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Evaluation of PCR, Nested PCR, and Fluorescent Antibodies for Detection of *Giardia* and *Cryptosporidium* Species in Wastewater

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Giardiasis and cryptosporidiosis are diseases caused by the protozoan parasites *Giardia lamblia* and *Cryptosporidium parvum*. Waterborne transmission of these organisms has become more prevalent in recent years, and regulatory agencies are urging that source and finished water be screened for these organisms. A major problem associated with testing for these organisms is the lack of reliable methodologies and baseline information on the prevalence of these parasites in various water sources. Our study addressed both of these issues. We evaluated the presence and reduction of *Giardia* cysts and *Cryptosporidium* oocysts in sewage effluent by a combination of indirect fluorescent antibody (IFA) staining and PCR. Our results indicated a 3-log reduction of *Giardia* cysts and a 2-log reduction of *Cryptosporidium* oocysts through the sewage treatment process as determined by IFA. We developed a nested PCR to detect *Cryptosporidium* oocysts and used a double PCR to detect *Giardia* cysts. A 100% correlation was noted between IFA and PCR detection of *Giardia* cysts while correlation for *Cryptosporidium* oocysts was slightly less. On the basis of these results, PCR may be a useful tool in the environmental analysis of water samples for *Giardia* and *Cryptosporidium* organisms.

In recent years, there has been a dramatic increase in the incidence of waterborne disease outbreaks caused by the protozoan parasites *Giardia lamblia* and *Cryptosporidium parvum*. Both of these organisms can cause a severe gastroenteritis if ingested. In 1993, a waterborne outbreak of cryptosporidiosis occurred in Milwaukee, Wis., which resulted in 403,000 people being infected and included at least 70 fatalities (11). *Cryptosporidium* infections are particularly problematic for immunocompromised individuals since drug therapy to control or eliminate this organism from a human host is not yet available. Giardiasis, fortunately, is treatable by several therapeutic agents, and to date, *Giardia* spp. have been the more often isolated of the two organisms in waterborne transmission cases (13).

One of the major problems related to *Giardia* and *Cryptosporidium* detection in water supplies is the lack of an efficient methodology. The current method recommended by the American Society for Testing and Materials (ASTM) for drinking water is laborious and time-consuming, requiring filtration of at least 1,000 liters of water, intensive filter manipulation to release *Giardia* cysts and *Cryptosporidium* oocysts, and fluorescent antibody staining and long hours counting the stained organisms microscopically (2). Recovery efficiency by the ASTM method is low, ranging from 5 to 20% (26), and a recent study by Clancy et al. (3) has shown that contract laboratories reportedly using the ASTM method were often unable to correctly recover and identify *Giardia* cysts and *Cryptosporidium* oocysts in seeded samples. These problems highlight the need to explore and evaluate different methodologies to detect these pathogens in order to protect against future waterborne outbreaks.

In this current study, we evaluate several new methodologies to determine the presence and reduction of *Giardia* cysts and *Cryptosporidium* oocysts during sewage treatment. Since cross-contamination between sewage and drinking water could become a source of these organisms in outbreaks, we felt that it was of interest to determine baseline levels as well as reduction of these pathogens through the treatment process. In order to address recovery problems, we modified the recommended ASTM method and also incorporated PCR as an alternative detection strategy. Primary influent, primary effluent, and secondary effluent wastewater from a large metropolitan treatment facility were analyzed. Since sewage may be expected to contain higher amounts of cysts and oocysts than drinking water, we reduced the sample size volume. We also substituted vortex flow filtration (VFF) as the sample concentration method for secondary effluent prior to ASTM-recommended processing and staining by fluorescent antibody. Concurrently, the sewage samples were analyzed for *Giardia* and *Cryptosporidium* organisms by PCR. Primers reported by Mahbubani et al. (12) were used for *Giardia* detection while a nested PCR based on primers reported by Ranucci et al. (20) was developed for *Cryptosporidium* detection.

