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Comparison of Primers and Optimization of PCR Conditions for Detection of *Cryptosporidium parvum* and *Giardia lamblia* in Water

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Eight pairs of published PCR primers were evaluated for the specific detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. Detection sensitivities ranged from 1 to 10 oocysts or cysts for purified preparations and 5 to 50 oocysts or cysts for seeded environmental water samples. Maximum sensitivity was achieved with two successive rounds of amplification and hybridization, with oligonucleotide probes detected by chemiluminescence. Primer annealing temperatures and $MgCl_2$ concentrations were optimized, and the specificities of the primer pairs were determined with closely related species. Some of the primers were species specific, while others were only genus specific. Multiplex PCR for the simultaneous detection of *Cryptosporidium* and *Giardia* was demonstrated with primers amplifying 256- and 163-bp products from the 18S rRNA gene of *Cryptosporidium* and the heat shock protein gene of *Giardia*, respectively. The results demonstrate the potential utility of PCR for the detection of pathogenic protozoa in water but emphasize the necessity of continued development.

Waterborne outbreaks of cryptosporidiosis and giardiasis have been well documented (18, 21), and occurrence of the respective causative agents, *Cryptosporidium parvum* and *Giardia lamblia*, in water supplies is a critical issue for the water industry. Conventional methods for the detection of *C. parvum* and *G. lamblia* in water supplies are labor-intensive and time-consuming. The current techniques for isolating *Cryptosporidium* and *Giardia* from water involve filtration and centrifugation to concentrate and purify oocysts and cysts, followed by immunofluorescence microscopy (2). Objects with the correct shape, dimensions, and fluorescence are confirmed by observation of internal structures by differential interference contrast microscopy. The limitations of this procedure include loss of cysts or oocysts during the various stages of selective isolation, resulting in reported recovery efficiencies ranging from 80% to less than 1% for *Cryptosporidium* (22). An evaluation of commercial laboratories demonstrated average recovery efficiencies of 9% for *Giardia* cysts and 3% for *Cryptosporidium* oocysts (6). Other limitations include nonspecific antibody binding, interference by sample debris, and inability to determine viability (25). Considerable effort has been expended in evaluating different methods of cyst and oocyst isolation, yielding a wide variety of recovery efficiencies, mostly below 40% for *Cryptosporidium* (35). Various alternative technologies have also been investigated in efforts to provide more effective detection of waterborne *Cryptosporidium* and *Giardia*. These methods include flow cytometry (29), use of charge-coupled devices (5), UV-visible spectroscopy (23), enzyme-linked immunosorbent assay (7), and the PCR.

Rapid and sensitive pathogen detection methods are essential for the water quality industry. PCR has the potential to address many of the limitations of current methods. The advantages of PCR include greater sensitivity, rapid analysis of many samples, relatively low cost, simultaneous detection of multiple pathogens, and the ability to discriminate between species and strains if suitable primers are selected. PCR methods for the detection of both *Cryptosporidium* and *Giardia* in clinical and environmental water samples have been described. *Cryptosporidium*-specific primers used in these reports amplified 256- and 556-bp fragments (3) and a 435-bp product (11, 12) from the 18S rRNA gene; an 873-bp product from a repetitive oocyst protein gene (30); and 451- and 329-bp amplicons from different undefined genomic regions (8, 15, 32). Primers specific for *Giardia* targeted a 183-bp product from the small-subunit (SSU) rRNA gene (33); 218- and 171-bp amplicons from a giardin gene (19, 20); and a 163-bp product from a heat shock protein (HSP) gene (1).

Reported sensitivities for the various primers ranged from "<1" cyst for all of the *Giardia*-specific primers to 1 to 100 oocysts for *Cryptosporidium*-specific primers. However, not all the original reports of these primers determined the sensitivity. Many of the primer pairs amplified DNA from nontarget organisms such as *Cryptosporidium muris* or *Giardia muris*, but these products could be distinguished from those of target organisms (*C. parvum* and *G. lamblia*) by restriction digestion (3, 11), hybridization with oligonucleotide probes (34), or size differences (1). Other studies did not suggest methods for discriminating between the human pathogenic species and non-human pathogens.

A comparison of PCR with primers specific for the 18S rRNA gene with an immunofluorescence assay (IFA) for the detection of *Cryptosporidium* concluded that PCR could be useful in routine monitoring in the water industry, particularly with finished-water samples (11). Moreover, the same authors recently reported that PCR was as sensitive as IFA for the detection of *Cryptosporidium* in wastewater concentrates (12). However, for PCR-based detection of *Cryptosporidium* and *Giardia* to become routine, methods must be standardized and a consensus on which primers are the most appropriate to use must be reached. Therefore, we conducted a thorough assess-

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ment of PCR, evaluating a total of eight pairs of primers previously used for the detection of *Cryptosporidium* and *Giardia*. The variables investigated included primer annealing temperature (T_a), $MgCl_2$ concentration, specificity, sensitivity, and PCR additives. We also studied multiplex PCR for the simultaneous detection of *Cryptosporidium* and *Giardia* in environmental water samples. Because of its extreme sensitivity, PCR is prone to contamination by nonspecific DNA, *Cryptosporidium* DNA from previous extractions, and carryover contamination by previously amplified DNA. Precautions against contamination are thus a necessity. The substitution of dUTP for dTTP and the inclusion of uracil DNA glycosylase (UDG) in an amplification reaction mixture enzymatically eliminate this carryover contamination before amplification occurs. None of the previous descriptions of primers utilized this method of preventing carryover contamination. All the reactions described here used dUTP and UDG. This article also reviews previous publications on PCR detection of *Cryptosporidium* and *Giardia* and presents a detailed analysis of primer and target sequences.

