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Polymerase Chain Reaction Detection of Nonviable Bacterial Pathogens

K. L. JOSEPHSON, C. P. GERBA, and I. L. PEPPER*

Department of Soil and Water Science, University of Arizona, Tucson, Arizona 85721

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Polymerase chain reaction (PCR) methodologies for detection of pathogens in environmental samples are currently available. However, positive amplification products for any set of primers only signal that the appropriate target nucleic acid sequences were present in the sample. The presence of the amplification products does not imply that the target organisms were viable. Here we show that PCR will detect nonviable cells, as long as intact target nucleic acid sequences are available. In an environmental water sample, nucleic acids degraded quickly and were not detectable by PCR after 3 weeks even when stored at 4°C. However, these data show that there is a window of opportunity for PCR analyses to result in false positives with respect to viable cells. We further show that care must be taken in the way samples are stored for future PCR amplifications and that filter sterilization of media is not acceptable for long-term preservation of samples for PCR.

Polymerase chain reaction (PCR) methodologies to detect bacterial pathogens in environmental samples including soil (5, 9) and water (2) have recently been developed. Recent problems associated with the PCR detection of pathogens in environmental samples include the difficulty of the extraction of organisms or DNAs from the sample and subsequent purification of the sample to remove colloidal debris or humic substances which can inhibit PCR (5, 9, 12). In addition, sensitivity of PCR detection of pathogens decreases in environmental samples relative to that in pure culture assays (14). However, the use of culture methods to assay bacteria in environmental samples is also problematic because many of these organisms are difficult to culture. Despite unique ecosystems such as the rhizosphere, in which organic metabolites of plant origin are abundant, most bacteria in environmental samples exist under limited starvation because of the diverse nature and high-level efficiency of metabolism of bacterial communities (11). Such oligotrophic conditions coupled to other abiotic stresses result in many organisms not being able to be cultured under normal conditions. Pathogens introduced into soil or water also face abiotic stress but in addition are subject to biotic stress or biological competition for nutrients, growth substances, water, and favorable habitats. Such pathogens may also become viable but nonculturable (11). Overall, perhaps 99% of all organisms in environmental samples may be unable to be cultured (4). Finally, not all pathogens in unfavorable environmental samples are infectious. Because PCR analyses are based on detection of intact nucleic acids rather than intact viable cells, there exists the possibility that positive pathogen PCR amplifications may arise from either dead cells or noninfectious cells. More recently, researchers have attempted to resolve the issue of viability of cells by directly extracting and analyzing mRNA from environmental samples. However, such processing is difficult because of the instability of mRNA. In fact, because of the short half-life of mRNA, its detection indicates that such cells were viable or very recently were viable. Tsai et al. (13) demonstrated gene expression resulting from viable (or recently viable) bacteria through DNA-RNA hybridizations. Other researchers have used PCR analysis of 16S rRNA genes in phylogenic studies (10). Bej et al. (1) reported that PCR would detect only viable *Legionella pneumophila*, but other researchers have theorized that PCR should have the potential to detect nonviable cells. However, to date, no one has experimentally demonstrated this phenomenon. This paper illustrates the potential for PCR detection of nonviable cells.

Escherichia coli ATCC 15224, *Salmonella typhi* ATCC 6539, and *Shigella sonnei* ATCC 11060 were separately grown in nutrient both. Mid-log-phase broth cultures were exposed to different doses of UV radiation calculated as $i_{ave} = I_0 (0.96) \cdot [1-e^{(-\partial_e L)}]/\partial_e L$ where ∂_e is absorbance per centimeter (base e), L is depth of solution irradiated, I_0 is measured intensity, and I_{ave} is average intensity.

Exposure times for desired doses were calculated as exposure time = dose ($\mu W \cdot cm^2$)/I_{ave} ($\mu W \cdot$).