MATERIALS AND METHODS

Sample collection and processing. Samples of sewage influent, primary treated effluent, or secondary treated effluent were collected and processed over a period of several months. Primary treatment consisted of the addition of ferric chloride and anionic anions while secondary treatment consisted of activated sludge. The 11 1-liter samples of primary influent and 11 1-liter samples of primary effluent were collected in 1-liter sterile Nalgene bottles pretreated with 4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4, for 1 h at room temperature to help prevent cysts and oocysts from attaching to the sides of the bottles. Ten 15-liter samples of secondary effluent were collected in a 20-liter plastic bucket pretreated with 4% BSA. One-liter samples were centrifuged in BSA-coated bottles at 2,450 × *g* for 10 min at 4°C. Pellets were collected into a BSA-coated centrifuge tube and centrifuged as above. The pellet was measured and divided into 250-μl aliquots. Each 250-μl aliquot was resuspended in 20 ml of eluting solution (PBS containing 0.1% Tween 80 and 0.1% sodium dodecyl sulfate [SDS]), overlaid onto 20 ml of Percoll-sucrose (Sigma Chemicals, St. Louis, Mo.) at a combined specific gravity of 1.10 (45 ml of Percoll, 10 ml of 2.5 M sucrose, and 40 to 45 ml of water, depending on the hydrometer reading), and centrifuged at 1,050 × *g* for 10 min at 4°C. The upper aqueous layer plus the top 5 ml of the Percoll-sucrose layer was collected and washed in an equal volume of

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eluting solution. Following centrifugation at 2,450 × *g* for 10 min at 4°C, the supernatant was aspirated to allow the bottom 5 ml plus pellet to remain.

The 15-liter secondary effluent samples were concentrated with the Benchmark VFF system with a P-S2000 400-cm^2 filter cartridge (Membrex, Fairfield, N.J.). A 4% BSA solution was run through the Benchmark tubing 1 h prior to attaching the filter cartridge to decrease the attachment of cysts and oocysts to the tubing. It was not necessary to pretreat the filter cartridge with BSA since the vortices dislodge any particles that threaten to clog or attach to the filter surface. When the sample was concentrated to less than 1 liter, two 1-liter aliquots of deionized H_2O were concentrated through the VFF system to rinse the unit and were added to the concentrated sample. The sample was then processed as described for the 1-liter samples above.

Indirect fluorescent antibody (IFA) staining. Nitrocellulose (Millipore, Bedford, Mass.) and cellulose acetate (Sartorius, Yauco, Puerto Rico) filters were moistened with PBS, pH 7.4, and placed on a manifold support (Hoefer Scientific, San Francisco, Calif.). Two milliliters of a 1% BSA solution (1% BSA in sterile deionized water) was added to each well, and a vacuum was applied (2 to 3 in. [ca. 5 to ca. 8 cm] of Hg). Concentrated samples were serially diluted and filtered in 1-ml aliquots. Influent and secondary effluent were evaluated at a 1:100 dilution, and primary effluent was evaluated at a 1:10 dilution. Sample processing continued as directed in the ASTM-proposed test method, including use of the Meridian Hydro-Fluor antibody staining kit (Cincinnati, Ohio).

Seeding studies. *Giardia* cysts were obtained from S. Ramalingam of Oregon Health Sciences University (Portland), and *Cryptosporidium* oocysts were purchased from Parasitology Research Laboratories (Phoenix, Ariz.). One-liter volumes of primary effluent were seeded with 10^3, 10^4, or 10^5 *Giardia* cysts and/or 10^2, 10^3, or 10^4 *Cryptosporidium* oocysts. A second liter of primary effluent was collected to determine background counts which were later subtracted from the seeded effluent total counts. Seeded samples and matched background samples were processed by two protocols to compare recovery efficiencies between the methods. The ASTM-proposed gradient density (as described above) was compared with a denser gradient as described by Vesey et al. (25) and LeChevallier et al. (10). Their studies suggested changing the specific gravity of the Percoll-sucrose to 1.13. In addition, LeChevallier et al. (10) suggested increasing the centrifuge speed to 6,700 × *g* for 10 min at 4°C for all centrifugations except the one performed with the gradient (1,050 × *g*).

