JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1992, p. 74–78
0095-1137/92/010074-05$02.00/0

Vol. 30, No. 1

Differentiation of *Giardia duodenalis* from Other *Giardia* spp. by Using Polymerase Chain Reaction and Gene Probes

M. H. MAHBUBANI,[1]† A. K. BEJ,[1]‡ M. H. PERLIN,[1] F. W. SCHAEFER III,[2] W. JAKUBOWSKI,[2] AND R. M. ATLAS[1]*

Department of Biology, University of Louisville, Louisville, Kentucky 40292[1] and Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268[2]

Received 14 May 1991/Accepted 4 October 1991

***Giardia* spp. are waterborne organisms that are the most commonly identified pathogenic intestinal protozoans in the United States. Current detection techniques for *Giardia* species in water include microscopy and immunofluorescence techniques. Species of the genus *Giardia* are classified on the basis of taxonomic criteria, such as cell morphology, and on host specificity. We have developed a polymerase chain reaction- and gene probe-based detection system specific for *Giardia* spp., which can discriminate between the relevant species of the *G. duodenalis* type pathogenic to humans and other *Giardia* species that are not human pathogens. This method can detect a single *Giardia* cyst and is therefore sensitive enough for environmental monitoring.**

The waterborne protozoan *Giardia lamblia* is the most common cause of defined waterborne diarrhea in the United States (9). *Giardia* species have been found in the small intestines of almost all classes of vertebrates (14, 18, 22). Whether or not giardiasis is a zoonosis is debatable (5, 13). However, the infection of wild animals with *Giardia* species from humans in controlled studies demonstrates that these wild animals may at least serve as reservoirs for the *Giardia* species pathogenic to humans (10, 12).

Species of the genus *Giardia* have been differentiated on the basis different taxonomic criteria such as cell morphology, trophozoite dimensions, and host specificity (14). This has led to confusion regarding species classification and epidemiology of giardiasis (21). The genus *Giardia* has been classified by Filice (14) into three morphological types, *G. duodenalis*, *G. muris*, and *G. agilis* on the basis of on the shape of the median body. *G. muris* infects rodents; *G. agilis* infects amphibians, and *G. duodenalis* includes most other species that infect mammals (including *G. lamblia* in humans), rodents, birds, and reptiles (15, 18, 20, 22, 29). *Giardia* isolates from cats, dogs, and rats have been designated *G. cati*, *G. canis*, and *G. simoni*, respectively (18, 28). A newly described species, *Giardia ardeae*, was isolated from the intestine of a gray heron (*Ardea cinera*) (11). While *G. duodenalis* has two full tail flagella, *G. muris* and *G. ardeae* have one full tail flagellum and one rudimentary tail flagellum (11). *Giardia* isolates from humans are known as *G. duodenalis*, *G. intestinalis*, *G. lamblia*, and *Lamblia intestinalis*. A *Giardia* isolate from a human could be called *G. lamblia* or *G. duodenalis*, but the same organism isolated from a nonhuman source could only be called *G. duodenalis*.

Diagnosis of *Giardia* infections in humans is usually established by microscopic detection of cysts or trophozoites in fecal specimens. This method has been reported to be only 50 to 70% sensitive when single stool specimens are analyzed (6). Serodiagnosis does not provide evidence of active infection (7). For environmental surveillance of *Giardia* species, detection currently employs filtration and other concentration method and microscopy using stains such as Lugol's iodine and trichrome (16, 26) and fluorescent antibodies (23, 25, 27). Two DNA probe-based assays have been reported, one with a sensitivity of 1 ng of DNA or 5×10^3 trophozoites (7) and the other with a sensitivity of one cyst (1). The latter is a cDNA probe based on the 16S rRNA sequence of *G. lamblia* and does not differentiate *Giardia* species. Environmental detection of *Giardia* species is complicated by the need to distinguish species found in humans and nonhuman species.

In the present study, we describe a method based on polymerase chain reaction (PCR) amplification and gene probe methods for the differentiation of *G. duodenalis* from *G. muris* and *G. ardeae*.

