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

**PAUL A. ROCHELLE,** DONNA M. FERGUSON, TROY J. HANDOJO, RICARDO DE LEON, MIC H. STEWART, AND ROY L. WOLFE

Water Quality Laboratory, Metropolitan Water District of Southern California, La Verne, California 91750-3399

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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, 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 anticytosporidial 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
single viable oocyst seeded into environmental water concentrations (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 RI.95-2 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 antiparasitophial activity of maduramicin (34).

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, antibodies, 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 water-treatment plants 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 organisms from which DNA was extracted (Table 1), were determined by nephelometric turbidity units (NTU) (91; NTU: EWS5, 1.5 NTU; 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 0.96 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 fetal bovine serum (FBS; Hyclone, Logan, Utah). The cells were grown in

**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 15,000 × g for 30 min. Washed oocysts were subsequently decontaminated 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 Hank's 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 5 × 10⁵ oocysts/cm². The monolayers were inoculated at 35°C for 2 h to allow initiation of infection and were washed with 1 ml of PBS to remove infective parasites on the cell walls, among other toxicities toward the monolayers (9); they were then reincubated at 35°C for up to 96 h.

Purified RNA from infected cultures was obtained with a Qiagen kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. RNA from infected cultures was obtained by phenol-chloroform extraction, ethanol precipitation, and air drying. RNA was treated with DEPC before use. 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 TRIzol Reagent (Molecular Research Center, Cincinnati, Ohio) to each 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 TRIzol lysate by reprecipitation with ethanol and air drying. The RNA pellet was resuspended in 50 μl of DEPC-treated 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 same wells by addition of 1 ml of sodium dodecyl sulfate (Sigma) and incubation at room temperature for 5 min. The cell lysate DNA was simultaneously extracted and recovered from the TRIzol lysate by reprecipitation with ethanol and air drying. The DNA pellet was resuspended in 50 μl of DEPC-treated sterile distilled water. DNA was simultaneously extracted and recovered from the TRIzol lysate by reprecipitation with ethanol and air drying. The DNA pellet was resuspended in 50 μl of DEPC-treated sterile distilled water. DNA was simultaneously extracted and recovered from the TRIzol lysate by reprecipitation with ethanol and air drying. The DNA pellet was resuspended in 50 μl of DEPC-treated sterile distilled water.
was transferred to a 1.5-mL microcentrifuge tube and mixed with oligo(dT) cellusose, 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, cphsp2475F (5′-CTTGCTGCTCTTACCAGTAC), was selected to have broad specificity to recognize all species of Cryptosporidium. The CPHSP2 primer pair (33), cphsp2423F (5′-AAATTGTTGAGAATCCTCTGT) and cphsp2424R (5′-CTTGCTCCTTACCCAGTAC), 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.), were 54.6 and 51.7°C for CPHSP1 and CPHSP2, respectively. Oligonucleotide probes cphsp2423 (5′-AAATTGTTGAGAATCCTCTGT) and cphsp2475 (5′-CCATTATCACTCGGTGGTATG) 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 MgCl2 concentrations were determined to be 55 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 30529, Entamoeba histolytica sp. strain ATCC 30015, Entamoeba coli ATCC 30946, Babesia microti ATCC 30222, Endolimax nana ATCC 25092, Blastocystis hominis ATCC 30177, Trypanosoma theileri ATCC 30017, Cyclopors sp. (M. Artwood, Centers for Disease Control and Prevention, Atlanta, Ga.), Toxoplasma gondii ATCC 40050, Dientamoeba fragilis ATCC 30848, Eimeria maxima ATCC 40537, Acanthamoeba castellani ATCC 30010, Pneumocystis carinii ATCC 30938, and Escherichia coli sp. strain CDC:0291:V213. The bacteria were E. coli ATCC 22662, and along with the algae Chlorella vulgaris ATCC 30017, Chlorella ellipsoidea ATCC 30017, 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′-AGGGCTTCCGGCATGCTGA and 5′-GTATCGTGACCCGTCCGAG (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 MgCl2, 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, an annealing temperature of 55°C for 30 s, and 72°C for 1 min. A final extension incubation at 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 transcripits by PCR and RT-PCR with the CPHSP1 primer pair. The RT reaction mixtures contained 2.5 μL of murine leukemia virus RT, 2.5 μM random hexamers or oligo(dT)12 primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 1 μL each dATP, dCTP, dGTP, and dUTP, 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 MgCl2. 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% sarcosine, 0.1% sodium dodecyl sulfate, and 1× SSC (10 mM 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, with the membranes washed three times (two 15-min washes with 2× SSC and 0.1% sodium dodecyl sulfate and one 15-min wash with 0.1× SSC) and then fixed to the membrane, hybridized, washed, and detected by the methods described above for Southern blots.

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 viruses, 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 primer 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. lambia 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. lambia in reactions performed at 55°C with 1.5 mM MgCl₂ (Fig. 1C, lane 7). Multiplex PCR detected both C. parvum and G. lambia 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 as the template for reverse transcription, and PCR was performed on 20% of the RT reaction mixture (Fig. 2A). 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/cm², 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.

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 FBS in the cell growth medium or pretreatment of culture slides with collagen (10 µg/cm²) 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 of...
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
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 concentration 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 10³/cm², the typical maximum density of parasite developmental stages following a 48-h postinfection incubation was 3.5 × 10⁷ to 6 × 10⁷/cm². 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 10⁶ oocysts/cm². 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 ampiclons 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 incubum, 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.

**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 pu-
FIG. 4. Detection of Cryptosporidium oocysts used to seed concentrates of environmental water samples by PCR with the CPHSP2 primer pair. Amplions 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.

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 detect 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 polyvinylpyrroldone) 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 $3.7 \times 10^5$ 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
PCR-based viability assay has been demonstrated previously for environmental water concentrates. RT-PCR on cell cultures infected with oocysts was used to seed the gene as the target for an infectivity assay for waterborne Cryptosporidium 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 CPhISP2) (33), but in the present study we describe the detailed application and optimization 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 RT-PCR so that all infectious oocysts yield intracellular development in the large quantities of hsp70 mRNA, 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 CPhISP2) (33), but in the present study we describe the detailed application and optimization 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.

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