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Development of a PCR Protocol for Sensitive Detection of *Cryptosporidium* Oocysts in Water Samples

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The development of a reliable method of using PCR for detection of *Cryptosporidium* oocysts in environmental samples with oligonucleotide primers which amplify a portion of the sequence encoding the small (18S) subunit of rRNA producing a 435-bp product was demonstrated. The PCR assay was found to provide highly genus-specific detection of *Cryptosporidium* spp. after release of nucleic acids from oocysts by a simple freeze-thaw procedure. The assay routinely detected 1 to 10 oocysts in purified oocyst preparations, as shown by direct microscopic counts and by an immunofluorescence assay. The sensitivity of the PCR assay in some seeded environmental water samples was up to 1,000-fold lower. However, this interference was eliminated by either flow cytometry or magnetic-antibody capture. Sensitivity was also improved 10- to 1,000-fold by probing of the PCR product on dot blots with an oligonucleotide probe detected by chemiluminescence. Confirmation of the presence of *Cryptosporidium* oocysts in water samples from the outbreak in Milwaukee, Wis., was obtained with this technique, and PCR was found to be as sensitive as immunofluorescence for detection of oocysts in wastewater concentrates.

The protozoan genus *Cryptosporidium* is increasingly recognized as an important agent of gastrointestinal disease in several animal species, including cows, goats, lambs, and humans. Infections with this coccidian parasite can lead to a chronic, life-threatening condition in immunocompromised individuals and to acute gastroenteritis and diarrhea in healthy people (6). An effective therapy for treating infections is not available (7).

There have been at least six documented waterborne outbreaks of *Cryptosporidium* infection in North America since 1985. These have occurred in Texas (8), Ontario (27), Georgia (11), Oregon (19), Pennsylvania (23), and Wisconsin (20). The risk to other drinking-water supplies has been demonstrated by the widespread occurrence of oocysts in surface waters throughout the United States (17, 28) and the resistance of the oocysts to most disinfectants normally used in the production of drinking water (15, 26). Contamination of drinking water supplies with human or animal feces may lead to outbreaks of cryptosporidiosis if the levels of oocysts are high enough to overwhelm filtration barriers or disinfection levels. Rapid and effective monitoring methods are needed at drinking water facilities to determine the occurrence of oocysts in source and treated water. Development of such methods would improve decisions concerning treatment, contamination, and public health risks.

There are several different methods available for detecting *Cryptosporidium* oocysts in water. Direct and indirect microscopic visualization of oocysts is usually performed with vital stains, dyes, or fluorescent antibodies (5, 29). A widely used method for detection of oocysts in environmental samples is the immunofluorescence assay (IFA) (17, 18, 31), which relies on microscopic visualization of oocysts labeled with fluorescein-conjugated antibodies. The IFA is time-consuming and subject to variance in sensitivity and does not lend itself to batch processing of samples.

Genetic methods which are based on detection of *Cryptosporidium* nucleic acid, by hybridization and amplification techniques such as PCR, have been developed (13, 16). In the present study, an assay for detection of *Cryptosporidium* DNA by PCR was characterized in terms of its sensitivity and specificity and used to detect oocysts in environmental samples. The assay was used for detection of the parasite in concentrates from wastewater, surface waters, and drinking water. Inhibition of sensitivity of the PCR assay caused by uncharacterized components in samples was examined. Flow cytometry (33, 34), dot blot (1, 13, 14), and magnetic antibody capture (4, 10, 24, 25) have been demonstrated to be useful in improving the sensitivity of molecular assays. These methods were analyzed for their usefulness in reducing or circumventing the problem of PCR inhibition.

MATERIALS AND METHODS

Oocysts. *Cryptosporidium parvum* oocysts were obtained in highly purified form from Alexon, Inc. (Mountain View, Calif.), from the University of Arizona (in a semipurified form), and in feces from naturally infected calves at Webster, Fla. (sieved and stored in 2.5% potassium dichromate). Oocysts were prepared from the calf fecal samples by the method of Arrowood and Sterling (2).

***Cryptosporidium* PCR assay.** Cow fecal or other environmental samples stored in dichromate or Formalin preservatives were washed four times with PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 3.5 mM $MgCl_2$) by centrifugation. For seeded experiments with purified oocysts, environmental samples were spiked, and serial dilutions (1:10) were made in PCR buffer. A 20% (wt/vol in water) Chelex 100 solution (Bio-Rad Laboratories, Richmond, Calif.) was mixed with each sample and each set of dilutions (20 μl of Chelex stock to 100 μl of undiluted or diluted sample). Samples were then subjected to six cycles of freezing and thawing to release the target DNA from oocysts. A dry ice-ethanol bath was used for freezing, and a water bath (98°C) was used for thawing; samples were incubated for 1 to 2 min in each bath. Sample debris was pelleted by centrifugation, and supernatants (50 μl) were used as the template in the PCR assay.

