

Detection of *Giardia* Cysts with a cDNA Probe and Applications to Water Samples

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Giardiasis is the most common human parasite infection in the United States, causing a lengthy course of diarrhea. Transmission of *Giardia* species is by the fecal-oral route, and numerous waterborne outbreaks have been documented. The Environmental Protection Agency has regulated *Giardia* species in drinking water through the Surface Water Treatment Rule. Current methods for detection of *Giardia* species in water rely primarily on microscopic observation of water concentrates with immunofluorescence techniques. We evaluated the efficacy of using a gene-specific probe for the detection of *Giardia* species in water. A cDNA probe, 265 bp long, from the small subunit of rRNA of *Giardia lamblia* was used for detection of cysts. The replicative form of the M13 vector with an insert was isolated from lysed host *Escherichia coli* XL1-Blue and used for production of the cDNA probe by nick translation with ³²P-labeled nucleotides. Six different protocols were tested for extracting nucleic acids from the cysts. With the most efficient procedure, disrupting *Giardia* cysts with glass beads in the presence of proteinase K, as few as 1 to 5 cysts per ml can be detected in water sample concentrates with dot blot hybridization assays.

The flagellated protozoan parasite *Giardia lamblia* causes the most common parasite infection identified in the human population in the United States and the world (3, 16) and causes a watery diarrhea spread by the fecal-oral route. *Giardia* species in the cyst stage are detected in 16 to 41% of fecally contaminated surface waters (7); there has been a dramatic increase in waterborne giardiasis in the United States, with 92 documented outbreaks since 1965 (4) accounting for 19.9% of all waterborne outbreaks since 1978 (1, 4).

The Environmental Protection Agency has been mandated to control *Giardia* contamination in drinking water through revisions to the Safe Drinking Water Act. This has led to promulgation of the Surface Water Treatment Rule, which addresses filtration of surface waters (18). Routine monitoring for *Giardia* species in water has not been recommended, primarily because the methods are inefficient, time consuming, and costly (7, 9).

Large-volume filtration (100 to 400 liters) is currently used for recovery of cysts from water. The filter is then washed to recover the cysts, the wash water is centrifuged, and the resulting pellets are subjected to density gradients or flotations to assist in clarification of the sample before microscopic observation. Methods for cyst detection utilize light microscopy or immunofluorescence microscopic techniques. Limitations include unknown antibody specificity for numerous animal isolates, potential for nonspecific antibody binding (13, 15), interference with cyst detection by contaminating debris, nonconfirmation of a positive sample, time required to examine each sample, problems in obtaining a sufficient representative volume of each sample, need for specialized equipment (epifluorescence microscope), and inability to determine viability of the cysts (7, 13).

A more rapid method for detection of *Giardia* cysts in the environment would enhance our ability to evaluate the

quality of waters. Gene-specific probes have recently been shown to be very useful in the detection of a number of microorganisms. The purpose of this study was to evaluate the potential of a gene-specific probe as a rapid method for *Giardia* cyst detection in water samples and to compare the procedure with immunofluorescence detection.

MATERIALS AND METHODS

Protozoan stocks and immunofluorescence detection. *G. lamblia* and *Giardia muris* were obtained in cyst form; they were generated through passage in gerbils from Swabby Gerbco, Inc. (Diane Swabby, Phoenix, Ariz.). The cysts were received immediately after excretion in a water suspension and stored at 4°C. Cysts were used consistently between 1 and 8 weeks after receipt. For some experiments, cysts were further purified through a cesium chloride step gradient (8). Recovered cysts were suspended in distilled water, washed twice by centrifugation, and finally suspended in distilled water. The unpurified preparation was used for most of the trials. *Giardia* cysts in all samples were identified and counted by using an indirect immunofluorescence procedure on membrane filters (13) with a *Giardia* monoclonal antibody (Meridian Diagnostic Inc., Cincinnati, Ohio) and fluorescein isothiocyanate-labeled, affinity-purified goat anti-mouse immunoglobulins A, G, and M (heavy and light chains) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.). Epifluorescence was observed with a BHTu epifluorescence microscope (Olympus, New Hyde Park, N.Y.). Trophozoites of *Giardia duodenalis* isolated from humans and beavers and an isolate from a great blue heron (5) were generously provided by Stanley L. Erlandson, University of Minnesota, Minneapolis.

