a 590-bp fragment from genotype 2. Base pair positions are relative to the entire 18S rRNA gene of *C. parvum* isolates carrying genotype 1 (GenBank accession no. AF093491) and genotype 2 (GenBank accession no. AF164102).

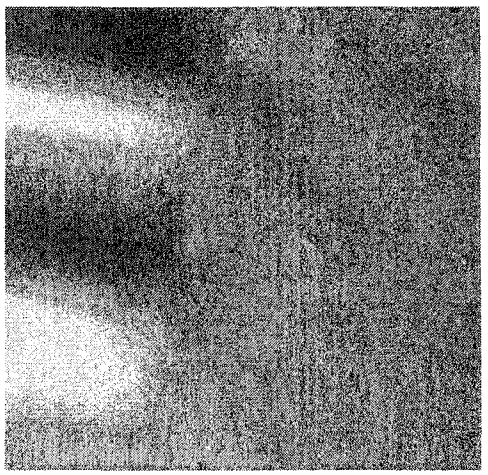

FIG. 1. Differential interference contrast microscopy of a single *C. parvum* oocyst during micromanipulation. Magnification, ×400. FITC-labeled oocysts were located using epifluorescence microscopy and isolated with 100× phase microscopy using a manually pulled glass micropipette (≈20-μm-inner-diameter tip opening) together with a micropump stabilized by a micromanipulator.

The external PCR master mixture incorporated a 200 nM concentration of each primer (ExCry1 and ExCry2), 1× PCR Gold buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl) (PE Applied Biosystems), 2 mM $MgCl_2$ (PE Applied Biosystems), 200 μM (each) dATP, dCTP, dGTP, and dTTP (Promega, Madison, Wis.), 1 U of *Taq* polymerase (Promega), and purified, sterile water. Ten microliters (approximately 50 ng) of purified *C. parvum* template DNA, genotype 1 or 2, was added to give a total volume of 50 μl. The nested PCR master mixture was essentially the same as the external PCR master mixture with the exception of nested primers (NesCry3 and NesCry4) and 1.5 mM $MgCl_2$. Two microliters of the amplified external reaction mixture was transferred to 48 μl of the nested master mixture.

PCR parameters used in the external reaction included an initial denaturation at 95°C for 5 min and a 10-min hold at 80°C (*Taq* polymerase was added at this step), followed by 40 cycles of 94°C for 45 s, 53°C for 75 s, and 72°C for 45 s. Final extension was carried out at 72°C for 7 min. The nested-reaction parameters were the same except that 35 cycles were performed at an annealing temperature of 65°C and dehybridization, annealing, and extension time periods were 25 s each. PCRs were performed in an Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, N.Y.). PCR amplicons were visualized and photographed on 1.2% agarose gels stained with ethidium bromide (0.5 μg/μl) following UV transillumination. Two negative-control tubes, containing 10 μl of sterile double-distilled H_2O instead of DNA, were the first and last samples completed with each PCR round.

Primer evaluation. The nested primer set was evaluated for *C. parvum* specificity. Isolates of *C. parvum* (KSU-1), *Cryptosporidium serpentis* (KSU-2), *Cryptosporidium andersoni* (KSU-3), *C. parvum* (KSU-4), *Cryptosporidium muris* (108735), and *Hammondia heydorni* were provided by Steve Upton (Kansas State University). *Cryptosporidium baileyi* was provided by Byron Blagburn (Auburn University). An isolate of *Neospora caninum* was provided by David Lindsay (Virginia Polytechnic and State University) via S. Upton. Isolates of *Cyclospora cayetanensis* (Robert Gilman, Johns Hopkins), *Eimeria neischulzi* (Don Duszyn-

ski, University of New Mexico), *Encephalitozoon (septata) intestinalis* (ATCC 50603), *Giardia duodenalis* (CDC0284), *Bacillus subtilis* (ATCC 27370), and *Escherichia coli* (ATCC 15224) were also tested. DNA from previously purified organisms was isolated using freezing and thawing and phenol-chloroform-ethanol extraction. DNA from organisms still in fecal material was isolated using the QIAamp DNA stool minikit (Qiagen Inc., Valencia, Calif.) in accordance with the manufacturer's instructions.

PCR sensitivity. Testing nested-PCR sensitivity with low numbers of oocysts consisted of transferring 1, 2, 3, 4, 5, 7, or 10 oocysts into PCR tubes containing 10 μl of 1× PCR buffer. In total, 50 samples for each number of oocysts were subjected to PCR.

The 10-μl 1× PCR buffer solution containing the single oocyst or multiple oocysts was subjected to six freeze/thaw cycles (2 min in liquid nitrogen followed by 2 min in a 98°C water bath). A 30-s centrifugation (14,000 × *g*) was added between the third and forth cycles. PCR conditions were those described above and consisted of adding 40 μl of the external master mixture directly to the 10 μl of 1× PCR buffer containing the ruptured single or multiple oocysts. Again, 2 μl of the amplified external reaction mixture was added to 48 μl of the nested master mixture.

RFLP analysis. Restriction sites within the nested amplicons allow for differentiation between *C. parvum* genotypes 1 and 2 (*Vsp*I) (23) and between *C. parvum* isolates from *C. baileyi* and *C. serpentis* (*Dra*II). In 20-μl reaction volumes, 15 μl of the nested PCR amplicon was digested with 6 U of *Vsp*I (Promega) or 4 U of *Dra*II (Hoffmann-La Roche Inc., Nutley, N.J.) in the supplied buffer for 6 h at 37°C. Digested products were visualized on 2% agarose gels stained with ethidium bromide (0.5 μg/μl) following UV transillumination.