MATERIALS AND METHODS

Sources of organisms. *Giardia* sp. strain MR4 and *G. ardeae* trophozoites were obtained from Stanley Erlandsen (University of Minnesota, Minneapolis); *G. lamblia* WB was from Frances Gillin (University of California, San Diego); *G. lamblia* Human-1-Portland, Human-2-Portland, and Portland-Beaver and *Giardia* sp. strain Be-1 were from Ernest Meyer (Oregon Health Sciences University, Portland); *G. lamblia* CDC:0284:1, UNO/04/87/1, and Egyptian strain were from Govinda S. Visvesvara (Centers for Disease Control, Atlanta, Ga.); *G. lamblia* RS, LT, CM, AB, JH, and N were from Theodore E. Nash (National Institutes of Health, Bethesda, Md.); and *G. intestinalis* New Orleans-1 (ATCC 50137) and KS (ATCC 50114), and *G. cati* UPV: 0685:1 (ATCC 50163) were from the American Type Culture Collection (Rockville, Md.). An inoculum to start the *G. muris* cycle was obtained from D. P. Stevens (Case Western Reserve University, Cleveland, Ohio). *Acanthamoeba castellani* (ATCC 30011), *Blastocystis hominis* (ATCC 50177), *Dientamoeba fragilis* (ATCC 40948), *Entamoeba coli* HU-1 (ATCC 30946), *Entamoeba histolytica* HM-1 (ATCC 30459) and HK-9 (ATCC 30015), *Hexamita inflata* (ATCC 50268), and *Trichomonas vaginalis* C-1 (ATCC 30001) were obtained from the American Type Culture Collection. *Cryptosporid-*

* Corresponding author.
† Present address: Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294.
‡ Present address: Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294.

ium parvum oocysts were provided by Charles Sterling (University of Arizona, Tuscon). An inoculum to start the *Cryptosporidium muris* cycle came from M. Iseki (Osaka City University Medical School, Osaka, Japan). Steve Upton (Kansas State University, Manhattan) KS provided *Eimeria nieschultzi* oocysts. *Chlorella* sp., *Scenedesmus* sp., and *Chlorcoccum* sp. were obtained from Robert Safferman (U.S. Environmental Protection Agency, Cincinnati, Ohio).

Escherichia coli (ATCC 12345), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (ATCC 12022), and *Salmonella typhimurium* (ATCC 19585) were obtained from the American Type Culture Collection. *Legionella pneumophila*, *Pseudomonas fluorescens*, *Pseudomonas alcaligenes*, *Pseudomonas cepacia*, *Pseudomonas diminuta*, *Xanthomonas maltophilia*, and *Alcaligenes faecalis* were obtained from the Centers for Disease Control. *Saccharomyces cerevisiae* was obtained from the Yeast Genetics Stock Center (Berkeley, Calif.), and human DNA was from Sigma Chemical Co. (St. Louis, Mo.). *Ustilago violacea* (pink strain) was from E. Garber (University of Chicago, Chicago, Ill.).

Giardia trophozoites were maintained at 35°C in Keister's modified TYI-S-33 medium (17). *G. muris*, *G. lamblia* CDC:0284:1, and *Cryptospordium muris* were cycled through female CF-1 mice, female Mongolian gerbils, and female CF-1 mice, respectively, after the procedures of Roberts-Thomson et al. (24).

Nucleic acid release. Nucleic acids were released from *Giardia* cysts and trophozoites by being heated in 50 μl of 0.1% diethylpyrocarbonate-containing water to 90°C for 5 min and then cooled to 4°C for 2 min. For specificity studies, DNA was isolated from *Giardia* strains by the method described by Butcher and Farthing (7). Cysts were collected from animals and sedimented by centrifugation. Approximately 10^5 cysts were used for tests of PCR detection. Trophozoites were sedimented by centrifugation from cultures and washed; approximately 10^5 trophozoites were used for PCR testing. DNA was recovered and purified from bacterial strains by the procedure described by Ausubel et al. (3).

PCR amplification. A 171-bp region of the *Giardia* giardin gene, based upon the sequence reported by Baker et al. (4), was amplified by using a 20-mer primer GGL639-658 (5'-AA GTGCGTCAACGAGCAGCT-3') and a 21-mer primer GGR 789-809 (5'-TTAGTGCTTTGTGACCATCGA-3'). Primer GGL639-658 was located between bp 639 and 658, and primer GGR789-809 was located between bp 789 and 809 of the giardin gene. A 26-mer gene probe GGP751-776 (5'-TCG AGGACGTCGTCTCGAAGATCCAG-3') was used for detection of the 171-bp amplified DNA. Primer GGL405-433 (5'-CATAACGACGCCATCGCGGCTCTCAGGAA-3'), a 29-mer oligonucleotide, and primer GGR592-622 (5'-TTTGT GAGCGCTTCTGTCGTGGCAGCGCTAA-3'), a 31-mer primer, amplified a 218-bp region of the giardin gene which was probed with a 28-mer oligonucleotide probe, GGP510-537 (5'-AGCTCAACGAGAAGGTCGCAGAGGGCTT-3').

