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Review article

Cryptosporidium parvum and *Cyclospora cayetanensis*: a review of laboratory methods for detection of these waterborne parasites

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Abstract

Cryptosporidium and *Cyclospora* are obligate, intracellular, coccidian protozoan parasites that infest the gastrointestinal tract of humans and animals causing severe diarrhea illness. In this paper, we present an overview of the conventional and more novel techniques that are currently available to detect *Cryptosporidium* and *Cyclospora* in water. Conventional techniques and new immunological and genetic/molecular methods make it possible to assess the occurrence, prevalence, virulence (to a lesser extent), viability, levels, and sources of waterborne protozoa. Concentration, purification, and detection are the three key steps in all methods that have been approved for routine monitoring of waterborne oocysts. These steps have been optimized to such an extent that low levels of naturally occurring *Cryptosporidium* oocysts can be efficiently recovered from water. The filtration systems developed in the US and Europe trap oocysts more effectively and are part of the standard methodologies for environmental monitoring of *Cryptosporidium* oocysts in source and treated water. Purification techniques such as immunomagnetic separation and flow cytometry with fluorescent activated cell sorting impart high capture efficiency and selective separation of oocysts from sample debris. Monoclonal antibodies with higher avidity and specificity to oocysts in water concentrates have significantly improved the detection and enumeration steps. To date, PCR-based detection methods allow us to differentiate the human pathogenic *Cryptosporidium* parasites from those that do not infect humans, and to track the source of oocyst contamination in the environment. Cell culture techniques are now used to examine oocyst viability. While fewer studies have focused on *Cyclospora cayetanensis*, the parasite has been successfully detected in drinking water and wastewater using current methods to recover *Cryptosporidium* oocysts. More research is needed for monitoring of *Cyclospora* in the environment. Meanwhile, molecular methods (e.g. molecular markers such as intervening transcribed spacer regions), which can identify different genotypes of *C. cayetanensis*, show good promise for detection of this emerging coccidian parasite in water.

Keywords: Waterborne; *Cryptosporidium*; *Cyclospora*; Detection; Environment

1. Introduction

Cryptosporidium and *Cyclospora* are obligate, intracellular, coccidian protozoan parasites that infest the gastrointestinal tract of humans and animals causing severe diarrhea illness. Both organisms produce

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environmentally resistant oocysts, which are excreted in the feces of infected individuals. *Cryptosporidium* is recognized worldwide as a waterborne pathogen, and the species *Cryptosporidium parvum* is the major cause of cryptosporidial infections in humans and livestock (O'Donoghue, 1995). Waterborne transmission of the oocysts and outbreaks of cryptosporidiosis either through drinking water or recreational use are well documented and have been listed by several authors (Solo-Gabriele and Neumeister, 1996; Rose et al., 1997; Smith and Rose, 1998; Oppenheimer et al., 2000; Fayer et al., 2000). More than half (56%) of the 75 waterborne cryptosporidiosis outbreaks between 1984 and 1999 were associated with drinking water, while 33 outbreaks (44%) were related to use of recreational water facilities including pools, rivers, and lakes (Fayer et al., 2000).

While *C. parvum* appears to be predominantly waterborne, *Cyclospora cayetanensis* is transmitted more often via contaminated produce (Sterling and Ortega, 1999; Rose and Slifko, 1999; Mota et al., 2000). Sturbaum et al. (1998) and Sherchand et al. (1999) provide evidence that contaminated drinking water and irrigation water are potential sources for cyclosporiasis. To date, however, only two waterborne *Cyclospora* outbreaks have been associated with the consumption of contaminated drinking water (Rabold et al., 1994; Huang et al., 1995).

Cryptosporidium and *Giardia* (a related enteric protozoa) occur widely in surface and drinking water supplies in the United States. In an extensive monitoring, LeChevalier and Norton (1995) reported that the occurrence of *Cryptosporidium* and *Giardia* in raw water samples was 60% and 54%, respectively. Examination of filtered drinking water showed that *Cryptosporidium* oocysts were detected in 13% of the water samples and *Giardia* cysts were detected 17% of the time.

The effectiveness of treatment processes for removing both pathogens is evaluated through monitoring (Rose et al., 1988; LeChevalier et al., 1991a,b; Rose, 1991; Jakubowski et al., 1996; LeChevalier and Norton, 1995; Hass et al., 1999). The United States Environmental Protection Agency's ''Interim Enhanced Surface Water Treatment Rule'' (IESWTR) stipulates zero as the goal for the maximum contaminant level of protozoan parasites in water. Compliance is defined by performance requirements for water treatment plants and by monitoring indices (e.g. turbidity, performance of individual filters) that aim to optimize the filtration process and in some cases the disinfection process. The regulation was published in December 1998, the final revision was published in January 2001, and the rule will be effective January 2002. Key provisions in the IESWTR establish a Maximum Contaminant Level Goal (MCLG) of zero for *Cryptosporidium* and require a $2-\log_{10}$ (99%) *Cryptosporidium* removal when using filtration only. The IESWTR applies to public water systems that use surface water or ground water under the direct influence of surface water and serve 10,000 or more people.

