APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1993, p. 3488–3491
0099-2240/93/103488-04$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 59, No. 10

Simple Method of Concentrating Enteroviruses and Hepatitis A Virus from Sewage and Ocean Water for Rapid Detection by Reverse Transcriptase-Polymerase Chain Reaction

YU-LI TSAI,[1]* MARK D. SOBSEY,[2] LOUIS R. SANGERMANO,[1] AND CAROL J. PALMER[1]

Environmental Sciences Laboratory, County Sanitation Districts of Orange County, Fountain Valley, California 92728,[1] and Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina 27599[2]

Received 12 May 1993/Accepted 29 July 1993

A rapid and simple method was developed to detect enteroviruses and hepatitis A virus (HAV) in sewage and ocean water. Sewage samples were concentrated by Centriprep-100 and Centricon-100 at 1,000 × *g*. Samples collected from estuary and near-shore surf zone ocean water in Southern California were concentrated by vortex flow filtration and microconcentration. Reverse transcriptase-polymerase chain reaction (RT-PCR), with enterovirus primers or HAV capsid-specific primers, was used to detect enteroviruses or HAV in all concentrated samples. A nonradioactive internal probe was used to confirm the amplified products. Results of seeding experiments indicated that at 4°C, HAV was more persistent than poliovirus in seawater and both HAV and poliovirus persisted longer at 4°C than at 25°C. RT-PCR was at least 500-fold more sensitive than cell culture. Results were obtained within 5 h by RT-PCR, in contrast with the 5 days to 3 weeks required for cell culture.

Historically, viruses have been recovered from water and wastewater by conventional adsorption to and elution from microporous filters followed by traditional cell culture methods (1). Since only small numbers of viruses are usually found in environmental water samples, the concentrating steps are necessary to recover viruses from a large volume of water. Because viruses propagate slowly on specific cell lines, it takes several weeks to detect the presence of viruses by plaque assay or observation of cytopathic effects. There are numerous problems associated with traditional cell culture. The sensitivity of detection by cell culture is low (9), and the method is labor-intensive and tedious. Moreover, some enteric viruses, such as Norwalk virus, rotaviruses, and hepatitis A virus (HAV), are difficult or impossible to cultivate by cell culture. To improve the detection sensitivity, enzyme-linked immunosorbent assay (18), radioimmunoassay (6), radioimmunofocus assay (10), and nucleic acid hybridization (7, 23) have been used to identify viruses from sewage or shellfish samples, but these assays require the use of radioisotopes or cumbersome procedures.

Recently, reverse transcriptase-polymerase chain reaction (RT-PCR) has been successfully used to detect enteroviruses, polioviruses, rotavirus, Norwalk virus, and HAV from stool, oyster, and environmental samples (2–5, 8, 9, 14, 22). The RT-PCR is a rapid and highly sensitive detection method which can circumvent the disadvantages of traditional cell culture methods. The traditional virus concentration procedures use beef extract to elute viruses from cartridge filters and either acid or polyethylene glycol (PEG) to precipitate viruses from filter eluates (1, 11, 23). One major problem related to the traditional concentration methods is that the beef extract and PEG are not eliminated from the concentrate, RT-PCR is inhibited, and further cleanup steps are required to obtain positive results (4, 15). Sewage and surface water samples concentrated by alum flocculation also inhibit the RT-PCR (9). In addition, humic substances in environmental extracts were also found to inhibit the PCR procedure (17, 19). Although methods have been developed to overcome the problem of humic interference (20, 21), the beef extract and PEG still pose problems when trying to perform RT-PCR to detect viruses.

In the present study, we successfully applied a simple ultrafiltration method to concentrate viruses, followed by RT-PCR to detect enteroviruses and HAV from the undiluted concentrated sewage and ocean samples without using beef extract or PEG. Vortex flow filtration and centrifugal microconcentration were used to concentrate viruses from environmental samples. The inhibitory effects caused by humic substances, beef extract, and PEG on RT-PCR were avoided.

