APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1990, p. 307–314
0099-2240/90/020307-08$02.00/0
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Vol. 56, No. 2

Detection of Coliform Bacteria in Water by Polymerase Chain Reaction and Gene Probes

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Received 21 June 1989/Accepted 30 October 1989

Polymerase chain reaction (PCR) amplification and gene probe detection of regions of two genes, *lacZ* and *lamB*, were tested for their abilities to detect coliform bacteria. Amplification of a segment of the coding region of *Escherichia coli lacZ* by using a PCR primer annealing temperature of 50°C detected *E. coli* and other coliform bacteria (including *Shigella* spp.) but not *Salmonella* spp. and noncoliform bacteria. Amplification of a region of *E. coli lamB* by using a primer annealing temperature of 50°C selectively detected *E. coli* and *Salmonella* and *Shigella* spp. PCR amplification and radiolabeled gene probes detected as little as 1 to 10 fg of genomic *E. coli* DNA and as few as 1 to 5 viable *E. coli* cells in 100 ml of water. PCR amplification of *lacZ* and *lamB* provides a basis for a method to detect indicators of fecal contamination of water, and amplification of *lamB* in particular permits detection of *E. coli* and enteric pathogens (*Salmonella* and *Shigella* spp.) with the necessary specificity and sensitivity for monitoring the bacteriological quality of water so as to ensure the safety of water supplies.

Coliform bacteria are used for monitoring the bacteriological safety of water supplies on the basis of the realization that the presence of coliform bacteria in water is an indicator of potential human fecal contamination and therefore the possible presence of enteric pathogens (4, 15). The traditional methods for detecting coliform bacteria rely upon culturing on a medium that selectively permits the growth of gram-negative bacteria and differentially detects lactose-utilizing bacteria, e.g., using MacConkey, m-Endo, eosin methylene blue, or brilliant-green-lactose-bile media (13). By using these media and an incubation temperature of 37°C, total coliform bacteria, which include members of the genera *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella* among others, are enumerated; by using an elevated incubation temperature, e.g., 44.5°C, fecal coliform bacteria, principally *Escherichia coli*, are enumerated. Because *E. coli* is primarily associated with human feces, it is a useful indicator of human fecal contamination of water and the appropriate focus of monitoring for indicators of potential enteric pathogens in environmental and potable waters (9).

There are several problems with viable culture methods used for routine monitoring of the bacteriological safety of water supplies, including maintaining the viability of bacteria between the time of collection and enumeration, lack of growth of viable but nonculturable bacteria (25) such as those stressed by chemicals in the water, time required for detection and confirmation of enteric bacteria (days), and lack of specificity for detection of true fecal coliforms (*E. coli*).

Recently, tests based on detection of β-D-glucuronidase, such as the Colilert test (Access Analytical Systems, Branford, Conn.), have been suggested as alternate approaches for detecting coliforms (10–12). Enzymatic transformation of the fluorogenic substrate 4-methylumbelliferyl-β-glucuronide or the colorimetric substrate *p*-nitrophenyl glucuronide is indicative of the presence of *E. coli*, but detection of such enzymatic activity still requires culturing of bacteria. Also Chang et al. (7) have recently reported that basing a test on β-D-glucuronidase activity may fail to detect a significant proportion (circa 30%) of fecal coliform bacteria in some cases because of occurrences of high incidences of β-D-glucuronidase-negative *E. coli*. High proportions of β-D-glucuronidase-negative *E. coli*, however, have not been found in the U.S. Environmental Protection Agency equivalency tests of the Colilert test system.

To avoid the problems inherent in methods based on the culturing of viable cells for the detection of coliforms, we have begun to develope a nonculture, genetically based procedure for the environmental detection of coliforms based on the recovery of DNA, amplification of target nucleotide sequences specifically associated with coliform bacteria by using the polymerase chain reaction (PCR), and detection of the amplified DNA with gene probes. PCR has been successfully used to detect microorganisms of clinical interest (1, 6, 14, 18, 20, 21, 23, 24, 26–29) and genetically engineered microorganisms (30). PCR has been shown to permit the detection of a single cell (19). The potential applicability of PCR for environmental monitoring of coliforms and enteric pathogens has, however, not been previously reported.

As a target for PCR amplification we selected a region of *lacZ* because conventional coliform monitoring is based on detection of the activity of the gene product (β-galactosidase) of *lacZ* produced by coliform bacteria. We also chose as a target for PCR amplification a region of the *malB* gene that codes for maltose transport protein, because this region includes *lamB* which encodes a surface protein that is recognized specifically by the *E. coli*-specific bacteriophage lambda. The hypothesis was that PCR amplification of *lacZ* would occur for all (total) coliforms and that PCR amplification of *lamB* would be specific for the fecal coliform *E. coli*. Our aim was to achieve the necessary specificity and sensitivity to make this a relevant method for environmental monitoring purposes. Current monitoring protocols can detect one viable (culturable) coliform bacterium per 100 ml

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(13), but the efficiency of detection relative to numbers of nonculturable coliforms is unknown.

