Detection of *Escherichia coli* in Sewage and Sludge by Polymerase Chain Reaction

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A method in which the polymerase chain reaction (PCR) was used was developed to amplify either a *uidA* gene fragment or a 16S rRNA gene fragment from *Escherichia coli* in sewage and sludge. Because of interference caused by humic acidlike substances, crude DNA extracts were purified with a Sephadex G-200 spun column before the PCR was begun. A Southern analysis in which a nonradioactive chemiluminescent method was used was performed to confirm the presence of PCR products. The sensitivity of detection for PCR products when the chemiluminescent method was used was determined to be 30 pg of *E. coli* genomic DNA template. In seeded sludge, the PCR amplified the target DNA from 80 *E. coli* cells per g of sludge and 50 *Shigella dysenteriae* cells per g of sludge. Because only 0.05 aliquot of a sludge extract was used for the PCR, we deduced that the PCR detected target DNA equivalent to the DNA of 2.5 to 4 cells in the extract. The PCR amplified the *uidA* fragment from diluted sewage influents and effluents containing *E. coli* cells. Therefore, the PCR performed with a chemiluminescent gene probe can be used to detect the presence of potentially pathogenic microorganisms in sewage and sludge. This technique can be expanded to permit direct detection of pathogenic microorganisms in water samples, thus leading to enhanced public health protection.

The polymerase chain reaction (PCR) has recently become a powerful tool with which to explore microbial activities and identities in environmental microbiology. Because of its superior sensitivity and specificity, the PCR has been used to (i) detect specific microorganisms in water, soils, and sediments without the need for cell cultivation (2, 3, 6, 9, 11, 15, 16, 18), (ii) detect viable but nonculturable organisms, such as *Vibrio vulnificus*, from a microcosm (4), and (iii) amplify mRNA (by an RNA PCR) to distinguish live *Giardia* cysts from dead cysts (10) in water samples.

The use of the PCR for identifying specific organisms obtained from environmental samples has been problematic because of the presence of various interfering substances, such as humic acids, metal ions, and other substances. Methods such as diluting crude extract templates (18), performing the PCR with preamplified products (11), and removing interfering substances (19) have been developed to overcome these problems when soil or sediment samples are used for the PCR.

Raw sewage and raw sludge contain relatively high concentrations of humic acidlike substances compared with soils. This has prevented the use of the PCR with sewage and sludge samples. If the interfering substances could be removed from these samples, the PCR could be used to detect low numbers of pathogenic microorganisms, such as *Legionella* spp., *Mycobacterium* spp., and enteric viruses, including hepatitis A virus, that are of public health concern.

The ability to detect low numbers of microorganisms would be valuable for wastewater agencies that reuse treated wastewater for irrigation of parks, golf courses, and other public greenways. In the future, if sludge is proven to be microbiologically safe, it may be used as a fertilizer for important food crops. Monitoring treated wastewater and sludge by the PCR would provide increased public health protection since the PCR is much more sensitive for detecting specific pathogenic microorganisms than traditional culture techniques are.

In this study, we adapted a rapid DNA extraction method (17) and a rapid purification procedure (19) and combined them with the PCR to detect *Escherichia coli* in sewage and sludge. The PCR was successfully used to amplify from both sewage and sludge a fragment of the *E. coli* *uidA* gene that codes for the β-D-glucuronidase.

**MATERIALS AND METHODS**

**Sampling and DNA extraction.** Raw sludge, digested sludge, primary influent, and effluent sewage samples were collected from the treatment facilities of the County Sanitation Districts of Orange County, Fountain Valley, Calif., and were processed within 4 h after sampling. The raw sludge contained 95% water, 2.7% total solids, and 1.6% volatile solids. The digested sludge consisted of 90% water, 6.5% total solids, and 4.6% volatile solids. The digested sludge was obtained from a primary digester that was fed with raw sludge.

Total DNA was extracted from 1 g (wet weight) of sludge by a rapid freeze-thaw, phenol-chloroform extraction method (17) and was resuspended in 200 μl of sterile TE buffer (10 mM Tris-HCl, 1.0 mM EDTA; pH 8.0). Portions (10 ml) of primary influent, primary effluent, and dilutions (10⁻³ to 10⁻⁶) were filtered through 0.5-μm-pore-size FHLP Teflon filters (diameter, 13 mm) by using a Swinnex filter holder (Millipore, Bedford, Mass.). Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) was used as the diluent. Crude DNA extracts from bacterial cells were obtained as described by Bej et al. (3).