MATERIALS AND METHODS

Protozoal stocks. Purified preparations of *C. parvum* oocysts and *G. lamblia* cysts were obtained from two commercial laboratories (Parasitology Research Laboratories, Phoenix, Ariz., and Waterborne, Inc., New Orleans, La.). *G. muris* cysts and *C. muris* oocysts were obtained from Parasitology Research Laboratories and the U.S. Environmental Protection Agency (USEPA; Cincinnati, Ohio), respectively, and *Cryptosporidium baileyi* oocysts were supplied by B. Blagburn (Auburn University, Auburn, Ala.). Cysts and oocysts were supplied either as purified preparations stored in antibiotic solution or as unpurified concentrates and were stored at 4°C. Cyst and oocyst densities were determined by hemocytometer counting, and lower densities were obtained by serial dilution of concentrated stocks. DNA was extracted from purified cysts and oocysts by freezing in liquid nitrogen for 2 min, followed by thawing at 95°C for 5 min. DNA was extracted from unpurified samples by resuspending cysts or oocysts in 500 μl of 0.5% sodium dodecyl sulfate containing 100 μg of proteinase K per ml and incubating for 30 min at 37°C. After addition of 100 μl of 5 M NaCl and 80 μl of hexadecyl-trimethylammonium bromide (CTAB; Sigma Chemical Co., St. Louis, Mo.), the samples were incubated for 15 min at 65°C. Organic extractions were performed with 680 μl of phenol-chloroform-isoamyl alcohol (25/24/1), and DNA was precipitated from the aqueous phase with 0.6 volumes of isopropanol and washed with 70% ethanol. After the DNA had been dried in a vacuum desiccator, it was resuspended in 100 μl of sterile distilled water.

Primer analysis and DNA amplification. In this investigation, primers were assigned designations comprising the first three letters of the primary author's last name and the numerical position of the 3′ nucleotide in the corresponding gene sequence. All PCR primers and probes were synthesized by a commercial laboratory (National Biosciences, Inc. [NBI], Plymouth, Minn.). Theoretical analysis of primers and design of additional probes were accomplished with an analysis software package (OLIGO version 5.0; NBI). Calculated optimum annealing temperatures ($T_{a_{Copt}}$ and T_p) were determined with OLIGO and the formula $T_p = 22 + 1.46[2(G+C) + (A+T)]$°C (36), respectively (see Table 1). The melting temperatures (T_m) of the individual primers were calculated with the formula $T_m = 2(A+T) + 4(G+C)$°C. DNA sequences of SSU rRNA genes from a variety of organisms were obtained by e-mail server from the Ribosomal Database Project (14), and other sequences were obtained from GenBank (4).

The designations and sequences of PCR primers and probes used for *Cryptosporidium* were as follows: AWA722F (AGTGCTTAAAGCAGGCAACTG), AWA1235R (CGTTAACGGAATTAACCAGAC), and AWA995F (TAGAGA TTGGAGGTTGTTCCT), AWA1206R (CTCCACCAACTAAGAACGGCC), targeting the 18S rRNA gene (3), and the probe CPRNA1097 (ACGGAAGG GCACCACCAGGA) (designed as part of the present study); LAX469F (CCG AGTTTGATCCAAAAAGTTACGAA), LAX869R (TAGCTCCTCATATGC CTTATTGAGTA), targeting a chromosomal fragment, and probes LAX520 (CTCAAAGCGAAGATGACCTT) and LAX681 (GAATTAACCTATAGGA ACCT) (15); WEB110F (ATCTTCACGCAGTGCGTGGT), WEB401R (CAT CAGCCGGTAGATGTCGA), and the internal oligonucleotide probe WEB123 (ACGTAGCGCCGGACGACAGCAGCAGCGCGT), also targeting an undefined chromosomal region (32). The primer and probe designations and sequences for *Giardia* were as follows: ABB97F (AGGGCTCCGGCATAACTTTCC), ABB220R (GTATCTGTGACCCGTCCGAG), targeting an HSP (1), and the probe GLHSP184 (CAGGCCTTGGCGTTCCCGAAG) (this study); MAH433F (CATAACGACGCCATCGCGGCTCTCAGGAA), MAH592R (TTTGTGAG CGCTTCTGTCGTGGCAGCGCTAA), and the probe MAH510 (AGCTCAA CGAGAAGGTCGCAGAGGGCTT), amplifying DNA from the giardin gene (20); MAH658F (AAGTGCGTCAACGAGCAGCTC), MAH789R (TTAGTG CTTTGTGACCATCGA), and the probe MAH751 (TCGAGGACGTCGTCT CGAAGATCCAG) (20); WEI1270F (GCGCACCAGGAATGTCTTGT), WEI 1414R (TCACCTACGGAATACCTTGTT), and the probe RDR34 (AGGGA CGCGTCCGGCG), targeting the SSU rRNA gene (33). (Amplicon sizes are given in Table 2.)

The basic amplification reaction mixture contained 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.01% gelatin; 0.2 to 0.5 μM each primer; 200 μM each dATP, dCTP, dGTP, and dUTP; and 2 U of DNA polymerase (Amplitaq; Perkin-Elmer Corp., Foster City, Calif.) in a 100-μl volume with 2 to 10 μl of template DNA. Prior to amplification, the reaction mixtures were treated with UDG according to the recommendations of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to prevent carryover contamination. The reaction mixtures were overlaid with either 75 μl of sterile mineral oil or wax beads (Ampliwax PCR Gems; Perkin-Elmer). The T_a varied depending on the primers used. The reactions were performed in a DNA thermal cycler (model 480; Perkin-Elmer). Reaction mixtures were generally denatured at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C, annealing for 1 min, and extension at 72°C for 2 min. A final extension incubation of 5 min at 72°C was included, followed by a 5-min incubation at 5°C to stop the reactions. The T_a depended on the T_m of the primers. Negative control reaction mixtures contained sterile distilled water in place of template DNA.

Environmental water concentrates. Three environmental water samples (EWS1, -2, and -3) were used for this study, all from treatment plant influents. The values for volume, turbidity, and pH of these water samples were as follows: EWS1, 569 liters, 0.55 nephelometric turbidity units (NTU), pH 8.32; EWS2, 641 liters, 0.65 NTU, pH 8.32; EWS3, 568 liters, 1.2 NTU, pH 8.38. Samples were collected at a flow rate of 3.1 to 3.4 liters/min, concentrated, and analyzed for *Cryptosporidium* and *Giardia* with the proposed USEPA Information Collection Rule method based on the American Society for Testing and Materials method (2). Packed pellet volumes of the concentrated samples were as follows: EWS1, 1.3 ml; EWS2, 1.3 ml; EWS3, 1.1 ml. DNA was extracted from these unpurified packed pellets. For seeded extractions, packed pellets were inoculated with purified cysts or oocysts prior to DNA extraction.