Sewage samples, including primary influent, primary effluent, and secondary effluent, were collected from a major metropolitan sewage treatment plant. One hundred milliliters of sewage was concentrated to 4 ml with a Centriprep-100 (Amicon, Inc., Beverly, Mass.) concentrator at 1,000 × *g*. An equal volume (4 ml) of a chloroform-isoamyl alcohol (24:1) mixture (Amresco, Solon, Ohio) was added to the concentrated sample. After brief vortexing, the homogenate was centrifuged at 800 × *g* for 10 min to reach a biphasic state. The upper aqueous phase was further concentrated to 100 μl with a Centricon-100 (Amicon) microconcentrator at 1,000 × *g*. All sewage samples were processed at 4°C within 4 h after collection. Concentrated samples were stored at −20°C and analyzed within 1 week.

Ocean samples were collected from near-shore waters of Southern California. Sample sites included surf zone (HP), the brackish zone of a river (SAR), and a flood control channel (D2). Fifteen liters of each sample was concentrated to 100 ml with the use of a vortex flow filtration (VFF) device (Membrex Inc., Garfield, N.J.) at 5 to 6 lb/in^2 with a 100-kDa filter and a rotor speed of 1,000 to 1,500 rpm. These concentrated samples were further condensed to 100 μl with

* Corresponding author.

Centriprep-100 and Centricon-100 as described for the sewage samples.

Enteroviruses and HAV from the concentrated samples were detected by the RT-PCR method as described by De Leon et al. (4). HAV strain HM175 and poliovirus type 1 strain LSc were used as positive controls. The amplification was performed with a GeneAmp RNA PCR kit (Perkin Elmer-Roche, Norwalk, Conn.) with slight modifications. Briefly, 2 μl of concentrated sample was added to 16 μl of RT reaction mixture containing 5 mM $MgCl_2$; 50 mM KCl, 10 mM Tris-HCl; 1 mM each dGTP, dATP, dTTP, and dCTP; and 2.5 μM random hexamers. Each sample mixture was heated at 99°C for 5 min and cooled at 4°C before the addition of 1 μl of RT (50 U/μl) and 1 μl of RNase inhibitor (20 U/μl). All of the above reagents were provided in the RNA PCR kit. The reverse transcription was carried out in a thermocycler (GeneAmp PCR System 9600; Perkin Elmer Cetus, Norwalk, Conn.) by the following steps: 25°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 4°C for 10 min. The finished RT mixture (20 μl) was mixed with the PCR reaction mixture (80 μl) containing oligonucleotide primers (0.3 μM) and AmpliTaq polymerase (Perkin-Elmer) to obtain the optimal concentrations for PCR as suggested by the manufacturer. The primers for enteroviruses (EV-R, 5′-ACCG GATGGCCAATCCAA-3′; EV-L, 5′-CCTCCGGCCCCTGA ATG-3′) and primers for HAV capsid (HAVC-R, 5′-CTCCA GAATCATCTCCAAC-3′; HAVC-L, 5′-CAGCACATCAG AAAGGTGAG-3′) were used during PCR (15) to amplify 196- and 192-bp cDNA fragments, respectively. The internal oligonucleotide probes for enteroviruses and HAV were EV-IN (5′-ACTACTTTGGGTGTCCGTGTTTC-3′) and HAVC-IN (5′-TTGCTCCTCTTTATCATGCTAT-3′), respectively (4). Oligonucleotides were synthesized by a DNA/RNA synthesizer (model 392; Applied Biosystems, Inc., Foster City, Calif.). The three-temperature protocol (95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 40 cycles) as described by De Leon et al. (4) was adapted to amplify the target cDNA sequences of enteroviruses or HAV. To simplify the amplification steps, a two-temperature RT-PCR protocol (95°C, 40 s; 60°C, 40 s; 40 cycles) using the thermostable rTth RT (Perkin-Elmer) was also tested on positive control samples. The PCR products were identified by electrophoresis on a 2% SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gel stained with ethidium bromide (0.5 μg/ml). The Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) was used to label EV-IN and HAVC-IN internal probes by using the protocols described by the manufacturer. The amplified DNA was transferred onto Hybond-N+ positively charged nylon membranes (Amersham, Arlington Heights, Ill.) by a Minifold I dot blotter (Schleicher & Schuell, Keene, N.H.) or a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.). The dot blot, Southern analysis, and DNA hybridization were performed as previously described (21). The low-salt washes (0.1× SSC in 0.1% sodium dodecyl sulfate) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were performed at 50°C for EV-IN and at 47°C for HAVC-IN.

