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Waterborne Protozoan Pathogens

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INTRODUCTION

Infectious diseases, once expected to be eliminated as public health problems, remain the leading cause of death worldwide. Dramatic changes in society, technology, travel, and the environment, together with the diminished effectiveness of certain approaches to disease control, have propelled the United States and the rest of the world into a new era. The spectrum of infectious diseases is expanding, and the incidence of many infectious diseases once thought conquered is increasing (38a).

Several developments have affected the prevalence and detection of intestinal parasites in the United States. These include (i) increased immigration from southeast Asia, the Caribbean, and Central America; (ii) the AIDS epidemic and the prevalence of opportunistic infections in these patients; (iii) rapid expansion of the day care industry, with increased recognition of *Giardia lamblia* and *Cryptosporidium parvum* as common pathogens in that setting; (iv) the development of improved stool examination techniques to identify *Cryptosporidium* species; and (v) recognition of these parasites as pathogens of immunologically competent individuals (92).

The U.S. public health system is being challenged by many newly identified pathogens and syndromes, such as cryptosporidiosis, microsporidiosis, and cyclosporiasis. These emerging infections, which may be transmitted by contaminated foods, recreational waters, surface water, and groundwater intended for drinking, place entire communities at risk. In the spring of 1993, contamination of a municipal water supply with *Cryptosporidium* spp. caused the largest recognized outbreak of waterborne illness in the history of the United States (115).

An increasing percentage of our population is elderly, and a growing number of persons are immunosuppressed because of human immunodeficiency virus (HIV) infection, organ transplantation, or cancer chemotherapy. These populations are at increased risk for emerging infections. Specifically, these populations are highly susceptible to opportunistic infections, and an ever-expanding array of such infections is being seen in patients with AIDS and other forms of immunosuppression. The identification of certain opportunistic pathogens in immunocompromised populations has also led to the recognition of these agents in persons with normal immunity; this has happened with *Cryptosporidium* spp. (38a).

Since 1971, the Centers for Disease Control and Prevention (CDC), in collaboration with the Environmental Protection Agency (EPA), have tabulated data concerning waterborne disease outbreaks (WBDOs) separately from those for foodborne disease outbreaks. In 1989, responsibility for the surveillance system shifted within CDC to the Division of Parasitic Diseases, Parasitic Diseases Branch, primarily because of the prominent role of *Giardia lamblia* as the etiologic agent in WBDOs (81). Tables 1 and 2 summarize WBDOs from 1984 to present.

Identification of the etiologic agent of a WBDO is dependent on timely outbreak recognition so that appropriate clinical and environmental samples can be obtained. The interests and expertise of the investigators and the routine practices of local laboratories also influence the identification of the causative agent. For example, in most laboratories, routine stool examination for ova and parasites does not include the special procedures needed to identify *Cryptosporidium* spp., microsporidia, or the newly recognized *Cyclospora* sp. Based on routine diagnostic methods, the number of WBDOs probably represents only a fraction of the total number that occur. The likelihood of an outbreak coming to the attention of health authorities varies considerably from one locale to another and depends largely on consumer awareness, physician interest, clinical laboratory procedures, and disease surveillance activities of state and local health and environmental agencies.

The lack of surveillance and limited availability of appropriate diagnostic tests have hindered public health efforts to prevent and control outbreaks. *Cryptosporidium parvum* was first recognized as a significant human pathogen in the early 1980s but has not received adequate public health attention. Cases do not have to be reported to CDC's National Notifiable Disease Surveillance System, and most state laboratories either do not routinely carry out testing to detect *Cryptosporidium* spp. or perform such testing only if specifically requested to do so (92).

Protozoal parasites were the most frequently identified eti-

TABLE 1. Recently reported WBDOs associated with water intended for drinking

Yr (reference)	No. of outbreaks and cases for protozoan parasite:			
	Cryptosporidium parvum	*Cyclospora cayetanensis*	*Entamoeba histolytica*	*Giardia lamblia*
1984 (36)	1 outbreak, 117 cases		1 outbreak, 4 cases	6 outbreaks, 879 cases
1985 (36)				3 outbreaks, 741 cases
1986–1988 (106)	1 outbreak, 13,000 cases			9 outbreaks, 1,169 cases
1989–1990 (81, 82)		1 outbreak, 21 cases		7 outbreaks, 697 cases
1991–1992 (130)	3 outbreaks, 3,551 cases			4 outbreaks, 123 cases
1993–1994 (98)	5 outbreaks, 403,246 cases[a]			5 outbreaks,[b] 385 cases
Total	10 outbreaks, 419,914 cases	1 outbreak, 21 cases	1 outbreak, 4 cases	34 outbreaks, 3,994 cases

[a] 403,000 cases were from one outbreak.
[b] *E. histolytica* was detected in some stool specimens.

ologic agents in WBDOs in 1991 to 1992 (130) and 1993 to 1994 (98). From 1978 through 1991, *Giardia lamblia* was the most commonly implicated pathogen. In 1992, the same numbers of outbreaks of giardiasis and cryptosporidiosis were reported. The increased identification of cryptosporidiosis may be due to heightened awareness that the organism does cause WBDOs. Outbreaks caused by *Cryptosporidium* spp., however, are still underrecognized. An important factor in the recognition of the outbreaks in 1992 was routine screening of stool specimens for *Cryptosporidium* spp. by certain local laboratories (130). In the Milwaukee outbreak of 1993, two laboratories identified *Cryptosporidium* oocysts in stool samples from seven adult area residents: no evidence of increased or unusual patterns of isolation of any other enteric pathogen were reported. This information, along with an evaluation of the city's water treatment plant records, pointed to the water supply as the likely source of infection and led to a "boil water" advisory (115).

The magnitude of the Milwaukee outbreak (130), coupled with its association with water obtained from a municipal water plant that was operating within existing state and federal regulations, emphasized the need for (i) improved surveillance by public health agencies to detect and prevent such outbreaks, (ii) reporting of cryptosporidiosis to CDC, and (iii) the submission by health care providers of stool specimens for examination for persons who have symptoms compatible with cryptosporidiosis. The importance of coordination among interested groups and agencies to respond appropriately to such outbreaks was also emphasized (5, 90). Not surprisingly, clinical laboratory procedures are well ahead of the water-testing methodologies in detecting the presence of such organisms. Because the potential threat of infection via the waterborne route is just being recognized for many of these organisms, it is imperative that the water industry also turn its attention to finding ways to detect these emerging and well-recognized protozoan pathogens in water.

TABLE 2. Reported WBDOs associated with recreational water[a]

Yr (reference)	No. of outbreaks and cases for protozoan parasite:		
	Cryptosporidium parvum	*Giardia lamblia*	*Naegleria* spp.
1984 (36)			
1985 (36)		2 outbreaks, 24 cases	
1986–1988 (106)		2 outbreaks, 1,169 cases	
1989–1990 (81, 82)		7 outbreaks, 697 cases	3 cases
1991–1992 (130)	2 outbreaks, 526 cases	4 outbreaks, 123 cases	6 cases
1993–1994 (98)	6 outbreaks, 663 cases	4 outbreaks, 141 cases	1 case
Total	8 outbreaks, 1,189 cases	12 outbreaks, 499 cases	10 cases

[a] Swimmer's itch is not included in this table.

This review is designed to give information on waterborne protozoan pathogens in a format that will be useful to clinical microbiologists and interested public health personnel. Parasite detection and identification present a challenge for the microbiologist. Correct organism identification depends on personnel training and experience, particularly involving microscopy. This crucial area of microbiology has become more important as clinical laboratories are under pressure to screen more samples, be constantly aware of cost containment, and train and update personnel. In many ways, the clinical laboratory is the first line of defense in providing a warning that an infectious disease is threatening the community. This review addresses the history, life cycle, incubation period, symptoms, incidence, therapy, current detection methods, and detection methods under development for each relevant parasite. It also addresses the water quality testing regulations for *Giardia* spp. and those proposed for *Cryptosporidium* spp. to give a complete picture of the complex issues dealing with waterborne parasites.

GIARDIA LAMBLIA

History

Leeuwenhoek in 1681 described parasites similar to *G. lamblia* in his own feces, but the first report was credited to Lambl in 1859. *G. lamblia* is the most commonly diagnosed flagellate in the intestinal tract. There is still debate over the appropriate classification and nomenclature of *Giardia* species. Three groups related to structural variations have been suggested by Filice: *Giardia* spp., the agilis group from amphibians; the muris group from rodents, birds, and reptiles; and the intestinalis group from a variety of mammals (including humans), birds, and reptiles (64). Within the United States, the term "*Giardia lamblia*" has been commonly used by health care workers, and it will probably continue to be reported as such (68).

Life Cycle

The cycle is composed of two stages: an actively multiplying trophozoite and a resistant cyst. Cysts survive in food and

water. When ingested, the cyst passes through the stomach, where the acid environment triggers excystation, which usually takes place in the duodenum. The trophozoites attach to the duodenal or proximal jejunal mucosa, probably via contraction of the ventral disk, and replicate by repeated binary division. Cyst formation takes place as the trophozoites move through the colon (68, 107).

