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Comparison of PCR and Cell Culture for Detection of Enteroviruses in Sludge-Amended Field Soils and Determination of Their Transport

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PCR and cell culture assays for enteroviruses were conducted on soil samples collected from an experimental farm that had received mesophilic anaerobically digested sludge for the past 7 years. Of 24 samples assayed, 21 samples were positive by PCR, implying that at least some viral nucleic acid sequences remained intact. However, these viral particles were unable to infect the Buffalo Green Monkey cell line used in subsequent cell culture assays. It is significant that positive PCR detection of nucleic acid sequences occurred even though the most recent sludge application was 3 months prior to soil sampling. Viral nucleic acid sequences were detected by PCR at points vertically and laterally displaced from sludge injections, illustrating significant transport of viruses. Rainfall and irrigation events may have contributed to viral transport.

Data on the potential for groundwater contamination by enteric viruses after land application of municipal sewage sludge are limited. Lysimeter studies performed by Damgaard-Larsen et al. (3) demonstrated that poliovirus and echovirus were not isolated from leachates but were recovered from the sludge-amended soil layer up to 6 months after these viruses were seeded into the sludge. Laboratory column studies of Damgaard-Larsen et al. (3). Other studies conducted by Jakubowski et al. (8) and Bitton et al. (2) reinforced the hypothesis that viruses in sludge are adsorbed to particulate matter and are inactivated before transport of these viruses can occur.

In these reported studies, the seeded virus of choice was poliovirus or echovirus. Studies conducted in which wastewater effluent was used for groundwater recharge demonstrated that these viruses do not migrate significant distances (less than 10 cm) after 10 days (7) and that adsorption of these viruses to soil can be 99% or greater (6). In contrast, other enteroviruses such as the coxsackie B3 virus have been isolated 18 m below the soil surface after wastewater recharge (5). Conclusions concerning the transport of these and other viruses from sludge-amended soil cannot be drawn, since studies with these viruses have not been conducted. In addition, viruses such as coxsackie A viruses and the Norwalk virus are difficult to study since they do not multiply in cell culture.

PCR detection can allow a broader study of the fate of enteroviruses in sludge-amended soil. Primers and probes have been designed to take advantage of the 5′ noncoding region common to most of the enteroviruses, including poliovirus, echovirus, and group A and B coxsackieviruses (1, 10). Although the techniques for detection of enteroviruses by PCR cannot distinguish between infectious and noninfectious virions, they nonetheless provide an opportunity to detect those enteroviruses which do not replicate in cell culture. PCR also overcomes the difficulty of detecting enteroviruses in environmental samples, since it only detects intact nucleic acid sequences. For cell culture detection, environmental viral isolates may require several blind passages to adapt to cell culture and produce an observable cytopathic effect (11).

PCR methods have been optimized for the detection of enteroviruses in sludge-amended soil (12). The purpose of this study was to apply these methods to detect enteroviruses in soil core samples collected from an experimental farm that had received municipal sewage sludge applications for the past 7 years and to determine if these viruses were transported from the sludge-amended soil layer.

Sensitivity studies were conducted with poliovirus type 1 (LSc) to estimate the minimum number of infectious virus particles per gram (dry weight) that could be detected by PCR. These studies were performed with autoclaved mesophilic anaerobically digested sludge and Pima Clay Loam soil to avoid detection of enteroviruses that may have been present in the sludge. The titer of the virus stock was determined by the PFU method described by Straub et al. (13). To ensure that the sensitivity data would represent realistic conditions encountered in environmental samples where the sample cannot be diluted, each 10-fold dilution of virus was first seeded into an aliquot of autoclaved sewage sludge. Each aliquot was then applied to a sterile soil sample. Each amended soil sample containing a given dilution of the virus was eluted with 3% beef extract (Beef Extract V; Becton Dickinson, Cockeysville, Md.) and processed for PCR with the Sephadex G-50 (Pharmacia; Piscataway, N.J.)-and-Chelex-100 (Bio-Rad; Hercules, Calif.) method described by Straub et al. (12). Finally, each sample was subjected to reverse transcription (RT)-PCR and seminested PCR, using the protocol described by Straub et al. (12). Primer sequences for PCR and seminested PCR were previously described by Abbaszadegan et al. (1). Sensitivity was based on the calculated initial concentration of virus per gram (dry weight) of sludge-amended soil in the soil sample before processing for PCR. The concentrations tested ranged from 2 PFU/g to 2×10^4 PFU/g in 10-fold increments. As a negative control for the sensitivity studies, autoclaved sludge that was not seeded with poliovirus was amended to sterile soil, processed, and subjected to RT-PCR and seminested PCR as described previously. Lack of amplification for negative controls and negative environmental samples was confirmed by seeding 10^3 PFU of poliovirus type 1 (LSc) into 1-ml aliquots of the beef extract eluates, treated with Sephadex G-50 and