In the United Kingdom, the sampling and analysis requirements outlined in the Water Supply (water quality) (amendment) regulations, 1999 (London: Stationary Office, 1999) dictate that treated water supplies be monitored daily for *Cryptosporidium*. The regulation stipulates a legally enforceable maximum of 10 oocysts per 100 l, and is process (filtration) based. Detection of *Cryptosporidium* oocysts at any level above 10 per 100 l constitutes a criminal offense (Fairley et al., 1999). No current regulations exist, however, for *Cyclospora* levels in water samples, and routine methods for environmental monitoring of *Cyclospora* are not available. In 1999, the American Water Works Association Research Division and USEPA identified *Cyclospora* as a protozoan of concern (AWWA Research Division Microbial Contaminants Research Committee, 1999). Routine and accurate detection, enumeration, speciation, and viability assessment of waterborne protozoan parasites remains an ambitious goal in environmental microbiology.

2. Current methods for detection of waterborne *Cryptosporidium*

The first recorded outbreak of giardiasis, which occurred in Aspen, CO in 1965, spurred the development of methods to detect protozoan parasites in water (Rose et al., 1988; Rose, 1991; Hass et al., 1999). Following the first waterborne outbreak of cryptosporidiosis in the 1980s, various approaches to recover and detect *Cryptosporidium* were developed and tested. The most common methods for detecting and quantifying *Cryptosporidium* oocysts in the environment were

adapted from those originally designed to determine the occurrence of *Giardia* cysts in environmental samples (Ongerth and Stibbs, 1987; Musial et al., 1987; Rose, 1988; Rose et al., 1989). The standard recovery and detection method involved three basic steps: (i) concentration of the water sample (e.g. filtration) to recover low numbers of parasites typically found in the environment; (ii) purification (e.g. density gradients); and (iii) immunofluorescent staining, which enhanced our ability to detect oocysts microscopically in filtered sample concentrates (Sauch, 1985; Ongerth and Stibbs, 1987; Musial et al., 1987; Rose, 1988; Jakubowski et al., 1996).

Initially, cartridge filtration (Musial et al., 1987) and membrane filtration (Ongerth and Stibbs, 1987) were used to recover oocysts from water samples. The oocysts were then differentiated from nontarget organisms such as free-living protozoans, bacteria, and algae via an immunofluorescent assay (Rose et al., 1989). The criteria for identifying *Cryptosporidium* oocysts included: (i) the fluorescence of environmental oocysts, which must be at least 50% of that seen in controls of fresh oocysts seeded into environmental samples; (ii) a distinct fluorescence around the oocyst wall; (iii) a general spherical shape, with a diameter of 4–6 μm; and in some cases (iv) a folding in the *Cryptosporidium* oocyst wall. Phase contrast microscopy was used to visualize internal morphology, the sporozoite, and four nuclei.

In the early 1990s, the American Society for Testing and Materials (ASTM) proposed a test method for detecting *Cryptosporidium* oocysts and *Giardia* cysts in low-turbidity water (ASTM, 1991), and the United Kingdom Standing Committee of Analysis provided a tentative method (“Blue Book” method) for detecting the same waterborne organisms (Anonymous, 1990). Both methods were based on similar procedures: cartridge filtration, a Percoll-sucrose step for selective separation of (oo)cysts from debris, and an immunofluorescent–antibody-based detection method for identification and enumeration of (oo)cysts. LeChevalier et al. (1991a) reported an average oocyst recovery efficiency of 25.3% using the ASTM method while the Scottish Parasite Diagnostic Laboratory cited a recovery that ranged between 3% and 29% (Smith and Hayes, 1997).

Nieminski et al. (1995) compared the ASTM method or cartridge filter system and an alternate method involving membrane filtration. Major advantages of the ASTM method included the ability to sample large volumes of water and to identify fluorescing (oo)cysts. Major disadvantages were its relatively high cost and the amount of time required to complete the analysis. The membrane method was less expensive and required less time than the ASTM method. The method was limited, however, because it did not differentiate (oo)cysts from algae. The authors recommended trials of a hybrid method that combined the most efficient steps from both the ASTM and the alternate method.

During the 1990s, the USEPA approved a method for detection and quantification of *Cryptosporidium* oocysts in water samples as part of a monitoring rule known as the “Information Collection Rule”. The method was called the ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure (US-EPA, 1996). The ICR method is still used in different laboratories throughout the US to monitor protozoan parasites in reclaimed effluents, surface and ground water sources, and drinking water from utilities. Alternative methodologies and new standard procedures, however, have enabled laboratories to improve the efficiency of recovery of waterborne parasites. Overviews of the standard and alternative detection methods for *Cryptosporidium* in water are given in Tables 1 and 2, respectively.

