

Confirmed detection of *Cyclospora cayetanesis*, *Encephalitozoon intestinalis* and *Cryptosporidium parvum* in water used for drinking

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ABSTRACT

Human enteropathogenic microsporidia (HEM), *Cryptosporidium parvum*, *Cyclospora cayetanesis*, and *Giardia lamblia* are associated with gastrointestinal disease in humans. To date, the mode of transmission and environmental occurrence of HEM (*Encephalitozoon intestinalis* and *Enterocytozoon bieneusi*) and *Cyclospora cayetanesis* have not been fully elucidated due to lack of sensitive and specific environmental screening methods. The present study was undertaken with recently developed methods, to screen various water sources used for public consumption in rural areas around the city of Guatemala. Water concentrates collected in these areas were subjected to community DNA extraction followed by PCR amplification, PCR sequencing and computer database homology comparison (CDHC). All water samples screened in this study had been previously confirmed positive for *Giardia* spp. by immunofluorescent assay (IFA). Of the 12 water concentrates screened, 6 showed amplification of microsporidial SSU-rDNA and were subsequently confirmed to be *Encephalitozoon intestinalis*. Five of the samples allowed for amplification of *Cyclospora* 18S-rDNA; three of these were confirmed to be *Cyclospora cayetanesis* while two could not be identified because of inadequate sequence information. Thus, this study represents the first confirmed identification of *Cyclospora cayetanesis* and *Encephalitozoon intestinalis* in source water used for consumption. The fact that the waters tested may be used for human consumption indicates that these emerging protozoa may be transmitted by ingestion of contaminated water.

Key words | *Cryptosporidium parvum*, *Cyclospora cayetanesis*, drinking water, *Encephalitozoon intestinalis*, microsporidia

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INTRODUCTION

Cyclospora cayetanesis and human enteropathogenic microsporidia (HEM) are emerging pathogenic protozoa that are known to cause aggressive forms of gastrointestinal disease (Curry & Canning, 1993; Weber & Bryan, 1994; Weber *et al.*, 1994; Connor, 1997; Koumans *et al.*, 1998; Madrid *et al.*, 1998; Herwaldt & Beach, 1999). To date, little information on the environmental occurrence of these emerging pathogens is available, primarily

due to lack of effective environmental detection methodologies (Dowd *et al.*, 1998). *Cyclospora cayetanesis* has been identified as the causative agent of water-washed foodborne disease and is suspected in three waterborne outbreaks (Sturbaum *et al.*, 1998), yet, to date, it has not been detected in drinking water. HEM, namely *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi*, are rapidly becoming recognized as the aetiological agents of

traveller's diarrhoea (Raynaud *et al.*, 1998) and in the past their presence has been confirmed in source waters (Dowd *et al.*, 1998).

Cyclospora cayetanesis may be spread by ingestion of fecal-contaminated water or food (Connor, 1997). Outbreaks of cyclosporiasis have been linked to various types of fresh produce (Madrid *et al.*, 1998). It has been determined that *Cyclospora* spp. need time (days or weeks) after being passed in a bowel movement to sporulate and become infectious (Ortega *et al.*, 1998). Because of this it is very unlikely that *Cyclospora* can be transmitted person to person. Thus, *Cyclospora* probably has an environmental developmental stage that occurs in water. Sturbaum *et al.* (1998) identified *Cyclospora* sp. in raw sewage, yet to date there has been no direct confirmation of *Cyclospora cayetanesis* in drinking water to add evidence to the hypothesis that it may be a waterborne pathogen. *Cyclospora* has been identified as the causative agent of several cases of traveller's diarrhoea (Koumans *et al.*, 1998; Nassef *et al.*, 1998). Sturbaum *et al.* (1998) stated

'The infection is under-recognized because our methods for diagnosis are rudimentary and insensitive. The mechanisms by which the parasite causes disease, the range of animal hosts, and the natural reservoir are unknown. *Cyclospora* is a unique coccidian parasite that has just begun to emerge; as yet, we have no clue as to where it comes from or where it hides.'