DNA extraction. Four milliliters of concentrated sample (1 ml each in 1.5-ml microcentrifuge tubes) was centrifuged at 1,400 × *g* for 5 min at 4°C, and the supernatant was discarded. To each pellet, 500 μl of proteinase K (20 mg/ml) (Boehringer-Mannheim, Indianapolis, Ind.) in 0.15 M NaCl–0.1 M EDTA was added, and the tubes were inverted two to three times to mix. The samples were incubated for 2 h at 65°C. Following incubation, 500 μl of lysis buffer (10% SDS in 0.1 M NaCl and 0.5 M Tris-HCl) was added to each tube. The tubes were briefly vortexed. The cells were lysed by a freeze-thaw method consisting of a dry ice and ethanol slurry for 2 min followed by 65°C for 5 min. This cycle was repeated five additional times. After the freeze-thaw cycles, 500 μl of Tris-saturated phenol (pH 8) (Amresco, Solon, Ohio) was added to each tube. The tubes were mixed by inversion and centrifuged at 5,700 × *g* for 5 min. In subsequent treatments using phenol-chloroform (25:24), chloroform, and 100% isopropyl alcohol, each upper aqueous layer was immediately transferred to a clean tube with an equal volume of the next reagent and centrifuged at 5,700 × *g* for 5 min. Following the addition of the isopropyl alcohol, the tubes were incubated for 1 h at −20°C. Samples were combined and centrifuged at 17,500 × *g* for 15 min, and then pellets were rinsed in 500 μl of 75% ethanol, centrifuged at 17,500 × *g*, and vacuum dried. The pellets were resuspended in 200 μl of high-pressure liquid chromatography water and purified by Centricon-100 Concentrators (Amicon, Beverly, Mass.), according to the manufacturer's instructions. As a further purification step, samples were run through Sephadex 200 (Pharmacia, Piscataway, N.J.) columns.

PCR parameters. PCR parameters for the 171-bp *Giardia* giardin product were those described by Mahbubani et al. (12). Slight modifications to cycle time, cycle number, and reaction mixture volume were made. The three-temperature amplification (94, 60, and 72°C) was reduced to 30 s, the GeneAmp PCR mixture (Perkin-Elmer, Foster City, Calif.) volume (including primers and template) was reduced to 50 μl, and the number of cycles was increased to 40. In addition, a double PCR was performed to enhance detection. One microliter of PCR product from the first reaction mixture was used as template for the second PCR.

A nested PCR was performed to detect *Cryptosporidium* oocysts. PCR primers (Cry-5 and Cry-6), as described by Ranucci et al. (20) for an oocyst cell wall protein, resulted in a 753-bp product. Modifications to the parameters included decreasing the annealing temperature to 54°C, decreasing the denaturing and extension cycling times to 30 s, and increasing the number of cycles to 40. Nested PCR primers (NCRYP.1, GGCTCCAAGGCCAATTTGTG, and NCRYP.2, GCATGCCCTGCAGGATATGC), which amplified a 283-bp product within the first amplicon, were developed by using Oligo 4.1 (National Biosciences, Plymouth, Minn.). The primers were added to GeneAmp PCR mixture (Perkin-Elmer) at a final concentration of 0.3 μM each. AmpliTaq polymerase (Perkin-Elmer) was used as per the manufacturer's directions. The $MgCl_2$ final concentration was 2 mM. The template, which was 1 μl of the previous PCR product, was added to a tube with reaction mixture, bringing the final volume to 50 μl per tube. The amplification parameters were as follows: 2 min at 94°C; 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 40 cycles; 3.5 min at 72°C; hold at 4°C. Template volume was 1 μl for all reaction mixtures. All PCR amplifications were completed on the Perkin-Elmer 9600 GeneAmp PCR System. Positive (*Giardia* or *Cryptosporidium* DNA) and negative (reaction mixture and primers without sample) controls were run with every PCR batch.