DNA extraction from environmental water samples and amplification. Total DNA was extracted from seeded and unseeded concentrates of source water samples (100 to 500 μl of packed pellet) by lysis in 50 mM Tris-HCl–20 mM EDTA, pH 8, containing 2 mg of proteinase K per ml and 0.5% Sarkosyl, incubated at 37°C for 1 h. Then, 5 M NaCl was added to give a final concentration of 1 M, and CTAB was added to a concentration of 1%. Following incubation at 65°C for 30 min, one freeze-thaw cycle, and phenol-chloroform extraction, the DNA was precipitated by the addition of 0.6 volumes of isopropanol, and the DNA pellet was washed with 70% ethanol. After desiccation, the DNA pellet was resuspended in 100 μl of sterile distilled water. PCR conditions were generally the same as for DNA from purified preparations, except that a variety of PCR additives were included in some reaction mixtures to enhance amplification. The additives used were 10 μg of bovine serum albumin (BSA; Boehringer Mannheim) per ml, 5% glycerol (Sigma), 5% dimethyl sulfoxide (DMSO; Sigma), and 5 μg of T4 gene 32 protein (T4G32; Boehringer Mannheim) per ml.

Product analysis and oligonucleotide hybridization. PCR products (15% of the amplification reaction mixture) were detected by standard agarose gel electrophoresis and ethidium bromide staining (26). The M1 and M2 molecular size markers used were obtained from Promega (Madison, Wis.) and Boehringer Mannheim, respectively. DNA was transferred from agarose gels to positively charged nylon membranes (Boehringer Mannheim) by either rapid (4-h) capillary transfer with 0.4 M NaOH buffer or overnight transfer of denatured DNA in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA was cross-linked to the membranes by UV irradiation (120 mJ for 2 min), followed by drying at 80°C. Prior to overnight transfer, DNA was denatured by incubation for 30 min in 0.4 M NaOH–0.6 M NaCl, followed by incubation in 1.5 M NaCl–0.5 M Tris-HCl, pH 7.5, at room temperature. The membranes were prehybridized for 1 h in 20 ml of hybridization solution (1 to 5× SSC containing 1% blocking reagent [Boehringer Mannheim]) at 5°C below T_m. Then 20 pmol of 5′-fluorescein-labeled oligonucleotide probe was added to 20 ml of fresh preheated hybridization solution, and the membrane was incubated for 18 h at T_m minus 5°C in a rotary hybridization oven (model 310; Robbins Scientific, Sunnyvale, Calif.). Stringency washes containing 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate, and 0.1 to 5× SSC were performed at T_m twice for 15 min each. The concentration of SSC used for hybridization and stringency washing depended on the T_m of the probe but was varied to allow the hybridizations and washes to be done below 65°C without using formamide. Hybridized probe was detected with an antifluorescein-alkaline phosphatase conjugate and a chemiluminescent substrate. The membranes were washed for 5 min in 0.3% Tween 20, followed by a 20-min incubation in 1% blocking reagent. Both of these solutions were made up in 0.1 M maleic acid–0.15 M NaCl, pH 7.5, and all incubations were at 23°C. Antifluorescein-alkaline phosphatase (1.5 U; Boehringer Mannheim) was added to the membranes in 0.1 M Tris-HCl (pH 7.5)–0.15 M NaCl–1% blocking reagent and incubated for 30 min. The membranes were washed in 0.3% Tween 20 and incubated for 15 min at 37°C with Lumigen-PPD (Boehringer Mannheim) in 0.1 M Tris–0.1 M NaCl–50 mM $MgCl_2$, pH 9.5. X-ray film (Fuji RX; Fisher Scientific Co., Tustin, Calif.) was exposed to the membranes at room temperature, for periods ranging from 30 s to 10 min, after light production reached a steady state (about 3 h after addition of the substrate).