For the microconcentration recovery and RT-PCR sensitivity test, 100 μl of poliovirus ranging from 10^3 to 10^{-4} PFU/100 μl was seeded into 2 ml of sterile distilled water and reconcentrated with Centricon-100 to a final volume of 100 μl. In the seeding experiment, 600 μl of serially diluted HAV or polioviruses (10^1 to 10^3 PFU/100 μl) was inoculated into 12 ml of nonsterile HAV- and enterovirus-negative ocean water (5 - 500 PFU/ml final concentration) and incubated at two different temperatures (4 and 25°C) in the dark for 28 days. Two-milliliter aliquots from each temperature were collected at 0, 1, 7, 14, 21, and 28 days, concentrated into 100 μl as described previously, and amplified by RT-PCR. The poliovirus and HAV stocks (10^6 PFU/ml) were grown and plaque assayed in BGMK (African green monkey kidney-derived) cells and in FRhK-4 (fetal rhesus kidney-derived) cells, respectively (15). The cell culture method took 5 days to 3 weeks to complete the plaque assay as described in *Standard Methods* (1). The virus density (PFU per 100 μl) of each serially diluted sample was derived from the virus stocks. Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) was used as the diluent.

VFF was previously used by Paul et al. (12) to concentrate bacterial phages and dissolved DNA from freshwater and seawater. In the present study we have combined VFF and microconcentration to concentrate seawater 10^5-fold for RT-PCR detection of viruses. On the basis of the RT-PCR results, the VFF in combination with microconcentration recovered the same order of magnitude of polioviruses as was seeded into 15 liters of sterile ocean water. However, the preliminary cell culture results indicate the total PFU counts in the VFF concentrate were less than, but within the same order of magnitude of, the initial seeded counts. The discrepancy could be due to the inactivation of the viral particles by the accumulated inhibitory compounds, by the operating temperature, or by the mechanical disturbance of VFF during the concentration process, but those inactivated virions were still detected by RT-PCR. Additional studies are under way to better define recovery efficiency. The sensitivity of detection did not change when the virus concentrates were stored at −20°C for 2 months. However, the stability of viruses in the concentrates using cell culture methods was not determined.

Figure 1A shows RT-PCR-amplified target DNA fragments (196 bp) of enteroviruses from concentrated poliovirus virions, sewage, and ocean samples. Southern hybridization analysis illustrates that sensitivity of detection for polioviruses was 10^{-1} PFU/100 μl (Fig. 1B, lane 5) compared with 1 PFU/100 μl (Fig. 1A, lane 4) on the ethidium bromide-stained gel. The detection level was at the same order of magnitude when a serially diluted stock of poliovirus was tested as the RT-PCR control (data not shown). No positive signals were found from samples collected from filtrates (permeates). This indicated that polioviruses were recovered primarily in the retentate after microconcentration. Because only 2 μl of concentrated retentate (100 μl) was used as a template for RT-PCR, the results suggest the PCR is capable of detecting 0.002 PFU per reaction, which implies the detection of the nonculturable polio viral particles. Thus, it is apparent that the RT-PCR with subsequent DNA hybridization is much more sensitive (500-fold) than cell culture for the detection of enteroviruses. Additionally, because of rapid enzymatic amplification in the RT-PCR, the presence of viral RNA was detected within 5 h, whereas 48 h to 10 days was required to determine the presence of poliovirus by cell culture methods (1). The sensitivity of detection by using the two-temperature thermostable rTth RT-PCR was found to be 1/10 that of three-temperature RT-PCR. Therefore, the three-temperature RT-PCR was used throughout this study.

Positive signals for enteroviruses were observed in raw sewage (primary influent), treated sewage (primary effluent and secondary effluent), and one flood control channel (D2) (Fig. 1B, lanes 9 to 15). Negative results were found in the brackish river water (SAR) and near-shore, surf zone marine

