Detection of a Single Viable *Cryptosporidium parvum* Oocyst in Environmental Water Concentrates by Reverse Transcription-PCR

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Current methods for detection of *Cryptosporidium parvum* oocysts in water are time-consuming and difficult. We have developed a reverse transcription (RT)-PCR which can detect the presence of a single viable oocyst spiked into concentrated environmental water samples. The test is based on the detection of mRNA from a *C. parvum* heat shock protein (hsp). The synthesis of hsp was induced by a short 45°C incubation followed by oocyst lysis by a freeze-thaw process. *Hsp70* mRNA, produced only from viable oocysts, was then isolated by hybridization to oligo(dT)₂₅-coated magnetic beads. Detection was achieved by RT-PCR amplification of a 590-bp region of *hsp70* mRNA specific for *C. parvum*. To test the method, samples of reticulated, reservoir, bore, and river water were concentrated by chemical flocculation and Percoll-sucrose gradient centrifugation and then spiked with dilutions of oocysts. In all four of the water types examined, the detection of single oocysts was possible by RT-PCR combined with Southern hybridization. RT-PCR products were not obtained from formalin-inactivated oocysts. An RNA internal positive control fragment was synthesized that was included with each reaction to guard against RT-PCR false-negative results that may be caused by the presence of inhibitory substances. However, when the magnetic beads were used to extract and concentrate mRNA, no inhibition was observed. The technique is versatile, straightforward, and rapid (1 day) and provides a sensitive and economic means of screening concentrated water samples for the presence of *C. parvum*.

Cryptosporidium parvum is a significant waterborne pathogen responsible for several serious outbreaks of illness worldwide (23, 25). The parasite can be transmitted through water in an oocyst form that is resistant to many environmental stresses and water treatment practices (28). In addition, while the numbers of oocysts in environmental waters are generally very low, the minimum infectious dose is small (7). Thus, sensitive detection methods are required to ensure a safe drinking water supply. A further requirement is the ability to distinguish live from dead cysts to enable a better determination of water treatment effectiveness and understanding of health risk. Current detection methods rely on microscopic examination of water concentrates by immunofluorescence methods, in which oocysts are specifically stained with fluorescently labelled monoclonal antibodies to obtain a total count (4). Viability can be ascertained by additional staining of a sample with fluorogenic vital dyes (9). However these methods are often difficult to perform with environmental water samples, because coconcentrated particulates can severely interfere with the microscopy.

Recently, flow cytometry with fluorescence-activated cell sorting has been used to separate oocysts from coconcentrated background material and alleviate problems of interference (33). This is a promising advance, but current high equipment and running costs limit the widespread application of this technology.

Several PCR-based methods for direct detection of *Cryptosporidium* spp. have been described (5, 13, 17–19, 35, 36), but

the successful translation of these methods to water samples has been hindered by the presence of enzyme inhibitors such as humic acids (32). These inhibitors are copurified with the target nucleic acid and greatly reduce the efficiency of the PCR. One way of alleviating this problem is by the use of magnetic beads coated with an oligonucleotide to capture nucleic acids specific for the target organism (14, 16).

The aim of this study was to develop a sensitive method suitable for testing a range of environmental water types for viable *C. parvum*. Our approach was to use oligo(dT)₂₅-coated magnetic beads to capture mRNA transcripts for a *C. parvum* heat shock protein (hsp), induced by exposure of oocysts to elevated temperature. The presence of mRNA has been correlated with the viability of an organism (1, 6, 24) and hsps are known to be synthesized with a high level of efficiency and the transcripts are present in large numbers in stressed organisms (21). Thus, induction of a heat shock response provides both a level of amplification to increase detection sensitivity and an index of viability.

An RNA internal standard was synthesized for inclusion in each reverse transcription (RT)-PCR, thus making it possible to monitor reaction efficiency, an important feature when examining samples likely to contain inhibitors of RT-PCR.

MATERIALS AND METHODS

Oocyst stock preparation. Viable *C. parvum* oocyst stocks were obtained from freshly voided calf fecal specimens, semipurified by Percoll-sucrose density gradient centrifugation, and preserved in 2.5% potassium dichromate at 4°C. Total oocyst concentration was determined by immunofluorescent (IF) staining with the Hydrofluor-Combo indirect immunofluorescence detection kit (Meridian Diagnostics), while the concentration of viable oocysts was enumerated by concurrent staining with 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) according to established methods (9). Epifluorescence was observed with an Axioskop microscope (Zeiss, Germany).