**Organisms and culture media.** *E. coli* ATCC 35346 and *Shigella dysenteriae* ATCC 49552 were obtained from the American Type Culture Collection, Rockville, Md., and were maintained on nutrient agar (Difco, Detroit, Mich.). Mid-log-phase *E. coli* and *S. dysenteriae* cells were collected, washed twice with phosphate-buffered saline, and...
serially diluted (10^{-2} to 10^{-5}); the serial dilutions of each strain were seeded into sterile 1-g sludge samples and enumerated by using heterotrophic plate count techniques (1, 2). Xylose lyase (the deoxycholate agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and mFC agar (Difco) were used as selective media for *Shigella* spp. and *E. coli*, respectively. Environmental isolates were identified by using the API 20E system (Analytab Products, Plainview, N.Y.). Genomic DNA was extracted from *E. coli* cultures by using a G NOME kit (Bio 101, La Jolla, Calif.) according to the manufacturer’s instructions.

**DNA purification by gel filtration chromatography.** Crude DNA extracts obtained from sludge and sewage were purified with Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) spun columns by using a previously described method (19). Briefly, 100 μl of a crude DNA extract was loaded onto a Sephadex G-200 column, which was then centrifuged twice for 10 min at 1,100 × g to obtain 100 μl of eluent.

**PCR.** Primers UAL-754 (5’-AAAAACGCAAGAGAAAAAG CAG-3’) and UAR-900 (5’-ACCGTGTTAGCATCGTCTT GCCG-3’), which have been described previously by Bej et al. (2), were used to amplify a 147-bp coding region of the uidA gene. All of the oligonucleotides were synthesized commercially (Genosys Biotechnologies, The Woodlands, Tex.). Eluents (10-μl aliquots) from Sephadex G-200-purified sewage and sludge samples were used as templates for the PCR. DNA amplification was completed by using native Taq polymerase (Perkin-Elmer, Norwalk, Conn.) and the protocol described by Bej et al. (2). The PCR products were analyzed by electrophoresis on a 2% SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) gel stained with ethidium bromide (0.5 μg/ml) and were identified by using a gel image analysis system (Ultra-Violet Products, San Gabriel, Calif.).

**DNA hybridization.** Internal oligonucleotide probe UA-IN (5’-TGCGGGATCCTAGCGCTAATT-3’) was used to verify the identities of the PCR products. The oligonucleotide was 3’ end labeled with digoxigenin-11-dUTP by using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). The labeled DNA was transferred onto Hybond-N+ positively charged nylon membranes (Amersham, Arlington Heights, Ill.) by using a MiniBond II slot blotter (Schleicher & Schuell, Keene, N.H.) (5) or a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) for the Southern analysis (14). DNA was fixed to the nylon membranes by UV irradiation at 254 nm for 2 min by using a UV Stratalinker 2400 apparatus (Stratagene). Hybridization was performed at 60°C in the presence of a labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2× SSC in 0.1% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0]) at room temperature and then twice with a low-salt solution (0.1× SSC in 0.1% sodium dodecyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim) was used to prepare the hybridized filters for chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N.Y.) by using autoradiography (12).

**RESULTS**

The results obtained with the total DNAs extracted from primary sewage influent and raw sludge are shown in Fig. 1A. Less fragmentation of DNA was found in the sewage extracts (Fig. 1A, lanes 2 and 3) than in the sludge extracts (lanes 4 and 5). DNA extracts from the samples were either pale yellow or brownish, indicating the presence of impurities, mainly humic acid-like substances. The concentration of the impurities in the sludge extracts was approximately 5 μg (dry weight) per μl of extract. A 1-μl portion of sludge extract was enough to block the PCR, as reported previously (19). After Sephadex G-200 column purification, the purified extracts (eluents) were colorless and suitable for the PCR.

Figure 1B shows the inhibitory effects on PCR amplification observed with unpurified crude DNA extracts containing 5 μg of humic acid-like substances (Fig. 1B, lanes 2 through 5). A distinct PCR product (371 bp) from sewage and sludge (Fig. 1B, lanes 6 through 9) was observed when the purified extracts were used as templates. The amplified target fragment (positions 1169 through 1539 on the *E. coli* 16S rRNA gene) indicated that *E. coli* was present in the sewage and sludge samples.