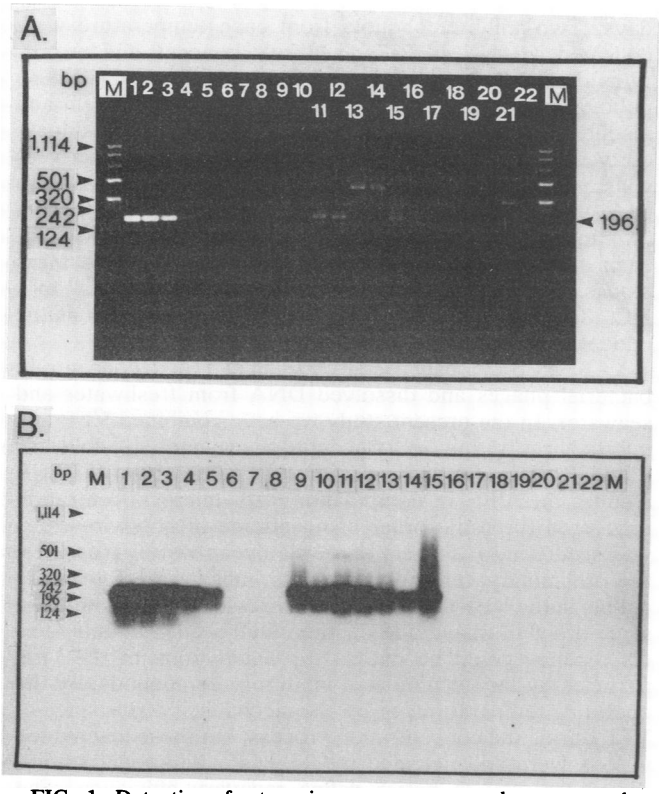

FIG. 1. Detection of enteroviruses on sewage and ocean samples by RT-PCR. Lanes: M, DNA molecular weight marker VIII (Boehringer Mannheim); 1 to 8, poliovirus (1, 10^3 PFU/100 μl [concentrate]; 2, 10^2 PFU/100 μl; 3, 10^1 PFU/100 μl; 4, 1 PFU/100 μl; 5, 10^{-1} PFU/100 μl; 6, 10^{-2} PFU/100 μl; 7, 10^{-3} PFU/100 μl; 8, 10^{-4} PFU/100 μl; 9, primary influent concentrate, undiluted; 10, primary influent concentrate, 10^{-1} dilution; 11, primary effluent concentrate, undiluted; 12, primary effluent concentrate, 10^{-1} dilution; 13, secondary effluent concentrate, undiluted; 14, secondary effluent concentrate, 10^{-1} dilution; 15, D2 flood control channel concentrate, undiluted; 16, D2 concentrate, 10^{-1} dilution; 17, HP surf zone concentrate, undiluted; 18, HP concentrate, 10^{-1} dilution; 19, SAR river concentrate, undiluted; 20, SAR concentrate, 10^{-1} dilution; 21, RNA positive control from GeneAmp RNA PCR kit (Perkin-Elmer); 22, reaction mixture (no RNA template). (A) RT-PCR products on an ethidium bromide-stained 2% SeaKem agarose gel. (B) Autoradiogram of panel A produced by DNA-DNA hybridization using a chemiluminescent internal probe (EV-IN).

water (HP) (Fig. 1B, lanes 17 to 20). Additional PCR products (500 bp) were obtained from secondary effluent, but they did not hybridize with internal probes, indicating they are due to amplification of nucleic acids from background organisms or viruses. No inhibitory effects on RT-PCR were observed when undiluted concentrates were used for testing (Fig. 1). Positive signals for HAV were found in all three sewage samples (Fig. 2), but not in D2, SAR, and HP samples (data not shown). RT-PCR tests on additional sewage and ocean samples are under way to determine the occurrence of HAV and enteroviruses in these samples. However, the plaque assay on concentrated environmental samples for enteroviruses and HAV resulted in less than 1 PFU/100 μl (i.e., culture negative) in 3 to 4 weeks. This could be because only 100 ml of sewage samples was concentrated and this volume is not sufficient for culturable virus detection by plaque assay. Similarly, 15 liters of sea

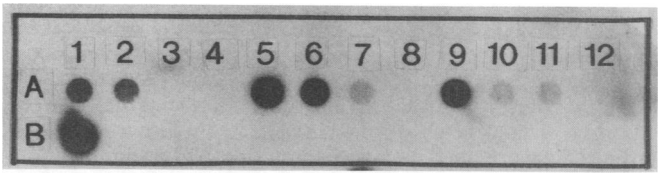

FIG. 2. Dot blot analysis of HAV RT-PCR using concentrated treated sewage samples. Dots: A-1, primary influent (PI), undiluted concentrate; A-2, PI (10^{-1}); A-3, PI, (10^{-2}); A-4, PI (10^{-3}); A-5, primary effluent (PE), undiluted; A-6, PE, (10^{-1}); A-7, PE (10^{-2}); A-8, PE (10^{-3}); A-9, secondary effluent (SE), undiluted concentrate; A-10, SE, (10^{-1}); A-11, SE (10^{-2}); A-12, SE (10^{-3}); B-1, HAV positive control; B-2, RNA positive control from Perkin Elmer RNA PCR kit; B-3, reaction mixture (no template). The dilutions of concentrated samples used for RT-PCR are indicated in parentheses.

water might not be enough for virus plaque assay because of the low sensitivity of detection by cell culture methods. It was, however, an adequate volume for RT-PCR detection of enteroviruses and HAV.

