APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1999, p. 3427–3432 Vol. 65, No. 8
0099-2240/99/$04.00+0

Detection of Infectious *Cryptosporidium parvum* Oocysts in Surface and Filter Backwash Water Samples by Immunomagnetic Separation and Integrated Cell Culture-PCR

GEORGE D. DI GIOVANNI,[1]* F. HELEN HASHEMI,[1] NANCY J. SHAW,[1] FELICIA A. ABRAMS,[1] MARK W. LECHEVALLIER,[2] AND MORTEZA ABBASZADEGAN[1]

American Water Works Service Co., Inc., Belleville, Illinois,[1] and American Water Works Service Co., Inc., Voorhees, New Jersey[2]

Received 21 January 1999/Accepted 19 May 1999

A new strategy for the detection of infectious *Cryptosporidium parvum* oocysts in water samples, which combines immunomagnetic separation (IMS) for recovery of oocysts with in vitro cell culturing and PCR (CC-PCR), was field tested with a total of 122 raw source water samples and 121 filter backwash water grab samples obtained from 25 sites in the United States. In addition, samples were processed by Percoll-sucrose flotation and oocysts were detected by an immunofluorescence assay (IFA) as a baseline method. Samples of different water quality were seeded with viable *C. parvum* to evaluate oocyst recovery efficiencies and the performance of the CC-PCR protocol. Mean method oocyst recoveries, including concentration of seeded 10-liter samples, from raw water were 26.1% for IMS and 16.6% for flotation, while recoveries from seeded filter backwash water were 9.1 and 5.8%, respectively. There was full agreement between IFA oocyst counts of IMS-purified seeded samples and CC-PCR results. In natural samples, CC-PCR detected infectious *C. parvum* in 4.9% (6) of the raw water samples and 7.4% (9) of the filter backwash water samples, while IFA detected oocysts in 13.1% (16) of the raw water samples and 5.8% (7) of the filter backwash water samples. All CC-PCR products were confirmed by cloning and DNA sequence analysis and were greater than 98% homologous to the *C. parvum* KSU-1 *hsp70* gene product. DNA sequence analysis also revealed reproducible nucleotide substitutions among the *hsp70* fragments, suggesting that several different strains of infectious *C. parvum* were detected.

The current U.S. Environmental Protection Agency Information Collection Rule method (23) and proposed method 1622 (21) for the detection of *Cryptosporidium* recovered from water samples do not specifically detect the human pathogen *Cryptosporidium parvum* or determine the viability or infectivity of recovered oocysts. Recently, several PCR-based methods for the detection of *C. parvum* have been described (4, 6, 7, 9, 20, 24); these include an infectivity assay based on in vitro cell culturing of the parasite with Caco-2 cells and detection of *C. parvum*-infected cells by targeting *C. parvum hsp70* mRNA by use of reverse transcription (RT)-PCR (18). We have developed another strategy for a *C. parvum* infectivity assay which integrates immunomagnetic separation (IMS) to recover *Cryptosporidium* oocysts from water samples with in vitro cell culturing with human ileocecal adenocarcinoma HCT-8 cells and detection of *C. parvum*-infected cells by targeting *C. parvum hsp70* DNA by use of standard PCR (CC-PCR) (5). IMS is superior to flotation in removing debris and allows larger equivalent volumes of water concentrate to be assayed. The final IMS-purified sample volume is typically 50 μl, compared to 5 ml for flotation-purified samples. This aspect is critical for the CC-PCR assay, since large-volume samples are not amenable to analysis. Rochelle et al. reported the detection of a single infectious oocyst by using an integrated cell culture-RT-PCR strategy (18) with oocyst-seeded finished water concentrates. Our previous report of a detection limit of less than five purified infectious oocysts for our CC-PCR *C. parvum* infectivity assay (5) was confirmed in this study. The growth of *C. parvum* in HCT-8 cell cultures has been reported to yield an approximate ratio of 17.9:1 HCT-8 cell culture infectious foci per *C. parvum* oocyst (19). Our CC-PCR strategy permits the assay of raw, filter backwash, and finished water samples and uses in vitro cell culturing in a 96-well format for high sample throughput. Standard PCR detection of genomic *C. parvum hsp70* DNA is less time-consuming than mRNA extraction and RT-PCR. Quantitation of the parasite by RT-PCR is hampered by the fact that the amount of *hsp70* mRNA is variable and dependent on the physiologic state of the parasite (13). Thus, standard PCR which targets the single copy of the *hsp70* gene per *C. parvum* genome (10) is more feasible for quantitation studies.

The objective of this work was to field test our CC-PCR *C. parvum* infectivity assay by using raw source water and filter backwash water grab samples obtained from 25 sites in the United States. Oocyst-seeded raw and filter backwash water samples were used to evaluate the method recovery efficiencies and the performance of the CC-PCR protocol with different water quality matrices. In addition, samples were processed by Percoll-sucrose flotation and oocysts were detected by an immunofluorescence assay (IFA) as a baseline method. CC-PCR-positive samples were confirmed by DNA sequence analysis of the *hsp70* PCR products and compared to investigate the potential genetic heterogeneity of waterborne infectious *C. parvum*.