The amplification reaction included forward and reverse oligonucleotide primers which hybridized to *Cryptosporidium* sequences encoding 18s rRNA. This resulted in amplification of a segment of genomic DNA of 435 bp. The forward primer (5′-AAGCTCGTAGTTGGATTTCTG-3′; CPB-DIAGF) corresponded to nucleotides 601 to 621, and the reverse primer (5′-TAAGGTGCTGAAGG AGTAAGG-3′; CPB-DIAGR) corresponded to nucleotides 1015 to 1035 of the

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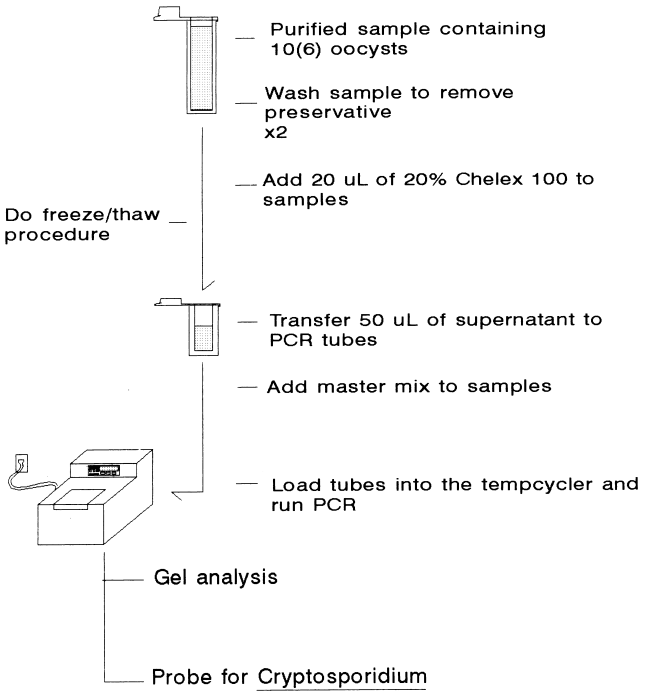

FIG. 1. *Cryptosporidium* PCR assay flowchart.

reported sequence (GenBank accession number L16996). The PCR mixture (50 μl of sample and 50 μl of master mix) contained sample (template), the four deoxynucleoside triphosphates (each at 150 μM), forward and reverse primer (200 nM each), 1× PCR buffer, and 2 U of thermostable polymerase (AmpliTaq; Perkin Elmer Corp., Norwalk, Conn.). Positive and negative controls for each batch of samples included template consisting of water with and without, respectively, 2 pg of plasmid A1 DNA. This plasmid contained, as 1/10 of its total size, the target sequence from *C. parvum* (provided by N. Pieniazek). Samples were incubated in a thermocycler (model 60 TempCycler [Coy Laboratory Products, Grass Lake, Mich.] or DNA Thermal Cycler model 480 [Perkin Elmer]) for 39 cycles with the following programmed profile: 5 min at 80°C, initial denaturation for 30 s at 98°C, and 39 cycles of amplification (annealing for 30 s at 55°C, extension for 1 min at 72°C, and denaturation for 30 s at 94°C). The final extension segment was prolonged to 10 min, and the samples were then cooled to 4°C.

The reaction products were detected on ethidium bromide-stained agarose gels by visualization of a fluorescent band with UV light (3, 30). Reaction products were also detected by dot blot hybridization (14) with an oligonucleotide probe. The sequence of the 38-nucleotide probe (5′-GGGGATCGAAGAC GATCAGATACCGTCGTAGTCTTAAC-3′) was homologous to an internal portion of the *C. parvum* PCR product, as determined previously by amplifying, cloning, and sequencing the product derived from *C. parvum* oocysts (provided by N. Pieniazek; GenBank accession number L16996). The probe was obtained from the DNA Synthesis Lab, University of Florida, Gainesville, Fla., as an oligonucleotide covalently tagged with biotin.

Nitrocellulose (BA85; Schleicher and Schuell) filters were used to prepare dot blots. The filters were prehybridized in 6× SSPE (20× SSPE stock is 3.6 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA [pH 7.4])–0.3% sodium dodecyl sulfate (SDS)–1.0% nonfat dry milk for 1 h at 65°C. The biotinylated probe was then added (to 0.5 nM), and the filter was incubated overnight at 37°C. Following hybridization, blots were washed four times for 5 min each in 4× SSPE at 65°C. An equivalent blot was subjected to prehybridization and hybridization without added probe to control for nonspecific detection (presence of environmental biotin).

Detection was done with the Southern Light chemiluminescence detection system (Version M protocol; Tropix, Inc., Bedford, Mass.), and the results were visualized with Kodak X-AR film (see Fig. 1 for the assay flowchart).

