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An Assay Combining Cell Culture with Reverse Transcriptase PCR To Detect and Determine the Infectivity of Waterborne *Cryptosporidium parvum*

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The presence of *Cryptosporidium* in drinking water supplies is a significant problem faced by the water industry. Although a variety of methods exist for the detection of waterborne oocysts, water utilities currently have no way of assessing the infectivity of detected oocysts and consequently are unable to accurately determine the risks posed to public health by waterborne *Cryptosporidium*. In this paper, the development of an infectivity assay for waterborne *Cryptosporidium parvum* is described. Oocysts were inoculated onto monolayers of Caco-2 cells and grown on microscope slides, and infections were detected by *C. parvum* specific reverse transcriptase PCR of extracted mRNA, targeting the heat shock protein 70 (*hsp70*) gene. A single infectious oocyst was detected by this experimental procedure. The use of concentrated samples obtained from 250 liters of finished water had no observable effect on the integrity of cell monolayers or on the infectivity of oocysts seeded into the concentrate. Intracellular developmental stages of the parasite were also detected by using fluorescently labeled antibodies. One pair of PCR primers targeting the *hsp70* gene was specific for *C. parvum*, while a second pair recognized all species of *Cryptosporidium* tested. The *C. parvum*-specific primers amplified DNA from 1 to 10 oocysts used to seed 65 to 100 liters of concentrated environmental water samples and were compatible with multiplex PCR for the simultaneous detection of *C. parvum* and *Giardia lamblia*. This paper confirms the utility of PCR for the detection of waterborne *C. parvum* and, most importantly, demonstrates the potential of an in vitro infectivity assay.

Cryptosporidium was confirmed as a significant waterborne public health threat by the 1993 outbreak in Milwaukee, Wis., in which an estimated 400,000 people became ill (23). Since 1983, there have been at least 31 reported outbreaks of cryptosporidiosis associated with either drinking or recreational water in the United States, the United Kingdom, Canada, Australia, and Japan, and over 25,000 cases in Canada and Japan were linked to drinking water in the first 6 months of 1996. Up to 87% of untreated raw water and 24% of finished drinking water contained *Cryptosporidium* oocysts, according to one survey (19), and analysis of 347 surface water samples between 1988 and 1993 demonstrated that 60% of the samples contained oocysts (20). The oocysts are resistant to chlorine at concentrations commonly used for drinking water treatment (typical residual concentrations range from 0.5 to 2 mg/liter), there is currently no effective anticryptosporidial agent for afflicted individuals (although cryptosporidiosis is usually self-limiting in otherwise healthy individuals), and the disease has potentially fatal consequences for immunocompromised individuals who become infected. As few as 30 oocysts caused infection in 20% of healthy volunteers tested, and the 50% infectious dose was determined to be 132 oocysts (8). Consequently, reliable detection methods would better enable water utilities to control this organism in both source and finished waters.

The detection method currently used in the United States, and included in the recently promulgated Information Collection Rule (39), is an indirect immunofluorescence assay (IFA) (2). However, this method has a variety of limitations. It underestimates oocyst numbers, with recovery efficiencies ranging from 0 to 140% (13), and it recognizes a diversity of *Cryptosporidium* species because of the broad specificity of the primary antibody. Only *Cryptosporidium parvum* is recognized as a human pathogen; therefore, species-specific detection is necessary to avoid reporting of, and subsequent remediation based on, false-positive results. Also, the IFA method cannot determine the viability or infectivity of detected oocysts. Consequently, a variety of alternative technologies have been proposed or developed to overcome the limitations of the IFA procedure.

A technique that has received much attention and the one that offers the greatest potential for the detection of a wide range of microorganisms in water is PCR. PCR primers targeting a variety of *Cryptosporidium* genes have been described (4–6, 14, 18, 30, 33, 35, 40, 41), and PCR-based *Cryptosporidium* detection assays have been reported for a variety of environmental and clinical samples. These include water (14, 32, 33), human and bovine feces (5, 21), wastewater (25), and milk (17). One of the benefits of PCR compared to IFA is the potential to determine the viability of detected oocysts; two different types of PCR-based viability assay have been described for *C. parvum*. The first of these assays involved DNase I digestion of oocyst suspensions to remove extracellular DNA, in vitro excystation, and subsequent amplification of a 451-bp amplicon from an undefined chromosomal region (10) or an 873-bp product from a repetitive oocyst protein (40). In both studies, it was reported that only viable oocysts produced amplification products. The second approach involved reverse transcriptase PCR (RT-PCR) of mRNA transcripts from an induced heat shock protein 70 (*hsp70*) gene, which detected a

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single viable oocyst seeded into environmental water concentrates (35).

However, if the water industry is to make accurate assessments of the risk to public health posed by waterborne *C. parvum* oocysts, it must be able to determine not just their presence and viability but also whether the oocysts are capable of causing infection. An infectivity assay will also provide the water industry with a tool to measure the efficacy of disinfection protocols. Human volunteer studies or animal infectivity models are impractical for use on a routine basis, but in vitro cell culture offers a promising alternative for the development of a *C. parvum* infectivity assay. Methods for in vitro infection of *C. parvum* in cell cultures have been reported, but there is no general consensus on the most appropriate cell line. The development of all stages of the life cycle of the organism was demonstrated in RL95-2 cells (31), the ability to support infection was compared for 11 continuous cell lines (37), and infection in Caco-2 cells proved useful for demonstrating the anticryptosporidial activity of maduramicin (3).

The objectives of this investigation were to develop an in vitro infectivity assay for *C. parvum* by using cell culture combined with RT-PCR to detect the infectious organisms and to develop *C. parvum*-specific PCR primers that would detect oocysts in environmental water samples and would be compatible with multiplex PCR for the simultaneous detection of *Cryptosporidium* and *Giardia.*

MATERIALS AND METHODS

Organisms and chemicals. Purified human- and bovine-derived oocyst preparations of *C. parvum* were obtained from Waterborne, Inc. (New Orleans, La.), and Parasitology Research Laboratories (Phoenix, Ariz.). *C. baileyi* and *C. muris* oocysts were generously supplied by B. Blagburn (Auburn University, Auburn, Ala.) and J. Owens (U.S. Environmental Protection Agency, Cincinnati, Ohio). *Giardia lamblia* and *G. muris* were obtained from Parasitology Research Laboratories. All other protozoa, bacteria, mammalian cell lines, and algae were obtained from the American Type Culture Collection. Chemicals, reagents, antibiotics, and cell culture media were supplied by Sigma Chemical Co. (St. Louis, Mo.) or Boehringer Mannheim (Indianapolis, Ind.) unless stated otherwise. All PCR and reverse transcription reagents were obtained from Perkin-Elmer (Foster City, Calif.).

Environmental water samples. Environmental water samples were collected at various locations throughout Southern California and concentrated by filtration and centrifugation, following the Information Collection Rule method (39). The turbidities of the various source and finished waters at the time of collection, and the volumes of water from which DNA was extracted, were as follows: EWS4, 6.2 nephelometric turbidity units (NTU), 91 liters; EWS5, 1.5 NTU, 80 liters; EWS6, 2.6 NTU, 68 liters; EWS7, 12.5 NTU, 76 liters; EWS8, 2.5 NTU, 75 liters; EWS9, 0.4 NTU, 69 liters; EWS10, 2.4 NTU, 66 liters; EWS11, 0.7 NTU, 64 liters; EWS12, 2.0 NTU, 102 liters; EWS13, 1.6 NTU, 86 liters; EWS14, 0.5 NTU, 75 liters; EWS15, 1.7 NTU, 73 liters; EWS16, 2.2 NTU, 81 liters; and EWS17, 0.9 NTU, 100 liters. Finished-water concentrates (FWC1, FWC2, and FWC3) were obtained from 1,000 liters of treatment plant effluents. The turbidities of these three samples ranged from 0.06 to 0.08 NTU.

