

New Insights into Human Cryptosporidiosis

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INTRODUCTION

An excellent review of cryptosporidiosis that summarized the history, classification, and life cycle of this parasite has previously appeared in this journal (30). The present article will serve as an update to that review and will focus on topics relevant to the clinical microbiologist, particularly those pertaining to human disease and to the *Cryptosporidium* species pathogenic to humans, *Cryptosporidium parvum*.

Cryptosporidium species are intracellular protozoan parasites that have emerged as an important cause of diarrhea among humans and animals. *Cryptosporidium* exists in the environment as a hearty, 5- μ m-diameter oocyst, which contains four sporozoites. Humans and animals are infected by ingesting these oocysts, which travel through the gut lumen to the small intestine, where they rupture, releasing the sporozoites. The sporozoites are motile, 5- by 1- μ m forms which adhere to and invade the absorptive epithelial cells which line the gastrointestinal tract. This invasion process is likely to involve molecules discharged from parasite organelles (rhoptries, micronemes, and dense granules) found in the apical end of the sporozoite. Thus, *Cryptosporidium* belongs to the phylum Apicomplexa, which contains its relatives, *Toxoplasma*, *Eimeria*, and *Plasmodium*. Soon after attachment and discharge of these organellar contents, *Cryptosporidium* focally disrupts the microvilli which cover the host cell and slides into the host cell, enveloping itself in the host cell membrane in the process. The parasite quickly establishes an intracellular niche which is unique to *Cryptosporidium*, in which the parasite and the surrounding parasitophorous vacuole bulge into the gut lumen and are separated from the host cell cytoplasm by a fascinating electron-dense structure. Here, the parasite replicates into

eight merozoites, which then rupture out of the host cell, infect other host cells, and complete the asexual stage of the life cycle. At some point, merozoites differentiate into gamonts, which undergo sexual reproduction within the same host to ultimately regenerate oocysts, which are excreted in the feces (29, 37, 137, 138, 140).

CLINICAL FEATURES

HIV-Infected Individuals

Unlike immunocompetent adults, in whom cryptosporidiosis is usually self-limited, people with AIDS are susceptible to a devastating form of cryptosporidiosis manifested by chronic, voluminous diarrhea (46, 114, 119). The factors which predispose these people to chronic cryptosporidiosis rather than self-limited illness appear to be immunologic (118). While a human immunodeficiency virus (HIV)-positive individual may acquire cryptosporidiosis at any point in the viral infection, the most severe, chronic form is limited to people with markedly impaired immune systems. Flanigan et al. have found that patients with CD4 cell counts of greater than 180 cells/mm³ cleared the infection spontaneously whereas 87% of patients with CD4 cell counts less than 180 cells/mm³ had persistent disease (40).

Although AIDS-related cryptosporidiosis often manifests as a severe, persistent diarrheal illness, there is actually marked variability in the clinical presentation, including asymptomatic infection (10, 52, 62, 88, 116, 125, 153). Manabe et al. have identified four general clinical categories of AIDS-related cryptosporidiosis: a cholera-like illness requiring intravenous rehydration therapy (33%), a chronic diarrheal illness (36%), an intermittent diarrheal illness (15%), and a transient diarrheal illness (15%) (82). These clinical manifestations are independent of the general immune status, since all patients in this study were markedly immunosuppressed. In addition to

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the obvious morbidity associated with AIDS-related cryptosporidiosis, mortality is also increased (20, 27, 61, 82, 144).

Although the intestinal tract is the primary site of cryptosporidiosis, other involved organ systems have been described, including the lungs, middle ear, biliary tract, pancreas, and stomach (34, 43, 49, 78, 123, 136, 146). These sites most probably represent luminal extension of a primary infection of the intestine rather than a primary extraintestinal infection or a disseminated infection. The biliary tract is the most common, clinically relevant site of extraintestinal infection. Vakil et al. found that HIV-positive patients exposed to a waterborne outbreak of cryptosporidiosis were at increased risk for biliary symptoms and death within 1 year if their CD4 cell counts were <50 cells/mm³ (143).

Since the introduction of highly active antiretroviral therapy (HAART) to the treatment of HIV-infected individuals, the overall morbidity and mortality due to opportunistic infections has dramatically declined (111). Several studies have specifically documented a decreased prevalence of cryptosporidiosis subsequent to introduction of HAART (67, 75, 92). Also, it appears that reconstitution of immune system function with HAART may lead to the resolution of existing cryptosporidiosis in some patients (see "Treatment" below).