Evaluation of specificity. The specificity of the PCR assay was evaluated with heterologous genomic DNAs from *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerevisiae* ATCC 26108, *Pichia polymorpha* ATCC 34438, *Pichia canadensis* Y11912 (Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill.), *Schizosaccharomyces pombe* ATCC 24843, *Rhodotorula glutinis* ATCC 26207, *Aspergillus nidulans*, *Aspergillus flavus*, *Alternaria* spp., *Sporothrix schenki*, *Histoplasma capsulatum* F182-678-1A, *Entamoeba histolytica* HM-1:IMSS clone 6, *Acanthamoeba castellanii* Neff, *Giardia lamblia*, *Trypanosoma brucei*, *Toxoplasma gondii* (with some human host genomic DNA), *Eimeria tenella*, *Eimeria gallapovonis* 893, *Eimeria vermiformis* 897, *Eimeria maxima* 68, *Pneumocystis carinii*, *Taenia saginata* 622, and calf thymus, salmon sperm, and rat GH3 DNA.

The heterologous genomic DNA preparations were obtained from colleagues (see Acknowledgments) with the following exceptions: salmon sperm and calf thymus DNAs were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Hoefer Scientific Instruments (San Francisco, Calif.), respectively; DNAs from the *Escherichia*, *Pseudomonas*, and *Rhodotorula* strains were prepared by standard methods (3); and DNAs from the *Pichia*, *Saccharomyces*, and *Schizosaccharomyces* strains were prepared from the strains obtained from W. L. Adair and Rosalyn Irby (University of South Florida, Tampa) by standard methods (3). The concentration of DNA in each of the samples was verified by fluorometry (TKO100 fluorometer; Hoefer) and by agarose gel electrophoresis.

PCRs were run with heterologous DNAs (2 ng of each) both with and without *Cryptosporidium* (2 pg of plasmid A1) template, and the reaction products were checked by gel electrophoresis. Controls included identical reactions with bacteriophage lambda-specific primers substituted for the *Cryptosporidium* genus-specific primers, with and without lambda DNA template, with the lambda-specific profile (Perkin Elmer control protocol, supplied with the AmpliTaq enzyme).

Radiolabeled primers were also used to detect nonspecific PCR amplification products. Forward and reverse primers were labeled separately by the kinase reaction (T4 polynucleotide kinase; Promega Corp., Madison, Wis.) (30) and [γ-^{32}P]ATP. Unincorporated nucleotide was reduced by spun-column chromatography. PCR mixes contained radiolabeled primers (0.6 ng of each primer; specific activity, about 10^4 cpm/ng) and unlabeled primers (about 110 ng each). The PCR products were subjected to electrophoresis, and sizes were determined by visualization of unlabeled marker fragments in the gels by ethidium bromide staining prior to autoradiography.

Effects of preservatives and oocyst age on the PCR assay. A cow fecal preparation of *C. parvum* oocysts was split, pelleted, and stored in potassium dichromate (2.5%) and Formalin (10%) for 6 months at 4°C. Samples were then washed and diluted, and oocysts were enumerated by IFA and detected by the PCR assay. IFA counts were obtained in 100 μl from the 10^{-3} dilutions, and oocysts were examined by differential interference contrast microscopy for internal features. Fifty microliters from each dilution level was used in the PCR assay. Naturally occurring oocysts concentrated from wastewater were also evaluated after storage.

Use of PCR assay with environmental samples. Environmental water concentrates were seeded with purified oocysts or sieved cow feces containing large numbers of oocysts. All samples were evaluated by the PCR assay as previously described. The water samples were filter concentrates (pellets) of 40 to 400 liters of water (28). Unseeded water sample pellets from surface waters (used by major metropolitan water utilities as a source for drinking water), cistern water, and wastewater were also evaluated by the PCR assay. IFA counts were obtained by using sucrose-Percoll flotation and monoclonal antibodies. Equivalent numbers of purified oocysts without any water (concentrated pellet) were also evaluated as an additional control.

Effect of flow cytometry on PCR sensitivity. Purified oocysts were added in equivalent numbers to samples containing different amounts of environmental pellets in identical final volumes, so that the ratio of oocysts to environmental pellet was varied. Samples were stained directly with fluorescein-conjugated *Cryptosporidium* antibody (Cell Labs, Sydney, Australia) and sorted by flow cytometry with an EPICS ELITE flow cytometry system (Coulter Corp., Hialeah, Fla.) (33, 34). Sorted samples recovered in microcentrifuge tubes were split, and oocysts were detected by both microscopy (counts) and the PCR assay (presence or absence). Influent oocyst numbers were obtained by fluorescence microscopy prior to flow cytometry.