MATERIALS AND METHODS

Recovery of DNA. Two methods were used to recover DNA from bacterial cells. During development of a PCR amplification procedure for coliform detection, total genomic DNA was extracted from cultures by the procedure of Ausubel et al. (2). Using this procedure, DNA from bacterial cells in 1.5-ml samples of overnight cultures were released by alkaline lysis with sodium dodecyl sulfate (SDS) treatment. Proteinase K (Sigma Chemical Co., St. Louis, Mo.) and hexadecyltrimethyl ammonium bromide (CTAB-NaCl) were used to remove proteins and carbohydrates, and the DNA was further purified by using chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (24:24:2) extractions followed by precipitation with isopropanol. After centrifugation at 12,000 × *g* for 15 min, the pelleted DNA was washed once with cold 70% alcohol and dried under vacuum. Using this procedure, we were able to recover 100 to 150 μg of purified genomic DNA from each sample.

The simpler direct lysis method of Li et al. (19) was also used for recovery of DNA from bacterial cells. Cells were recovered from 100 ml of water sample to which bacterial cells had been added. Cells were collected by centrifugation at 10,000 × *g* for 15 min. The cells were transferred to 0.6-ml Eppendorf tubes and after centrifugation for 5 min at 12,000 × *g*, the cell pellets were suspended in 20 μl of lysis solution containing 1× PCR buffer, 0.05 mg of proteinase K per ml, 20 mM dithiothreitol, and 1.8 μM SDS; the samples were vortexed for 15 s and incubated at 37°C for 1 to 1.5 h, after which they were heated to 85 to 90°C for 5 min to inactivate the proteinase K. Then an additional 10 μl of PCR buffer, dNTPs, *Taq* polymerase, and primers were added, and PCR was performed as described below.

PCR amplification. PCR amplification was performed by using a DNA thermal cycler and GeneAmp kit with native *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The PCR solution used contained 1× PCR amplification buffer (10× buffer contains 50 mM KCl, 100 mM Tris hydrochloride [pH 8.13], 15 mM $MgCl_2$, and 0.1% [wt/vol] gelatin), 200 μM each of the dNTPs, 0.2 to 1 μM of each of the primers, 1 ag (10^{-18} g) to 1 μg template DNA, 2.5 U of *Taq* DNA polymerase, and double-distilled water containing 0.1% diethylpyrocarbonate. Template DNAs were initially denatured at 94°C for 1 to 3 min. Then a total of 25 to 40 PCR cycles were run under the following conditions: denaturation at 94°C for 0.5 to 1 min, primer annealing at 40, 50, 60, or 70°C for 0.5 to 1 min, DNA extension at 72°C for 1 to 2 min. Oligonucleotide primers were synthesized by using a DNA synthesizer (Systec, Minneapolis, Minn.) and purified using an oligonucleotide purification cartridge (Applied Biosystems, Foster City, Calif.) for small samples and reversed-phase high-performance liquid chromatography with a C-8 3-μm reversed-phase column (Perkin-Elmer) for large samples.

An 876-base-pair (bp) region of the *E. coli lacZ* gene, based on the sequence reported by Kalnins et al. (17), was amplified by using the 24-mer primers ZL-1675 (5′-ATGAAA GCTGGCTACAGGAAGGCC) and ZR-2548 (5′-CACCAT GCCGTGGGTTTCAATATT). Primer ZL-1675 was located between bp 1675 and 1698 and primer ZR-2548 was located between bp 2525 and 2548 within the coding sequence of the *lacZ* gene of *E. coli*. Another 24-mer primer, ZR-2025 (5′GGTTTATGCAGCAACGAGACGTCA), was used along with primer ZL-1675 to amplify a shorter 326-bp region of *lacZ*. Primer ZR-2025 was located between bp 2001 and 2025 which is a region closer to the amino terminal of the *E. coli lacZ* gene than the primer sequence ZR-2548.

A 554-bp sequence downstream from the sequence encoding the lambda attachment site peptide of *lamB*, based on the sequence reported by Bedouelle et al. (3), was amplified by using two different 24-mer primers. Primer BL-4899 (5′-GG ATATT TCTGGTCCTGGTGCCGG) was located between bp 4899 and 4922, and primer BR-5452 (5′-ACTTGGTGCC GTTGTCGTTATCCC) was located between bp 5429 and 5452. Another set of 24-mer primers was also used to amplify a 309-bp segment of the coding region of the *lamB* gene of *E. coli*. These primers were designated BL-4910 (5′-CTGATC GAATGGCTGCCAGGCTCC), which was located between bp 4910 and 4933, and BR-5219 (5′-CAACCAGACGATAG TTATCACGCA), which was located between bp 5195 and 5219.

In some experiments, regions of *lacZ* and *lamB* were amplified simultaneously by using mixtures of primers ZL-1675 and ZR-2548 for *lacZ* and BL-4899 and BR-5452 for *lamB*. In these experiments, 50 ng to 1 μg of target genomic DNAs and various relative concentrations of primers (0.125 to 1.0 μM of each primer) were used.

Detection of amplified DNAs. PCR-amplified DNAs were detected by using gel electrophoresis and radiolabeled gene probes. The DNAs were separated by using either a 0.8 to 1% horizontal agarose gel or a 5% vertical polyacrylamide gel. Agarose gels were run in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA [pH 8.0]). Polyacrylamide gels were run in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA [pH 8.0]) at 5.7 to 9.0 V/cm for 2 to 4 h. The gels were stained in 2 × 10^{-4}% ethidium bromide solution, visualized with a Photo/PrepI UV transilluminator (Fotodyne, Inc., New Berlin, Wis.), and photographed.