Detection of amplified products. PCR products were electrophoresed on a 2% agarose gel with ethidium bromide (0.6 μg/ml) and visualized with the UVP Transilluminator and Imagestore 5000 System (Ultra Violet Products, San Gabriel, Calif.). The PCR products were denatured, neutralized, and transferred to a Hybond N^+ membrane (Amersham, Arlington Heights, Ill.) via Southern blotting with the Posiblot System (Stratagene, La Jolla, Calif.) overnight at 80 lb/in^2. Prehybridization and hybridization were performed at 60°C for 2 h each. The *Giardia* internal probe has been previously described by Mahbubani et al. (12). The internal sequence probe for *Cryptosporidium* organisms (CRYPip, CTGATTCTGTCCCAGCTGTGCCTA) was designed in our laboratory with Oligo 4.1 software, synthesized on an ABI 392 DNA/RNA Synthesizer (Applied Biosystems, Foster City, Calif.), and end labeled with the Genius 5 Kit (Boehringer-Mannheim). The *Cryptosporidium* internal probe was designed to hybridize with both of the amplicons. All fragments were detected with the Genius 3 Kit (Boehringer-Mannheim), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Giardia and *Cryptosporidium* organisms are highly infective, and ingestion of as few as 10 cysts (1) or 30 oocysts (5) can result in human infection. Both cysts and oocysts are known to be strongly resistant to disinfection, including chlorination (6, 19), and are difficult to remove by standard filtration (25). The failure of filtration to remove these parasites may be attributed to recently presented data which showed that *Cryptosporidium* oocysts have the ability to change shape and squeeze through pores smaller than the size (ca. 5 μm) of the oocysts (15). This may apply to *Giardia* cysts as well. Thus, they can easily become a problem for water utilities in both source and finished waters. This is meaningful from a public health standpoint since treated sewage is often discharged into surface waters or ocean waters or may be used in reclamation projects. Many environmental reservoirs of *Giardia* and *Cryptosporidium* organisms have been defined. LeChevallier et al. (8) reported that 97% of the raw waters examined in the northeastern United States contained *Giardia* or *Cryptosporidium* organisms while Ongerth et al. (13, 14) found both parasites in relatively pristine surface waters of the northwestern United States. Studies of drinking water collected from 14 different states showed that *Giardia* cysts and *Cryptosporidium* oocysts could be detected in 17 and 27%, respectively, of the samples tested (9).

Recently promulgated regulations, including the Surface Water Treatment Rule and the Information Collection Rule, are focused on the removal of these parasites during water treatment in order to protect the public against future outbreaks. A major problem with instituting these regulations is lack of an efficient methodology with which to test water for the presence of these organisms and lack of studies which show baseline levels in different water sources. Our study has evaluated removal of cysts and oocysts during sewage treatment, which is an important medium because of the growing trend in water reuse. We have also evaluated new methodologies and modifications of the Environmental Protection Agency-recommended ASTM detection method in order to enhance *Giardia* and *Cryptosporidium* detection.

Cyst and oocyst reduction through the wastewater treatment process as measured by IFA staining. After primary sewage treatment, only a 1-log reduction was observed for both cysts and oocysts. Secondary sewage treatment resulted in a further 2-log reduction of *Giardia* cysts and 1-log reduction of *Cryptosporidium* oocysts (Table 1). Counts from the different samples showed a large variation; however, removal rates averaged 99.92% for *Giardia* cysts and 98.87% for *Cryptosporidium* oocysts through the sewage treatment process. Although we did not perform excystation studies to determine viability, in num-

TABLE 1. Average reduction of *Giardia* cysts and *Cryptosporidium* oocysts through the sewage treatment process as determined by fluorescent antibody staining

Organism	No./liter		
	Primary influent (n = 11)	Primary effluent (n = 11)	Secondary effluent (n = 10)
Giardia cysts	1.3×10^4	2.6×10^3	1.1×10^1
Cryptosporidium oocysts	1.5×10^3	1.1×10^2	1.7×10^1

bers alone, neither cysts nor oocysts may have been removed to a level below that which would be acceptable for facilities discharging into surface or groundwaters. Regulations contained in the Surface Water Treatment Rule state that *Giardia* cysts in raw waters need to be reduced or inactivated by 99.9% in finished water. LeChevallier et al. (8), however, have calculated that, if *Giardia* levels in raw water are greater than 0.7 cysts per 100 liters, a 99.9% reduction will not produce safe finished water. Therefore, in our study, parasite levels detected after secondary treatment (11 cysts per liter and 17 oocysts per liter) indicate that secondary treated water discharged without additional treatment may be an inappropriate source for further utilization, especially since recovery rates normally do not exceed 50% and underestimate the populations of *Giardia* and *Cryptosporidium* organisms.

Seeding and recovery. Poor recovery efficiency is a hallmark of *Giardia* and *Cryptosporidium* detection. In our study, recovery rates utilizing ASTM-recommended Percoll-sucrose specific gravity of 1.10 (method 1) ranged from 0 to 38% (average, 19%) for *Giardia* cysts and 10 to 28% (average, 19%) for *Cryptosporidium* oocysts (Table 2). Increasing the specific gravity to 1.13 and increasing the centrifugation speed to 6,700 × *g*, as proposed by Vesey et al. (25) and LeChevallier et al. (10) (method 2), resulted in a doubling of the *Giardia* recovery rates and a notable improvement of 93% recovery of *Cryptosporidium* oocysts. However, the less-than-optimal recovery rates plus the wide ranges in recovery efficiency highlight the lack of consistency in performing routine detection of these organisms. Thus, a none-detected determination means little at the current recovery levels.

