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Polymerase Chain Reaction-Gene Probe Detection of Microorganisms by Using Filter-Concentrated Samples

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To detect low levels of microorganisms in environmental samples by using polymerase chain reaction (PCR)-gene probe detection, samples were concentrated by filtration. Fluoropore (Millipore Corp.) filters were compatible with PCR DNA amplification, whereas various other filters including nitrocellulose and cellulose acetate filters inhibited PCR amplification. By concentrating cells on Fluoropore filters and releasing the DNA by freeze-thaw cycling, PCR DNA amplification could be performed without removing the filter. Concentration with Fluoropore FHLP and FGLP filters permitted the detection of single cells of microorganisms in 100-ml samples by PCR-gene probes.

Environmental monitoring of microorganisms to detect potential sources of pathogens for preventative public health and epidemiological purposes requires a high degree of sensitivity. Often concentrations of pathogens and indicator microorganisms of less than 1/ml must be detected in water and air samples to provide adequate protective surveillance. U.S. federal regulations, for example, require that the concentration of coliform bacteria in potable waters be less than 1/100 ml in at least 95% of the water samples tested (1, 13) so that there is a statistical safety margin that water supplies will not serve as a conduit for waterborne disease transmission. Even lower concentrations of waterborne pathogens must be detected if direct detection methods are employed. For example, to ensure that water does not contain the protozoan pathogen *Giardia duodenalis*, 100- to 1,000-gal (1 gal = 3.785 liters) samples are analyzed (17, 19, 25).

Filtration methods typically are used to concentrate microorganisms for analyses requiring detection of <1 microorganism per ml (8, 16). Analysis often is completed by placing a filter directly on a nutritive medium and observing the growth of microorganisms on the filter (7, 11, 20) or by direct counting of cells on the filter which may be stained with specific fluorescent antibody stains (1, 9, 13, 22, 23, 26, 28). Conventionally, a series of biochemical tests are done for specific identification of an indicator microbe or a microbial pathogen, which take several days to perform (18). Even when the detection requirements are >1 microorganism per ml, such as for monitoring cooling towers for *Legionella* species, volumes of water of >1 ml usually are analyzed (1, 10). In some cases, large volumes are directly analyzed or samples are concentrated by centrifugation (12, 14).

We have been developing gene probe methods for the detection of microorganisms in environmental samples using the polymerase chain reaction (PCR) (27) to amplify the DNA from target genes (2, 5, 6, 21) so that specific and sensitive detection can be accomplished rapidly. If PCR-gene probe methods are to be applied for environmental monitoring of microorganisms, such as *Escherichia coli*, *Giardia* species, or other pathogens, it is essential that even a single target gene be detected in samples of 100 ml or even ≥100 gal. While PCR permits detection of single cells in a sample, environmental samples must be concentrated to detect concentrations of microorganisms of <1/ml. Target genes must not be lost or destroyed during the concentration of cells from the environment, and substances used in the concentration procedure must not interfere with DNA amplification or detection. In this communication, we report on the development of filtration methods compatible with PCR detection of microorganisms in environmental waters.

MATERIALS AND METHODS

Bacterial cell recovery by filtration. Serially diluted bacterial cells in 100 ml of dechlorinated (treated with 0.1% [wt/vol] sodium thiosulfate [Sigma]) potable water were filtered through various 13-mm-diameter filters by using a Millipore filter manifold with a vacuum pressure of 17 to 20 lb/in^2. The following bacterial species were used in various tests: *E. coli* ATCC 11775 and ATCC 12575, *Shigella flexneri* ATCC 12022, *Salmonella typhimurium* ATCC 19585, *Klebsiella pneumoniae* ATCC 13883, *Citrobacter freundii* ATCC 33128, *Enterobacter cloacae* ATCC 13047, *Enterobacter aerogenes* ATCC 13048, *Legionella pneumophila* CDC (serogroup 1), *Legionella bozemanii* CDC, and 16 environmental isolates of *E. coli*. Depending on the concentration of the bacterial cells in a sample (1 to 10^6), filtering time varied between 15 and 40 min.