The results of HAV seeding experiments show that HAV viral RNA was not detected after a 7-day incubation at 25°C (Fig. 3). The level of detection from the day zero sample was 0.1 PFU/μl (10 PFU/100 μl; Fig. 3, A4) which was less than that of polioviruses seeded into sterile distilled water (Fig. 1A, lane 5). This indicated that degradation of viruses occurred in nonsterile ocean water during the concentration process. However, HAV was detected at 4°C throughout the 28-day incubation period. These results suggest that degradation of HAV occurred rapidly at 25°C, but it persisted well at 4°C for the entire 28-day period. Therefore, temperature affects the persistence rate of HAV in the marine environment because HAV persisted better at a low temperature (4°C) than at ambient temperature.

In poliovirus seeding experiments, polioviruses were not detected after 7 days of incubation at 25°C nor were they detected after 21 days at 4°C (data not shown). These results

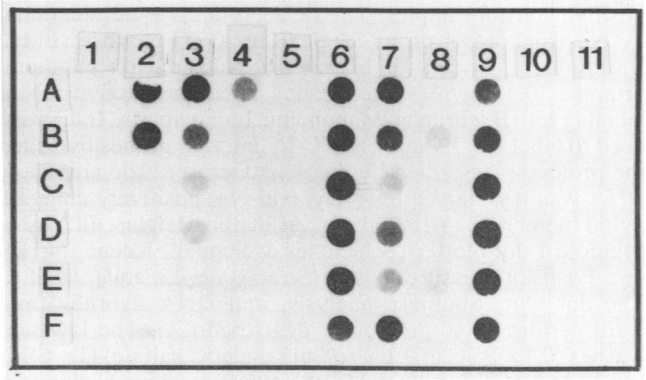

FIG. 3. Dot blot analysis of HAV viral RNA survival in nonsterile ocean water at two different temperatures. Conditions (columns): 1, 25°C, unseeded; 2, 25°C, seeded, 10^3 PFU/100 μl (concentrate) (500 PFU/ml); 3, 25°C, 10^2 PFU/100 μl (50 PFU/ml); 4, 25°C, 10^1 PFU/100 μl (5 PFU/ml); 5, 4°C, unseeded; 6, 4°C, seeded, 10^3 PFU/100 μl (concentrate) (500 PFU/ml); 7, 4°C, 10^2 PFU/100 μl (50 PFU/ml); 8, 4°C, 10^1 PFU/100 μl (5 PFU/ml); 9, HAV positive control; 10, RNA positive control from Perkin Elmer RNA PCR kit; 11, reaction mixture only (no template). Final concentrations of HAV in seeded ocean water are shown in parentheses. Rows: A, day 0; B, day 1; C, day 7; D, day 14; E, day 21; F, day 28.

suggest that ambient temperature promoted degradation of poliovirus viral RNA in seawater, while it took 3 weeks to degrade at 4°C. Clearly, HAV is more resistant than poliovirus in seawater at 4°C. On the basis of study of logarithm changes in virus titer, Sobsey et al. (16), using the tissue culture technique, reported that HAV were more persistent than poliovirus in nonsterile environmental waters at 25°C. Our results using RT-PCR confirm this finding.

In the present study, in order to prevent RNA degradation by RNase activity, the RNA virions were kept intact until they were burst open by heat denaturation immediately before RT-PCR. Because RNA was easily degraded in the seawater (13), it was unlikely that the amplification products resulted from the free viral RNA. Only 1/25th (15 liters/375 liters) of the volume of ocean water needed to perform the conventional adsorption-elution method was concentrated through VFF and successfully used for microconcentration for virus detection. Minimal equipment and labor were required during the concentration. Because neither pH adjustment nor beef extract was employed, the concentrated samples were immediately ready for RT-PCR. The sensitivity of detection by RT-PCR was much higher than that of the traditional cell culture method, and RT-PCR proved to be a powerful and sensitive method for virus detection in environmental water samples.