For Southern blots, the DNAs were transferred onto nylon membranes (ICN Biomedicals, Costa Mesa, Calif., or Bio-Rad Laboratories, Richmond, Calif.) by using 0.4 M NaOH solution and fixed onto the membranes either by baking for 1 h at 80°C or by UV irradiation. For dot blots, the double-stranded amplified DNAs were denatured by adding a denaturing solution containing 0.1 volume of 3 M NaOH and 0.1 M disodium EDTA, incubated at 60°C for 15 min to 1 h, and neutralized with 1 volume of cold 2 M ammonium acetate; the samples were then spotted onto Zeta probe nylon membranes (Bio-Rad) by using a Bio-Rad dot blot manifold at a vacuum pressure of 4 to 5 lb/in^2.

The amplified DNAs immobilized on the nylon membranes were prehybridized with a hybridization solution containing 5× SSPE (1× SSPE is 10 mM sodium phosphate [pH 7.0], 0.18 M NaCl, 1 mM disodium EDTA), 0.5% SDS, 5% Denhardt solution, and 100 μg of phenol-extracted, denatured, salmon sperm DNA (Sigma) per ml or 50 μg of type X Baker's yeast tRNA (Sigma) per ml; prehybridization was at 55 to 60°C for 20 to 30 min. The prehybridization buffer was removed, and the membranes were hybridized with fresh hybridization solution containing 200 to 500 ng of denatured radiolabeled gene probes and incubated at 55 to 60°C for 3 to 16 h. The blots were washed twice in 2× SSPE–0.5% SDS at room temperature for 10 min each and once in 0.1× SSPE–0.1% SDS at 55°C for 3 to 5 min with gentle agitation. To detect ^{32}P-labeled DNAs, the blots were covered with Saran Wrap (Fisher Biochemical, Pittsburg, Pa.) and X-ray film (Kodak X-AR film; Eastman Kodak Co.,