Treatments for the liberation of nucleic acids from *Giardia* cysts. Six methods were tested to determine the most efficient procedure for extracting nucleic acids from *G. lamblia* cysts. The same stock of purified and unpurified cysts was used for each method. At the beginning of each procedure, a

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1-ml sample from a purified cyst preparation and a second 1-ml sample from an unpurified cyst preparation were counted by using an immunofluorescence procedure, and the cyst concentrations were adjusted to approximately 10^4 cysts per ml with distilled water. These samples were then diluted from 10^{-1} to 10^{-4} (0.1 ml of sample to 0.9 ml of distilled water), and the original sample and each dilution were extracted by using one of the six procedures. This was repeated two to three times for each of the extraction methods. The resulting *Giardia* nucleic acid extracts were boiled for 10 min and then spotted under vacuum with a dot blot apparatus (BRL, Gaithersburg, Md.) onto nylon membranes (Gene Screen Plus; DuPont, Boston, Mass.), air dried, and baked in a vacuum oven (Napco, Portland, Oreg.) for 2 h at 80°C.

(i) **Enzyme digestion of *Giardia* cysts.** Cellulase (6.7 U/mg; Sigma, St. Louis, Mo.) was added to a concentration of 12.5 mg/ml, and collagenase (0.28 U/mg; Boehringer Mannheim, Indianapolis, Ind.) was added, to a concentration of 2.5 mg/ml, to the cyst suspensions. The cysts were digested for 4 h at 45°C, and then proteinase K (14.3 U/mg; Sigma) was added to the suspensions to a concentration of 80 µg/ml; the cysts were then incubated for 30 min at 65°C.

(ii) **Baking.** Dilutions (1:10) of the *Giardia* cyst suspension were directly spotted onto a nylon membrane, air dried, and baked in a vacuum oven for 2 h at 80°C.

(iii) **Phenol-chloroform extraction.** Standard procedures for phenol-chloroform extraction were performed on purified and unpurified cyst preparations (10). An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) was added to the *Giardia* suspensions, and the suspensions were mixed thoroughly and centrifuged in a microspin centrifuge (model 24 S; Sorvall, Boston, Mass.) for 3 min. Next, chloroform-isoamyl alcohol (24:1, vol/vol) was added to the aqueous phase and thoroughly mixed. This was repeated to ensure removal of phenol from the aqueous phase.

(iv) **Deionized formamide extraction.** The *Giardia* cyst suspension was heated to 65°C, and then an equal volume of hot deionized formamide (65°C) was added. The solution was mixed for 2 min, boiled for 10 min, and centrifuged in a microcentrifuge for 1 min. The total volume was recovered and spotted.

(v) **French press.** One milliliter of an ice-cold *Giardia* cyst suspension was passed through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in² three times to disrupt the cysts. The suspension was then boiled and spotted onto the membrane.

(vi) **Glass bead method.** Glass beads (212 to 300 µm; Sigma) were washed for 5 min in 50 ml of commercial bleach, rinsed with distilled water, and then treated with 1 M hydrochloric acid for 5 min. These washing steps were repeated twice. The beads were washed in 95% ethanol for 5 min twice and then rinsed with distilled water. The glass beads were stored in Tris-EDTA buffer (pH 7.5) until use.

The treated glass beads were added to 1 ml of the *Giardia* cyst suspension (200 µl, approximately 0.2 g), and proteinase K (Boehringer Mannheim) was added to a concentration of 100 µg/ml. Mixtures of cysts, glass beads, and proteinase K were agitated by attaching microfuge tubes to the blade of an electric jigsaw (Sears and Roebuck, Chicago, Ill.), which was operated for 10 min at full power to disrupt *Giardia* cysts in the sample. Afterward, samples were incubated for 30 min at 65°C and centrifuged in a microcentrifuge for 1 min. The pellet of debris and glass beads was discarded; the supernatant was collected, boiled, and spotted onto a nylon membrane.