Design and testing of Chsp PCR. Nucleotide sequences corresponding to *hsp70* genes from *C. parvum* were obtained from the GenBank and EMBL databases (GenBank accession no. U11761). *C. parvum*-specific *hsp70* (Chsp)

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TABLE 1. Specificity of the Chsp PCR for the detection of *C. parvum* oocysts

Organism (source)	Presence of 590-bp PCR product
<i>Cryptosporidium parvum</i> (M. O'Callaghan, DPI, SA ^a)	+
<i>Cryptosporidium muris</i> (G. Vesey, AWT ^b)	— ^c
<i>Cryptosporidium baileyi</i> (G. Vesey, AWT)	—
<i>Giardia lamblia</i> (M. Eaton, Gribbles Pathology)	—
<i>Plasmodium falciparum</i> (S. Cramer, Monash University)	—
Hookworm (K. Denster, Melbourne University)	—
<i>Taenia</i> species (K. Denster, Melbourne University)	—
<i>Entamoeba coli</i> (K. Denster, Melbourne University)	—
<i>Hymenolepis nana</i> (K. Denster, Melbourne University)	—
<i>Trichuris trichiura</i> (K. Denster, Melbourne University)	—
<i>Endolimax nana</i> (K. Denster, Melbourne University)	—
<i>Clonorchis</i> species (K. Denster, Melbourne University)	—
<i>Ascaris lumbricoides</i> (K. Denster, Melbourne University)	—
<i>Enterobacter aerogenes</i> ATCC 13048	—
<i>Enterococcus faecalis</i> ATCC 19433	—
<i>Aeromonas sobria</i>	—
<i>Pseudomonas aeruginosa</i> ATCC 10145	—
<i>Shigella sonnei</i>	—
<i>Klebsiella pneumoniae</i>	—
<i>Proteus mirabilis</i>	—
<i>Citrobacter freundii</i> ATCC 8090	—
<i>Salmonella derby</i>	—
<i>Salmonella salford</i>	—
<i>Legionella pneumophila</i> ATCC 43111	—
<i>Escherichia coli</i> ATCC 11775	—
<i>Yersinia enterocolitica</i>	—
<i>Campylobacter jejuni</i> NCTC 11351	—
<i>Salmonella infantis</i>	—
<i>Salmonella orientalis</i>	—
<i>Salmonella typhimurium</i>	—
<i>Homo sapiens</i>	—

^a DPI, SA, Department of Primary Industry, South Australia.^b AWT, Australian Water Technologies.^c This organism also tested negative by Chsp RT-PCR.

PCR primer Chsp1 (sequence 5'-AGCAATCCTCTGCCGTACAGG-3') corresponds to nucleotide numbers 2431 to 2451, and primer Chsp4 (sequence 5'-AAGAGCATCCTTGATCTTCT-3') corresponds to nucleotide numbers 3001 to 3020. These primers produce a PCR product with a size of 590 bp from *C. parvum*. Primer sequences were tested for possible homology to other nucleotide sequences (including hsp sequences) by BLASTN analysis (2). Primers were also tested for possible self-annealing and nonspecific binding within the *C. parvum* hsp70 gene with the computer program Amplify PCR (B. Engels, 1992, University of Wisconsin, Madison). Chsp PCR was performed with a range of other organisms to check primer specificity for *C. parvum* (Table 1). DNA was extracted from these organisms by boiling the isolates in the presence of InstaGene (Bio-Rad) according to the manufacturer's directions. For isolates contained in fecal specimens, PCR analysis was performed in duplicate, with the second reaction mixture containing a low concentration spike of *C. parvum* DNA in addition to the sample DNA to check for inhibition of the PCR. If inhibition was observed, the sample DNA preparation was further purified by phenol-chloroform-isoamyl alcohol extraction, and then Chsp PCR was repeated.