3. Comparison of recovery efficiencies and evaluation of *Cryptosporidium* detection methods

Dufour et al. (1999) reported that the ICR method usually underestimates the occurrence and levels of *Cryptosporidium* sp. They also criticized the method for being costly, complex, and difficult to perform; yielding highly variable results; and demonstrating differential response in a variety of water matrices. Specific limitations of the ICR method include low recovery efficiencies (as low as 3% for *Cryptosporidium* oocysts), nonspecific antibody binding, interferences by sample debris, and inability to determine viability, infectivity, and strain of isolated oocysts (Rose, 1991; LeChevalier et al., 1995; Juranek et al., 1995; Schaefer, 1997; Rochelle et al., 1997b; Dufour et al., 1999). These limitations notwithstand-

Table 1
Standard techniques for recovery, concentration, and detection of *Cryptosporidium* oocysts from environmental waters

Concentration technique	General approach for concentration and detection procedures	Recovery efficiency (%)
Cartridge filtration (ASTM, 1991; USEPA, 1996)	100 l (raw water source), 1000 l (finished) Water sample filtered through a 10-in.-long polypropylene yarn-wound cartridge filter, 1.0 μm, nominal porosity Filter cut apart and particles eluted. Stomacher Eluting solution consists of: • Phosphate buffered saline (PBS), pH 7.4 • 0.1% polyoxyethylensorbitan mono-oleate (Tween-80) • 0.1% Sodium dodecyl sulfate (SDS) Sample concentrated by centrifugation ($1050 \times g$, 10 min) Percoll–sucrose density-gradient centrifugation used to purify concentrated sample Oocysts detected by IFA and confirmed by DIC microscopy	0–100
Method 1622/1623: (USEPA, 1999a,b)	10–1000 l water samples Water sample filtered through the Envirochek™ HV sampling capsule Particles eluted through wrist action agitation Eluting buffer consists of: • Laureth-12 • 1 M Tris pH 7.4 • 0.5 M EDTA, 2 Na, pH 8.0 • Antifoam A • Deionized water Sample concentrated by centrifugation ($1000–1100 \times g$ for 15 min) to a final pellet Dynal IMS used to purify concentrated samples Oocysts detected by IFA and confirmed by DIC microscopy and vital dye staining characteristics (4′,6-diamidino-2-phenylindole [DAPI])	12–93 (21–100)*
Standard operating protocol (SOP) for the monitoring of *Cryptosporidium* oocysts in treated water supplies 1999, SI No. 1524 (Anon., 1999)	Water samples filtered through Genera Filta-Max™ filter membranes Particles eluted using appropriate wash station Eluting buffer consists of: • Phosphate buffered saline (PBS) • 0.1% polyoxyethylene (20) sorbitan monolaurate Sample concentrated by centrifugation ($1100 \times g$ for 15 min) Dynal IMS procedure Oocysts detected by IFA and confirmed by DIC microscopy and vital dye staining characteristics (4′,6-diamidino-2-phenylindole [DAPI])	30–50**

* USEPA acceptance criteria.
** SOP acceptance criteria.

ing, the ICR method has been useful in outbreak investigations for characterizing the sources of contamination and for determining the extent of watershed contamination and the effectiveness of water treatment process on removal of the organisms (Jakubowski et al., 1996).

In a comprehensive evaluation of the ICR method, LeChevalier et al. (1995) found that losses of oocysts occurred during each step of sample processing. To reduce oocyst loss, they suggested modifications in the key method components (sampling, processing, staining or detection). The changes included the uses

of a higher specific gravity gradient for sample clarification and a higher centrifugation speed for sample concentration. Their recommendations were relatively easy to implement and improved the efficiency of the method.

In 1996, the USEPA initiated an effort to identify new and innovative technologies for protozoan monitoring and analysis. After evaluating potential alternatives to the then-current method through literature searches, discussions with research and commercial laboratories, and meetings with experts in the field, the Engineering and Analysis Division within the Office of Science and Technology within EPA's Office of Water developed draft Method 1622 for *Cryptosporidium* detection. This *Cryptosporidium*-only method was validated through an interlaboratory study in August 1998, and was revised and approved as a valid method in January 1999. In October 1998, the USEPA validated a method for simultaneous detection of *Cryptosporidium* and *Giardia* and designated the new combined procedure as Method 1623: *Cryptosporidium* and *Giardia* in water by filtration, immunomagnetic separation (IMS) and immunofluorescence assay (FA) microscopy.

Methods 1622 and 1623 require filtration, immunomagnetic separation of the (oo)cysts, and an immunofluorescence assay for determination of (oo)cyst concentrations, with confirmation through vital dye staining (4′ 6-diamidino-phenylindole (DAPI)) and differential interference contrast (DIC) microscopy (USEPA, 1999a,b). The interlaboratory validation of Methods 1622 and 1623 used Gelman (Envirochek™) capsule filtration, Dynal immunomagnetic separation (IMS), and Meridian staining protocol. Alternate procedures are allowed, provided that required quality control tests are performed and all quality control acceptance criteria in these methods are met.

The Envirochek capsule protocol was chosen for validation because the capsule could handle the specified volume (10 l) without clogging and was easy to use in both field and laboratory settings (Matheson et al., 1998; Clancy et al., 1999). The original Envirochek capsules, which were designed for 10-l volumes of water, contained a Supor polyethersulphonate membrane with a 1-μm absolute pore size. An Envirochek capsule for sampling large volumes of water (500 l or more) is now available (Envirochek™ HV). Envirochek™ HV incorporates a new 1-μm track-etched membrane, which is designed to process high volumes of treated water while maintaining high recovery characteristics and meeting the USEPA requirements. Preliminary results in our laboratory have demonstrated a recovery efficiency of *Cryptosporidium* oocysts ranging from 30% to 56% for the high volume filter.