The HEM as a group are obligate intracellular parasites that infect members of almost every major phyla of the animal kingdom including humans (Weber & Bryan, 1994). HEM are ubiquitous in their geographical distribution (Weber *et al.*, 1994) and have been documented as disease agents on most continents including North and South America, Europe, Asia, Africa and Australia. Two human enteropathogenic species of microsporidia, *Enterocytozoon bienersi* and *Encephalitozoon intestinalis*, are of particular interest, because they have been identified in association with various water sources (Dowd *et al.*, 1998).

HEM are currently listed in the emerging pathogen-research priority lists of the United States Environmental Protection Agency (USEPA), the National Institute of Health (NIH) and the Centers for Disease Control and Prevention (CDC), and are included in the EPA's 1999 drinking water Contaminant Candidate List (CCL). There

is increasing concern over HEM due to improvements in diagnostic procedures, increased cases of traveller's diarrhoea caused by HEM (Van Gool *et al.*, 1997; Raynaud *et al.*, 1998) and the fact that their presence has been confirmed in US source waters (Dowd *et al.*, 1998). Unfortunately, there is only limited documentation both for the methods of recovery and detection of HEM in environmental source waters (Dowd *et al.*, 1999) and for the prevalence of HEM in source or treated waters (Dowd *et al.*, 1998).

MATERIAL AND METHODS

In order to provide the initial evidence for the hypothesis that *Cyclospora cayetanesis* and HEM are waterborne pathogens, we have used molecular methods developed in our laboratory to screen drinking water sources obtained from Guatemala, which is the source of raspberries implicated in several foodborne outbreaks (Koumans *et al.*, 1998). Water concentrates from these sources were purified by density gradient flotation and community DNA was subsequently extracted. Following this, HEM-specific and *Cyclospora* sp.-specific PCR screening was performed, resulting in the generation of separate PCR amplicons for each of these organisms. These amplified PCR products were then sequenced and computer database homology comparison (CDHC) performed (Dowd *et al.*, 1999). This allowed simultaneous confirmation of the detection and determination at species level for each protozoan.

A total of 12 water samples were screened in this study. MERTU/CDC in Guatemala had initially collected these water samples for microbial analysis from small reservoirs fed by springs or wells. Local communities use these reservoirs as sources of drinking water. The samples were chosen based upon an initial screening for *Giardia* spp. and *Cryptosporidium* spp. Samples positive for either of these protozoa as determined by immunofluorescent analysis (IFA) (data not shown) were then screened for *Cyclospora* spp. and HEM.

Water samples were collected using the USEPA Information Collection Rule (ICR) method for the concentration and detection of protozoan parasites

(USEPA, 1998). Briefly, surface water was collected using a portable water pump connected by a two-way valve to a hose with a Snap-tite quick connect (LEGO). The hose was connected to a plastic filter holder (Kenmore, Maryland) that in turn was connected to a flow meter ($1/2 \times 1/2$ in (13×13 mm)) (Kent, Ocala, Florida). The two-way valve was used to slow water flow to 2 gal (7.56 l) per min, while the end of a screened six-foot intake hose was submerged in the water source. A 1DPPY cartridge filter (Cuno, Meriden, Connecticut) in a plastic filter holder was used to trap the parasites.

Filters were placed in plastic bags and shipped to the laboratories. The filters were cut apart and washed using a Stomacher 400 (Labsystem Seward) in the elution solution (USEPA, 1998) for a total of 10 min at high speed, to release particulates, including protozoan spores, cysts and oocysts, trapped within the filter. After washing, the elution solution was concentrated by centrifugation in 750-ml plastic centrifuge bottles at 3,600 rpm ($2,000 \times g$) for 10 min in a swinging bucket rotor centrifuge. To help ensure that the pellets formed remained intact no brake was applied. Sample supernatant was aspirated and discarded. Pellets were resuspended in an appropriate volume of elution solution (Walford & Noah, 1999), pooled into one sample and re-centrifuged, at 2,100 rpm ($990 \times g$) for 10 min. Supernatants were again removed and the final pellet was resuspended in an equal volume of 10% formalin and stored at 4°C until further processing.