Water samples. To test the sensitivity and applicability of the detection method in a range of water types and when using a chemical flocculation method routinely employed for oocyst concentration from water, a 20-liter sample (each) was collected from a river, a reservoir, a bore, and a tap site. The physicochemical quality of each sample was determined by *Standard Methods for the Examination of Water and Wastewater*, 18th ed. (3), and each sample was concentrated to a final volume of 10 ml by calcium carbonate flocculation (34) and Percoll-sucrose density centrifugation (4). This involved addition of 200 ml of 1 M CaCl₂ and 200 ml of 1 M NaHCO₃ to a 20-liter water sample. The pH was adjusted to between 9.9 and 10.1 with 0.2 M NaOH and allowed to settle for 4 h. The supernatant was carefully removed by vacuum, and the flocc pellet was dissolved in 500 ml of 10% NH₂SO₃H, washed four times in a 0.01% Tween 80 solution, and resuspended in elution fluid (0.01% Tween 80, 1% sodium dodecyl sulfate [SDS] in phosphate-buffered saline [PBS]) to a final volume of 20 ml. Further purification was then performed by underlaying the pellet with a Percoll-sucrose solution (specific gravity, 2.10) and centrifuging the mixture at 2,000 × g for 10

min. The interface was recovered and resuspended in PBS to produce a final volume of 10 ml for use in subsequent spiking experiments.

Preparation of spiked samples. Fresh dilutions of oocysts were prepared for each of the five spiked samples (Table 2). A concentration of 200 oocysts μl⁻¹ was prepared for use in seeding experiments by washing 100 μl of the oocyst stock once in PBS to remove the preservative. The washed oocysts were resuspended in 100 μl of water, and a dilution series was prepared comprising two serial dilutions, three doubling dilutions, and a final 10-fold dilution to obtain viable oocyst concentrations of approximately 1,000, 100, 50, 25, 12, and 1 oocyst, respectively, per 50-μl volume. A series of six 1-ml volumes, prepared in screw-cap microtubes, of the sample concentrates were inoculated with 50 μl of each of the six dilutions. An additional 1-ml volume was inoculated with 50 μl of sterile water as a negative control. A 50-μl volume of each dilution was also enumerated by IF-DAPI-PI methods, as described above, to obtain an accurate count of each inoculum.

Induction of heat shock response and oocyst lysis. The 1-ml volumes of spiked concentrate were centrifuged at 5,000 × g for 3 min to pellet oocysts and resuspended in 200 μl of InstaGene matrix (Bio-Rad). Samples were vortexed briefly to resuspend the pellet and then incubated at 45°C for 20 min to induce production of hsp70 mRNA. After heat shock treatment, 200 μl of lysis-binding buffer (100 mM Tris-HCl [pH 8.0], 500 mM LiCl, 10 mM EDTA [pH 8.0], 1% lithium dodecyl sulfate, 5 mM dithiothreitol) was added to each sample, and this mixture was then subjected to freeze-thaw treatment to rupture oocysts and release nucleic acid. This involved five cycles of freezing samples in liquid nitrogen for 1 min and then thawing at 65°C for 1 min. Samples were processed immediately after lysis for mRNA extraction.

Extraction of mRNA and hybridization. Extraction and isolation of *C. parvum* hsp70 mRNA was achieved with oligo(dT)₂₅-linked magnetic beads (Dyna beads; Dynal, Oslo, Norway), essentially according to the protocol of the manufacturer. Beads were prepared on the day of use in 1.5-ml Eppendorf tubes. Four microliters (20 μg) of beads was used per sample. These were washed once with a magnetic concentrator (MPC-E; Dynal) with 500 μl of PBS containing 0.1% bovine serum albumin to remove the preservative. Washed beads were resuspended in 4 μl of lysis-binding buffer and stored at 4°C. After freeze-thaw lysis, the sample was spun at 17,000 × g to pellet the debris, and the supernatant, containing the nucleic acid, was transferred to a clean Eppendorf tube containing the prepared oligo(dT)₂₅ beads. Hybridization was performed at 30°C with gentle mixing by rolling for 30 min. By using the magnetic particle concentrator to pellet the beads, the lysis solution was removed and the beads were washed once with 200 μl of wash buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1.0 mM EDTA) and once with 200 μl of 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl). After the final wash, all traces of liquid were removed with a micropipettor, and the beads were resuspended in 3 μl of pyrocarbonic acid diethyl ester (DEPC)-treated water to be used directly in RT-PCR, or 6 μl when an additional 3 μl was tested directly by PCR in experiments to detect carryover DNA. When DNase and RNase treatments were performed, mRNA was first eluted from the magnetic beads according to the protocol described by the manufacturer (Dyna). To elute mRNA, the magnetic beads and hybridized mRNA were resuspended in elution buffer (2 mM EDTA) and incubated at 65°C for 2 min. The beads were pelleted with the magnetic concentrator, and the supernatant containing the mRNA was removed and used in subsequent experiments.