PCR and hybridization detection results for *Giardia* and *Cryptosporidium* organisms. Although technically more challenging, another method of screening for *Giardia* cysts and *Cryptosporidium* oocysts is the use of molecular methodologies. We have previously shown that PCR is a useful tool for detecting pathogens in environmental samples (7, 16–18, 23, 24) and offers a more sensitive and specific detection method for analyzing water samples. A commonly expressed drawback of PCR is that it does not determine viability; however, neither does the currently accepted IFA method. In fact, the antibodies recommended for the IFA method have been shown to antigenically cross-react with various species of algae in environmental samples (22). *Giardia* cysts and *Cryptosporidium* oocysts were detected by PCR in over half of the sewage samples representing all phases of treatment. In PCR detection of *Giardia* cysts, the double PCR afforded greater sensitivity since signals were detected in more lanes than when only single PCR was used (data not shown). Inhibitory factors, such as humic and fulvic acids present in sewage, may explain why a double PCR was needed to detect the *Giardia* amplicon in this study. Mahbubani et al. (12), who reported on the development of these primers, performed PCR detection of *Giardia* organisms on river water, which would not be expected to have the high amounts of potential PCR inhibitors found in sewage. Thus, in our study of sewage, a single PCR did not always detect the desired target but double PCR increased the chance of obtaining a positive result and resulted in a stronger signal. We also noted that, in many cases, signals were observed at higher dilutions but not at lower dilutions, indicating that inhibitors needed to be diluted out before a signal could be obtained. This suggests that better sample preparation methods need to be developed in order for PCR to be efficient at lower dilutions in some environmental sample types.

Detection of *Cryptosporidium* organisms by PCR required a nested step since the primers published by Ranucci et al. (20) for the 753-bp amplicon resulted in nonspecific amplification, as evidenced by the numerous nontarget bands in Fig. 1a, and did not allow visualization of the target 753-bp band. Typically, target bands for the 753-bp amplicon were never observed after the first PCR amplification steps, but the target band (283 bp) with the second primers was observed after performing the nested PCR. Figure 1a shows results from seeding dilutions of *Cryptosporidium* oocysts into primary effluent. Clearly, the first PCR (top section of the gel) did not amplify the seeded *Cryptosporidium* oocysts to a detectable level but the nested reaction detected as few as 10^2 oocysts per liter (bottom section of the gel). These results were confirmed on the corresponding Southern blot (Fig. 1b). These primers may have the ability to

TABLE 2. IFA recovery efficiency for *Giardia* cysts and *Cryptosporidium* oocysts seeded into 1 liter of primary effluent: comparison of ASTM method proposing a specific gravity of 1.10 and method with Vesey et al. (25)-LeChevallier et al. (10)-proposed specific gravity of 1.13 and increased centrifugation of 6,700 × *g*

Method[a]	Type of result	Result for organism					
		Giardia cysts			*Cryptosporidium* oocysts		
		Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
1	Background no.	6.0×10^3	2.0×10^3	6.8×10^3	3.5×10^2	5.0×10^1	1.5×10^2
	No. seeded	1.2×10^4	1.1×10^4	1.1×10^4	1.0×10^4	4.5×10^3	4.5×10^3
	No. recovered	8.2×10^3	1.8×10^3	1.1×10^4	1.3×10^3	8.5×10^2	1.4×10^3
	% Recovery	18	0	38	10	18	28
	Avg % recovery for 3 samples		19			19	
2	Background no.	4.7×10^3	6.5×10^3		1.5×10^2	1.5×10^3	
	No. seeded	2.6×10^5	1.2×10^5		3.5×10^4	5.0×10^3	
	No. recovered	5.3×10^4	8.4×10^4		3.5×10^4	6.0×10^3	
	% Recovery	19	65		96	90	
	Avg % recovery for 2 samples	42			93		

[a] Method 1, ASTM-recommended specific gravity; method 2, modified specific gravity and centrifugation speed.