Effect of magnetic-antibody capture on PCR sensitivity. Purified oocysts were seeded into both water (high-performance liquid chromatography) and an environmental water pellet (1-ml volume). Replicate tubes of both were processed by both the standard PCR protocol and a magnetic-antibody capture PCR protocol. The secondary antibody (goat polyclonal anti-mouse immunoglobulin M [IgM]) linked to a ferric ion (BioMag), the magnetic cell-sorting device, and the cell-sorting protocols (sorting protocol and preload protocol) were obtained from Perseptive Diagnostics, Inc. (Cambridge, Mass.). Sample (1 ml) was suspended in 10 ml of 1× phosphate-buffered saline (PBS), and a blocking agent (nonfat dried milk) was added to a concentration of 5%. Prior to addition of the antibodies to the sample, the antibodies were loaded (coupling of the secondary antibody to the primary one prior to addition of antibodies to the sample) by the Perseptive Diagnostics preloading protocol, and the following concentrations of antibodies: 1 ml of secondary antibody (BioMag) was preloaded to 2 ml of a 1:20 dilution of the primary antibody (monoclonal mouse IgM anti-*Cryptosporidium* oocyst; Meridian Diagnostics, Inc., Cincinnati, Ohio). The samples were then processed by the Perseptive Diagnostics magnetic-antibody cell-sorting protocol.

To remove the antibody complexes from the oocysts (to prevent binding of template by the ferric ions during the freeze-thaw procedure), the concentrated samples were rapidly vortexed for 30 to 45 s. The samples were then immediately put into the magnetic sorter, and after 2 min, the supernatant containing the oocysts was transferred to another tube. The supernatant was centrifuged at approximately 10,500 × *g* and resuspended to a volume of 100 μl. Dilutions were

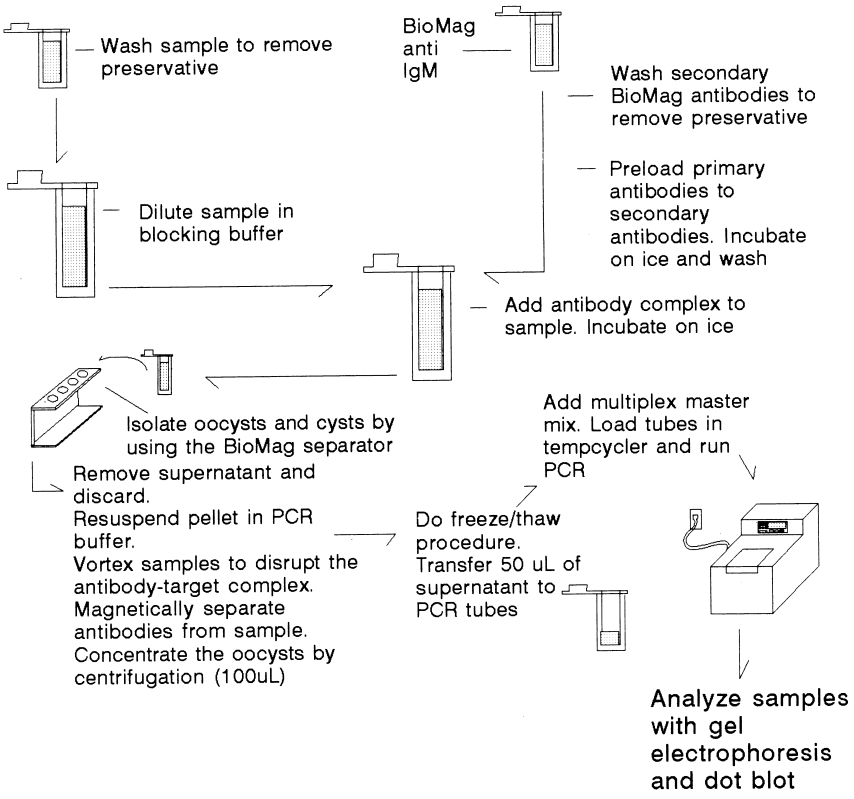

FIG. 2. Magnetic-antibody capture PCR assay flowchart.

completed, and the samples were then processed by the standard PCR assay protocol (see Fig. 2 for the magnetic-antibody capture assay flowchart).

RESULTS

Sensitivity of the PCR assay. A standardized PCR assay for detection of *Cryptosporidium* oocysts in environmental water concentrates was developed. The sensitivity of the PCR assay was found to vary from about 1 to 200 oocysts in purified samples, depending on the source and age of the sample.