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 innately 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 view of the need to determine the true limit of PCR for detecting whole oocysts, the combination of an ultramicropump attached to a micromanipulator was used to deliver the desired number of oocysts (1 or 10 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%) singly isolated oocysts were detected. In no case were additional

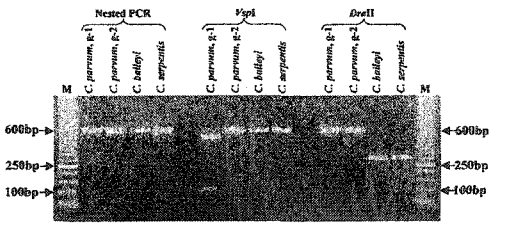

FIG. 2. Nested PCR amplification and restriction enzyme digestions with *Vsp*I and *Dra*II of a segment within the 18S rRNA of *Cryptosporidium* species; g-1 and g-2, *C. parvum* genotypes 1 and 2, respectively. Lanes M, 50-bp molecular marker (Pharmacia, Piscataway, N.J.).

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 593- and 590-bp fragments from genotype 1 and genotype 2, respectively (Fig. 2). Isolated DNA from test organisms *C. parvum* (KSU-1), *C. parvum* (KSU-4), *C. andersoni*, *C. baileyi*, *C. muris*, *C. serpentis*, *H. heydorni*, *N. caninum*, *Cyclospora cayetanensis*, *E. neischulzi*, *E. (septata) intestinalis*, *G. duodenalis*, *B. subtilis*, and *E. coli* was also subjected to PCR and RFLP digestion. PCR with the external primers amplified products from *C. parvum* (KSU-1), *C. parvum* (KSU-4), *C. baileyi*, *C. serpentis*, *Cyclospora cayetanensis*, *E. neischulzi*, and *N. caninum* DNA (Table 1). With the exception of that from *C. parvum*, *C. baileyi*, and *C. serpentis*, amplified DNA from the external-primer reaction was not amplified with the nested-primer set (Table 1). Differentiation between *C. parvum* genotypes 1 and 2 was accomplished with restriction enzyme *Vsp*I (Fig. 2). Genotype 1 contains the restriction site producing 503- and 90-bp fragments, while genotype 2 does not contain the restriction cut site. Differentiation of *C. parvum* from *C. baileyi* and *C. serpentis* was accomplished with restriction enzyme *Dra*II (Fig. 2). The *Dra*II digestion site within *C. baileyi* produced 295- and 284-bp fragments (indistinguishable within the agarose gel) (Fig. 2). The *Dra*II digestion site within *C. serpentis* produced 298- and 284-bp fragments (indistinguishable within the agarose gel) (Fig. 2).

The amplification of DNA from *C. baileyi* and *C. serpentis* lowers the specificity of this primer set at the species level, but the *Vsp*I and *Dra*II restriction digestion sites readily differentiate between *C. parvum* genotypes and other *Cryptosporidium* species, respectively. Unfortunately, due to the difficulty in acquiring oocysts, DNA from *Cryptosporidium meleagridis* could not be evaluated using these primers.

Finally, nested PCR sensitivity was evaluated with single and multiple *C. parvum* genotype 2 oocysts. PCR amplification rates were as follows: 100% for PCR tubes with 10 oocysts, 94% for tubes containing 7 oocysts, 92% for tubes containing

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

Test organism (strain, genotype)	PCR product (size[b] [bp]) obtained from:		Results of RFLP digestion with:	
	External primers	Nested primers	*Vsp*I	*Dra*II
C. parvum (Peru, 1)	+ (844)	+ (593)	+	−
C. parvum (Iowa, 2)	+ (840)	+ (590)	−	−
C. parvum (KSU-1, 2)	+ (840)	+ (590)	−	ND
C. serpentis (KSU-2)	+ (836)	+ (583)	−	+
C. andersoni (KSU-3)	−	−	ND	ND
C. parvum (KSU-4, 2)	+ (840)	+ (590)	−	ND
C. baileyi	+ (831)	+ (579)	−	+
C. muris (108735)	−	−	ND	ND
Cyclospora cayetanensis	+ (883)	−	ND	ND
Eimeria neischulzi	+ (885)	−	ND	ND
Encephalitozoon intestinalis	−	−	ND	ND
Giardia duodenalis	−	−	ND	ND
Neospora caninum	+[c]	−	ND	ND
Bacillus subtilis	−	−	ND	ND
Escherichia coli	−	−	ND	ND

[a] +, amplification or digestion; −, no amplification or digestion; ND, not done.
[b] Amplicon size based on the following representative GenBank accession numbers: *C. parvum* genotypes 1 and 2, AF093491 and AF164102, respectively; *C. baileyi*, L19068; *C. serpentis*, AF151376; *C. cayetanensis*, AF111183; *E. neischulzi*, U40263.
[c] 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 five 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