RNase and DNase treatment of extracted hsp70 mRNA. The extracted mRNA from 5,000 oocysts was eluted from the magnetic beads in a 20-μl volume of elution buffer, and 5-μl aliquots were treated with 10 U of RNase 1 (Promega), incubated at 42°C for 15 min, and 10 U of RQ1 DNase (Promega) supplemented with 2 mM MgCl₂ and incubated at 37°C for 15 min, while the remaining mRNA was left untreated. RNase and DNase were inactivated by phenol-chloroform-isoamyl alcohol extraction with isopropanol precipitation. The mRNA pellets were resuspended in 6 μl of DEPC-treated H₂O, with 3-μl volumes analyzed separately by both RT-PCR and PCR.

RT. RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin-

TABLE 2. Sensitivity of the Chsp RT-PCR assay for detection of *C. parvum* oocysts in four water types as determined by silver-stained polyacrylamide gel electrophoresis

Water type	Result with oocyst spike in 1 ml of water concentrate ^a						
	1,000	100	50	25	10	1	0
Insta-Gene	+	+	+	+	+	+	—
Reticulated ^b	+	+	+	+	+	+	—
Reservoir	+	+	+	+	+	+ ^c	—
River	+	+	+	+	+	+ ^c	—
Bore	+	+	+	+	+	+	—

^a Oocyst concentrations represent the average inoculum from five experiments.

^b Tap sample from a municipal water supply.

^c This sample was positive only after Southern hybridization analysis.

Elmer). RT was specifically primed with the Chsp4 primer. Three microliters of prepared sample was reverse transcribed in a 30- μ l volume containing 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM deoxynucleoside triphosphates (dNTPs), 1 U of RNasin, 2.5 U of murine leukemia virus reverse transcriptase, 25 mM Chsp4, and 1 μ l of internal positive control (IPC) RNA equivalent to approximately 25 oocysts, constructed as described below. Samples were incubated in an FTS-960 thermal sequencer (Corbett Research, Sydney, Australia) at 42°C for 30 min, and then the enzymes were inactivated at 90°C for 5 min. Samples were held at 4°C until the PCR was performed.

PCR. A 10- μ l sample from the RT reaction mixture was added directly to a fresh tube supplemented with 1.25 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 U of *ampli-Taq* DNA polymerase (Perkin-Elmer), 2.5 mM upstream primer Chsp1, and water to make the final volume 20 μ l. Amplification was performed in a Corbett FTS-960 thermal sequencer with a touchdown protocol (12) which consisted of 5 cycles at 94°C for 15 s and 68°C for 30 s; 5 cycles at 90°C for 5 s and 64°C for 30 s; 25 cycles at 90°C for 5 min, 58°C for 15 s, and 72°C for 15 s; and 1 cycle at 72°C for 1 min. In experiments in which PCR was performed directly, 3 μ l of prepared sample was amplified in a 20- μ l volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μ M dNTPs, 0.2 μ M (each) primers Chsp1 and Chsp4, 2.5 mM $MgCl_2$, and 1 U of *ampli-Taq* DNA polymerase. Thermal cycling was carried out as described for RT-PCR.

Polyacrylamide gel electrophoresis. After PCR amplification, 5- μ l aliquots from each reaction mixture were loaded onto 5.5% polyacrylamide gels with the mini-Protein II vertical electrophoresis cell (Bio-Rad). Electrophoresis was carried out in 1 $\times$ TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 200 V for 35 min. PCR products were visualized by staining with silver nitrate (8).

Agarose gel electrophoresis. Ten-microliter aliquots from each PCR were run in 1.2% horizontal agarose gels (Promega) in 1 $\times$ TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 60 mA for 30 min. PCR products were visualized by staining with ethidium bromide (1 μ g ml⁻¹) with UV transillumination.