Children in Developing Countries

Cryptosporidium is a recognized cause of diarrhea, particularly among children, in developing countries (36, 77, 79, 93, 102–104, 124, 126). Several studies have suggested that cryptosporidiosis is most common in children younger than 1 year and is associated with malnutrition. Because these studies were largely incidence studies, it was not clear if malnutrition predisposed children to cryptosporidiosis, if cryptosporidiosis led to malnutrition, or both. Checkley et al. observed a cohort of Peruvian children, aged 0 to 3 months on recruitment, for 2 years to address this question (22). The incidence of cryptosporidiosis in this cohort was high (45%); however, neither wasting nor low weight was a significant risk factor for cryptosporidiosis. Children with symptomatic cryptosporidiosis grew less during the first month of infection than did children without diarrhea who were not infected. Interestingly, this study identified a large percentage of asymptomatic infections (63%). The effect of asymptomatic cryptosporidiosis was less severe, but these children also gained less weight than the controls did. Consequently, this study suggests that cryptosporidiosis leads to malnutrition in previously normal children. The factors which determine whether a primary infection will be symptomatic or asymptomatic are undefined.

In a study of Brazilian children, Agnew et al. have identified a possible mechanism for malnutrition subsequent to cryptosporidiosis (2). In a case-control study of children monitored from birth, children younger than 1 year were found to experience excessive and protracted (nearly 2 years) episodes of diarrheal illness, which was not due to the initial episode or recurrent episodes of cryptosporidiosis. Only 14% of these children were initially coinfecting with a second pathogen (including *Salmonella* species, *Shigella flexneri*, *Ascaris lumbricoides*, *Trichuris trichiura*, and *Giardia lamblia*). The mechanism of this subsequent diarrheal disease is unclear but may involve persistent malabsorption due to *Cryptosporidium*-induced intestinal injury or enhanced susceptibility to other enteric pathogens. Alternatively, immunologic abnormalities may be present in these children, analogous to the increased delayed mortality associated with high-titer measles vaccination in low socioeconomic populations.

EXPERIMENTAL HUMAN INFECTIONS

A group of investigators in Texas has pioneered experimental studies of cryptosporidiosis in human volunteers. In their first study, 29 healthy adults, previously unexposed to *C. parvum* (seronegative), were challenged with different doses of the Iowa isolate of *C. parvum*. In these studies, the percentage of infected individuals (those subsequently excreting oocysts in stool) was directly related to the dose of oocysts administered, ranging from 20% for 30 oocysts to 100% for $>1,000$ oocysts. Of the 18 individuals who excreted oocysts, 11 had enteric symptoms and 7 had diarrhea plus one other enteric symptom (35). Volunteers with diarrheal illness tended to excrete more oocysts over the course of infection (21). Linear regression analysis determined that the median infectious dose was very low (132 oocysts). Trials comparing the infectious dose of three different isolates suggest that isolates may differ significantly in virulence (108).

To examine the susceptibility and serologic response to reinfection, 19 of the previously infected volunteers were rechallenged with 500 oocysts 1 year after the primary infection. Fewer subjects shed oocysts after the second exposure (16%), but the rates of diarrhea were similar, although the diarrhea was less clinically severe. The number of anti-*Cryptosporidium* immunoglobulin G (IgG) and IgA seroconversions increased after secondary exposure, but the serologic response did not correlate with symptoms or the presence of oocysts in the stool (109). Further studies examining serologic responses to specific *Cryptosporidium* antigens suggested that increases in specific antibody reactivity were more prevalent among symptomatic individuals and that persons with preexisting antibodies may be less likely to develop illness (101). Specifically, individuals with preexisting IgG antibody to a 27-kDa *Cryptosporidium* antigen excreted fewer oocysts than did those without this antibody. Also, IgG reactivity to a 17-kDa antigen and IgM reactivity to the 27-kDa antigen were higher prior to infection for asymptomatic individuals than for symptomatic individuals. Therefore, previous exposure of immunocompetent adults to *Cryptosporidium* is not entirely protective but may decrease the severity of disease and the number of oocysts shed.

DIAGNOSIS

The diagnosis of cryptosporidiosis rests on the identification of the 5- μ m spherical oocysts (or oocyst components) in stool or the intracellular stages within biopsy specimens of human gastrointestinal mucosa. In tissue sections, a simple hematoxylin-and-eosin stain should suffice to identify the morphology of the intracellular life stages of the parasite in its unique apical location within the intestinal epithelial cell (47, 53, 74, 78). Mucosal biopsies may also identify important, treatable copathogens such as cytomegalovirus. In one study, the duodenal mucosa was infected in 93% of AIDS patients with cryptosporidiosis; consequently, this appears to be a reasonable site for biopsy in these patients (78).