Reaction of the *Giardia*-specific cDNA probe with a variety of microorganisms. The *Giardia*-specific cDNA probe was tested for cross-hybridization with nucleic acids from other microorganisms (protozoa, helminths, and bacteria) that might be found in water, including other enteric protozoa such as *Entamoeba* and *Cryptosporidium* species or bacteria commonly found in polluted waters such as *Escherichia coli*. The following microorganisms (at a concentration of approximately 10^4 cells per ml) were treated by the glass bead method, and the resulting nucleic acids were treated as previously described: *Cryptosporidium muris* (oocysts), *Cryptosporidium parvum* (oocysts), *Entamoeba histolytica* (cysts), *Trichomonas vaginalis* (trophozoites), *Trichomonas foetus* (trophozoites), *Saccharomyces cerevisiae*, *Candida albicans*, *Ascaris lumbricoides* (ova), *Ascaris suum* (ova), *Hymenolepis nana* (ova), *Clonorchis* species (ova), hookworm (egg), *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.), *E. coli* ATCC 15597, and *Pseudomonas aeruginosa* ATCC 27853.

Preparation of cDNA. The cDNA probe was a 265-bp fragment (between base pairs 636 and 900) of the small subunit 16S-like rRNA of the protozoan *G. lamblia* (14). This cDNA clone was generously provided by Mitchell L. Sogin, National Jewish Center, Denver, Colo.). The fragment of rRNA-cDNA was an M13mp18 clone at the *KpnI* site, and the replicative form (double-stranded circular form) of M13mp18 with the insert was isolated and purified from lysed host *E. coli* XL1-Blue by banding in a cesium chloride-ethidium bromide gradient by the method of Maniatis et al. (10). The M13 vector with the insert or the excised 265-bp cDNA segment was used for production of the probes. The cDNA was labeled with both [³²P]dATP and [³²P]dCTP (specific activity, 3,000 Ci/mmol; New England Nuclear) by nick translation (12) to a specific activity of 1.0×10^9 cpm/µg of DNA. The labeled cDNA was purified by chromatography through a Sephadex G-50 column (Pharmacia, Piscataway, N.J.) and denatured by heating for 10 min in a boiling water bath.

Hybridization procedure. After the samples were spotted, the membrane was air dried and baked in a vacuum oven for 2 h at 80°C to fix the nucleic acid on the membrane. Prehybridization conditions were with constant agitation in a Seal-a-Meal bag (Dazey Corp., Industrial Park, Kans.) for 3 h at 52°C. Prehybridization buffer contained 50% deionized formamide, 1% sodium dodecyl sulfate, 5% dextran sulfate, 5× SSPE buffer (0.75 M NaCl, 0.05 M NaH₂PO₄, 5 mM EDTA), and 45 µg of sheared, denatured salmon sperm DNA per ml (all ingredients from Sigma) (final pH, 7.4). In the hybridization solution the concentration of deionized formamide was lowered to 45% and the salmon sperm DNA concentration was decreased to 2 µg/ml. Between 10 and 20 ng of ³²P-labeled probe (average specific activity, 1.0×10^9 cpm/µg of DNA) was added to the sealable plastic bag, and the preparation was hybridized for 36 h at 52°C (17).

Quality control for hybridization assays of environmental samples. To evaluate and eliminate false-positive signals in hybridization assays, *Giardia*-positive and -negative samples were assayed along with environmental samples. Cysts were filtered out from *Giardia*-contaminated gerbil fecal material by a membrane containing mixed esters of cellulose (5.0-µm pore size; Nuclepore Corp., Pleasanton, Calif.). This filtrate and *Giardia*-negative environmental samples, determined with the immunofluorescence method, were used as negative controls. All solutions and diluents utilized during the processing of samples were tested for possible nonspecific binding of the cDNA probe. Solutions were spotted onto mem-

TABLE 1. Comparison of different treatments for the liberation of nucleic acids from *Giardia* cysts for hybridization assays

Sample treatment	Detection of the indicated no. of cysts reacting with the cDNA probe									
	Purified ^a					Unpurified ^b				
	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
Enzyme digestion	-	-	-	-	-	-	-	-	-	-
Baking of sample	+	+	+	-	-	-	-	-	-	-
Phenol-chloroform	+	+	-	-	-	+	+	-	-	-
Formamide	+	+	+	+	+	+	+	+	+	+
French press	+	-	-	-	-	-	-	-	-	-
Glass bead method	+	+	+	+	+	+	+	+	+	+

^a Cysts were purified on a cesium chloride gradient.

^b Cysts from gerbil feces.

branes at two times the volume used for processing environmental samples. The cDNA fragment within the vector (10 to 0.1 ng) was used as a positive control.