Incidence

G. lamblia is the most commonly isolated intestinal parasite throughout the world and is especially prevalent in children in developing countries (29). In a recent study to document patterns of intestinal parasitism in the United States, *G. lamblia* was the most frequently identified parasite (92). Using microscopy, rates of detection for this parasite vary between 2 and 5% in industrialized countries and between 20 and 30% in developing nations. In contrast to the general population, pockets of high or low prevalence have been described, and they appear to be more common in urban areas than in rural populations (66).

Symptoms

The incubation period is usually 1 to 2 weeks. Onset usually begins with a feeling of intestinal uneasiness, followed by nausea and anorexia. Low-grade fever and chills may also be early symptoms. Subsequent symptoms may include explosive, watery, foul-smelling diarrhea; marked abdominal gurgling and distension associated with the passage of foul gas; and perhaps belching, with a foul taste. Upper or mid-epigastric cramps may also occur. The acute stage, which lasts 3 or 4 days, can resemble other causes of traveler's diarrhea and is often not recognized as being due to giardiasis (197).

Therapy

Quinacrine, metronidazole, tinidazole (not available in the United States), furazolidone, and paromomycin are all commonly used treatment regimens for giardiasis (4).

Current Detection Methods

Examination of fresh stool for cysts and trophozoites and examination of a permanent stained smear are still the most widely used detection methods. Because the parasites mechanically adhere to the intestinal mucosa by the ventral disk, a series of five to six stools may be examined without recovering the organism (68). Procedures using the enzyme immunoassay (EIA) have been developed to detect *Giardia* spp. antigen in feces. An evaluation of a commercially available EIA kit by using stool specimens from diapered children attending a day care center compared microscopic examination of stool specimens for parasites with the EIA. The EIA was at least as sensitive for *G. lamblia* as were microscopic wet examinations, and it was 100% specific. Because many specimens can be read by a single technician in a short period, the EIA is also potentially less expensive than microscopic examination of stools (6). A direct fluorescent-antibody (DFA) method using monoclonal antibodies can detect very low numbers of organisms in a shorter examination time than that needed for examination of permanent-stained smears. No false-positive results were reported (69). This assay can be used for detecting both *Giardia* and *Cryptosporidium* spp. on the same slide. Table 3 summarizes the DFA and EIA commercial kits.

Detection Methods under Development

Nucleic acid-based detection of *G. lamblia* cysts and trophozoites in fecal specimens has proven to be a challenge due in part to the difficulty of lysing cysts and, more importantly, the large amount of other DNA and inhibitory substances present in these clinical specimens. Only one reported study has applied PCR to the evaluation of human fecal samples. The sensitivity of the small-subunit rRNA PCR assay resulted in some false-negative and false-positive results compared with microscopic detection (193).

Association with Water

Giardia cysts have been detected in 81% of raw water samples (103) and 17% of filtered water samples (104) in the United States. *G. lamblia* continues to be the most frequently identified etiologic agent in WBDOs. As in the past, outbreaks of giardiasis are associated with ingestion of unfiltered, inadequately chlorinated surface water or groundwater influenced by a surface water (82). Waterborne transmission has been implicated as a cause of giardiasis in travelers. Backpackers who drink unfiltered stream water are also at risk (4). Swimming pool-associated outbreaks of gastroenteritis have also been attributed to *Giardia* spp. (130).

NAEGLERIA FOWLERI

Life Cycle

There are three stages to the *Naegleria* life cycle: the trophozoite, the flagellate, and the cyst (184). Trophozoites are active and usually elongated with broadly rounded processes called lobopodia. Their cytoplasm is granular and contains vacuoles, and they feed on bacteria such as *Escherichia coli* (123, 124, 184). The flagellate stage is pear shaped and motile and eventually reverts to the trophic stage (25, 123, 124, 184). Cysts are usually spherical, smooth, double walled, and refractile, measuring about 10 μm in diameter (124, 184). Adverse environmental conditions cause the organisms to encyst (123). The portals of entry for human infection are the olfactory neuroepithelium and nasal passages, which are usually exposed to the flagellate stage during periods of swimming or bathing in hot baths or hot springs (95, 123, 184). Infection can also occur by breathing infectious cysts present in dust or soil particles (123, 124, 185). Once the organism has been inhaled, excystation occurs and the trophozoite penetrates the nasopharyngeal mucosa, migrates to the olfactory nerves, and invades the brain through the cribriform plate (25).

Incidence

While there are six species in the genus, *Naegleria fowleri* is the primary human pathogen, producing primary amebic meningoencephalitis (PAM), a rapidly progressing meningoencephalitis which is almost always fatal. *N. australiensis* may be pathogenic to a lesser extent (95, 123, 184). To date, there have been more than 192 reported cases of disease worldwide and more than 64 cases in the United States. While the numbers of cases may appear low, exposure to the organisms may be relatively common since antibodies to *Naegleria* spp. are widespread in human sera (25, 164).

Symptoms

No predisposing factors are necessary for human infections to occur (124). After a 2- to 7-day incubation period, the

TABLE 3. Comparison of commercial assays for *G. lamblia* detection

Kit name (reference)	Specificity/ sensitivity (%)	Comparison method	Time	Advantages	Disadvantages
ProSpecT Giardia EZ Microplate Assay (EIA) (Alexon)					
6	100/98	Microscopic exam	Not reported	Visual interpretation	Not recommended to replace ova and parasite exam
157	100/96	Microscopic exam	Not reported	Easy to run	
163	100/95	Microscopic exam	Not reported	Good screening technique	
206	99.8/97	Trichrome/microscopy, Merifluor	Not reported	Easy to test large numbers of specimens	
Merifluor (DFA) (Meridian)					
69	100/100	Routine ova and parasite microscopic exam	Exam time, 20–30 s/specimen	Short exam time	Centrifugation of sample
9	100/99.2	Chlorazole black E stain	2 min/slide	No concentration step Rapid scanning of stained slides	Possible false-negative without centrifugation step
206	99.8/100	Trichrome stain, ProSpecT	Not reported	Detects *C. parvum* at the same time	Concentration step
Giardia-CEL IF (DFA) (Bradsure Biologicals Ltd.)					
178	NR[a]	Thompson's stain	Not reported	Detected 10 times more cysts	

[a] NR, not reported in conventional terms.

symptoms of PAM are evident. Onset is abrupt, with rapidly progressive headaches, fever, nausea, vomiting, pharyngitis, and nasal obstruction or discharge (123, 124). As the symptoms persist, lethargy, confusion, and stiff neck develop. Convulsions may also occur, with progressive deterioration to coma and death within 1 to 14 days. The mean time interval from onset to death is 6.4 days (95, 114, 123, 184, 186). Other symptoms include abnormalities of taste and smell; seizures; cerebellar ataxia; nuchal rigidity; photophobia; palsies of the third, fourth, and sixth cranial nerves; and increased intracranial pressure. Cardiac abnormalities may also develop (114). Subclinical infections are possible in healthy people when these protozoa colonize the nose and throat (114, 124).

Therapy

Amphotericin B has been used successfully in two human infections (164).

Current Detection Methods

Naegleria spp. can be isolated from water by centrifugation and filtration (59, 120). With environmental monitoring as a form of prevention, the objective is to distinguish pathogenic *N. fowleri* from nonpathogenic thermophilic *Naegleria* spp. (174, 175). Identification of *N. fowleri* from water is based on morphology, temperature tolerance, pathogenicity (114), use of monoclonal antibodies (175), isoenzyme electrophoretic profiles, and DNA restriction fragment length polymorphisms (RFLP) (95). While these tests can discriminate between species, they are expensive and time-consuming and require the presence of large numbers of organisms (95, 174).

Clinical diagnosis of *N. fowleri* and subsequent patient survival depends most importantly on an initial awareness of the organism and accurate laboratory interpretation of the cerebrospinal fluid (CSF) wet mount (25, 26). A diagnosis of PAM should be suspected in every case of purulent meningoencephalitis in which no bacteria are found. Examination of fresh CSF is mandatory when the CSF pressure is not high enough to contraindicate a lumbar puncture. Motile amebas may be seen in CSF which appears cloudy and slightly hemorrhagic with increased numbers of erythrocytes and leukocytes, especially polymorphonuclear neutrophils (114). In wet mounts, active movement of the 8- to 20-μm trophozoites: needle-like acanthopodia can be observed, and sometimes the pear-shaped flagellate form can be seen swimming in the CSF (124, 184). The protein level in CSF is usually increased; glucose levels are normal or slightly reduced (114, 123).

Alternatively, a Cytospin instrument (Shandel Scientific) may be used to prepare the smears, which can be fixed in Schaudinn's fixative for up to 1 h. Staining with Giemsa stain reveals trophozoites (114, 123, 184). When stained, the ameba can be easily confused with monocytes and lymphocytes, and so it is important to note that the leukocytes have larger nuclei and lymphocytes have scant cytoplasm. Other fixatives and stains such as the modified Wheatley or Masson trichrome, hematoxylin-eosin, and iron hematoxylin may also be used (114, 124). Routine Gram stain is not usually helpful (124). *N. fowleri* cysts are not likely to be seen in tissue or CSF, because infection is rapidly fatal and the patient dies before the trophozoite can encyst (114).