A variety of diagnostic options are available for the detection of *Cryptosporidium* in clinical stool samples (Table 1). The particular assay used by a laboratory will depend on a number of factors, particularly cost containment and level of staff training. Auramine-rhodamine screening of stool sediment smears followed by modified Ziehl-Neelsen (acid-fast) staining is a sensitive and specific approach for the identification of *Cryptosporidium* oocysts in stool (81) and is utilized at my institution. Stringent morphologic criteria must be applied to the diagnosis to avoid confusion with other oocysts, such as *Cyclospora* oocysts, which are significantly larger (10 μ m). While this

TABLE 1. Diagnostic options for *Cryptosporidium* detection in clinical samples

Technique	Advantages	Disadvantages
Acid-fast stain (Ziehl-Neelsen, Kinyoun) Auramine-rhodamine	Low cost Rapid screening	Low sensitivity and specificity Low sensitivity and specificity; high cost (equipment)
EIA	Sensitive (32.9 ng of <i>Cryptosporidium</i> protein [Meridian Diagnostics, Inc., Cincinnati, Ohio]); minimal training required	High cost
IFA	Sensitive (100 oocysts/ml [Meridian Diagnostics, Inc., Cincinnati, Ohio])	High cost
PCR	Sensitive (1 oocyst) (150); permits genotyping	Nonstandardized methods; high cost; specialized training and equipment required

assay is usually adequate to diagnose cryptosporidiosis in symptomatic HIV-infected individuals who are excreting billions of oocysts (9), additional sensitivity may be desirable when screening immunocompetent individuals, asymptomatic individuals, or environmental samples; consequently, additional tests have been developed to improve upon the acid-fast stain. It is also likely that cryptosporidiosis is underdiagnosed because clinicians fail to consider this diagnosis in patients with diarrheal illnesses (particularly immunocompetent adults and children) and do not request stool analysis for *Cryptosporidium*, a test not normally included in routine stool analysis. Ideally, laboratories should have ongoing communication with public health departments and water utilities in order to recognize outbreaks and should have mechanisms in place to screen all specimens for *Cryptosporidium* in such settings.

Immunoassays

Several immunofluorescence assay (IFA) kits that are commercially available for the detection of *Cryptosporidium* in stool and environmental samples are listed in Table 2. Commercially available enzyme immunoassays (EIAs) for the detection of *Cryptosporidium* are also listed in Table 2. A recent comparative study by Garcia and Shimizu found high EIA (ProSpecT [Alexon] and Meridian Premier *Cryptosporidium* [Meridian Diagnostics]) sensitivity (98 to 99%) and specificity (100%) compared with the Meridian MERIFLUOR *Cryptosporidium*/*Giardia* kit (45). All IFA results (TechLab, Meridian MERIFLUOR) gave similar results, with 100% sensitivity and specificity. Graczyk et al. have found that an EIA (ProSpecT [Alexon]) and two IFAs (MERIFLUOR and Hydrofluor [Ensys, Inc.]) were very sensitive for the detection of *C. parvum* (100%) but that both tests showed some cross-reactivity for non-*parvum* *Cryptosporidium* oocysts (54). While such cross-reactivity is probably not significant in clinical samples, it may

misidentify *Cryptosporidium* species which are nonhuman pathogens within environmental samples (54).

PCR

PCR-based detection of microbes in clinical samples is attractive due to its extreme sensitivity and specificity. Additionally, the genetic information obtained from the sample may permit nonhuman pathogens to be distinguished from human pathogens. Methods for PCR-based detection of *Cryptosporidium* in clinical samples and drinking water have recently been reviewed (44, 100, 150). Several published PCR-based detection systems for *Cryptosporidium* in stool are summarized in Table 3. Some investigators have found high sensitivity for PCR-based assays (one oocyst) and suggest that these assays are more sensitive than microscopic analysis of acid-fast smears (98); unfortunately, no large comparative study has been performed to determine the ideal primers, PCR conditions, or stool extraction methods to use with clinical samples.

