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PCR for Bioaerosol Monitoring: Sensitivity and Environmental Interference

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The PCR technique has potential for use in detection of low concentrations of airborne microorganisms. In this study, the sensitivity of PCR and its susceptibility to environmental interference were assessed with *Escherichia coli* DH1 as the target organism. Air samples, containing environmental bioaerosols, were collected with AGI-30 samplers and seeded with *E. coli* DH1 cells. Parallel studies were performed with cells seeded into the sampler prior to collection of air samples to determine the effects of environmental inhibition and sampling stress on the PCR assay. Baseline studies were also performed without environmental challenge or sampling stress to compare two protocols for cell lysis, solid phase and freeze-thaw. Amplification of a plasmid target sequence resulted in a detection limit of a single bacterial cell by the freeze-thaw and solid-phase methods within 5 and 9 h, respectively. With a genomic target, the sensitivity of the solid-phase method was 10-fold lower than that of freeze-thaw. Samples which contained 10^3 to 10^4 CFU of environmental organisms per m^3 inhibited amplification; however, a 1/10 dilution of these samples resulted in successful amplifications. No difference in sensitivity of the PCR assay was obtained as a result of sampling stress, although a 10-fold decrease in culturability was observed. A field validation of the protocol with genomic primers demonstrated the presence of airborne *E. coli* and/or *Shigella* spp. in outdoor samples. This study indicates that the PCR method for detection of airborne microorganisms is rapid and sensitive and can be used as an alternative method for air quality monitoring.

Airborne microorganisms are a potential source of a wide variety of public and industrial health hazards. Of particular significance are bioaerosols associated with wastewater treatment processes (4), nosocomial infections (1), fermentation facilities (14), biological warfare (19), and the release of genetically engineered microorganisms to enhance agricultural productivity (17). Also, the airborne transmission of pathogenic microorganisms (e.g., *Legionella pneumophila* [23] and *Mycobacterium tuberculosis* [7]) and the appearance of newly recognized pathogens with an airborne transmission route (e.g., hantavirus [9]) are of growing concern.

Effective monitoring of bioaerosols requires the efficient collection of microorganisms from the air. In addition, an appropriate technique for analysis of air samples must be selected. The variety and complexity of bioaerosol pollutants complicate monitoring and exposure assessment research. While monitoring for airborne microorganisms has traditionally focused on the collection of fungal spores and bacterial cells and the analysis of samples by total-count and culture techniques, these methods have several limitations (12). The total-count enumeration methods are laborious, and identification of microorganisms is problematic. Culture methods assume that the organisms will grow and produce classical characteristics within a specified period. However, organisms that are not culturable under the specific growth conditions imposed in the laboratory remain undetected yet may be capable of inducing adverse health effects (6, 10). This is particularly a problem with organisms subjected to the stress of aerosolization and sampling, which can result in a loss of culturability (5, 8, 22). These losses are difficult to assess and may vary within and between species. In addition, culture-based analysis methods can take several days to weeks to perform. Therefore, rapid, accurate means to monitor airborne microorganisms are needed to overcome the constraints encountered with traditional culture-based methods.

An alternative method for detection of microorganisms in environmental samples is the PCR assay (15). PCR permits the detection of target nucleic acid sequences of DNA, thereby eliminating the requirement for growth to detect and identify microorganisms. The specificity, sensitivity, and reduced processing time of this technique are suitable for applications in aerobiological monitoring for the detection of small numbers of targeted microorganisms. Previously, detection of a genetically modified bacterium by PCR was shown in greenhouse aerosolization experiments (2). Solid-phase PCR amplification detected airborne bacterial cells when the traditional culture assay was negative. Detection of target sequences at low concentrations in sampling buffer indicated that PCR amplification could be used to detect microorganisms retrieved during aerobiological monitoring by liquid impingement.

Because air samples may contain compounds inhibitory to the amplification assay, determination of environmental interferences is necessary to assess the utility of the PCR for field screening. It was reported previously that trace amounts of humic substances, noncharacterized coextracted substances, and high concentrations of nontarget DNA can inhibit polymerase activity, thus causing false-negative results and a decrease in the sensitivity of detection (11, 18, 21). Concerns for the possibility of interference to the amplification process owing to airborne environmental contaminants prompted this study.