For culture of CSF sediment, brain biopsy samples, or environmental samples, 1.5% nonnutrient agar is preseeded with a lawn of *E. coli* and will allow the growth of trophozoites (124, 184). Plates are incubated for 10 days at 37°C. In 4 to 5 days, trophozoites may be observed under ×10 magnification. As bacterial feeder cells are depleted, cysts may also be observed (124). *N. fowleri* will also grow in peptone-yeast-extract broth without added bacteria (axenic culture) and in mammalian cell cultures such as human fibroblasts (MRC-5), Vero monkey kidney cells, and HEp-2 cells (114, 120). In cell culture, a cytopathic effect is seen; this has been mistaken for virus-

induced cytopathic effect (114, 124). Retrospective diagnosis can be made on tissue specimens obtained at autopsy by using the immunofluorescent-antibody assay (IFA) or immunoperoxidase or standard stains (124). Serologic testing provides no help in the diagnosis of PAM (114).

A distinctive feature of *N. fowleri* is its ability to convert into a pear-shaped flagellate form. To distinguish between *Naegleria* and *Acanthamoeba* spp., the flagellate form can be induced by mixing a loopful of growth containing ameba from the agar plate with 1 ml of distilled water. In water, the majority of *N. fowleri* trophozoites will transform into free-living flagellates (123, 124).

Detection Methods under Development

Routine methods for detecting, identifying, and quantitating *Naegleria* spp. from environmental samples may be replaced by methods such as endonuclease digestion of DNA, PCR, flow cytometry, or DNA probes (96, 114). Limitations of PCR include environmental factors such as interference by humic acids, which are breakdown products of organic matter in the soil (174). The API ZYM product (Analytab Products) has been used to assay for isoenzyme differences to distinguish among the three thermophilic species (*N. fowleri*, *N. lovanensis*, and *N. australiensis*) (120).

Association with Water

The preferred environment for *N. fowleri* is the soil; however, heavy rains and runoffs introduce this organism into lakes, ponds, and surface waters (59, 120). *Naegleria* spp. are distributed worldwide in thermally polluted streams, and they tolerate temperatures of 40 to 45°C (25, 123, 174, 184). They can also be found in coastal water, freshwater, sewage, heating and ventilation units, poorly chlorinated swimming pools, artificial lakes, and warm water near discharge outlets of power plants (123). In Australia, one fatal case of PAM led to the detection of *Naegleria* spp. in the household water supply. This case emphasizes that PAM may be associated with washing and bathing as well as with swimming (120). Vertical distribution in freshwater has been correlated with physical, chemical, and biological parameters. Significant numbers of *Naegleria* spp. were found in water layers containing filamentous cyanobacteria and eubacteria, which serve as food sources. In addition, large numbers of organisms have been isolated from water with increased iron and manganese concentrations. As expected, *Naegleria* spp. are found in increased numbers in waters contaminated with coliforms. In addition, some species of *Naegleria* interact with *Legionella* spp. and are thought to play a possible role in the dissemination of *Legionella* in water (114).

ACANTHAMOEBA SPP.

Life Cycle

The life cycle of *Acanthamoeba* spp. has two stages, the trophozoite and the cyst. Trophozoites are slender, with spine-like processes called acanthopodia, which protrude from the surface and enable the organisms to undergo slow gliding movements. *Acanthamoeba* spp. are slightly larger than *Naegleria* spp., measuring 10 to 50 μm in diameter, and they divide by binary fission (25, 123, 124, 184). There is no flagellate stage (184). Cysts are wrinkled, double walled, star shaped, hexagonal, polygonal, or spherical and about 15 to 20 μm in diameter with two or more pores, opercula, or ostioles (123, 124, 184).

Incidence

Acanthamoeba spp. are opportunistic pathogens that produce a multifocal encephalitis called granulomatous amebic encephalitis (GAE), a chronic central nervous system disease of immunocompromised hosts (120), and various other disease states including keratitis and pneumonitis (11, 25, 124). A recent history of contact with water is usually not seen in patients with GAE. Some sources cite the lower respiratory tract as one possible route of invasion through inhalation of cysts from air, aerosols, or dust. Another route may be via skin infections (25, 70, 123, 124). Keratitis is the only water-related syndrome caused by *Acanthamoeba* spp. (184).

Antibodies in human sera are widespread, correlating with the ubiquitous nature of this organism (25). *A. castellani*, *A. culbertsoni*, and *A. polyphaga* are most commonly associated with keratitis and GAE (185). Organisms have been found as members of the normal flora and have been cultured from the upper airways of apparently healthy people, suggesting that infection may be common and self-limited in a competent host (11, 123).

Symptoms

Contact lens wearers may develop keratitis, a chronic ulceration of the corneal epithelium, producing symptoms that may be nonspecific and falsely diagnosed as herpes simplex virus keratitis. The severity of the condition may fluctuate and then rapidly progress as a corneal abscess characterized by a unique ring-shaped morphology with a relatively clear central area (114, 123). The incubation period varies depending on a number of host factors. When an eye infection involves previous trauma, the onset appears to be more rapid than in infections related to the use of contact lenses (68).

Therapy

Treatment regimens include ketoconazole and clotrimazole for some strains; propamidine isothionate is used to treat keratitis (68, 123).

Current Detection Methods

Processing, storage, and culture of specimens or environmental samples for *Acanthamoeba* spp. are similar to those described for *Naegleria* spp. (68, 124, 184). Direct smears and cultures of corneal scrapings can be performed to detect the organism (123). *Acanthamoeba* trophozoites are slightly larger than those of *Naegleria* spp. and are characterized by finely granular cytoplasm with vacuoles, a single nucleus surrounded by a clear halo and containing a large dense central nucleolus, and a large karyosome or endosome. The motility is sluggish (114, 123, 124). Giemsa and Gram stains are typically used to stain the organism. With Gram stain, trophozoites are only faintly visible and have an uneven cellular contour. If basic fuchsin is used in the counterstain step and the stain is overstained for 2 min, the trophozoites become more visible (25). Cysts are wrinkled, double walled, and refractile; star-shaped, hexagonal, polygonal, or spherical; and about 15 to 20 μm in diameter. They stain faintly or not at all (25, 114, 123, 124, 184). Other stains used are hematoxylin-eosin, periodic acid-Schiff, Gomori methenamine silver, Wright's stain, methylene blue, Congo red, Janus green, Masson trichrome, Wheatley's trichrome, and acridine orange (25). Calcofluor white stain has also been used successfully because its affinity to chitin and cellulose reveals the cyst wall as fluorescent green and the protoplasm as red-orange (25, 87, 114). The calcofluor method

has also been used to reveal numerous *Acanthamoeba* cysts adherent to contact lens surfaces, establishing a diagnosis in cases in which other smears were negative. Its use correlates well with culture techniques. By using this method, a fragment of contact lens is excised with sterile scissors, stain is added, and the lens is rinsed in tap water and mounted on a microscope slide under a coverslip (87).

In monoxenic culture with *E. coli*, the organism usually grows in 2 to 3 days, but cultures should be incubated for 2 weeks before results are reported as negative (114). In addition, one can perform cultures in E6 monkey kidney cells and human embryonic lung cells (184). Although *Acanthamoeba* spp. can grow on blood agar and chocolate agar, producing tracks in the agar surface, these tracks can be easily overlooked without microscopic examination of the plates (25). Because of these difficulties, the typical monoxenic culture method for *Acanthamoeba* spp. continues to be 1.5% nonnutrient agar preseeded with a lawn of *E. coli* (112, 177, 190).

Detection Methods under Development

There have been reports outlining the value of filtering corneal scrapings suspended in Page's ameba saline (13) placing the filter on a lawn of *E. coli*, and examining daily for trophozoites at ×10 magnification (25). In another recently described monoxenic culture technique, corneal scrapings are placed into 2.5 ml of sterile physiological saline which has been preseeded with 0.5 ml of a turbid *Stenotrophomonas* (previously *Xanthomonas*) *maltophilia* suspension. The trophozoites are visualized more easily in the liquid medium than on solid medium, and wet mounts of the liquid can be prepared and examined at higher magnifications than can the agar culture counterpart (25, 114). Unfortunately, identification by morphology is not always possible, since cyst morphology may vary with culture conditions. Nonmorphological criteria used for identification include isoenzyme electrophoresis, banding patterns of electrophoretically separated proteins, RFLP of mitochondrial or genomic DNA, PCR, and immunofluorescence (25, 174, 183, 189).

Association with Water

Acanthamoeba spp. pervade the entire environment and can be found in tap, fresh, coastal, and bottled mineral water; contact lens solutions and eyewash stations; soil, dust, and air; sewage; heating, ventilation, or air-conditioning units; dialysis machine and dental units; hot tubs; and gastrointestinal washings (123, 124, 166). Most episodes of keratitis occur during warm weather and often follow water exposure or a history of swimming in lakes and ponds while wearing contact lenses (25, 184). *Acanthamoeba* infections were also linked to nonsterile home-made saline solutions for contact lenses and were prevalent until Food and Drug Administration warnings increased awareness and decreased the incidence of disease (184).