* Corresponding author. Mailing address: American Water Works Service Co., Inc., Quality Control and Research Laboratory, 1115 S. Illinois St., Belleville, IL 62220. Phone: (618) 239-0518. Fax: (618) 235-6349. E-mail: gdigiova@bellevillelab.com.

MATERIALS AND METHODS

Oocysts and microscopy. Purified viable *C. parvum* oocysts (bovine isolate LA-1) were obtained from Waterborne, Inc. (New Orleans, La.). Oocyst stocks

and Percoll-sucrose flotation-purified oocysts were enumerated by IFA microscopy as described in the *ICR Microbial Laboratory Manual* (22). Oocysts purified by IMS (method described below) were enumerated by fluorescence microscopy as follows. IMS-purified samples (40 μl of sample plus 10 μl of deionized water wash from a microcentrifuge tube) or purified oocyst stocks (50 μl) were placed into individual wells of polylysine-treated three-well microscope slides (Meridian Diagnostics, Inc., Cincinnati, Ohio) and dried at 42°C. Samples were fixed with 1 drop of room-temperature methanol and air dried; then, 75 μl of fluorescein isothiocyanate-labeled anti-*Cryptosporidium* monoclonal antibody (Waterborne, Inc.) was added to each well. Slides were incubated at 37°C for 30 min in a humid chamber. Excess fluorescein isothiocyanate-labeled monoclonal antibody was removed by aspiration with a micropipette followed by a single wash with 1 drop of deionized water and aspiration. Slides were placed in the dark until dry; then, 10 μl of mounting medium (10% glycerol, 80% phosphate-buffered saline [PBS], 5% 5 M NaCl, 5% formalin, 2.5% 1,4-diazabicyclo[2.2.2]octane [pH 8.6]) was added to each well. A coverslip was applied, and slides were examined by epifluorescence microscopy at a magnification of ×200. Presumptive oocysts were confirmed by 400×-magnification epifluorescence microscopy and 1,000×-magnification Nomarski differential interference contrast microscopy to determine internal morphology.

Recovery of oocysts from environmental water samples. Approximately 10-liter grab samples of raw water and filter backwash water were concentrated by centrifugation at 1,800 × *g* and 4°C for 10 min. Seeded 10-liter samples were spiked with 1,615 to 2,880 purified viable *C. parvum* oocysts prior to concentration. *Cryptosporidium* oocysts were recovered from up to three 0.5-ml packed-pellet portions of each water sample concentrate by IMS (Dynabeads anti-*Cryptosporidium*; Dynal A.S., Oslo, Norway). IMS was performed according to the manufacturer's suggestions, with the exception of the dissociation step. The manufacturer's acidified Hanks' balanced salt solution (AHBSS, pH 2.75) dissociation protocol for viability testing was originally used but was found to have a variable end-point pH (ca. 3.0 to 6.0) due to residual IMS SL buffer A. To remove residual IMS buffer, a second wash with 1× PBS (pH 7.2) was performed after the samples had been separated with a microcentrifuge tube magnetic particle concentrator (MPC-M; Dynal).

To improve recovery, a combined acid-enzymatic dissociation method which may digest the antibody-oocyst complexes directly off the IMS beads and serve as an excystation trigger for subsequent cell culturing was used. For each IMS reaction, 200 μl of AHBSS–1% trypsin (type II-S porcine pancreas; Sigma Chemical Co., St. Louis, Mo.) was added, and samples were incubated at 37°C for 1 h with 10 s of vortexing every 15 min. Following separation of the samples in the MPC-M, the supernatants containing the dissociated oocysts were transferred to microcentrifuge tubes. To ensure recovery of oocysts, a second wash of the IMS beads with 100 μl of AHBSS–1% trypsin was performed. Dissociated samples were neutralized with 0.5 N NaOH (4.0 μl), and replicate IMS reactions were pooled, centrifuged at maximum speed in a microcentrifuge for 2 min with no brake and aspirated down to 20 μl for CC-PCR or 40 μl for microscopy.

IMS oocyst recovery trials comparing 0.1 N HCl and AHBSS–1% trypsin dissociation methods were performed with deionized water and replicate 0.5-ml packed-pellet portions of raw water and filter backwash water sample concentrates. Water concentrates were seeded with oocysts, and IMS was performed as described above. The 0.1 N HCl IMS dissociation step was performed as previously described (1).

Percoll-sucrose flotation for the recovery of *Cryptosporidium* oocysts was performed with up to 2.0 ml of a packed pellet of each water sample concentrate as described in the *ICR Microbial Laboratory Manual* (22), with the exception that a 1.15-specific-gravity Percoll-sucrose solution was used.