Several factors complicate the PCR-based detection of *C. parvum* in stool. Standard fixation in 10% buffered formalin may reduce the sensitivity of the PCR, particularly if fixation occurs over an extended period. Also, extended formalin fixation may alter the buoyancy of *C. parvum* oocysts, interfering with standard methods for purification of *C. parvum* oocysts from stool. PCR detection of oocysts from frozen stool is also possible, but the sensitivity may be reduced, probably due to rupture of oocysts during thawing. One method for oocyst purification from stool commonly used in the research laboratory involves density gradient centrifugation of stool (135, 139). While this method provides purified oocysts, ideal for PCR analysis, it is more suitable for a research laboratory than a clinical laboratory and may not be useful for specimens containing few oocysts. The PCR can be inhibited by numerous substances, including some stool components. Several investi-

TABLE 2. Commercially available assays for *Cryptosporidium* detection

Assay	Manufacturer
IFA	
Meridian MERIFLUOR <i>Cryptosporidium</i> / <i>Giardia</i>	Meridian Diagnostics, Inc., Cincinnati, Ohio
DetectIF <i>Cryptosporidium</i>	Northumbria Biologicals, Ltd., Northumbria, United Kingdom
TechLab <i>Giardia</i> /Crypto IF kit	TechLab, Blacksburg, Va.
TechLab Crypto IF kit	TechLab, Blacksburg, Va.
EIA	
Alexon ProSpecT <i>Cryptosporidium</i> Rapid Assay	Alexon, Inc., Sunnyvale, Calif.
Alexon ProSpecT <i>Cryptosporidium</i> Microplate Assay	Alexon, Inc., Sunnyvale, Calif.
Meridian Premier <i>Cryptosporidium</i>	Meridian Diagnostics, Inc., Cincinnati, Ohio

TABLE 3. PCR-based methods for detection of *Cryptosporidium* in stool

Reference	Oocyst purification	Extraction method	Target
Laxer et al. (71)	Sucrose/Percoll centrifugation	Prot K/Ph/Chl ^a	Anonymous (71)
Ranucci et al. (120)	Centrifugation	Boiling	COWP-190
Webster et al. (148)	NaCl centrifugation	Prot K/Ph/Chl	Anonymous (148)
Awad-El-Kariem et al. (7)	NaCl centrifugation	Prot K/Ph/Chl	SSU ^c rRNA
Leng et al. (76)	None	Prot K/PCR spin column	Anonymous (71)
Morgan et al. (97)	None	Boiling	Anonymous (97)
Benigno Balabat et al. (8)	None	Prot K/Ph/Chl	Anonymous (71)
Gobet et al. (48)	NaCl centrifugation	Boiling	Anonymous (71)
Zhu et al. (154)	None	Glass beads/boiling/EtOH ppt ^b	SSU rRNA

^a Proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation.

^b Ethanol precipitation.

^c SSU, small subunit.

gators have developed nucleic acid extraction methods for stool to remove these inhibitors (48, 76, 154). Unfortunately, many of these methods are quite complex, and detailed comparative studies have not been performed to identify the most useful technique.

TREATMENT

One of the most biologically intriguing, and clinically frustrating, features of cryptosporidiosis is its resistance to antimicrobial drugs. Unlike many of its relatives (*Toxoplasma gondii*, *Eimeria*, and *Plasmodium*), there is no curative therapy for cryptosporidiosis, despite in vitro and in vivo testing of hundreds of compounds. One possible explanation for this is that *Cryptosporidium* establishes a compartment within the host cell, which is morphologically different from the setting used by the related parasites. This unique parasitophorous vacuole may somehow shelter the parasite from antimicrobial drugs (56).

Because the clinical course of cryptosporidiosis depends largely on the immune status of the host, treatment options vary accordingly (55). In immunocompetent adults and children, no specific therapy is indicated, since the disease is self-limiting; however, as in any diarrheal illness, hydration must be carefully monitored. In individuals with persistent diarrhea, an underlying immunodeficiency (HIV infection, congenital immunodeficiency, etc.) should be considered. In developing countries, children with cryptosporidiosis often have associated (or subsequent) malnutrition, which should be addressed.

In immunocompromised hosts, particularly AIDS patients with CD4 cell counts below 200/mm³, cryptosporidiosis can be life-threatening and must be treated aggressively. Initially, the nutritional, hydration, and electrolyte status of the patient should be assessed and corrected with intravenous hydration, if necessary. Antimotility agents, such as opiates and somatostatin analogues, may also be used. In people with AIDS, the ideal treatment involves partial restoration of immune function with HAART. Several case reports have demonstrated the resolution of cryptosporidial diarrhea coincident with a rise in CD4 cell count upon combination antiretroviral therapy (12, 14, 42). While laboratories should be poised to respond to cases of cryptosporidiosis among individuals failing to respond to HAART, we have seen few such cases; most of our cases of AIDS-related cryptosporidiosis occur in patients who have never received HAART.