DNA extraction from environmental water samples and pure cultures. Total DNA was extracted from 0.5 ml of these concentrated samples by a previously described method (32). The DNA pellet was resuspended in 100 μl of sterile distilled water. The same method was used to extract DNA from pure cultures of protozoa, bacteria, and algae. Extracted DNA was examined by agarose gel electrophoresis, using standard techniques (34). The DNA was purified on Wizard spin columns as specified by the manufacturer (Promega, Madison, Wis.).

In vitro culture of mammalian cell lines. Caco-2 cells (ATCC HTB 37) were grown in Eagle's minimal essential medium supplemented with 4 mM L-glutamine, 30 mM HEPES (pH 7.34), 100 U of penicillin per ml, 0.1 mg each of streptomycin and kanamycin per ml, 0.25 μg of amphotericin B per ml, and 15% fetal bovine serum (FBS; Hyclone, Logan, Utah). The cells were grown in SuperCell one- or four-well culture slides (Erie Scientific, Portsmouth, N.H.) in a humidified incubator at 35°C in an atmosphere containing 5% CO_2. Some slides were pretreated with collagen (10 μg/cm^2) for 24 h at 35°C to improve the attachment of cells to the slides. The cells were inoculated onto slides to achieve an initial density of 10^5 cells/cm^2, and the cultures were incubated until the monolayers reached 75 to 100% confluence (48 to 120 h). HCT-8 (ATCC CCL 244), MDBK (ATCC CCL 22), and Vero (ATCC CCL 81) cell lines were grown in Eagle's minimal essential medium or RPMI 1640 medium supplemented with antibiotics and 5 to 10% FBS.

Treatment of oocysts and environmental samples prior to infection. *C. parvum* oocysts were obtained as purified live suspensions in saline solution containing various antibiotics or 2.5% potassium dichromate. The oocysts were washed by resuspension in 2.5 volumes of ice-cold, sterile, distilled water and pelleted at 1,500 × *g*. Washed oocysts were simultaneously decontaminated and prepared for excystation by an adaptation of a previously published method (36). The oocysts were pelleted for 3 min at 5,000 × *g* at 4°C, resuspended in freshly prepared ice-cold 10% bleach (final concentration of hypochlorite, 0.5%) in phosphate-buffered saline (PBS), and incubated on ice for 10 min. The oocysts were washed twice by successive pelleting and resuspension in sterile, ice-cold PBS. The decontaminated oocysts were resuspended in 1 ml of cell culture growth medium.

To induce in vitro excystation, purified oocysts were resuspended in 1.1% hypochlorite and incubated on ice for 10 min. The oocysts were washed three times in cold PBS and incubated in PBS for 1 h at 37°C; they were then incubated for 2 h at 37°C, with gentle agitation, in prewarmed PBS containing 0.25% trypsin and 0.75% taurocholic acid (36). Excysted sporozoites were recovered by filtration through a 2-μm syringe filter and washed in Hanks' balanced salt solution. Excystation efficiencies ranged from 36 to 59% by this method.

In vitro infection of cell cultures. Decontaminated oocysts or sporozoites were resuspended and diluted in cell culture growth medium and inoculated onto 75 to 100% confluent monolayers of Caco-2 cells to achieve average densities ranging from 1 to 2 × 10^5 oocysts/cm^2. The monolayers were incubated at 35°C for 2 h to allow initiation of infection and were washed with 1 ml of PBS to remove uninfective parasites, empty oocyst walls, and compounds exhibiting toxicity toward the monolayers (9); they were then reincubated at 35°C for up to 96 h.

Pellets of finished-water concentrates (FWC) were seeded with *C. parvum* oocysts and decontaminated in 0.5% hypochlorite for 10 min at 4°C. The samples were washed five times by successive pelleting and resuspension in PBS before inoculation onto Caco-2 monolayers grown on four-well chamber slides. The monolayers were inoculated with these seeded water concentrates at densities equivalent to volumes of water ranging from 25 to 250 liters per well.

In some cases, the oocysts were labeled with fluorescein prior to inoculation onto the cell monolayers to allow differentiation of de novo infectious stages from original-inoculum oocysts (3). The oocysts were washed with 0.1 M sodium acetate-buffered saline (pH 5.5) and then incubated in 10 mM sodium periodate at 4°C for 20 min. After being washed in sodium acetate-buffered saline, the oocysts were resuspended in 240 μM fluorescein thiosemicarbazide at room temperature for 60 min (3). Infection of the monolayers was assessed by three methods: (i) application of fluorescent antibodies directed at various infectious stages of *C. parvum*, (ii) amplification of *hsp70* gene fragments, and (iii) detection of *hsp70* mRNA by RT-PCR.

Detecting infectivity with anti-*Cryptosporidium* antibodies. Infected monolayers were washed with PBS and fixed in 100% methanol for 5 min at room temperature. All antibody incubations were performed at room temperature. The monolayer was incubated in 1% blocking reagent (Boehringer Mannheim) for 30 min. It was washed twice with PBS and then incubated with 0.2 μg of anti-*C. parvum* sporozoite immunoglobulin M (IgM) antibody (MAb2H2; ImmuCell, Portland, Maine) per ml in a humidified chamber for 30 min. The monolayer was washed twice with PBS and then incubated with 0.5 ml of anti-mouse IgM (10 μg/ml) conjugated to the fluorochrome Cy3 (Jackson ImmunoResearch, West Grove, Pa.) or fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, Ala.) for 30 min in a humidified chamber protected from light. The Caco-2 cells were stained with a commercial counterstain (MeriFluor; Meridian Diagnostics, Cincinnati, Ohio) during incubation with the secondary antibody. The monolayers were washed twice with PBS, and following removal of the chamber assembly, the cells were mounted in glycerol containing 2% 1,4-diazabicyclo[2,2,2]octane. Fluorescence was observed by epifluorescence microscopy with the following filter combinations: (i) excitation at 450 to 490 nm, dichroic mirror at 510 nm, and emission at 515 nm for FITC (BH2 filter) and (ii) excitation at 510 to 560 nm, dichroic mirror at 575 nm, and emission at 590 nm for Cy3 (G2A filter).

RNA and DNA extraction from infected-cell cultures. All glassware used for RNA extraction reagents was baked at 180°C overnight, and all reagents were either treated with 1% diethylpyrocarbonate (DEPC) or made with DEPC-treated sterile distilled water. DEPC was removed from the water and reagents by autoclaving after incubation at room temperature for 8 h. Following incubation, the growth medium was removed from the infected-cell cultures and the monolayer was washed with PBS. Total RNA was extracted by adding 1 ml of TriReagent (Molecular Research Center, Cincinnati, Ohio) to each slide well and homogenizing the cells with a sterile pipette tip. The cell lysate was then transferred to a microcentrifuge tube, and the extraction was performed as specified by the manufacturer. Following ethanol precipitation and air drying, the RNA pellet was resuspended in 50 μl of DEPC-treated sterile distilled water. DNA was simultaneously extracted and recovered from the TriReagent lysis as recommended by the supplier, and the final pellet was resuspended in 100 μl of sterile distilled water.

mRNA was extracted directly, using oligo(dT) cellulose, without prior extraction of total RNA. The monolayers were washed briefly with PBS, and the cells were lysed in the slide wells by addition of 1 ml of sodium dodecyl sulfate lysis reagent (Sigma) and incubation at room temperature for 5 min. The cell lysate

was transferred to a 1.5-ml microcentrifuge tube and mixed with oligo(dT) cellulose, and the mRNA was recovered with a small-scale isolation kit as recommended by the supplier (Sigma).