Irradiated cultures were subsequently analyzed by (i) cultural dilution and plating, (ii) total direct counts, and (iii) PCR analysis. After UV irradiation, care was taken to maintain samples in the dark to prevent photoreactivation. Cultural enumerations were done on cell suspensions diluted in 0.1% peptone and plated on nutrient agar. Triplicate plates of appropriate dilutions were incubated in the dark at 37°C for 24 h, and cells were counted as CFU per milliliter. For direct counts, cell suspensions were incubated in 0.05% acridine orange and applied to a 0.2-μm-pore-size Nuclepore black polycarbonate membrane. Filter counts were made with a Zeiss epifluorescent UV microscope. Appropriate dilutions were made to yield 20 to 100 counts per field, with a total of 10 fields counted for each membrane. For PCR detection of strains, *lamB* primers were used to detect *E. coli* or *Shigella sonnei*, while *phoP* was used to detect *Salmonella typhi* (14). Cells were lysed by boiling, and 25 cycles of PCR amplification were performed as described earlier (5, 14).

Figure 1 illustrates detection of each organism by the three methodologies. For each organism, viable cell counts (CFU) decreased with increased UV dosage. However, detection of cells by acridine orange staining remained constant for all samples (Fig. 1). Likewise, positive PCR amplifications of equal intensity were obtained from all samples even when no viable cells remained (Fig. 1). Samples were checked for the presence of viable but nonculturable organisms by incubat-

* Corresponding author.

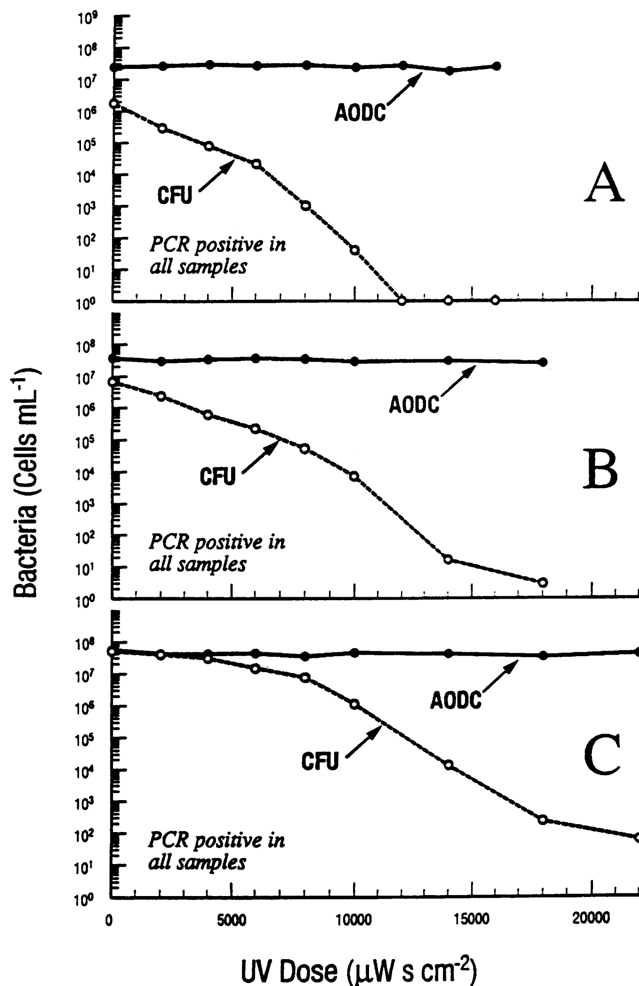

FIG. 1. Detection of *E. coli* (A), *S. typhi* (B), and *S. sonnei* (C) by culture methods (CFU), acridine orange direct counts (AODC), or PCR analyses. The limits of detection for culturable counts and direct counts are 10 CFU/ml and 3×10^3 cells per ml, respectively.

ing bacteria in nalidixic acid (7). This inhibits cell division in viable gram-negative bacteria, causing elongation of growing viable cells, whereas nongrowing cells remain short. In our samples, no viable but nonculturable organisms were found.