While research in our laboratory previously demonstrated the use of PCR for detection of an airborne microorganism, the precision, comparability, and limitations of this technique

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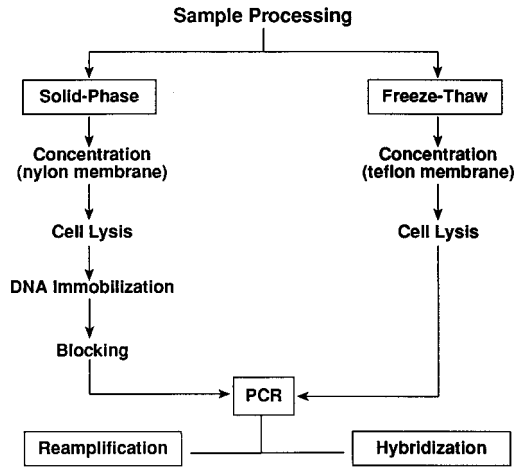

FIG. 1. Diagram of cell lysis protocols.

for measurement of airborne microbial contaminants had not been fully assessed. The purpose of this study was to (i) determine the detection limits of PCR for field monitoring of airborne bacteria; (ii) optimize the PCR process, including the bacterial lysis and amplification steps; (iii) examine environmental interference to the method; and (iv) demonstrate that the methods developed can be used for rapid detection of low concentrations of airborne microorganisms.

MATERIALS AND METHODS

Target organism. *Escherichia coli* DH1 containing a plasmid (pWTALA5′) derived from pBR322 was used as the target organism (2, 20). This plasmid has a 437-bp insert from *Bombyx mori* coding for $tRNA_2^{Ala}$ and constitutes a unique marker for the identification of the DH1 cells. The *E. coli* DH1 cells were cultured on Luria-Bertani agar (pH 7.5) (Difco Laboratories, Detroit, Mich.) supplemented with 50 μg of ampicillin and 15 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml (LBAT agar) and incubated at 37°C for 24 h. For seeding experiments, the organism was prepared as follows. Bacterial cells were grown in LBAT broth at 37°C until the optical density at 600 nm was ca. 0.6. The culture was harvested by centrifugation, washed twice, and serially diluted in sterile phosphate buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4). Cell concentration (CFU per milliliter) was determined by spread plating onto LBAT agar.

Cell lysis. Two methods were evaluated with respect to release of DNA of the target bacterium prior to PCR amplification (Fig. 1). The solid-phase method involved lysing the bacterial cells as previously described by Alvarez et al. (2), with modifications. Serially diluted bacterial cells were filtered through 13-mm-diameter Magna nylon filters (pore size, 0.45 μm; MSI, Inc., Westboro, Mass.) by using a filter manifold (Millipore, Inc., Bedford, Mass.) with positive pressure. The membrane was removed from the manifold, and the bacterial cells were lysed by sequentially placing membranes face up consecutively for 10 min onto 3MM filters (Whatman Inc., Clifton, N.J.) soaked with (i) 10% sodium dodecyl sulfate (SDS), (ii) 0.5 M NaOH–1.5 M NaCl (steamed above a boiling-water bath), (iii) 1.0 M Tris-HCl (pH 7.4)–1.5 M NaCl, and (iv) 0.3 M NaCl. The liberated nucleic acids were then fixed onto the nylon membrane by baking in an oven at 80°C for 2 h. The nonspecific sites were blocked by incubating the membranes in 1% bovine serum albumin (fraction V; Sigma) for 2 h in a rotary incubator at 40°C. Entire membranes were placed face inward inside microcentrifuge tubes and subjected to PCR amplification.

Bacterial cells were also lysed by the freeze-thaw method described by Bej et al. (3), with some modifications (Fig. 1). *E. coli* DH1 cells were filtered onto a Teflon membrane (diameter, 13 mm; pore size, 0.45 μm; Gelman Sciences, Ann Arbor, Mich.) under positive pressure. Each filter was placed into a microcentrifuge tube with 100 μl of sterile distilled water and vortexed for 30 s to dislodge the cells. The filter was then removed from the tube and discarded. Tubes containing the suspended cells were subjected to a series of seven alternating freeze-thaw cycles consisting of an ethanol-dry ice bath for 1 min followed by a 65°C water bath for 1 min for each freeze-thaw cycle. Following lysis of the cells, samples were heated to 85°C for 5 min to denature any nucleases or proteases and subjected to PCR amplification.