If HAART therapy is not possible, several antibiotics that have some efficacy against *Cryptosporidium* (paromomycin, nitazoxanide, azithromycin) have been reported and should be considered. Of these, paromomycin has been the most widely

used and has consistently displayed at least partial activity in experimental systems and clinical trials (39, 41, 139). A combination of paromomycin and azithromycin has also been proposed for the treatment of cryptosporidiosis (129). Other experimental therapies, like bovine hyperimmune colostrum, may also be considered (28). In patients with severe disease, infection with a copathogen such as cytomegalovirus should also be considered and treated.

Nitazoxanide (NTZ) is the latest drug to be widely tested against human cryptosporidiosis. NTZ is a nitrothiazole benzamide with broad antimicrobial activity. An open-label study of NTZ in 15 Mexican AIDS patients with cryptosporidiosis found parasite clearance in nearly 100% of patients, triggering larger studies in the United States (38). Another small, uncontrolled African study of NTZ also suggested that it has some efficacy (32). Unfortunately, one larger clinical trial and in vivo animal studies have been less encouraging (31, 139). Controlled clinical trials (ACTG 336) to determine the efficacy of NTZ in treating cryptosporidiosis are under way.

The best approach to prevention of cryptosporidiosis in HIV-infected individuals is the maintenance of immune system function by using HAART, since chronic cryptosporidiosis occurs only in severely immunosuppressed individuals. Also, it has been suggested that antibiotic regimens containing clarithromycin, aimed at preventing mycobacterial infections in severely immunosuppressed individuals, may inadvertently have a protective effect against cryptosporidiosis (60, 63). Avoidance of tap water has been touted in the AIDS community, but no clinical trial has confirmed the efficacy of this approach. Despite this, numerous commercial filters are available to remove oocysts from drinking water. Bottled water has also been advocated to prevent cryptosporidiosis, but few regulations are in place to guarantee these products.

PATHOPHYSIOLOGY

In general, diarrhea develops when intestinal absorption is impaired or secretion is enhanced. Both of these processes are regulated by the intestinal epithelial cells which are infected by *Cryptosporidium* (26). Several investigators have identified impaired glucose-stimulated Na⁺ and H₂O absorption and/or increased Cl⁻ secretion in experimental models of cryptosporidiosis (4, 5, 94). In addition to these transport defects, abnormalities in the barrier properties of the intestinal epithelium, mediated in part by intercellular junctional complexes, contribute to *Cryptosporidium* diarrhea. Two groups have found evidence of permeability defects and decreased resistance across *C. parvum*-infected intestinal cell lines (1, 57). In addition, both groups found that *C. parvum* infection of these

monolayers resulted in the release of cytoplasmic lactate dehydrogenase, consistent with cellular injury, which ultimately resulted in cell death. Another group has suggested that *Cryptosporidium* induces apoptosis in biliary epithelial cells, but this mechanism of cell death has not been confirmed in vivo (25). Malabsorption and abnormal intestinal permeability (decreased vitamin B₁₂ absorption, decreased D-xylose absorption, abnormal lactulose/mannitol permeability test) have been confirmed in people with AIDS and cryptosporidiosis (53, 133). One mechanism for the induction of intestinal secretion by *Cryptosporidium* may involve the stimulation of prostaglandin production by intestinal epithelial cells (70).

BIOLOGY

Biochemistry

Like other protozoan parasites, *Cryptosporidium* appears incapable of de novo purine synthesis and relies on salvage pathways for hypoxanthine, guanine, and adenine. Studies with radiolabeled purine precursors (formate and glycine) indicate that these compounds are incorporated into host cells but not intracellular *C. parvum*. Enzymatic activity necessary for purine salvage (hypoxanthine, guanine, and xanthine phosphoribosyltransferase) was identified in *C. parvum* sporozoites and may localize to a single enzyme. Such an enzyme may serve as an antiparasitic drug target (33). Keithly et al. have identified a polyamine biosynthesis pathway in *C. parvum* which is found chiefly in plants and some bacteria but not mammalian cells (64). The lead enzyme of this pathway, arginine decarboxylase, is sensitive to a specific, irreversible arginine decarboxylase inhibitor, which reduces the intracellular growth of *C. parvum*. Another potential drug target is the shikimate pathway, in which (in plants) chorismate is converted to *p*-aminobenzoic acid, folate, and other aromatic compounds (121). *Cryptosporidium* and other Apicomplexan parasites were found to be sensitive to glyphosate, an inhibitor of the shikimate pathway. This inhibition also provides circumstantial evidence for the existence of a plastid-like organelle in *Cryptosporidium*, similar to that described for *Plasmodium* and *Toxoplasma* (68, 87).