DNA release from collected bacterial cells. A 100-μl sample of diethyl pyrocarbonate (Sigma)-treated autoclaved sterile water was added to each tube, and the bacterial cells collected by filtration were subjected to six cycles of freeze-thaw lysis. The gentle release of total genomic DNA from bacterial cells by freeze-thaw lysis in the presence of lysozyme has been described by Grossman and Ron (15) and Ron et al. (24). In our experiments, each sample was frozen in an ethanol-dry ice bath for 1 min and then thawed by transferring it to a 50°C water bath for 1 min without lysozyme. The samples were vortexed vigorously for 10 to 15 s after every two cycles of freeze-thawing to release the cells or nucleic acids from the filter surface. The samples were then transferred to a DNA Thermal Cycler (Perkin-Elmer Cetus) and heated to 85°C for 3 to 5 min to inactivate proteases and

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nucleases to protect the Ampli*Taq* DNA polymerase enzyme, primers, or target nucleic acids from possible destruction or damage. The samples were then cooled to room temperature and spun for 2 to 3 s in an Eppendorf microcentrifuge to collect any condensation from heating the samples. The PCR mix was added to each sample, and primer-directed amplification of the target DNA was performed without further purification.

To determine the efficiency of the freeze-thaw lysis method, an exponentially growing *E. coli* culture (10^5 cells per ml) was centrifuged and washed once in sterile water, and the cells were resuspended in 100 μl of diethyl pyrocarbonate-treated dechlorinated autoclaved distilled water. Aliquots (0.1 ml) of the resuspended samples were subjected to up to 10 freeze-thaw cycles in an ethanol-dry ice bath and 50°C water bath. After each freeze-thaw cycle, three of the aliquots were separated. One of the aliquots was plated on nutrient agar and grown for 48 h at 37°C to determine viable CFU. The second aliquot was used for acridine orange direct count (9) determination of total cell number. The third aliquot was used for PCR amplification. Initial cell number in the culture was determined from the number of colonies which appeared on the plate prior to freeze-thaw cycles. Percent cell lysis was calculated from the initial number of colonies appearing on the agar plates (or determined by direct microscopic observation) at zero freeze-thaw cycle − number of colonies appearing on the agar plates (or determined by direct microscopic observation) after a given freeze-thaw cycle/initial number of colonies appearing on the agar plates (or determined by direct microscopic observation) at zero freeze-thaw cycle × 100. All tests were performed in triplicate.

PCR amplifications. For detection of total coliform bacteria, a 264-bp region located closer to the amino-terminal end of the *lacZ* gene of *E. coli* was amplified by using two 24-mer primers, LZL-389 (5′-ATGAAAGCTGGCTACAGGAAGG CC-3′) and LZR-653 (5′-GGTTTATGCAGCAACGAGACG TCA-3′), as previously reported by Bej et al. (6). A 346-bp segment of the coding region of the *lamB* gene of *E. coli* was amplified by using two 24-mer primers, LBL-445 (5′-CTG ATCGAATGGCTGCCAGGCTCC-3′) and LBR-790 (5′-CA ACCAGACGATAGTTATCACGCA-3′), for the detection of *E. coli* and other enteric pathogens such as *Salmonella* and *Shigella* species (6). For more specific detection of *E. coli* and *Shigella* species, 154 bp of the regulatory region of *uidA* gene, designated *uidR*, which is located upstream of the *uidA* structural gene, were amplified by using the 22-mer primers URL-301 (5′-TGTTACGTCCTGTAGAAAGCCC-3′) and URR-432 (5′-AAAACTGCCTGGCACAGCAATT-3′) (4). Triplex PCR amplification was performed with primers for the *lacZ*, *lamB*, and *uidR* genes of *E. coli*. In triplex PCR amplification, typically 1 ng of purified genomic DNA of *E. coli* was amplified with equimolar quantities (0.5 μM) of each of the three sets of *lacZ*, *lamB*, and *uidR* primers at a annealing temperature of 60°C for a total of 30 cycles.