PCR primers, probes, and DNA amplification. Two sets of PCR primers were designed, based on the sequence of the *C. parvum hsp70* gene (16) obtained from the GenBank database (accession no. U11761). An alignment of *hsp70* gene sequences from a range of organisms was created with the CLUSTAL V software package (12), and unique sequences within the *C. parvum hsp70* gene were identified. The CPHSP1 set of primers, cphsp2386F (5′-CTGTTGCTTATGGT GCTGCTG) and cphsp2672R (5′-CCTCTTGGTGCTGGTGGAATA), was selected to have broad specificity to recognize all species of *Cryptosporidium*. The CPHSP2 primer pair (33), cphsp2423F (5′-AAATGGTGAGCAATCCTCTG) and cphsp2764R (5′-CTTGCTGCTCTTACCAGTAC), was designed to be specific for *C. parvum*. The theoretical optimum annealing temperatures of the primer pairs, determined with the OLIGO primer analysis package (National Biosciences, Inc., Plymouth, Minn.), were 54.6 and 51.7°C for CPHSP1 and CPHSP2, respectively. Oligonucleotide probes cphsp2423 (5′-AAATGGTGAG CAATCCTCTGCCG) and cphsp2475 (5′-CCATTATCACTCGGTTTAGA) were designed to target internal regions of the amplicons obtained with CPHSP1 and CPHSP2, respectively. Amplification products from the CPHSP1 and CPHSP2 primer pairs were 307 and 361 bp, respectively. All primers and 5′-fluorescein-labeled oligonucleotide probes were synthesized by a commercial service (National Biosciences, Inc.). Through empirical evaluation of the primers, the optimum annealing temperatures and $MgCl_2$ concentrations were determined to be 53 and 55°C and 2.5 and 1.5 mM for CPHSP1 and CPHSP2, respectively. The specificity of the primers was tested on DNA extracted from a range of other organisms. The protozoa tested were *C. parvum*, *C. muris*, *C. baileyi*, *G. lamblia*, *G. muris*, *Hexamita* sp. strain ATCC 50329, *Entamoeba histolytica* ATCC 30015, *Entamoeba coli* ATCC 30946, *Babesia microti* ATCC 30222, *Endolimax nana* ATCC 50293, *Blastocystis hominis* ATCC 50177, *Trypanosoma theileri* ATCC 30017, *Cyclospora* sp. (M. Arrowood, Centers for Disease Control and Prevention, Atlanta, Ga.), *Toxoplasma gondii* ATCC 40050, *Dientamoeba fragilis* ATCC 30948, *Eimeria maxima* ATCC 40357, *Acanthamoeba castellani* ATCC 30010, *Pneumocystis carinii* ATCC 50385, and *Encephalitozoon* sp. strain CDC:0291:V213. The bacteria were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, *Aeromonas hydrophila* ATCC 7965, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 13883, *Bacillus subtilis* ATCC 6051, *Synechococcus* sp. strain ATCC 27145, and *Synechocystis* sp. strain ATCC 27150. The four mammalian cells described above were also tested, along with the algae *Chlorella vulgaris* ATCC 30581, *Selenastrum capricornutum* ATCC 22662, and *Scenedesmus obliquus* ATCC 11457 and the yeast *Saccharomyces cerevisiae.*

The sensitivity of the primers was determined by performing PCR on serial dilutions of oocysts. The *C. parvum*-specific primer pair (CPHSP2) was also designed to be compatible with primers targeting a heat shock protein gene in *G. lamblia*, to allow multiplex PCR for the simultaneous detection of both parasites. The sequences of the *G. lamblia* primers were 5′-AGGGCTCCGGCATAACT TTCC (forward) and 5′-GTATCTGTGACCCGTCCGAG (reverse) (1).

Amplification reaction mixtures contained 2.5 U of AmpliTaq, 1× PCR buffer, 0.25 μM each forward and reverse primer, 1.5 to 3.5 mM $MgCl_2$, and 200 μM each dATP, dCTP, dGTP, and dUTP in a 100-μl volume, overlaid with 75 μl of sterile mineral oil. To prevent carryover contamination, the reaction mixtures were treated with 1 U of uracil DNA glycosylase prior to amplification, as recommended by the manufacturer (Boehringer Mannheim). PCR was performed in a model 480 DNA thermal cycler (Perkin-Elmer) with the following temperature cycle: 94°C for 2 min followed by 40 cycles of 94°C for 30 s, annealing for 30 s, and 72°C for 1.5 min. A final extension incubation of 72°C for 5 min was followed by 5 min at 5°C. Amplification reactions on DNA extracted from environmental water samples were performed with reaction mixtures containing 10 μg of bovine serum albumin per ml.

Detection of *hsp70* gene fragments and mRNA transcripts by PCR and RT-PCR with the CPHSP2 primer pair. The RT reaction mixtures contained 2.5 U of murine leukemia virus RT, 2.5 μM random hexamers or oligo$(dT)_{16}$ primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP, and dTTP, and 1 U of RNase inhibitor in a 20-μl reaction volume. The reaction mixtures were incubated at 42°C for 20 to 60 min. PCR was performed on 10 to 20 μl of the RT reaction under the same general reaction conditions described above. The *C. parvum*-specific primers were used, and the reaction was annealed at 55°C in the presence of 1.5 mM $MgCl_2$. Negative controls consisted of RNA extractions performed on sterile distilled water and uninfected Caco-2 cells and reverse transcription and PCR performed on sterile distilled water. Following agarose gel electrophoresis, PCR products were analyzed by hybridization with the cphsp2475 oligonucleotide probe.

Oligonucleotide hybridization. PCR products were transferred to positively charged nylon membranes (Boehringer Mannheim) by a method described previously (32). Hybridization solutions contained 1% blocking reagent, 0.1% sarcosyl, 0.02% sodium dodecyl sulfate, and 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) for the cphsp2475 probe or 0.3× SSC for the cphsp2423 probe. The membranes were hybridized in 20 ml of hybridization solution, containing 50 pmol of 5′-fluorescein-labeled probe, for 18 h at 59°C in a rotary hybridization oven. Stringency washes (two 15-min washes at 57°C) were performed with 20 mM Tris (pH 7.4)–0.01% sodium dodecyl sulfate–1× SSC for cphsp2475 or 0.3× SSC for cphsp2423. Hybridized probe was detected with an anti-fluorescein antibody conjugated to alkaline phosphatase and the chemiluminescent substrate Lumigen or CDP-Star, as recommended by the supplier (Boehringer Mannheim). For gels containing digoxigenin-labeled molecular size markers (Boehringer Mannheim), membranes were incubated with an anti-digoxigenin antibody-fluorescein conjugate for 20 min prior to addition of the anti-fluorescein antibody-alkaline phosphatase conjugate. X-ray film (Fuji RX; Fisher Scientific, Tustin, Calif.) was exposed to the membranes at room temperature for periods ranging from 30 s to 10 min. Prior to application to nylon membranes by using a slot blot manifold, 50-μl volumes of the completed amplification reaction mixtures were denatured at 95°C for 10 min and diluted in 0.125 M NaOH–0.125× SSC. The diluted DNA was left on the membrane for 20 min, and the liquid was removed by application of vacuum pressure for 1 min. The DNA was fixed to the membrane, hybridized, washed, and detected by the methods described above for Southern blots.

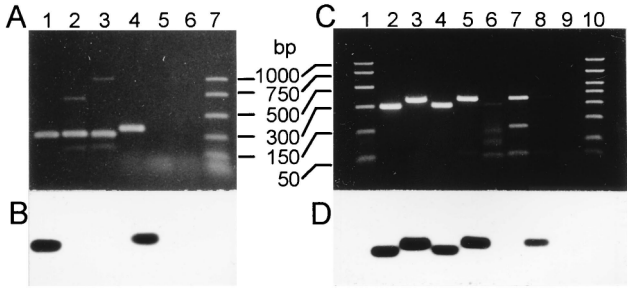

FIG. 1. (A) Agarose gel electrophoresis of PCR products obtained with primer pairs CPHSP1 (307 bp) and CPHSP2 (361 bp) from *C. parvum* (lanes 1 and 4), *C. muris* (lanes 2 and 5), and *C. baileyi* (lanes 3 and 6). Lane 7 contains molecular size markers. (B) Southern blot of the DNA in panel A hybridized with probe cphsp2475. (C) Agarose gel electrophoresis of CPHSP1 (301-bp) and CPHSP2 (361-bp) amplicons from *C. parvum* oocysts (lanes 2 and 3) and a mixture of *C. parvum* oocysts and *G. lamblia* cysts (lanes 4 and 5). Multiplex PCR was carried out on a mixture of cysts and oocysts with primers targeting a *G. lamblia* heat shock protein gene combined with primer pairs CPHSP1 (lane 6) and CPHSP2 (lane 7). Lanes 8 and 9 contain negative controls for CPHSP1 and CPHSP2, respectively. Lanes 1 and 10 contain molecular size markers. (D) Southern blot of the DNA in panel C hybridized with probe cphsp2475. Lane 10 contains digoxigenin-labeled molecular size markers (700, 500, 400, 300, and 200 bp).