The heterotrophic plate counts were 2.6 × 10^8 ± 0.2 × 10^8 CFU/ml for raw sewage and 1.6 × 10^10 ± 0.8 × 10^10 CFU/g for raw sludge. The *E. coli* counts on mFC agar were 8.6 × 10^1 ± 6.0 × 10^2 CFU/ml and 1.2 × 10^6 ± 0.4 × 10^6 CFU/g for raw sewage and raw sludge, respectively. *Shigella* spp. were not recovered from either sample.

The PCR detection limit for the uidA gene was determined by using *E. coli* genomic DNA as the template. Figure 2A shows 147-bp fragments amplified from the uidA-coding region in *E. coli* genomic DNA when amounts of DNA ranging from 3 ng to 3 μg were used. The amplified products were visualized on an ethidium bromide-stained agarose gel under UV light when the initial concentration of the DNA template was as low as 30 fg per reaction. A Southern blot analysis showed that the PCR products were detected when 30 ng of genomic DNA was used as the template (Fig. 2B). Long PCR fragments (147 to 400 bp) were found at template concentrations greater than 3 fg per reaction.

Figure 3A shows the hybridization signals of amplified 147-bp *uidA* fragments from raw sludge and digested sludge when the internal probe was used. Since *E. coli* was abundant in both samples (data not shown), positive PCR results were expected. However, amplified products were observed only when purified sludge extracts were used for PCR (Fig. 3A, slots 2, 4, 6, 8, 10, and 12). The heterotrophic plate counts for raw sludge and digested sludge in this experiment were 4.0 × 10^8 ± 2.0 × 10^8 and 4.1 × 10^8 ± 2.3 × 10^8 CFU/g, respectively.

The results of an experiment to determine the sensitivity of detection of *E. coli* in seeded sterile sludge when the PCR was used are shown in Fig. 3B. *E. coli* genomic DNA was used to amplify the 147-bp *uidA* fragment. The amplified product was detected in sludge seeded with as few as 80 cells per g. No amplified products were detected in sterile unseeded sludge (Fig. 3B, slot 1). Similarly, the amplified *uidA* fragment was detected in sterile sludge seeded with a minimal density of 50 *S. dysenteriae* cells per g (data not shown). Because only 5% (10 of 200 μl) of each purified extract was used for the PCR, the sensitivities of detection were assumed to be 4 and 2.5 cells for *E. coli* and *S. dysenteriae*, respectively.

Membrane filtration followed by freeze-thaw cycles (3) was used to extract total DNAs from influent and effluent sewage samples. Prior to the PCR, the crude DNA extracts were purified with a Sephadex G-200 spin column. No PCR products were detected when unpurified extracts were used for amplification. The hybridization signals revealed the presence of an amplified *uidA* fragment in diluted sewage
samples at dilutions ranging from $10^{-3}$ to $10^{-6}$. No amplification was detected when we examined the Sephadex G-200 column eluents that did not contain DNA templates (Fig. 3C, slots 1 and 6). The heterotrophic plate counts for the influent and effluent samples were $7.1 \times 10^6 \pm 2.1 \times 10^6$ and $5.1 \times 10^6 \pm 1.9 \times 10^6$ CFU/ml, respectively. No Shigella spp. were recovered from influent and effluent sewage samples. The *E. coli* counts were not determined.

**DISCUSSION**

The initial problem associated with using the PCR with sewage and sludge samples (i.e., interfering substances) was eliminated. A Sephadex G-200 spun column was used to purify all crude extracts before amplification. The removal of interfering substances allowed the use of larger volumes (10 to 50 µl) of extracts as templates during the PCR (total volume, 100 µl), resulting in increased sensitivity. Spun columns have been used successfully by other researchers to remove interfering components from beef extract-glucine ferric chloride virus concentrates before the PCR was performed (6, 13). This illustrates the efficacy of using gel filtration chromatography to purify environmental samples used for PCR amplification. Because *E. coli* is commonly found in sewage and sludge samples, detection of *E. coli* 16S rRNA genes or the *uidA* gene by the PCR was expected. Although the *uidA* gene may be found in some *Shigella* spp. (2), it is most commonly present in *E. coli* (8).