Percoll (Sigma-Aldrich Inc.)—sucrose density flotation was employed to separate protozoa from denser particulate matter. Pellets resuspended in 20 ml of elution solution were placed in 50-ml conical centrifuge tubes. This sample was carefully underlain with Percoll-sucrose and centrifuged at 2,800 rpm ($1,050 \times g$) for 10 min. The upper aqueous layer (20 ml) containing the protozoa was then aspirated off with an additional 5 ml from the interface. This sample was transferred to a new 50-ml conical centrifuge tube and the volume was adjusted to 50 ml. This was then centrifuged at 2,800 rpm ($1,050 \times g$) for 10 min and the supernatant removed, leaving 1 ml of the supernatant in addition to the pelleted sample.

For the purpose of community DNA extraction the pellet was resuspended and transferred to a microcentrifuge tube, washed twice with molecular grade water

by centrifugation of the pellet at 14,000 rpm ($16,000 \times g$) for 2 min and resuspended in 100 μ l of sterilized reagent grade water. These washing steps remove excess formalin and suspended DNA material, ensuring that only the genetic material from intact spores and oocysts was extracted. To lyse the parasites and release their genetic material the microcentrifuge tube was placed in a boiling water bath for 10 min. Following this boiling lysis procedure the sample was once again centrifuged at 14,000 rpm ($16,000 \times g$) for 10 min, to pellet cell and other debris, and the supernatant used for PCR analysis.

Both sets of PCR primers were designed such that they did not form excessive primer dimers during the PCR. The formation of dimers during PCR is believed to be a major factor contributing to the inhibition of PCR during analysis of environmental samples (Dowd *et al.*, 1999). Primers for *Cyclospora* spp. were designed to differentiate between the various *Cyclospora* spp. and between *Eimeria* spp. This was done because *Eimeria* spp. and *Cyclospora* spp. have at least 98% similarity between their ribosomal subunit sequences and only two hypervariable regions exist to allow differentiation. The primers for *Cyclospora*, which produce, approximately, a 500 bp product, amplify across all of the hypervariable regions available allowing for species confirmation. The forward primer for *Cyclospora* was designated CycF1 (5' CGG CTA CCA CAT CTA AGG AAG G 3') and the reverse primer designated CycR1 (5' TAA AAT ACG AAT GCC CCC AAC TGT 3'). Similar to the *Cyclospora* primers, the HEM primers were also designed to be specific for the human pathogenic microsporidia and when combined with CDHC have been shown to confirm identification of PCR amplified microsporidia to the species level (unpublished data). These primers were also shown to be highly sensitive when tested in spiked environmental and clinical samples and do not exhibit serious primer or primer–primer dimerizations as predicted by primer design software (DNAsar, Madison, Wisconsin) or actual testing (data not shown). The forward HEM primer was designated MicF1 (5' AGG TTG ATT CTG CCT GAC 3') and the reverse primer was designated MicR1 (5' GCG CCT GCT GCC RTC CT 3'). These primers form, approximately, a 400 bp product.

All PCR conditions were as follows: *Taq* Gold (Perkin-Elmer Corp., Norwalk, Connecticut)—induced

hot start cycling conditions consisting of 10 min of denaturation at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 30 s and extension at 72°C for 1 min. A final extension step consisting of 5 min at 72°C was also included.

Species confirmation using PCR-sequencing and CDHC was performed as described by Dowd *et al.* (1998). PCR products were purified by using a QIAquick PCR purification kit (Qiagen) and were resuspended in reagent grade water. The forward PCR primer was then used for dye termination PCR sequencing, which was performed at the University of Arizona's Laboratory of Molecular Systematics and Evolution sequencing facility. Sequence analyses were performed with advanced BLAST 2.0 (1) on the National Center for Biotechnology Information's web site (<http://www.ncbi.nlm.nih.gov>). Following initial BLASTn identification, query sequences and high scoring pair (HSP) database sequences were re-aligned using pair-wise sequence alignment (DNASTAR, Madison, Wisconsin) and a basepair-by-basepair sequence comparison performed to confirm the results. At least 99% homology was required for species determination of *Cyclospora cayetanesis* because of the similarity in the sequences of other species of *Cyclospora* found in the database; 97% homology was required for species determination of HEM.

