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Detection of Infectious Enteroviruses by an Integrated Cell Culture-PCR Procedure

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Rapid detection of infectious enteroviruses in environmental samples was made possible by utilizing an integrated cell culture-reverse transcriptase PCR approach. By this method, the presence of infectious enterovirus was confirmed within 24 h, compared with ≥3 days by cell culture alone. The combined methodology eliminated typical problems normally associated with direct reverse transcriptase PCR by increasing the equivalent volume of environmental sample examined and reducing the effects of inhibitory compounds.

Currently, the standard method for the detection of enteroviruses in environmental samples involves cell culture assay, which is expensive and time-consuming (10). Cell culture of environmental samples is further complicated by the presence of organic and inorganic materials that are toxic to the cell. An alternative method for the detection of viruses in environmental samples is PCR (1, 3, 4, 15). The decreased time and cost and increased sensitivity of PCR allow the detection of the small numbers of target DNAs and RNAs usually found in environmental samples (17, 19). However, one cannot distinguish between amplification of infectious and noninfectious viral sequences. Furthermore, direct PCR is limited by small reaction volumes (13). Typical PCR sample volumes are 10 to 50 μl; however, typical water sample concentrates are 20 to 30 ml, which can easily be accommodated by cell culture. The use of a combined cell culture-PCR technique utilizes the major advantages of the separate methodologies while overcoming many of their disadvantages. Previous studies in our laboratory have revealed that inhibition can be overcome with higher target concentrations (18). Therefore, dilution of the sample by cell culture media, coupled with an increase in infectious virus concentration, reduces the effect of inhibitory substances often associated with direct reverse transcriptase PCR (RT-PCR) analysis. The combined cultural and molecular technique reduces the effects of toxic compounds on cell culture and inhibitory compounds on PCR while indirectly increasing the PCR equivalent volume examined and the chance for the rapid detection of infectious viruses.

The integrated cell culture-PCR technique involved inoculation of the concentrated sample onto cell monolayers, which were then incubated for a minimum of 24 h; this was followed by RT-PCR analysis of the cell culture lysate. All experiments were repeated twice, with similar data found on each trial. Poliovirus type 1 (strain LSc-2ab) was used to evaluate detection limits and sensitivity of RT-PCR with infected cell culture lysates. Primary sewage effluent from the Sand Island Treatment Plant, serving Honolulu, Hawaii, was also evaluated for viable enteroviruses by RT-PCR with cell culture lysates before and after visible cytopathic effect (CPE). Twenty liters of primary sewage samples was adjusted to pH 3.5 with 1 M HCl and a final concentration of 0.0015 M $AlCl_3$ (5). The sample was then filtered under positive pressure with a 10-in. (25.4-cm), 0.45-μm-pore-size electronegative pleated cartridge filter (6) with a total surface area of 2,800 cm^2 (Filterite Corp., Timonium, Md.). One liter of 0.1 M NaCl (pH 3.5) was passed through the filter to enhance virus elution. Subsequently, viruses adsorbed to the filters were recovered by two passages of a 1-liter volume of 1.5% beef extract V–0.05 M glycine, pH 9.5. Eluents were stored at −20°C for further processing.