In vitro cell culturing of *C. parvum*. Human ileocecal adenocarcinoma (HCT-8; ATCC CCL-244) cells were cultivated as previously described (25). Cell culture maintenance medium consisted of RPMI 1640 with L-glutamine (Gibco BRL, Grand Island, N.Y.), 5% fetal bovine serum (pH 7.2), 15 mM HEPES buffer, 100,000 U of penicillin G liter^{-1}, 100 mg of streptomycin liter^{-1}, 700 μg of amphotericin B liter^{-1}, and 12.5 mg of tetracycline liter^{-1}. Growth medium used for the in vitro development of *C. parvum* contained 10% fetal bovine serum, 15 mM HEPES buffer, 50 mM glucose, 35 mg of ascorbic acid liter^{-1}, 1.0 mg of folic acid liter^{-1}, 4.0 mg of 4-aminobenzoic acid liter^{-1}, 2.0 mg of calcium pantothenate liter^{-1}, 100,000 U of penicillin G liter^{-1}, 100 mg of streptomycin liter^{-1}, 700 μg of amphotericin B liter^{-1}, and 12.5 mg of tetracycline liter^{-1}. At 24 h prior to inoculation, 96-well cell culture microplates were seeded with 5 × 10^4 HCT-8 cells per well. Plates were incubated at 37°C in a 5% CO_2 humidified incubator to allow for the development of 100% confluent monolayers. Just prior to inoculation of monolayers, 50 μl of maintenance medium was removed. Samples for CC-PCR were resuspended in 180 μl of prewarmed growth medium immediately following IMS dissociation and used to inoculate two HCT-8 cell monolayers (100 μl of inoculum for each). AHBSS–1% trypsin-treated *C. parvum* oocysts were used as positive controls, and single-cycle freeze-thaw killed oocysts were used as negative controls. The inoculated cell monolayers were incubated at 37°C in a 5% CO_2 humidified incubator for 72 h. After incubation, the cell monolayers were washed five times with 200 μl of PBS to remove nonexcysted oocysts. Cell monolayers were harvested by the addition of 200 μl of 1× Tris-EDTA (pH 8.0) buffer, and resuspended cells were transferred to microcentrifuge tubes. Harvested cells were centrifuged at maximum speed in a microcentrifuge for 2 min, aspirated down to a 5- to 10-μl volume, and frozen at −20°C until PCR analysis.

CC-PCR detection of infectious *C. parvum* oocysts. Harvested HCT-8 cells and *C. parvum* oocyst PCR-positive controls were lysed by eight cycles of freezing in liquid nitrogen and thawing in a 98°C heated block. Aliquots of lysed samples were used directly for PCR without further purification. PCR primers specific for the *C. parvum hsp70* gene resulted in a 361-bp product (18). PCR was performed with a Perkin-Elmer model 9600 thermal cycler (PE Applied Biosystems, Foster City, Calif.). Each 50-μl PCR mixture contained 5.0 μl of 10× amplification buffer with Mg (1.5 mM final concentration; Boehringer Mannheim Biochemicals, Indianapolis, Ind.); 200 μM each dATP, dTTP, dCTP, and dGTP (Boehringer); 200 nM each forward and reverse CPHSP2 primer; 2.5 μl of bovine serum albumin (30 mg ml^{-1}; Sigma); and various amounts of *C. parvum* template DNA. Amplification conditions were as follows: initial denaturation at 95°C for 5 min; samples kept at 80°C while 2.0 U of *Taq* DNA polymerase (Boehringer) was added (hot start); 40 cycles of denaturation at 94°C for 30 s; annealing at 59°C for 1 min; extension at 72°C for 30 s; a single final extension at 72°C for 10 min; and a 4°C soak. Amplification products were separated by horizontal gel electrophoresis on a 2.0% agarose gel (Amresco, Solon, Ohio) containing 0.5 μg of ethidium bromide (Sigma) ml^{-1} and visualized under UV light. Gel images were captured with a gel documentation system (UVP, Inc., Upland, Calif.).

Cloning and DNA sequence analysis of CC-PCR products. CC-PCR products were cloned and sequenced for confirmation of homology to the *C. parvum hsp70* gene. Products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Cloned products were sequenced commercially (ACGT, Northbrook, Ill., and DNA Sequencing Service, The University of Arizona, Tucson), and sequence homology to the *C. parvum* KSU-1 *hsp70* gene (10) was confirmed with Gene Runner version 3.0 (Hastings Software, Inc., Hastings, N.Y.). Duplicate clones of each PCR product were sequenced and analyzed to identify potential sequencing errors. Clones obtained from independent CC-PCR products from the same sample were analyzed to exclude random nucleotide misincorporation by *Taq* DNA polymerase.

Statistical analyses. Statistical analyses of seeded-sample oocyst recoveries were performed with a two-sample *t* test and SYSTAT version 8.0 software (SPSS Inc., Chicago, Ill.). Results are given at the 95% confidence level.

Nucleotide sequence accession numbers. The *C. parvum* KSU-1 *hsp70* reference sequence used in this study has GenBank accession no. U11761. The *hsp70* sequences described in this study have the following GenBank accession numbers: *C. parvum* LA-1, AF150831; *C. parvum* AWS-1, AF150816; *C. parvum* AWS-2, AF150817; *C. parvum* AWS-3, AF150818; *C. parvum* AWS-4, AF150819; *C. parvum* AWS-5, AF150820; *C. parvum* AWS-6, AF150821; *C. parvum* AWS-7, AF150822; *C. parvum* AWS-8, AF150823; *C. parvum* AWS-9, AF150824; *C. parvum* AWS-10, AF150825; *C. parvum* AWS-11, AF150826; *C. parvum* AWS-12, AF150827; *C. parvum* AWS-13, AF150828; *C. parvum* AWS-14, AF150829; and *C. parvum* AWS-15, AF150830.