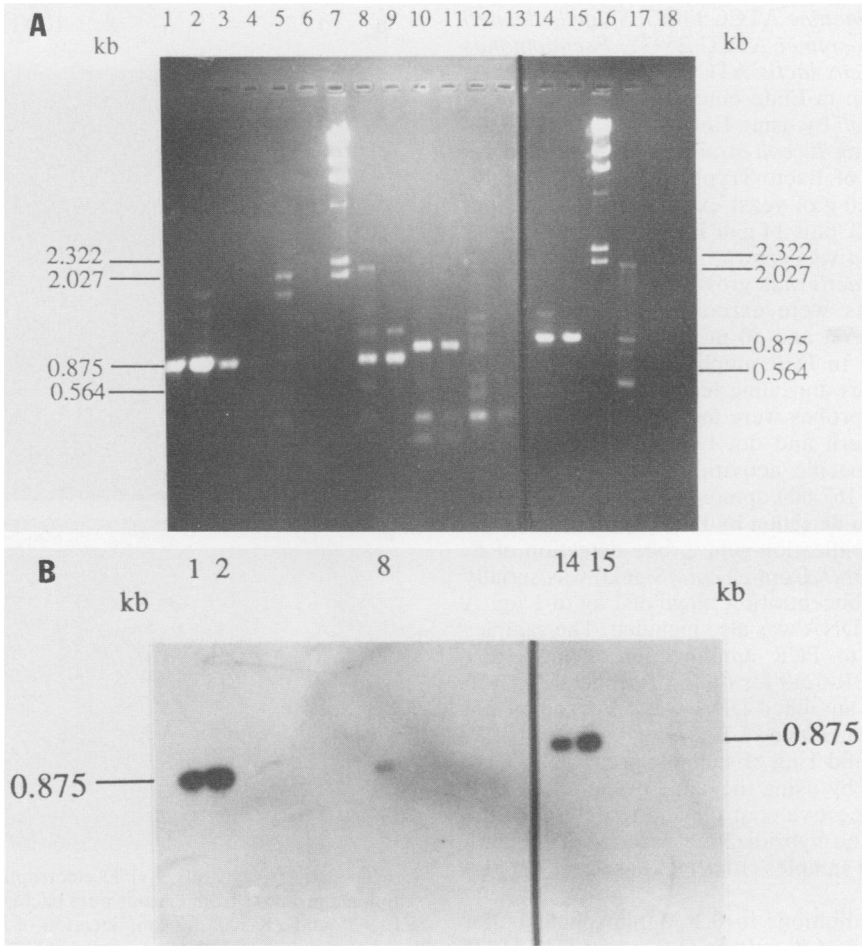

FIG. 1. (A) Agarose gel (1%) electrophoretic analysis of PCR-amplified products from various pure bacterial DNAs, using primers ZL-1675 and ZR-2548 for amplification of *lacZ*. Lanes: 1, *E. coli* ATCC 11775 DNA with a primer annealing temperature of 50°C; 2, *E. coli* ATCC 11775 DNA with a primer annealing temperature of 40°C; 3, *L. lactis* ATCC 19435 DNA with a primer annealing temperature of 40°C; 4, *L. lactis* ATCC 19435 DNA with a primer annealing temperature of 50°C; 5, *Enterobacter aerogenes* ATCC 13045 DNA with a primer annealing temperature of 40°C; 6, *Enterobacter aerogenes* ATCC 13045 DNA with a primer annealing temperature of 50°C; 7, lambda + *Hin*dIII size standard; 8, *K. pneumoniae* ATCC 13883 DNA with a primer annealing temperature of 40°C; 9, *K. pneumoniae* ATCC 13883 DNA with a primer annealing temperature of 50°C; 10, *C. freundii* ATCC 33128 DNA with a primer annealing temperature of 40°C; 11, *C. freundii* ATCC 33128 DNA with a primer annealing temperature of 50°C; 12, *S. typhimurium* ATCC 19585 DNA with a primer annealing temperature of 40°C; 13, *S. typhimurium* ATCC 19585 DNA with a primer annealing temperature of 50°C; 14, *Enterobacter cloacae* ATCC 13047 DNA with a primer annealing temperature of 40°C; 15, *Enterobacter cloacae* ATCC 13047 DNA with a primer annealing temperature of 50°C; 16, lambda + *Hin*dIII size standard; 17, *P. putida* mt-2 (cured) DNA with a primer annealing temperature of 40°C; 18, *P. putida* mt-2 (cured) DNA with a primer annealing temperature of 50°C. Lanes 1 to 13 were run in one gel and lanes 14 to 18 in a second gel. (B) Southern blot analysis, using gene probe LZ-1, of the gel shown in panel A.

Rochester, N.Y.) was placed over them; film exposure was at −70°C for 1 to 48 h.

The 50-mer gene probe LZ-1 (5′-TGACGTCTCGTTGC TGCATAAACCGACTACACAAATCAGCGATTTCCATT) was used for detection of amplified *lacZ*, and the 50-mer gene probe LB-1 (5′-TGCGTGATAACTATCGTCTGGTTG ATGGCGCATCGAAAGACGGCTGGTTG) was used for detection of amplified *lamB*. Both gene probes hybridize to target sequences located within the respective regions of amplified DNAs. The gene probes were 5′-end radiolabeled with [γ-^{32}P]ATP (>3,000 Ci/mmol) by a modified version of the forward reaction described by Ausubel et al. (2). The 30-μl reaction solution used in this procedure contained 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol (Sigma), 1 mM KCl, 1 to 10 μg of oligonucleotide gene probe, 120 pmol [γ-^{32}P]ATP (specific activity, >3,000 Ci/mmol) (New England Nuclear Corp., Boston, Mass.), 1 mM spermidine (disodium salt), and 20 U of T4 polynucleotide kinase (U.S. Biochemical Corp., 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 hydrochloride [pH 7.6], 1 mM disodium EDTA).

Specificity of coliform detection by PCR and gene probes. To determine the specificity of coliform detection by PCR amplification of regions of *lacZ* and *lamB*, tests were conducted with the following bacterial strains: *E. coli* ATCC 11775, *E. coli* ATCC 10798, *E. coli* ATCC 15224, *E. coli* ATCC 25404, *E. coli* (*lamB*) ATCC 23556, *E. coli* (*lamB*) ATCC 23737, *E. coli* (*lamB*) ATCC 23739, *E. coli* (*lamB*) ATCC 12435, *Enterobacter cloacae* ATCC 13047, *Salmonella typhimurium* ATCC 19585, *Citrobacter freundii* ATCC

33128, *Klebsiella pneumoniae* ATCC 13883, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, *Pseudomonas putida* mt-2, *Lactococcus lactis* ATCC 19435, and 32 environmental isolates from m-Endo enumeration plates which were identified as *E. coli* by using Enterotubes (Roche). All enterobacteria, including *E. coli* strains, were grown in 2× YT liquid broth (10 g of Bacto-Tryptone [Difco Laboratories, Detroit, Mich.], 10 g of yeast extract, 5 g of NaCl per liter), TYE agar (2× YT plus 14 g of Bacto-Agar [Difco] per liter) at 35°C; *P. putida* was grown on *Pseudomonas* isolation agar at 30°C; *L. lactis* was grown on litmus milk agar (Difco) at 37°C. DNAs were extracted from 12- to 16-h cultures of these bacteria and 50 ng to 1 μg of recovered DNAs were subjected to PCR amplification as previously described, using various annealing temperatures during the PCR procedure; gene probes were used to detect amplified DNAs by both Southern and dot blot procedures as described above. The specific activities of the radiolabeled probes were 68,000 to 167,000 dpm/μg of DNA.

Sensitivity of coliform detection by PCR. To determine the sensitivity of PCR amplification-gene probe detection of *E. coli*, 1 μg of genomic DNA from *E. coli* (Sigma) was serially diluted to establish a concentration range of 1 ag to 1 μg. A control with no *E. coli* DNA was also included. The samples were then subjected to PCR amplification, using either primers BL-4910 and BR-5219 for *lamB* or primers ZL-1675 and ZR-2548 for *lacZ*; amplified DNA was analyzed by dot blot, using gene probes LB-1 and LZ-1. One microgram of *P. putida* genomic DNA and 1 μg of salmon sperm DNA were also subjected to PCR by using the same primers and PCR conditions as for the negative controls and to determine the background signal of the hybridization reaction. One-tenth of each of the amplified samples (10 μl) was used for dot blot analysis.