PCR amplification was performed using a DNA Thermal Cycler and GeneAmp kit with Ampli*taq* DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The PCR solution contained 1× PCR amplification buffer (10× buffer containing 50 mM KCl, 100 mM Tris-Cl [pH 8.13], 15 mM $MgCl_2$, and 0.1% [wt/vol] gelatin), 200 μM (each) of the deoxynucleoside triphosphates, 0.5 μM (each) of the primers, 1 fg to 1 μg of template DNA, 2.5 U of Ampli*taq*, and double distilled water treated with 0.1% diethylpyrocarbonate. Template DNAs were initially denatured at 94°C for 1 to 3 min. A total of 25 PCR cycles were performed during which DNAs were denatured at 94°C for 1 min and the primers were annealed at 60°C for 1 min and extended at 72°C for 1 min. Oligonucleotide primers were synthesized using an Applied Biosystems DNA Synthesizer (Foster City, Calif.) and purified using an Oligonucleotide Purification Cartridge (Applied Biosystems) for small quantities and reverse-phase high-pressure liquid chromatography with a C-8 reverse-phase column (pore size, 3 μm) (Perkin-Elmer), for large amounts.

Detection of amplified DNAs. PCR-amplified DNAs were detected by using gel electrophoresis and radiolabeled gene probes by standard methods (3). The DNAs were separated using either 4% Nu Sieve 3:1 horizontal agarose gels or 5% vertical polyacrylamide gels. Agarose gels were run in TAE buffer (0.04 mM Tris-acetate, 0.001 mM EDTA [pH 8.0]). Polyacrylamide gels were run in TBE buffer (0.089 mM Tris-borate, 0.089 mM boric acid, 0.002 mM EDTA [pH 8.0]) at 5.7 to 9.0 V cm^{-1} for 2 to 4 h. The gels were stained in a solution of ethidium bromide (2 μg/ml) and visualized with a UV transilluminator (Fotodyne Inc., New Berlin, Wis.).

For Southern blots, the DNAs were transferred onto nylon membranes (ICN Biomedicals, Costa Mesa, Calif., or Bio-Rad Laboratories, Richmond, Calif.) using 0.4 M NaOH. The amplified DNAs immobilized on the ICN nylon membranes were prehybridized with a solution containing 5× SSPE (1× SSPE is 10 mM sodium phosphate [pH 7.0], 0.18 mM NaCl, and 1 mM EDTA), 0.5% sodium dodecyl sulfate SDS, 5% Denhardt's solution, and 100 μg of phenol-extracted, denatured, salmon sperm DNA (Sigma) per ml or 50 μg of baker's yeast tRNA type X (Sigma) per ml. Prehybridization was at 55 to 60°C for 15 to 20 min. After removal of the prehybridization buffer, the membranes were hybridized with fresh hybridization solution containing 200 to 300 ng of denatured radiolabeled gene probe and incubated at 55 to 60°C for 3 to 16 h with gentle shaking. The blots were washed twice in 2× SSPE–0.5% SDS at room temperature for 10 min and once in 0.1× SSPE–0.1% SDS at 55 to 60°C for 3 to 5 min with gentle agitation. To detect ^{32}P-labeled DNAs, the blots were covered with Saran Wrap (Fisher Biochemical, Pittsburgh, Pa.) and Kodak X-AR film (Eastman Kodak Co., Rochester, N.Y.) was placed over the blots. All films were exposed at −70°C for 1 to 48 h and developed with an automatic film developer (AFP Imaging 14 TE X-ray film processor; Packer International, Chicago, Ill.).