RESULTS

Of the 12 water samples screened by the PCR method, six showed amplification of microsporidia SSU-rDNA and five showed amplification of *Cyclospora* 18S-rDNA. All water samples screened in this study had been previously confirmed positive for *Giardia* spp. using the ICR method (USEPA, 1998). Positive samples were subjected to CDHC and the results of these sequence analyses are shown in Table 1. The species of HEM identified in all cases was *Encephalitozoon intestinalis* but the species of *Cyclospora cayetanesis* was confirmed in only three of the five samples. This is because two of the samples that showed amplification failed to provide adequate sequence data.

As quality controls for PCR reactions at least two negative controls were always run. All negative controls

were negative even after 2 × -PCR (PCR reaction that is purified and used as a template in a second PCR) thus indicating lack of contamination. The PCR facilities used included compartmental isolation of the pre-PCR lab, which is an amplified PCR product free zone, dedicated pipettes and aerosol resistant pipette tips. In addition, PCR set-up and purifications were performed in laminar flow hoods pre-sterilized by UV radiation, ethanol and chlorine bleach, and only after analysis of all environmental samples were two positive control samples created and analysed by spiking purified water concentrates with *Cyclospora cayetanesis* oocysts. Both positive control samples returned results similar to those seen for the actual environmental samples. In addition, negative controls run with the positive controls also showed lack of contamination.

One of the questions raised by molecular analysis is related to the potential of microsporidia-specific PCR to detect other species of microsporidia in water, especially those species that infect fish, insects and amphibians but not humans. The same is true for molecular detection of *Cyclospora* spp., especially PCR, which has the potential to amplify the rRNA not only of the newly identified *Cyclospora* species (GenBank accession numbers AF111185, AF111186 and AF111187) but also those of *Eimeria* spp. and *Isospora* spp. However, the ability of PCR sequencing and database searching to distinguish between very closely related species of microsporidia has already been shown (Dowd *et al.*, 1999). Thus, the inherent ability of CDHC to confirm the identity of these organisms to the species level justifies the use of the PCR/CDHC for analyses of environmental samples. In those cases where comparisons of the amplicons and database sequence by pair-wise alignment showed less than 100% homology to the database sequences when using BLASTn, it was because the computer base calling software was unable to identify bases. An example of this would be when the base calling software incorporates an 'N' in place of an actual base due to a weak or covered signal from the actual base. It was felt, however, that manually calling the bases would introduce experimental bias into the analyses even when performed blindly prior to alignment.

In addition to molecular detection of *Cyclospora cayetanesis* by PCR and confirmation by CDHC, the samples

Table 1 | Guatemalan drinking water samples screened for emerging pathogenic protozoa by PCR and the identities of the organisms as determined by sequence analysis

Sample/source	<i>Cyclospora</i> spp. (PCR)	<i>Cyclospora</i> spp. (A, S, Sh, I)	HEM	<i>Giardia</i> spp. (IFA)/ <i>Cryptosporidium</i> (IFA)
	BLASTn ID		BLASTn ID	
1	<i>C. cayetanesis</i>	+	Not detected	+ / -
2	<i>C. cayetanesis</i>	+	<i>E. intestinalis</i>	+ / +
3	Not detected		Not detected	+ / +
4	Not detected		Not detected	+ / -
5	Not detected		Not detected	+ / -
6	Not detected		<i>E. intestinalis</i>	+ / +
7	+ No sequence*		Not detected	+ / -
8	<i>C. cayetanesis</i>	+	<i>E. intestinalis</i>	+ / +
9	Not detected		Not detected	+ / -
10	Not detected		Not detected	+ / -
11	+ No sequence	+	<i>E. intestinalis</i>	+ / -
12	Not detected		<i>E. intestinalis</i>	+ / -

*Initial PCR was positive but subsequent PCR purification and sequencing generated no results. Thus only presumptive detection is indicated.
A, S, Sh, I=autofluorescence, size, shape, internal structures.