Additionally, serial dilutions in 0.1 M phosphate buffer (pH 7.2) of an overnight (16 h) culture of *E. coli* ATCC 11775 grown in 2× YT broth at 37°C were used to determine the sensitivity of PCR amplification of *lamB* for coliform detection. Dilutions were performed by using 100 ml of autoclave-sterilized tap water treated with 0.1% sodium thiosulfate for dechlorination. In addition to the *E. coli* cells from the serial dilutions, ca. 10^9 cells of *P. putida* were added to each dilution blank to serve as a nontarget background population and to facilitate collection of bacterial cells from the samples. Bacterial cells were collected by centrifugation at 12,000 × *g* for 10 min in a Sorvall RS-5 centrifuge; the pellets were suspended in a small volume of sterile dechlorinated tap water, transferred to 1.5-ml microcentrifuge tubes, and sedimented by centrifugation at 12,000 × *g* for 5 min. The cells were suspended in PCR buffer, and PCR amplifications and gene probe detections were performed as described above for *lamB*.

To determine the number of viable *E. coli* cells in each dilution, replicate samples of the serial dilutions were plated onto m-Endo agar. CFUs of the target *E. coli* cells were determined by counting colonies with typical coliform appearance after 24 h of incubation at 37°C. Direct counts were performed by using the acridine orange direct count procedure (8).

RESULTS AND DISCUSSION

Specificity of coliform detection by PCR amplification of *lacZ*. PCR amplification using primers ZL-1675 and ZL-2548 and a primer annealing temperature of 40°C produced positively amplified DNA bands for both coliform and noncoliform bacterial target DNAs (Fig. 1). Using *Citrobacter* DNA as a template, the amplified DNA was larger than when *E. coli* DNA was the template, indicating a difference between the target *lacZ* genes between these organisms. The differences were also indicated by the differential responses to increasing the primer annealing temperature between *E. coli* and the other bacterial species tested. Raising the primer annealing temperature to 50°C to increase the stringency of PCR eliminated amplification for all noncoliforms, e.g., *P. putida* and *L. lactis*, but also eliminated amplification of some coliforms, e.g., *Enterobacter aerogenes* and *C. freundii*. Using a primer annealing temperature of 50°C, *lacZ* amplification occurred with *E. coli*, *Enterobacter cloacae*, and to a lesser extent with *K. pneumoniae* (Fig. 1A). Southern blots showed hybridization with the LZ-1 gene probe, indicating amplification of the target *lacZ* only with *E. coli*, *Enterobacter cloacae*, and *K. pneumoniae*, even when a nonstringent primer annealing temperature of 40°C was employed (Fig. 1B). Amplification and hybridization for *lacZ* also occurred for *E. coli* and *Enterobacter cloacae* with primer annealing temperatures of 60 and 70°C. All strains of *E. coli* tested, including the 32 environmental isolates, showed positive DNA amplification and hybridization with

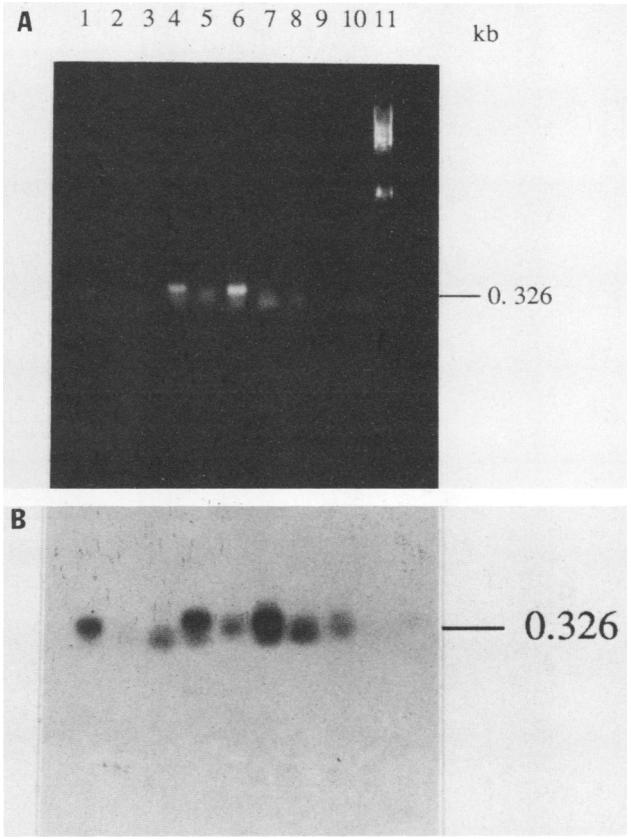

FIG. 2. (A) Agarose gel (1%) electrophoretic analysis of PCR-amplified products from various pure bacterial DNAs, using primers ZL-1675 and ZR-2025 for amplification of *lacZ*. PCR amplification used a primer annealing temperature of 50°C. Lanes: 1, *E. coli*; 2, *S. typhimurium*; 3, *Shigella flexneri*; 4, *K. pneumoniae*; 5, *Enterobacter aerogenes*; 6, *Enterobacter cloacae*; 7, *C. freundii*; 8, *Shigella sonnei*; 9, *P. aeruginosa*; 10, *L. lactis*; 11, lambda + *Hin*dIII size standard. (B) Southern blot analysis, using gene probe LZ-1, of gel shown in panel A.