Following filtration, elution, and centrifugation, any oocysts that were present in the sample are purified by immunomagnetic separation (IMS). IMS allows for more efficient separation of organisms from other debris, results in cleaner slide preparations for microscopic examination, and reduces the number of false-positives (Connell et al., 2000). Connell et al. (2000) also reported that differences in (oo)cyst detection rates are related to differences in the purification process used in the ICR method and methods 1622 and 1623. The density-gradient flotation procedure used in the ICR method is based on the specific gravity of the oocysts. Nontarget particles with the same specific gravity are coextracted, however, and when transferred to a membrane, they can cause misidentification and false positives. In contrast, methods 1622 and 1623 use the IMS procedure and anti-*Cryptosporidium* antibodies to extract target organisms, i.e. oocysts, from the sample. The probability for misidentification is reduced since fewer nontarget particles are transferred to the slide. McCuin et al. (2001) reported high capture efficiency for the target organism (*Cryptosporidium*) and demonstrated that the IMS method can recover low numbers of oocysts from environmental samples.

Most researchers agree that there are fewer false-positives with methods 1622 and 1623 than with the ICR method. It is important to note, however, that high concentrations of dissolved iron may have an inhibitory effect on the IMS–IFA portion of EPA methods 1622 and1623. Yakub and Stadterman-Knauer (2000) indicated that the recovery of *Cryptosporidium* sp. was reduced at threshold concentrations of dissolved iron between 4 and 20 mg/l. They also observed incomplete FITC staining, which suggested that the interaction between dissolved iron and the oocyst surface is a significant source of interference.

While it appears that methods 1622 and 1623 perform better than ICR, further modifications and alternatives to both methods have been proposed. For example, Simmons et al. (2001) reported 42–46%

recovery for *Cryptosporidium* oocysts when they used a hollow-fiber ultrafilter system to concentrate oocysts in seeded surface and reagent water. The disposable ultrafilter consists of a series of polysulfone hollow fibers contained within a polycarbonate housing, which is portable and easy to use. Large volumes of water can be processed, and the concentration procedure is compatible with the subsequent purification and detection steps in EPA method 1622.

Genera Technologies and Seven Trent Water have developed another novel filter system, the Crypto-Dtect compressed filter system. The Crypto-Dtect system uses multiple layers of open cell, reticulated foam rings, which when compressed, act as very efficient filters, but when decompressed, allow simple removal of the entrapped particles. The compressed foam filter system is conveniently sized, easy to handle, simple to install, and very efficient at entrapping *Cryptosporidium* oocysts from water and releasing them upon elution. Sartory et al. (1998) reported a mean recovery of 90% from both uncleaned tap water concentrates and river water. More recently, Genera Technologies has patented the Filta-Max™ system for collection and recovery of oocysts as part of their complete detection process, along with Puri-Max™ (immunomagnetic separation) and Quanti-Max™ (automated detection).

An alternative collection method for the recovery of *Cryptosporidium* oocysts from large volumes of water involves continuous flow (CF) centrifugation. CF centrifugation concentrates particles by size weight, and recovery of oocysts averages 14% (Swales and Wright, 2000). Processing time is rapid, but this intensive equipment-based technique is not field applicable.

Current methods for recovery, concentration, and detection of *Cryptosporidium* oocysts in water samples still rely on filtration, clarification, and microscopy. The methods of choice include EPA method 1623 in the US and the Standard Operating Protocol 1999, SI No. 1524 (Genera Filta-Max, Dynal IMS, and Celllab FITC) in the UK. Both methods rely on immunofluorescence assays for detection of oocysts.

Lot-to-lot variation in commercially available antibodies and significant differences in properties such as avidity and cross-reactivity with algae and non-*parvum* species have been reported. Hoffman et al. (1999) identified differences among four of the currently used antibodies and indicated that while none is without its advantages and disadvantages, meticulous quality control and quality assurances are needed for routine detection of parasites in water. Ferrari et al. (1999) used different classes of monoclonal antibodies (IgG1, IgG3, and IgM) for environmental monitoring of *Cryptosporidium*. Their results revealed that staining with IgG1 antibodies generally produced fewer unwanted fluorescent particles than staining with IgM and IgG3 antibodies. It appears that IgG1 antibodies have higher avidity and specificity to oocysts in water concentrates than other commercially available antibodies (Weir et al., 2000).

Modifications to immunofluorescence detection of *Cryptosporidium* include the use of additional nonspecific fluorescent dyes such as 4′,6-diamidino-2-phenylindole (DAPI) (Rochelle et al., 1995). The combination of DAPI with immunofluorescence provides an easy reproducible method for detecting sporulated oocysts in water samples and minimizes the need for DIC or phase contrast optics (Grimason et al., 1994).

Flow cytometry with fluorescence activated cell sorting (FACS) improves the sensitivity of the immunofluorescence assay, and the combination of FACS with epifluorescence microscopy enables enumeration of low numbers of *Cryptosporidium* oocysts in environmental samples (Vesey et al., 1993, 1994, 1997a; Medema et al., 1998). In this technique, fluorescing oocysts are separated from most other particles, which means that the oocysts can be detected easily and rapidly. It should be noted, however, that the presence of large numbers of fluorescent particles may still cause difficulties during microscopic confirmation of suspect oocysts. Interference from autofluorescent particles can be minimized when a FITC fluorochrome is used (Vesey et al., 1997b). CY3, phycoerythrin, and tetramethylrhodamine B isothiocyanate were the best fluorochromes for drinking water samples (Vesey et al., 1997b). More recently, Ferrari et al. (2000) tested a two-color immunofluorescence flow cytometric assay, which could potentially increase the specificity of current detection methods while aiding confirmation steps. They evaluated six different combinations of *Cryptosporidium*-specific monoclonal antibodies and found that the combination containing CRY104-FITC and CRY104-PE produced a highly specific assay.