For detection of the total genus *Legionella*, a 104-bp region of the 5S rRNA gene was amplified by using two 20-mer primers, *L*5SR9 (5′-ACTATAGCGATTTGGAAC CA-3′) and *L*5SR93 (5′-GCGATGACCTACTTTCGCAT-3′) (21). A 650-bp macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* was amplified with two 21-mer primers, *Lmip*L920 (5′-GCTACAGACAAGGATAAGTTG-3′) and *Lmip*R1548 (5′-GTTTTGTATGACTTTAATTCA-3′), for detection of all serogroups of *L. pneumophila* (21). For the detection of genus *Legionella* and/or *L. pneumophila*, a duplex amplification with *Lmip*L920 and *Lmip*R1548 for the *mip* gene and *L*5SL9 and *L*5SR93 for the 5S rRNA gene as targets was performed. The *mip* and 5S rRNA primer sets were used in a ratio of 5:1, respectively, when duplex amplifications were performed.

A total volume of 150 μl of PCR mix containing 15 μl of 10× reaction buffer (500 mM Tris-Cl [pH 8.9], 500 mM KCl, 25 mM [in some cases up to 80 mM] $MgCl_2$), 24 μl of deoxynucleoside triphosphate (dNTP) mix (final concentration of 200 μM each dNTP) (Perkin-Elmer Cetus), 0.2 to 1.0 μM each primer, and 5 U (1 μl) of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) was added to each tube. The samples were spun again for 2 to 3 s to collect any liquid on the wall or on the cap of the tube. Sterile mineral oil (80 μl) (Sigma) was added at the top of the sample. To prevent contact between the mineral oil and the filter, the edge of the filter was folded with a sterile needle and submerged into the aqueous phase before adding the mineral oil.

The target DNA was amplified in a DNA Thermal Cycler (Perkin-Elmer Cetus), using initial denaturation of the target DNA at 95°C for 3 min and then 25 to 45 cycles (depending on the expected number of target bacteria in water samples) of three-step PCR amplifications consisting of denaturation at 94°C for 1 min and primer reannealing at 60°C (for *E. coli*) or 45°C (for *Legionella* species) for 1 min and primer extension at 72°C for 1 min. The samples were kept at 72°C for 3 min at the end of the amplification cycles to complete the extension and to make sure that the amplified DNAs were double stranded.

Generally, PCR was run with the filter used to collect cells present in the PCR. Each filter was folded and transferred with sterile forceps into a 0.5-ml GeneAmp (Perkin-Elmer Cetus) tube with the cell-coated surface facing inside. In some tests, the cells were washed from the filters and the filters were removed before PCR. The Teflon Fluoropore filters FHLP and FGLP (Millipore Corp.) were soaked in ethanol, and all other filters including FHLC were soaked in sterile water until they became transparent (<1 min). Each filter was then transferred to a Swinnex filter holder (Millipore) for filtration. For Fluoropore FHLP 0013 and 0025 and FGLP 0013 and 0025 filters, the laminated surfaces and for polycarbonate filters the shiny surfaces were used for collecting the bacterial cells. The hydrophobic Durapore HVHP and GVHP filters (Millipore) and several other hybridization nylon and nitrocellulose membranes from various sources (Table 1) were also treated with 1× or 10× Denhardt's solution (100× Denhardt's solution contained 2% Ficoll [molecular weight, 400,000], 2% polyvinylpyrrolidone [molecular weight, 360,000], and 2% bovine serum albumin [nuclease free]) (3) at 45°C for 1 h with gentle shaking, washed with Tris-EDTA buffer (pH 8.0) (3), and used for the filtration of the samples containing bacterial cells.