This study is based upon work supported by the National Water Research Institute under Awards WQ-91-01 and HR-92-06. Matching fund supports were received from County Sanitation Districts of Orange County and the Water Environment Foundation.

We thank Bich Tran for technical assistance and G. Fred Bonilla for sample collection and concentration. We are also grateful to Moy Yahya, Marilyn Koval, and Charles McGee for performing plaque assays. Special thanks go to Patrick McNelly for editing the manuscript.

REFERENCES

1. **American Public Health Association.** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
2. **Atmar, R. T., T. G. Metcalf, F. H. Neill, and M. K. Estes.** 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. Appl. Environ. Microbiol. **59:**631–635.
3. **De Leon, R., S. M. Matsui, R. S. Baric, J. E. Herrmann, N. R. Blacklow, H. B. Greenberg, and M. D. Sobsey.** 1992. Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. J. Clin. Microbiol. **30:**3151–3157.
4. **De Leon, R., C. Shieh, R. S. Baric, and M. D. Sobsey.** 1990. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p. 833–853. *In* Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, San Diego, Calif. American Water Works Association, Denver.
5. **Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z.-Y. Fang.** 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. **28:**276–282.
6. **Hejkal, T. H., R. L. Labelle, B. H. Keswick, C. P. Gerba, B. Hafkin, and Y. Sanchez.** 1982. Viruses in a small community water supply associated with a gastroenteritis epidemic. J. Am. Water Works Assoc. **74:**318–321.
7. **Jiang, X., M. K. Estes, and T. G. Metcalf.** 1987. Detection of hepatitis A virus by hybridization with single-stranded RNA probes. Appl. Environ. Microbiol. **53:**2487–2495.
8. **Jiang, X., J. Wang, D. Y. Graham, and M. K. Estes.** 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J. Clin. Microbiol. **30:**2529–2534.
9. **Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J. M. López-Pila.** 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol. **59:**1213–1219.
10. **Lemon, S. M., L. N. Binn, and R. H. Marchwiki.** 1983. Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. J. Clin. Microbiol. **17:**834–839.
11. **Lewis, G. D., and T. G. Metcalf.** 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl. Environ. Microbiol. **54:**1983–1988.
12. **Paul, J. H., S. C. Jiang, and J. B. Rose.** 1991. Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl. Environ. Microbiol. **57:**2197–2204.
13. **Pichard, S. L., and J. H. Paul.** 1991. Detection of gene expression in genetically engineered microorganisms and natural phytoplankton populations in the marine environment by mRNA analysis. Appl. Environ. Microbiol. **57:**1721–1727.
14. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
15. **Schwab, K. J., R. De Leon, R. S. Baric, and M. D. Sobsey.** 1991. Detection of rotaviruses, enteroviruses and hepatitis A virus by reverse transcriptase-polymerase chain reaction, p. 475–491. *In* Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, Orlando, Florida. American Water Works Association, Denver.
16. **Sobsey, M. D., P. A. Shields, F. S. Hauchman, A. L. Davis, V. A. Rullman, and A. Bosch.** 1988. Survival and persistence of hepatitis A virus in environmental samples, p. 121–124. *In* A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.
17. **Steffan, R. J., J. Goksoyr, A. K. Bej, and R. M. Atlas.** 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54:**2908–2915.
18. **Steinman, J. C.** 1981. Detection of rotavirus in sewage. Appl. Environ. Microbiol. **41:**1043–1045.
19. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
20. **Tsai, Y.-L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
21. **Tsai, Y.-L., C. J. Palmer, and L. R. Sangermano.** 1993. Detection of *Escherichia coli* in sewage and sludge by polymerase chain reaction. Appl. Environ. Microbiol. **59:**353–357.
22. **Wilde, J., R. Van, L. Pickering, J. Eiden, and R. Yolken.** 1992. Detection of rotaviruses in the day care environment by reverse transcriptase polymerase chain reaction. J. Infect. Dis. **166:**507–511.
23. **Zhou, Y.-J., M. K. Estes, X. Jiang, and T. G. Metcalf.** 1991. Concentration and detection of hepatitis A virus and rotavirus from shellfish by hybridization tests. Appl. Environ. Microbiol. **57:**2963–2968.