Molecular Genetics

Karyotypic analysis suggests that *C. parvum* contains eight chromosomes, ranging in size from 0.945 to 2.2 Mb, giving a total haploid genome size of approximately 10.4 Mb (11, 59). Also, one group has identified a low-molecular-weight molecule which may correspond to the 35-kb circular, extrachromosomal DNAs (plastids) found in other Apicomplexan parasites (11).

Recently, significant progress has been made toward understanding the *C. parvum* genome through expressed sequence tag (EST) and genome sequence survey (GSS) DNA-sequencing projects, as well as a genome-mapping project. To date, the EST project has isolated and partially sequenced 567 ESTs, with 37% of the unique clones demonstrating significant homology to GenBank sequences. A summary of this data can be found on the World Wide Web (31a). Two projects to sequence random fragments of *Cryptosporidium* genomic DNA are under way. One project has determined the sequence of 1,507 fragments, totaling more than 888,000 bp of new sequence; 27% of these sequences demonstrated homology to GenBank sequences. The second GSS project has sequenced 654 fragments, totaling more than 324,700 bp, with 16% of the unique sequences demonstrating homology to GenBank sequences. In addition to these sequencing projects, a complete

map of the eight *C. parvum* chromosomes has recently been completed (115). All of these projects will greatly facilitate future studies of this organism.

rRNA gene structure is central to the phylogenetic classification and genotyping of microbial organisms; therefore, the recent characterization of the *C. parvum* rRNA gene organization by Le Blancq et al. was an important milestone in *Cryptosporidium* research (72). These investigators found that the small- and large-subunit rRNAs are 1.7 and 3.6 kb, respectively; a 151-bp putative 5.8S rRNA was also identified. Like other eukaryotes, the rDNA unit is arranged as a 5' small-subunit rRNA-internal transcribed spacer 1 (ITS1)-5.8S rRNA-ITS2-large-subunit rRNA 3' complex. There appear to be five copies of the rDNA per haploid genome, which are not organized in the conventional head-to-tail arrangement but, rather, are dispersed throughout the genome to at least three different chromosomes. Interestingly, there are two distinct types of rDNA units (four copies of type A and one copy of type B) which contain marked differences in the ITS regions. Knowledge of this intraorganismal heterogeneity is crucial when interpreting PCR-based genotyping of *C. parvum* isolates based on rRNA heterogeneity. Similar rDNA organization is found in the Apicomplexan protozoa *Plasmodium*, *Babesia*, and *Theileria*. In *Plasmodium*, the two classes of rRNAs are differentially expressed during the life cycle of the parasite. Such information on developmental expression is not yet available for *C. parvum*. Curiously, another Apicomplexan parasite, *T. gondii*, has an entirely different rDNA organization, containing multiple copies of tandemly arrayed rDNAs. The phylogenetic and biologic consequences of these differences have not been resolved.

C. parvum was recently found to contain two small extrachromosomal, cytoplasmic, virus-like double-stranded RNAs (65, 66). These RNAs (1,786 and 1,374 nucleotides) each contain a single open reading frame which encodes a putative RNA-dependent RNA polymerase and a protein with limited homology to mammalian mitogen-activated c-Jun NH₂-terminal protein kinases, respectively. Virus-like particles were not observed within sporozoites by electron microscopy, but other data suggested that the RNAs may be unencapsidated. Although there are several examples of protozoan viruses which infect *Giardia*, *Trichomonas*, and *Leishmania*, these viruses do not resemble the *C. parvum* virus-like RNAs. To date, these RNAs have been identified in many laboratory isolates and commercial samples of *C. parvum* but have not been found in five non-*C. parvum* members of the genus (66).

GENETIC DIVERSITY

Paralleling the clinical diversity of cryptosporidiosis is increasing evidence of molecular heterogeneity among *C. parvum* isolates. Western blot analysis of *C. parvum* oocyst antigens with a *Cryptosporidium*-specific monoclonal antibody revealed heterogeneity among several human, calf, and lamb isolates (105). A second study of *C. parvum* antigens also found heterogeneity among human isolates and between human and animal isolates when using polyclonal and monoclonal antibodies against *C. parvum* antigens in Western blots (106). Two-dimensional gel electrophoresis of *C. parvum* sporozoite proteins from five different isolates has revealed a 106-kDa peptide which differed in its isoelectric point in several of the isolates and a 40-kDa protein in one isolate which was not present in the others (89). Finally, isoenzyme typing of *C. parvum* isolates from different geographical locations revealed two isoenzymes of phosphoglucosyltransferase and hexokinase which segregated according to human or animal origin (6).