Environmental pellets (stored in 3.7% formaldehyde) recovered from secondarily treated sewage and river waters were processed by the glass bead extraction method and hybridized with the gene-specific probe as described above.

Autoradiography. Positive hybridized signals were detected by autoradiography. This was carried out at -70°C by exposure to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a Lightning-Plus intensifying screen (DuPont, Wilmington, Del.) for 24 to 48 h.

RESULTS AND DISCUSSION

The *Giardia* cyst is the infectious stage, and the development of approaches for the use of gene-specific probes in environmental samples necessitates the development of efficient methods for extraction of nucleic acids directly from cysts. Procedures that bypass the purification steps of environmental concentrates would be advantageous because they would reduce cyst losses and sample preparation time.

The data outlined in Table 1 show the efficiencies of the six different treatments for liberating or exposing the nucleic acid directly from the cysts for assay by the gene-specific probe method.

The enzyme digestion method for disrupting *Giardia* cysts was inefficient for extraction of nucleic acids as evaluated by the hybridization assay. No positive signals were detected with this method. Examination of these enzyme-digested cysts by immunofluorescence microscopy revealed that 80% of the cysts were opened up by a combination of cellulose, collagenase, and proteinase K enzymes. The inefficiency of nucleic acid extraction may be due to the presence of contaminating RNases in fecal material, and the impurity of the cellulase and collagenase may have contributed to degradation of nucleic acids released from the cysts during the long incubation (4 h) required for complete digestion of cyst cell walls.

Although the baking procedure was the easiest, it would not be applicable to unpurified samples. Water concentrates that contain fecal material or other debris would not efficiently filter through a nylon membrane. We also noticed that during prehybridization the accumulated material on the membrane came off and floated in the solution, resulting in loss of the sample. The sensitivity obtained with this method was 10^2 cysts per ml in the purified sample.

The direct phenol-chloroform extraction was capable of

detecting 10^3 cysts per ml in the purified cyst samples. However, for unpurified samples, the complete extraction had to be repeated two to three times to ensure removal of the denatured proteins. This tedious procedure resulted in a greater loss of the nucleic acids. Also, any residual phenol-chloroform in the aqueous sample causes shrinking and melting of the nylon membrane as well as damage to the dot blot apparatus.

Between 1 and 5 cysts per ml were detected from both purified and unpurified cyst preparations when hot, deionized formamide was used for extraction of nucleic acids. The nylon membrane and dot blot apparatus were not affected by the 50% formamide present in the sample. Therefore, the nucleic acid-enriched supernatant recovered from purified or unpurified samples was spotted directly on the hybridization membrane. This method of sample preparation was efficient and quick compared with the phenol-chloroform extraction method. The efficiency of the extraction method for either purified or unpurified cyst preparations was similar as determined with the hybridization assay; however, the procedure was inconsistent, occasionally resulting in sensitivities of 10 to 100 cysts per ml.

The French press method detected 10^3 purified cysts per ml. This method was time consuming, and approximately one-third of the 1-ml sample volume was lost during processing.

Disruption of *Giardia* cysts by vigorous agitation with glass beads resulted in a consistent and reproducible method for extracting cyst nucleic acids in purified and unpurified *Giardia* cyst preparations, with a detection limit of 1 to 5 cysts per ml. This extraction method would eliminate the purification steps for processing water samples, which offers a distinct advantage over the current methods for microscopic detection of *Giardia* cysts in water.

The result of dot blot hybridization assays between the *Giardia*-specific cDNA probe and nucleic acids from other microorganisms listed in Materials and Methods indicated that there was no detectable cross-hybridization between this cDNA probe and the nucleic acids of the microorganisms. It would be very time consuming to test every type of microorganism. This brief evaluation, however, demonstrates that the stringency of the hybridization conditions used are adequate to prevent false-positive results. The cDNA probe in our study was derived from a segment of the 16S-like rRNA and has a G+C content of approximately 69%. This probe has a melting temperature of 74°C in the hybridization buffer used (17), and the stringency used in the hybridization reactions ($T_m - 22^{\circ}\text{C}$) was designed to minimize annealing to nontarget sequences (2, 11, 19).