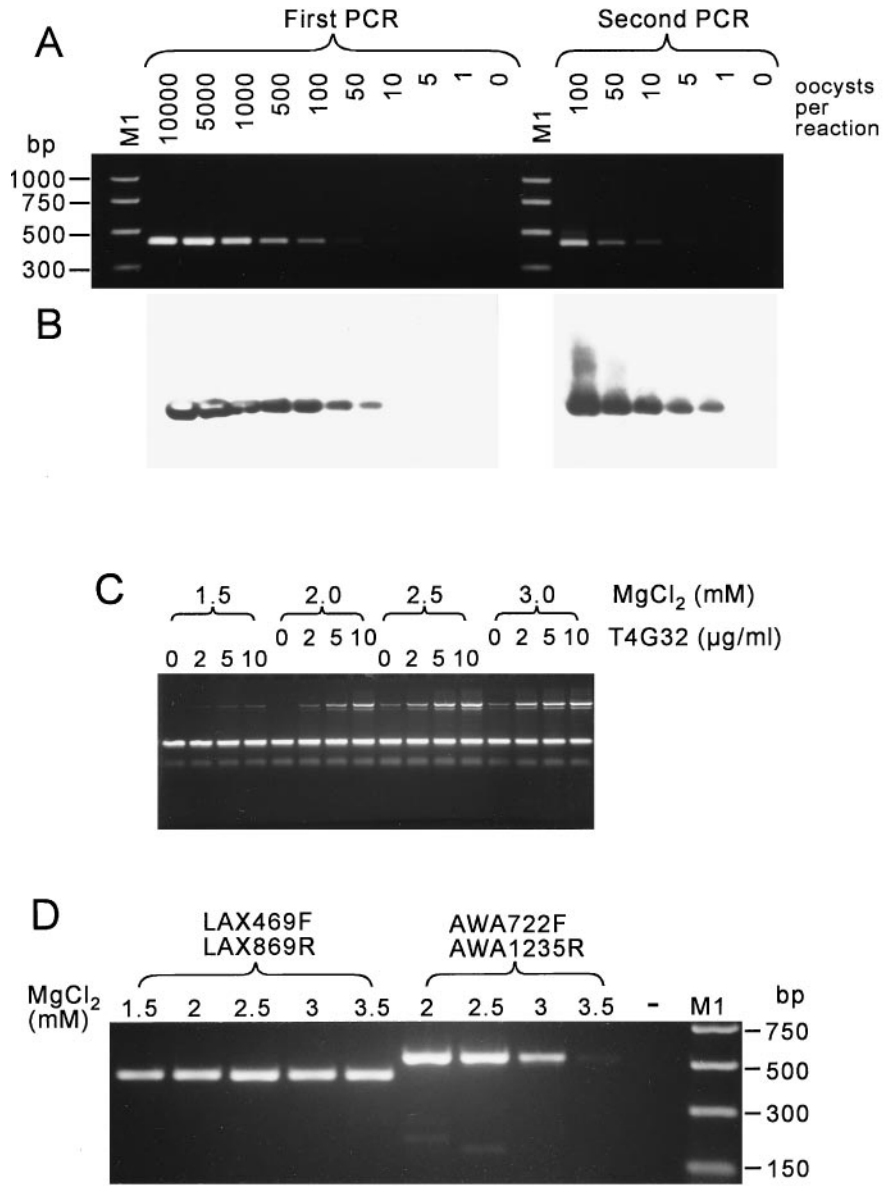

FIG. 1. PCR detection of *Cryptosporidium* and *Giardia* DNA from purified oocyst and cyst preparations. (A) Ethidium bromide-stained amplification products (451 bp), obtained with primers LAX469F and LAX869R from a serial dilution of *C. parvum* oocysts, in a 1.5% agarose gel. Reaction conditions were 2.5 mM $MgCl_2$ annealed at 52°C. Wells contained 15% of the total amplification reaction mixture, M1, molecular size marker. (B) Southern blot of the gel in panel A hybridized with a mixture of the LAX520 and LAX681 probes at 64°C in 5× SSC and washed at 61°C in 1× SSC. The chemiluminescent detection procedure is described in Materials and Methods. (C) Effects of T4G32 protein and $MgCl_2$ on amplification of a 163-bp product (ABB97F-ABB220R) from *G. lamblia*. (D) Effects of $MgCl_2$ on amplification of *C. parvum* DNA. −, negative control; M1, molecular size marker.

For probe analysis of larger amplicon volumes, 50 μl of amplification product was applied to positively charged nylon membranes by a slot blot manifold and was probed according to the protocol described above.

Amplification products were purified with Wizard PCR columns (Promega). DNA was eluted in 50 μl of sterile distilled water, and 5 μl of purified product was digested with 10 U of *Sty*I, *Hae*III, or *Scr*FI at 37°C for 4 h in a 25-μl volume. Digested DNA was separated in a 2.5% agarose gel.

RESULTS AND DISCUSSION

Primer analysis and PCR optimization. When this work was initiated, a literature search revealed four pairs of primers each for *Cryptosporidium* and *Giardia*. All of these primers (a total of eight pairs) were selected for evaluation to determine which, if any, were the most suitable for use in the detection of waterborne protozoa. The primers specific for *Cryptosporidium* amplified 556- and 256-bp products from the 18S rRNA gene (3) and 451- (Fig. 1A and B) and 329-bp fragments from undefined genomic regions (15, 32). Primers specific for *Giardia* targeted a 163-bp product from the HSP gene (1), 218- and 171-bp amplicons from the giardin gene (19, 20), and a 183-bp product from the SSU rRNA gene (33).

In this paper the calculated optimum annealing temperatures, determined by computer analysis, and actual optimum annealing temperatures, determined by empirical testing, are referred to as $T_{a_{Copt}}$ and $T_{a_{opt}}$, respectively. Primer sequences were analyzed with DNA analysis software (OLIGO; NBI) to determine their $T_{a_{Copt}}$ and calculated maximum annealing tem-

TABLE 1. Thermal characteristics and cited thermal cycling parameters of PCR primers for amplification of *Cryptosporidium*- and *Giardia*-specific DNA

Primer pair	T_p[a] (°C)	T_m[b] (°C)	$T_{a_{Copt}}$[c] ($T_{a_{Cmax}}$[d])(°C)	Step and conditions (°C/min)[e]	No. of cycles[f]
Cryptosporidium					
LAX469F, LAX869R	74.6, 74.6	72.5,72.5	51.9 (71.0)	D, 94/1; A, 45/2; E, 72/3	35
WEB110F, WEB401R	67.3, 67.3	62.5,62.5	56.0 (72.0)	D, 94/1.5; A, 50/1.5; E, 72/2	40
AWA722F, AWA1235R	67.3, 65.8	62.5,60.5	53.3 (67.8)	D, 94/0.75; A, 47/1; E, 72/3	38
AWA995F, AWA1206R	65.8, 70.2	60.5,66.5	53.7 (66.9)	D, 94/0.75; A, 47/1; E, 72/3	38
Giardia					
WEI1270F, WEI1414R	67.3, 64.3	62.0,58.0	60.2 (67.7)	D, 95/1; A, 50/1; E, 72/1	50
ABB97F, ABB220R	70.2, 68.7	66.0,64.0	55.4 (72.0)	D, 94/0.75; A, 55/0.5; E, 72/0.75	30
MAH433F, MAH592R	89.2, 92.1	92.0,96.0	64.6 (72.0)	D, 94/1; A, 60/1; E, 72/1	25
MAH658F, MAH789R	67.3, 65.8	62.0,60.0	57.4 (70.8)	D, 94/1; A, 60/1; E, 72/1	25

[a] Optimum PCR temperature (±5°C) for each primer, calculated by the formula $T_p = 22 + 1.46[2(G+C) + (A+T)]$°C. The first temperature reported corresponds to the first primer in the pair, and the second temperature corresponds to the second primer.
[b] T_m calculated by the 2(A+T) + 4(G+C)°C method for each primer. Temperatures are reported as in footnote *a*.
[c] Optimum annealing temperature for primer pair calculated by OLIGO version 5.0.
[d] Maximum annealing temperature for primer pair calculated by OLIGO version 5.0.
[e] Actual denaturation (D), annealing (A), and extension (E) temperatures and times used in cited publications.
[f] Number of cycles used in original publications.

peratures ($T_{a_{Cmax}}$). The $T_{a_{Copt}}$ was up to 10°C higher than the T_a used in the original descriptions of the primers (Table 1). The $T_{a_{opt}}$ were determined empirically by performing amplifications at the $T_{a_{Copt}} \pm 6$°C in 2°C increments. These empirically determined $T_{a_{opt}}$ ranged from 52 to 65°C (Table 2) and were generally very close to the $T_{a_{Copt}}$. The $T_{a_{opt}}$ was defined as that temperature which resulted in the highest yield of specific product and a low yield of primer artifacts (but not necessarily the lowest yield of artifacts). For most of the primer pairs (those with a $T_{a_{max}}$ of >70°C), two-step PCR was recommended by primer analysis software. However, combining the annealing and extension steps of the amplification cycle at 68°C reduced the yield of amplification products.