TABLE 4. Genotyping of human *Cryptosporidium* isolates

Reference	Genetic marker ^d	No. of <i>C. parvum</i> isolates (no. of HIV ⁺ persons)	No. of genotype 1 isolates/ total no. of human isolates
Carraway et al. (15)	rDNA	5 (2)	2/2
Bonnin et al. (13)	Repetitive DNA fragment	23 (13)	6/13
Carraway et al. (16)	Poly(T)	15 (2)	6 ^a /6
Peng et al. (112)	TRAP-C2	39 (1)	13 ^b /16
Spano et al. (132)	COWP	14 (NI ^c)	5/7
Morgan et al. (95)	rDNA	47 (NI)	28/32
Morgan et al. (99)	18S rDNA, acetyl-CoA synthase	27 (NI)	6/7
Widmer et al. (152)	Poly(T), COWP, RNR	13 (13)	10/13
Widmer et al. (151)	β -Tubulin	6 (4)	3/4 ^b
Spano et al. (131)	TRAP-C1	3 (NI)	3/3
Spano et al. (130)	TRAP-C1, COWP, poly(T), RNR	28 (2)	12/17
Sulaiman et al. (134)	TRAP-C2	92 (21)	41/50

^a Three mixed genotype 1 and 2 isolates.

^b Two hybrid genotypes.

^c NI, not indicated.

^d Abbreviations: CoA, coenzyme A; TRAP, thrombospondin-related adhesive protein; COWP, *Cryptosporidium* oocyst wall protein; RNR, ribonucleotide reductase.

Recent genetic studies of *Cryptosporidium* support the evidence of isolate diversity obtained by analysis of sporozoite proteins and suggest that there are at least two subtypes of human isolates. Restriction fragment length polymorphism (RFLP) analysis of *C. parvum* genomic DNA from three bovine and three human isolates revealed polymorphism between the human and bovine isolates and among the human isolates (110). Another technique, the random amplification of polymorphic DNA (RAPD) (149), has also revealed genetic heterogeneity among *C. parvum* isolates. According to one study, RAPD analysis of 25 *C. parvum* isolates revealed two genotypes; one genotype was unique to human isolates, while the other was found predominantly in isolates from calves and lambs (96). Another recent study examining five *C. parvum* isolates by RAPD also found genetic heterogeneity among human and animal isolates (15). Finally, RFLP analysis of a repetitive DNA sequence in 23 human and calf *C. parvum* isolates revealed the same profile in all calf isolates but two patterns among the human isolates, one of which was identical to the profiles in the calf isolates (13). Sequence analysis of three *C. parvum* genes (encoding dihydrofolate reductase-thymidylate synthase, α -tubulin, and β -tubulin) from a calf isolate and a human isolate has also identified an unexpectedly high level of polymorphism (145).

More recently, several investigators have used RFLP and DNA sequencing of polymorphic genes to determine the genotype of larger numbers of *C. parvum* isolates from animals, humans infected in outbreaks, and people with AIDS. These studies continue to support the concept that humans can be infected by two genetically distinct types of *Cryptosporidium*, designated genotype 1 (human type) and genotype 2 (bovine type). The results of several recent studies are summarized in Table 4. These studies suggest that cattle are exclusively infected by genotype 2 isolates and that most human infections are caused by genotype 1 parasites. Several of the human infections caused by genotype 2 isolates appear to be zoonotic, with an identifiable bovine source. Unfortunately, division of *C. parvum* isolates into two discrete groups is probably an oversimplification. First, several investigators have found evidence of isolates containing a mixture of genotype 1 and genotype 2 alleles (16). Second, multilocus genotype determination has failed to identify recombinant genotypes, suggesting that most genotype 1 and genotype 2 isolates are reproductively isolated populations (130); however, the same investigators have identified polymorphism in the β -tubulin gene intron

which might have arisen from a recombination event (151). Although these studies have contributed significantly to our understanding of genetic diversity among *Cryptosporidium* isolates, most have been small studies with geographically diverse isolates from sporadic outbreaks. Larger studies focused on specific populations, such as people with AIDS or children in developing countries, are necessary before we can have a more complete understanding of the molecular epidemiology of cryptosporidiosis.

Several investigators have speculated that *Cryptosporidium* isolates may also vary in virulence, in part explaining the clinical diversity observed in cryptosporidiosis. One study has suggested that genotype 1 isolates are less virulent than genotype 2 isolates in in vitro assays measuring the disruption of intestinal cell monolayers (monolayer resistance and cytoplasmic lactate dehydrogenase release) (152). Also, experimental data indicates that the host ranges for genotype 1 and 2 isolates are different, with genotype 1 isolates being infectious mainly to humans and primates and genotype 2 isolates being infectious to most mammals (112, 117, 152).