The filters which failed to amplify for target cells or purified target DNA were treated with the following chemicals in an attempt to block target DNA or primers from binding to the filters: yeast tRNA (Sigma) (1 μg/μl, 37°C, 1 to 4 h); phenol-extracted salmon sperm DNA (Sigma) (1 μg/μl, 37°C, 1 to 4 h); bovine serum albumin type V (Sigma) (5% at 60°C for 15 min); Nonidet P-40 (0.01% at 25°C for 30 min); Tween 20 (0.01% at 25°C for 30 min); Nonidet P-40 plus Tween 20 (0.01% each at 25°C for 30 min); sodium dodecyl sulfate (SDS) (0.001% at 25°C for 30 min); Denhardt's solution (1× or 10× at 42°C for 30 min).

Besides serially diluted bacterial cells, various concentrations of purified genomic DNA (1 μg to 1 ag) (following the procedure described by Ausubel et al. [3]) either from *E. coli* or from *L. pneumophila* were added to GeneAmp reaction

TABLE 1. PCR amplification in the presence of various filters[a]

Filter				Intensity of PCR products[b]							
				Purified genomic DNA[c]				No. of cells/100 ml[d]			
Product	Material	Pore size (μm)	Manufacturer	1 ng	1 pg	1 fg	1 ag	10^6	10^3	10^2	10^1
Fluoropore FHLP	PTFE[e], hydrophobic	0.5	Millipore	+++	++	+	−	+++	+++	++	+
Fluoropore FGLP	PTFE (bonded to high-density polyethylene), hydrophobic	0.2	Millipore	+++	++	+	−	+++	+++	+++	++
Fluoropore FHLC	PTFE, hydrophilic	0.5	Millipore	++	+	−	−	++	+	−	−
Fluoropore FHUP	PTFE, unlaminated, hydrophobic	0.5	Millipore	−	−	−	−	−	−	−	−
Durapore HVLP	Polyvinylidene difluoride, hydrophilic	0.5	Millipore	+	−	−	−	+	−	−	−
Durapore GVHP[f]	Polyvinylidene difluoride, hydrophilic	0.2	Millipore	++	+	−	−	+++	++	+	−
Durapore HVHP[f]	Polyvinylidene difluoride, hydrophilic	0.4	Millipore	++	+	−	−	++	+	−	−
MF, type HAWP	Mixed esters of cellulose	0.45	Millipore	−	−	−	−	−	−	−	−
MF, Type PHWP	Mixed esters of cellulose, hydrophilic	0.3	Millipore	−	−	−	−	−	−	−	−
MF, type GSWP	Mixed esters of cellulose, hydrophilic	0.22	Millipore	−	−	−	−	−	−	−	−
Isopore HTTP	Polycarbonate, hydrophilic	0.4	Millipore	−	−	−	−	−	−	−	−
Polycarbonate PVPC	Hydrophilic	0.4–0.45	Nuclepore	+	−	−	−	+	−	−	−
Polycarbonate PCTE	Hydrophilic, PVP free	0.4	Poretics	−	−	−	−	−	−	−	−
Silver AG45	Pure silver metal, hydrophilic	0.45	Millipore	−	−	−	−	−	−	−	−
Flotronics FM-45	Silver metal	0.45	Selas	−	−	−	−	−	−	−	−
Immobilon N[f]	Nylon	0.45	Millipore	−	−	−	−	−	−	−	−
Hybond N[f]	Nylon	0.45	Amersham	−	−	−	−	−	−	−	−
Biotrans N[f]	Nylon	0.2	ICN	−	−	−	−	−	−	−	−
Zetaprobe[f]	Nylon	0.45	Bio-Rad	−	−	−	−	−	−	−	−
Bio-Dot[f]	Nitrocellulose	0.45	Bio-Rad	−	−	−	−	−	−	−	−