RESULTS

Evaluation of CPHSP primers. A search of the GenBank database revealed that primer pair CPHSP2 had no homology within any sequences other than the *C. parvum hsp70* gene. The specificity of the primers was evaluated with DNA extracted from a range of 16 non-*Cryptosporidium* protozoa, 8 bacteria, 3 algae, 4 mammalian cell lines (including Caco-2 cells), and 1 yeast. Weak amplification was obtained with DNA from some of these sources with primer pair CPHSP2 annealed at 53°C but not at 55°C. These weak amplicons were not the correct size, and the combination of PCR with CPHSP2, followed by hybridization with the *C. parvum*-specific oligonucleotide probes, was negative for all the test organisms. Parallel amplification reactions were performed on DNA extracted from all these organisms, seeded with *C. parvum* DNA from 100 oocysts, to ensure that negative results were not the result of PCR inhibition. None of the DNA extracts inhibited the PCR. Primer pair CPHSP2 amplified the expected 361-bp product from *C. parvum* but did not amplify DNA from *C. muris* or *C. baileyi* (Fig. 1A). Therefore, primer pair CPHSP2 was specific for *C. parvum*. Primer pair CPHSP1 amplified the expected 307-bp product from all three species of *Cryptosporidium* tested, but only the *C. parvum* amplicon hybridized with the cphsp2475 probe (Fig. 1B). The CPHSP1 primers also amplified DNA from *S. cerevisiae*, but the amplicon did not hybridize with the cphsp2475 probe. The DNA fragments obtained from CPHSP1 amplification on *C. muris* and *C. baileyi*

are currently being sequenced to allow the design of primers and probes specific for the *hsp70* gene of these organisms. Both sets of primers (CPHSP1 and CPHSP2) detected a single oocyst when DNA was extracted from purified preparations of oocysts.

Although much attention is currently focused on *C. parvum*, the protozoan parasite *G. lamblia* is still a concern to the water industry, since it is the most common parasite of humans in the United States (11). The current assay (IFA) for detection of waterborne *Cryptosporidium* also detects *Giardia*, and such simultaneous detection methods offer time- and labor-saving advantages over single-organism assays. We therefore designed primer pair CPHSP2 to be compatible with primers targeting an *hsp* gene of *Giardia* (1). This multiplex PCR resulted in amplification of a 361-bp fragment from *C. parvum* and a 163-bp product from *G. lamblia* in reactions performed at 55°C with 1.5 mM $MgCl_2$ (Fig. 1C, lane 7). Multiplex PCR detected both *C. parvum* and *G. lamblia* seeded into environmental water concentrates. Primer pair CPHSP1 was not compatible with multiplex PCR (Fig. 1C, lane 6).

Assessment of infectivity by RT-PCR. Control RT-PCRs were performed to ensure that the amplicons obtained were the result of RNA amplification rather than of contaminating DNA. Reactions in which the RT was omitted did not yield amplification products, and DNase I treatment of mRNA templates did not inhibit RT-PCR. RNase digestion of RT-PCR templates prevented amplification. When total RNA extracted with TriReagent was used as the template, it was necessary to treat the samples with DNase I prior to RT-PCR because it was difficult to recover the RNA without traces of DNA contamination. The mRNA extracted with oligo(dT)-cellulose was free of contaminating DNA. Negative RT and PCR controls, containing sterile distilled water in place of RNA or cDNA template, were always included with each set of reactions.

C. parvum-specific *hsp70* mRNA in Caco-2 monolayers infected with a single oocyst was detected following a 48-h incubation at 37°C and extraction of mRNA with oligo(dT)-cellulose (Fig. 2B, lane 2). Oocyst densities were determined by using the average of triplicate hemacytometer counts, and lower oocyst densities were obtained by dilutions in PBS. Hemacytometer counts are more accurate than IFA staining, because the primary antibody used for the standard IFA procedure does not bind to all oocysts and thus underestimates oocyst densities. Positive hybridization was obtained following *C. parvum*-specific RT-PCR on mRNA extracted from monolayers seeded with 1, 5, 10, 50, 100, 500, or 1,000 oocysts (Fig. 2). The detection sensitivity was substantially increased by incubating the RT reaction mixture for 1 h, using random hexamers for cDNA synthesis, and performing 40 cycles of amplification (Fig. 2B), compared to the use of a 45-min incubation and $oligo(dT)_{16}$ primers for cDNA synthesis and 35 cycles of amplification (Fig. 2A). RT-PCR products were also obtained from purified sporozoites (Fig. 2A, lanes 9 and 10) and from heat-induced oocysts (Fig. 2B, lane 9). Oligo(dT)-linked magnetic beads have also been used for the recovery of mRNA directly from samples without prior extraction of total RNA (35), but since we achieved a sensitivity of a single infectious oocyst with oligo(dT)-cellulose, it was not considered necessary to compare the two methods.

When amplifying DNA extracted from oocysts with primer pair CPHSP2, a 361-bp amplicon was generated (Fig. 2A, lane 8), as expected from sequence analysis. However, RT-PCR of mRNA from infected-cell cultures resulted in the 361-bp amplicon and a second, larger amplicon (about 500 bp). Both amplicons hybridized with the cphsp2475 probe when mRNA was extracted from cell cultures infected with a high density of oocysts. However, with mRNA extracted from monolayers infected with <100 oocysts/cm^2, the larger amplicon became predominant. Uninfected cells never gave amplicons of any size with these primers. This larger amplicon may be the result of (i) reduced primer specificity in RT-PCRs, (ii) partial dimerization of specific products, or (iii) inefficient mRNA processing in sporozoites and other developmental stages of the parasite. We are currently sequencing the amplicon so that we can determine its origin.