TABLE 2. Empirically determined optimal conditions and performance of PCR primers

Primer pair	Product size (bp)	Optimal $[MgCl_2]$ (mM)	$T_{a_{opt}}$ (°C)[a]	Sensitivity[b]
C. parvum				
AWA722F-AWA1235R	556	2	52	10
AWA995F-AWA1206R	256	2	54	1
LAX469F-LAX869R	451	2–3.5	52	1
WEB110F-WEB401R	329	ND[c]	ND	ND
G. lamblia				
ABB97F-ABB220R	163	2.5	55	1
MAH433F-MAH592R	218	1.5	65	10
MAH658F-MAH789R	171	1.5	62	1
WEI1270F-WEI1414R	183	ND	ND	ND
Multiplex				
AWA995F-AWA1206R	256	1.5	55	1[d]
ABB97F-ABB220R	163			

[a] Experimentally determined $T_{a_{opt}}$.
[b] Sensitivity determined in this report as the number of cysts or oocysts per reaction mixture.
[c] ND, not determined because of inefficient amplification in the initial evaluations.
[d] One oocyst and one cyst.

Optimal $MgCl_2$ concentrations were generally 1.5 to 2.5 mM, except for the LAX primers, which had no optimum between 2 and 3.5 mM (Table 2). For most of the other primers, increasing the $MgCl_2$ concentration resulted in an increased yield of both specific and nonspecific products. At $MgCl_2$ concentrations above 2.5 mM, nonspecific products (about 800 bp) were produced by the ABB primer pair. The yield of these nonspecific products was greatly increased by the addition of T4G32 at concentrations ranging from 2 to 10 μg/ml (Fig. 1C). T4G32 is a single-stranded, helix-destabilizing protein that reportedly increases the efficiency of thermostable DNA polymerases (Boehringer Mannheim). At the published T_a of 60°C, the MAH433F-MAH592R primer pair produced a dimer of about 60 bp with the same efficiency as the expected product, even at 1.5 mM $MgCl_2$. Raising the T_a to 64°C reduced the amount of dimer formed but did not completely eliminate it. Sequence analysis showed that the reverse primer (MAH592R) contained a significant hairpin loop (T_m = 86°C), and both primers formed a 10-base dimer. These factors were responsible for the stable dimer formed by these primers. In contrast to all of the other primer pairs, the yield of specific product from the AWA722F-AWA1235R primer pair decreased at $MgCl_2$ concentrations greater than 2 mM (Fig. 1D). Various nonspecific products of different sizes, as well as a primer-dimer, were obtained with these primers, but the specific (556-bp) and nonspecific products disappeared at higher $MgCl_2$ concentrations (>3.5 mM). The primer-dimer was produced at $MgCl_2$ concentrations of 1 mM and above. The dimer was most pronounced when the primers were annealed at the published temperature of 47°C (3) but was reduced by increasing the T_a to 52°C ($T_{a_{opt}}$ = 53.3°C [Table 1]). Increasing $MgCl_2$ concentrations resulted in an increased yield of the LAX PCR

product, whereas the yield of the AWA722F-AWA1235R primer pair decreased (Fig. 1D).

In initial evaluations, the WEB primer pair, targeting an undefined region of the *Cryptosporidium* genome, amplified a product of the correct size (329 bp) with very low efficiency. The yield of amplification product was not increased by raising $MgCl_2$ concentrations to 5.5 mM. Three weak, nonspecific products of various sizes were also observed. Adding BSA (10 μg/ml) or T4G32 (10 μg/ml) to the reaction mixture increased the yield of amplification products slightly, but not sufficiently to warrant further evaluation. These primers were reported to have a detection sensitivity of 20 oocysts under ideal conditions following hybridization with a radiolabeled probe (32). They detected *C. parvum* and *C. baileyi*, but these products were discriminated by hybridization with an internal probe.

The WEI primer pair, targeting the *Giardia* SSU rRNA gene, did not amplify DNA at all in initial reaction mixtures containing 1.5 to 8.5 mM $MgCl_2$ annealed at 50 to 65°C. Analysis of the primer and expected product sequences revealed a 29°C difference between the product and primer T_m as a result of the high G+C content of the sequence bounded by the primers (72%). This was noted in the original description of these primers, and the author indicated that successful PCR was only achieved when 10% glycerol and 10% DMSO were included in the reaction mixture to reduce the stability of the amplification product (34). The reported detection sensitivity for these primers was one cyst or fewer. In our investigation, inclusion of 10% glycerol and 5 to 10% DMSO in the amplification reaction mixture resulted in inefficient amplification of the correct-size product (183 bp) from purified cysts and no amplification from cysts seeded into environmental water samples. The performance of these primers was confirmed in three experiments. Consequently, these primers were also excluded from further evaluation. They had previously been used to divide 35 *G. lamblia* isolates into three subgroups, but no correlation between geographic origin and group was demonstrated (33). In addition to *G. lamblia* and *G. muris*, these primers also produced amplification products of various sizes from *Entamoeba histolytica*, *Leishmania major*, and *Trypanosoma brucei*, but only *G. lamblia* amplicons hybridized to the internal oligonucleotide probe (33).

The incorporation of dUTP into PCR products and the treatment of reaction mixtures with UDG to prevent carryover contamination of subsequent reaction mixtures did not affect the yield of specific amplicons but did reduce the yield of primer artifacts. The recommended concentration of dUTP was 600 μM (Boehringer Mannheim), but there was no difference in amplicon yield from reaction mixtures containing dUTP ranging in concentration from 200 to 800 μM, so 200 μM was used routinely. The inclusion of UDG in a PCR mixture is possible only when the T_a is greater than 50°C, as residual UDG activity may degrade target amplicons at temperatures lower than 50°C. Although dUTP and dTTP can both be incorporated into single amplicons at proportions of uracil greater than 50% and still be degraded by UDG (9), we included 100% uracil to ensure complete amplicon degradation. Negative controls never produced amplification products during these evaluations.

Amplification product confirmation by restriction digestion. Incorporation of dUTP into amplicons did not affect the amplification yield but did have important consequences for analysis of the products. Some of the original reports on these primers described the use of restriction endonuclease digestion to confirm the identity of amplicons. Digestion of the 556-bp AWA product with *Mae*I was used to discriminate between *C. parvum* and *C. muris* (3), and *Sty*I was used to confirm the identity of the 451-bp LAX product in conjunction with two oligonucleotide probes (15). *Dra*I, *Nde*I, *Rsa*I, and *Sst*I digestion distinguished between 435-bp amplicons from the 18S rRNA genes of *C. parvum*, *C. muris*, and *C. baileyi* (10). The recognition sequences for all of these enzymes included thymine, so PCR products incorporating uracil would not contain the necessary restriction sites. It has been reported that incorporation of more than 50% dUTP into amplification products dramatically reduces the efficiency of digestion by enzymes that have a thymine in their recognition sequences (9). Therefore, in the present study, restriction enzymes *Hae*III (GGCC) and *Scr*FI (CCNGG) were selected for product confirmation. *Sty*I could still be used for digestion of the LAX product, because the target sequence contained the CCAAGG recognition site.