The 5' ends of the gene probes were radiolabeled with [γ-^{32}P]ATP (specific activity of >3,000 Ci $mmol^{-1}$) (New England Nuclear Corp., Boston, Mass.) by a modified version of the forward reaction described by Ausubel et al. (3). The 30-μl reaction solution used in this procedure contained 50 mM Tris-Cl [pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (Sigma), 1 mM KCl, 10 μg of oligonucleotide gene probe (120 pmol of [γ-^{32}P]ATP; specific activity of >3,000 Ci $mmol^{-1}$), 1 mM spermidine (disodium salt), and 20 U of T4 polynucleotide kinase (U.S. Biochemical Co., Cleveland, Ohio). The reaction mixture was incubated at 37°C for 1 h, and the radiolabeled probes were purified by using a Sephadex G-50 column and TE buffer (10 mM Tris-Cl [pH 7.6], 1 mM EDTA [disodium salt]) or by using a Centricon 10 microconcentrator (Amicon Corp., Danvers, Mass.).

Specificity testing. To determine the specificity of PCR for *Giardia* and *G. duodenalis* diagnosis, nucleic acids were recovered and subjected to PCR from the following *Giardia* spp.: *G. lamblia* CDC:0284:1, Human-1-Portland, Human-2-Portland, Portland-Beaver, WB, Egyptian, RS, LT, CM,

AB, JH, and N; *Giardia* sp. strains MR-4 and Be-1; *G. intestinalis* New Orleans-1 (ATCC 50137), KS (ATCC 50114), and UNO/04/87/1 (ATCC 50184); *G. cati* UPV:0685:1 (ATCC 50163); *G. muris*; and *G. ardeae*. Nontarget human, protozoan, fungal, algal, and bacterial DNAs were also tested from the following sources: *Acanthamoeba castellani* (ATCC 30011), *Entamoeba histolytica* HM-1 (ATCC 30459) and HK-9 (ATCC 30015), *E. coli* HU-1 (ATCC 30946), *Cryptosporidium parvum*, *Cryptosporidium muris*, *Dientamoeba fragilis* (ATCC 40948), *Blastocystis hominis* (ATCC 50177), *Hexamita inflata* (ATCC 50268), *Trichomonas vaginalis* (ATCC 30001), *Eimeria nieschulzi*, *Chlorella*, *Scenedesmus*, *Chlorococcum*, *E. coli*, *Legionella pneumophila*, *Salmonella typhimurium*, *Shigella*, *P. cepacia*, *P. diminuta*, *P. fluorescens*, *P. aeruginosa*, *Alcaligenes faecalis*, *Xanthomonas maltophilia*, *Saccharomyces cerevisiae*, *Ustilago violacea*, and human DNA.

Sensitivity testing. Single cysts of *G. lamblia* and *G. muris* were collected using a Zeiss Axiovert 35 inverted microscope and a deFonbrune pneumatic micromanipulator. The cysts were transferred to Gene-Amp tubes (Perkin Elmer-Cetus), nucleic acids were released, and PCR was performed with a Perkin Elmer thermal cycler.

Cysts (10 to 1,000) were also added to 100-ml environmental water samples from the Ohio River and to 100-ml potable water samples from the municipal Louisville Water Co. Also, samples concentrated by conventional filtration from 4 to 400 liters of Ohio River water were tested for *Giardia* species. Some of the samples were spiked with 1,000 to 100,000 *Giardia* cysts as positive controls for these environmental sensitivity tests. In these tests, the cysts were collected by filtration with 0.5-μm-pore-size Fluoropore filters. The filters were placed into PCR buffer, and PCR was performed.

RESULTS

Amplification with primers GGL639-658 and GGR789-809 gave a 171-bp PCR amplification product with all *Giardia* sp. tested. No amplification was seen with any of the non-*Giardia* sp. tested. Southern blot analysis using radiolabeled probe GGP 751-776 confirmed that the PCR amplification using these primers was specifically diagnostic for all *Giardia* spp. tested and did not detect non-*Giardia* spp. (Fig. 1).

PCR amplification with primers GGL405-433 and GGR592-622 gave a 218-bp product for *G. duodenalis* which showed hybridization with probe GGP510-537 (Fig. 2). Amplification was achieved with all *G. duodenalis* strains. No 218-bp PCR-amplified product was obtained with *G. muris* or *G. ardeae*, nor with any non-*Giardia* spp. *Giardia* sp. strain Be-1 and *G. cati*, which have been classified within the *G. duodenalis* group (20), showed positive PCR amplification with primers GGL405-433 and GGR592-622. Hence, primers GGL405-433 and GGR592-622 were specific for the *G. duodenalis* group, while primers GGL639-658 and GGR789-809 detected all strains of the genus *Giardia* tested.

Multiplex PCR using both primer sets gives two bands with *G. duodenalis* and one band with *G. muris* or *G. ardeae* (Fig. 3) when amplified DNA is electrophoresed on an agarose gel. This enables the detection of both the genus and the species in a single reaction.