Air sample collection. Air sampling was performed by liquid impingement with AGI-30 samplers (Ace Glass Co., Vineland, N.J.) operated in nonsterile field environments. The AGI-30 samplers, each containing 20 ml of collection buffer (0.1 M phosphate buffer), were operated outdoors at the manufacturer's suggested flow rate of 12.5 liters/min for 10 min. After the samplers were returned to the laboratory, the inlet of each sampler was rinsed with 5 ml of sterile phosphate buffer to collect cells trapped in the curved inlet tube. Wash buffer was added to the collection buffer, resulting in a total volume of 23 to 24 ml. Multiple samples were pooled for seeding experiments. Total numbers of culturable airborne environmental microorganisms per cubic meter of air sampled were determined by filter concentration of a 10-ml aliquot of the sample onto a 37-mm-diameter mixed cellulose ester filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filter was then placed on tryptic soy agar (Difco Laboratories) and incubated at 28°C for 24 to 48 h. The remainder of the sample was subdivided for seeding experiments.

A field validation of the modified freeze-thaw method was also performed with bioaerosol monitoring. After bioaerosol sampling in nonsterile environments, 10-ml aliquots of the sampling buffer of pooled AGI-30 samples were processed by the freeze-thaw cell lysis method as described above and subjected to PCR amplification with primers for a genomic target specific for the detection of *E. coli* and *Shigella* spp. (3).

PCR amplification. For amplification of target DNA, two sets of primers were used. The sequences of the primers used for the amplification are 5′CTGTTGG CATCTTTTAGATTAAGTG3′ (PWF1) and 5′AAGTGCAAATTGAATTGA ATCG3′ (PWR3). These sequences are specific for the pWTALA5′ plasmid insert, and the size of the amplification product is 394 bp. For detection of *E. coli*, a 154-bp fragment of the regulatory region of the *uidA* gene was amplified with primers 5′TGTTACGTCCTGTAGAAAGCCC3′ (URL301) and 5′AAAACT GCCTGGCACAGCAATT3′ (URR432) (3).

A total volume of 200 μl of PCR mix was added to each tube. All PCR conditions (final concentrations) were as follows: 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 1.5 mM MgCl, 200 μM each deoxynucleoside triphosphate, 0.25 μM each primer, and 4 U of *Taq* polymerase (Promega, Madison, Wis.). A mineral oil overlay to completely cover the solution was added to prevent condensation. PCR amplification was conducted with a DNA thermal cycler (TempCycler II; Coy, Grass Lake, Mich.), using an initial denaturation step of 95°C for 2 min followed by 35 cycles consisting of denaturation at 95°C, annealing at 55°C, and primer extension at 72°C (1 min each), with a final extension at 72°C for 5 min. Aliquots (20 μl) of the PCR products were separated by gel electrophoresis through a 2% horizontal agarose gel at 50 V in 0.5× TBE buffer (16) for 1 h. Gels were stained with ethidium bromide and visualized under UV transillumination.

To reamplify plasmid DNA, 5-μl samples of the original amplification products were transferred to tubes containing 45 μl of the reaction mixture described above except that the oligonucleotide primer PWF1 was replaced by primer PWF2 (5′CACTCATTCCGTTTCAAAAGTG3′), which recognizes sequences contained within the plasmid insert sequence amplified by PWF1 and PWR3. The *uidA* gene target DNA was reamplified with the same set of primers. Samples were amplified for an additional 25 cycles as described above.

Blotting and hybridization conditions. For slot blot analysis, 50-μl aliquots of amplified samples were denatured by the addition of 100 μl of 0.5 M NaOH–25 mM EDTA, incubated for 30 min at room temperature, and neutralized with 120 μl of 2 M ammonium acetate. The samples were loaded into wells of a manifold (Bio-Dot SF Microfiltration Apparatus; Bio-Rad, Hercules, Calif.) fitted with a nylon membrane (Zeta Probe; Bio-Rad) previously wetted in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]). Each well was rinsed with 0.4 ml of 2× SSC, and the DNA was immobilized by baking for 2 h at 80°C.

PCR-generated biotinylated probes were synthesized as described by Lo et al. (13), with the following modifications. After a PCR amplification, 5-μl samples of the amplification products were transferred to tubes containing 95 μl of the same reaction mixture described above except that the dCTP concentration was reduced to 150 μM and 51 μM bio-11-dCTP (Enzo Biochem, Farmingdale, N.Y.) was included. Twenty-five cycles of PCR were performed with primers PWF2 and PWR3 (plasmid probes) or primers URL301-URR432 (genomic probes) as described above. Probe purification with spin columns (IBI Nu-Clean D50; Eastman Kodak Co., New Haven, Conn.) was performed as specified by the manufacturer, and probes were stored at −20°C.