1. **Aldom, J. E., and A. H. Chagla.** 1995. Recovery of *Cryptosporidium* oocysts from water by a membrane filter dissolution method. Lett. Appl. Microbiol. **20:**186–187.
2. **Arrowood, M. J., and K. Donaldson.** 1996. Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J. Eukaryot. Microbiol. **43:**89S.
3. **Awad-El-Kariem, F. M., D. C. Warhurst, and V. McDonald.** 1993. Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. Parasitology **109:**19–22.
4. **Bukhari, Z., R. M. McCuin, C. R. Fricker, and J. L. Clancy.** 1998. Immunomagnetic separation of *Cryptosporidium parvum* from source water samples of various turbidities. Appl. Environ. Microbiol. **64:**4495–4499.
5. **Carraway, M., S. Tzipori, and G. Widmer.** 1997. A new restriction fragment length polymorphism from *Cryptosporidium parvum* identifies genetically heterogeneous parasite populations and genotypic changes following transmission from bovine to human hosts. Infect. Immun. **65:**3958–3960.
6. **Clark, D. P.** 1999. New insights into human cryptosporidiosis. Clin. Microbiol. Rev. **12:**554–563.
7. **Deng, M. Q., D. O. Cliver, and T. W. Mariam.** 1997. Immunomagnetic capture PCR to detect viable *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **63:**3134–3138.
8. **Fayer, R., C. A. Speer, and J. P. Dubey.** 1997. The general biology of *Cryptosporidium*, p. 1–41. *In* R. Fayer (ed.), *Cryptosporidium* and cryptosporidiosis. CRC Press, Inc., New York, N.Y.
9. **Hallier-Soulier, S., and E. Guillot.** 2000. Detection of cryptosporidia and *Cryptosporidium parvum* oocysts in environmental water samples by immunomagnetic separation-polymerase chain reaction. J. Appl. Microbiol. **89:**5–10.
10. **Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:**1423–1428.
11. **Laxer, M. A., B. K. Timblin, and R. J. Patel.** 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **45:**688–694.
12. **Lowery, C. J., J. E. Moore, B. C. Millar, D. P. Burke, K. A. McCorry, E. Crothers, and J. S. Dooley.** 2000. Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. J. Med. Microbiol. **49:**779–785.
13. **MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addis, K. R. Fox, J. B. Rose, and J. P. Davis.** 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:**161–167.
14. **Peng, M. M., L. Xiao, A. R. Freeman, M. J. Arrowood, A. A. Escalante, A. C. Weltman, C. S. Ong, W. R. MacKenzie, A. A. Lal, and C. B. Beard.** 1997. Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. Emerg. Infect. Dis. **3:**567–573.
15. **Rochelle, P. A., R. De Leon, A. Johnson, M. H. Stewart, and R. L. Wolfe.** 1999. Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **65:**841–845.
16. **Shepherd, K. M., and A. P. Wyn-Jones.** 1996. An evaluation of methods for the simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts from water. Appl. Environ. Microbiol. **62:**1317–1322.
17. **Smith, H. V., and J. B. Rose.** 1998. Waterborne cryptosporidiosis: current status. Parasitol. Today **14:**14–22.
18. **Spano, F., L. Putignani, S. Guida, and A. Crisanti.** 1998. *Cryptosporidium parvum*: PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of *Cryptosporidium*-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. Exp. Parasitol. **90:**195–198.
19. **Sterling, C. R.** 2000. Cryptosporidiosis: the treatment dilemma. J. Med. Microbiol. **49:**207–208.
20. **Sulaiman, I. M., L. Xiao, and A. A. Lal.** 1999. Evaluation of *Cryptosporidium parvum* genotyping techniques. Appl. Environ. Microbiol. **65:**4431–4435.
21. **Sulaiman, I. M., L. Xiao, C. Yang, L. Escalante, A. Moore, C. B. Beard, M. J. Arrowood, and A. A. Lal.** 1998. Differentiating human from animal isolates of *Cryptosporidium parvum*. Emerg. Infect. Dis. **4:**681–685.
22. **Wiedenmann, A., S. Steuer, P. Kruger, and K. Botzenhart.** 1997. A simple procedure for an exact evaluation of the sensitivity of the selective detection of viable *Cryptosporidium* oocysts by *in vitro* excystation and PCR, p. 109–113. *In* C. Fricker and P. Rochelle (ed.), Proceedings of the 1997 International Symposium on Waterborne *Cryptosporidium*. American Water Works Association, Denver, Colo.
23. **Xiao, L., L. Escalante, C. Yang, I. Sulaiman, A. Escalante, R. Montali, R. Fayer, and A. Lal.** 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. **65:**1578–1583.
24. **Yang, S., S. K. Benson, C. Du, and M. C. Healey.** 2000. Infection of immunosuppressed C57BL/6N adult mice with a single oocyst of *Cryptosporidium parvum*. J. Parasitol. **86:**884–887.

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.

Applied and Environmental Microbiology. 2002 Jun;68(6):2991-6.

To: Journals Dept.
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Sturbaum GD, Reed C, Hoover PJ, Jost BH, Marshall MM, Sterling CR
Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts.
Appl Environ Microbiol. 2001 Jun;67(6):2665-8.
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Appl Environ Microbiol. 2002 Jun;68(6):2991-6.
PMID: 12039759 [PubMed - indexed for MEDLINE]

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

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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 seed doses of 5, 10, and 15 oocysts. IMS-FA detected oocysts at concentrations at or below the three nominal oocyst seed doses, illustrating that IMS-FA is sensitive enough to detect low oocyst numbers. However, the species of the oocysts could not be determined with this technique. IMS-PCR, targeting the 18S rRNA gene 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, (ii) 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).