In order to reduce the volume of beef extract from the primary eluates to approximately 30 ml, samples were reconcentrated by organic flocculation within 48 h, as previously described (7, 11, 20). Reconcentrated samples were vigorously mixed for 15 min with equal volumes of Freon (1,1,2-trichlorotrifluoroethane; Aldrich, Milwaukee, Wis.) to reduce toxicity and other microbial contaminants before addition to the cell culture; this was followed by 15 min of centrifugation at 3,000 × *g*. The aqueous phase was collected and treated with antibiotics (penicillin-streptomycin, mycostatin, kanamycin, and gentamicin: 100 μg/ml) for 30 min at 37°C prior to storage at −80°C until further processing. Buffalo green monkey kidney continuous cell line cultures (BGM) were used exclusively in this study since they are one of the most efficient cell lines for isolating enteroviruses in waters and wastewaters (9, 14). Poliovirus type 1 (strain LSc-2ab) stocks were diluted, and a 1.0-ml inoculum from each dilution was added to five individual cell culture flasks of 4-day-old BGM cells, each with a growth area of 25 cm^2. Four dilutions with estimated virus concentrations of 28, 2.8, 0.28, and 0.028 PFU/ml were assayed. Tris-buffered saline (Sigma Chemicals, St. Louis, Mo.) was used as a negative control inoculum. After allowing adsorption for 60 min, 1× Eagle's minimal essential maintenance medium with 2% fetal bovine serum was added to the flasks (Irvine Scientific Co., Santa Ana, Calif.). Flasks were incubated at 37°C and observed for up to 10 days for CPE. Individual flasks for each dilution were collected for early growth detection by the integrated approach on days 1, 2, 3, 5, and 10. Flasks to be used for the integrated RT-PCR amplification were frozen and thawed three times at −80 and 37°C, respectively. Poliovirus was pu-

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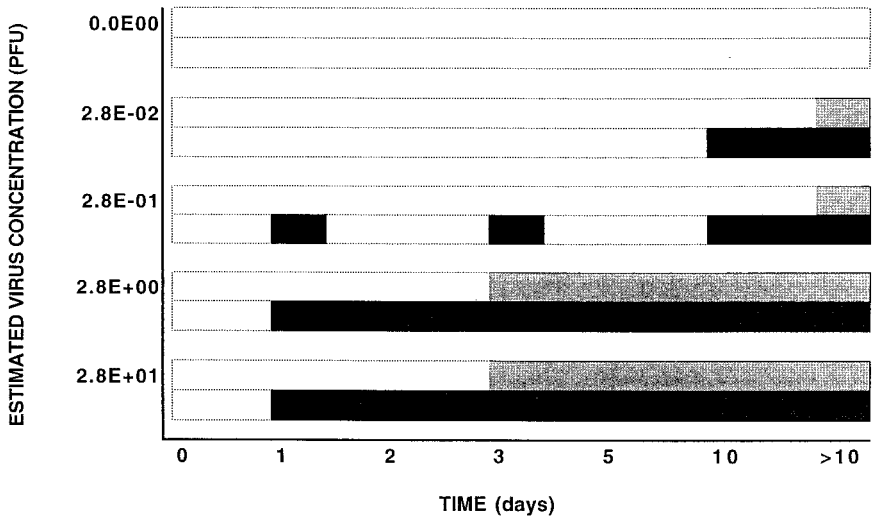

FIG. 1. Detection of poliovirus in distilled water by the integrated cell culture–RT-PCR technique. Various concentrations of poliovirus type 1 (strain LSc-2ab) were inoculated into distilled water and detected by conventional cell culture (grey bars) and a new integrated cell culture–RT-PCR technique (black bars). E, exponent (e.g., 2.8E−02 = 2.8×10^{-2}).

rified from lysed cells either with Sephadex G-200–Chelex 100 columns, as previously described (16, 18), or by equal-volume Freon extraction followed by low-speed centrifugation ($3,000 \times g$ for 15 min). Undiluted primary sewage concentrate and dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were likewise assayed on BGM monolayers. Individual flasks were, however, incubated for 1, 2, 3, 5, 10, and 14 days for each dilution prior to RT-PCR analysis. On the specified day, inoculated cells were lysed, Freon extracted, and centrifuged as described above. Lysates which were negative by primary cell culture but positive by RT-PCR were assayed a second time on BGM cells by adding 1 ml of primary lysate to fresh 25-cm^2 flasks of BGM monolayers and observing them for another 14 days for CPE. Each experiment was repeated twice in its entirety, and the most-probable-number technique was used to determine the concentration of virus in cell culture samples (8).