RESULTS

Oocyst recoveries and CC-PCR with seeded water samples. Flotation and IMS recoveries of oocysts from raw and filter backwash water samples seeded with viable *C. parvum* LA-1 are summarized in Table 1. Results of CC-PCR analysis of IMS-purified seeded samples are also included in Table 1. While IMS had higher mean recoveries than flotation for both raw and filter backwash water samples, differences were not significant (*P* values were 0.235 and 0.362, respectively). Mean IMS and flotation oocyst recoveries were lower for filter backwash water than for raw water. This result was most likely due to interference by the large amounts of debris in the filter backwash water samples. However, it is important to note that IMS oocyst recoveries for several filter backwash water samples (A, D, and E) were higher than the recoveries for two raw water samples (C and D).

CC-PCR results agreed with all IFA oocyst counts for IMS-purified seeded samples (Table 1). These results included a positive CC-PCR assay for seeded filter backwash water sample C, which had an IMS-IFA count of only three oocysts. IMS-purified filter backwash water samples B and F had no detectable oocysts by IFA and were found negative by CC-PCR. Deionized water negative controls ($n = 8$) were all found negative by both IFA and CC-PCR.

Effect of IMS dissociation method on oocyst recovery. IMS trials revealed significantly higher oocyst recoveries from deionized (*P*, 0.025), raw (*P*, 0.005), and filter backwash (*P*, 0.020) water samples with the 0.1 N HCl dissociation method

TABLE 1. Oocyst recoveries from seeded water samples and CC-PCR results

Sample	% Oocyst recovery with:		CC-PCR result[b]
	Flotation-IFA[a]	IMS-IFA[a]	
Raw water A	18.3	56.6	Positive
Raw water B	14.9	33.7	Positive
Raw water C	14.2	10.4	Positive
Raw water D	22.3	6.9	NA
Raw water E	13.6	27.8	Positive
Raw water F	16.1	21.0	Positive
Filter backwash water A	9.4	17.2	Positive
Filter backwash water B	<7.7	<1.7	Negative
Filter backwash water C	<3.9	6.2	Positive
Filter backwash water D	2.0	7.2	NA
Filter backwash water E	<1.2	19.8	Positive
Filter backwash water F	<10.9	<2.9	Negative
Deionized water negative controls ($n = 8$)	ND[c]	ND	Negative

[a] Mean ± standard deviation percent recoveries for raw water and filter backwash water samples were 16.6 ± 3.0 and 5.8 ± 3.7, respectively. Mean ± standard deviation percent recoveries for raw water and filter backwash water samples were 26.1 ± 16.5 and 9.1 ± 6.9, respectively.

[b] Results showed 100% agreement with IFA results. NA, not available; CC-PCR assays were invalid due to a power failure during PCR (raw water sample D) and an incubator malfunction (filter backwash water sample D), respectively.

[c] ND, None detected.

TABLE 3. Sites found positive for *Cryptosporidium* oocysts by flotation-IFA and infectious *C. parvum* by CC-PCR

Site found positive[a] by:			
Flotation-IFA		CC-PCR	
Raw water	Filter backwash water	Raw water	Filter backwash water
2 (2)	11 (2)	7	3
3	13	9	8
4	17	11[b]	9
5	25 (3)[b]	12	10
8		13	15
9		14	17 (2)
11[b]			21
14			25[b]
16			
18			
20			
21			
25			

[a] Numbers in parentheses indicate the numbers of samples found positive if there was more than one.

[b] From only sites 11 and 25 did the same sample test positive by both flotation-IFA and CC-PCR.

than with the AHBSS–1% trypsin dissociation method (Table 2).

Detection of *Cryptosporidium* oocysts by flotation-IFA and infectious *C. parvum* by CC-PCR in environmental water samples. A total of 122 raw water and 121 filter backwash water samples were analyzed. Flotation-IFA detected oocysts in 13.1% ($n = 16$) of the raw water and 5.8% ($n = 7$) of the filter backwash water samples, while CC-PCR detected infectious *C. parvum* in 4.9% ($n = 6$) of the raw water and 7.4% ($n = 9$) of the filter backwash water samples. Raw water mean equivalent volumes assayed were 1.57 liters by flotation-IFA and 2.68 liters by IMS. Filter backwash water mean equivalent volumes assayed were 0.75 liter by flotation-IFA and 1.01 liters by IMS. Sites with samples found positive for *Cryptosporidium* oocysts by flotation-IFA and infectious *C. parvum* by CC-PCR are listed in Table 3. Several sites had multiple positive samples, and overall *Cryptosporidium* was detected at 19 of the 25 sites. From only sites 11 and 25 did the same sample test positive by both flotation-IFA and CC-PCR.