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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 (8, 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, 2.7 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

***C. parvum* oocysts.** *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 dispensed into 25 ml of 1% Tween 20 so that 1-ml 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 0.007 to 0.04 oocyst/liter (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 Envirochek sampling capsules (Pall Gelman Laboratory, Ann Arbor, Mich.) at a flow rate of 0.5 gal per min. Particulate matter was eluted by using EPA Method 1623 (25). The final centrifuge pellet was measured and resuspended in 20 ml of double-distilled H_2O. One-milliliter aliquots (each equivalent to 1 liter of sample test water) were dispensed into 125-mm flat-sided Leighton tubes (Dynal 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-μm-pore-size 25-mm-diameter black polycarbonate membrane filters on a fritted glass support. Three replicates were tested for each oocyst dose. The oocysts were labeled with fluorescein isothiocyanate by using a Merifluor C/G detection kit for *Cryptosporidium* and *Giardia* (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 1-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 H_2O, 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 dissociation steps (50 μl each). Microscopic replicates for each nominal oocyst value were scanned at a magnification of ×200 by using fluorescent microscopy, and presumptive 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 dissociation suspensions were transferred into 0.6-ml thin-wall PCR tubes, frozen at −4°C, and shipped to the University of Arizona.

PCR. The nested PCR primers 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 dissociation suspensions were washed three times in 100 μl of 1× PCR buffer (10 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 resuspended in a solution containing 25 μl of 1× PCR buffer and 5 μl of InstaGene matrix (Bio-Rad, Hercules, Calif.). This 30-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 30-s 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 as the PCR DNA template. PCRs were performed with a Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, N.Y.). PCR amplicons were visualized on 1.2% ethidium bromide-stained agarose gels with UV transillumination and photodocumented. Two negative control tubes, containing 10 μl of sterile double-distilled H_2O instead of template, were the first and last samples completed for each PCR round.

RFLP analysis. A 10-μl aliquot of nested PCR amplicon was subjected to digestion with *Dra*I (Roche Molecular Biomedicals, Nutley, N.J.), *Dra*II (Roche Molecular Biomedicals), or *Vsp*I (Promega, Madison, Wis.) by following the manufacturer's recommendations. Digested PCR products were visualized on 2% ethidium bromide-stained agarose gels with UV transillumination.

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.

TABLE 1. Seeded oocyst detection and confirmation by IMS-FA when EPA Method 1623 was used

No. of seeded oocysts/ml[a]	Replicate	No. of oocysts/ml			
		Membrane verified[b]	IMS-FA		
			Deionized water	Dowdy Lake	Cache la Poudre River
0	1	NA[c]	ND[d]	ND	ND
5	1	3	3	4	3
	2	4		3	6
	3	3		2	4
10	1	5	7	9	6
	2	8		5	4
	3	10		7	8
15	1	8	15	7	11
	2	17		7	13
	3	16		13	12

[a] Nominal number of seeded oocysts per milliliter.
[b] Verified number of seeded oocysts per milliliter as determined on 25-mm-diameter black polycarbonate membranes.
[c] NA, not applicable.
[d] ND, none detected.

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 oocyst 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 replicate 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 number 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 value 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 H_2O and naturally occurring waters are summarized in Table 1. In all but one case,

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

Sample	No. of oocysts/ml	Replicate	PCR detection	RFLP digest[a]			Identity determined by sequencing[b]
				*Dra*I	*Dra*II	*Vsp*I	
Deionized H_2O	0	1	−	ND[c]	ND	ND	ND
	5	1	−	ND	ND	ND	ND
	10	1	+	−	−	−	ND
	15	1	+	−	−	−	ND
Dowdy Lake	0	1	+[d]	+	−	−	*Gymnodinium* spp.
	5	1	+	−	−	−	*C. parvum*
		2	+	−	−	−	*C. parvum*
		3	+[d]	+	−	−	*Gymnodinium* spp.
	10	1	+[d]	+	−	−	*Gymnodinium* spp.
		2	+	−	−	−	*C. parvum*
		3	+	−	−	−	*C. parvum*
	15	1	+	−	−	−	*C. parvum*
		2	+	−	−	−	*C. parvum*
		3	+	−	−	−	*C. parvum*
Cache la Poudre River	0	1	+	−	+	−	*C. muris*
	5	1	+	−	−	−	*C. parvum*
		2	+	−	−	−	*C. parvum*
		3	+	−	−	−	*C. parvum*
	10	1	+	−	−	−	*C. parvum*
		2	+	−	−	−	*C. parvum*
		3	+	−	−	−	*C. parvum*
	15	1	+	−	+	−	Undetermined
		2	+	−	−	−	*C. parvum*
		3	−	ND	ND	ND	ND

[a] +, RFLP-positive digestion; −, RFLP-negative digestion.
[b] Identity as determined by nucleotide sequencing and BlastN search.
[c] ND, not done.
[d] The PCR amplicon (approximately 625 bp) was larger than the expected 590-bp amplicon of *C. parvum*.

IMS-FA detected less than the nominal oocyst value. The six oocysts detected and microscopically confirmed in replicate 2 of Cache la Poudre water seeded with 5 oocysts/ml can be attributed to introduced standard deviation error (see above) or to the presence of naturally occurring oocysts in the river, as *Cryptosporidium* spp. have been previously detected in this water source (Gertig, personal communication). These results indicate that IMS-FA is able to efficiently recover and detect low numbers of oocysts seeded into natural waters of low turbidity.

The results of IMS-PCR detection, RFLP digestion, and DNA sequencing analyses are summarized in Table 2. Like microscopic detection, IMS-PCR detection proved to be sensitive for detecting the different nominal oocyst doses in both natural waters and deionized H_2O. The negative results for the 5-oocyst/ml dose in deionized H_2O and for the third replicate of the Cache la Poudre sample seeded with a 15-oocyst/ml dose can most likely be explained by losses that occurred during repeated centrifugation of oocysts. In this protocol, oocysts recovered by IMS were washed three times by centrifugation in order to reduce naturally occurring PCR inhibitors. During this 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 amplified *C. parvum* positive control DNA (Fig. 1A). While the positive amplification results were not a surprise for the Cache la Poudre River water, since *Cryptosporidium* spp. have been detected previously in this water (Gertig, personal communication), the positive PCR results for Dowdy Lake water were unexpected. Subsequently, the Cache la Poudre River and Dowdy Lake background PCR amplicons were DNA sequenced and determined to be 584 and 624 bp long, respectively. BlastN searches (1) identified the 584-bp Cache la Poudre amplicon as a *Cryptosporidium muris* amplicon (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). Microscopy analysis of Dowdy Lake water identified the dinoflagellates *Ceratium* spp. and *Peridinium* spp. but not *Gymnodinium* spp.