The specificity of the cDNA probe within the genus *Giardia* was examined. Cysts from *G. lamblia*, the species that infects humans, and from *G. muris*, the species that infects mice, were reacted with the probe by using the formamide and glass bead procedures. Triplicate dot blots were spotted with nucleic acid from *G. lamblia* and *G. muris*, and each was hybridized to one of the following: a ³²P-labeled isolated *Giardia* cDNA segment, ³²P-labeled M13mp18 with the *Giardia* segment and the vector alone, or ³²P-labeled M13mp18. The results for both extraction procedures were the same. The M13mp18 vector (phage DNA) did not hybridize to *Giardia* nucleic acid, but both species reacted with the probe with equal sensitivities (Table 2; Fig. 1).

Different *Giardia* isolates from the trophozoite stage from humans, beavers, and a bird were also examined. The cDNA probe reacted with all isolates but with various sensitivities.

TABLE 2. Reaction of the cDNA probe with nucleic acids from *Giardia* cysts^a

Probe	Species	Detection of the indicated no. of cysts reacting with the cDNA probe				
		10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
Segment alone	<i>G. lamblia</i>	+	+	+	+	+
	<i>G. muris</i>	+	+	+	+	+
Segment and vector	<i>G. lamblia</i>	+	+	+	+	+
	<i>G. muris</i>	+	+	+	+	+
Vector alone	<i>G. lamblia</i>	-	-	-	-	-
	<i>G. muris</i>	-	-	-	-	-

^a Cysts were treated with the glass bead method.

Generally the probe was able to detect 100 trophozoites (Table 3). The highest degree of sensitivity was obtained with extracts of trophozoites from two human isolates. The sensitivity obtained from the bird isolate was the lowest, 100-fold less than that from the human isolates.

Species classification within the genus *Giardia* is ill defined. *Giardia* was classified into three species in 1952 (6), *G. muris*, found in rodents; *G. agilis*, found in amphibians; and

TABLE 3. Reaction of the *Giardia*-specific probe with different isolates of trophozoites^a

<i>Giardia</i> isolate	Detection of the indicated no. of trophozoites reacting with the cDNA probe				
	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
Human isolate 1	+	+	+	+	-
Human isolate 2	+	+	+	-	-
Human isolate 3	+	+	+	+	-
Human isolate 4	+	+	+	-	-
Beaver isolate 1	+	+	+	-	-
Beaver isolate 2	+	+	+	-	-
Bird isolate 1	+	+	-	-	-

^a Nucleic acids were extracted with the glass bead procedure. The isolates from humans and beavers are *G. duodenalis*, and the isolate from the bird is *G. ardeae* (5).

G. duodenalis, found in mammals. The species from humans is also known as *G. duodenalis* or *G. lamblia*. A new species from birds, *G. ardeae*, has been described (5). The genetic diversity within the genus *Giardia* is presently unknown. Probes that are specific to isolates capable of initiating human infections may be desirable for evaluating human health hazards. However, for evaluating water treatment efficiency, it may be more desirable to detect any cyst that passes the treatment barriers.

Four environmental concentrates from secondary or tertiary treated sewage or surface waters were screened for *Giardia* contamination by using an immunofluorescence technique and a gene-specific probe method. The sewage was domestic wastewater originating from a community of 600,000 persons. The surface waters were rivers with no sewage discharges. A 1-ml sample of the pellet recovered from the filters and/or 1 ml of the pellet remaining after clarification by potassium citrate density gradient centrifugation was assayed with the cDNA probe. Samples and 10-fold dilutions were subjected to the glass bead procedure described above. A monoclonal antibody directed against *Giardia* cysts (Meridian Diagnostics) was used in an indirect immunofluorescence procedure to stain samples after clarification and filtration through membrane filters (13-mm diameter, 5.0- μ m pore size) (13). Filters were examined by using epifluorescence, and cysts were enumerated.

Between 100 and 400 liters of water was collected originally, and the equivalent of 0.2 to 226 liters of the concentrated pellet was examined. For the cDNA probe the equivalent volume equal to 1 ml of pellet was processed along with 10-fold dilutions. For immunofluorescence detection, between 2 and 10 ml of pellet was processed and examined, representing between 40 and 300 liters of original sample. Positive results were observed for all wastewater samples and in one surface water sample (Table 4). Positive signals were observed in samples without flotations at dilutions 10-fold greater than those in which positive signals were observed after flotation. It appeared that gene probe detection was slightly more sensitive than microscopic detection of *Giardia* cysts for wastewater samples. In one surface water sample no positive results were found with the cDNA probe or with immunofluorescence; to obtain a volume equivalent to that used for the immunofluorescence assay, 10 1-ml replicate samples would need to be processed for the gene-specific probe assay.