Table 2
Alternative techniques used for concentration of *Cryptosporidium* oocysts from environmental waters

Technique	Concentration and detection procedures	Recovery efficiency (%)	Reference
Membrane filtration	20-l water samples Filtration through polycarbonate membranes (293 mm, pore sizes 5 and 1 μm) Purification through density gradient (40% potassium citrate) Detection by IFA	5–25	Ongerth and Stibbs, 1987
Calcium carbonate flocculation method	10-l water samples Calcium chloride (1 M) Sodium hydrogen carbonate (1 M) Sulfamic acid (10%) Purification and detection through flow cytometry and IFA	69	Vesey et al., 1993
Membrane dissolution method	10-l water samples Fi1tration through 142-mm-diameter, 1.2-μm-pore size mixed esters of cellulose membrane filter Purification by Percoll–sucrose density gradient flotation or Dynal IMS Detection by IFA	50	Aldom and Chagla, 1995; McCuin et al., 2001
Compressed foam filter system	10–20 l water samples Filta-Max Purification by Chemunex modified Dynal IMS Detection by ChemScan	49–73	Sartory et al., 1998; Rushton et al., 2000
Continuous flow centrifugation	100 l water samples Continuous flow centrifuge Clarification through Percoll discontinuous gradients or flow cytometry Detection by IFA	13–15	Swales and Wright, 2000
Size-selective continuous flow filtration	100 l water samples Size-selective continuous flow filtration apparatus Detection by IFA	0–85	Oda et al., 2000

Laser scanning devices (e.g. ChemScan RDI, Chemunex, Paris, France) have also been used to detect oocysts (Rushton et al., 2000). After concentration with the Genera Crypto-Dtect filter system and purification by IMS, the final extract is absorbed onto a carrying membrane and labeled with FITC-monoclonal antibody. The membrane is then scanned on the ChemScan (CS), which records the location of every possible oocyst using the parameters of fluorescence, size, and shape. Subsequently, a conventional fluorescence microscope, fitted with an electronic stage, can be used to view the membrane. In parallel trials, CS performed consistently better with a 73% average recovery efficiency as compared with 49% for manual fluorescence microscopy (MM). The ChemScan is rapid, easy to operate, and unaffected by the presence of debris in the final concentrate.

4. Molecular approaches for detection of *Cryptosporidium* oocysts in water

The development of sensitive and specific molecular detection methods such as the polymerase chain reaction (PCR) has greatly increased our knowledge about the type of *Cryptosporidium* in the environment. Many PCR assays for detecting waterborne oocysts have been described (Johnson et al., 1995;

Mayer and Palmer, 1996; Stinear et al., 1996; Rochelle et al., 1997a,b; Shiana et al., 1998; Chung et al., 1999; Kostrzynska et al., 1999). Wiedenmann et al. (1998) compared different PCR assays and demonstrated that problems inherent to the technique such as PCR inhibition, the requirement for extreme sensitivity, and viability assessment were all solved. In other words, Wiedenmann et al. (1998) demonstrated the suitability of PCR-based assays for routine environmental monitoring of *Cryptosporidium*. Some of the molecular approaches that have been developed to improve conventional detection methodologies are summarized in Table 3.

One advantage of the molecular assays is that the PCR and RT-PCR products can be used to determine the genus, species, and genotype of the parasite. The genotype information can then be used to determine the specific strain or outbreak source. Recently, Orlandi and Lampell (2000) developed an extraction-free, filter-based protocol to prepare DNA templates that could be adapted to detect *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Encephalitozoon intestinalis* by PCR. The filters preserve DNA integrity and the technique eliminates potential sources of target DNA losses, as additional purification steps are not needed. This method has not been tested in water samples, but the authors suggested that it could be easily adapted to detect diverse protozoan pathogens from a variety of clinical, food, and environmental sources.

Other PCR-based detection methods such as those described by Xiao et al. (2000, 2001a,b) have been used successfully to detect *Cryptosporidium* in storm water, surface water, and wastewater samples. These methods, which rely on a small-subunit rRNA-based nested PCR-restriction fragment length polymorphism (RFLP) technique, allow us to differentiate the human pathogenic *Cryptosporidium* parasites from those that do not infect humans, and to track the source of oocyst contamination in the environment.

The feasibility of PCR-based detection methods for environmental monitoring of *Cryptosporidium* oocysts was recently described by Sturbaum et al. (2001). The authors were interested in detection sensitivity, and used micromanipulation techniques to deliver a desired number of oocysts (1 or 10) into PCR tubes for subsequent DNA liberation and PCR detection. They then demonstrated by using nested PCR–RFLP primers that the amplification rates increased from 38% to 100% for test samples containing 1 and 10 oocysts, respectively. These results suggest that PCR-based detection methods may be sensitive enough to detect the low numbers of oocysts in environmental samples.