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. *Dra*II 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. 1C), 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

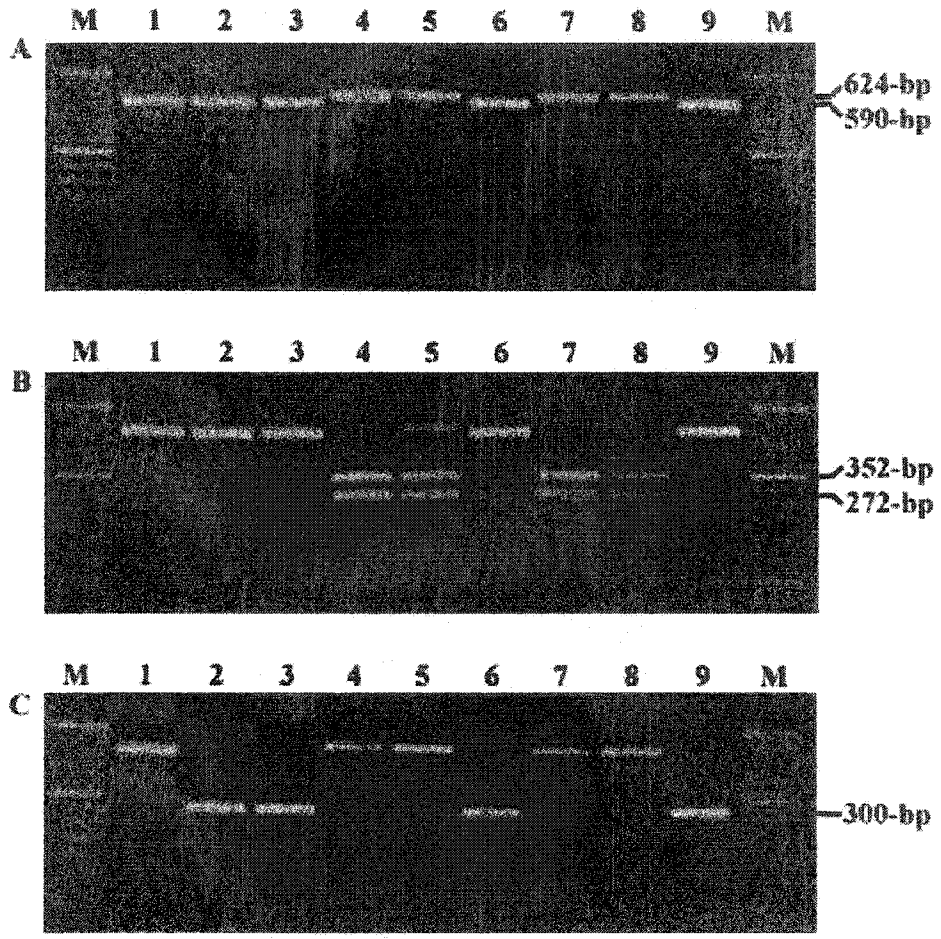

FIG. 1. Nested PCR (A) and RFLP digestion with *Dra*I (B) or *Dra*II (C) of *Cryptosporidium* spp. and two natural water matrixes with or without seeded *C. parvum* oocysts following IMS. Lanes1 to 4, amplicons from known isolates; lanes 5 to 9, amplicons from water matrixes; lane 1, *C. parvum* Iowa isolate; lane 2, *C. andersoni*; lane 3, *C. muris*; lane 4, *G. fuscum*; 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. *Dra*I 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 *Dra*I, *Dra*II, and *Vsp*I. Restriction digestion with *Vsp*I was performed to confirm that PCR amplicons were from *C. parvum* genotype 2 and not genotype 1, and subsequent *Vsp*I digestions of all background and seeded water samples were negative (Table 2). However, three samples were digested with either *Dra*I or *Dra*II; replicate 3 of Dowdy Lake water seeded with 5 oocysts (Dowdy-5-3) was digested with *Dra*I, replicate 1 of Dowdy Lake water seeded with 10 oocysts (Dowdy-10-1) was digested with *Dra*I, and replicate 1 of Cache la Poudre River water seeded with 15 oocysts (Poudre-15-1) was digested with *Dra*II (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 93%. 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 *Dra*II 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 *Dra*I or *Dra*II followed amplification. Both *C. andersoni* and *C. muris* were PCR positive, and digestion with *Dra*II was able to discriminate these organisms from the *C. parvum* positive control (Fig. 1A and C). Isolated DNA from *G. fuscum* also was amplified, and digestion with *Dra*I 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, Sporozoa; apicomplexans), Ciliophora (ciliates), and Dinozoa (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% 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 recommendation 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.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Arrowood, M. J., and K. Donaldson.** 1996. Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J. Eukaryot. Microbiol. **43**:89S.
3. **Bukhari, Z., R. M. McCuin, C. R. Fricker, and J. L. Clancy.** 1998. Immunomagnetic separation of *Cryptosporidium parvum* from source water samples of various turbidities. Appl. Environ. Microbiol. **64**:4495–4499.
4. **Bukhari, Z., and H. V. Smith.** 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. J. Clin. Microbiol. **33**:2592–2595.
5. **Cavalier-Smith, T.** 1993. Kingdom Protozoa and its 18 phyla. Microbiol. Rev. **57**:953–994.
6. **Cavalier-Smith, T.** 1999. Zooflagellate phylogeny and the systematics of protozoa. Biol. Bull. **196**:393–396.
7. **Deng, M. Q., D. O. Cliver, and T. W. Mariam.** 1997. Immunomagnetic capture PCR to detect viable *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **63**:3134–3138.
8. **Di Giovanni, G. D., F. H. Hashemi, N. J. Shaw, F. A. Abrams, M. W. LeChevallier, and M. Abbaszadegan.** 1999. Detection of infectious *Cryptosporidium parvum* oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. Appl. Environ. Microbiol. **65**:3427–3432.
9. **Drozd, C., and J. Schwartzbrod.** 1996. Hydrophobic and electrostatic cell surface properties of *Cryptosporidium parvum*. Appl. Environ. Microbiol. **62**:1227–1232.
10. **DuPont, H. L., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose, and W. Jakubowski.** 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. **332**:855–859.
11. **Gajadhar, A. A., W. C. Marquardt, R. Hall, J. Gunderson, E. V. Ariztia-Carmona, and M. L. Sogin.** 1991. Ribosomal RNA sequences of *Sarcocystis muris, Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary

relationships among apicomplexans, dinoflagellates, and ciliates. Mol. Biochem. Parasitol. **45:**147–154.

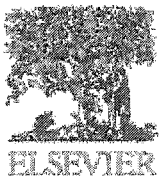
12. **Hallier-Soulier, S., and E. Guillot.** 2000. Detection of cryptosporidia and *Cryptosporidium parvum* oocysts in environmental water samples by immunomagnetic separation-polymerase chain reaction. J. Appl. Microbiol. **89:**5–10.
13. **Hallier-Soulier, S., and E. Guillot.** 1999. An immunomagnetic separation polymerase chain reaction assay for rapid and ultra-sensitive detection of *Cryptosporidium parvum* in drinking water. FEMS Microbiol. Lett. **176:**285–289.
14. **Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:**1423–1428.
15. **LeChevallier, M., W. Norton, and R. Lee.** 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl. Environ. Microbiol. **57:**2610–2616.
16. **Lowery, C. J., J. E. Moore, B. C. Millar, D. P. Burke, K. A. McCorry, E. Crothers, and J. S. Dooley.** 2000. Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. J. Med. Microbiol. **49:**779–785.
17. **McCuin, R. M., Z. Bukhari, J. Sobrinho, and J. L. Clancy.** 2001. Recovery of *Cryptosporidium* oocysts and *Giardia* cysts from source water concentrates using immunomagnetic separation. J. Microbiol. Methods **45:**69–76.
18. **Monis, P. T., and C. P. Saint.** 2001. Development of a nested-PCR assay for the detection of *Cryptosporidium parvum* in finished water. Water Res. **35:**1641–1648.
19. **Reynolds, D. T., R. B. Slade, N. J. Sykes, A. Jonas, and C. R. Fricker.** 1999. Detection of *Cryptosporidium* oocysts in water: techniques for generating precise recovery data. J. Appl. Microbiol. **87:**804–813.
20. **Rochelle, P. A., R. De Leon, A. Johnson, M. H. Stewart, and R. L. Wolfe.** 1999. Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **65:**841–845.
21. **Rose, J., J. Lisle, and M. LeChevallier.** 1997. Waterborne cryptosporidiosis: incidence, outbreaks, and treatment strategies, p. 93–109. *In* R. Fayer (ed.), *Cryptosporidium* and cryptosporidiosis. CRC Press, Inc., New York, N.Y.
22. **Smith, H. V., and J. B. Rose.** 1998. Waterborne cryptosporidiosis: current status. Parasitol. Today **14:**14–22.
23. **Sterling, C. R.** 2000. Cryptosporidiosis: the treatment dilemma. J. Med. Microbiol. **49:**207–208.
24. **Sturbaum, G. D., C. Reed, P. J. Hoover, B. H. Jost, M. M. Marshall, and C. R. Sterling.** 2001. Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. **67:**2665–2668.
25. **United States Environmental Protection Agency.** 1999. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Publication EPA-821-R-99-006. United States Environmental Protection Agency, Washington, D.C.

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.



Gregory Dean Sturbaum
University of Arizona

20 September 2003
Our ref: HW/ct/sep 03.J068

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Short communication

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

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Cryptosporidium 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 Gilman, Johns Hopkins University and Asociacion Benefica PRISMA, Lima, Peru), HFL-6 and HM5-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 ATTATTTTTACGTTCCTTCCACTTG 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 (GenBank AY129677–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

Abbreviations: bp, base pairs; *C. parvum*, *Cryptosporidium parvum*; Cpgp40/15, *C. parvum* 40/15 glycoprotein; GP900, *C. parvum* 900-kDa glycoprotein; HSP70, *C. parvum* 70-kDa heat shock protein gene; kDa, kilodaltons; p23, *C. parvum* 23-kDa glycoprotein; rRNA, ribosomal RNA; type I, *C. parvum* genotype I; type II, *C. parvum* genotype II

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[22]. 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 three 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 primer set Cpgp40/15-24 ATTACTCTCCGTTATAGTCTCC and Cpgp40/15-936 GATTGCAAAAACGGAAGGAACG 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 Ia [23], isolates 278 and 339 were subtype Id [23], and isolates 1487 and 1531 were subtype Ie [28]. In addition, subtype Ie isolates differ from the other type I subgenotypes by containing Asp instead of Glu in the second position of the putative Gp40/Gp15 cleavage site (E-E) [23,24]. US isolates HFL-6 and HM5-C contained only minor amino acid differences from each other, and were classified as the subtype Ib [23]. Predicted N-linked glycosylation sites were conserved among subtype isolates, but differed in location between the subtypes. Additionally, isolates HFL-6 and HM5-C did not contain a putative glycosylation site.