TABLE 1. Reproducibility of the PCR assay for detecting purified calf oocyst isolates[a]

Test	Sample[b]	Trial no.	Signal[a] with the following no. of oocysts:			
			0.9	9.0	90	900
Gel	UA2	1	−[c]	−	−	++
		2	−	−	−	++
		3	−	−	−	++
	UA5	1	−	−	+++	++++
		2	−	−	+++	+++++
		3	−	−	+++	+++++
Blot	UA2	1	−	−	++++	+++++
		2	−	−	+++	+++++
		3	−	+	+++++	+++++
	UA5	1	+++	++++	++++++	++++++
		2	−	+++++	++++++	++++++
		3	−	+++++	++++++	++++++

[a] The positive control (A1 plasmid template) gave heavy signals on both gel and blot. The negative control (no template) gave no signals.
[b] UA2 and UA5 were dilution sets of purified oocysts.
[c] ++++++, heavy signal; +++++, medium signal; ++++, medium light signal; +++, light signal; ++, very light signal; +, ultralight signal; −, no signal.

Table 1 illustrates the sensitivity and reproducibility of the assay. Triplicate samples were taken from two preparations of oocysts (UA2, 12 months old, and UA5, 9 months old) and evaluated by PCR. The maximum level of sensitivity with the older sample (UA2) was 90 oocysts by electrophoresis and 9 oocysts by dot blot. The maximum level of sensitivity with the younger sample (UA5) was 90 oocysts by electrophoresis and 0.9 oocysts by dot blot. This experiment demonstrated that the assay could reproducibly detect 9 to 90 oocysts. When evaluating preparations that were ≤2 months of age, 0.9 oocysts were consistently detected.

Specificity of the PCR assay. The PCR assay was found to be highly specific for the *Cryptosporidium* template sequence in tests with heterologous genomic DNAs. In a PCR mix containing 2 ng each of 26 different heterologous DNAs as the template, no band was detected on an ethidium bromide-stained agarose gel. An otherwise identical reaction mix in which *Cryptosporidium* template (2 pg of plasmid A1) was included gave rise to the specific product. Bacteriophage lambda DNA (2 ng) was amplified in the presence of the heterologous DNAs (2 ng each) and lambda-specific primers when the standard profile for lambda amplification was used (Perkin Elmer control protocol, supplied with AmpliTaq enzyme). This demonstrated that all signals and bands seen could be attributed to primer specificity and that there was no detectable inhibition of the PCR assay.

To provide a more sensitive test for specificity, autoradiography was used to detect radiolabeled PCR products. When radiolabeled primers were used in PCRs with heterologous DNAs with or without added A1 template or with freeze-thaw-

TABLE 2. Effects of storing oocysts for 6 months in different preservatives on PCR assay[a]

Preservative	IFA		PCR at dilution:									
	No. of oocysts (10^{-3} dilution)	% Empty oocysts	10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}	
			No.	Signal	No.	Signal	No.	Signal	No.	Signal	No.	Signal
Potassium dichromate (2.5%)	70	12	700	++++	70	+++	7	++	0.7	+	0.07	−
Formalin (10%)	52	10	520	−	52	−	5	−	0.5	−	0.05	−
PBS	23	33	230	+++	23	−	2	−	0.2	−	0.02	−
Saline	21	27	210	+++	21	−	2	−	0.2	−	0.02	−

[a] Equivalent oocyst numbers for each dilution used in the PCR assay were calculated from the IFA counts shown. Fifty-microliter samples were used in the IFA and PCR assays. The percentage of empty oocysts (without visible sporozoites) was determined by observation of a minimum of 50 oocysts from each preserved sample by differential interference contrast microscopy. For PCR, the number of oocysts at each dilution and the corresponding signal are shown. See Table 1, footnote *c*, for definitions.

extracted cow feces lacking or containing oocysts, a *Cryptosporidium*-specific band was detected only in those reaction mixes to which the specific template (A1) or oocysts were added.

Effect of preservatives and age. A strong inhibitory effect of formaldehyde (Formalin) and potassium dichromate on the PCR was found. Strong inhibition of the positive control template (A1) was observed when potassium dichromate was present in the range from 1.25 to 12.5 ppm and when Formalin was present in the range of 5 to 50 ppm. With oocyst preparations, the results were similar, but the inhibition associated with potassium dichromate could be removed by washing the sample prior to the freeze-thaw procedure.

Oocyst preparations from semipurified calf feces were stored for 6 months in potassium dichromate (2.5%), Formalin (10%), PBS, and saline. Oocyst levels ranged from 2.1×10^4 to 7.0×10^4/ml, and 10 to 33% of the oocysts were empty (devoid of internal contents or sporozoites). Table 2 shows that potassium dichromate was able to maintain the integrity of the preparation for detection by PCR (sensitivity of 1 to 10 oocysts in samples <6 months old). Storage in Formalin drastically decreased PCR detection in a matter of a few days or weeks.