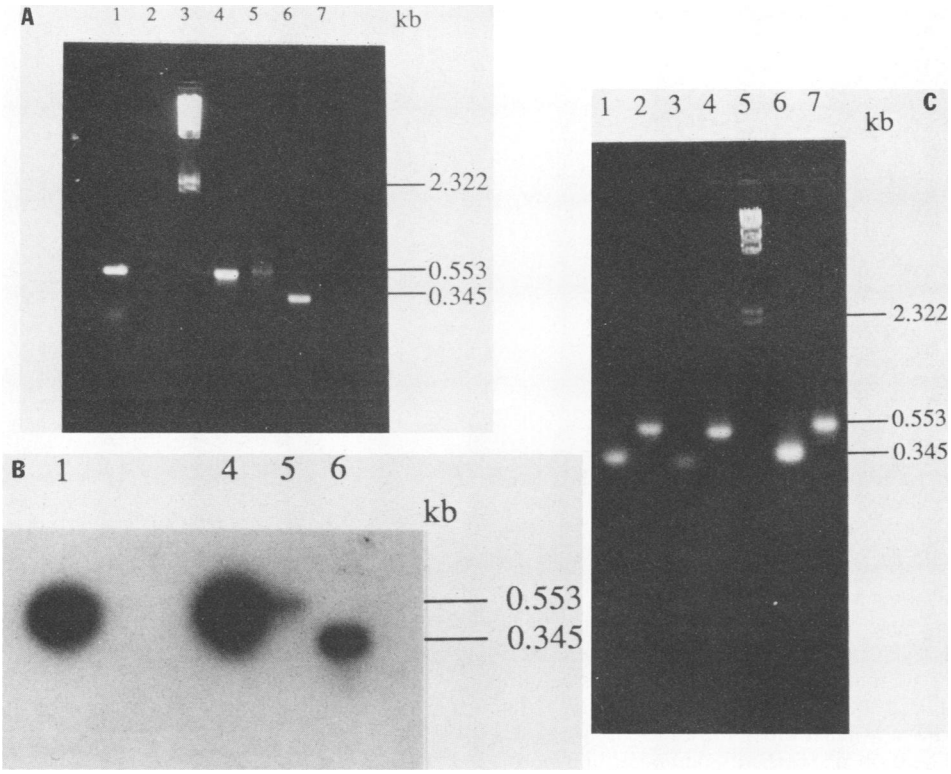

FIG. 3. (A) Agarose gel (1%) electrophoretic analysis of PCR-amplified products from various pure bacterial DNAs, using primers of *lamB*. Lanes: 1, *E. coli* DNA with a primer annealing temperature of 70°C, using primers BL-4899 and BR-5452; 2, *S. typhimurium* DNA with a primer annealing temperature of 70°C, using primers BL-4899 and BR-5452; 3, lambda + *Hin*dIII size standard; 4, *E. coli* DNA with a primer annealing temperature of 60°C, using primers B-4899 and BR-5452; 5, *S. typhimurium* DNA with a primer annealing temperature of 60°C, using primers BL-4899 and BR-5452; 6, *E. coli* DNA with a primer annealing temperature of 60°C, using primers BL-4910 and BR-5219; 7, *S. typhimurium* DNA with a primer annealing temperature of 60°C, using primers BL-4910 and BR-5219. (B) Southern blot analysis, using gene probe LB-1, of gel shown in panel A. (C) Agarose gel (1%) electrophoretic analysis of PCR-amplified products from *E. coli* and *Shigella* DNAs, using primers for *lamB* amplification. Lanes: 1, *Shigella flexneri* DNA with a primer annealing temperature of 60°C, using primers BL-4910 and BR-5219; 2, *Shigella flexneri* DNA with a primer annealing temperature of 60°C, using primers B-4899 and BR-5452; 3, *Shigella sonnei* DNA with a primer annealing temperature of 60°C, using primers BL-4910 and BR-5219; 4, *Shigella sonnei* DNA with a primer annealing temperature of 60°C, using primers B-4899 and BR-5452; 5, lambda + *Hin*dIII size standard; 6, *E. coli* DNA with a primer annealing temperature of 60°C, using primers BL-4910 and BR-5219; 7, *E. coli* DNA with a primer annealing temperature of 60°C, using primers BL-4899 and BR-5452.

the gene probe for *lacZ* at the predicted position for 875 bp. *Shigella* DNA also was amplified and detected.