5. Viability and infectivity assays for *C. parvum*

Fluorescent antibody-based detection methods do not distinguish *C. parvum* from other *Cryptosporidium* oocysts that have no public health significance. In addition, IFA provides no information about the viability, infectivity, and virulence of recovered oocysts. These types of data are required to assess the public health risks of waterborne transmission of *Cryptosporidium* (Rochelle et al., 1997b; Matheson et al., 1998; Widmer et al., 1999; Fayer et al., 2000).

The classical definition of viability is the ability of an organism to reproduce, metabolize, and in the case of obligate parasites, to infect. Seven methods have been used to evaluate the viability of enteric protozoa including *C. parvum*. These include: (i) in vitro methods such as excystation (Bingham and Meyer, 1979; Smith and Smith, 1989; Rose, 1990; Robertson et al., 1993; Black et al., 1996; Schaefer, 1997; Vesey et al., 1997b); (ii) inclusion or exclusion of fluorogenic dyes (Schupp and Erlandsen, 1987; Campbell et al., 1992, 1993 Robertson et al., 1992); (iii) nucleic acid stains (Belosevic et al., 1997a,b; Neumann et al., 2000); (iv) reverse transcriptase polymerase chain reaction (RT-PCR) (Stinear et al., 1996; Rochelle et al., 1997b; Kaucher and Stinear, 1998; Widmer et al., 1999; Jenkins et al., 2000); (v) fluorescence in situ hybridization (Vesey et al., 1995, 1998); (vi) infectivity methods using mice models (Roberts-Thomson et al., 1976; Korich et al., 1990; Enriquez and Sterling, 1991; Finch et al., 1993; Tzipori, 1998); and (vii) cell culture (Slifko et al., 1997; Rochelle et al., 1997b; Di Giovanni et al., 1999). Fluorogenic dyes and cell culture have the greatest application to environmental samples.

Mice models have been used in the past to determine infectivity for *C. parvum* genotype 2 (Korich et al., 1990). The animal infectivity method is, however, tedious, difficult, and expensive, and is not readily amenable to normal laboratory analysis in the water

Table 3
Molecular approaches for detection of *Cryptosporidium* oocysts in water

Limit of detection	Target sequence	Approach	Reference
1 oocyst	*C. parvum hsp70*	Seeded water samples were concentrated by calcium carbonate flocculation and oocysts purified by Percoll–sucrose density centrifugation. Release of nucleic acid was achieved by freeze and thaw cycles. Further steps included oligo(dT)$_{25}$-coated magnetic beads to isolate mRNA, RT-PCR.	Stinear et al., 1996
10 oocysts	*C. parvum hsp70* (hsp mRNA)	Finished water concentrates (ICR method) seeded with *C. parvum* oocysts, in vitro cell culture (Caco-2 cells), RNA extraction (from cell culture) by: homogenization with TriReagent kit (Molecular Research Center, Cincinnati, OH), ethanol precipitation, Oligo (dT) cellulose kit. RT-PCR.	Rochelle et al., 1997b
1–10 oocysts	Gene fragment CPR1 encoding a repetitive *C. parvum* oocysts cell wall protein	Municipal water samples were processed by the membrane filter dissolution method. Water concentrates were spiked with suspension of oocysts. DNA extraction: lysis in TE–sarcosyl–proteinase K-buffer, 10 cycles of freeze and thaw, additional proteinase K and further DNA purification using QIAmp spin columns (Qiagen, Chatsworth, CA, USA). PCR, nested PCR, detection by Digene SHARP Signal™ System Assay.	Chung et al., 1999
	C. parvum hsp70	Grab samples (concentrated by centrifugation at 1800 × *g*), Dynal IMS plus acidified Hanks' balanced salt solution–1% trypsin were used to isolate oocysts. Purified oocysts were inoculated into cell lines, (HCT-8 cells), nucleic acid released by freeze and thaw cycles of harvested cells. PCR.	Di Giovanni et al., 1999
1 oocyst	Unknown genomic region	Seeded samples concentrated by method 1622 including Dynal IMS for selective separation of oocysts. Extraction of nucleic acid: DNA released in the presence of 25% (w/v) Chelex 100 (BioRad, Hercules, CA, USA), freeze and thaw cycles plus centrifugation at 13,000 × *g*. Single-tube nested PCR test and dot blot hybridization with an internal digoxigenin-labelled probe used for identification.	Haller-Soulier and Guillot, 1999
1 oocyst/l	dsRNA	Seeded samples obtained by filtration (ICR method). DsRNA from *C. parvum* extracted through Xtra Bind Capture System (Xtrana, Denver, CO) plus further extraction and purification procedures. Nested set RT-PCR amplification. Lateral flow chromatography format for detection.	Kozwich et al., 2000

industry (Neumann et al., 2000). Moreover, the *C. parvum* human genotype (genotype 1) will not infect standard animal models (Widmer et al., 2000). It is only recently that serial propagation of type 1 *Cryptosporidium parvum* in gnotobiotic piglets has been successful (Widmer et al., 2000). The same can be said for *Cyclospora cayetanensis* since US researchers have just developed animal and tissue culture models for propagation of the parasite (Hanes et al., 2000).