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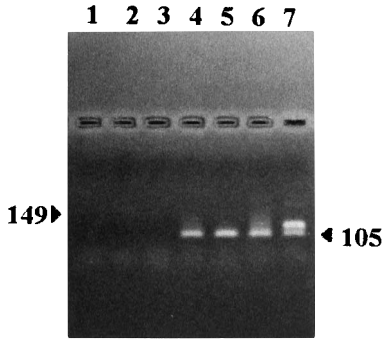

FIG. 1. Sensitivity of PCR detection of poliovirus type 1 (LSc) in sludge-amended soil after seminested PCR. Lanes: 1, reagent negative control; 2, autoclaved sludge-amended soil negative control; 3, 20 PFU/g; 4, 200 PFU/g; 5, 2.0×10^3 PFU/g; 6, 2.0×10^4 PFU/g; 7, poliovirus type 1 positive control. The sample volume for all PCRs was 10 μl. Reaction products are indicated (with sizes in base pairs) by arrowheads.

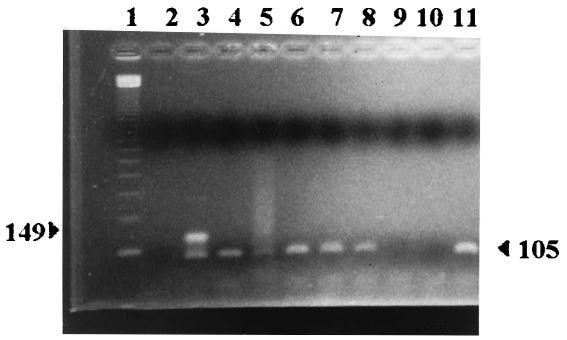

FIG. 2. PCR detection of enteroviruses in soil samples collected from plots receiving no sludge applications. Lanes: 1, 123-bp marker; 2, negative control; 3, poliovirus positive control; 4, 150- to 200-cm-depth core (site 2); 5, 150- to 200-cm-depth core (site 1); 6, 100- to 150-cm-depth core (site 2); 7, 100- to 150-cm-depth core (site 1); 8, 50- to 100-cm-depth core (site 2); 9, 50- to 100-cm-depth core (site 1); 10, surface to 50-cm-depth core (site 2); 11, surface to 50-cm-depth core (site 1). Reaction products are indicated (with sizes in base pairs) by arrowheads.

Chelex-100 as previously described, and subjecting these to RT-PCR (30 cycles only). All negative controls and negative environmental samples treated in this manner were positive. Positive controls used in all PCRs to ensure that the reagents functioned properly consisted of poliovirus type 1 (LCs) that had been purified by polyethylene glycol (molecular weight, 8,000; Sigma Chemical Co., St. Louis, Mo.) and titrated to 10^3 PFU per PCR.

The sensitivity of virus detection by PCR in sludge-amended soil was 2,000 PFU/g for RT-PCR only (data not shown) and 200 PFU/g (dry weight) of sludge-amended soil for seminested PCR (Fig. 1). These were the concentrations of virus before processing the sample for PCR. We chose to use the actual number of PFU seeded into the sludge-amended soil sample before sample processing to provide a conservative estimate of the sensitivity of enterovirus detection by PCR. Recovery efficiency studies based on plaque assay data were performed, and the results indicated that the overall recovery efficiency, including elution and sample purification with Sephadex G-50 and Chelex-100, was 38%. When the positive control was subjected to seminested PCR, two bands could be visualized after agarose gel electrophoresis and visualization by UV transillumination. The larger band (149 bp) was the product from the first reaction, and the smaller band (105 bp) was the product from the seminested PCR.

Environmental soil core samples were collected from an experimental farm which had received sewage sludge for the past 7 years. The farm was divided into plots approximately 5 by 100 m according to the number of sludge applications per year. The categories were (i) plots which had not received sludge, (ii) plots which had received one sludge application per year, and (iii) plots which had received three sludge applications per year. A total of 24 soil core samples were assayed for enteroviruses both by PCR and by cell culture on Buffalo Green Monkey cells. The samples assayed were taken from two different locations on the farm, from plots in all three application categories, and from four depths. The depths sampled were (i) surface to 50 cm, (ii) 50 to 100 cm, (iii) 100 to 150 cm, and (iv) 150 to 200 cm. Between sampling periods, all equipment was thoroughly cleaned and then sanitized with 10 mg of free chlorine per liter; this was followed by rinsing with 95% ethanol. In the field, the corer used to collect depth samples was sanitized with ethanol, and soil was collected from the center of the core to minimize contact with the sides of the corer. In addition, after the surface core had been taken, soil from around the surface core was removed to prevent sludge-amended soil from contaminating the deeper corings. For negative-control environmental soil samples, duplicate soil core samples, taken at a depth of 150 to 200 cm, were collected from a nearby field that had not received sludge for the past 7 years. All environmental soil core samples were processed by elution of 10 g of soil with 50 ml of sterile 3% beef extract by the methods described by Straub et al. (13). For infectivity studies, samples were assayed directly on Buffalo Green Monkey cells (a minimum of 3 ml per sample, depending on sample toxicity). For PCR, aliquots from these same samples were treated with Sephadex G-50 and Chelex-100 and subjected to RT-PCR and seminested PCR as described previously (12).