ENTAMOEBA HISTOLYTICA

History

The trophozoite form of *Entamoeba histolytica* was described by Lösch in 1875 from organisms found in a patient with chronic dysentery. The clinical evidence of the association of this organism with dysentery was described by Councilman and LaFleur in 1891. Quincke and Roos described the cyst form in 1893, and Schaudinn named the organism *Entamoeba histolytica* in 1903 (27). In 1925, Brumpt showed that quadrinucleate cysts of *E. histolytica* were a complex of two species. He differentiated a pathogenic, invasive form from a nonpathogenic, noninvasive one that he called *Entamoeba dispar*. His explanation gained little support until recently, when Sargeaunt and Williams performed studies of isoenzyme typing that differentiated between pathogenic and nonpathogenic *E. histolytica* strains (160). Clark and Diamond reexamined Brumpt's claim in light of recent biochemical, immunological, and genetic studies. They redescribed the invasive parasite, retaining the name *E. histolytica*, and set it apart from the noninvasive parasite, *E. dispar* (43–45).

Life Cycle

The life cycle, as described by Dobell, includes trophozoite, precyst, cyst, metacyst, and metacystic trophozoite stages. The cyst form is the infective form for humans. The cysts survive in water and food. After cysts are ingested, no changes occur in an acid environment, but when the pH becomes neutral or slightly alkaline, the encysted organism becomes active, with the outcome being the emergence of small metacystic trophozoites. These organisms develop into normal trophozoites when established in the large intestine. Cyst formation occurs only within the intestinal tract. The one-, two-, and four-nucleated cysts are the infective stage transmitted from one host to another (27, 68).

Incidence

Infections with *E. histoloytica* occur worldwide, and it has been suggested that 12% of the world's population is infected with this organism. About 10% of those infected every year have clinical symptoms; 80 to 98% of these patients have symptoms related to the intestinal mucosa, and in the remaining 2 to 20%, the amebae invade beyond the intestinal mucosa. Once the infection is cleared, recurrence of invasive colitis or amebic abscess is unusual (27). With the exceptions of malaria-causing plasmodia and schistosomes, *E. histolytica* causes more deaths than any other parasite (152). Groups at high risk for amebiasis in developed countries include travelers, immigrants, migrant workers, imunocompromised individuals, institutionalized individuals, and sexually active male homosexuals (27).

Symptoms

The incubation period may vary from a few days to months, depending on the area of endemicity. Normally, the time frame ranges from 1 to 4 months. *E. histolytica* is unique among the intestinal amebae because it is able to invade tissues. The presentation of disease may range from an asymptomatic infection to a disseminated fatal disease. The four major intestinal syndromes caused by infection are asymptomatic colonization; acute amebic colitis, which usually presents with lower abdominal pain, frequent bloody stools over a period of several weeks, and fever; fulminant colitis, which occurs most often in children who present with diffuse abdominal pain, profuse bloody diarrhea, and fever; and ameboma, which presents as a completely asymptomatic lesion or as a tender mass accompanied by symptomatic dysentery (152).

Therapy

Iodoquinol and diloxanide furoate act on organisms in the intestinal lumen but are not highly effective against organisms in tissue. Metronidazole, chloroquine, and dehydroemetine are effective in the treatment of invasive amebiasis (68, 152).

Current Detection Methods

Laboratory diagnosis is by examination of a minimum of three stool specimens, using concentration and permanent stain techniques. The importance of the stained slide cannot be overemphasized, particularly when there are so few organisms in the stool specimen (27).

Detection Methods under Development

With the recognition of the pathogenic *E. histolytica* and the nonpathogenic *E. dispar*, nucleic acid-based probe assays have been developed. Several genes that contain DNA sequences differing between the pathogenic and nonpathogenic strains are the basis for DNA-based assays to detect and identify *E. histolytica*. Two assays have been tested with patient samples in limited studies. A PCR assay was used to study the epidemiology of pathogenic and nonpathogenic strains in a rural community in Mexico. Formalin-fixed stool samples were used for extraction of DNA. The PCR amplifications were performed with two sets of primers that discriminated between pathogenic and nonpathogenic strains. This assay had a sensitivity of 96% and a specificity of 98% (3). The second PCR method can be performed at room temperature in a 1.5-ml microcentrifuge tube format. No laborious hybridizations or use of radionucleotides is required. The entire procedure can be performed in 1 day. One trophozoite per mg of stool sample could be detected and differentiated in 0.1 g of specimen (93). An EIA also has been reported to distinguish the pathogenic and nonpathogenic strains directly from stool samples. Monoclonal antibodies directed against cross-reactive and *E. histolytica*-specific epitopes of the amebic galactose adhesin were used to detect both *E. dispar* and *E. histolytica*, giving a specificity and sensitivity of 97 and 87%, respectively (78). This EIA is commercially available from TechLab, Inc., Blacksburg, Va. Another commercially available EIA (Alexon, Inc. Sunnyvale, Calif.) detects antigen from pathogenic and nonpathogenic strains. Recently, a commercial antigen detection kit (TechLab, Inc.) designed to rapidly detect and differentiate *E. histolytica* from *E. dispar* in stool samples was tested. The kit was able to differentiate *E. dispar* from *E. histolytica* by the *E. histolytica*-specific test and was 95% sensitive and 93% specific compared with zymodeme analysis (77). No reports were available at this writing on comparisons and evaluations of these kits with respect by clinical procedures.

Association with Water

Transmission by water is common in developing countries, where much of the water supply for drinking is untreated and fecally contaminated. The use of human feces for fertilizer is also an important source of infection (27). In the United States, six WBDOs were attributed to *E. histolytica* between 1946 and 1980 (108).

CRYPTOSPORIDIUM PARVUM

History

In 1910, Tyzzer proposed the genus name *Cryptosporidium* for the protozoan parasite he frequently found in the gastric glands of laboratory mice. In 1912, he suggested a new species, *C. parvum* for a smaller organism that developed only in the small intestine of mice. In 1976, two independent groups reported the first cases of human cryptosporidiosis (52).

Life Cycle

When mature thick-walled oocysts in food or water are ingested, excystation of the oocyst occurs within the small intestine and the released sporozoites parasitize epithelial cells of the gastrointestinal tract. The excystation procedure usually requires reducing conditions, pancreatic enzymes, and bile salts; however, it is possible for *Cryptosporidium* oocysts to excyst in warm aqueous solutions without any special stimuli. Sporozoites penetrate enterocytes and develop into trophozoites. The trophozoite stage is intracellular beneath the host cell membrane but is extracytoplasmic. This stage divides by asexual multiplication into forms with six to eight nuclei, each maturing into a merozoite of type 1 meronts. This merozoite stage can either develop into a type II meront and initiate sexual multiplication and oocyst development or can reinitiate asexual multiplication into a type I meront. Zygotes formed after fertilization develop into environmentally resistant thick-walled oocysts that undergo sporogony to form sporulated oocysts containing four sporozoites. These sporulated oocysts are released in feces and can transmit infection from one host to another. Approximately 20% of the zygotes develop into thin-walled oocysts, which represent autoinfective life cycle forms that can maintain the parasite in the host. This stage and the persistent meronts are believed to be responsible for life-threatening disease in immunodeficient persons who do not have repeated exposure to environmentally resistant forms (52, 176).

Incidence

The incidence of cryptosporidiosis is unknown. The disease is generally not reportable to local and state health departments, and many physicians are unfamiliar with the parasite. Also, many persons do not seek medical attention for diarrheal illness (22). The mean prevalence rate for *Cryptosporidium* infection is between 1 and 3% in Europe and North America but is considerably higher in underdeveloped continents, ranging from 5% in Asia to approximately 10% in Africa (51). Of interest to clinical personnel is that several nosocomial infections have been reported (66, 159). In one study, nosocomial infection occurred in more than 10% of the patients and two of three infected children died (159). Patients with late-stage AIDS, regardless of age, are highly susceptible to cryptosporidiosis, and after exposure, most develop prolonged, severe, life-threatening diarrhea (51).

Symptoms

The incubation period is 5 to 28 days with a mean of 7.2 days. Diarrhea, which characteristically may be choleralike, is the most common symptom. Other symptoms include abdominal pain, nausea, fever, and fatigue.