TABLE 3. Specificity of PCR primers used for the detection of *Cryptosporidium* and *Giardia*

Primer pair	PCR product obtained from[a]:				
	C. parvum	*C. muris*	*C. baileyi*	*G. lamblia*	*G. muris*
AWA722F-AWA1235R	+	+	+	−	−
AWA995F-AWA1206R	+	+	+	−	−
LAX469F-LAX869R	+	−	−	−	−
WEB110F-WEB401R	+	−	+	−	−
ABB97F-ABB220R	−	−	−	+	+[b]
MAH433F-MAH592R	−	−	−	+	−
MAH658F-MAH789R	−	−	−	+	+
WEI1270F-WEI1414R	−	−	−	+	+

[a] +, positive PCR; −, negative PCR.
[b] Product from *G. muris* was 526 bp compared to 163-bp product from *G. lamblia*.

Specificity and sensitivity. The specificities of all of the primers for unrelated species (e.g., non-*Cryptosporidium* or non-*Giardia* protozoa and bacteria) were determined in the original descriptions of the primers and so are not reported here. However, the specificities of all the primer pairs were determined with closely related species in the present study. Only one *Cryptosporidium* or *Giardia* primer pair was specific for *C. parvum* or *G. lamblia*, respectively (Table 3). In the original reports describing these primers, PCR products from organisms other than *C. parvum* or *G. lamblia* were discriminated by restriction digestion, product size, or probing. However, a PCR assay based on primers that are specific for just *C. parvum* or *G. lamblia* would be more rapid and convenient to use on a routine basis, since post-PCR processing for species identification would be eliminated.

The inability of primers targeting the 18S rRNA gene to discriminate between different species of *Cryptosporidium* is not surprising, in light of the similarity between these genes from some strains of *C. parvum* and *C. muris*. Sequence comparisons showed that, whereas 18S rRNA genes from different strains of *C. parvum* demonstrated 92.1 to 99.6% similarity, one strain of *C. parvum* showed 99.6% similarity with *C. muris*. A sequence similarity of 99% is equivalent to only 17 different bases in the entire 18S rRNA gene (total length, 1,740 bp). Consequently, there are very few regions within the *Cryptosporidium* 18S rRNA gene that permit the design of species-specific primers or probes. In comparison, there is only about 80% sequence similarity between the SSU rRNA genes of *G. lamblia* and *G. muris*, corresponding to about 290 different bases over the length of the gene. Therefore, it should be possible to design primers targeting the SSU rRNA gene that are specific for *G. lamblia*.

A sensitivity of 1 to 10 cysts or oocysts per reaction mixture was achieved with purified preparations (Table 2), but this was

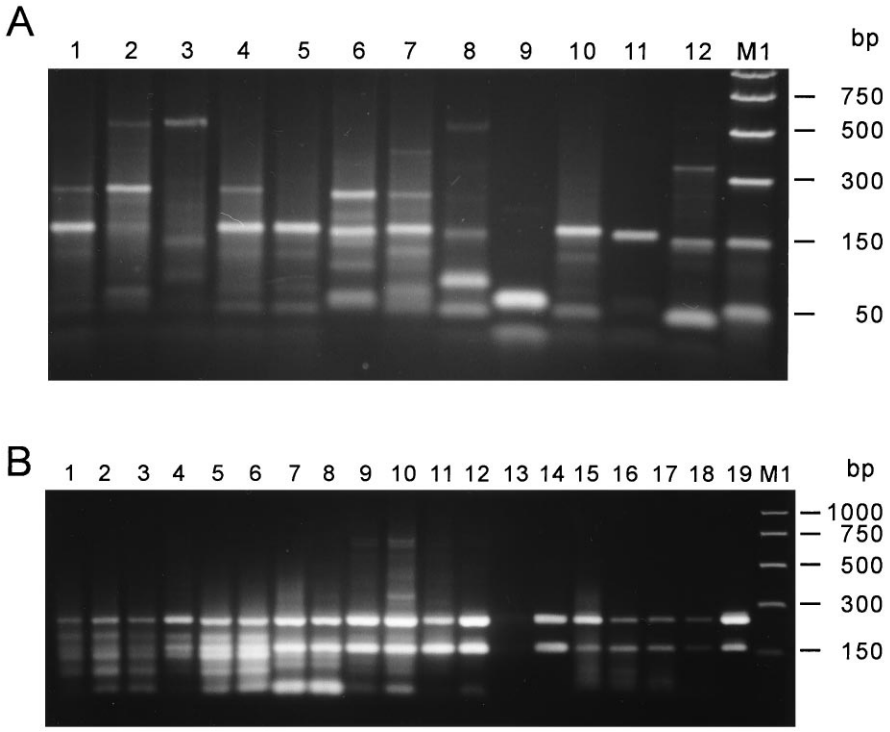

FIG. 2. Multiplex PCR for the simultaneous detection of *Cryptosporidium* and *Giardia* in a single reaction mixture. (A) Various combinations of primers were tested for their ability to produce specific amplicons of the correct size. Lanes: 1, AWA722F-AWA1235R and MAH658F-MAH789R; 2, AWA722F-AWA1235R and MAH658F; 3, AWA995F-AWA1206R and MAH433F-MAH592R; 4, LAX and MAH433F-MAH592R; 5, AWA1235R and MAH658F-MAH789R; 6, AWA995F-AWA1206R and ABB; 7, AWA995F-AWA1206R and MAH658F-MAH789R; 8, AWA722F-AWA1235R and ABB; 9, AWA722F-AWA1235R and MAH433F-MAH592R; 10, LAX and MAH658F-MAH789R; 11, LAX and ABB; 12, AWA722F-AWA1235R, MAH433F-MAH592R, and ABB. M1, molecular size marker. (B) Multiplex amplification of 256- and 163-bp fragments from *C. parvum* and *G. lamblia* with primers AWA995F-AWA1206R and ABB97F-ABB220R under various reaction conditions. Lanes: 1 to 4, 3.5 to 1 mM $MgCl_2$, 50°C annealing; 5 and 6, 1.5 and 2.5 mM $MgCl_2$, respectively, 52°C; 7 and 8, 2.5 mM $MgCl_2$, 55°C; 9 and 10, 1.5 mM $MgCl_2$, 54°C; 11 and 12, 1.5 mM $MgCl_2$, 55°C; 13, empty; 14, purified ABB and AWA products amplified individually; 15, DNA extracted from purified cysts and oocysts by the same extraction protocol as that used for environmental samples; 16 to 18, environmental water sample (EWS1) seeded with 1,000, 500, and 100 cysts and oocysts, respectively, combined in equal proportions; 19, 1,000 purified cysts and oocysts, 1.5 mM $MgCl_2$, 55°C, *Tth* polymerase. M1, molecular size marker.