that were positive were confirmed visually. Additional aliquots of the archived water samples were re-purified by density gradient flotation and subsequently filtered onto 22 mm 0.45 µm pore size membranes, which were then mounted on glass slides and screened by fluorescent microscopy. *Cyclospora cayetanesis* has characteristic autofluorescent properties, a distinctive size and a distinctive shape. These properties were used during microscopic analyses of the samples. Results of these analyses showed that four of the five samples contained organisms displaying correct fluorescence, shape and size. Because of the debris in the samples, visualization of internal structures was hindered. However, for each sample at least one organism was found to possess internal structures similar to *Cyclospora cayetanesis*. Visual identification of HEM

was not performed by either IFA or other microscopic methods because attempts at such analysis have been shown (Dowd *et al.*, 1998, 1999) to be virtually useless. The only microscopic method shown to have any utility in the study of the microsporidia is transmission electron microscopy (Curry & Canning, 1993; Weber *et al.*, 1994), which is a technology not easily, or feasibly, applied to such environmental samples (Dowd *et al.*, 1999).

DISCUSSION

The presence of *Encephalitozoon intestinalis* and *Cyclospora cayetanesis* in drinking water could have been

anticipated, as they are both suspected to be waterborne pathogens (Curry & Canning, 1993; Connor, 1997; Enriquez *et al.*, 1997; Dowd *et al.*, 1998; Sturbaum *et al.*, 1998; Sterling & Ortega, 1999). However, the importance of these findings should not be underestimated. The finding by IFA of *Giardia* sp. in these water samples was less surprising because *Giardia* sp. is a recognized waterborne agent. Another interesting observation was that two of the water samples contained all four of the protozoan pathogens (*Cryptosporidium* spp. and *Giardia* spp. as determined by IFA, and *Cyclospora cayetanesis* and *E. intestinalis* as determined by PCR). This shows that these pathogens co-occur in environmental samples and previous reports have shown that clinical diagnoses of gastrointestinal diseases can miss co-infections. This may be because *Cryptosporidium parvum* is an easily recognized, widely publicized protozoan pathogen, and well-documented methodologies are in place for its clinical diagnosis. Because of these reasons, it is one of the first organisms screened for when patients present with profuse watery diarrhoea. Thus, co-infections with other protozoa may be overlooked if *Cryptosporidium* spp. infection is initially diagnosed. In addition, a study by Raynaud *et al.* (1998) noted:

'It is concluded from the present study that cyclosporiasis is quite similar to cryptosporidiosis and both oocysts have affinity to acid fast stain so the present recommendations are that all laboratories, screening stool for *Cryptosporidium*, should measure the oocysts, to distinguish between these different parasites.'

In comparison, HEM such as *Encephalitozoon intestinalis* are so small (diameter 0.5–1.5 μm) that co-infections with these parasites are almost sure to be overlooked when patients are initially diagnosed with the larger *Cryptosporidium* spp. (diameter 5–7 μm). The only cases in which HEM are now regularly identified are when patients present with profuse watery diarrhoea with 'no apparent cause'. With such cases it would be unlikely for clinical microbiologists to screen for HEM unless the patient has AIDS or unless they are conducting a survey. Thus, the prevalence of HEM and *Cyclospora cayetanesis* infections may be dramatically underestimated.

There is a definite need for better understanding of the epidemiology of *Cyclospora cayetanesis* and HEM and for better management and control measures to ensure the

safety of water used for drinking and water-washed produce (Herwaldt & Beach, 1999). Confirmation of these water sources as a potential source of disease may rapidly lead to the development of better management practices being implemented by populations in developing countries, in order to reduce the prevalence of these parasites in waters used for consumption and irrigation. Little is known about the occurrence or prevalence of HEM and *Cyclospora cayetanesis* in relation to immunocompetent humans and wild and domestic animals. Both *Cyclospora cayetanesis* and HEM are being increasingly identified as the causative agent of traveller's diarrhoea, probably through the consumption of contaminated food or water. However, the environmental occurrence of these two emerging pathogens has not been adequately documented because of a previous lack of effective methodologies.

CONCLUSIONS

This study represents the first confirmation of *Cyclospora cayetanesis* and *Encephalitozoon intestinalis* in waters used for drinking. This study also greatly emphasizes the need for further research to document the occurrence of these protozoan pathogens in source and treated water. Finally, this study has further advanced the hypothesis that these two emerging pathogenic protozoa are potential waterborne aetiological agents of disease in humans.

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