These data suggest that culture methods detect only viable culturable cells, whereas acridine orange and PCR methods detect both viable culturable cells and dead cells. Presumably, the latter two methods would also detect viable but nonculturable organisms, as long as intact nucleic acid sequences were present in the sample. Thus, if the aim is to determine the presence of viable pathogens, PCR analysis could result in false positives. However, if determination of the potential for gene transfer were the aim, then culture methods could give false-negative results, whereas the PCR analysis would result in an appropriate positive result. Normally, once a cell dies in an environmental sample, its nucleic acids would be expected to be rapidly degraded and not detected by PCR. However, DNA can be adsorbed and protected by colloidal material (8), and such sorbed DNA can subsequently become available for PCR amplifications or act as a transforming agent (6).

TABLE 1. Detection of *E. coli* in a surface pond water sample

Time (wk)	*E. coli* CFU/ml		PCR result[a]	
	Boiled	Live	Boiled	Live
0	0	2.9×10^7	++	++
1	0	1.8×10^6	++	++
2	0	8.4×10^4	+	++
3	0	6.5×10^3	0	+

[a] ++, strong amplification; +, weak amplification; 0, no amplification.

To illustrate that degradation of nucleic acids normally occurs in environmental samples, we conducted an additional experiment utilizing a surface pond water sample collected from the natural pond Agua Caliente, close to Tucson, Ariz. Live or boiled *E. coli* cells were added to pond water samples at an initial concentration of 2.9×10^7 cells per ml. There were no indigenous *E. coli* organisms in the pond water sample. Samples were stored at 4°C for 3 weeks. At specific time intervals, aliquots of the sample were plated on mFC medium (Difco, Detroit, Mich.), which is specific for *E. coli*. Aliquots were also subjected to PCR amplifications. The sample with boiled *E. coli* cells added had 0 CFU of *E. coli* per ml. The initial population in the sample with live cells added decreased from 2.9×10^7 to 6.5×10^3 CFU/ml after 3 weeks (Table 1). Boiled *E. coli* cells were detectable by PCR for 2 weeks but were PCR negative by week 3. Thus, even when samples were stored at 4°C, there was sufficient microbial activity to cause degradation of nucleic acids. Hence, in environmental samples at ambient temperatures of 20°C or greater, degradation would be expected to proceed very rapidly. Thus, the rate of degradation of nucleic acids will be dependent on the specific environmental sample, but in any environmental sample there will be a window of opportunity for false positives resulting from PCR amplifications of nucleic acids from dead cells prior to degradation.

The fact that positive PCR amplifications are dependent on intact nucleic acids prompted us to evaluate the best method of storing bacterial isolates for future analyses. Specifically, we grew *E. coli* cultures to mid-log phase in nutrient broth. Cells were harvested by centrifugation and resuspended in either sterile H_2O, 0.1 M Tris at a pH of 7.3, or 0.1% peptone. The H_2O and the peptone were sterilized by autoclaving, whereas the Tris was filter sterilized. Cells were then either lysed by boiling prior to incubation at 4°C or incubated at 4°C without lysing. Samples were analyzed by culture, direct counts, and PCR at time zero, at weekly intervals for 6 weeks, and again at 10 and 16 weeks.

Figure 2A illustrates the number of bacteria determined by plate counts. Of course, when cells were boiled, no CFU were detected. However, even when the cells were stored live, the number of CFU was dependent on the storage medium. Throughout the experiment, counts of viable cells remained highest for the peptone-treated cells, followed by the water-treated cells. After 16 weeks, both showed an approximately 3-log reduction in CFU from time zero. Storage in Tris buffer had the most deleterious effect on CFU, with a 5-log reduction after 1 week and no detection by week 4.