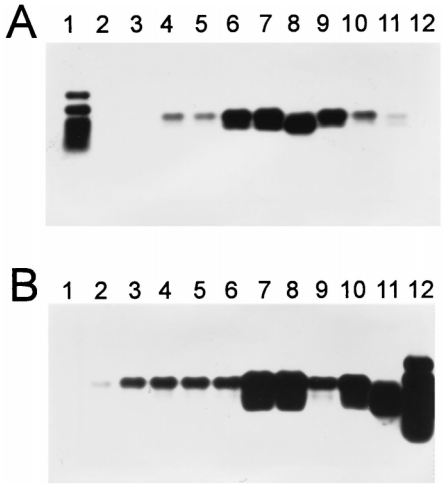

FIG. 2. Detection of *C. parvum* infection in Caco-2 cell monolayers by RT-PCR of extracted mRNA and subsequent hybridization with fluorescein-labeled oligonucleotide probes. (A) Southern blot of a 1% agarose gel containing RT-PCR products from mRNA extracted from monolayers (48 h postinfection) infected with 10 to 500 oocysts (lanes 4 to 7), sporozoites obtained by in vitro excystation of 10^5 and 10^4 oocysts (lanes 9 and 10), and cell culture medium removed from a monolayer immediately following inoculation with 10^3 oocysts (lane 11). Lanes 8 and 12 contain PCR products from DNA extracted from 10^5 oocysts and 10^5 Caco-2 cells, respectively. Lane 3 is the negative control. Lanes 1 and 2 contain digoxigenin-labeled (50- to 1,000-bp) and unlabeled molecular size markers, respectively. mRNA was reverse transcribed at 42°C for 45 min with $oligo(dT)_{16}$ primers in a 20-μl final volume. Half of the extracted mRNA was used as the template. PCR was performed on 50% of the RT reaction mixture with primer pair CPHSP2, annealed at 55°C for 35 cycles. Each lane of the gel contained 20% of the resulting amplification reaction. DNA was transferred, hybridized, and detected by the procedures described in Materials and Methods. The probe was cphsp2475. X-ray film was exposed for 1 min. (B) Caco-2 monolayers were infected with 0, 1, 5, 10, 50, 100, 500, or 1,000 *C. parvum* oocysts (lanes 1 to 8), and RT-PCR was performed on mRNA extracted 48 h after initiation of infection. Lanes 9 and 10 contain RT-PCR products obtained from mRNA extracted from 1,000 heat-induced oocysts and the sporozoites resulting from an in vitro excystation of 10^5 oocysts, respectively. Lane 10, PCR of DNA extracted from 10^5 oocysts. Lane 12 contains digoxigenin-labeled molecular size markers. mRNA was reverse transcribed at 42°C for 60 min with random hexamers in a 40-μl final volume. Half of the extracted mRNA was used as the template for reverse transcription, and PCR was performed on 20 μl of the reverse transcription reaction mixture with primer pair CPHSP2 annealed at 55°C for 40 cycles. The hybridization conditions, probe, and X-ray exposure were the same as those used in panel A.

Cell monolayers that were incubated for 10 days prior to inoculation with oocysts supported higher levels of infectivity than did 5-day-old cells, as determined by staining with fluorescent antibodies, but no overall differences were detected based on comparisons of the intensity of the hybridization signal following RT-PCR of extracted RNA. The absence of 5% FBS in the cell growth medium or pretreatment of culture slides with collagen (10 μg/cm^2) did not affect the infectivity of the oocysts. However, these conclusions were based on semiquantitative comparisons of hybridization signals. Development of quantitative RT-PCR with these primers will allow accurate measurements of infectivity. An earlier investigation reported that the use of 10% FBS resulted in the development

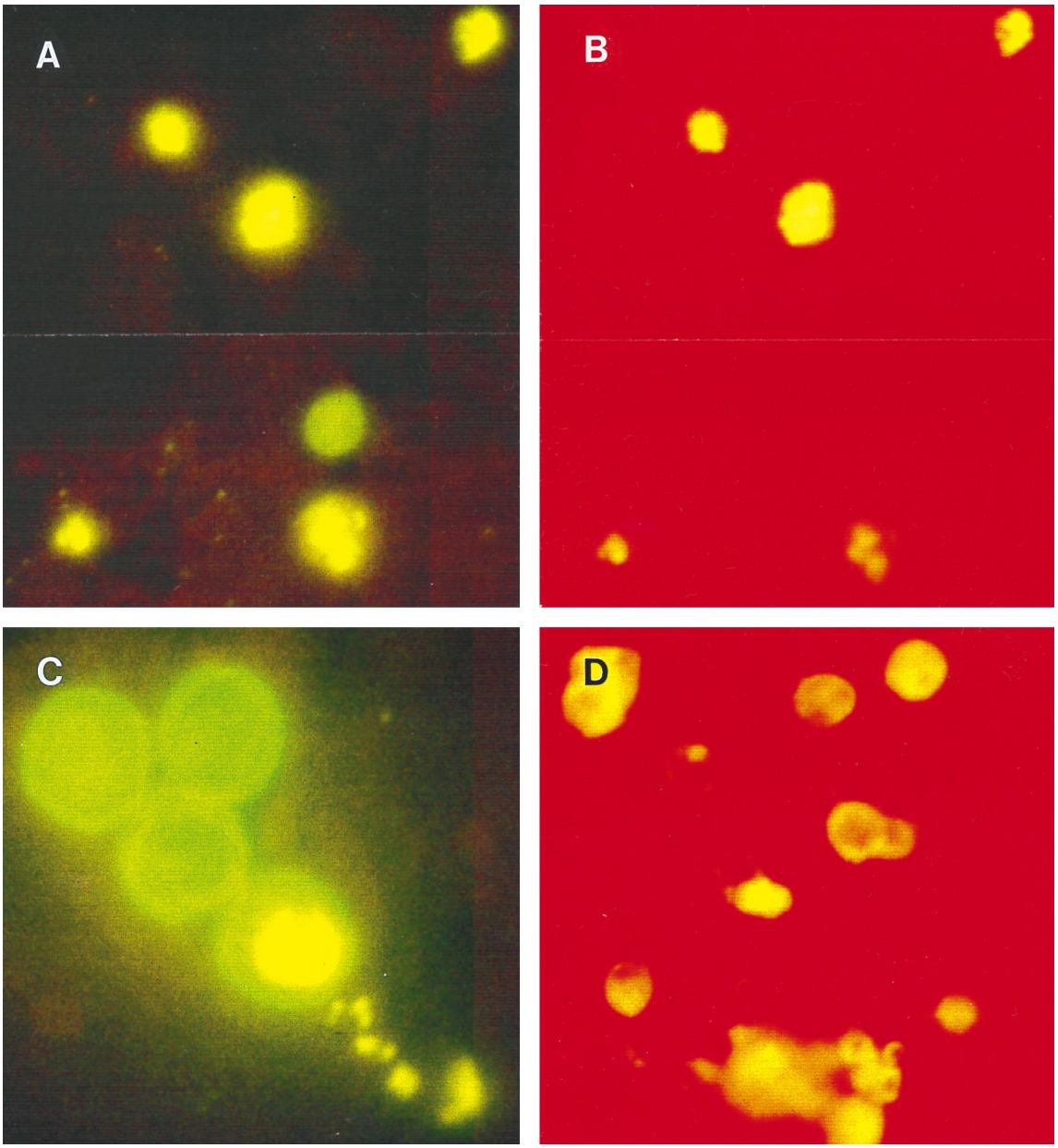

FIG. 3. Immunofluorescence detection of *C. parvum* infection in Caco-2 monolayers. (A) Original-inoculum oocysts (green fluorescence) were labeled with FITC by periodic acid oxidation, while various developmental stages (yellow fluorescence) were detected with an anti-sporozoite IgM as the primary antibody and anti-IgM antibody conjugated to Cy3 as the secondary antibody. The cells were observed microscopically at a magnification of ×400 with a BH2 filter. (B) The same image as in panel A, observed with the G2A filter. (C) Four inoculum oocysts labeled with FITC. A single oocyst was infectious and capable of excysting and releasing sporozoites, which were detected with anti-sporozoite IgM and anti-IgM-Cy3, observed with a BH2 filter at a magnification of ×1,000. (D) Intracellular developmental stages in a monolayer inoculated with an FWC seeded with *C. parvum* oocysts. Infectious stages were detected with anti-sporozoite IgM and anti-IgM-Cy3. G2A filter, magnification ×400.

of twice as many parasite stages compared to the use of 5% FBS (38).

Assessing infectivity with fluorescent antibodies. Fluorescently labeled antibodies were used to monitor the status of infections as an intermediate step in the development of the RT-PCR detection method. *C. parvum* infectious foci were readily detected with the anti-sporozoite IgM and Cy3-labeled anti-IgM antibodies when the original-inoculum oocysts were directly labeled with FITC. Under microscopic observation with the BH2 filter, inoculum oocysts fluoresced green while sporozoites, merozoites, and other stages in the life cycle of *C. parvum* fluoresced yellow (Fig. 3A and C). FITC-labeled inoculum oocysts were not observed when the G2A filter was used (Fig. 3B). This combination of fluorescent labels was also used to detect infectious stages in monolayers inoculated with FWCs seeded with *C. parvum* oocysts (Fig. 3D). Dual fluorescent labels for discrimination between inoculum oocysts and de novo oocysts have been used previously in a cell culture assay studying the effects of anticryptosporidial agents (3). The disadvantage of this approach is that any other organisms that are in the original inoculum, apart from *C. parvum*, will also be labeled with FITC during periodic acid treatment. This may be