Because amplification with primers ZL-1675 and ZL-2548 did not form the basis for total coliform detection equivalent to total coliform enumeration by viable count procedures, a shorter region of *lacZ* closer to the amino terminal and the active site was amplified by using primers ZL-1675 and ZR-2025 and a primer annealing temperature of 50°C. The region amplified with these primers appears to be sufficiently conserved to provide a basis for PCR and gene probe detection of total coliforms (Fig. 2). There was some variation, however, among the various coliforms species tested with regard to the sizes of amplified DNAs, indicating some heterogeneity in the gene sequences. The use of *lacZ* amplification permitted detection of *Shigella* sp. but not of *Salmonella* sp., so that some enteric pathogens would not be directly detected by this method. As for *Salmonella* sp., noncoliforms were not amplified or detected.

Specificity of coliform detection by PCR amplification of *lamB*. PCR amplification of *lamB*, like that of *lacZ*, produced additional nonspecific DNA amplification, including amplification of noncoliform DNAs when primer annealing temperatures were below 50°C. Use of primers BL-4899 and BR-5452 and primer annealing temperatures of 60°C, however, limited the range of bacteria showing positive DNA amplification; *E. coli* (including all *lamB*-negative strains tested), *S. typhimurium*, and *Shigella* spp. were the only bacteria that showed amplification of *lamB* when a primer annealing temperature of 60°C was used, as detected by hybridization with gene probe LB-1 (Fig. 3). Raising the primer annealing temperature still further, to 70°C, eliminated amplification of *S. typhimurium*, but still permitted amplification of *E. coli* and *Shigella* spp.

When the second set of primers, BL-4910 and BR-5219, and a primer annealing temperature of 50°C were used, only *E. coli* (including all *lamB*-negative strains tested), *S. typhimurium*, and *Shigella* spp. showed amplification of the target 309-bp region that hybridized with gene probe LB-1. *S. typhimurium* DNA failed to amplify with these primers when a primer annealing temperature of 60°C was used, whereas *E. coli* (including the 32 environmental isolates) and *Shigella* spp. still showed amplification of target DNAs (Fig. 3). Thus, use of these primers for *lamB* and a primer annealing temperature of 60°C during PCR permitted detection of *E. coli* and *Shigella* spp. (Fig. 2); use of the lower primer annealing temperature of 50°C permitted detection of these

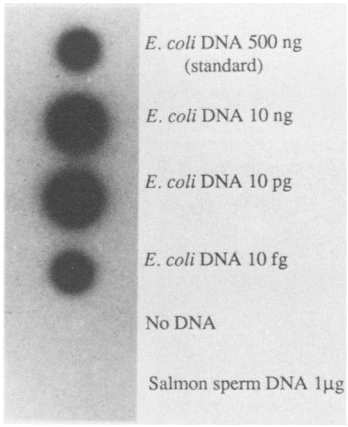

FIG. 4. Dot blot analysis after PCR amplification of various amounts of genomic *E. coli* DNA, using primers ZL-1675 and ZR-2548 for *lacZ* amplification. Controls included no added target DNA and nontarget salmon sperm DNA. A standard of 500 ng of unamplified *E. coli* DNA also was included.

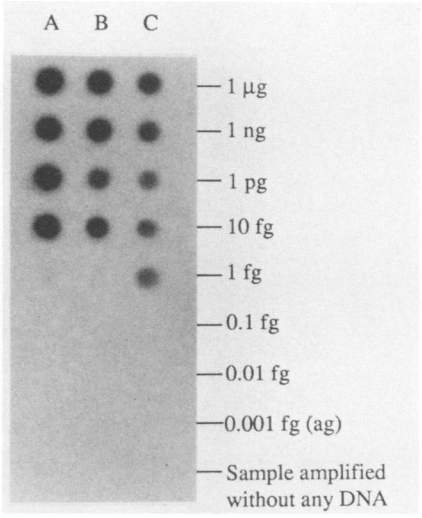

FIG. 5. Dot blot analysis after PCR amplification of various amounts of genomic *E. coli* DNA, using primers BL-4910 and BR-5219 for *lamB* amplification. Controls included no added target DNA and nontarget salmon sperm DNA. Three replicates (panels A, B, and C) were run for each DNA concentration.

enteric bacteria and also *Salmonella* spp. (Fig. 3). These results indicate that the primer annealing temperature should be close to the T_m to minimize nonspecific amplification. They also suggest that besides *E. coli*, *Salmonella*, and *Shigella* spp. may have at least a portion of the *lamB* gene. Hence, PCR amplification of *lamB*, as demonstrated here, provides a means of monitoring the indicator bacterial species of fecal contamination (*E. coli*) and also of the principal enteric bacterial pathogens that cause waterborne disease outbreaks (*Salmonella* and *Shigella* spp.). Therefore, the bacteria associated with human fecal contamination of waters (both indicator and pathogens) could be detected by PCR amplification and gene probes for *lamB*.