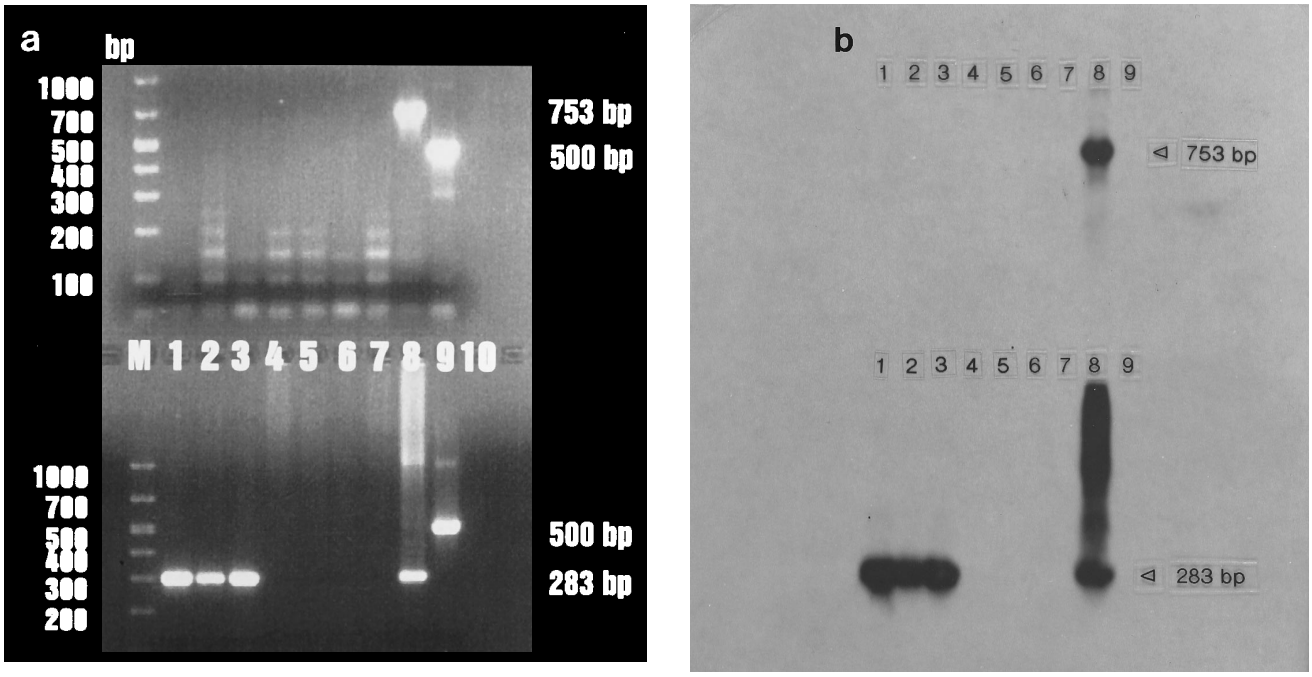

FIG. 1. PCR products of *Cryptosporidium* oocysts seeded into primary effluent. (a) Results on an agarose gel. Upper section, first PCR; bottom section, nested PCR. Lanes 1 to 7, samples seeded with 3.7×10^4, 3.7×10^3, 3.7×10^2, 3.7×10^1, 3.7×10^0, 0, and 0 oocysts, respectively. Lanes 8, *Cryptosporidium* DNA-positive control; lane 9, 500-bp lambda DNA control; lane 10, negative control. Lane 8 shows the 753-bp amplicon on the top section of the gel and the 283-bp amplicon on the bottom section. (b) Corresponding Southern blot of the agarose gel.

be used alone without the first PCR. Further studies need to be completed to evaluate this primer set with other *Cryptosporidium* spp. and closely related species.

Correlation of PCR and IFA. There was a 100% agreement between a positive PCR amplification of the *Giardia* 171-bp target and visual detection by IFA in all three phases of the sewage treatment process; however, discrepancies were found when comparing PCR and IFA for *Cryptosporidium* detection (Table 3). Of the 11 primary influent samples that were tested for *Cryptosporidium* organisms, four were both PCR and IFA positive, three samples were both PCR and IFA negative, three samples were PCR negative but IFA positive, and one sample was positive by PCR but negative by IFA (Table 3). In primary effluent samples, four samples were positive by both PCR and IFA, four samples were negative by both PCR and IFA, and three samples were PCR negative but IFA positive. Ten secondary effluent samples were compared. Six samples were both PCR and IFA positive, one sample was PCR and IFA negative, one sample was PCR negative but IFA positive, and two samples were PCR positive but IFA negative.