TABLE 1. Infectivity in monolayers inoculated with *C. parvum*-seeded FWCs

Water sample	Detection of *C. parvum* infection in monolayers with FWC at[a]:							
	0 liters[b]		10 liters		50 liters		100 liters	
	IFA[c] (10^3)	RT-PCR[d]	IFA (10^3)	RT-PCR	IFA (10^3)	RT-PCR	IFA (10^3)	RT-PCR
FWC1	3.2 ± 0.4	++	3.1 ± 0.4	++++	4.7 ± 1.2	+++	4.7 ± 0.3	+
FWC2	2.8 ± 0.6	++++	3.1 ± 0.5	+++	2.1 ± 0.9	+++	2.8 ± 0.1	+++

[a] Caco-2 monolayers were inoculated with FWCs seeded with 10^5 oocysts.
[b] The monolayers were inoculated with an equal volume of growth medium in place of FWC.
[c] Intracellular developmental stages (indicative of infection) were detected by IFA with anti-sporozoite IgM and FITC-conjugated anti-IgM. Values are the mean and standard deviation for duplicate monolayers of infectious foci/cm^2.
[d] RT-PCR was performed on the old medium removed from the monolayer at 2 h postinfection. ++++, very strong hybridization signal; +++, strong hybridization signal; ++, intermediate hybridization signal; +, weak hybridization signal.

particularly problematic when detecting infectious *C. parvum* in concentrated water samples, since these samples contain a range of organisms. Although oocyst preparations were decontaminated in 0.5% hypochlorite prior to infection of monolayers, we observed bacteria and yeast cells labeled with FITC. Both types of organism were easily discriminated from *C. parvum* by their size, shape, and intensity of fluorescence. When inoculum oocysts were not directly labeled and indirect immunofluorescence (anti-sporozoite IgM followed by anti-IgM conjugated to FITC) was used to detect infectious stages, the different stages were differentiated on the basis of size. Inoculum oocysts were 5 to 8 μm in diameter, whereas stages that were ≤3 μm were considered to be intracellular developmental stages of the parasite.

It has previously been reported that HCT-8 cells supported more *C. parvum* developmental stages than did 10 other cell lines tested (37). Upton et al. (37) reported that HCT-8 cells supported 2.2-fold more parasite stages and needed only 14 h of incubation prior to inoculation with oocysts compared to Caco-2 cells, which needed 120 h of preincubation. However, in our laboratory, Caco-2 cells were the easiest to handle on a routine basis, supported higher densities of infectious organisms (results not shown), and allowed easier observation of infectious foci with fluorescently labeled antibodies. Caco-2 cells also have the advantage of supporting in vitro infection by a variety of other organisms that are important to the water industry, such as enterotoxigenic *Escherichia coli* (7), *Shigella* (27), *Giardia* (15), and enteric viruses (29).

Infection of monolayers with seeded FWCs. FWCs obtained by filtration and centrifugation of up to 250 liters of water had no adverse effect on the integrity of Caco-2 monolayers as determined by microscopic observation of monolayers both before and after fixation. Inoculation with FWCs also had no detrimental effect on the infectivity of *C. parvum* oocysts (Table 1).

Although monolayers were inoculated with *C. parvum* oocysts at a density of 10^5/cm^2, the typical maximum density of parasite developmental stages following a 48-h postinfection incubation was 3×10^3 to 6×10^3/cm^2. Considering that each oocyst contained four sporozoites capable of initiating infection, this represented an approximately 100-fold decrease. Possible explanations for this include (i) an infectivity rate of only 1% for the *C. parvum* oocysts we tested; (ii) inhibition of infection by compounds in the monolayer or growth medium; (iii) removal of potentially infectious, unexcysted oocysts after 2 h of incubation; and (iv) failure of the antisporozoite antibody to bind to all developmental stages. A 1% infectivity rate was discounted since *C. parvum*-specific mRNA was detected by RT-PCR from monolayers infected with a single oocyst (Fig. 2). The significance of the third scenario at one critical step in the in vitro infection procedure was investigated. Monolayers were infected with two different FWC (obtained from 10 to 250 liters of water) seeded with 10^5 oocysts/cm^2. At 2 h postinfection, the growth medium was removed from the monolayer and replaced with fresh medium (0.25 ml in four-well slides). The old medium was concentrated by centrifugation, and total RNA was extracted. RT-PCR was performed on this RNA, using random primers for the RT reaction and primer pair CPHSP2 for the amplification. The results for both FWC1 and FWC2 indicated that large quantities of mRNA-producing oocysts were removed from the monolayer during the 2-h postinfection wash (Table 1). The variable intensities of RT-PCR amplicons following hybridization with the cphsp2475 probe indicated that the loss of oocysts was not consistent for all samples. Extending the initial incubation period to 3 h increased the density of developmental stages. Further confirmation was provided by the ability of the removed medium to initiate infection in fresh monolayers. In this experiment, inhibition of infection by compounds in the FWC could be discounted since the infectivity in the control monolayer, which received no FWC, was the same as that in monolayers which did. A 3-h postinfection wash was reported to enhance *C. parvum* infectivity in MDBK cells by removing toxic compounds released by the inoculum, unexcysted oocysts, and oocyst remnants (9). However, in our in vitro infectivity assay, a 2-h postinfection wash appeared to be detrimental. We are currently investigating the effects of prolonging the initial incubation period, or of removing it altogether, on the infectivity of oocysts seeded into environmental water concentrates. Variable staining intensities of the anti-sporozoite and anti-oocyst antibodies (results not shown) indicated that the anti-sporozoite antibody did not recognize all intracellular developmental stages of the parasite.

Despite this loss of potentially infectious oocysts during monolayer manipulation, we were able to detect mRNA from monolayers infected with a single oocyst, as described above (Fig. 2). It has been demonstrated that supplementation of growth medium with 10% FBS and four vitamins (ascorbic acid, calcium pantothenate, folic acid, and *para*-aminobenzoic acid) resulted in the development of 10^6 parasite stages in cell cultures inoculated with 10^6 oocysts (38). We will therefore investigate the effects of various medium supplements on the generation of *C. parvum* developmental stages in infected monolayers.

Detection of *C. parvum* DNA in seeded environmental water concentrates. DNA was extracted from concentrates of environmental water samples obtained by filtration and centrifugation of 65 to 100 liters of untreated water. These extracted DNA samples showed different degrees of PCR inhibition, but amplification was significantly improved by spin column puri-

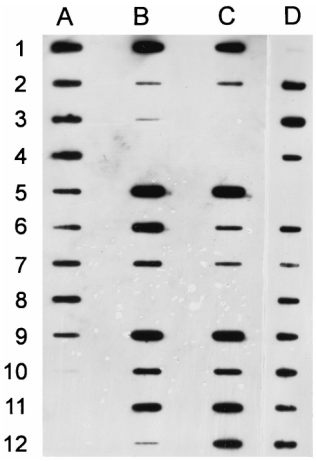

FIG. 4. Detection of *C. parvum* oocysts used to seed concentrates of environmental water samples by PCR with the CPHSP2 primer pair. Amplicons were applied to a nylon membrane by using a slot blot manifold and hybridized with probe cphsp2475 by the procedures described in Materials and Methods. DNA was extracted from 1,000 purified oocysts (A1), EWS6 seeded with 5,000 to 1 oocysts (A2 to A11), and unseeded EWS6 (A12). Slots B1 to C12 are described as follows: sample designation, number of oocysts with which the sample was seeded, and whether the DNA was purified (P) or not (UP) with spin columns prior to PCR. B1, EWS4, 100, P; B2, EWS4, 10, P; B3, EWS4, 100, UP; B4, EWS4, 10, UP; B5, EWS8, 100, P; B6, EWS8, 10, P; B7, EWS8, 100, UP; B8, EWS8, 10, UP; B9, EWS5, 100, P; B10, EWS5, 10, P; B11, EWS5, 100, UP; B12 EWS5, 10, UP; C1, EWS7, 100, P; C2, EWS7, 10, P; C3, EWS7, 100, UP; C4, EWS7, 10, UP; C5, EWS10, 100, P; C6, EWS10, 10, P; C7, EWS10, 100, UP; C8, EWS10, 10, UP; C9, EWS9, 100, P; C10, EWS9, 10, P; C11, EWS9, 100, UP; C12, EWS9, 10, UP. D1, negative control; D2, positive control; D3 and D4, FWC3 seeded with 100 and 10 oocysts, respectively; D5, empty. Samples in slots D6 to D12 were each seeded with 100 oocysts: D6, EWS11; D7, EWS12; D8, EWS13; D9, EWS14; D10, EWS15; D11, EWS16; D12, EWS17. The DNA templates for slots D1 to D4 were not purified, whereas the extracted DNA for slots D6 to D12 was purified on spin columns.

fication of the DNA prior to PCR. Addition of unpurified DNA extracted from the equivalent of 6.8 liters of EWS6 allowed the detection of five oocysts by PCR but inhibited the detection of one oocyst (Fig. 4, slots A2 to A11). Purification of extracts on spin columns allowed the detection of a single oocyst with this sample.