Therapy

No chemotherapy is available for this organism. Supportive care with oral or intravenous rehydration is the treatment of choice for immunocompetent patients. For immunocompromised individuals, the disease can be life-threatening. Attempts to treat infection with numerous antiparasitic and antimicrobial drugs have not proven effective. Initial reports suggested that spiramycin might be effective, but in a controlled trial of 54 patients with AIDS and cryptosporidiosis, efficacy was not demonstrated (52). A double-blind trial in AIDS patients has suggested that paromomycin treatment may result in improvement in both clinical and parasitologic param-

TABLE 4. Comparison of commercial assays for *Cryptosporidium* detection

Kit name (reference)	Specificity/ sensitivity (%)	Comparison method	Time	Advantages	Disadvantages
ProSpecT Microtiter Assay (EIA) (Alexon)					
53	98/98	Acid-fast and IFA stains	130 min/plate	Preserved or fresh specimens	Retesting in borderline zone
1	99.5/96[a]	Merifluor	Not reported	No centrifugation step	Difficult to interpret visually
94	99/94.5	Merifluor, acid-fast stain, ColorVue	120 min/plate, 17 min hands-on	Batch testing	
ProSpecTR (EIA) (Alexon)					
144	98.5/100	Acid-fast stain	10–15 min total	No sample concentration Easy to use	One sample per test
Color Vue (EIA) (LMD Laboratories; Seradyn, Inc.)					
133	99.8/66.3 diarrheic stools: (100/87.9)[b]	Acid-fast and auramine stains	150 min for 24 samples	Easy to interpret visually Specimens in preservatives	Low sensitivity for routine testing Retesting needed due to low sensitivity
156	99/93[a]	Merifluor indirect	135 min		
1	100/72[a]	Merifluor	Not reported	No centrifugation step	
94	100/94.5	Merifluor, ProspecT, acid-fast stain	120 min/plate, 25 min hands-on	Batch testing	
Merifluor (DFA) (Meridian)					
94	100/94.5	Acid-fast stain ProSpecT, ColorVue	35 min, 6 min hands-on time; exam time, <1 min/specimen	Easy to read 10 times more sensitive than permanently stained slides	Concentration step Equipment costs (fluorescence microscope)
69	100/100	Modified acid fast stain	Exam time, 20–30 s/specimen		
9	100/93	Modified acid-fast stain	Exam time, 2 min/specimen	Rapid screening of slides	More expensive than conventional staining methods
Crypt o-CEL IF (DFA) (Bradsure Biologicals Ltd.)					
178	NR[c]	Modified acid-fast, phenol-auramine, Giemsa stains	Not reported	Detected five times more oocysts than Ziehl-Neelson	

[a] These values are based on retesting of samples with equivocal results.
[b] Values in parentheses are for diarrheic stools.
[c] NR, not reported in conventional terms.

eters (195). Clinical trials involving immunotherapy are currently in progress.

Current Detection Methods

Routine diagnostic methods include fecal concentration with Sheather's sugar formulation or formalin-ethyl acetate and acid-fast stained smears performed multiple times (68). This approach is labor-intensive and is limited by the skill of the microscopist. The need for rapid and sensitive diagnostic techniques has seen the advent of commercial detection kits. These kits use the EIA and the DFA techniques. Table 4 provides a summary of published studies of these commercial kits (1, 53, 69, 74, 94, 133, 156, 178) and lists the advantages and disadvantages of each as discussed in the references indicated.

Detection Methods under Development

A reverse passive hemagglutination assay has been developed by Farrington et al. The assay involves an anti-oocyst monoclonal antibody coupled to stabilized sheep erythrocytes. It is reported that this procedure could be used as a screening test; however, it has not been compared with EIA or IFA testing procedures (12, 60). In 1991, Laxer et al. developed a specific and highly sensitive procedure involving PCR tech-

niques for the identification and detection of *C. parvum* and reported in 1992 that it was possible to detect *C. parvum* DNA in paraffin-embedded tissue by PCR (100, 101). Webster et al. reported on the ability to reproducibly detect 20 oocysts under optimal conditions by his PCR procedure (191). At this time, no PCR assay to detect *Cryptosporidium* spp. in a clinical setting has been described. Weiss reports that the chief advantages of nucleic acid-based detection techniques are their sensitivity for detecting pathogens and the speed at which they can definitively identify an organism (193). Application of a PCR diagnostic assay will depend on many factors, including cost, speed, ease of performance, availability, sensitivity and specificity in comparison with other diagnostic assays, the volume or size of the sample that can be economically analyzed, and the need to directly detect and identify the parasite (158). Ortega et al. determined whether sensitive RFLP analysis of genomic DNA could distinguish *C. parvum* isolates of human and bovine origin. A 4.3-kbp fragment was present in all human isolates and absent in the bovine isolates (141). Another PCR-based technique, randomly amplified polymorphic DNA (RAPD) analysis, has demonstrated the feasibility of distinguishing between *C. parvum* isolates and may find application for epidemiological studies (34).

Association with Water

Oocysts of *Cryptosporidium* spp. are widely distributed in water. LeChevallier et al. found oocysts in 87% of raw water samples (103) and 27% of drinking water samples (104). *Cryptosporidium* spp. have been responsible for eight WBDOs associated with water intended for drinking in the United States (22, 82, 130). These outbreaks have occurred in water systems that used well and spring water treated solely by chlorination and in surface water systems that have been filtered. *Cryptosporidium* spp. accounted for the greatest number of individuals becoming ill from water in which the etiologic agent of the outbreak could be identified. In the massive Milwaukee outbreak, investigators found that the severity of illness in Milwaukee residents, as defined by more frequent abdominal cramping, nausea, vomiting, and fever, was greater than in previously described sporadic cases. In addition, the severity of illness was greater in visitors than in Milwaukee residents. Of both visitors and residents with laboratory-confirmed cryptosporidiosis, 39% had recurrent bouts of watery diarrhea that lasted an average of 2 days after having had normal stools for 2 to 14 days (115, 116, 171). Swimming pool-associated outbreaks of cryptosporidiosis have also been widely reported (19, 39, 86, 125, 173). The highly transmissible nature of *Cryptosporidium* spp. underscores the risk for acquiring cryptosporidial diarrhea in areas of high endemicity, especially by travelers from regions where the rate of exposure to the parasite is lower (51).

CYCLOSPORA CAYETANENSIS

History

Cyclosporans were first noted by Eimer in 1870 in the intestine of moles. Since then, they have been found in moles, other rodents, and snakes (140). Cyclospora-like organisms in humans were first observed in 1979 in three individuals in Papua New Guinea (14). The oocysts closely resembled those of *Cyclospora* spp. but were thought to represent a new species of *Isospora*. Starting in 1985, organisms 8 to 10 μm in size, staining red with modified acid-fast stains, and autofluorescing under UV light were isolated with increasing frequency from humans worldwide. They were described frequently as cyanobacterium-like bodies (blue-green algae) or coccidian-like bodies (CLBs) and were deemed responsible for diarrheal illness (110, 138).

Because of the resemblance of "CLBs" to unsporulated coccidians in fresh feces from Peruvian children, attempts were successfully made to sporulate and excyst the organism, leading to the classification of the oocysts in the coccidian genus *Cyclospora*. The morphological characteristics, patient symptoms, and failure of conventional antimicrobial therapy linked this newly described *Cyclospora* organism to previous reports of CLB infections in humans in different parts of the world (37, 38, 110, 146, 169). This link between CLBs and *Cyclospora* spp. was confirmed by experiments in which CLBs from different parts of the United States and the rest of the world were shown to sporulate and excyst, thus identifying them as *Cyclospora* spp.

Life Cycle

Cyclospora cayetanensis belongs to the subphylum Apicomplexa, family Eimeriidae. The species name was derived from the university where it was initially studied (Universidad Peruana Cayetano Heredia). Spheroid oocysts are 8 to 10 μm in diameter, and each oocyst has two ovoid sporocysts with two sporozoites each. The sporozoites have structures typical of coccidians as shown by electron microscopy studies (140).

The life cycle of this cyclosporan species has yet to be completely elucidated. Unsporulated oocysts are shed in the feces, and up to 40% of the oocysts will sporulate within 7 to 13 days under optimal laboratory conditions (140). In intestinal biopsy samples from patients excreting CLBs, parasites with definite coccidian characteristics have been observed within jejunal enterocytes (21). The life cycle variations between *Cyclospora* species make it difficult to predict the complete life cycle for *C. cayetanensis* of humans, although exogenous sporulation closely resembles that of *C. talpae* (140). It also remains to be determined whether humans are the only natural host.

Incidence

Organisms similar in appearance to *Cyclospora* spp. have been found in patients with protracted diarrheal illness in North, Central, and South America; the Caribbean; Africa; Bangladesh; Southeast Asia; Australia; England; and Eastern Europe. Most infections have been reported in tourists or expatriates visiting countries that normally have high diarrheal disease rates (7, 23, 48, 56, 71, 84, 89, 102, 111, 126, 145, 148, 153, 169, 170, 182), although indigenous *Cyclospora* infections in many countries and in U.S. patients with no travel history have also been described (40, 91, 122, 135). Not surprisingly, recently reported cases of cyclosporiasis include immunocompromised patients with AIDS (91, 149, 161, 200). In 1996, more than 850 cases of laboratory-confirmed cyclosporiasis were reported to the CDC. Most of the outbreaks were from states east of Rocky Mountains. These outbreaks were epidemiologically linked to ingestion of berries (40). In studies conducted in Lima, Peru, *C. cayetanensis* and *Cryptosporidium parvum* were detected in vegetables purchased from a market in an area of endemic infections by both organisms (139).