Filter concentrates, collected from untreated domestic wastewater and secondarily treated effluents from a sewage treatment facility, were evaluated by IFA and PCR for the detection of naturally occurring oocysts (Table 3). Sixty-five percent (13 of 20) of the samples were positive by IFA, with 1 to 4 oocysts detected in 300 to 1,800 μl of pellet. The pellets were stored for 11 to 16 months in 2.5% potassium dichromate at 4°C and assayed by PCR. Positive results were obtained for 90% (9 of 10) of the samples that were ≤12 months of age, while no positive results were obtained for samples that were stored for more than 12 months. Three samples that were negative by IFA were positive by PCR, and one sample that was positive by IFA was negative by PCR in the ≤1-year-old samples. For the seeded samples, to which known concentrations of oocysts were added back, positive PCR results were noted for all of the undiluted samples (25,000 oocysts), with inhibition noted for 2 of the 20 samples at the 10^{-1} dilution level (2,500 oocysts).

Use of PCR assay with environmental samples. The sensitivity of the PCR assay was reduced as much as 100- or 1,000-fold for oocyst-seeded environmental samples compared with purified oocysts. In six trials, low levels of oocysts were not detected by PCR in environmental pellets (Table 4). However, when oocysts were separated from other particulates with a flow cytometer prior to extraction for the PCR assay, detection was possible regardless of the amount of environmental pellet added.

Washed environmental water pellets and the supernatants obtained from these washes were tested separately for inhibitory activity in the PCR assay with cow fecal samples containing large numbers of oocysts. It was found that the pellets usually contained more than three times as much inhibitory activity as the supernatants.

Table 5 and Figure 3 show the results of the use of magnetic-antibody capture for removing inhibitors associated with the environmental samples. In this experiment, PCR detection of seed levels as high as 25,000 oocysts was inhibited by environmental pellet components. Positive results were noted only for samples diluted to 1:100 (no detection in the undiluted sample or the 1:10 dilution). However, with the antibody capture procedure, the inhibition was removed, and detection was noted in the undiluted sample.

A total of 67 water sample concentrates (unseeded) have been tested to date by the PCR assay, as shown in Table 6 (standard PCR assay, not magnetic-antibody capture). Of the tests, 31.9% were positive overall, ranging from a high of 54.2% positive for wastewater to a low of 8.7% positive for coastal waters. Cistern waters used for drinking purposes in the American Virgin Islands were found to be positive for *Cryptosporidium* oocysts in 16.7% of the samples. During the outbreak investigation in Milwaukee, approximately 21.4% of the drinking-water samples were shown to contain *Cryptosporidium* oocysts, and the results for all 14 samples tested were available in less than 24 h.

DISCUSSION

A method utilizing PCR for the rapid detection of *Cryptosporidium* oocysts in contaminated water samples has been developed. Over 60 samples have now been screened by this procedure, and the ability for batch processing allowed rapid confirmation of the contamination event during the waterborne outbreak in Milwaukee (20). This procedure uses pretreatment, a simple freeze-thaw procedure, and detection by hybridization with a chemiluminescent probe and has demonstrated excellent specificity, reproducibility, and sensitivity.

Previous work had examined DNA probes, PCR, and IFA for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in environmental samples (1, 13). Direct probing was found to be insensitive, and false-positive results were observed, while initial results demonstrated the feasibility of using PCR. Mahbubani et al. (21) also reported on the use of PCR for detection of *Giardia* cysts and found that detection was possible with water samples of 100 ml to 4 liters but that concentrations as high as 10^5 cysts could not be detected when samples were concentrated from 400 liters of water. We have also reported on this interference associated with concentrates derived from 100 to 400 liters of water. In this study, washed environmental water pellets and the supernatants obtained from these washes

TABLE 3. Analysis of aged samples by PCR[a]

Sample no.	Sample age (mo)	IFA analysis		PCR analysis				
		No. of oocytes	Vol of pellet examined (μl)	Dilution	Vol of pellet examined (μl)	Blot results[b]		No. seeded
						Unseeded	Seeded	
5-1e	16	0	1,800	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
5-2e	16	3	1,800	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
6-1s	15	0	300	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
6-2e	15	0	600	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
7-1s	15	1	800	10^0	50	−	++++	25,000
				10^{-1}	5	−	++	2,500
7-2e	15	4	840	10^0	50	−	++++	25,000
				10^{-1}	5	−	++	2,500
8-1s	14	3	600	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
8-2e	14	0	48	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
9-1s	14	2	1,000	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
9-2e	14	0	178	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
10-1s	12	2	400	10^0	50	+	++	25,000
				10^{-1}	5	−	+	2,500
10-2s	12	0	200	10^0	50	+	++	25,000
				10^{-1}	5	−	+	2,500
11-1s	11	1	400	10^0	50	+	++++	25,000
				10^{-1}	5	−	++	2,500
11-2s	11	1	320	10^0	50	+	++++	25,000
				10^{-1}	5	−	++	2,500
12-1s	11	1	500	10^0	50	−	++	25,000
				10^{-1}	5	−	+	2,500
12-2e	11	1	480	10^0	50	+	++	25,000
				10^{-1}	5	−	+	2,500
13-1s	11	3	721	10^0	50	+	++++	25,000
				10^{-1}	5	−	+	2,500
13-2e	11	1	778	10^0	50	+	++++	25,000
				10^{-1}	5	−	+	2,500
14-1s	11	1	40	10^0	50	+++	+	25,000
				10^{-1}	5	++	−	2,500
14-2e	11	0	1,488	10^0	50	++	+	25,000
				10^{-1}	5	−	−	2,500