High-stringency hybridization and detection were performed essentially as described in the Kodak Chemiluminescent Detection System Technical Manual (Eastman Kodak Co.). Membranes were prehybridized in 5× SSPE (25 mM NaH_2PO_4, 2.5 mM EDTA, 0.45 M NaCl)–0.1% SDS–0.5% blocker–5% dextran sulfate–50 μg of denatured salmon sperm DNA per ml for 30 min at 60°C. Hybridization was performed overnight at 65°C in the same solution containing the biotin-labeled oligonucleotide (10 μl of PCR-labeled biotinylated probe per 50 cm^2 of filter). Posthybridization washes were sequentially performed once each for 15 min at 60°C in 2× SSPE–0.1% SDS, 0.5× SSPE–0.1% SDS, and 0.1× SSPE–0.1% SDS followed by two brief rinses in TBS (100 mM Tris, 150 mM NaCl [pH 7.5]). After incubation in blocking solution (0.5% blocker, 1× TBS) for 45 min at 42°C, streptavidin-horseradish peroxidase conjugate (final concentration, 1/5,000) was added, and the mixture was incubated for 30 min at room temperature. Membranes were washed three times for 5 min at room temperature in TBS–1% Triton and twice for 5 min at room temperature in TBS, equilibrated, and exposed to XAR film (Eastman Kodak Co.) in cassettes at room temperature for 30 min.

Seeding experiments. A study was designed to facilitate the analysis of samples for detection of bacteria by increasing the sensitivity of PCR and reducing the

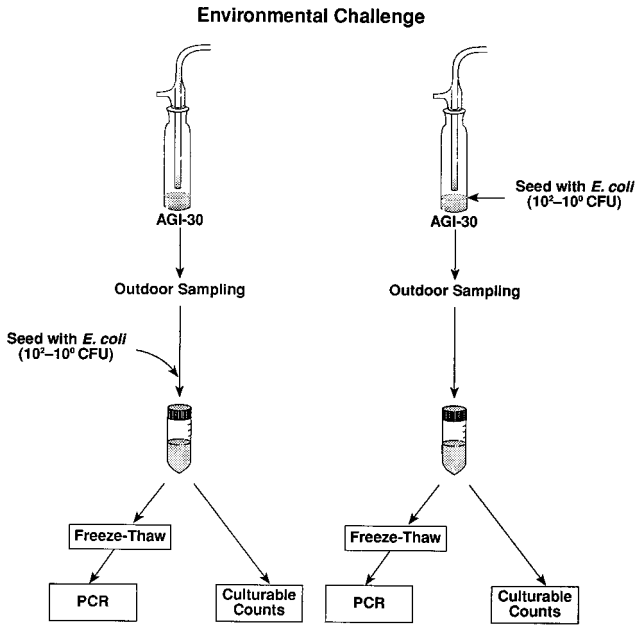

FIG. 2. Schematic representation of environmental challenge seeding experiments.

time required for analysis. Aliquots of *E. coli* DH1 dilutions were seeded into tubes containing 10 ml of sterile phosphate buffer. The concentration of *E. coli* cells in the vials ranged from 10^2 to 10^{-1} CFU per tube as determined by dilution and spread plate culture methods. These samples were then processed and analyzed for detection of the target cells by both solid-phase and freeze-thaw PCR methods as described above (Fig. 1). The sensitivity of the PCR amplification was assessed by using primers for plasmid insert as well as genomic (*uidA*) detection of the *E. coli* DH1 target. Reamplification and slot blot hybridization of aliquots from the amplification products were performed as described above. The sensitivity levels of the two methods were then compared.

Concern over the possibility of interferences with the PCR amplification owing to environmental contaminants prompted a series of environmental challenge experiments (Fig. 2) with AGI-30 samplers, as described above. Different outdoor sites were selected for which different concentrations of airborne microorganisms were expected. The total number of culturable airborne environmental microorganisms was determined by culturing an aliquot of the buffer on TSA and incubating it at 28°C for 24 to 48 h. The remainder of the sample was subdivided for PCR analysis. Suspensions of the test organism were seeded (10^2 to 10^0 CFU per tube) after sample collection into tubes containing 10 ml of the environmental challenge samples. The DNA was released by the freeze-thaw cell lysis method as described above. Experiments were repeated with seeding performed before the collection of environmental air samples to determine the effect of sampling stress on culturability of the seeded organism (Fig. 2).