[a] Identical results were found for *L. pneumophila* and *E. coli*.
[b] −, no detectable signal; +, faintly detectable signal; ++, moderate signal; +++, intense signal.
[c] Amount of genomic DNA initially calculated from spectrophotometric analysis followed by serial dilution; data represent maximum number of occurrences in a total of 30 sets of samples.
[d] As determined by viable plate counts and acridine orange direct counts (9); data represent maximum number of occurrences in a total of 30 sets of samples.
[e] Polytetrafluoroethylene bonded to high-density polyethylene.
[f] Membranes treated with 1× or 10× Denhardt's solution prior to filtration.

tubes (Perkin-Elmer Cetus), and PCR amplifications were performed in the presence of each type of filter to determine the sensitivity of PCR detection.

Detection of amplified DNAs. Typically, 1/10th of each of the total PCR-amplified DNA samples (15 μl) was used for gel electrophoresis or for dot-blot DNA-DNA hybridization. For detection of *mip*-amplified DNA, a 50-mer oligonucleotide probe, *Lmip*-1 (5′-TTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAA-3′), and for the detection of 5S rRNA-amplified DNA, a 50-mer oligonucleotide probe, *L5*SL9 (5′-CTCGAACTCAGAAGTCAAACATTTCCGCGCCAATGATAGTGTGAGGCTTC-3′), were used (21); for the detection of *lacZ*-amplified DNA, a 25-mer oligonucleotide probe, ELZ-1 (5′-CAGGATATGTGGCGGATGAGCGGCA-3′), was used; for *uidR*-amplified DNA, a 40-mer oligonucleotide probe, UAR-1 (5′-CAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATT-3′) (4), was used; and for *lamB*-amplified DNA, a 25-mer oligonucleotide probe, ELB-1 (5′-ACTGGGATATTTCTGGTCCTGGTGC-3′), was used.

The oligonucleotide probes were radiolabeled at their 3′-OH ends with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) (Dupont, NEN Research Products, Boston, Mass.) by using terminal deoxyribonucleotidyl transferase (IBI) or at their 5′ ends with [γ-^{32}P]ATP (specific activity, >3,000 Ci/mmol) (NEN Research Products) by using polynucleotide kinase (U.S. Biochemical Corp.) following the procedure described by Ausubel et al. (3). The specific activity of each probe was 5×10^7 to 1×10^9 cpm/μg of DNA as determined by a Beckman LS5000TD scintillation counter. For each hybridization, 200 to 300 ng of the respective radiolabeled oligonucleotide probe was used.

PCR-amplified DNAs were detected by using gel electrophoresis and radiolabeled gene probes. The DNAs were separated by using a 0.8 to 1% horizontal agarose gel, a 4% Nusieve-Seakem (3:1) (FMC BioProducts, Rockland, Maine) agarose gel with Tris-acetate-EDTA buffer (3), or a 10% vertical polyacrylamide gel with Tris-borate-EDTA buffer (3). The gels were stained in 2×10^{-4}% ethidium bromide solution, visualized with a Photo/PreplI UV transilluminator (Fotodyne, Inc., New Berlin, Wis.), and photographed.

For Southern and dot-blot hybridizations, the DNAs were denatured and transferred onto Zetaprobe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.) by following the procedure described by the manufacturer. All hybridizations