DNA sequence analysis of CC-PCR products. All CC-PCR products were confirmed by cloning and DNA sequence analysis to be >98% homologous to the *C. parvum* KSU-1 *hsp70* gene. Five of the CC-PCR products were 100% homologous to the *C. parvum* KSU-1 *hsp70* reference sequence, while the other products contained nucleotide substitutions which represented six different *hsp70* genotypes (Fig. 1). The sequence of *C. parvum* LA-1, which was used as our laboratory quality control strain, differed from the *C. parvum* KSU-1 *hsp70* reference sequence at three positions, and a single environmental *C. parvum* strain (AWS-11) had this *hsp70* genotype (Fig. 1).

TABLE 2. Comparison of IMS oocyst recoveries with 0.1 N HCl and AHBSS–1% trypsin dissociation

Sample[a]	Mean (range) % oocyst recovery with:	
	0.1 N HCl	AHBSS–1% trypsin
Deionized water	94.3 (85.4–104.0)	61.8 (56.0–77.3)[b]
Raw water	57.3 (56.1–58.8)	36.3 (33.3–40.4)
Backwash water	25.4 (21.9–29.8)	13.8 (11.7–15.3)

[a] Oocyst seed was 114 ± 9; $n = 3$ unless otherwise noted; 0.5-ml packed-pellet volumes for raw water and backwash water samples.

[b] $n = 6$.

DISCUSSION

To allow the water industry to make accurate human health risk assessments, it is crucial to have methods to detect viable, infectious *C. parvum* oocysts in water samples. Here we present field test results of an integrated CC-PCR *C. parvum* infectivity assay. This study is the first to report the recovery of naturally occurring *C. parvum* oocysts from environmental water samples with IMS and the determination of their infectivity.

Comparison of oocyst recoveries from seeded samples with Percoll-sucrose flotation and IMS revealed that although mean IMS oocyst recoveries were higher than flotation oocyst recoveries for both raw water and filter backwash water samples, the differences were not statistically significant (P values were 0.235 and 0.362, respectively) due to sample-to-sample variation in IMS oocyst recoveries. The IMS oocyst recoveries in this study are not comparable to those previously reported by others with the Dynal IMS system (1, 2, 16), since the previous studies did not include sample concentration. Thus, the IMS oocyst recoveries in this study are the first reported for the entire IMS method (concentration of seeded 10-liter samples and IMS). In previous studies, IMS recoveries from 10-ml deionized water samples seeded directly with *C. parvum* oocysts and dissociated with 0.1 N HCl were approximately 91% (2) and 77% (1). Our trials revealed significantly higher oocyst recoveries from deionized, raw, and filter backwash water samples with 0.1 N HCl dissociation than with AHBSS–1% trypsin dissociation (Table 2). Therefore, it is important to note that if the 0.1 N HCl dissociation method had been used for the IMS recovery trials in this study, it is likely that the IMS oocyst recoveries would have been significantly higher than the flotation recoveries. One possible explanation for the differences in oocyst recoveries from deionized water among the laboratories

