APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 531–537 Vol. 61, No. 2
0099-2240/95/$04.00+0

Concentration and Purification of Beef Extract Mock Eluates from Water Samples for the Detection of Enteroviruses, Hepatitis A Virus, and Norwalk Virus by Reverse Transcription-PCR

KELLOGG J. SCHWAB,[1] RICARDO DE LEON,[2] AND MARK D. SOBSEY[1]*

Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina 27599-7400,[1] and Department of Environmental Analysis and Design, University of California at Irvine, Irvine, California 92717-5150[2]

Received 20 June 1994/Accepted 16 November 1994

In this study we developed a concentration and purification procedure to facilitate reverse transcription (RT)-PCR detection of enteric viruses in water sample concentrates obtained by conventional filter adsorption-elution methods. One liter of beef extract-glycine eluate with or without humic acid and seeded with poliovirus type 1, hepatitis A virus, and Norwalk virus was used as a model system, and the eluent was further processed for RT-PCR compatibility. The sample concentration and purification procedures which we used included polyethylene glycol precipitation, Pro-Cipitate precipitation, a second polyethylene glycol precipitation, spin column chromatography, and ultrafiltration. The sample volumes were reduced from 1 liter to 20 to 50 μl, and the samples were purified enough so that viruses could be detected by the RT-PCR. The ability to detect low levels of enteric viruses by molecular techniques was compared directly with the ability to detect enteric viruses by cell culture infectivity procedures. As little as 3 PFU of poliovirus type 1 in an initial 1 liter of mock eluate was detected by the RT-PCR.

Surface water and groundwater continue to be subjected to fecal contamination from a variety of sources associated with human activities in both urban and rural settings. The methods currently used to assess the microbial quality of drinking water and to determine water potability are based on analyses for indicator bacteria belonging to the coliform group. Several studies have demonstrated that treated water supplies which are considered acceptable on the basis of levels of indicator bacteria may still contain viruses and other pathogens (6, 13). As there are no generally accepted microbial indicators for enteric viruses, there is a clear need for methods that can be used for direct virological examination of water supplies in order to better assess health risks due to enteric viruses. However, the traditional methods used to isolate enteric viruses from water rely on animal cell cultures and are technically difficult, time consuming, inefficient, and expensive (4, 7, 10, 26, 37). Virus detection methods based on targeting viral nucleic acids via hybridization reactions (gene probes) (12, 17, 18, 24, 27, 31, 33) and, more recently, in vitro nucleic acid amplification (PCR) (1, 8, 20, 47, 49) offer advantages for detecting individual enteric viruses or groups of enteric viruses in environmental samples. In particular, PCR in conjunction with reverse transcription (RT) can detect as little as 0.01 PFU per sample.

Because the levels of viruses of public health concern typically found in environmental water and drinking water are low, viruses must be concentrated from hundreds to thousands of liters of water. In the most widely used concentration methods, viruses are first concentrated by adsorption to microporous filters and then eluted with approximately 1 liter of eluent. However, this technique also effectively concentrates a variety of other solutes, such as humic acids and proteins, which may interfere with methods used subsequently to detect viruses (14, 39). In particular, many naturally occurring inorganic and organic solutes inhibit the nucleic acid polymerases used for amplification of target genomes (reverse transcriptase and *Taq* polymerase) (43–45). Nucleases and proteases may also degrade virus genomes before they can be amplified. In addition, various proteins, carbohydrates, and other organic compounds may bind magnesium ions and nucleotides required by nucleic acid polymerases, and some solutes may be toxic to these polymerases (11, 16, 19, 20). Beef extract, the agent that is used most widely to elute adsorbed viruses from filters and other media, contains high concentrations of poorly characterized components (15, 42) which may interfere with molecular detection methods. While recognizing the problems associated with the use of beef extract as an elution medium, in this study we focused on beef extract, because it has worldwide acceptance and its use is compatible with cell culture techniques used for virus detection (5, 15, 28). Furthermore, methods that could be developed to reduce the interference caused by beef extract could also remove or neutralize other interfering substances found in environmental water samples.

Therefore, the objective of this study was to develop and evaluate methods that can be used to detect low levels of enteric viruses in beef extract eluates by RT-PCR amplification and subsequent analysis of the amplified viral cDNA by oligoprobe hybridization. The ability to detect low levels of enteric viruses by these molecular techniques was compared directly with the ability to detect enteric viruses by cell culture infectivity procedures.