To determine whether the filterable fraction caused inhibition of the PCR assay, a second seeding experiment in which 10^2 *E. coli* cells were added to 10 and 1 ml of unfiltered and filter-sterilized (0.22-μm-pore-size HVLP filters, Millipore, Bedford, Mass.) buffer from previously collected air samples was performed. PCR amplification and product detection were performed as described above.

RESULTS

To determine the sensitivity of the cell lysis methods and primers for detection of the target microorganism, PCR was performed on *E. coli* DH1 cells serially diluted in sterile phosphate buffer solution. Table 1 shows results of the amplification both for plasmid and genomic target DNA. Results illustrate that sensitivity of detection for the plasmid target when using the freeze-thaw method was 10^0 CFU, corresponding to approximately one bacterial cell, compared with 10^1 CFU for the solid-phase lysis on the ethidium bromide-stained gel. However, when the presence of amplified bacterial plasmid DNA was identified after reamplification or slot blot hybridization with a biotin-labeled oligonucleotide, the sensitivity was at the same order of magnitude for both cell lysis methods. Samples seeded with 10^{-1} CFU were negative for both lysis methods.

TABLE 1. Comparison of the sensitivity of two cell lysis protocols for the detection of *E. coli* cells by PCR

Method	Detection procedure	Sensitivity with plasmid target[a]:				Sensitivity with genomic target[a]:			
		10^2	10^1	10^0	10^{-1}	10^2	10^1	10^0	10^{-1}
Freeze-thaw	Amplification[b]	+	+	+	−	+	+	−	−
	Reamplification[c]	ND[d]	+	+	−	+	+	+	−
	Hybridization[e]	ND	+	+	−	ND	+	+	−
Solid phase	Amplification	+	+	−	−	+	+	−	−
	Reamplification	ND	+	+	−	+	+	−	−
	Hybridization	ND	+	+	−	ND	+	−	−

[a] Numbers indicate the number of CFU seeded per sample.
[b] Thirty-five cycles of PCR amplification.
[c] Twenty-five cycles of amplification with 5 μl of initial product.
[d] ND, not determined.
[e] Slot blot hybridization with 50 μl of initial product.

In contrast to plasmid amplification, the results of the PCR assay for the genomic target sequence were negative initially for 10^0 CFU when lysed by the freeze-thaw method (Table 1). After reamplification or hybridization of these samples, positive results were obtained. This indicates that reamplification and hybridization further increase the sensitivity of PCR by allowing the detection of a small number of target genes which cannot be visualized by ethidium bromide staining alone. The sensitivity of detection by using solid-phase lysis and genomic primers was 10-fold lower than that of the freeze-thaw protocol, even after reamplification or hybridization. A detection limit of 10^1 CFU was routinely obtained with this protocol.

Data obtained from environmental interference trials are shown in Fig. 3 to 6. Positive results were obtained at 10^2 and 10^1 CFU dilutions in the seeded samples (Fig. 3), as shown by a distinct band corresponding to the 394-bp target sequence (plasmid target). Although the 10^0 CFU dilutions were initially negative, positive results were obtained for both 10^0 dilutions after reamplification (data not shown). No difference in the level of sensitivity after PCR amplification was observed between samples seeded before and after air sampling. The positive result in the 10^0 dilution indicated detection of approximately one target bacterium per seeded sample. No amplification was observed in negative controls, consisting of non-

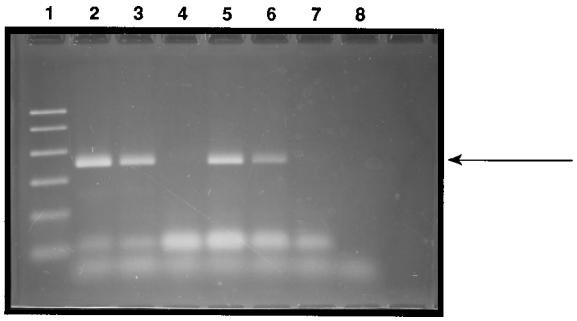

FIG. 3. Electrophoretic separation of PCR amplification products from cells seeded in 10-ml aliquots of environmental challenge samples (2.1×10^2 CFU of culturable airborne microorganisms per m^3). Lanes: 1, PCR marker (Promega) (bands from top to bottom, 1,000, 750, 500, 300, 150, and 50 bp); 2 through 4, 10^2, 10^1, and 10^0 cells, respectively, seeded after outdoor sampling; 5 through 7, 10^2, 10^1, and 10^0 cells, respectively, seeded before outdoor sampling; 8, negative control.