IMMUNOLOGY

Much of what is known about the immune response to *Cryptosporidium* has been learned from experimental murine models. While such models are useful, they have several limitations. First, immunocompetent adult mice are not susceptible to *C. parvum* infection. For unclear reasons, only immunocompetent mouse pups (younger than 26 days) are susceptible to infection (107). Second, few murine models completely mimic human infection, since infected mice do not typically develop diarrhea. Recent exceptions are the gamma interferon (IFN- γ) knockout mice, which can be infected by relatively few oocysts and will experience weight loss and wasting and eventually die (58, 90). Mice with severe combined immunodeficiency (SCID mice) are also susceptible to *C. parvum* infection, and have been widely used to study the immunology of cryptosporidiosis (86).

The two key immune components necessary for prevention and/or resolution of cryptosporidiosis as shown by these studies are CD4⁺ lymphocytes and IFN- γ (23, 24, 85, 113, 141). Depletion of IFN- γ by intraperitoneal injection of anti-IFN- γ antibodies resulted in a shortened prepatent period, increased oocyst excretion, and early disease and death (85). Also, a human case of protracted cryptosporidiosis in a patient with

IFN- γ deficiency has been reported (51). Selective immune cell reconstitution experiments have defined an important role for CD4⁺ lymphocytes in the prevention or resolution of cryptosporidiosis (23, 85, 113) as well as a possible role for CD8⁺ lymphocytes (85). Similarly, mice which lack functional CD4⁺ lymphocytes (major histocompatibility complex class II-deficient mice) were more susceptible to infection than were control mice and were unable to clear the infection; CD8-deficient mice (major histocompatibility complex class I-deficient mice) resolved the infection (3). Also, mice deficient in T-cell receptor α (present on most CD4⁺ lymphocytes) were more susceptible to infection than were controls. Gamma/delta-T-cell-deficient neonatal mice were more susceptible than control mice but were able to clear the infection (147). Since the cytokine interleukin-12 can induce IFN- γ production, it is not surprising that treatment of mice with interleukin-12 prevented or greatly reduced the severity of infection (142). In experimental murine infections, neither tumor necrosis factor nor natural killer cells were important in resolving infection (24, 85, 122). The mechanisms by which *Cryptosporidium*-infected intestinal epithelial cells initiate immune responses are not entirely clear. One apparent mechanism in human cells involves the production of tumor necrosis factor alpha, interleukin-8, and C-X-C chemokines by infected mucosa (69, 127).

OUTBREAKS OF WATERBORNE INFECTION

Outbreaks of cryptosporidiosis due to drinking-water contamination, including a massive outbreak in Milwaukee, Wis. (80), have been increasingly recognized. Several recent reviews have dealt with this important public health concern (44, 83, 128). There are four major factors that contribute to these outbreaks: (i) the prevalence of *Cryptosporidium* in source water is high; (ii) *Cryptosporidium* oocysts are refractory to chlorine treatment of drinking water; (iii) coarse filtration methods normally performed on surface drinking waters do not efficiently remove *Cryptosporidium* oocysts, due to their small diameter; and (iv) the infectious dose of *Cryptosporidium* for humans is extremely low (73). Although water treatment deficiencies have been identified in some outbreaks, at least one outbreak has occurred in association with a modern treatment facility where treatment was well documented and unremarkable (50). In the United States, routine testing of drinking water is mandatory for all surface water utilities serving populations of >100,000 persons; however, the current methods for *Cryptosporidium* detection may underestimate parasite numbers (44). In several situations, outbreaks were caused by contamination of recreational water in swimming pools and sprinklers (19, 84). In addition to drinking water, food contamination has been implicated in several outbreaks of cryptosporidiosis (17, 18, 91). Presumably these outbreaks were due to fecal contamination of food by infected animals or food workers. Such incidents may lead to increased regulation of potentially infected food products imported into the United States. In the future, sensitive PCR-based assays may facilitate the detection and genotyping of *Cryptosporidium* in environmental sources.

CONCLUSIONS

Cryptosporidium is a highly infectious cause of diarrheal illness around the world. The human host range is broad and includes people with AIDS, children in developing countries, and outbreaks among immunocompetent individuals. Within many of these groups, the manifestations of disease are diverse, ranging from asymptomatic infections to life-threatening

illness. Recent evidence suggests that humans can be infected by at least two genetically different types of *Cryptosporidium*. This diversity may, in part, explain the clinical spectrum of cryptosporidiosis, but other factors, including host differences, are also likely to be important.

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