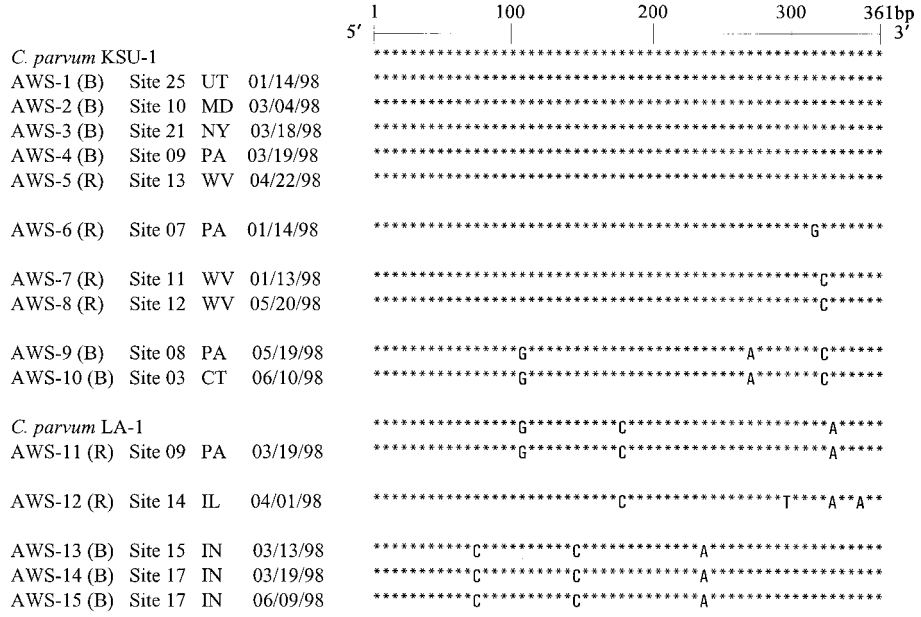

FIG. 1. DNA sequence alignment of *hsp70* CC-PCR products of infectious *C. parvum* recovered from raw (R) and filter backwash (B) water samples. States (postal abbreviations) from and dates (month/day/year) on which samples were recovered are indicated. *C. parvum hsp70* PCR products were 361 bp long (18), and the *C. parvum* KSU-1 *hsp70* sequence was the reference sequence (nucleotides [nt] 2423 to 2783). *C. parvum* LA-1 served as the laboratory quality control strain. Nucleotide substitutions from the 5′ end were as follows: nt 2507, T→C; nt 2528, A→G; nt 2563, T→C; nt 2591, T→C; nt 2646, C→A; nt 2682, G→A; nt 2714, C→T; nt 2731, A→G; nt 2740, T→C; nt 2747, G→A; and nt 2762, G→A. GenBank accession numbers for *C. parvum* sequences obtained in this study are provided in Materials and Methods.

may be the protocols used for microscopic enumeration. Recoveries reported by Campbell et al. (2) (91%) were based on both hemacytometer and well slide IFA counts, while those of Bukhari et al. (1) were based on well slide IFA counts with multiple washes. The well slide protocol used by our laboratory minimizes the numbers of washes, since washing steps may cause loss of oocysts.

The AHBSS–1% trypsin dissociation method was used in this study instead of the 0.1 N HCl method because of its compatibility with the CC-PCR infectivity assay. AHBSS-trypsin dissociation also served as an excystation trigger and pretreatment for cell culturing and was anticipated to have a less adverse impact than 0.1 N HCl on the infectivity of environmentally stressed oocysts. Recently, it was reported that 0.1 N HCl-treated *C. parvum* oocysts retained their in vitro cell culture infectivity (17), although large numbers (1,000) of fresh oocysts were used and infections were not quantitated. In another study at the same laboratory, sporozoites released (without the use of 0.1 N HCl) from IMS-recovered fresh oocysts (>100) retained their infectivity, but the infections were not quantitated (16). Recent results obtained with a quantitative infectivity assay in our laboratory (5a) revealed a significant reduction (range, 51 to 69%) in the infectivity of 0.1 N HCl-treated oocysts compared to AHBSS-trypsin-treated oocysts. Since 0.1 N HCl has an adverse effect on the infectivity of freshly purified oocysts, the effect is likely more pronounced with environmentally stressed oocysts. Therefore, the AHBSS-trypsin dissociation method is preferred for CC-PCR, while the 0.1 N HCl dissociation method is recommended for IMS-IFA studies.

The unique chemical and physical properties (matrix) of a water sample may have an effect on oocyst recovery with IMS. We attempted to investigate what appeared to be matrix effects which resulted in low oocyst recoveries from some water samples by performing additional IMS recovery trials. We used a replicate, unseeded sample concentrate of backwash water sample F (which had <2.9% recovery by IMS; Table 1). When oocysts were added directly to the sample concentrate and immediately processed by IMS, a mean recovery of 67.4% (n = 3; range, 56.0 to 77.3%) was obtained. However, when the sample concentrate was seeded, centrifuged, and resuspended prior to IMS, the mean recovery was only 1.0% (n = 3; range, 0.7 to 1.3%). These results suggested that during centrifugation, the oocysts became associated with particulates, a situation which hampered oocyst recovery.

Despite the similar oocyst recoveries with flotation and IMS for seeded samples in this study (Table 1), several advantages of IMS over flotation are critical for the CC-PCR infectivity assay. First, IMS is superior to flotation in removing debris and allows larger equivalent volumes of water concentrate to be assayed. This difference was particularly evident for the raw water samples assayed in this study. This difference was not as evident for the filter backwash water samples assayed in this study, since the experimental design limited IMS to the purification of 1.5 ml of a packed pellet and flotation to 2.0 ml of a packed pellet. Flotation was more effective at removing the large debris particles from the filter backwash water samples than the fine particles from the raw water samples. Another advantage of IMS over flotation is that the final IMS-purified sample volume is typically 50 μl, compared to 5 ml for flotation-purified samples. This factor is critical for the CC-PCR assay, since large-volume purified samples are not amenable to analysis. CC-PCR results for IMS-purified seeded samples agreed with all IFA oocyst counts. These results included a positive CC-PCR assay for a seeded filter backwash water sample which had an IMS-IFA count of only three oocysts. In addition, no cytotoxic effects for cell monolayers were observed for any of the samples due to trace water debris present in IMS-purified samples.

It is difficult to compare IFA and CC-PCR, since each

method detects different types of oocysts. IFA detects all oocysts (including those of other *Cryptosporidium* species), dead, viable, or infectious. In contrast, the CC-PCR assay detects only infectious *C. parvum* oocysts. Therefore, it was anticipated that there would be a larger number of IFA-positive samples than of CC-PCR-positive samples. Indeed, IFA detected oocysts in 13.1% of the raw water samples; CC-PCR detected infectious *C. parvum* in 4.9% of the raw water samples. Unexpectedly, more filter backwash water samples were found positive for infectious *C. parvum* by CC-PCR (7.4%) than to contain total oocysts by IFA (5.8%). Only 2 of the 15 CC-PCR-positive water samples were IFA positive for oocysts (Table 3). These results are likely a reflection of the splitting of samples containing very small numbers of oocysts between the IFA and CC-PCR assays; the high fluorescent background interference of flotation-purified samples; the similar volumes of filter backwash water samples examined by IFA and IMS (0.75 and 1.01 liters, respectively); and the high sensitivity of the CC-PCR assay.