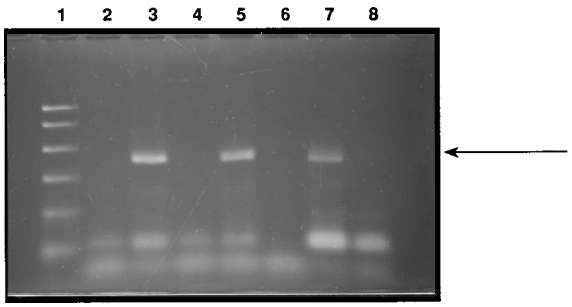

FIG. 4. Agarose gel (2% agarose) electrophoresis of PCR amplification products from cells seeded in 10-ml aliquots of environmental challenge samples (1.6×10^4 CFU of culturable airborne microorganisms per m^3). Lanes: 1, PCR marker; 2, 4, and 6, 10^2, 10^1, and 10^0 cells, respectively, seeded after sampling; 3, 5, and 7, 1/10 dilution of 10^2, 10^1, and 10^0 cells, respectively, seeded after sampling; 8, negative control.

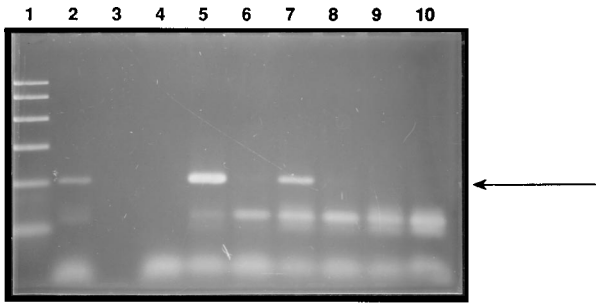

FIG. 6. Agarose gel (2% agarose) electrophoresis of PCR amplification products from cells seeded in 10-ml aliquots of environmental challenge samples (8×10^3 CFU of culturable airborne microorganisms per m^3). Lanes: 1, PCR marker; 2, field validation results; 4, 6, and 8, 10^2, 10^1, and 10^0 cells, respectively, seeded after sampling; 5, 7, and 9, 1/10 dilution of 10^2, 10^1, and 10^0 cells, respectively, seeded after sampling; 10, negative control.

seeded buffer samples. Although these results indicate that environmental interference may not affect PCR detection of airborne bacteria, only 2.1×10^2 CFU of culturable airborne microorganisms per m^3 was measured in the outdoor air during this trial. In contrast, when the outdoor airborne culturable-microorganism concentration was 10-fold higher (4×10^3 CFU/m^3), the results of the amplification assay were negative at 10^2 to 10^0 CFU dilutions in seeded samples (data not shown). However, positive results were obtained in subsequent trials by performing a 1/10 dilution of the samples. With an airborne-microorganism concentration of 1.6×10^4 CFU/m^3, results were positive at 10^2 through 10^0 CFU in seeded samples after a 1/10 dilution, while undiluted samples were negative (Fig. 4). Performing the 1/10 dilution after the freeze-thaw lysis step was determined to be more effective than performing it prior to lysis (data not shown).

The requirement for dilution to eliminate interference in the seeded samples could be attributed to undefined substances or to nontarget DNA in the sample. To determine if the inhibition was related to background airborne microbial concentrations, 10- and 1-ml aliquots of collection buffer that had been filtered (pore size, 0.22 μm) after air sampling (outdoor airborne-microorganism concentration, 10^4 CFU/m^3) were seeded with *E. coli* DH1 cells (10^2 CFU) and amplification results were compared with those of nonfiltered buffer samples. The results are shown in Fig. 5. Positive results were observed in both filtered samples and in the 1-ml nonfiltered samples but not in the 10-ml nonfiltered seeded sample. This demonstrates the influence of high concentrations of nontarget DNA on the inhibition of the amplification reaction.

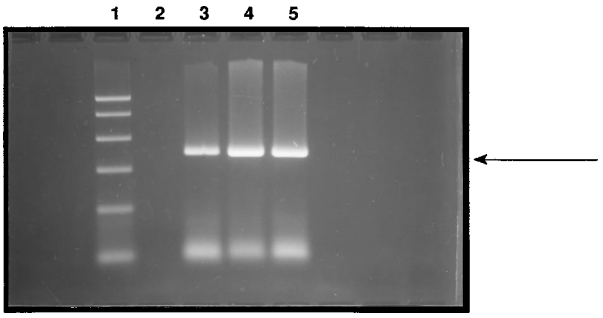

FIG. 5. PCR amplification products of *E. coli* cells seeded in filtered sterilized (pore size, 0.22 μm) and nonfiltered collection buffer after air sampling (10^4 CFU of outdoor airborne microorganisms per m^3). Lanes: 1, PCR marker; 2 and 3, 10^2 cells seeded in 10- and 1-ml aliquots, respectively, of nonfiltered collection buffer; 4 and 5, 10^2 cells seeded in 10- and 1-ml aliquots, respectively, of filtered collection buffer.