Specific enterovirus sequences present in the cell culture lysates were amplified by RT-PCR. Fifty microliters of total PCR mixture was used to accommodate a lysate sample volume of 5 μl per reaction (5 μl = 0.0071% of the total lysate volume). A master mix of 1.5 μl of 10× buffer, 3.5 μl of 25 mM $MgCl_2$, and 4 μl of 10 mM deoxynucleotide triphosphate was made for each 50-μl RT reaction mixture. Potential target viral RNA was heat extracted, reverse transcribed, and PCR amplified, for 30 cycles, by using the method and enterovirus primer sequences previously described (1). Samples which were negative by single PCR (30 cycles) were subsequently analyzed by seminested PCR (18). For seminested PCR, 5 μl of the single-PCR mixture was added to 50-μl reaction mixtures of fresh reagents with the upstream external primer and a downstream primer internal to the sequence amplified by single PCR. Amplification conditions were identical to those used in single PCR for an additional 30 cycles.

Amplified PCR products were visualized by 1.6% low-electroendosmosis agarose gel electrophoresis (Fisher Scientific, Fair Lawn, N.J.). Twenty microliters of the PCR product was combined with 2 μl of Ficoll loading buffer (20% Ficoll, 1% sodium dodecyl sulfate, 0.25% bromphenol blue, and 0.1 M EDTA, pH 8.0) and subjected to electrophoresis at 100 V for 1.5 h. The gel was stained with ethidium bromide solution for 15 min and destained in distilled water for 45 min. Presumptive and confirmed product bands were visible at the 149- and 105-bp standard markers, respectively (123-bp ladder; Gibco BRL, Gaithersburg, Md.), when exposed to a 302-nm UV transilluminator. PCR results were evaluated on the basis of the visual presence or absence of amplification product bands.

Figures 1 and 2 compare the ability to detect poliovirus type 1 (strain LSc-2ab) in distilled water and enteroviruses in primary sewage effluent, respectively, by using CPE and the integrated cell culture-PCR procedure. Each sample was also analyzed by direct RT-PCR (data not shown). Figure 1 shows the detection limit of poliovirus type 1 (strain LSc-2ab) in distilled water over time by using CPE and the integrated cell culture-PCR technology. PCR analysis of cell culture lysates on day 0 of the inoculum was negative, indicating that the level of enteroviral sequences, from either particles or PFU, was not detectable by PCR prior to their growth in cell culture. By the combined cell culture-PCR technique, ≥2.8 PFU/ml was detected after only 1 day of growth on BGM cells, compared with 3 days with cell culture alone. At very low concentrations of virus (<1 PFU/ml), it is possible to have a PCR with no virus particle, because only 5 μl of 20 ml of cell culture lysate is being sampled for the PCR. However, over time (≥10 days) virus concentration increases and PCR becomes positive. Similarly, at concentrations of <1 PFU/ml, each individual cell culture flask may not be inoculated with a virus and thus remains negative when analyzed by the integrated technique as well as cell culture alone. The combined methodology did detect a virus concentration of 0.028 PFU/ml after 10 days of growth on cells. Concentrations of <2.8 PFU/ml were detectable by conventional cell culture only after secondary passage on new BGM cell monolayers, requiring >10 days for visible CPE. Therefore, the combined technique had the advantage of being more rapid than cell culture alone for the detection of enteroviruses. Furthermore, since samples containing enterovirus sequences were positive, by the integrated approach, only after >24 h of incubation in cell culture, PCR detection was likely due to infectious virus replication and a concomitant increase in the target nucleic acid concentration. Even at these low initial concentrations, the integrated approach confirmed the presence of enteroviruses after 10 days of incubation whereas the cell culture alone required a secondary passage for