Previous studies have shown that *Cryptosporidium* oocysts present in raw surface water may still be present in treatment plant filter backwash water (3, 8, 15). Oocysts present in raw surface water may be concentrated by water treatment with sand filtration; the degree of concentration has been reported to range from less than 1 (8) up to 3 orders of magnitude (3, 15). In this study, IFA-positive raw water samples had a mean concentration of 190 oocysts/100 liters (range, 37 to 1,463), while filter backwash water samples had a mean concentration of 220 oocysts/100 liters (range, 37 to 556). If the IFA data are adjusted on the basis of oocyst recovery efficiencies determined from seeded samples, then positive filter backwash water samples contained about 3.3 times as many oocysts as positive raw water samples.

DNA sequence analysis of the CC-PCR products revealed a total of six different *C. parvum hsp70* genotypes (Fig. 1). It is possible that the single nucleotide substitution observed for AWS6 was due to random misincorporation by *Taq* DNA polymerase. The sequence of our laboratory quality control strain, *C. parvum* LA-1, differed from the *C. parvum* KSU-1 *hsp70* reference sequence at three nucleotide positions. These differences were reproducible with different lots of oocysts obtained from the supplier and harvested from different experimentally infected immunosuppressed mice. These results suggested that *hsp70* sequences may be useful for differentiating strains of *C. parvum*. Five of the CC-PCR products (AWS-1 to AWS-5) were identical to the *C. parvum* KSU-1 *hsp70* reference sequence and came from samples from five different states, suggesting widespread occurrence of this *C. parvum hsp70* genotype. Similarly, AWS-9 and AWS-10, which represented a novel *C. parvum hsp70* genotype, came from samples collected in two different states. In contrast, other *C. parvum hsp70* genotypes appeared to have limited geographic distributions. For example, the *C. parvum hsp70* genotype represented by AWS-13, AWS-14, and AWS-15 came only from samples collected in Indiana. There was also evidence of the occurrence of mixed *C. parvum hsp70* genotypes within watersheds. Two samples from site 9 (AWS-4 and AWS-11) were collected at the same time yet contained two different *C. parvum hsp70* genotypes. In contrast, two samples from site 17 (AWS-14 and AWS-15) were collected at different times and contained identical *C. parvum hsp70* genotypes. These results exemplify the complex ecology of *C. parvum*, and analysis of additional environmental strains is necessary to clarify the significance of our findings.

Using the PCR primers of Johnson et al. (7), we attempted to amplify *Cryptosporidium* 18S rRNA genes from the genomic DNA present in the completed *hsp70* CC-PCR samples to gain further information about the infectious *C. parvum* detected in this study. Sequence polymorphisms within this amplified region differentiate *C. parvum* from *C. baileyi*, *C. muris*, and *C. wrairi* (7) as well as from human and animal *C. parvum* strains (14, 27). Thus far, we have been able to successfully amplify and sequence only one sample (AWS-12), and the 18S rRNA gene fragment was identical to that of a novel human *C. parvum* strain characterized by colleagues at the Centers for Disease Control and Prevention (26). The AWS-12 *C. parvum* strain also represented a novel *hsp70* genotype (Fig. 1).

The detection of infectious *C. parvum* in filter backwash water samples with the CC-PCR assay in this study is significant in that it provides the first evidence that infectious *C. parvum* may penetrate water treatment barriers. Previous studies revealed that oocysts may be present in treatment plant effluents (11, 12), including two sites from this study which had samples positive for infectious *C. parvum*. Determination of the occurrence of infectious *C. parvum* in finished drinking water is critical for the water industry to make accurate human health risk assessments. The results of this study support the utility of IMS for oocyst recovery and purification of samples of diverse water qualities and the sensitivity and specificity of the *C. parvum* CC-PCR infectivity assay. Future research is aimed at the optimization of oocyst recovery and the application of the CC-PCR infectivity assay to finished water samples.

ACKNOWLEDGMENTS

This research was supported by grants from the American Water Works Research Foundation and the American Water Works Service Co., Inc.

We gratefully acknowledge the efforts of Ramon Aboytes-Torres and Dale Young in sample processing.