The sensitivity testing showed that when one or more cysts were present PCR amplification of giardin DNA gave a positive signal. In 10 repetitive trials, single *Giardia* cysts recovered by using a micromanipulator gave PCR amplification product, whereas no amplification was observed in blank water controls lacking cysts (Fig. 4).

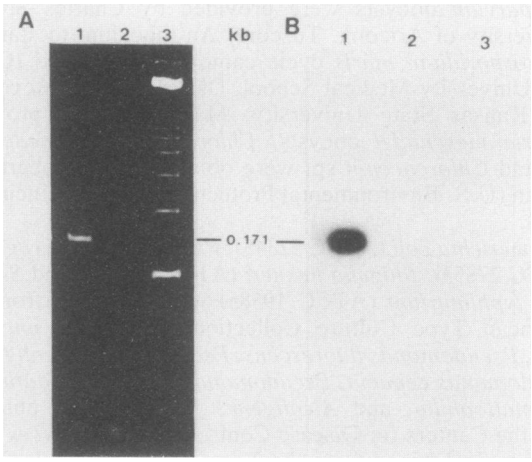

FIG. 1. (A) Ethidium bromide-stained polyacrylamide gel electrophoresis analysis of amplified DNA with primers GGL639-658 and GGR789-809. Lane 1, *G. lamblia*; lane 2, *Cryptosporidium parvum*; lane 3, 123-bp DNA ladder. (B) Southern blot (DNA-DNA) hybridization of the gel in panel A with radiolabeled probe GGP751-776.

PCR detected cysts in 100-ml Ohio River and 100-ml potable water samples at concentrations less than 1 per ml. PCR detected *Giardia* cysts in 4-liter Ohio River samples at a concentration of ≥0.25 cysts per ml. However, the PCR detection of *Giardia* species in the Ohio River samples that had been concentrated from 400 liters was not successful, even when 10^5 cysts were present in the sample.

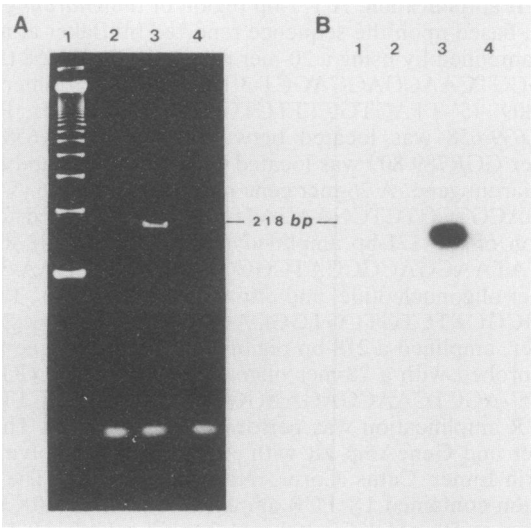

FIG. 2. (A) Ethidium bromide-stained polyacrylamide gel electrophoresis analysis of amplified DNA with primers GGL405-433 and GGR592-622. Lane 1, 123-bp DNA ladder; lane 2, *G. muris*; lane 3, *G. lamblia*; lane 4, *Entamoeba histolytica*. (B) Southern blot (DNA-DNA) hybridization of the gel in panel A with radiolabeled probe GGP510-537.