Of 14 environmental samples with turbidity values ranging from 0.4 to 12.5 NTU, only 1 (EWS7, 12.5 NTU) failed to yield an amplification product when seeded with 100 oocysts without prior purification of the DNA (Fig. 4, slot C3). Following spin column purification of the DNA, amplicons were obtained from all environmental samples seeded with 10 oocysts. For some samples (EWS9, 0.4 NTU), DNA purification was not necessary to obtain amplification from 10 oocysts. Generally, the samples with the lowest turbidity gave the strongest hybridization signals.

DISCUSSION

Following publication of the *C. parvum hsp70* gene sequence (16), primers targeting the gene were recently described for an RT-PCR-based viability assay (35) and for detection of *C. parvum* in cell culture (26, 33). The reported detection sensitivity in one of these assays was a single viable oocyst used to seed 2 liters of four environmental water samples with turbidities ranging from 1.2 to 12 NTU (35). Although the authors of that report reported successful amplification (35), detailed analysis of the primers by the authors of the present paper revealed an excessive difference (23°C) between the melting temperatures (T_m) of the amplification product and reverse primer. Also, the difference in T_m of the primers was 12.5°C. Such large differences in T_m may lead to inefficient amplification in some environmental samples. In an earlier evaluation of primers targeting *Cryptosporidium* and *Giardia*, those with large T_m differences were less effective for amplification of specific amplicons than were primers with closely matched T_m (32). The T_m differences for the primers described in the present paper were 0.4 and 4.9°C for primer pairs CPHSP1 and CPHSP2, respectively. Primers amplifying a 643-bp fragment from *C. parvum hsp70* mRNA were also recently described (26).

In previous reports of PCR-based *Cryptosporidium* detection in water, oocysts used to seed environmental samples were purified by immunomagnetic capture (14) or density gradient centrifugation (35) prior to extraction of DNA and subsequent PCR. Despite the advantage of precleaning of environmental samples, both of these methods may place limitations on detection assays. Density gradient centrifugation may not recover all of the oocysts in a sample and may therefore decrease the sensitivity of detection. In an evaluation of the standard IFA procedure for detection of waterborne *Cryptosporidium*, only 27% of oocysts were recovered from Percoll-sucrose density gradients with a specific gravity of 1.1 (28). Both procedures selectively recover the target organism, either by specific antibody binding or by sedimentation at a particular specific gravity. However, it would be advantageous to perform parallel detection assays for multiple pathogens on a single sample. This can be accomplished by extraction of total DNA from a concentrated water sample without selective recovery of a particular organism. Any manipulations performed after the DNA extraction, such as spin column purification, will not selectively target the DNA of any one organism.

In the present study, we demonstrated the detection by PCR of 1 to 10 oocysts in concentrates from 65 to 100 liters of untreated environmental water samples following DNA extraction and spin column purification. We are currently adapting the extraction procedure (e.g., addition of polyvinylpolypyrrolidone) to remove more of the PCR-inhibitory compounds so that we can consistently detect a single oocyst in 100-liter water concentrates, irrespective of the turbidity of the water. While most authors have reported detection sensitivities of 1 to 10 oocysts when purified oocyst preparations were used, the detection sensitivities in environmental samples have varied greatly. In a recent paper comparing different primers for the detection of *Cryptosporidium*, we demonstrated sensitivities ranging from 5 to 50 oocysts by using DNA extracted from environmental water concentrates and not purified further (32). In this earlier study, it was usually necessary to perform two amplification reactions, each comprising 40 cycles, to achieve this level of sensitivity. In the present study, spin column purification of extracted DNA obviated the requirement for multiple or nested reactions. Nested PCR, with primers targeting an oocyst wall protein gene, was necessary to detect 3.7×10^2 oocysts used to seed 1 liter of wastewater (25), and 500 oocysts were detected in 1 g of human feces by nested PCR targeting an undefined chromosomal region (5). In a recent study involving selective recovery of *Cryptosporidium* by immunomagnetic capture, RT-PCR detected a single viable oocyst used to seed concentrates from 2 liters of water (35), and 1 to 10 oocysts were detected in 20 ml of artificially contaminated milk (17).

It has been reported that there was no difference in the levels of mRNA in heat-induced oocysts and in uninduced oocysts (35), and the authors of that report speculated that other stress factors, such as storage of oocysts at 4°C, were responsible for this. However, it is possible that the *hsp70* gene

of *C. parvum* is constitutively expressed, since the 5′ noncoding part of the gene does not contain the ideal regulatory heat shock element common to most *hsp70* genes (16). Constitutive high levels of expression have been detected in several organisms (24).

Heat shock proteins are the major products of protein synthesis when an organism is subjected to heat stress (22), and it has been proposed that the heat shock response plays a fundamental role during host invasion by parasites (24). This makes *hsp* genes the ideal target for RT-PCR because, under certain conditions, organisms will contain large quantities of *hsp* mRNA. The utility of the *hsp70* gene as a target for a PCR-based viability assay has been demonstrated previously (35). In the present paper, we describe the use of the *hsp70* gene as the target for an infectivity assay for waterborne *C. parvum*, combining in vitro infection of Caco-2 cells with RT-PCR. Infections were monitored and assessed by RT-PCR of *hsp70* mRNA and the application of fluorescently labeled antibodies. We recently reported the sequences of the *C. parvum* specific primers (primer pair CPHSP2) (33), but in the present study we describe the detailed evaluation and application of the primers. This is the first description of *C. parvum*-specific RT-PCR on cell cultures infected with oocysts used to seed environmental water concentrates.

Although the *hsp70* gene has thus been shown to be an appropriate target for viability and infectivity assays, the design of the PCR primers should be given considerable attention. Some areas of the gene may offer greater specificity, while different combinations of primers may result in greater sensitivity and compatibility. The primers described in this paper were capable of detecting a single infectious parasite against a background of $>10^6$ mammalian cells and also of detecting infectious oocysts used to seed FWCs. These primers consistently detected 10 oocysts used to seed environmental water concentrates and were compatible with multiplex PCR for the simultaneous detection of *C. parvum* and *G. lamblia*. Further research must be conducted to optimize the in vitro infectivity assay so that all infectious oocysts yield intracellular developmental stages and to develop the quantitative aspects of the assay. Also, the methods must be simplified if they are to achieve broad application. However, the results presented in this paper confirmed the utility of PCR for detection of waterborne *C. parvum* and, most importantly, demonstrated the potential of an in vitro infectivity assay that may allow the water industry to make more accurate assessments of the risk posed to public health by waterborne *Cryptosporidium.*