REFERENCES

1. **Bukhari, Z., R. M. McCuin, C. R. Fricker, and J. L. Clancy.** 1998. Immunomagnetic separation of *Cryptosporidium parvum* from source water samples of various turbidities. Appl. Environ. Microbiol. **64:**4495–4499.
2. **Campbell, A. T., B. Gron, and S. E. Johnsen.** 1997. Immunomagnetic separation of *Cryptosporidium* oocysts from high turbidity water sample concentrations, p. 91–96. *In* C. R. Fricker, J. L. Clancy, and P. A. Rochelle (ed.), Proceedings of the 1997 AWWA International Symposium on Waterborne *Cryptosporidium*. American Water Works Association, Denver, Colo.
3. **Cornwell, D., and R. Lee.** 1993. Recycle stream effects on water treatment. American Water Works Association Research Foundation, Denver, Colo.
4. **Deng, M. Q., D. O. Cliver, and T. W. Mariam.** 1997. Immunomagnetic capture PCR to detect viable *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **63:**3134–3138.
5. **Di Giovanni, G. D., M. LeChevallier, D. Battigelli, A. Campbell, and M. Abbaszadegan.** 1997. Detection of *Cryptosporidium parvum* by enzyme immunoassay and the polymerase chain reaction. *In* Proceedings of the AWWA Water Quality Technology Conference. American Water Works Association, Denver, Colo. [on CD-ROM.]

5a. **Di Giovanni, G. D., et al.** Unpublished data.

6. **Gibbons, C., F. Rigi, and F. Awadelkariem.** 1998. Detection of *Cryptosporidium parvum* and *C. muris* oocysts in spiked backwash water using three PCR-based protocols. Protistologica **149:**127–134.
7. **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.
8. **Karanis, P., D. Schoenen, and H. M. Seitz.** 1996. *Giardia* and *Cryptosporidium* in backwash water from rapid sand filters used for drinking water production. Zentbl. Bakteriol. **284:**107–114.
9. **Kaucner, C., and T. Stinear.** 1998. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. Appl. Environ. Microbiol. **64:**1743–1749.
10. **Khramtsov, N., M. Tilley, D. Blunt, B. Monteleone, and S. Upton.** 1995. Cloning and analysis of a *Cryptosporidium parvum* protein with homology to cytoplasmic form HSP 70. J. Eukaryot. Microbiol. **42:**416–422.
11. **LeChevallier, M. W., and W. D. Norton.** 1993. Monitoring of *Giardia* and *Cryptosporidium* in the American water system. American Water Works Service Co., Inc., Voorhees, N.J.

12. **LeChevallier, M. W., W. D. Norton, and R. G. Lee.** 1991. *Giardia* and *Cryptosporidium* spp. in filtered drinking water supplies. Appl. Environ. Microbiol. **57:**2617–2621.
13. **Morgan, U., and R. Thompson.** 1998. PCR detection of *Cryptosporidium*: the way forward? Parasitol. Today **14:**241–245.
14. **Morgan, U. M., C. C. Constantine, D. A. Forbes, and R. C. Thompson.** 1997. Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. J. Parasitol. **83:**825–830.
15. **Richardson, A., R. Frankenberg, A. Buck, J. Selkon, J. Colbourne, J. Parsons, and R. Mayon-White.** 1991. An outbreak of waterborne cryptosporidiosis in Swindon and Oxfordshire. Epidemiol. Infect. **107:**485–495.
16. **Rochelle, P., R. De Leon, A. Johnson, M. Stewart, and R. Wolfe.** 1999. Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. Appl. Environ. Microbiol. **65:**841–845.
17. **Rochelle, P. A., R. De Leon, M. H. Stewart, and R. L. Wolfe.** 1998. Infectivity of waterborne *Cryptosporidium* oocysts recovered using USEPA draft method 1622. *In* Proceedings of the AWWA Water Quality Technology Conference. American Water Works Association, Denver, Colo. [on CD-ROM.]
18. **Rochelle, P. A., D. M. Ferguson, T. J. Handojo, R. De Leon, M. H. Stewart, and R. L. Wolfe.** 1997. An assay combining cell culture with reverse transcriptase PCR to detect and determine the infectivity of waterborne *Cryptosporidium parvum*. Appl. Environ. Microbiol. **63:**2029–2037.
19. **Slifko, T. R., D. Friedman, J. B. Rose, and W. Jakubowski.** 1997. An in vitro method for detecting infectious *Cryptosporidium* oocysts with cell culture. Appl. Environ. Microbiol. **63:**3669–3675.
20. **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. (Erratum, **63:**815, 1997.)
21. **U.S. Environmental Protection Agency.** 1997. Draft method 1622: *Cryptosporidium* in water by filtration/IMS/FA. Office of Research and Development, U.S. Government Printing Office, Washington, D.C.
22. **U.S. Environmental Protection Agency.** 1996. ICR microbial laboratory manual. Office of Research and Development, U.S. Government Printing Office, Washington, D.C.
23. **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.
24. **Wagner-Wiening, C., and P. Kimmig.** 1995. Detection of viable *Cryptosporidium parvum* oocysts by PCR. Appl. Environ. Microbiol. **61:**4514–4516.
25. **Woods, K. M., M. V. Nesterenko, and S. J. Upton.** 1995. Development of a microtitre ELISA to quantify development of *Cryptosporidium parvum* in vitro. FEMS Microbiol. Lett. **128:**89–94.
26. **Xiao, L.** 1998. Personal communication.
27. **Xiao, L., I. Sulaiman, R. Fayer, and A. A. Lal.** 1998. Species and strain-specific typing of *Cryptosporidium* parasites in clinical and environmental samples. Mem. Inst. Oswaldo Cruz **93:**687–692.