The 63% (7 of 11), 72% (8 of 11), and 70% (7 of 10) agreement between PCR and IFA results for *Cryptosporidium* detection in primary influent, primary effluent, and secondary effluent, respectively, was encouraging but not as good as the 100% agreement between PCR and IFA for *Giardia* detection. The discrepancies caused by IFA-positive–PCR-negative re-

TABLE 3. Comparison of IFA staining and PCR for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in sewage effluent

Sample	Result											
	Primary influent				Primary effluent				Secondary effluent			
	Giardia		*Cryptosporidium*		*Giardia*		*Cryptosporidium*		*Giardia*		*Cryptosporidium*	
	PCR	IFA	PCR	IFA	PCR	IFA	PCR	IFA	PCR	IFA	PCR	IFA
1	+	+	−	+	+	+	+	+	+	+	−	−
2	+	+	−	+	+	+	+	+	+	+	+	−
3	+	+	−	+	+	+	−	−	+	+	+	+
4	+	+	+	+	+	+	−	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	−	−	+	+	+	+
7	+	+	+	+	+	+	−	+	+	+	−	+
8	+	+	−	−	+	+	+	+	+	+	+	+
9	+	+	+	−	+	+	−	−	+	+	+	+
10	+	+	−	−	+	+	−	−	+	+	+	−
11	+	+	−	−	+	+	−	+	NT[a]	NT	NT	NT

[a] NT, not tested.

sults could be due to the tendency of the IFA method to cross-react with nontarget organisms such as algae or to inhibitory effects on PCR enzymes caused by interfering substances, such as humic acids, present in the sewage. It is important to note that, because of the specificity of targeting actual genetic sequences unique to a given organism, the PCR method is less likely to show cross-reactivity. This allows for a distinct advantage over the IFA method. The PCR-positive and IFA-negative results could have been caused by the greater sensitivity of PCR over IFA or the difficulties in reading results under the microscope by IFA staining. The tagged organisms could have been hidden under debris. It is difficult to pinpoint the exact causes in these cases.

In summary, sewage is a difficult medium to concentrate and analyze because of the myriad of organisms and other particulates normally present in the sample. Using the VFF concentration method, we were able to successfully concentrate sewage samples for further staining with fluorescent antibodies and analysis by PCR. The VFF may be equally useful for drinking water samples for which larger amounts of water need to be processed. A study by Whitmore and Carrington (26) also evaluated the VFF method for *Cryptosporidium* detection and reported that recovery rates from seeded river water averaged around 30%. Another modification of value was the increased specific gravity and centrifugation speeds suggested by Vesey et al. (25) and LeChevallier et al. (10) which may prove to enhance recovery when the standard recommended ASTM method is used. Lastly, we found a 100% correlation between the use of IFA and PCR for detection of *Giardia* organisms, and to a lesser extent in detection of *Cryptosporidium* organisms, in the sewage samples. Because PCR is less cumbersome and time-consuming than the IFA method, the PCR could prove useful as a screening tool to test water samples for these organisms.

Although the sewage treatment process reduced the levels of *Giardia* cysts and *Cryptosporidium* oocysts, they were not completely eliminated after secondary treatment. Since these organisms are known to survive for months in the environment (4, 21), there is a need to explore better treatment methods, such as ozonization, which may result in more efficient protozoan removal rates so as to ensure public health protection and prevent further waterborne outbreaks caused by these two medically important organisms.