Southern blot hybridization. Southern blot hybridization was performed with the 590-bp Chsp PCR product labelled with digoxigenin. The probe was prepared by PCR amplification of the fragment with a digoxigenin dNTP labelling mix containing digoxigenin-11-dUTP (Boehringer Mannheim). After amplification, the labelled PCR product was purified with Prep-A-Gene (Bio-Rad) and resuspended in sterile H₂O at a concentration of approximately 5 ng μ l⁻¹. PCR products, separated by agarose gel electrophoresis, were transferred to positively charged nylon membranes (Hybond N+; Amersham), by alkaline (0.4 M NaOH) capillary transfer (30). The membrane was then rinsed briefly in 2 $\times$ SSC (0.15 M NaCl, 0.015 M trisodium citrate) and prehybridized for 30 min in 50 ml of prehybridization solution (5 $\times$ SSC, 1% SDS, 1% blocking reagent, 0.1% Sarkosyl) according to the *Digoxigenin Protocol Manual* (Boehringer Mannheim) at 68°C. Hybridization was performed in 5 ml of prehybridization solution with 20 ng of digoxigenin-labelled probe ml⁻¹ overnight at 68°C. After hybridization, the membranes were washed twice in wash solution 1 (2 $\times$ SSC, 0.1% SDS) for 5 min at room temperature and then twice in wash solution 2 (0.1% SSC, 0.1% SDS) for 15 min at 68°C. After the posthybridization washes, probe detection was performed with anti-digoxigenin alkaline phosphatase conjugate Fab fragments (Boehringer Mannheim) according to the manufacturer's protocol.

Construction of an RNA IPC fragment. In order to monitor the efficiency of RT-PCR and to detect the presence of inhibitors, an RNA mimic fragment was produced that could be added to each test. A DNA fragment was produced by the method of Ross et al. (29) with the two modifying primers Chsp1/link (5'-CCTCTGCCGTACAGGAGAGTGCCAAATTAC-3') and Chsp4/link (5'-CATCCTTGATCTTCTATGTGAGTTGTGACC-3'). These were designed to amplify a 412-bp product from dengue virus type 2 (DEN2) NS3 cDNA (dengue virus cDNA kindly supplied by M. J. Pryor) and to introduce 3'-terminal primer homology for PCR amplification with Chsp1 and Chsp4. The fragment was approximately 140 bp smaller than the target Chsp fragment, was flanked by the Chsp4 and Chsp1 primer sequences, and contained a nonhomologous internal sequence of DEN2 NS3 cDNA that was coreverse transcribed and amplified with the target fragment, appearing as a band at approximately 453 bp (Fig. 1). This intervening DNA sequence was chosen because it did not share homology with the Chsp target sequence or with any of the other PCR targets used in the laboratory. This maximized the potential of the mimic to be used in quantitative RT-PCR (29) and prevented contamination problems through cross-reaction with other routinely used PCR tests. To produce an RNA copy, the DNA product was purified with Prep-A-Gene (Bio-Rad) and cloned with the pGEM-T vector system (Promega) according to the manufacturer's instructions. The Chsp PCR was used to check that recombinants contained the IPC fragment, and the correct orientation was confirmed by restriction enzyme analysis with *XhoI* and *ScaI*. Plasmid clones shown to contain the IPC fragment in the correct orientation were linearized by restriction enzyme digestion with *SaI* and were transcribed with the riboprobe T7 in vitro transcription system (Promega). The transcription product was treated with RQ1 DNase (Promega) to remove DNA, and then the RNA was purified by phenol-chloroform-isoamyl alcohol extraction. The absence of DNA in the preparation was confirmed by direct PCR. The RNA IPC concentration was estimated by A_{260} readings and then diluted 1:20,000, such that its inclusion in RT-PCR would still permit detection of very small numbers of oocysts. Comparison of the IPC against dilutions of oocysts indicated an IPC RT-PCR product equivalent to the Chsp RT-PCR product from 25 oocysts. IPC RNA was stored in 50- μ l aliquots at -70°C.

FIG. 1. Sensitivity of *C. parvum*-specific *hsp70* RT-PCR for bore water samples, incorporating the RNA IPC. Dilutions of viable *C. parvum* oocysts were spiked into 1-ml volumes of bore water concentrate, as described in Materials and Methods. (A) Silver-stained polyacrylamide gel. (B) Ethidium bromide-stained agarose gel. (C) Southern hybridization of the gel in panel B with digoxigenin-labelled Chsp PCR product. Lanes: 1, 600 oocysts; 2, 58 oocysts; 3, 40 oocysts; 4, 20 oocysts; 5, 8 oocysts; 6, 1 oocyst; 7, 0 oocysts (negative control); M, *HaeIII* ϕ x174 DNA size marker (Promega).