Sensitivity of coliform detection by PCR amplification of *lacZ* and *lamB*. Besides appropriate selectivity for target coliforms, to be useful for monitoring purposes, a PCR-gene probe approach must provide sufficient sensitivity to ensure the safety of potable water supplies. A target sensitivity of one cell per 100 ml, which is as good as viable culture methods, is desirable. The sensitivity of detection was found to depend on the PCR conditions. Raising the primer annealing temperature, which as already discussed enhanced the selectivity of DNA amplification to target cells, lowered the sensitivity of detection. Thus, using a primer annealing temperature of 70°C permitted amplification and detection of *lacZ* when more than 100 fg of genomic *E. coli* DNA (ca. 1 ag of target DNA) was present but not with lower amounts of DNA. In contrast, as little as 1 to 10 fg of *E. coli* DNA could be detected by PCR amplification of *lacZ* when a primer annealing temperature of 40°C was used (Fig. 4); the same detection limit was found using *lamB* amplification (Fig. 5). The detection of 10 fg of genomic DNA was reliable. At 1 fg of genomic DNA, approximately 22% of the samples gave positive signals, which closely corresponds to the expected Poisson distribution of the target gene at that concentration of genomic DNA. Concentrations of *E. coli* DNA below 1 fg did not show positive amplification and detection by hybridization with gene probes. The sensitivity of detection achieved by amplification of *lacZ* and *lamB* coupled with ^{32}P-labeled gene probes is equivalent to <0.01 ag of target DNA, i.e., single genome copy (single-cell) detection. This detection level is as sensitive as that reported for eucaryotic cells (16, 19) and more sensitive than the detection of 1 fg of target DNA from enteropathogenic bacteria by PCR and gene probe analysis reported by Olive et al. (22).

The direct lysis procedure using cells recovered by centrifugation also indicated sensitive detection of *E. coli* by PCR and gene probe analysis. As few as one to five viable cells per 100-ml water sample were detected (Fig. 6). Similarly as few as one viable cell in a sample was detected by using amplification of *lamB* (Fig. 7). We cannot, however, eliminate the possibility that some dead cells or viable but nonculturable cells were detected by PCR. Direct counts of overnight cultures were 2 orders of magnitude higher than

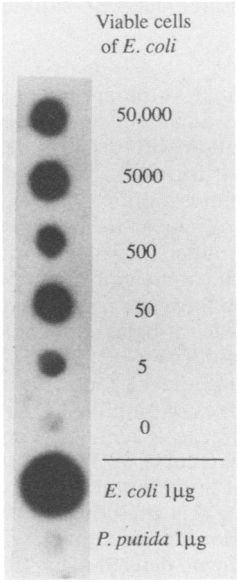

FIG. 6. Dot blot analysis after PCR amplification of serial dilutions of a 16-h *E. coli* culture, using primers for *lacZ* amplification. Viable cells were determined by viable plate count. Controls included pure *E. coli* and *P. putida* DNAs.

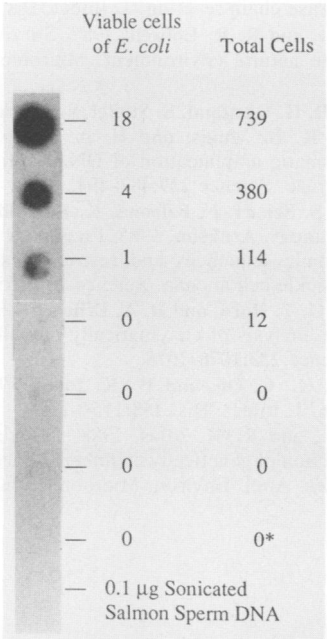

FIG. 7. Dot blot analysis after PCR amplification of serial dilutions of a 16-h *E. coli* culture, using primers for *lamB* amplification. Viable cells were determined by viable plate count. Total cells were determined by acridine orange direct count. Controls included a dilution blank with no added cells (indicated by *) and nontarget salmon sperm DNA.

the viable counts. The PCR-detectable signals corresponded to the viable counts and not to the direct counts. When the sample was diluted below the level of detectable viable cells by the plating procedure, no signal was detected by the PCR procedure.

Some variability in the amount of amplified DNA produced, as evidenced by the relative intensities of the spots on the autoradiograms, could be due to efficiency of cell recovery. Increased sensitivity and more reliable collection of cells may be achieved by filtering larger volumes of water; tests of filter recovery coupled with PCR are currently being investigated in our laboratory. Also PCR may permit the detection of bacterial cells in Formalin-preserved samples (5), which would simplify sample handling and permit centralized water quality analyses.

In conclusion, the use of PCR and gene probes permits both the specificity and sensitivity necessary for monitoring coliforms as indicators of human fecal contamination of waters. PCR amplification of *lacZ* by using primers ZL-1675 and ZR-2025 and an annealing temperature of 50°C permits the detection of most coliforms, and PCR amplification of *lamB* by using primers BL-4910 and BR-5219 and an annealing temperature of 60°C enables the specific detection of low levels of the enteric indicator organism *E. coli* and of the enteropathogenic bacteria of concern, *Salmonella* and *Shigella* spp. With some simplification of the DNA extraction method and the development of an appropriate nonisotropic gene probe detection technique, PCR can permit a rapid and reliable means of assessing the bacteriological safety of waters and should provide an effective alternative methodology to conventional viable culture methods. PCR may also permit sufficient sensitivity and specificity for the direct detection of pathogens in environmental samples, rather than the current practice of relying on the indirect detection of indicator organisms.

ACKNOWLEDGMENTS

Steven Cuskey supplied the *P. putida* mt-2 (cured) strain. R. Scott Hoffman and Martin Boyce provided technical assistance. Will Bloch critically reviewed the manuscript and provided invaluable constructive criticism.

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