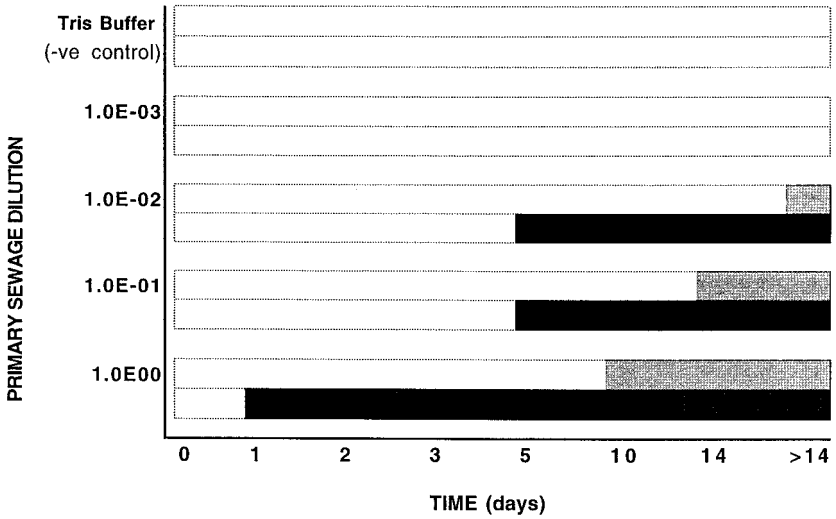

FIG. 2. Detection of enteroviruses in primary sewage effluent by the integrated cell culture–RT-PCR technique. Various dilutions of primary sewage effluent were analyzed for infectious enteroviruses by conventional cell culture (grey bars) and a new integrated cell culture–RT-PCR technique (black bars). E, exponent (e.g., $1.0E{-}03 = 1.0 \times 10^{-3}$); −ve, negative.

presumptive positive results. The integrated cell culture-PCR technique was also more sensitive than direct PCR. By direct RT-PCR, the poliovirus detection limit was 0.28 PFU per PCR volume (data not shown). The integrated approach, however, indirectly increased the overall detection sensitivity to 0.028 PFU per original flask inoculum by increasing the number of infectious virus particles present in the PCR mixture volume.

The integrated cell culture-PCR technique produced similar results with primary sewage effluent (Fig. 2). Detection of enteroviruses by the combined technique again produced results more rapidly than conventional cell culture alone. Infectious enterovirus presence was evident, in the undiluted primary sewage, after only 1 day by the combined method, compared with up to 10 days by using CPE. Likewise, infectious enteroviruses were detected in 10- and 100-fold dilutions of the primary sewage after only 5 days by the integrated technique but 14 days of incubation, and a secondary passage was required for detection by conventional cell culture alone. Furthermore, by direct RT-PCR, primary sewage effluent samples were enterovirus negative for all dilutions, presumably because of the presence of inhibitory factors. Such inhibition, due to metals, humic acids, and other organic matter, has been reported previously (2, 12). Therefore, the combined methodology was able to detect infectious enterovirus in primary sewage samples which appeared negative by primary cell culture or direct PCR alone. In addition, the integrated technique allowed identification of the infectious agent using specific primer sequences in the PCR analysis. Although all lysates which were positive by RT-PCR eventually produced CPE during the secondary cell culture assay, the integrated approach could detect infectious enteroviruses up to 9 days faster than cell culture alone.

In conclusion, the integrated cell culture-PCR approach allowed for more rapid and sensitive detection of low levels of enteric viruses in large volumes of water concentrates compared with that by primary cell culture alone. The reduced time that samples were contacted with cells decreased toxicity effects commonly observed in cell culture, thereby reducing costs of further purification and repeat analysis. Incubation of the sample on cells allowed multiplication of infectious viruses present, which were subsequently detected by PCR. This increases the chance of detecting an infectious virus particle. In addition, PCR analysis of cell culture harvests provided greater sensitivity than did direct RT-PCR by allowing indirect evaluation of a larger sample volume and overcoming the effects of inhibitory compounds.

It is essential for a routine virus analysis technique to target only those viruses which are infectious and potentially capable of causing deleterious public health effects. The integrated cell culture-PCR technique has the ability to greatly reduce the time involved for routine analysis of water samples for infectious viruses and may also allow specific identification and quantitative detection of human pathogenic viruses which do not produce typical CPE, such as hepatitis A virus and rotavirus.

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