Symptoms

The onset of illness caused by *Cyclospora* spp. has been reported as abrupt in 68% of adult patients, with symptoms lasting an average of 7 weeks (35, 84, 170). In AIDS patients, infections tend to last up to 4 months (80). The symptoms are

similar to those caused by cryptosporidiosis, including nausea, anorexia, abdominal cramping, watery diarrhea, and weight loss (143). Diarrhea alternating with constipation has been commonly reported.

Therapy

Trimethoprim-sulfamethoxazole (TMP-SMX) appears to be the drug of choice. Cessation of symptoms and oocyst excretion in Peruvian patients (one adult and four children) occurred 1 to 3 days posttreatment (117). A study of 43 AIDS patients with cyclosporiasis showed that TMP-SMX at 960 mg (160 mg of TMP/800 mg of SMX) four times a day for 10 days was effective. Diarrhea and abdominal pain stopped after 2.5 days. Recurrence of symptoms was observed 1 to 3 months posttreatment in 44% of patients (143). In a double-blinded, placebo-controlled trial of TMP-SMX for *Cyclospora* infections in 40 expatriates in Nepal, oocysts and symptoms cleared after 7 days of treatment in 94% of the patients. After 7 days of crossover therapy, 91% of the patients in the placebo group cleared the infection (83).

Current Detection Methods

Unsporulated oocysts are found in freshly collected stool samples. Oocysts can be observed by light microscopy. Accurate size measurements of oocysts of *C. cayetanensis* (8 to 10 μm) distinguish them from oocysts of *Cryptosporidium parvum* (4 to 6 μm). *Cyclospora* oocysts stain variably by a modified acid-fast technique; they stain best by the modified carbol fuchsin method (199). Oocysts can be stained by the Kinyoun, Ziehl-Neelsen, and safranin methods, but they do not stain well with iron hematoxylin, trichrome, Grocott-Gomori methenamine silver nitrate, iodine, or periodic acid-Schiff stains. Oocysts autofluoresce green under UV epifluorescent illumination with a 450 to 490 excitation filter and fluoresce blue with a 365 excitation filter (140). Oocysts can be concentrated by sequential differential centrifugation steps, formalin-ethyl acetate, or sedimentation and sucrose flotation in Sheather's solution (48). A PCR method (202) has been used in some research areas, but protocols have not yet been reported for the clinical or environmental laboratories.

Association with Water

While waterborne transmission of *C. cayetanensis* remains unproven except for a single reported case (151), it is highly suspect as a major mode of transmission. Most reports of infection have come from cities or regions in countries that are predominantly coastal, near both freshwater and salt water. The prolonged sporulation time, 1 to 2 weeks, further supports the idea that this organism can be transmitted through water (142). Case reports lend increased evidence that water is involved in disease transmission. Some patients reported consumption of untreated water or reconstituted milk (35, 75, 82, 84, 199). In a 1990 outbreak involving 20 individuals in a Chicago hospital, most of whom were resident physicians, epidemiological evidence suggested that water from a rooftop reservoir was responsible for the infections (37). In Utah, a man became infected after cleaning his flooded basement following heavy rains (75). In another incident, a child became ill and passed CLBs in his feces 1 week after swimming in Lake Michigan. Water samples taken from the inlet of the Chicago municipal water supply system showed the presence of organisms resembling CLBs, but their identity was not confirmed. In Nepal, 12 of 14 British soldiers and their dependents developed diarrhea and 6 were confirmed to be harboring *Cyclospora* spp. A 2-liter sample collected from a water storage tank at the time of the outbreak was concentrated, and oocysts were observed. This is the single confirmed report of *Cyclospora* transmission through water (151). Other potential sources of infection include travelers or natives infected with *Cyclospora* spp., contaminated food, and possibly insects as transport hosts. Epidemiological studies will help to sort out these issues of transmission and to delineate control and treatment strategies against infection with *Cyclospora* spp.

ISOSPORA BELLI

History

Isospora belli is a coccidian parasite taxonomically related to *Cryptosporidium* spp., *Cyclospora* spp., and *Toxoplasma* spp. (119). It is thought to be the only *Isospora* species that infects humans, which are the organism's only known host (68). First described in 1915, it is implicated in traveler's diarrhea, most often in tropical areas of endemicity such as South America, Africa, and Southeast Asia (119, 196). Intestinal involvement and symptoms are usually transient unless the patient is immunocompromised (119).

Life Cycle

After an infectious sporulated oocyst is ingested, it will excyst, releasing sporozoites into the small intestine, where they penetrate mucosal intestinal epithelial cells of the distal duodenum and enterocytes of the proximal jejunum and develop into trophozoites (68, 119, 196). Both sexual and asexual stages of development occur. Oocysts are produced, passed in the feces, and then mature outside the body in 2 to 3 days, depending on environmental conditions (196). Oocysts are very hardy and can remain viable in the environment for months (68).

Incidence

I. belli has been associated with outbreaks of diarrhea in mental wards, day care centers, World War II veterans from the Pacific, immigrants, and persons with human immunodeficiency virus infection. Infection rates vary from 0.2 to 3% in AIDS patients in the United States to 8 to 20% in AIDS patients from Haiti and Africa (20, 119). These rates may be underestimated (68, 119).

Symptoms

The incubation period in a host is a few days (164). Approximately 1 week after oocyst ingestion, a variety of symptoms including diarrhea, weight loss, abdominal pain, cramping, and low-grade fever occur (68, 196). In the immunocompetent host, the cardinal symptom of isosporiasis is profuse diarrhea with 6 to 10 watery, soft, foamy, and offensive-smelling bowel movements per day, suggestive of a malabsorption process (68). Clinically, the disease is almost indistinguishable from giardiasis, crytosporidiosis, and microsporidiosis, presenting as diarrhea without blood or leukocytes (68, 196). The disease is usually self-limiting within a 2- to 3-week period; however, oocyst shedding may persist for 2 to 3 weeks after the patient recovers (68, 99, 196). Occasionally, chronic illness occurs in infants or in otherwise healthy adults (20). Immunocompromised hosts, especially AIDS patients, exhibit more severe symptoms which can persist for months, years, or indefinitely; dehydration and debilitation can be life-threatening (20, 68, 119). Atypical presentations such as acalculous cholecystitis

and reactive arthritis can also occur (20, 73). The organism is thought not to disseminate, but ingestion of increased numbers of oocysts may create what appears to be a disseminated infection (119, 121).

Therapy

The drug of choice is TMP-SMX (68).

Current Detection Methods

Direct or concentrated wet smears of fresh or preserved stool specimens, rather than permanent stained smears, are recommended for examination because the thin, transparent oocyst walls are difficult to detect in polyvinyl alcohol-preserved stool specimens (68, 196). Even in wet smears, oocysts are pale, transparent, and easily overlooked under normal microscopy conditions. Reducing the level of light on the microscope will help to demonstrate the oocysts. Freshly shed oocysts are oval, measure 20 to 33 μm by 10 to 19 μm, and generally contain only one or two immature sporonts. Continued development outside the body yields a mature oocyst containing two sporocysts, each containing four crescent-shaped sporozoites. This stage is occasionally recovered from the stool and is the infectious form of the parasite (68, 119, 196). The acid-fast stain is another proven detection method. With this stain, oocysts stain red. Auramine-rhodamine stains can also be helpful in diagnosis, but identification must be confirmed with wet smears or acid-fast stains, especially if the stool contains other cells or excess artifacts (68, 119). An intestinal biopsy sample is sometimes the only specimen in which the organism is found, because of the small numbers and the intermittent shedding of these organisms in the stool (68, 119). Currently, no serologic tests are available (119). *I. belli* oocysts will exhibit autofluorescense under UV epifluorescent illumination using a 450 to 490 excitation filter (139).

Association with Water

Poor sanitation and fecal contamination of food and water are the most likely explanations for the spread of infection by *I. belli* (47, 68, 119, 196). Prevention of disease centers around improved personal hygiene and sanitation to eliminate possible fecal-oral transmission from food, water, or environmental surfaces (68).

MICROSPORIDIA

History

The term "microsporidia" is a nontaxonomic term used to describe organisms in the order Microsporidia of the phylum Microspora. This phylum contains over 100 genera and 1,000 species that are ubiquitous in nature and infect a wide range of vertebrate and invertebrate hosts. Until recently, awareness of the significance of these organisms as human pathogens was lacking. Since the first documented case in 1985, five genera have been implicated in human disease: *Encephalitozoon*, *Enterocytozoon*, *Septata*, *Pleistophora*, and *Vittaforma* (because of DNA analysis, *Septata intestinalis* may be placed in the genus *Encephalitozoon*, and based on ultrastructural studies *Nosema corneae* has been reclassified as *Vittaforma corneae*) (30, 79). A sixth, catch-all grouping, called microsporidium, is used to accommodate microsporidia that have yet to be classified (28, 137). Host specificity is considered only moderately selective, since human infections with nonmammalian genera have been documented. Likewise, tissue and organ specificity does not appear to be very restrictive, with one exception. To date, *Enterocytozoon bieneusi* appears to be the only human microsporidium with some degree of tissue specificity, having been documented only in the human intestinal tract (28, 33, 109).