As a field validation of the lysis amplification protocol, PCR amplification was performed with primers specific for the *uidA* gene on unseeded AGI-30 collection buffer after sampling outdoor air adjacent to livestock corrals. Positive amplification of this target was observed by ethidium bromide staining alone (Fig. 6), indicating the presence of *E. coli* and/or *Shigella* spp. in the air sample collected.

Genomic DNA was used as a target in environmental interference experiments with an 8×10^3-CFU/m^3 culturable-airborne-microorganism concentration. Positive amplification was obtained with 1/10 dilutions from seeded samples with a total concentration of 10^2 and 10^1 CFU/10-ml sample (Fig. 6). The positive result in the diluted 10^1-CFU seeded sample indicated detection on an agarose gel of genomic DNA corresponding to approximately one bacterial cell. Negative results were observed for nonseeded samples, processed side by side with the environmental challenge samples. Amplification of undiluted, seeded samples by PCR was not possible, even after reamplification (data not shown).

Although no difference was obtained in the sensitivity of detection by PCR of samples seeded before and after air sampling (Fig. 3), it was observed throughout the study that sampling stress reduced the culturability of the seeded *E. coli* cells in 10-min AGI-30 samples. A decrease in culturable counts of at least 1 order of magnitude was obtained from samples seeded prior to air sampling compared with those seeded after air sample collection (Table 2).

DISCUSSION

This study demonstrates the sensitivity of the PCR assay for the rapid detection of low concentrations of airborne bacteria. To develop an efficient protocol for the detection of airborne microorganisms, the sensitivity of PCR amplification was evaluated, cell lysis protocols were modified, and environmental interferences in the protocol were determined. Processes investigated included (i) plasmid versus genomic amplification, (ii) freeze-thaw versus solid-phase lysis, and (iii) slot blot hybridization versus reamplification. Detection of 1 to 10 target organisms was achieved with primers specific for plasmid or genomic targets, with enhanced results obtained by both slot blot hybridization and reamplification.

The difference in sensitivity observed between plasmid and genomic detection is probably the result of the presence of

TABLE 2. PCR results and the effect of sampling stress on culturable-cell counts for trials with *E. coli* cells seeded prior to air sampling

Trial	*E. coli* concn (CFU/ml, seeded)	Culture counts[a] (CFU/ml, after sampling)	PCR result[b]
1	140	7	+
	14	1	+
2	101	36	+
	14	6	+
3	180	9	+
	180	6	+

[a] Incubation time, ca. 24 h.
[b] Analysis time, ca. 5 h.

multiple copies of the pWTALA5′ plasmid in the target cells. This factor should be considered and may be used advantageously when selecting amplification targets. Multiple copy number, the equivalent of multiple amplification targets in an organism, improves the possibility of detection of a low concentration of target organisms.

To maximize the lysis of bacterial cells, different temperatures and additional lysis steps were tested. For the freeze-thaw protocol, the temperature during the thawing step was increased from 50 to 65°C and the number of freeze-thaw cycles was increased. Optimal conditions per organism must be determined because too many or too few lysis cycles may result in false-negative results due to DNA degradation or failure to release the target DNA, respectively. Although removing the filters prior to cell lysis may result in loss of some cells, routine detection of 1 to 10 target cells was observed. For the solid-phase lysis protocol, an additional steaming step was added, and this alteration increased the sensitivity of detection. Therefore, an optimized PCR protocol requires not only characterization of specific primers and amplification parameters but also empirical determination of optimum cell lysis conditions.

Total processing time for amplification of targeted cells, from cells lysis to gel electrophoresis, was approximately 5 h for the freeze-thaw protocol and 9 h for the solid-phase protocol. The modified freeze-thaw lysis protocol was used throughout the remainder of the study because it allowed for faster processing. However, solid-phase PCR remains an alternative option. Advantages of developing solid-phase PCR over other direct detection techniques include the fact that the DNA present in the original sample becomes bound to the filter, permitting multiple processing and detection of several cell types in succession (20). Therefore, the solid-phase PCR technique permits a single sample to be repeatedly amplified, a feature not available with the freeze-thaw or other analytical methods.