Soil core samples from the experimental farm were screened for enteroviruses by PCR and cell culture assays. Figure 2 displays the PCR results, after seminested PCR, for the plots from the experimental farm which did not receive sludge. The results for the remaining samples from the experimental farm were similar, in that all but three samples were positive after seminested PCR. The results demonstrated that significant transport of viruses occurred to depths greater than that reported in previous studies (2, 3, 9). In addition, viruses were found laterally displaced from sludge-amended soil.

Cotton is routinely planted annually at the experimental farm during the month of April. Before planting, sludge is applied as a liquid up to a depth of 10 cm during the months of January and February. During the late winter to early spring of 1993, rainfall was particularly heavy in the desert Southwest. At the experimental farm a total of 7.1 cm of rainfall was recorded during the months of February and March. During the month of April, the field was irrigated with well water to a depth of 10 cm of water prior to seeding. After planting, the field was irrigated regularly to maintain field capacity. An additional 1.6 cm of rainfall was recorded in May, when the samples were collected. Therefore, the combination of rainfall and irrigation would have allowed viruses to be transported through the soil matrix. It is also important to note that positive PCR detection of enteroviruses occurred 3 months after sludge amendment. This implies that at least some of the viral nucleic acids were still intact.

When cell culture assays were conducted with samples collected from the experimental farm, none contained cell culture-infectious enterovirus. On the basis of the PCR results, the culture data can be interpreted in several ways. The first

possibility is that viruses were inactivated but at least the portion of the viral genome that was amplified in the PCR reaction was intact, perhaps within a damaged but intact capsid. The second possibility is that the enteroviruses detected by PCR were enteroviruses which do not multiply in the Buffalo Green Monkey cell line that was used. Either possibility is likely in terms of the data presented by Enriquez et al. (4), who reported that enteroviruses could be detected by nucleic acid hybridization a minimum of 45 days after negative cell culture results.

Because of the number of positive results obtained after seminested PCR, it was necessary to confirm these results using soil samples that had not been contaminated with human sewage. To this end, we collected duplicate soil core samples from a nearby farm that had not received sewage sludge for a minimum of 7 years. After seminested PCR, no amplification products were observed. To confirm that the negative results for these samples and the three samples from the experimental farm were not due to the presence of an inhibitor, 1-ml aliquots of the beef extract eluate were seeded with poliovirus, processed, and subjected to PCR as previously described. These seeded samples yielded positive PCR products after 30 cycles of amplification. This confirms that the PCR-negative results from the control farm and the experimental farm were valid.

To date, replicated field studies concerning the fate and transport of enteroviruses in sludge-amended soil are limited and largely inconclusive. In the study reported by Van Sluis et al. (14), the investigators did not recover viruses from soil samples collected immediately after sludge amendment, despite the fact that the sludge used to amend the soil was positive for enteroviruses as determined by cell culture. Bitton et al. (2) recovered enteroviruses up to 7 days after sludge amendment of field soil columns. However, no viruses were detected in the leachate throughout the study.

Regardless of the inability of PCR to distinguish infectious from noninfectious viruses and to provide a quantitative result, the current study is the first to document than at least some enteroviruses do not remain adsorbed to sludge or soil as previously hypothesized by Damgaard-Larsen et al. (3), Bitton et al. (2), and Pancorbo et al. (9). In contrast, the PCR results demonstrated that the viruses migrated vertically and laterally, as observed in the soil samples assayed from experimental plots that did not receive sludge.

The results also challenged the hypothesis that virus inactivation in sludge or soil is due to the release of nucleic acid and the subsequent degradation of the genome (15, 16, 17). If inactivation results from minor changes in the capsid, then PCR results and cell culture data will not be well correlated, as was observed in the present study. More recent studies conducted by Enriquez et al. (4) lend support to this latter hypothesis. In light of the present study, more research is needed to determine the state and ultimate fate of virus particles in soils.

In conclusion, sensitivity studies suggested that the techniques used to process the samples would only be capable of detecting 200 PFU/g of soil and only after seminested PCR. However, this sensitivity was based on cell culture-adapted viruses for which the ratio of infectious to defective particles can be as great as 1:10 (11). This ratio can decrease to $1:10^4$ for environmental samples and may decrease further if inactivation mechanisms only involve minor structural changes as opposed to destruction of nucleic acid sequences as determined by PCR. Therefore, PCR represents a significant advance in the analytical techniques used to detect enteroviruses in sludge-amended soil, in that the likelihood of detection is greater with PCR than with current cell culture techniques.

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