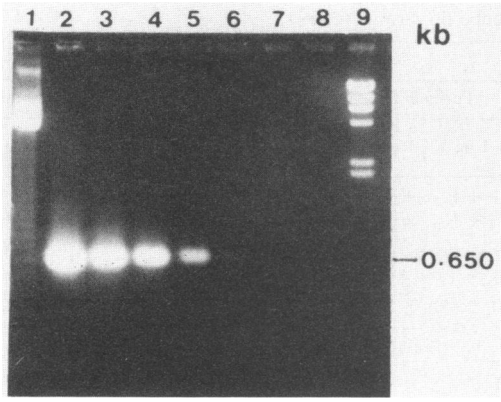

FIG. 1. Ethidium bromide-stained 1% agarose gel electrophoresis analysis of PCR-amplified DNA from *L. pneumophila*. Various numbers of *L. pneumophila* cells were collected on a Fluoropore FHLP filter, lysed, and PCR amplified with *Lmip*L920 and *Lmip*R1548 primers for the *mip* gene. Lanes: 1, 123-bp DNA ladder as a size standard; 2, 10^4 cells; 3, 10^3 cells; 4, 10^2 cells; 5, 1 to 10 cells; 6, 10^0 cells; 7, dilution blank; 8, *Pseudomonas putida*. Similar results were observed when a Fluoropore FGLP filter was used.

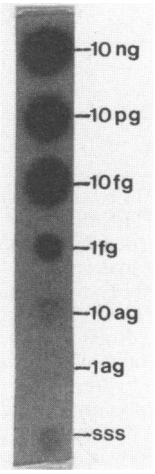

FIG. 2. DNA-DNA dot-blot hybridization analysis of PCR-amplified DNA, using various amounts of genomic DNA from *L. pneumophila* as a target. PCR amplification was performed in the presence of a Fluoropore FHLP filter, and the hybridization was done with a radiolabeled *Lmip*-1 oligonucleotide probe. sss, single-stranded salmon sperm nonspecific hybridization control.

were performed at 55 to 60°C for 16 h, using a hybridization solution described by the manufacturer (Bio-Rad). The blots were washed twice in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) 0.5% SDS at 45°C for 10 min each and once in 0.1× SSPE–0.1% SDS at 53°C for 3 to 5 min with gentle agitation. To detect ^{32}P-radiolabeled DNAs, the blots were covered with Saran Wrap (Fisher Biochemical, Pittsburgh, Pa.), and X-ray film (X-AR film; Eastman Kodak Co., Rochester, N.Y.) was placed over them; film exposure was at −70°C for 1 to 16 h.

RESULTS

Effectiveness of freeze-thaw lysis and DNA release. The freeze-thaw lysis method resulted in efficient lysis of all *E. coli*, *Legionella*, and other coliform bacteria strains tested including several environmental isolates. Cell lysis was high after the first lysis cycle (ca. 70 to 75%) as measured by viable plate count and acridine orange direct count procedures, and all cells were lysed within six cycles of freeze-thawing. No PCR-amplified DNA bands were observed from samples containing target bacterial cells not subjected to at least one freeze-thaw lysis cycle.

Efficiency of PCR amplification in the presence of filter. Filtration with Fluoropore filters (FHLP or FGLP) and DNA release by freeze-thawing permitted the recovery and PCR amplification of DNA from low numbers (1 to 10) of bacteria in 100-ml water samples (Fig. 1). Using radiolabeled gene probes, single cells were detected in approximately 18% of the samples, which corresponds with the expected Poisson distribution. Successful amplification with the highest sensitivity occurred when Fluoropore (FHLP or FGLP) filters were used (Table. 1). A lower sensitivity of detection, 100 to 1,000 bacterial cells, was attained when a water-wettable Fluoropore FHLC filter was used for collecting the bacterial cells, which were then lysed by freeze-thawing. Two hydrophobic Durapore membranes, GVHP and HVHP, showed positive PCR amplification at 10^3 and 10^2 cells, respectively, after they were treated with 10× Denhardt's solution and then PCR amplification. Treatment with 1× Denhardt's solution and then PCR amplification with these filters showed detection at the next lower order of magnitude. Positive PCR-amplified DNA bands were observed with Durapore or polycarbonate (Nuclepore or Millipore) filters only when at least 10^5 to 10^6 or more cells were filtered and PCR amplified. No PCR amplification was observed when other membranes were used. None of the filters after treatment with DNA-binding blocking agents showed improved amplification. Likewise, increasing the $MgCl_2$ concentration to 8 mM did not help in PCR amplification with these filters.