Both reamplification and slot blot hybridization with biotin-labeled probes increased the sensitivity of the PCR assay, and no difference in detection level was observed between the two methods. As with the cell lysis protocols, the decision of which protocol to use must be based on the particular needs of the project. Processing time can also be a factor in this decision. Nonradioactive hybridization required 5 h to overnight for processing compared with 3 h for reamplification.

Successful PCR amplification was achieved in a field validation of the protocol developed. Airborne *E. coli* and/or *Shigella* cells were detected by using AGI-30 samplers with the freeze-thaw release of DNA and PCR amplification. The major obstacle in using the PCR assay for environmental samples is the presence of components that inhibit polymerase activity or the primer binding and reduce the sensitivity of detection. Although no interference was noted in the first outdoor challenge trial, the concentration of airborne microorganisms was low (10^2 CFU/m^3). Outdoor concentrations of airborne microorganisms are often much higher, and PCR detection of seeded target cells was inhibited in environmental samples averaging 10^3 to 10^4 CFU/m^3. However, a 10-fold dilution of the crude DNA extract after freeze-thaw lysis eliminated interference with the amplification process. PCR amplification of undiluted, seeded samples was not possible. Although the dilution method can attenuate the inhibition effect, it can also reduce the sensitivity. However, a sensitivity equivalent to one organism, even after dilution, was attained throughout this study. This may be due to the presence of multiple copies of the target sequence and to the fact that the dilution was done after the cell lysis step.

A high concentration of nontarget DNA is a major inhibitory component of PCR amplification. The presence of nontarget DNA coextracted with target DNA from soil samples was reported to inhibit the PCR assay (11). In the presence of indigenous total DNA in soils, PCR amplification was inhibited when the DNA concentration in soil exceeded 1 μg/ml in the reaction mixture. Our results in which a simple filtration step attenuated the inhibition previously observed seem to confirm these results.

Because aerosolization and sampling stress may result in the loss of culturability of vegetative bacterial cells (5, 8, 14), it is expected that the PCR analysis method, which permits the detection of cells regardless of their metabolic state, may be orders of magnitude more sensitive than culturable-cell detection methods. This is especially important since allergic reactions and other adverse health effects can be caused by nonculturable microorganisms. The 10-fold reduction in viability as a result of sampling stress observed in our study underscores the need for analytical techniques, such as PCR, that can be used in addition to traditional culture techniques.

The detection of target sequences at low concentrations in sampling buffer indicates that PCR amplification could be used to detect one cell retrieved during aerobiological monitoring by liquid impingement. The retrieval of one cell with an AGI-30 sampler at 12.5 liters/min for 10 min and subsequent PCR amplification corresponds to a detection limit of ca. 9 cells per m^3 of air. In comparison, retrieval of a single culturable bacterial cell with Andersen impactor sampler (Graseby Instruments, Inc., Atlanta, Ga.) operating at a flow rate of 28.3 liters/min for 2 min corresponds to a detection limit of ca. 18 culturable bacteria per m^3 and requires a minimum of 48 h for detection. Theoretically, by increasing the Andersen sampler run time to 5 min, a lower detection limit of 7 culturable bacteria per m^3 could be achieved, which would approximate the detection limit of the PCR method. However, Buttner and Stetzenbach (5) demonstrated decreasing measurements of culturable *Pseudomonas syringae* cells with increasing sampling time. The decreased number of bacterial cells per cubic meter was attributed to decreased viability caused by stress incurred during prolonged sampling time. The PCR method does not rely on culturability of the organism for detection and therefore is unaffected by extended sampling time.

While the feasibility of detecting airborne organisms was addressed in this study, other important issues must be further investigated to evaluate PCR for analysis of bioaerosols: quantitation of airborne organisms, the use of alternative collection methods, and the ability to distinguish between living and dead organisms. PCR may be used to enhance the detection of airborne microorganisms for which culture methods are

lengthy, inconvenient, a biosafety hazard, or simply unavailable. The array of potentially airborne pathogenic microorganisms encompasses bacteria, parasites, fungi, and viruses. These methods will also be of great benefit to scientists tracking genetically engineered microorganisms released into natural environments. In summary, the PCR amplification assay enhances the detection of airborne microorganisms by lowering the level of detection, reducing the analysis time required, and eliminating the need for costly and time-consuming identification procedures.

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