generally 5- to 10-fold lower with DNA from environmental samples. Lower sensitivities with some primer pairs (AWA722F-AWA1235R and MAH433F-MAH592R) may have resulted from amplification of primer artifacts or other nonspecific products or from primer secondary structure and stability. A sensitivity of one cyst or oocyst was achieved with 40 PCR cycles followed by hybridization with a specific oligonucleotide probe (Fig. 1A and B). However, a second PCR of 40 cycles with 5 μl of the product from the first reaction mixture as the template resulted in detection of a single oocyst without subsequent hybridization. The conditions for the second round of amplification reactions were the same as for the first round, except that UDG was not included because it would degrade the amplicons.

A recent report described the use of the LAX primers, annealed at 55°C, for detection of *Cryptosporidium* in bovine feces (16). The authors reported a sensitivity of 1 to 10 oocysts per reaction mixture with purified oocysts, which represented a 10^3- to 10^4-fold increase in sensitivity in comparison to an enzyme immunoassay. PCR detection was 100-fold more sensitive than enzyme immunoassay for fecal samples.

Other primers. Recent reports, published after this study began, described different primers for the detection of *Cryptosporidium* (12, 30). Primers targeting the 18S rRNA gene had a reported sensitivity of 1 to 10 oocysts with purified preparations (12). The forward and reverse primers corresponded to nucleotide positions 601 to 621 (CPB-DIAGF) and 1015 to 1035 (CPB-DIAGR), respectively; their use had been described previously, but the sequences had not been published (10, 11, 31). The three bases at the 5′ end of the reverse primer overlapped with the three bases at the 3′ end of AWA995F. Primers targeting the 18S rRNA gene were unable to discriminate between *C. parvum*, *C. muris*, and *C. baileyi*, but single representatives of each species could be differentiated by restriction digests (10). However, our analysis of four *C. parvum* 18S rRNA gene sequences and three from *C. muris* (all obtained from the Ribosomal Database Project) revealed that it was not possible to differentiate between the two species (by either restriction digests or probing) based on sequences spanned by CPB-DIAGF and CPB-DIAGR, because of the apparent genetic homogeneity within the *Cryptosporidium* genus. In this region of the 18S rRNA gene, there were only two nucleotide differences between one *C. parvum* and a *C. muris* sequence, and only one of these differences affected a restriction enzyme recognition sequence (*Tsp*E1, nucleotide position 679). Moreover, a second *C. muris* sequence matched the *C. parvum* sequence at this position. However, errors in the database sequences or incorrect identification of organisms from which sequences were obtained cannot be discounted as the cause of this apparent homogeneity within the *Cryptosporidium* genus. These discrepancies need to be investigated to determine the validity of the sequences.

A second report described primers (CP397 and CP1270) that amplified an 873-bp fragment from a repetitive oocyst

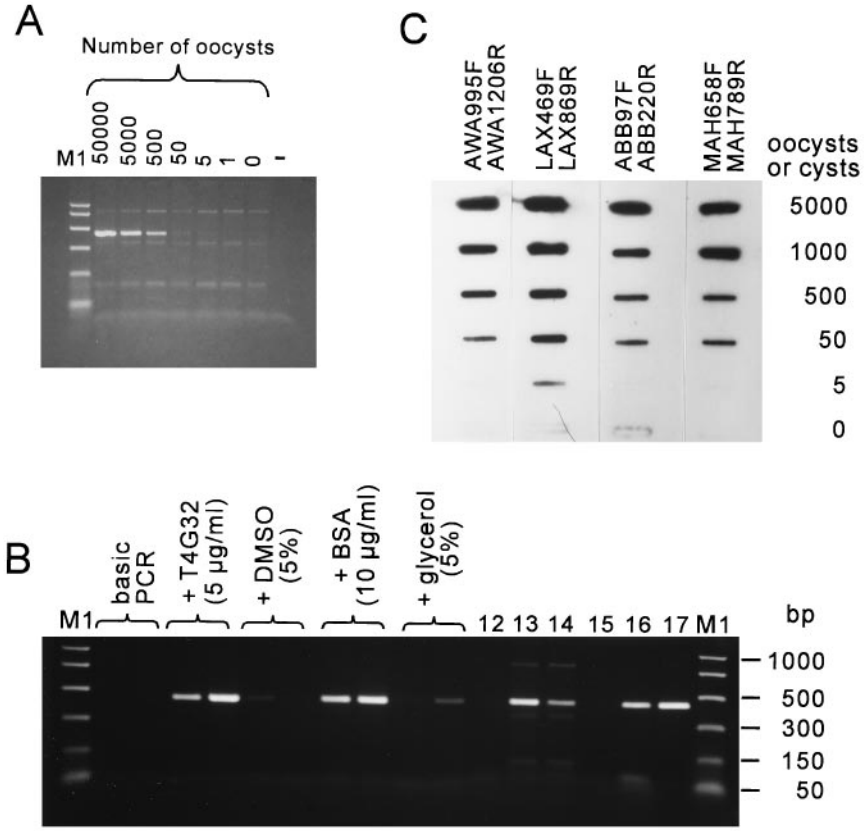

FIG. 3. PCR detection of *Cryptosporidium* and *Giardia* DNA in environmental water samples. (A) Amplification of a 451-bp product (LAX469F-LAX869R) from EWS3 seeded with *C. parvum* oocysts. DNA was extracted from a concentrated sample pellet equivalent to 49 liters of raw water and amplified by the basic PCR protocol described in Materials and Methods. −, negative control; M1, molecular size marker. (B) Amplification of the 451-bp LAX product from DNA extracted from EWS2, seeded with *C. parvum* oocysts, in the presence of various potential PCR enhancers. Lanes: 12, negative control; 13 and 14, same as the second and third lanes in panel A; 15, negative control; 16, basic PCR, purified oocysts; and 17, purified LAX product. M1, molecular size marker. (C) Slot blot analysis of PCR products from an environmental water sample (EWS2) seeded with 0 to 5,000 cysts or oocysts. Specific *Cryptosporidium* or *Giardia* DNA was amplified with the primers indicated, using the PCR protocol described in Materials and Methods with the addition of 10 μg of BSA per ml. Membranes were hybridized with the CPRNA1097, LAX681, GLHSP184, and MAH751 fluorescein-labeled oligonucleotide probes, respectively, from left to right.

protein gene (30). These primers did not amplify DNA from any non-*Cryptosporidium* organisms, but *C. parvum* was the only species of *Cryptosporidium* tested. There are no analogous DNA sequences available for other *Cryptosporidium* spp., so predictions could not be made about the actual specificities of these primers. The reported sensitivity of 10 sporozoites was achieved by two successive rounds of amplification, each comprising 30 cycles.