RESULTS

To determine the specificity of the Chsp primers, we tested the primers in a PCR with DNA extracted from organisms likely to be present in environmental water samples or likely to have some sequence homology with *C. parvum*. We also tested some other *Cryptosporidium* species (Table 1). For some organisms, faint amplification products were visible on silver-stained acrylamide gels, but in all cases these were of a different size than the Chsp PCR product and were considered unlikely to interfere with the efficiency of the test. In addition to PCR analysis, RT-PCR was performed with mRNA extracted from 100 viable *Cryptosporidium muris* oocysts. No amplification products were detected (Table 1).

To assess the sensitivity of the RT-PCR, dilutions of enumerated, viable *C. parvum* oocysts purified from a calf fecal specimen were seeded into 1-ml volumes of concentrated water samples, and RT-PCR was performed with extracted *hsp70* mRNA. Sensitivities were determined in four different water types to assess the general applicability of the method for detecting viable oocysts in a range of environmental water samples. In addition to the water samples, dilutions of viable oocysts were also seeded directly into InstaGene to assess the absolute recovery of oocysts in the absence of inhibitors likely to be present in the water concentrates. A separate dilution series was prepared for each of the five sensitivity experiments, the results of which are shown in Table 2.

At the highest dilution, in which the average viable oocyst number was 1, the Chsp RT-PCR produced a 590-bp band for all water types. For reticulated and bore waters, this band was clearly visible on silver-stained acrylamide gels. However, for

TABLE 3. Physicochemical quality of the four types of water examined in this study

Water source	pH	Color (PCU)	Amt of total organic carbon (mg liter ⁻¹)	EC ($\mu\text{S cm}^{-1}$)	Turbidity (nephelometric turbidity units)	Amt of iron (mg liter ⁻¹)
Reticulated	7.4	45	2.0	60	5.1	0.56
Reservoir	7.2	35	4.0	130	1.2	0.39
River	7.4	80	2.0	7,800	12	0.67
Bore	6.4	10	1.5	890	3.3	2.9

reservoir and river samples, this band could only be detected by hybridization. The sensitivity with reticulated or bore water samples was no different from that obtained by seeding oocysts directly into InstaGene, indicating that recovery of even single oocysts was achievable despite the likely presence of RT-PCR inhibitors. These water samples were tested prior to use in seeding experiments and were shown to be free of oocysts by IF microscopy. All four samples contained some level of bacterial contamination (data not shown). For all water types, there were no 590-bp bands produced unless oocysts were deliberately seeded, further attesting to the specificity of the RT-PCR for *C. parvum* oocysts. Some physicochemical parameters for each water type were also tested and are presented in Table 3. These give an indication of the wide variation in water quality between the dirty natural samples and demonstrate the ability of this assay to function with a high level of sensitivity even in water of poor physicochemical quality.

The results from seeding oocysts into concentrates from the bore water sample are shown in Fig. 1. Even for the lowest inoculum level, the 590-bp product is clearly visible on a silver-stained acrylamide gel (Fig. 1A). In contrast, the limit of detection with ethidium bromide staining of agarose gels is 10-fold higher (Fig. 1B). Southern hybridization confirms the identity of the Chsp RT-PCR product (Fig. 1C). The RNA IPC included in each reaction is clearly visible on the acrylamide gel and is just visible on the agarose gel. It is easily distinguishable from the Chsp RT-PCR product. We have used the RNA IPC at a deliberately low level to ensure it is not preferentially amplified over low levels of *C. parvum hsp70* mRNA. In these experiments, the Chsp product from a single oocyst is easily detectable, despite the inclusion of the RNA IPC.

The importance of the 45°C 20-min heat treatment step on RT-PCR detection sensitivity was investigated. Experiments were conducted with both high and low concentrations of oocysts (200 and 20 oocysts, respectively), and levels of *hsp* mRNA produced, as indicated by the intensity of the Chsp RT-PCR products from oocysts with and without heat treatment, were observed. There was no observable difference between *hsp* mRNA from oocysts subjected to heat treatment and that from oocysts without heat treatment (data not shown).

The relative intensities of the Chsp product shown in Fig. 1A reflect the starting concentrations of the two templates. The intensity of the Chsp product relative to the RNA IPC product decreases with decreasing numbers of *C. parvum* oocysts, particularly when the reduction is 10-fold or more. Since both bands are amplified by the same primers, a visual comparison of the intensities of the two bands can provide some information regarding the absolute numbers of viable oocysts present in the sample. From the gel shown in Fig. 1A, the RNA IPC product appears to be equivalent to the Chsp product from approximately 25 oocysts.