An ethidium bromide-stained gel could detect the PCR products amplified from 30 fg of *E. coli* genomic DNA (seven copies of the *uidA* gene). The sensitivity of detection was increased to 30 ag (less than one copy) when the chemiluminescent probe was used. The chemiluminescent detection limit is at the same order of magnitude as the detection limit reported by Bej et al. (3), who used radioactive $^{32}$P-labeled oligonucleotide probes with *Legionella pneumophila* genomic DNA. Because of its safety, low cost, and sensitivity equivalent to the sensitivity of the $^{32}$P method, the chemiluminescent method has distinct advantages over radioisotopic detection. Long fragments with defined primer ends were produced during the PCR (7). In the sensitivity test, the long fragments were found only when higher template concentrations were used for amplification. This suggests that optimization of template concentration is necessary to reduce the production of long fragments and to increase the amplification efficiency.

The hybridization results showed that the presence of humic acid-like substances completely inhibited the PCR. Amplified DNA was detected only after sample extracts were purified with Sephadex G-200 spun columns. The results of the sensitivity tests performed with seeded sterile sludge samples suggest that 80 *E. coli* cells per g and 50 *S. dysenteriae* cells per g can be detected by using the PCR and gene probe methods. Because mid-log-phase cells were used for seeding, amplified DNA from nonviable cells was minimized. However, the PCR products of unseeded native samples could be amplified from free DNA or from DNA from dead cells. The detection limit for unseeded sludge samples needs further investigation. Only the DNA direct extraction method (17) was used for obtaining the PCR DNA templates from sludge samples. The filtration method (3) was not suitable for determining PCR sensitivity for sludge samples because dilution procedures, which reduced the detection sensitivity, were necessary to prevent the filters from clogging.
Because raw sewage also contains other particulate materials, diluting the samples is necessary before filtration. Alternatively, centrifugation at 6,000 \( \times \) g for 10 min was used to obtain cell pellets from large volumes (50 to 100 ml) of undiluted sewage samples. Crude DNA extracts obtained by a freeze-thaw, phenol-chloroform extraction method (17) must be purified prior to the PCR.

Our results showed that DNA shearing was more prominent in sludge than in sewage after extraction. This could be caused by different components, such as more particulate matter present in sludge than in sewage, or by the action of contaminated DNase during the extraction. The filtration methods require an additional Sephadex G-200 purification step in order to obtain positive PCR results. *Shigella* spp. were not recovered from influent and effluent samples, suggesting that the amplified *uidA* gene fragments originated from *E. coli*.

In conclusion, the PCR and nonradioactive gene probe methods were successfully used to detect target microorganisms present in sewage and sludge samples. The PCR interfering substances were removed by using a Sephadex G-200 spun column. This purification procedure is crucial for environmental DNA extracts before PCR amplification. Because of their sensitivity and specificity, the PCR and gene probe techniques can be used to detect potentially pathogenic microorganisms in raw sewage and sludge. This should allow wastewater industries to evaluate the efficiency of treatments for removing pathogens.
FIG. 3. Slot blot hybridization signals for amplified *uidA* DNA obtained from sludge and sewage samples. (A) Raw sludge and digested sludge. Slots 1, 3, and 5, unpurified raw sludge extracts; slots 2, 4, and 6, purified raw sludge extracts; slots 7, 9, and 11, unpurified digested sludge extracts; slots 8, 10, and 12, purified digested sludge extracts; slot 13, *E. coli* genomic DNA control; slot 14, reaction mixture (no template). (B) Seeded sterile sludge. Slot 1, unseeded extract; slot 2, preparation seeded with 8.0 × 10^7 cells per g; slot 3, preparation seeded with 8.0 × 10^6 cells per g; slot 4, preparation seeded with 8.0 × 10^5 cells per g; slot 5, preparation seeded with 8.0 × 10^4 cells per g; slot 6, preparation seeded with 8.0 × 10^3 cells per g; slot 7, preparation seeded with 8.0 × 10^2 cells per g; slot 8, preparation seeded with 8.0 × 10^1 cells per g; slot 9, preparation seeded with 8.0 cells per g; slot 10, reaction mixture (no template); slot 11, *E. coli* genomic DNA. (C) Primary influent (slots 2 through 5) and effluent (slots 7 through 10). Slots 1 and 6, unseeded control (sterile diluent); slots 2 and 7, 10^-3 dilution; slots 3 and 8, 10^-4 dilution; slots 4 and 9, 10^-5 dilution; slots 5 and 10, 10^-6 dilution; slot 11, *E. coli* genomic DNA; slot 12, reaction mixture (no template).

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