Detection by acridine orange and PCR is shown in Fig. 2B. When stored live, cells were detectable by these two methods in both the peptone and the water treatments for the duration of the study. Storage in peptone and water showed approximately 1- and 2-log reductions, respectively, in direct

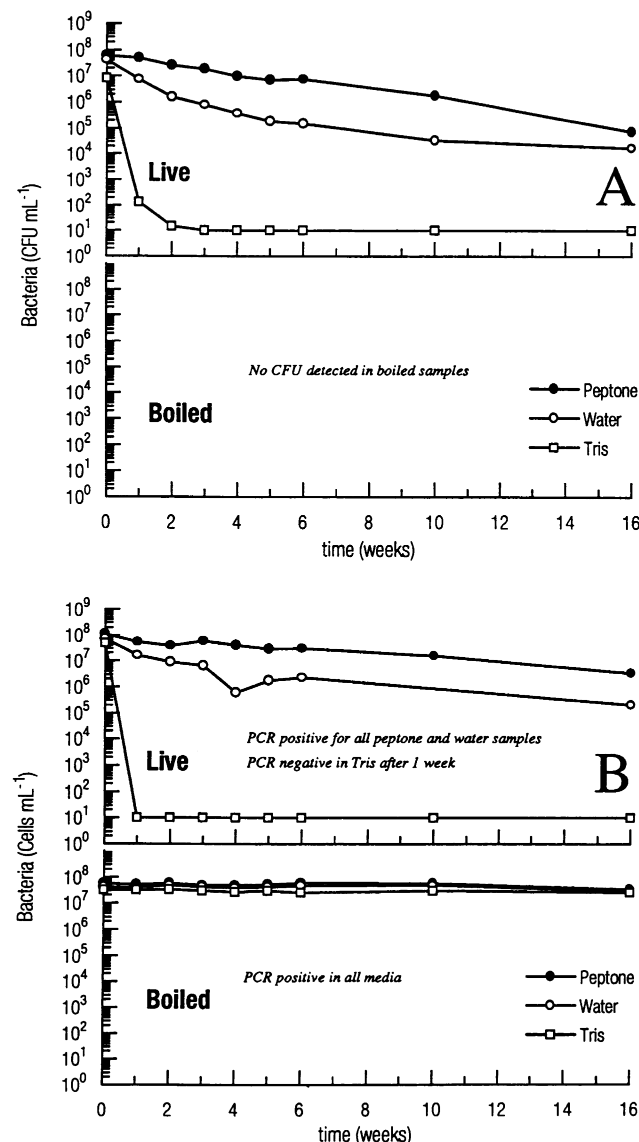

FIG. 2. Influence of boiling and type of medium on detection of *E. coli* by plate counts (A) or by direct counts or PCR (B). CFU plate counts of 10^1 indicate that samples were below the limit of detection of 10^1 CFU/ml. Direct counts in Tris designated 10^1 indicate that samples were below the limit of detection of 3×10^3 cells per ml.

counts after 16 weeks. However, live cells stored in Tris buffer were detectable only by PCR or direct counts at time zero and were not detected after a 1-week period by either method. The fact that cells stored in Tris buffer were not detectable by PCR or direct counts even after as short a period as 1 week is of interest. We speculate that since the Tris was filter sterilized rather than autoclaved, bacterial enzymes were still present in the Tris buffer, which allowed subsequent degradation of cells and nucleic acids. When cells were stored in Tris which had been autoclaved, nucleic acid sequences could be detected by PCR or direct counts, even after a 4-week period (data not shown). These data illustrate that filter sterilization is not acceptable for preparation of media for long-term preservation of samples for PCR.

For the cells boiled prior to incubation at 4°C, there were no differences in acridine orange counts for the three media and counts remained stable for the 16-week duration of the experiment, presumably because of inactivation of nucleases through heat treatment. All three media gave positive PCR amplifications after 16 weeks. These data clearly show that PCR analyses do detect nonviable bacterial pathogens. It is ironic that one of the major advantages of PCR, namely, its great sensitivity, can lead to erroneous conclusions with respect to the viability of pathogens. The data also point out the need for care when samples are to be preserved for future analyses. The method of choice for detection of pathogens may well be PCR amplification, because of its great sensitivity. However, care must be taken in the interpretation of such data with respect to viability of pathogens and the potential for disease transmission.

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