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REFERENCES

1. **Abbaszadegan, M., M. S. Huber, I. L. Pepper, and C. P. Gerba.** 1993. Detection of viable *Giardia* cysts in water samples using polymerase chain reaction, p. 529–548. *In* Proceedings of the American Water Works Association Water Quality Technology Conference. American Water Works Association, Denver, Colo.
2. **American Society for Testing and Materials.** 1991. Proposed test method for *Giardia* cysts and *Cryptosporidium* oocysts in low-turbidity water by fluorescent antibody procedure, p. 925–935. ASTM standards 11.01. American Society for Testing and Materials, Philadelphia, Pa.
3. **Arrowood, M. J., L.-T. Xie, and M. R. Hurd.** 1994. In vitro assays of maduramicin activity against *Cryptosporidium parvum*. J. Eukaryot. Microbiol. **41:**23S.
4. **Awad-El-Kariem, F. M., D. C. Warhurst, and V. McDonald.** 1994. Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. Parasitology **109:**19–22.
5. **Balabat, A. B., G. W. Jordan, Y. J. Tang, and J. Silva.** 1996. Detection of *Cryptosporidium parvum* DNA in human feces by nested PCR. J. Clin. Microbiol. **34:**1769–1772.
6. **Carraway, M., S. Tzipori, and G. Widmer.** 1996. Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. Appl. Environ. Microbiol. **62:**712–716.
7. **Darfeuille-Michaud, A., D. Aubel, G. Chauviere, C. Rich, M. Bourges, A. Servin, and B. Joly.** 1990. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. Infect. Immun. **58:**893–902.
8. **Dupont, H. L., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose, and W. Jakubowski.** 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. **332:**855–859.
9. **Eggleston, M. T., M. Tilley, and S. J. Upton.** 1994. Enhanced development of *Cryptosporidium parvum* in vitro by removal of oocyst toxins from infected cell monolayers. J. Helminthol. Soc. Wash. **61:**122–125.
10. **Filkorn, R. A., A. Wiedenmann, and K. Botzenhart.** 1994. Selective detection of viable *Cryptosporidium* oocysts by PCR. Zentralbl. Hyg. Umweltmed. **195:**489–494.
11. **Hibler, C. P., and C. M. Hancock.** 1990. Waterborne giardiasis, p. 271–293. *In* G. A. McFeters (ed.), Drinking water microbiology. Springer-Verlag, New York.
12. **Higgins, D. G., A. J. Bleasby, and R. Fuchs.** 1992. CLUSTAL V: improved software for multiple sequence alignment. CABIOS **8:**189–191.
13. **Jakubowski, W., S. Boutros, W. Faber, R. Fayer, W. Ghiorse, M. LeChevallier, J. B. Rose, S. Schaub, A. Singh, and M. H. Stewart.** 1996. Environmental methods for *Cryptosporidium*. J. Am. Water Works Assoc. **88:**107–121.
14. **Johnson, D. W., N. J. Pieniazek, D. W. Griffin, L. Misener, and J. B. Rose.** 1995. Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl. Environ. Microbiol. **61:**3849–3855.
15. **Katelaris, P. H., A. Naeem, and M. J. Farthing.** 1995. Attachment of *Giardia lamblia* trophozoites to a cultured human intestinal cell line. Gut **37:**512–518.
16. **Khramtsov, N. V., M. Tilley, D. S. Blunt, B. A. Monteleone, and S. J. Upton.** 1995. Cloning and analysis of a *Cryptosporidium parvum* gene encoding a protein with homology to cytoplasmic form HSP70. J. Eukaryot. Microbiol. **42:**416–422.
17. **Laberge, I., A. Ibrahim, J. R. Barta, and M. W. Griffiths.** 1996. Detection of *Cryptosporidium parvum* in raw milk by PCR and oligonucleotide probe hybridization. Appl. Environ. Microbiol. **62:**3259–3264.
18. **Laxer, M. A., B. K. Timblin, and R. J. Patel.** 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **45:**688–694.
19. **LeChevallier, M. W., W. D. Norton, and R. G. Lee.** 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl. Environ. Microbiol. **57:**2610–2616.
20. **LeChevallier, M. W., and W. D. Norton.** 1995. *Giardia* and *Cryptosporidium* in raw and finished water. J. Am. Water Works Assoc. **87:**54–68.
21. **Leng, X., D. A. Mosier, and R. D. Oberst.** 1996. Simplified method for recovery and PCR detection of *Cryptosporidium* DNA from bovine feces. Appl. Environ. Microbiol. **62:**643–647.
22. **Lindquist, S., and R. Petersen.** 1990. Selective translation and degradation of heat-shock messenger RNAs in *Drosophila*. Enzyme **44:**147–166.
23. **MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, and J. P. Davis.** 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:**161–167.
24. **Maresca, B., and L. Carratu.** 1992. The biology of the heat-shock response in parasites. Parasitol. Today **8:**260–266.
25. **Mayer, C. L., and C. J. Palmer.** 1996. Evaluation of PCR, nested PCR, and fluorescent antibodies for detection of *Giardia* and *Cryptosporidium* species in wastewater. Appl. Environ. Microbiol. **62:**2081–2085.
26. **Mead, J. R., M.-T. Bonafonte, M. J. Arrowood, and R. F. Schinazi.** 1996. In vitro expression of mRNA coding for a *Cryptosporidium parvum* oocyst wall protein. J. Eukaryot. Microbiol. **43:**84S.
27. **Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P. J. Sansonetti.** 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect. Immun. **57:**3727–3734.
28. **Nieminski, E. C., F. W. Schaefer III, and J. E. Ongerth.** 1995. Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl. Environ. Microbiol. **61:**1714–1719.
29. **Pinto, R. M., J. M. Diez, and A. Bosch.** 1994. Use of the colonic carcinoma cell line Caco-2 for in vivo amplification and detection of enteric viruses. J. Med. Virol. **44:**310–315.
30. **Ranucci, L., H.-M. Muller, G. LaRossa, I. Rechmann, M. A. G. Morles, F. Spano, E. Pozio, and A. Crisanti.** 1993. Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. Infect. Immun. **61:**2347–2356.
31. **Rasmussen, K. R., N. C. Larsen, and M. C. Healy.** 1993. Complete development of *Cryptosporidium parvum* in a human endometrial carcinoma cell line. Infect. Immun. **61:**1482–1485.
32. **Rochelle, P. A., R. De Leon, M. H. Stewart, and R. L. Wolfe.** 1997. Compar-

ison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. Appl. Environ. Microbiol. **63:**106–114.

33. **Rochelle, P. A., D. M. Ferguson, T. J. Handojo, R. De Leon, M. H. Stewart, and R. L. Wolfe.** 1996. Development of a rapid detection procedure for *Cryptosporidium*, using in vitro cell culture combined with PCR. J. Eukaryot. Microbiol. **43:**72S.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Stinear, T., A. Matusan, K. Hines, and M. Sandery.** 1996. Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by reverse transcription PCR. Appl. Environ. Microbiol. **62:**3385–3390.
36. **Upton, S. J., M. Tilley, M. V. Nesterenko, and D. B. Brillhart.** 1994. A simple and reliable method of producing in-vitro infections of *Cryptosporidium parvum* (Apicomplexa). FEMS Microbiol. Lett. **118:**45–50.
37. **Upton, S. J., M. Tilley, and D. B. Brillhart.** 1994. Comparative development of *Cryptosporidium parvum* (Apicomplexa) in 11 continuous host cell lines. FEMS Microbiol. Lett. **118:**233–236.
38. **Upton, S. J., M. Tilley, and D. B. Brillhart.** 1995. Effects of select medium supplements on in vitro development of *Cryptosporidium parvum* in HCT-8 cells. J. Clin. Microbiol. **33:**371–375.
39. **U.S. Environmental Protection Agency.** 1996. National primary drinking water regulation: monitoring requirements for public drinking water suppliers: *Cryptosporidium*, *Giardia*, viruses, disinfection byproducts, water treatment plant data and other information requirements. Fed. Regist. **61:**24354–24388.
40. **Wagner-Weining, C., and P. Kimmig.** 1995. Detection of viable *Cryptosporidium parvum* oocysts by PCR. Appl. Environ. Microbiol. **61:**4514–4516.
41. **Webster, K. A., J. D. E. Pow, M. Giles, J. Catchpole, and M. J. Woodward.** 1993. Detection of *Cryptosporidium parvum* using a specific polymerase chain reaction. Vet. Parasitol. **50:**35–44.