Excystation is a method that has been used in laboratory disinfection and survival studies, but cannot be used to study oocyst viability in environmental

samples because it requires large concentrations of oocysts (10^5 oocysts/ml or greater) for the analysis. In addition, in vitro excystation methods have limited use in samples where there is high microbial abundance and diversity (Neumann et al., 2000).

The inclusion or exclusion of vital dyes has been used as a marker of intact membranes in *Cryptosporidium* oocysts and as an indicator of the presence of internal features such as nuclei. The vital dyes are fluorogenic, which makes them amenable to the IFA and microscopic procedures, and they may be useful tools for assessing the viability and infectivity of small numbers of oocysts found in environmental samples (Smith et al., 1991; Campbell et al., 1992; Smith, 1996; Jenkins et al., 1997). One commonly used dye permeability assay tests the differential uptake of the fluorochromes 4′ -6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by the oocysts (Gasser and O'Donoghue, 1999). Sporozoite nuclei, which take up DAPI but fail to stain with PI, are viable, while nuclear material that stains with both fluorochromes is nonviable. The interpretation of viability from fluorogenic dye inclusion or exclusion must be undertaken cautiously since the dye tests are known to overestimate viability, and the staining can be variable with a portion of the oocysts not staining with either dye (Campbell et al., 1992; Smith, 1996; Jenkins et al., 1997; Black et al., 1996; Neumann et al., 2000). Nonetheless, results from dye permeability assays correlate well with results from in vitro excystation assays and the standard mouse infectivity assay.

Nucleic acid stains with SYTO dyes (SYTO-9, hexidium, and SYTO-59) have also been tested as tools for identifying infectious *C. parvum* oocysts in source drinking water (Belosevic et al., 1997a; Neumann et al., 2000). SYTO-59 is particularly useful because its fluorescence spectrum does not overlap with that of FITC. It may therefore be used in conjunction with commercially available FITC-labeled anti-*C. parvum* monoclonal antibodies to detect and determine the viability or infectivity of oocysts in environmental samples (Neumann et al., 2000).

RT-PCR is a molecular method that is based on the amplification of RNA and often specifically messenger RNA (mRNA) (Abbaszadegan et al., 1997; Wiedenmann et al., 1998; Widmer et al., 1999). The method involves several steps: (i) isolation of RNA from the oocyst, (ii) purification of the RNA, (iii) reverse transcription to a DNA complementary strand, (iv) amplification, and (v) detection of the amplified product. One of the advantages of RT-PCR is that it does not depend on a preceding biological process like in vitro excystation (Wiedenmann et al., 1998). RT-PCR suffers from many of the same disadvantages as PCR such as: (i) inefficient extraction of RNA from the cysts or oocysts, (ii) interferences in the transcription and amplification steps by environmental constituents, (iii) small processed volumes for RT-PCR, and (iv) nonquantitative nature of the test.

Cryptosporidium oocyst infectivity can also be determined by cell culture methods, which are sensitive to low numbers of oocysts (Slifko et al., 1997; Rochelle et al., 1997b; Slifko et al., 1999; Di Giovanni et al., 1999). Numerous cell lines and detection schemes have been used (Table 4), but most investigators use the human illeocecal adenocarcinoma cells (HCT-8) cell line. Standard filtration and IMS procedures have been used to prepare environmental

Table 4
Cell lines used to study *C. parvum* genotype 2 infectivity

Cell line (origin)	Isolate or outbreak source	Original isolating host	Infectivity detection method	Reference
Caco-2 (human)	Ames, IA	Bovine	RT-PCR	Rochelle et al., 1997b
BS-C-1 (African green monkey)	NA	Bovine	Giemsa stain	Deng and Cliver, 1998
BFTE (bovine)	Ames, IA	Bovine	SEM and TEM	Yang et al., 1996
HCT-8 (human)	KSU-1, Ames, IA, TAMU, UCP	Bovine, equine	ELISA, PCR, RT-PCR, Specific IF, FDM–MPN, In situ hybridization	Woods et al., 1996; DiGiovanni et al., 1999; Okhuysen et al., 1999; Slifko et al., 1997, 1999; Rochelle et al., 2000
MDBK (bovine)	GCH1, Ames, IA	Human, bovine	IF, PCR	Theodos et al., 1998
MDCK (canine)	Ames, IA	Bovine	Giemsa stain	Yang et al., 1996

concentrates for inoculation onto cell monolayers, where the oocysts undergo excystation and initiate infection in the cells (Di Giovanni et al., 1999). A quantitative procedure has been developed whereby the cells are fixed and infection is observed using labeled antibodies and microscopy (Slifko et al., 1997, 1999). This has been successfully used to study UV inactivation of *Cryptosporidium* oocysts (Huffman et al., 2000). Alternatively, the cells are extracted and PCR or RT-PCR is used to detect infection (Rochelle et al., 1997a; Di Giovanni et al., 1999).

Water laboratories have already demonstrated (through peer-reviewed literature) the high sensitivity of cell culture to a single oocyst (Di Giovanni et al., 1999; Rochelle et al., 1997b; Slifko et al., 1997, 1999). Other benefits of using cell culture to study *C. parvum* infectivity include the following: the method is applicable to environmental oocysts from treated and untreated waters; both genotypes 1 and 2 will infect HCT-8 cells; results are available in 48 h; and the method is fairly easy and less labor intensive than animal infectivity studies. The correlation between cell culture data and animal infectivity data is significant ($r = 0.78$) for both untreated and treated (disinfected) oocysts (Slifko, 2001).