[a] In sample numbers, e indicates domestic wastewater secondary effluent and s indicates domestic untreated sewage. Samples were stored in 2.5% potassium dichromate. All IFA results were obtained when samples were <7 days old.
[b] ++++, heavy signal; +++, medium heavy signal; ++, medium signal; +, light signal.

were tested separately for inhibitory activity in the PCR assay. Although the components have not been identified, the pellets exhibited more inhibitory activity than the dissolved components in the supernatants. Both absorption of target DNA and inhibition of the primer binding and enzyme activity are possible explanations.

Several procedures were used to counter the inhibition, including the use of Chelex, which has previously been found to enhance detection of PCR products (35). By far the best procedure for reversing inhibition was separation of the target organism from environmental debris prior to extraction by the freeze-thaw process. Both the flow cytometry and antibody capture techniques proved to be useful.

Flow cytometry has been used for routine analysis of *Cryptosporidium* oocysts in water (33). However, the cost of the instrument and the technical support needed to maintain and run the instrument may make this technique impractical for most laboratories. Antibody capture has recently been shown

TABLE 4. Separation of *Cryptosporidium* oocysts from interfering substances in environmental water samples by flow cytometry (FC) for PCR detection

Water sample[a]	Ratio of pellet to seeded oocysts	No. of oocysts after FC	Detection by PCR[b]	
			After FC	Before FC
Buffer control	0:1	47	+++	++
LA	1:1	69	+++	−
	2:1	68	+++	−
	10:1	120	+++	−
	1:0	0	−	−
BV	1:1	148	+++	−
	2:1	58	+++	−
	10:1	99	+++	−
	1:0	0	−	−

[a] The buffer control was 10 mM Tris (pH 7.5)–1 mM EDTA. LA and BV are concentrates obtained from two independent 400-liter filtered volumes of river water.
[b] +++, medium heavy signal; ++, medium signal; −, no signal.

TABLE 5. Separation of *Cryptosporidium* oocysts from interfering substances in environmental water samples by magnetic-antibody capture (MAC) for PCR detection[a]

Sample	Dilution	No. of oocysts		Detection by PCR[b]	
		After MAC (1 ml)	Before MAC (100 μl)	After MAC	Before MAC
Buffer control	10^0	250,000	25,000	++++++	++++++
	10^{-1}	25,000	2,500	++++++	++++++
	10^{-2}	2,500	250	+++++	+++++
	10^{-3}	250	25	+++	++++
Environmental	10^0	250,000	25,000	++++++	−
	10^{-1}	25,000	2,500	++++++	−
	10^{-2}	2,500	250	+++++	+++++
	10^{-3}	250	25	+++	+++

[a] The positive control (A1 plasmid) gave a heavy signal, and the negative control gave no signal. Oocyst numbers were calculated from original IFA counts on the undiluted seed stocks. Larger numbers of oocysts are detectable with the magnetic-antibody capture assay because of the ability to concentrate samples (1 ml for magnetic-antibody capture versus 100 μl for the standard protocol in this particular experiment).

[b] ++++++, heavy signal; +++++, medium signal; ++++, medium light signal; +++, light signal; −, no signal.

to be highly versatile, efficient, and useful for environmental samples. Bifulco and Schaeffer (4) reported 82% recovery of *Giardia* cysts from water by an antibody-magnetite method. In addition, an antigen capture procedure has been used to isolate hepatitis A virus from waste and shellfish samples (9). We confirm the usefulness of an antibody capture procedure for detection of *Cryptosporidium* oocysts. Not only did this procedure reduce interference, but it also allowed the use of a second concentration-purification step which is far superior to the flotation gradients currently used for IFA detection of oocysts. Because such small volumes are processed for PCR, this enhances the sensitivity of the assay, as it allows concentration of the sample from 1 to 10 ml down to 100 μl.

There are several issues to address when evaluating environmental samples for the presence of oocysts. We have shown that the age of the oocysts and the storage conditions will affect the PCR results. Potassium dichromate should be used for storage of the oocysts and environmental samples for assay by PCR. The sample, however, must be thoroughly washed prior to extraction, as potassium dichromate inhibits the PCR. Formalin storage is not recommended. In addition, after 12 months of storage, negative PCR results will predominate.