To ensure that RNA, and not DNA, was being amplified and detected, we isolated *hsp70* mRNA from a preparation of 1,000 *C. parvum* oocysts and subjected it to DNase and RNase treat-

ments. The following results confirm that the isolation and amplification were specific for mRNA and that DNA was not being carried over into the PCR. RT-PCR was positive with DNase and no treatment and negative with RNase. PCR was negative with DNase and RNase treatment and with no treatment. Some carryover DNA was occasionally observed when mRNA was extracted from preparations containing greater than 1,000 *C. parvum* oocysts. This is unlikely to be a problem in testing environmental water samples, unless gross contamination is suspected. In this event, a dilution of the concentrated sample or the inclusion of a DNase treatment may be necessary. No RT-PCR product was obtained with *C. parvum* oocysts fixed in formalin (data not shown), indicating that mRNA was produced only by live oocysts. Direct Chsp PCR performed with formalin-fixed oocysts produced a 590-bp band.

DISCUSSION

Direct PCR detection of microorganisms in environmental waters has been the goal of many laboratories but has been hampered by the presence of PCR-inhibiting substances which copurify with the target nucleic acid. Magnetic beads coated with specific antibodies have been used to immobilize intact microbes from food, water, and fecal material for subsequent removal of inhibitors, concentration, and analysis (17, 26). Recently, magnetic beads with attached oligonucleotides have been used to isolate specific target nucleic acid sequences (14, 16). Magnetic beads are simple to use, do not require expensive equipment, and demonstrate a high level of recovery (16). The method we have described in this report uses oligo(dT)₂₅-coated magnetic beads to selectively bind polyadenylated mRNA molecules, which were induced by heat stress and which were amplified by a *C. parvum*-specific RT-PCR to achieve single-oocyst sensitivity.

We have selected a region of a *C. parvum hsp70* gene as a target for the RT-PCR, because under conditions of heat stress, the *hsp* is the major product of protein synthesis, with 1,000- to 10,000-fold increase in *hsp70* mRNA (22). Thus, the large number of *hsp70* mRNA molecules likely to be induced from a single viable oocyst increases the chance of detecting small numbers of oocysts.

The observation that levels of *hsp* mRNA from oocysts without heat treatment were equivalent to those with heat treatment suggests that another stress factor was present. This may have been the 4°C oocyst stock storage temperature or the preservative solution in which the oocysts were stored. Further investigation of factors inducing *C. parvum* Hsp70 synthesis were not undertaken. Heat induction may prove to be an unnecessary step in this assay; however, given the current limited knowledge about stress responses in *C. parvum*, the inclusion of this treatment should ensure maximum expression of mRNA in oocysts isolated from real samples and thus should maintain maximum detection sensitivity.

Our results demonstrate that, with hybridization, the RT-PCR described here detects a single viable oocyst in all water

types tested and that RT-PCR products are only produced from viable oocysts. However, previous work with *Giardia* cysts has shown that mRNA for the giardin protein can be recovered under experimental conditions from cysts killed by heating or monochloramine exposure (24). While *hsp70* mRNA is highly stable during periods of heat stress, its instability and short half-life at ambient temperatures (11, 22) suggest it is unlikely to be preserved in dead oocysts from environmental sources and is therefore a suitable marker for oocyst viability. However, it may be necessary to determine the survival of *hsp70* mRNA in oocysts killed by various treatments to ensure that RT-PCR products directly correlate with the presence of viable oocysts.

As far as we can determine, the RT-PCR appears to be specific for *C. parvum*, although there are *Cryptosporidium* species which we have not yet been able to test. We are attempting to obtain viable oocysts from some of these other species for testing.