Within the type II isolates (AF528763–AF528767), all were identical with the exceptions that: 1), the Iowa isolate analyzed in this study was unique in that it contained Tyr at position 178, instead of the Asp residue observed in the other genotype II isolates studied (AAF78348, AAF78349, AAF78350, and AAF78351), and 2), the Moredun isolate contained an additional Ser within the Ser repeat region near the N-terminus of the protein. Furthermore, the single N-linked glycosylation site is conserved amongst all the isolates.

Due to the large size of *gp900* (approximately 5199 bp) and the troublesome nature of the threonine repeats in domains 2 and 5, we developed separate primer sets to amplify domains 1, 3 and 5 separately. Primers gp900D1-100 ATGAGAATTGAATCATCTGGTGC and gp900D1-880 ACTCCAACACATTTAGTGTATGG amplified an 803 bp fragment of *gp900* domain 1 from all thirteen isolates, of which 724 bp were successfully sequenced (GenBank AF527841–AF527853). The sequence data revealed one base transversion and 11 base transitions between the genotype I and II isolates (data not shown). Alignment of the deduced amino acid sequences revealed four amino acid changes that differentiated genotype I from II. Also, within this region of domain 1, four of the five cysteine residues as well as the reported putative L-V-D binding motif [21] were found to be conserved between all the isolates. The three predicted N-linked glycosylation sites were also conserved [21].

gp900 domain 3 was troublesome to amplify therefore separate primer sets were developed. Primer set gp900D3-1562 AAACTGAGAGTGTAATTAAACCTG and gp900D3-2031 TTGCGGTAGTCGTTGTTACG 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 ACCTGATGAATGGTGTTGGTTGG and gp900D3-2080 GTTGTAGTAGTTGTTGGTTGTCC were developed and used to amplify a 522 bp fragment from isolates HFL-6 and HM5-C. Isolates Y049 and 830 did not amplify with any combination of the above primers. Nucleotide alignments revealed no base pair changes between the type I and II isolates. Alignment of the amino acid sequences with the two published domain 3 sequences, AF068065 and U83169, revealed three 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 ACCAATTCCAGGTTCTCAAGCAGGACAAATAGC and gp900D5-5129 ATTTCTTTGCTGCCTCAGCTGCAACTGACTCCG 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 AF527854–AF527864), with the exceptions of HFL-6 and HM5-C, using primers gp900D5-2490 ACCAATTCCAGGTTCTCAAGCAGGACAAATAGC, gp900D5-2987 AGCAAACTCCATTCTCCCC, gp900D5-3343 ATGGATTCTTCATTTGCTGG, gp900D5-3843 TGGTAACATTATTAACCC, gp900D5-4400 CACAAGGTGAAAATGGAGG, and gp900D5-4711 GTAGATCCTCAGACTGGAG. 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, when compared with the 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 *C. parvum* 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 epidemiological transmission patterns. The data provided in this work provides further strong evidence for the demarcation between the genotype I and genotype II lineages 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 *cpgp40/15*, *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 of 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 Cpgp40/15 [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 *cpgp40/15* (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.

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References

[1] Griffiths JK. Human cryptosporidiosis: epidemiology, transmission, clinical disease, treatment, and diagnosis. Adv Parasitol 1998;40:37–85.

[2] O'Donoghue PJ. *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parsitol 1995;25:139–95.

[3] Petersen C. Cryptosporidiosis in patients infected with the human immunodeficiency virus. Clin Infect Dis 1992;15:903–9.

[4] Current WL, Reese NC, Ernst JV, Bailey WS, Heyman MB, Weinstein WM. Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. N Engl J Med 1983;308:1252–7.

[5] DuPont HL, Chappell CL, Sterling CR, Okhuysen PC, Rose JB, Jakubowski W. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N Engl J Med 1995;332:855–9.

[6] Widmer G, Akiyoshi D, Buckholt MA, Feng X, Rich SM, Deary KM, et al. Animal propagation and genomic survey of a genotype 1 isolate of *Cryptosporidium parvum*. Mol Biochem Parasitol 2000;108:187–97.

[7] Aiello A, Xiao L, Limor J, Liu C, Abrahamsen M, Lal A. Microsatellite analysis of the human and bovine genotypes of *Cryptosporidium parvum*. J Euk Microbiol 1999;46:46S–7S.

[8] Caccio S, Homan W, van Dijk K, Pozio E. Genetic polymorphism at the beta-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. FEMS Microbiol Lett 1999;170:173–9.

[9] Caccio S, Homan W, Camilli R, Traldi G, Kortbeek T, Pozio E. A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. Parasitol 2000;120:237–44.

[10] Spano F, Putignani L, Crisanti A, Sallicandro P, Morgan UM, Le Blancq SM, et al. Multilocus genotypic analysis of *Cryptosporidium parvum* isolates from different hosts and geographical origins. J Clin Microbiol 1998;36:3255–9.