Life Cycle

Microsporidia are obligate intracellular protozoa that form highly specialized, environmentally resistant spores that vary in size, shape, and method of cell division among different species. Human species are ovoid or pyriform and smaller than those isolated from other species, ranging in size between 1 and 2 μm in diameter (68).

The microsporidial life cycles, while varied, can be divided into three general phases: the infective stage, merogony, and sporogony. The infective stage begins with ingestion or possibly inhalation of spores by a susceptible host. Following ingestion, the spore is stimulated to extrude its coiled polar filament under the influence of environmental conditions such as pH shifts or changes in ionic concentration. Once extruded, the filament becomes a polar tubule, through which the organism can infect susceptible host cells by injecting them with infectious spore material known as the sporoplasm (28). Once injected, the sporoplasm undergoes merogony, developing into meronts, the proliferative stages of the organism. The meronts multiply by repeated binary fission or multiple fission, forming multinucleate plasmodial forms. Sporogony follows, as the meront cell membranes thicken to form sporonts, which divide and give rise to sporoblasts. The thick spore wall of the sporoblast, with an underlying layer of chitin, serves to protect the infective stage, which is thought to be ingested or inhaled by hosts (28, 68). Without undergoing further multiplication, the sporoblasts develop into mature spores, which accumulate in the infected cell until the cell ruptures, releasing the infectious spores. The combination of merogony and sporogony as a means of multiplication gives the microsporidia huge reproductive potential and results in heavy host infestations and subsequent environmental contamination. Released spores can reinfect other nearby cells or enter the environment via stool, urine, or respiratory secretions (28, 167).

Incidence

Reported cases of human infection are increasing. While some cross-reactivities between the microsporidia have been detected, serologic surveys suggest that latent human infections can occur (85, 136, 167). Although the role of microsporidia as human pathogens was once questioned, the organism continues to be isolated from 18 to 70% of stool specimens and duodenal and jejunal biopsy specimens from cases of otherwise unexplained chronic diarrhea in AIDS patients (16, 63, 97, 137, 150, 162). *E. bieneusi* has been reported in mixed infections with *Cryptosporidium parvum* in 28% of AIDS patients presenting with cryptosporidiosis (28, 127, 128, 132, 190).

Symptoms

Because the route of human infection has not been fully characterized, the incubation period is still unknown. Chronic diarrhea, dehydration, and weight loss greater than 10% of body weight are the most common symptoms of microsporidiosis. While rare cases of acute self-limiting infections in immunocompetent individuals exist, most infections are found in association with human immunodeficiency virus infection. Prolonged diarrhea for up to 48 months has been reported, and although it is not always the sole cause of death, mortality rates for infected patients are often greater than 56% (28, 150).

Infections appear to be associated with pronounced cellular immune deficiency as established by patient CD4 lymphocyte counts of less than or equal to 100 cells/ml (33, 147, 162). Other documented infections include keratitis, conjunctivitis, hepatitis, peritonitis, myositis, central nervous system infection, renal disease, sinusitis, and disseminated disease (31, 33, 129, 147, 150, 162). In cases of intestinal infection, 1 to 20 stools are passed per day; stools are watery, nonbloody, and fecal leukocyte negative. Abdominal pain, vomiting, and fever appear to occur when a commonly occurring concomitant biliary infection is present. Diarrhea is worsened by most foods and appears to be worse in the morning. Laboratory evidence for D-xylose and fat malabsorption is almost universal. *E. bieneusi* has also been isolated from patients without reported diarrhea, but in all cases in which a follow-up report existed, diarrhea did develop when the CD4 lymphocyte count dropped, and evidence of enteropathy has been found in patients in whom it was sought. It is thought that this parasite produces light infections in normal human populations or is present at low levels and becomes activated when an individual becomes immunocompromised (24, 28, 127, 128, 132, 190). The pathology of microsporidial infection varies depending on the organs that are affected (165).

Therapy

To date, albendazole is the only effective treatment to various degrees for *Encephalitozoon* spp. No obvious treatment success has been achieved with *Enterocytozoon* infections, although metronidazole, octreotide, pyrimethamine, and TMP-SMX have been tried with some success (15, 109, 113, 132). Topical fumagillin appears to be successful against microsporidial keratoconjunctivitis (155, 201).

Current Detection Methods

Microsporidiosis is difficult to diagnose. The organisms can be detected in tissue sections by using hematoxylin-eosin, Gram, periodic-acid Schiff, Gomori methenamine silver, Warthin-Starry, acid-fast, and Giemsa stains. Calcofluor white, Uvitex-2B, polarized light, and the most commonly used method, modified trichrome with Chromotrope 2R, are used for organism detection in stool specimens (28, 32, 54, 57, 68, 127, 128, 132, 136, 190, 198). Slides must be prepared with a very thin layer of specimen, stained, and examined under at least ×1,000 magnification. Spores are oval and stain light pink with the modified trichrome stain; unfortunately, so do yeasts and other debris (68). Examination of three stools a day for 3 days may be necessary to establish a diagnosis (17). Quantitation of spores in enteric specimens does not appear to correlate with patterns of diarrhea (46).

Pitfalls of diagnosis center around the size of the organisms. Liquid stool specimens must be centrifuged for a full 10 min at $500 \times g$ to sediment these organisms (68). Alternatively, a Cytospin instrument can be used to prepare smears. Historically, identification to the genus level required electron microscopy to determine the size of the spore and the characteristic number of polar filament coils (28, 50, 196).

Detection Methods under Development

Recently, Western blotting and RNA analysis have been used in combination with other techniques to help confirm the identity of the organism (28, 49, 50, 196, 203, 204). *E. bieneusi* is the most difficult microsporidium to diagnose. To date, it has been only transiently grown in cell culture and no animal models for human intestinal disease are known (136, 168). Currently, no specific antibodies are available for use in its detection; however, there is a reported cross-reactivity between *Encephalitozoon* antibodies and *E. bieneusi* antigens. Through this cross-reaction, polyclonal antibodies have been somewhat successfully used to demonstrate spores of *E. bieneusi* in stool and intestinal biopsy tissue (8, 28, 57, 194, 196, 205). Because no in vitro cultivation methods for *E. bieneusi* are available, it appears that the future of diagnosis for this organism may be limited to the use of diagnostic rRNA probes and PCR (55, 62, 65, 187, 203, 204). Monoclonal antibodies have been developed for other microsporidial species and may prove useful for their diagnosis (8, 18, 55, 172, 179, 188, 205).

Association with Water

Common environmental sources of microsporidia include ditch and other surface waters. Under routine environmental conditions, microsporidia are able to survive and maintain their infectivity for days to weeks and can infect nonhuman hosts through ingestion or direct inoculation. At 4°C, the organisms can survive in water for more than 1 year. To date, the species known to infect humans have not been isolated or identified from water sources (28, 33, 192). The infection route for the microsporidia is also unknown. It is postulated that *E. bieneusi* is a natural human parasite and, unlike other microsporida, does not have a zoonotic origin. A potential role for human-to-human transmission has been postulated, either through the environment via the fecal-oral route or the urinary-oral route or directly via eye inoculation, sexual transmission, or transplacental transmission (72, 131, 180). At this time, the route for acquiring respiratory infection is also unknown, but it may involve inhalation, aspiration of gastrointestinal contents, hematogenous dissemination, or other means (28).

WATER QUALITY PROTOZOAN TESTING AND MONITORING

American Society for Testing and Materials Method

Since the early 1980s, many large and small water utilities either have set up in-house water quality testing laboratories or have contracted with commercial water testing laboratories for the detection of *Giardia* and *Cryptosporidium* spp. in finished water. The proposed American Society for Testing and Materials (ASTM) analytical procedure (10) is considered to be the method of choice in the United States for detecting these parasites from source waters (42). The method is labor-intensive, time-consuming, technically complex, and dependent on the degree of quality control of the laboratory (5, 42, 105). Variations in sample collection, turbidity (water quality), and weather conditions may influence the results. The ASTM method involves sampling at least 380 liters of water through a 1.0-μm-nominal-porosity polypropylene yarn cartridge filter, extracting the particulate from the cartridge filter by cutting it apart and washing the fibers, and concentrating the extracted particulates by centrifugation. The concentrated particulates are then processed to selectively concentrate cysts and oocysts by flotation in 50-ml tubes on a Percoll-sucrose gradient. The recovered particulates are stained with fluorescently tagged antibodies on cellulose acetate filters and mounted on slides. These slides are scanned with a UV epifluorescence microscope for characteristic staining patterns of *Giardia* cysts and *Cryptosporidium* oocysts. This presumptive IFA identification is confirmed by differential interference contrast or phase-contrast optics to identify the internal morphological characteristics of the parasites.