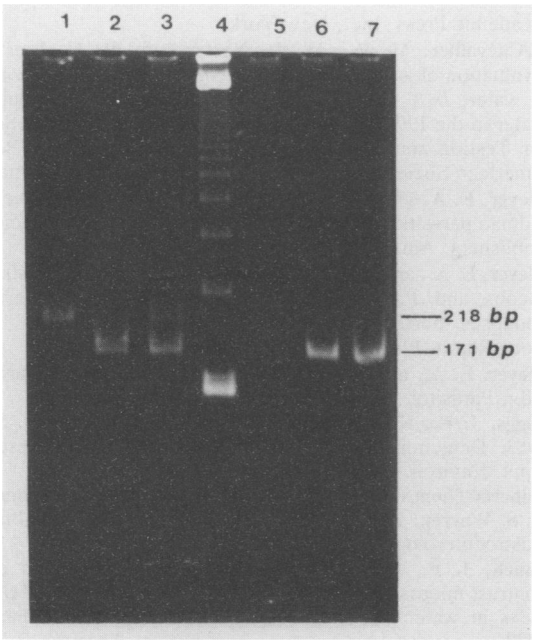

FIG. 3. Ethidium bromide-stained polyacrylamide gel electrophoresis analysis of multiplex amplified DNA. Lane 1, *G. lamblia* amplified with primers GGL405-433 and GGR592-622; lane 2, *G. lamblia* amplified with primers GGL639-658 and GGR789-809; lane 3, *G. lamblia* amplified with both sets of primers used in lanes 1 and 2; lane 4, 123-bp DNA ladder; lane 5, *G. muris* amplified with primers GGL405-433 and GGR592-622; lane 6, *G. muris* amplified with primers GGL639-658 and GGR789-809; lane 7, *G. muris* amplified with both sets of primers used in lanes 5 and 6.

DISCUSSION

The *G. duodenalis* group has been differentiated from *G. muris* on the basis of morphological features (14). *G. ardeae* may be included within the *G. muris* group on the basis of the variable median body feature (2, 18). It may also be classified within the *G. duodenalis* group on the basis of the clawhammer shape of the median bodies. *G. ardeae* shows phenotypic similarities with *G. muris* such as a single caudal flagellum and the deeply notched adhesive disc. The chromosomal migration patterns of *G. ardeae* and *G. muris* have been shown to be distinct from each other and from *G. duodenalis* derived from humans, beavers, and other domestic animals (8, 11). Clearly, classification based on morphological features alone is inadequate for determining the different *Giardia* species.

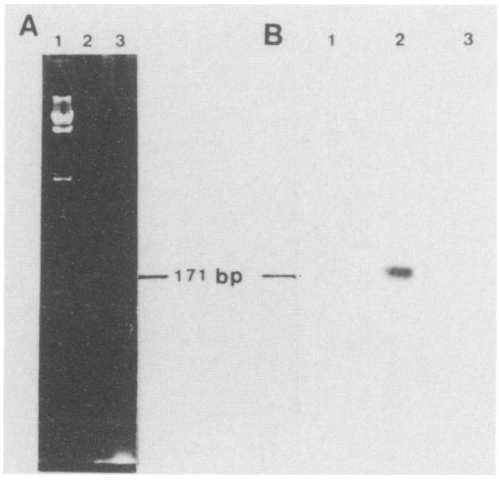

FIG. 4. (A) Ethidium bromide-stained polyacrylamide gel electrophoresis analysis of amplified DNA using primers GGL639-658 and GGR789-809. Lane 1, lambda *Hin*dIII DNA size standards; lane 2, a single *G. muris* cyst; lane 3, no *G. muris cysts*. (B) Southern blot (DNA-DNA) hybridization of the gel in panel A with radiolabeled probe GGP751-776.

The immunofluorescent-antibody technique currently used for detecting *Giardia* species in environmental waters does not distinguish the *G. duodenalis* type from other *Giardia* species. Round to oval objects (8 to 18 μm long by 5 to 15 μm wide) which fluoresce a brilliant apple green are classified as presumptive *Giardia* cysts. Fluorescence microscopy has the additional disadvantage of cross-reactivity of the primary or secondary antibody with other organisms such as algal cells. Confirmed *Giardia* cysts in environmental samples are determined by phase-contrast microscopic observation of internal structures, which may be difficult because debris may obscure the observation of the diagnostic internal structures (19). The sensitivity level of the immunofluorescent antibodies for environmental samples of *Giardia* species is undetermined.

Primers and probes developed in this study can detect *Giardia* specifically and can distinguish the *G. duodenalis* type from other *Giardia* species. The sensitivity of detection is a single *Giardia* cyst, a level of sensitivity suitable for clinical and environmental monitoring of *Giardia* species. Application of these primers and probes to the clinical detection of *Giardia* species has shown promising and reliable results (10a). While we have not tested all possible target *Giardia* and nontarget microorganisms found in water, we have tested sufficient numbers of *Giardia* and non-*Giardia* strains to conclude that the PCR and gene probe detection method can differentiate *Giardia* species and specifically the species relevant to humans in the *G. duodenalis* group.

ACKNOWLEDGMENTS

This study was supported by a cooperative agreement with the U.S. Environmental Protection Agency and by Perkin-Elmer Cetus Corp.

We thank W. B. Wead and Paul Matheson for assistance in recovering single *Giardia* cysts and Cathy M. Bernadino for culturing *Giardia* sp. trophozoites.

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