The inclusion of the RNA IPC in each reaction guards against the false-negative results which may arise from the concentration of PCR inhibitors during the isolation and purification of oocysts. We resuspended oocysts that had been spiked into concentrated water samples in InstaGene (a chelating resin) to help overcome the possibility of inhibition. In these experiments, the sensitivity of the RT-PCR was slightly lower for the reservoir and river waters. Samples of this nature may be expected to contain higher levels of PCR inhibitors than highly treated waters, chiefly in the form of humic acids, fulvic acids, salts, and heavy metals (32). Analysis of the reservoir and river samples showed that the quality of the river water was inferior to the others, particularly with regard to salt and metal ion concentrations. The reservoir sample, however, was no worse than either the bore or reticulated samples for most parameters, apart from total organic carbon. The higher level of total organic carbon in this sample, taken as a guide to the level of humic acid, may explain the slightly lower sensitivity seen with this sample. An alternative explanation for the slightly lower sensitivity observed with both the river and reservoir samples may be related to the difficulties in obtaining the same spike dose among the five sensitivity experiments, particularly when seeding in the range of a single oocyst. For each experiment, a new dilution series was prepared; hence, while the average count obtained by IF-DAPI-PI microscopy from the highest dilutions was one oocyst, for some of these dilutions, zero oocysts were counted. Nevertheless, the Chsp RT-PCR product was detected in the highest dilutions for each of the five samples, suggesting that the IF-DAPI-PI procedure underestimated the actual numbers of oocysts in each assay. This was probably due to oocyst losses in the multiple washing and centrifugation steps required by the staining method and reinforces the higher detection sensitivity of the RT-PCR assay.

This method compares favorably with the *C. parvum* viability assay described recently by Wagner-Wiening and Kimmig (35), who reported a reliable level of detection of 100 sporozoites with an excystation protocol coupled with PCR amplification. This sensitivity was achieved with purified sporozoites. These authors did not attempt to assess the sensitivity of their PCR on seeded environmental samples.

We have designed this method specifically to detect the small numbers of viable oocysts typically found in environmental waters. Our observation that carryover of DNA could occur when testing preparations containing greater than 1,000 oocysts has implications for adopting this method in analyzing those samples, such as fecal samples, which may contain very high numbers of viable oocysts. In cases such as these, a dilu-

tion of the concentrated sample or the inclusion of a DNase treatment may be necessary prior to mRNA isolation.

We did not observe any problems with RNase activity during the course of this study. Normal procedures for handling RNA were observed, which included DEPC treatment of all lysis and wash solutions where possible and the use of RNase- and DNase-free aerosol-resistant pipette tips. Gloves were worn for all manipulations. Once oocyst lysis had been performed, samples were processed promptly to limit the possibility of degradation of the *hsp70* transcript.

Existing methods for *Cryptosporidium* analysis require laborious immunofluorescence screening of water concentrates, which requires a high degree of operator expertise and is prone to subjective assessments. Therefore, it has a relatively high risk of error. The Chsp RT-PCR we have described may replace screening by microscopy or could be used as a primary screening tool so that only those samples shown to contain viable *C. parvum* oocysts by RT-PCR are then analyzed by immunofluorescence microscopy. One advantage of microscopy is that quantitation of oocysts is possible. With RT-PCR, visual comparison of the intensities of the IPC band and the 590-bp band can provide an estimation of the number of oocysts present in the sample. The use of densitometry or high-performance liquid chromatography to accurately measure the amount of each product in each reaction would increase the accuracy of this estimate, provided the PCR was still in the exponential phase (31). Alternatively, our RT-PCR method with a recently developed PCR quantitation system utilizing fluorescent reporter molecules (15) may provide accurate quantitation.

The Chsp RT-PCR method functions with a high level of sensitivity even in samples expected to contain high levels of PCR inhibitors, suggesting that it may be possible to analyze pellets from primary concentrates without the need for additional purification. Avoidance of clarification procedures such as density gradient centrifugation could dramatically increase the overall recovery of viable oocysts, because losses of up to 68% have been reported in addition to some evidence that dead oocysts may be preferentially concentrated (20).

The robust nature of the RT-PCR should also enable it to assay water concentrates obtained by other methods, such as with cartridge or membrane filters (27), providing these concentration methods do not damage viable oocysts. We are currently undertaking a comparative study of RT-PCR and immunofluorescence microscopy to assess the performance of each procedure when analyzing real (spiked and unspiked) environmental water samples, concentrated by calcium carbonate flocculation or cartridge filtration. Samples prepared by chemical flocculation are limited to a 4-h settling period to prevent loss of oocyst viability through prolonged exposure to high pH levels (10). Preliminary experiments we have conducted indicate no significant reduction in viable oocysts after overnight exposure to pH 10, when a fresh oocyst stock was used. It is possible, however, that environmentally stressed *Cryptosporidium* oocysts may be more sensitive to this treatment.

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