When 10^3 cells were filtered through these various filters, the filters were transferred to a GeneAmp tube, freeze-thawing was performed in the presence of water, and the supernatant was transferred to another GeneAmp tube for PCR amplification, positive PCR amplification was achieved in each case. Thus, the cells were adequately lysed in the presence of each of the filters to permit PCR DNA amplification. The filters, however, blocked DNA amplification by another mechanism.

Similar results were observed when different concentrations of purified genomic DNAs from *E. coli* or *Legionella* species, were used for PCR amplification in the presence of each type of filter described above (Table 1). Two Fluoropore filters, FHLP and FGLP, showed detection of amplified products from 1 fg of genomic DNA, which is equivalent to detecting one bacterial cell (6, 21) (Fig. 2). Although the amount of PCR-amplified DNA was found to be slightly higher when the Fluoropore FGLP filter was used, the filtration time was much longer than for the Fluoropore FHLP filter. Since the sensitivity of detecting one cell was not affected by the Fluoropore FHLP filter and the filtration time was within the acceptable range, it is desirable to use the FHLP filter. Moreover, when environmental water samples are filtered, the Fluoropore FGLP filter may clog quickly and as a result will not allow the required amount of water to pass through within an acceptable time frame.

Amplified DNA from 50 to 100 fg of genomic DNA, which is equivalent to the detection of approximately 50 to 100 bacterial cells, was detected with radiolabeled probes when a water-wettable FHLC filter was used. The smaller pore

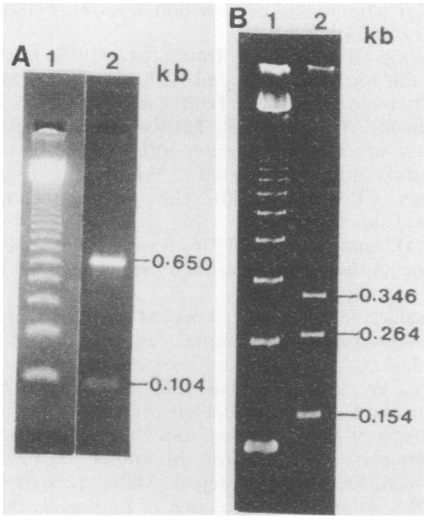

FIG. 3. Ethidium bromide-stained agarose gel electrophoresis of multiplex PCR amplification for the detection of multiple targets when amplified in the presence of a Fluoropore FHLP filter. (A) *L. pneumophila* DNA was amplified with *Lmip*L920 and *Lmip*R1548 for the *mip* gene and *L5*SL9 and *L5*SR93 for the 5S rRNA gene as targets. Lanes: 1, 123-bp DNA ladder as a size standard; 2, multiplex PCR amplification showing 0.650-kb amplified DNA from *mip* gene and 0.104-kb amplified DNA from 5S rRNA gene. (B) *E. coli* DNA was amplified with LZL-389 and LZR-653 for the *lacZ* gene, LBL-445 and LBR-790 for the *lamB* gene, and URL-301 and URL-432 for the *uidR* gene. Lanes: 1, 123-bp DNA ladder as a size standard; 2, multiplex PCR amplification showing 0.346-kb amplified DNA from *lamB* gene, 0.264-kb DNA from *lacZ* gene, and 0.154-kb amplified DNA from *uidR* gene.

size of the Fluoropore FGLP filter (0.2 μM) compared with the Fluoropore FHLP filter (0.5 μM) may permit more efficient cell recovery on the FGLP filter; thus, slightly higher amounts of amplified products were detected in the agarose gel when the FGLP filter was used as determined by agarose gel electrophoresis. No bands were observed and no Southern blot-positive bands were found when using 50 to 100 fg of genomic DNA for the other filters tested.