Multiplex PCR. The current standard method for the detection of *Cryptosporidium* and *Giardia* involves an indirect IFA that detects both organisms simultaneously (2). Simultaneous detection of *Cryptosporidium* and *Giardia* in a single reaction mixture would also be beneficial to a PCR-based method, because it would halve the number of amplification reactions necessary. Therefore, combinations of primers were tested for their ability to generate specific amplification products of the correct size under a variety of PCR conditions. The most suitable primers for multiplex detection were those targeting the HSP gene in *Giardia* (ABB97F-ABB220R, 163-bp product) and the 18S rRNA gene in *C. parvum* (AWA995F-AWA1206R, 256-bp product [Fig. 2A, lane 6]). This was the only primer combination that produced two amplicons of the correct size. Product identity was confirmed by restriction digestion with *Scr*FI and *Hae*III and probing. The optimum reaction conditions were 1.5 mM $MgCl_2$ with a T_a of 55°C (Fig. 2B), which was within 2°C of optimum for both primer pairs. $MgCl_2$ concentrations above 2.5 mM resulted in loss of the 163-bp product from *Giardia* and the appearance of nonspecific products. This primer combination detected one *Giardia* cyst and one *Cryptosporidium* oocyst in purified preparations. Using seeded water samples (EWS1), multiplex PCR detected 50 cysts and 50 oocysts (Fig. 2B, lane 18). Unfortunately, the *Cryptosporidium* primers with the best specificity (LAX469F-LAX869R) did not yield an amplification product when combined with *Giardia*-specific primers.

Detection of *Cryptosporidium* and *Giardia* in environmental water samples. Various procedures were tested for their ability to extract relatively pure, undegraded DNA from seeded and unseeded environmental water samples. Freezing-thawing in the presence of CTAB, followed by phenol-chloroform extraction, gave consistently high yields of undegraded DNA.

Although the LAX primers amplified the correct fragment from one water sample (EWS3) seeded with five *C. parvum* oocysts, they also produced three nonspecific amplification products at a constant concentration (Fig. 3A). With DNA extracted from a second environmental sample (EWS2), the LAX primers generated amplicons only when either T4G32 (5 μg/ml) or BSA (10 μg/ml) was added to the reaction mixture to overcome PCR inhibition resulting from DNA contaminants such as humic acids (Fig. 3B). The inclusion of 5% DMSO or

5% glycerol also relieved the inhibition slightly. However, when amplifying DNA from purified cysts with the ABB97F-ABB220R primer pair, T4G32 greatly increased the level of nonspecific products (approximately 800 bp), particularly at $MgCl_2$ concentrations greater than 2 mM (Fig. 1C). Consequently, BSA was the most appropriate PCR additive to overcome inhibition by contaminants in environmental samples. A previous report found no difference in the ability of BSA or T4G32 to overcome inhibition of PCR in environmental samples but concluded that BSA was the most appropriate, due to its cost effectiveness relative to T4G32 (13). BSA is approximately 100-fold less expensive than T4G32.

The detection sensitivity in seeded environmental water samples, determined by slot blots hybridized with fluorescein-labeled oligonucleotide probes, ranged from five *C. parvum* oocysts for the LAX primers to 50 *C. parvum* oocysts or *G. lamblia* cysts for the AWA722F-AWA1235R, ABB, and MAH658F-MAH789R primer pairs (Fig. 3C).

The ABB primer pair also amplified *Giardia*-specific DNA from unseeded environmental water samples (EWS1 and EWS2) containing <1.3 and 7 cysts per 100 liters, as determined by IFA. The positive reactions resulted from amplification of extracted DNA equivalent to approximately 24 liters of raw water.

A common problem encountered with PCR-mediated amplification of DNA extracted from environmental samples is inhibition by humic-type materials that coextract with the DNA (24, 27). Various methods for purification of environmental DNA prior to PCR, including preparative agarose gel electrophoresis (17), spun columns (28), and adsorption to glass particles (24), have been described. Techniques used specifically to enhance PCR detection of *Cryptosporidium* include flow cytometry and immunomagnetic separation of oocysts from water samples prior to PCR (12) and spin column purification of DNA extracted from feces (16).

In the present study, PCR inhibition was overcome by the addition of either BSA or T4G32 to the amplification reaction mixture, although BSA was the most effective reagent, since the addition of T4G32 resulted in amplification of nonspecific fragments with some primers. However, the highest turbidity of water samples in this study was 1.2 NTU. It is likely that DNA extracted from water with higher turbidities will need extra purification prior to PCR.

Conclusions. The results described in this paper indicate the potential effectiveness of PCR for the detection of waterborne *Cryptosporidium* and *Giardia* but demonstrate the necessity of primer evaluations and optimization to achieve suitable sensitivity and specificity. Not all primers successfully amplified their targets, even though they were previously reported to do so, as highlighted by the WEB and WEI primers. For the detection of *C. parvum*, the LAX primers demonstrated the greatest sensitivity combined with specificity. However, these primers were not compatible with any of the *Giardia* primers for multiplex PCR. The ABB primers detected a single *Giardia* cyst, and although they were not specific for *G. lamblia*, the amplicon from *G. muris* was easily distinguished from that of *G. lamblia* on the basis of size. None of the primers evaluated in this study had the ideal combination of sensitivity, specificity, and compatibility with multiplex PCR. Therefore, new primers targeting different genes or different regions of the same genes should be designed as the necessary sequence information becomes available. It would be beneficial to analyze the ever-increasing DNA sequence databases to allow the design of new primers that are specific for all strains of either *C. parvum* or *G. lamblia* and are also compatible with multiplex PCR. PCR should become a more widely accepted method of pathogen detection and monitoring within the water industry and should be used in parallel with conventional techniques to improve detection capabilities for existing and newly emerging pathogens.

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