A fluorescent in situ hybridization (FISH) technique developed by Vesey et al. (1998) shows considerable promise as an indicator of *C. parvum* oocyst viability. In these assays, a fluorescent DNA probe is targeted to the 18S rRNA of *C. parvum*. The 18S rRNA is usually present in viable organisms and is degraded by cellular RNases in dead or dying cells. Existing FISH techniques, however, are limited to measuring the viability of *C. parvum* oocysts and not their infectivity (Neumann et al., 2000).

Recently, other approaches for measuring viability of oocysts in environmental samples have been described. For example, Call et al. (2001) have developed a quantitative immunoassay that can detect low numbers of excystable, sporozoite-releasing *C. parvum* oocysts in turbid water samples. The CP7 viability assay uses a monoclonal antibody (CP7) to capture soluble *C. parvum* sporozoite antigen that has been released by in vitro excystation. The captured antigen is measured via electrochemiluminescence (ECL) using a ruthenium-labeled anti-rabbit antibody. The ECL counts derived from the CP7 viability assay are directly related to the number of viable oocysts. The configuration of the CP7 viability assay permits the evaluation of samples from turbid environmental water sources (as high as 200 NTU) at a detection limit as low as 50 viable oocysts/ml of concentrated sample.

6. Current research on detection of waterborne *C. cayetanensis*

Many of the methods that are used to recover and detect *C. parvum* in water have been successfully used to detect *Cyclospora* oocysts in drinking water and wastewater (Rabold et al., 1994; Sturbaum et al., 1998). For example, membrane filtration and light microscopy were used to recover and detect *Cyclospora* oocysts at the time an outbreak occurred amongst British soldiers and dependents stationed in a small military detachment in Nepal (Rabold et al., 1994).

To determine the occurrence of *Cyclospora* oocysts in Peru, wastewater samples were collected with the Envirochek capsule and Haniffin polypropylene cartridge filters and examined with UV epifluorescence and molecular methods (Sturbaum et al., 1998). Microscopic examination of the wastewater concentrates revealed autofluorescent unsporulated oocysts. Further identification steps included a 2-week incubation period in 2.5% potassium dichromate solution to check for sporulation (Sturbaum et al., 1998; Sherchand et al., 1999), and nested PCR–RFLP to determine the identity of the *Cyclospora* species (Sturbaum et al., 1998).

To our knowledge, no monoclonal antibodies are available to detect *Cyclospora* oocysts in environmental samples. Acid-fast staining techniques, which are used for the diagnosis of the organisms in stool specimens, are problematic and sometimes lead to misidentification of the parasite in the clinical setting (Marshall et al., 1997; Mota et al., 2000). One of the problems is that *C. cayetanensis* oocysts exhibit marked variability in the acid-fast from nonstaining to full staining. Therefore, acid-fast techniques are not recommended for detection of *Cyclospora* in environmental samples.

For *Cyclospora*, no vital dye assay is currently available, and while the excystation method provides a means for determination of viability, the time required for the oocysts to sporulate is relatively long

(1 and 2 weeks) (Ortega et al., 1994). Therefore, some researchers have developed new methods such as electrorotation for the rapid determination of oocyst viability and sporulation state, which is particularly important in assessing the risk associated with potentially contaminated water (Dalton et al., 2001).

Several molecular techniques have been described for the detection of *Cyclospora* oocysts in food-related samples (Jinneman et al., 1999; Adam et al., 2000). The approach described by Jinneman et al. (1999) involves an oligonucleotide-ligation assay that differentiates between *Cyclospora* and *Eimeria* PCR amplification products. The second technique involves the use of molecular markers such as intervening transcribed spacer (ITS) regions (Adam et al., 2000). This technique can be used to identify different genotypes of *C. cayetanensis* and is, therefore, of great epidemiological value. Both methods show good promise for monitoring *Cyclospora* oocysts in environmental samples.

Due to the inability to find an adequate animal model and obtain oocysts for research methods, development has been slow. Application of these methods to surveys evaluating occurrence of *Cyclospora* in the environment has been slower yet.

7. Concluding remarks

The rapid and accurate identification and enumeration of waterborne parasites remains an ambitious goal in environmental microbiology. Even though standard methodologies are currently available for detection of *Cryptosporidium* in environmental waters, alternative methods, which are simpler, more efficient, and reliable, are still being evaluated. Standard approaches are needed for other emerging parasites such as *Cyclospora*. Extremely sensitive molecular techniques, which are already available, are particularly important for low level of detection, and identification of species and source of contamination. Still, the most sensitive concentration and detection techniques required to assess virulence and viability/infectivity of waterborne parasites are either expensive or not practical for routine monitoring of waterborne parasites. Therefore, investments in new techniques for rapid, sensitive, and specific detection of microbial pathogens in water are needed. In addition, the development of a consensus approach for applying PCR methods to environmental samples is needed. Finally, application of these methods to a variety of environs for developing occurrence databases will be the ultimate test.

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