Multiplex PCR amplification for detection of multiple targets. Simultaneous detection of multiple targets by multiplex PCR amplification has been tested in the presence of all four Fluoropore (FHLP, FGLP, FHLC, and FHUP) filters. Using different molar quantities of *mip* and 5S rRNA primers for the simultaneous detection of two different targets, genus *Legionella* and/or *L. pneumophila*, it was possible to achieve distinctly visible amplified DNA, as determined by gel electrophoresis (Fig. 3A). The requirement for unequal molar quantities of these two primers for achieving uniform DNA-amplified bands may be due to the differences in the sizes of the two targets to be amplified, the differences in the T_a (primer-annealing temperature) values, and/or the initial large copy number of the 5S rRNA target. Use of different molar quantities of these two primers was found to be more efficient in producing equal amplifications of the two targets than the staggered amplification described previously by Bej et al. (5). In contrast to this, equimolar quantities of *lacZ*, *lamB*, and *uidR* primers were used to achieve almost equal quantities of amplified DNA as determined by gel electrophoresis (Fig. 3B). These multiplex PCR amplifications were achieved only in the presence of Fluoropore FHLP, FGLP, and FHLC filters. In either case, no nonspecific amplification was observed in ethidium bromide-stained agarose or polyacrylamide gels.

DISCUSSION

Microorganisms are often concentrated from environmental samples by filtration, permitting the detection of concentrations of microorganisms of <1/ml. Most commonly, cellulose acetate or polycarbonate filters are used (8, 16). We found that cells concentrated on such filters did not give positive PCR-gene probe signals in the presence of the filters. Such filters are inhibitory to PCR DNA amplification. Tests with purified DNA showed no or very inefficient PCR amplification in the presence of such filters. This may have been due to binding of DNA to the filters or to other interference. One solution to this problem would be to concentrate the cells by filtration, release them from the filter, and then remove the filters before cell lysis and PCR amplification. This approach may be fine for detection of moderate to high cell concentrations, but it is likely that some cells would be lost. If the application requires detection at the single-cell level in the sample, then removal of the filter is undesirable. Therefore, we sought other filters that were compatible with PCR DNA amplification in the presence of the filter.

Successful PCR amplifications were achieved by using freeze-thaw lysis of cells concentrated with Fluoropore filters (FHLP, FGLP) when *lacZ* primers for detection of all coliform bacteria, *lamB* primers for *E. coli*, *Salmonella typhimurium*, and *Shigella flexneri*, *uidR* primers for *E. coli* and *Shigella flexneri*, 5S rRNA primers for total genus *Legionella*, and *mip* primers for *L. pneumophila* were used. It is possible that the environmental and drinking waters contain more than one type of microbial pathogen in addition to the indicator microorganism. Use of multiplex PCR for amplification and detection of more than one target can be useful for monitoring multiple microbial pathogens in a single water sample. In this study, we showed that the presence of Fluoropore filters (FHLP and FGLP) does not interfere with the PCR amplification of multiple targets.

A single cell in 100 ml of water was detectable by using Fluoropore filter (FHLP and FGLP) concentration and then freeze-thaw release of DNA and PCR amplification. Freeze-thaw release of DNA was used as a robust means of disrupting cells. Samples of 100 ml of potable water could be filtered with 13-mm Fluoropore filters (FHLP and FGLP) within a few minutes. Even Ohio River water passed through the filters within 15 min. In more turbid samples, however, it may be necessary to use larger-diameter filters, requiring larger volumes of PCR mix, or to use prefiltration. In some cases, inhibitory substances concentrated along with cells on the filters may also inhibit PCR; this was not found to be a problem with potable and river-water samples.

In conclusion, PCR can be used for detecting very low concentrations of microorganisms (<1/ml) by using Teflon filters for concentrating the cells, releasing the DNA by repetitive freeze-thaw cycles, and performing PCR in the presence of the filter.

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