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REFERENCES

1. **Adam, R. D.** 1991. The biology of *Giardia* spp. Microbiol. Rev. **55:**706–732.
2. **American Society for Testing and Materials.** 1993. P229 proposed test method for *Giardia* cysts and *Cryptosporidium* oocysts in low turbidity water by a fluorescent antibody procedure, p. 899–909. *In* Annual book of ASTM standards, section 11, water and environmental technology, vol. 11.02. Water (II). American Society for Testing and Materials, Philadelphia.
3. **Clancy, J. L., W. D. Gollnitz, and Z. Tabib.** 1994. Commercial labs: how accurate are they? J. Am. Water Works Assoc. **86:**89–97.
4. **DeRegnier, D. P., L. Cole, D. G. Schupp, and S. L. Erlandsen.** 1989. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl. Environ. Microbiol. **55:**1223–1229.
5. **DuPont, H. L., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose, and W. I. Jakubowski.** 1995. The infectivity of *Cryptosporidium* in healthy volunteers. N. Engl. J. Med. **332:**855–859.
6. **Korich, D. G., J. R. Mead, M. S. Mador, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:**1423–1428.
7. **Lang, A. L., Y. L. Tsai, C. L. Mayer, and C. J. Palmer.** 1994. Multiplex PCR for detection of the heat-labile toxin gene and Shiga-like toxin I and II genes in *Escherichia coli* isolated from natural waters. Appl. Environ. Microbiol. **60:**3145–3149.
8. **LeChevallier, M. W., W. D. Norton, and R. G. Lee.** 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl. Environ. Microbiol. **57:**2610–2616. (Erratum, **58:**780, 1992.)
9. **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.
10. **LeChevallier, M. W., W. D. Norton, J. E. Siegel, and M. Abbaszadegan.** 1995. Evaluation of the immunofluorescence procedure for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl. Environ. Microbiol. **61:** 690–697.
11. **MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, and J. P. Davis.** 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:** 161–167.
12. **Mahbubani, M. H., A. K. Bej, M. H. Perlin, F. W. Schaefer III, W. Jakubowski, and R. M. Atlas.** 1992. Differentiation of *Giardia duodenalis* from other *Giardia* spp. by using polymerase chain reaction and gene probes. J. Clin. Microbiol. **30:**74–78.
13. **Ongerth, J. E., G. D. Hunter, and F. B. DeWalle.** 1995. Watershed use and *Giardia* cyst presence. Water Res. **29:**1295–1299.
14. **Ongerth, J. E., and H. H. Stibbs.** 1987. Identification of *Cryptosporidium* oocysts in river water. Appl. Environ. Microbiol. **53:**672–676.
15. **Owens, J. H., S. Y. Li, and F. W. Schaefer III.** 1995. Pressurized polycarbonate membranes demonstrate compressibility of *Cryptosporidium parvum* oocysts, abstr. Q-295, p. 452. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
16. **Palmer, C. J., G. F. Bonilla, B. Roll, C. Paszko-Kolva, L. R. Sangermano, and R. S. Fujioka.** 1995. Detection of *Legionella* species in reclaimed water and air with the EnviroAmp Legionella PCR kit and direct fluorescent antibody staining. Appl. Environ. Microbiol. **61:**407–412.
17. **Palmer, C. J., M. L. Lee, G. F. Bonilla, B. J. Javier, E. B. Siwak, and Y. L. Tsai.** 1995. Analysis of sewage effluent for human immunodeficiency virus (HIV) using infectivity assay and RT-PCR. Can. J. Microbiol. **41:**809–815.
18. **Palmer, C. J., Y. L. Tsai, C. Paszko-Kolva, C. Mayer, and L. R. Sangermano.** 1993. Detection of *Legionella* species in sewage and ocean water using polymerase chain reaction, direct fluorescent antibody, and plate count methods. Appl. Environ. Microbiol. **59:**3618–3624.
19. **Peeters, J. E., E. A. Mazas, W. J. Masschelein, I. V. de Maturana, and E. Debacker.** 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. **55:**1519–1522.
20. **Ranucci, L., H.-M. Muller, G. LaRosa, I. Rechmann, M. A. G. Morales, F. Spano, E. Pozio, and A. Crisanti.** 1993. Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. Infect. Immun. **61:**2347–2356.
21. **Robertson, L. J., A. T. Campbell, and H. V. Smith.** 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl. Environ. Microbiol. **58:**3494–3500.
22. **Rodgers, M. R., D. J. Flanigan, and W. Jakubowski.** 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Appl. Environ. Microbiol. **61:**3759–3763.
23. **Tsai, Y. L., M. D. Sobsey, L. R. Sangermano, and C. J. Palmer.** 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. Appl. Environ. Microbiol. **59:**3488–3491.
24. **Tsai, Y. L., B. Tran, L. R. Sangermano, and C. J. Palmer.** 1994. Detection of poliovirus, hepatitis A virus, and rotavirus from sewage and ocean water by triplex reverse transcriptase PCR. Appl. Environ. Microbiol. **60:**2400–2407.
25. **Vesey, G., P. Hutton, A. Champios, N. Ashbolt, K. L. Williams, A. Warton, and D. Veal.** 1994. Application of flow cytometric methods for the routine detection of *Cryptosporidium* and *Giardia* in water. Cytometry **16:**1–6.
26. **Whitmore, T. N., and E. G. Carrington.** 1993. Comparison of methods for recovery of *Cryptosporidium* from water. Water Sci. Technol. **27:**69–76.