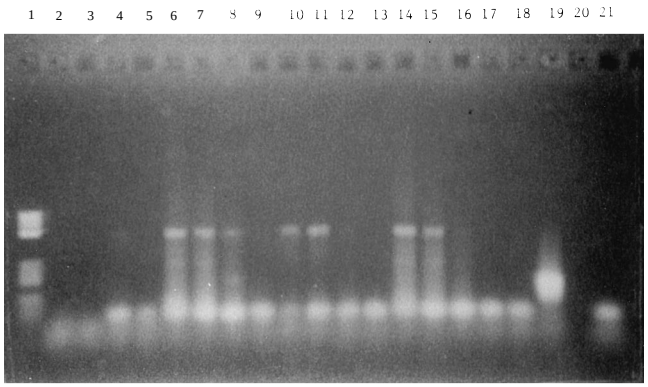

FIG. 3. Magnetic-antibody capture PCR. Lane 1, size markers (*Hae*III); lanes 2 through 5, dilution set (10^0, 10^{-1}, 10^{-2}, and 10^{-3}, respectively) of seeded environmental sample with the standard *Cryptosporidium* protocol; lanes 6 through 9, dilution set of seeded environmental sample with magnetic-antibody capture; lanes 10 through 13, dilution set of a purified seeded sample with the standard *Cryptosporidium* protocol; lanes 14 through 17, dilution set of a purified seeded sample with magnetic-antibody capture; lane 18, *Cryptosporidium* positive control (positive by dot blot [not shown]); lane 19, *Giardia* DNA, amplified with a *Giardia* HSP70 primer set; lane 20, empty lane; lane 21, negative control.

TABLE 6. PCR analysis of different environmental sample types

Sample description	No. of samples tested	No. of:		% Positive
		Positive results	Negative results	
Milwaukee outbreak	14	3	11	21.4
Cistern water	6	1	5	16.7
Wastewater	24	13	11	54.2
Coastal (fresh, salt, and brackish) waters	23	2	21	8.7

Empty oocysts may be found routinely in water concentrates (17). This presents a particular problem in regard to IFA analysis, as failure to determine internal morphology may cause false-positive results. This is not a concern in PCR analysis, as DNA is required for amplification.

Of major concern with both IFA and PCR is the inability to determine the viability of oocysts containing sporozoites. A positive PCR result may not indicate viability. Mahbubani et al. (22) found that PCR could not be used to assess the viability of *Giardia* cysts, and only through evaluation of mRNA with excystation procedures could live and dead cysts be distinguished. This has not been shown to be valuable for environmental concentrates. It may be possible to incorporate an excystation step into the existing protocols and use antibodies for capture or detection by targeting the emerging sporozoites. It may also be possible to address viability and improve the sensitivity of the PCR assay by incorporating a reverse transcription step prior to amplification of the genomic target; this may improve sensitivity by 100-fold (36).

Unlike for bacteria and viruses, there are currently no in vitro culture procedures which can be used to amplify viable and infectious *Cryptosporidium* oocysts prior to PCR. Although Upton et al. (32) recently reported a cell culture procedure, this would need to be tested for environmental samples and for concurrent use with PCR.

PCR and gene probes have been used to distinguish some *Giardia* species (21). Our *Cryptosporidium* primers are specific for at least four *Cryptosporidium* species, for which the sequence of the small rRNA is known (*C. baileyi*, *C. muris*, *C. parvum*, and *C. wrairi*). Only *C. parvum* is known to infect humans, so that differentiation of the species will be important. Although this was not the focus of this study, the PCR test can be expanded to distinguish the different species by single-strand conformation polymorphism and species-specific nested PCR primers (27a). Investigation of water supplies by this method will aid in understanding the specific risks to humans and the sources of contamination.

Application of the PCR (Table 6) test has shown that 54.2% of the wastewater samples, 21.4% of the Milwaukee samples, 17.0% of the cistern waters, and 8.7% of the coastal waters were positive for the presence of *Cryptosporidium* oocysts in 10- to 50-liter equivalent volumes. In the same set of samples, 58.3% of the wastewater samples were positive by IFA. The percentage of positive samples in the Milwaukee group was 42.8% by IFA. For the cistern and coastal waters, 66.7 and 34.7%, respectively, were positive by IFA (100-liter equivalent volumes). This may be explained by the differences in sensitivity between the two methods and the presence of empty oo-

cysts, as seen under microscopy. This was without magnetic-antibody capture. Use of this procedure will address some of the mismatch due to differences in sensitivity and sample interferences. Optimization of the magnetic-antibody capture for PCR is needed, including variables such as bead size and type and antibody titer and specificity. This should result in a marked increase in PCR sensitivity for application to environmental samples by allowing concentration and purification of the sample target.

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