In conclusion, we have developed a simple and efficient method for extraction of nucleic acid directly from *Giardia* cysts for dot blot hybridization and detection with ribosomal

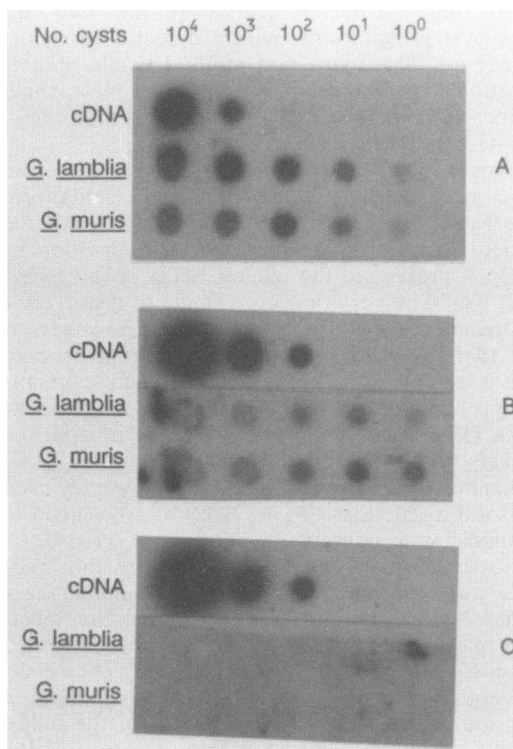


FIG. 1. Autoradiogram of dot blot analysis of possible cross-hybridization of M13 vector with *Giardia* target nucleic acids. (A) M13 vector with the *Giardia* insert used as the probe. The cDNA probe (0.1 to 10 ng) was used as a positive control. The specificity and sensitivity of the cDNA probe were the same for *G. lamblia* and *G. muris*. (B) *Giardia* segment alone used as the probe. The positive signals indicated the same sensitivity and specificity for *G. lamblia* and *G. muris* for this probe. (C) M13 vector used as the probe. The lack of signals was an indication of no cross-hybridization between the M13 probe and *Giardia* nucleic acids.

TABLE 4. Detection of *Giardia* species in water concentrates by immunofluorescence microscopy and cDNA hybridization

Sample	Reaction with the probe/equivalent volume examined (liters) ^a	No. of cysts detected by immunofluorescence/equivalent volume examined (liters)
Sewage effluent 1 ^b	+/18.9, +/1.9, +/0.2	ND ^c
Sewage effluent 1 (floated) ^d	+/27, +/2.7, -/0.3	21/56
Sewage effluent 2 ^c	+/23.6, +/2.4, -/0.2	1/47
Surface water 1	+/19, -/2	ND ^c
Surface water 1 (floated) ^d	-/100	1/143
Surface water 2	-/23	ND ^c
Surface water 2 (floated) ^d	-/130	0/261
Negative control ^f	-/0.01	0/0.01

^a Equivalent volume collected per the volume of pellet concentrate examined.

^b Secondary treatment.

^c ND, Not determined.

^d Sample clarified by using potassium citrate density gradients.

^e Tertiary treatment.

^f Cysts were filtered out from gerbil fecal material.

cDNA probes. The extraction procedure enables us to process samples contaminated with *Giardia* species rapidly and also eliminates the clarification steps required for microscopic immunofluorescence techniques. The sensitivity of the gene probe technique is between 1 and 5 cysts in 1 ml of concentrated samples. The use of a ribosomal cDNA probe makes it possible to detect the low numbers of *Giardia* cysts that are commonly found in water because of the high copy number of rRNA in each of the two trophozoites per cyst; this method appears comparable to the immunofluorescence system for cyst detection in environmental samples. Although neither the cDNA probe nor the immunofluorescence system can be used to determine cyst viability, it may be feasible to develop gene-specific probes to routinely monitor *Giardia* cyst contamination in water supplies, particularly as new nonradioactive labels are developed for nucleic acid probes.

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