TABLE 5. Diagnostic techniques for detection of waterborne protozoa in clinical samples

Protozoan parasite	Diagnostic stage(s)	Samples recommended
Cryptosporidium parvum	Oocyst (4–6 μm)	1 watery stool, 1–2 formed stools
Cyclospora cayetanensis	Oocyst (8–10 μm)	3–6 stools
Entamoeba histolytica	Trophozoite (10–60 μm) or cyst (5–20 μm)	3 stools
Giardia lamblia	Trophozoite (12–15 μm) or cyst (10–12 μm)	6 stools
Isospora belli	Oocyst (20–33 by 10–19 μm)	3–6 stools (may need intestinal biopsy)
Microsporidia	Spore (1–2 μm)	3 stools (may also be found in eye, respiratory, and tissue specimens)
Naegleria spp.	Trophozoite (8–20 μm)	CSF, brain
Acanthamoeba spp.[b]	Trophozoite (10–50 μm), cyst (15–20 μm)	Cornea, contact lenses

[a] Preferred method.
[b] For eye infections only.

Information Collection Rule

Protozoan monitoring, as part of the Information Collection Rule, proposed in the *Federal Register* Requirements for Public Drinking Water Supplies (61), is scheduled to begin in January 1997 for surface water systems that serve more than 100,000 people. This method differs from the ASTM method in the volume of raw water required and the use of Hoffman modulation or differential interference contrast microscopy instead of phase-contrast microscopy for the confirmation of morphological characteristics of the cysts and oocysts.

Monitoring Method Limitations

Limitations of the ASTM and Information Collection Rule methods are that recovery rates tend to be low (5, 42, 105) and that some commercial laboratories lack proficiency in testing for the parasites, which suggests that oocysts and cysts may go undetected. In 1993, Clancey et al. (42) conducted a blind survey of 16 commercial laboratories, testing their ability to assay for *Giardia* and *Cryptosporidium* spp. by the ASTM method. Filters seeded with cysts and oocysts were sent to the laboratories. Thirty-six percent of the laboratories reported false-negative results for *Giardia* spp. and 55% reported false-negative results for *Cryptosporidium* spp. The report recommended that improved methods for recovery of these parasites be developed. Recently, 54 algal species were tested for fluorescence by the ASTM *Giardia* and *Cryptosporidium* method, and 24 showed some degree of fluorescence. The addition of goat serum to the IFA mixture blocked the fluorescence of most nontarget organisms in this study (154); however, whether algal autofluorescent organisms fluoresced in these samples was not reported. Recently, Graczyk et al. evaluated commercial EIA and IFA kits for detection of *Cryptosporidium* spp. oocysts other than *C. parvum*. This report suggested that commercial diagnostic kits for *C. parvum* should be critically examined for cross-species identification before they are recommended or adopted for use in testing environmental samples (74).

Nieminski et al. (134) compared the ASTM method to an alternative method developed by Hansen and Ongerth (76). The alternative method involves filtering smaller sample volumes through a 2.0-μm polycarbonate membrane filter, recovering particles by rinsing and Squeegeeing, and concentrating by centrifugation. The cysts and oocysts are then selectively concentrated from other particulates by gradient flotation followed by detection by immunofluorescent-antibody staining. The findings of this study indicated that the main advantage of the ASTM method is its ability to confirm presumptively identified cysts and oocysts. The alternative method was attractive because of the membrane filter sampling method, small sample volumes, and flexibility. The major limitation is lack of a confirmation step. Another method suggested by Vesey et al. (181) for detection of *Cryptosporidium* and *Giardia* spp. in water is a flocculation concentration followed by flow cytometry by fluorescence-activated cell sorting. This technique alleviates many of the time-consuming, labor-intensive steps of the ASTM method. However, when the flow-cytometric technique is used, it is recommended that the results be confirmed by microscopy. The flow cytometer is also an expensive addition to any laboratory. An improvement of the ASTM methodology currently being evaluated incorporates a direct slide preparation and staining method to replace the ASTM cellulose acetate filter-staining procedure (67). Other technologies under development in the United Kingdom include an electrorotation assay method, vortex flow filtration and immunomagnetic separation, and immunomagnetizable particle separation (IMS) PCR. Another commercial product under evaluation is electrochemiluminescence. This technology is based on attracting target organisms to an electrode surface and marking them with reusable ruthenium molecules, which emit light at levels directly correlating with the number of target organisms in the sample (41). Methods for detection of *Giardia* and *Cryptosporidium* cysts and oocysts in water samples with cDNA probes and PCR applications are also in the developmental stage (2, 88, 118).

Even with improved water-testing methods for parasite detection, the test report indicates only the number of parasites in a specific water sample, but the viability of the organisms is unknown. At present, methodologies for assessing the viability of parasites found in water samples is being funded by the American Water Works Association. The viability issue is a major concern of the American Water Works Association and

TABLE 5—*Continued.*

Stains and related methods	Fluorescence techniques	EIAs	Developing technologies
Modified acid fast	DFA	Yes	PCR-based RFLP, DNA (RAPD) and reverse passive hemagglutination
Modified acid fast (carbol fuchsin),[a] will also stain with Kinyoun, Ziehl-Neelson and safranin modified acid-fast methods	Autofluorescence		PCR
Wheatley trichrome, iron hematoxylin		Yes	PCR
Wheatley trichrome, iron hematoxylin	DFA	Yes	PCR
Unstained direct or concentrated smear read with reduced light,[a] acid fast, auramine rhodamine			
Modified trichrome-Chromotrope 2R,[a] electron microscopy, from tissue (PAS, hematoxylin-eosin, methenamine silver, Gram's, acid fast [variable])	Calcofluor white		PCR, rRNA sequencing
Wet mount of CSF sediment,[a] Schaudinn's fixed smears stained with Wright-Giemsa, modified Wheatley, or Masson trichrome, hematoxylin-eosin, iron hematoxylin, agar or cell culture			PCR, IFA, immunoperoxidase, electron microscopy
Wet mount of CSF sediment.[a] Schaudinn's fixed smears stained with Wright-Giemsa, Wheatley, or Masson trichrome, hematoxylin-eosin, methenamine silver, methylene blue, Janus green, Congo red, acridine orange, agar or cell culture	Calcofluor white		PCR, IFA, RFLP, isoenzyme electrophoresis, immunoperoxidase, electron microscopy, fluorescent lectins

EPA, since in a recent study in which healthy volunteers received *C. parvum* oocysts, the median infective dose was calculated to be 132 (58). Ingestion of as few as 10 viable *Giardia* cysts is also known to cause infection (197).

Safe Drinking Water Act

All public water systems currently fall under the regulation of the Safe Drinking Water Act of 1974. The microbial content of drinking water is regulated by the EPA through the Total Coliform Rule and the Surface Water Treatment Requirements. A maximum contaminant level for total coliforms specifies the percentage of samples that may contain any coliforms during a month. The turbidity of finished water must meet specified maximum and monthly standards. All public systems using surface water or groundwater under the direct influence of surface water must provide disinfection. Systems must also filter the water unless they meet specific conditions, including source water quality criteria for turbidity and total or fecal coliforms and a watershed control program to minimize potential contamination by human enteric viruses and *Giardia* cysts (130).

CONCLUSIONS

At this time, the methodology available to commercial and utility water-testing laboratories may not accurately assess and detect *Cryptosporidium* and *Giardia* spp. in the water supply. Outbreaks do occur as a result of treated water supplies. The clinical laboratory may be the first to detect an increase in the number of stool samples positive for parasites. The detection methods available to clinical laboratories may have to be evaluated to meet the needs of identifying emerging infections in high-risk patient groups and monitoring these infections in the general patient population. Table 5 provides a summary of current clinical detection methods for waterborne pathogens.

Timely recognition of emerging infections requires early-warning systems to detect new threats to health before they develop into public health crises. Surveillance of selected infectious diseases in the United States is based on state laws and regulations that require reporting of these diseases to health departments. At a recently convened CDC Workshop on Waterborne Cryptosporidiosis, which included representatives from 40 states and from regulatory and public health agencies, water utilities, and advocacy groups, it was recommended that cases of *Cryptosporidium* infection be reportable as an infectious disease to the CDC National Notifiable Disease Surveillance System (5). This measure was supported and approved by the Council of State and Territorial Epidemiologists. Although such action might not improve diagnosis or reporting of cryptosporidiosis by physicians, it provides legal authority for collecting needed information. It is of interest that this type of surveillance is most likely to reflect the occurrence of cryptosporidiosis in immunocompromised populations, because health care providers are more likely to request that such patients who have diarrhea be tested for cryptosporidia. Another approach suggested was to monitor laboratory data for the detection of *Cryptosporidium* spp. This approach would again increase the detection of *Cryptosporidium* in patients with AIDS and would not provide information about the parasite in the general population, because physicians do not routinely ask for a *Cryptosporidium* test in addition to the routine ova and parasite examination (90).

As clinical laboratory directors assess methods for detecting the increasing number of emerging infections, they also need to increase the frequency of their dialog with health administrators, public health officials, physicians, and their laboratory personnel. Issues of cost, personnel efficiency, and equipment needs must be evaluated to ensure that the challenge of diagnosing these emerging infections will be met. Not surprisingly, the clinical laboratories are well ahead of the water industry in the ability to detect the presence of such organisms. Because the potential threat of infection via the waterborne route is just being recognized for many of these organisms, it is imperative that the water industry also turn its attention to finding ways to detect these emerging and well-recognized protozoan pathogens in water, particularly in finished water.

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