[11] Spano F, Putignani L, Guida S, Crisanti A. *Cryptosporidium parvum*: PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of Cryptosporidium-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. Exp Parasitol 1998;90:195–8.

[12] Peng MM, Xiao L, Freeman AR, Arrowood MJ, Escalante AA, Weltman AC, et al. Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. Emerg Infect Dis 1997;3:567–73.

[13] Gobet P, Toze S. Sensitive genotyping of *Cryptosporidium parvum* by PCR-RFLP analysis of the 70-kilodalton heat shock protein (HSP70) gene. FEMS Microbiol Lett 2001;200:37–41.

[14] Morgan U, Weber R, Xiao L, Sulaiman I, Thompson RC, Ndiritu W, et al. Molecular characterization of *Cryptosporidium* isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States. J Clin Microbiol 2000;38:1180–3.

[15] Spano F, Putignani L, McLauchlin J, Casemore DP, Crisanti A. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol Lett 1997;150:209–17.

[16] Morgan UM, Xiao L, Hill BD, O'Donoghue P, Limor J, Lal AA, et al. Detection of the *Cryptosporidium parvum* "human" genotype in a dugong (*Dugong dugon*). J Parasitol 1998;86:1352–4.

[17] Sulaiman IM, Xiao L, Yang C, Escalante L, Moore A, Beard CB, et al. Differentiating human from animal isolates of *Cryptosporidium parvum*. Emerg Infect Dis 1998;4:681–5.

[18] Riggs MW, Stone AL, Yount PA, Langer RC, Arrowood MJ, Bentley DL. Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J Immunol 1997;158:1787–95.

[19] Petersen C, Gut J, Doyle PS, Crabb JH, Nelson RG, Leech JH. Characterization of a >900,000-M(r) *Cryptosporidium parvum* sporozoite glycoprotein recognized by protective hyperimmune bovine colostral immunoglobulin. Infect Immun 1992;60:5132–8.

[20] Petersen C, Barnes DA, Gousset L. *Cryptosporidium parvum* GP900, a unique invasion protein. J Eukaryot Microbiol 1997;44:89S–90S.

[21] Barnes DA, Bonnin A, Huang JX, Gousset L, Wu J, Gut J, et al. A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Mol Biochem Parasitol 1998;96:93–110.

[22] Perryman LE, Jasmer DP, Riggs MW, Bohnet SG, McGuire TC, Arrowood MJ. A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Mol Biochem Parasitol 1996;80:137–47.

[23] Strong WB, Gut J, Nelson RG. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene

encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect Immun 2000;68:4117–34.

[24] Cevallos AM, Zhang X, Waldor MK, Jaison S, Zhou X, Tzipori S, et al. Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. Infect Immun 2000;68:4108–16.

[25] Nesterenko MV, Woods K, Upton SJ. Receptor/ligand interactions between *Cryptosporidium parvum* and the surface of the host cell. Biochim Biophys Acta 1999;1454:165–73.

[26] O'Connor RM, Thorpe CM, Cevallos AM, Ward HD. Expression of the highly polymorphic *Cryptosporidium parvum* Cpgp40/15 gene in genotype I and II isolates. Mol Biochem Parasitol 2002;119: 203–15.

[27] Leav BA, Mackay MR, Anyanwu A, RM OC, Cevallos AM, Kindra G, et al. Analysis of sequence diversity at the highly polymorphic Cpgp40/15 locus among *Cryptosporidium* isolates from human immunodeficiency virus-infected children in South Africa. Infect Immun 2002;70:3881–90.

[28] Peng MM, Matos O, Gatei W, Das P, Stantic-Pavlinic M, Bern C, Sulaiman IM, Glaberman S, Lal AA, Xiao L. A comparison of *Cryptosporidium* subgenotypes from several geographic regions. J. Eukaryot. Microbiol. 2001; 28S–31S.

[29] Sturbaum GD, Reed C, Hoover PJ, Jost BH, Marshall MM, Sterling CR. Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. Appl Environ Microbiol 2001;67:2665–8.

[30] Rochelle PA, Jutras EM, Atwill ER, De Leon R, Stewart MH. Polymorphisms in the beta-tubulin gene of *Cryptosporidium parvum* differentiate between isolates based on animal host but not geographic origin. J Parasitol 1999;85:986–9.

[31] Widmer G, Tchack L, Chappell C, Tzipori S. Sequence polymorphism in the β-tubulin gene reveals heterogeneous and variable population structures in *Cryptosporidium parvum*. Appl Environ Microbiol 1998;64:4477–81.

[32] Guyot K, Follet-Dumoulin A, Recourt C, Lelievre E, Cailliez JC, Dei-Cas E. PCR-restriction fragment length polymorphism analysis of a diagnostic 452-base-pair DNA fragment discriminates between *Cryptosporidium parvum* and *C. meleagridis* and between *C. parvum* isolates of human and animal origin. Appl Environ Microbiol 2002;68:2071–6.

[33] Ong CS, Eisler DL, Goh SH, Tomblin J, Awad-El-Kariem FM, Beard CB, et al. Molecular epidemiology of cryptosporidiosis outbreaks and transmission in British Columbia, Canada. Am J Trop Med Hyg 1999;61:63–9.

[34] Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, et al. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. *J Infect Dis 2001*;3:492–7.

[35] Carraway M, Tzipori S, Widmer G. Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. Appl Environ Microbiol 1996;62:712–6.

[36] Sestak K, Ward LA, Sheoran A, Feng X, Akiyoshi DE, Ward HD, et al. Variability among *Cryptosporidium parvum* genotype 1 and 2 immunodominant surface glycoproteins. Parasit Immun 2002;24:213–9.