* Corresponding author. Mailing address: Department of Environmental Sciences and Engineering, C.B. 7400, Rosenau Hall, Room 106, University of North Carolina, Chapel Hill, NC 27599-7400. Phone: (919) 966-7303. Fax: (919) 966-7141.

MATERIALS AND METHODS

Viruses and cells. Poliovirus type 1 (PV1) strain LSc was propagated in BGMK (African green monkey kidney-derived) cells, and the infectivity of PV1

was determined by the plaque technique or by the quantal assay for cytopathic effects (CPE). Hepatitis A virus (HAV) cytopathic strain HM175 (3) was grown and plaque assayed in FRhK-4 (fetal rhesus kidney-derived) cells. Viruses were harvested from infected cell lysates by freeze-thawing, fluorocarbon extraction, and polyethylene glycol (PEG) precipitation. Norwalk virus (NV) strain 8FIIA was obtained from human stool samples from infected volunteers by preparing a 20% stool slurry in 0.15 M phosphate-buffered saline (PBS) and then extracting the preparation with fluorocarbon. At the concentrations used for RT-PCR detection the virus stock preparations did not inhibit molecular detection (data not shown).

The quantal assay for PV1 CPE was performed with small sample volumes (ca. 10 to 50 μl) by increasing the sample volume to 1 ml with PBS and then inoculating 0.1-ml portions into 10 wells containing newly confluent cell layers in 24-well tissue culture dishes with the growth medium removed by aspiration. After 90 min of adsorption and addition of maintenance medium, the cultures were observed daily for CPE for 10 days. The most probable number of cytopathic units per milliliter was then estimated by using the Thomas equation (2).

PCR primers and oligoprobes. The oligonucleotide primer and probe sequences used for enteroviruses and HAV in this study were identical to those described previously (9), except for a single base change in the HAV oligonucleotide probe. The highly conserved 5′ untranslated region of enteroviruses was used as the target for the synthesis of a 197-bp panenterovirus cDNA (the 5′ primer was CCTCCGGCCCCTGAATG, the 3′ primer was ACCGGATGGCC AATCCAA, and the internal oligoprobe was TACTTTGGGTGTCCGTGTT TC). For HAV, the genomic region corresponding to the interface of the VP1 and VP3 capsid proteins was the target for a 192-bp cDNA (the 5′ primer was CAGCACATCAGAAAGGTGAG, the 3′ primer was CTCCAGAATCATCT CCAAC, and the internal oligoprobe was TGCTCCTCTTTATCATGCTATG). For NV the genomic region encoding the viral polymerase was the target for a 260-bp cDNA (7) (the 5′ primer was CAAATTATGACAGAATCCTTC, the 3′ primer was GAGAAATATGACATGGATTGC, and the internal oligoprobe was ATGTCATCAGGGTCAAAGAGG). The downstream or antisense 3′ primers were complementary to the (+) sense virion RNA, and the upstream 5′ primers were homologous to the (+) sense viral RNA. Internal oligomer probes were synthesized in the (+) sense orientation so that they hybridized only with cDNA or PCR products and not with viral genomic (+) sense RNA.

RT and enzymatic amplification (RT-PCR). An RNA PCR kit obtained from Perkin Elmer-Roche, Alameda, Calif., was used throughout this study. The manufacturer's instructions were followed, except that the reaction volume for RT was increased from 20 to 30 μl to accommodate a 10-μl virus sample. Viral RNA was released from virions by heating reaction mixtures at 99°C for 5 min. After the mixtures were chilled and reverse transcriptase (50 U) and RNase inhibitor (20 U) were then added, RT was carried out at 42°C for 1 h by using random primers, and then the tubes were heated to 99°C for 5 min to inactivate the enzyme. After chilling, the tubes were supplemented with 2.5 U of *Taq* polymerase and panenterovirus, HAV capsid, or NV polymerase primer pairs for PCR amplification. PCR amplification was performed for 40 cycles, with each cycle consisting of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min. A 15-μl portion of the PCR product was analyzed by electrophoresis on 2% agarose gels, which were stained with ethidium bromide and visualized by UV light.

Southern transfer and nonradioactive oligoprobe detection. The PCR product was transferred from agarose gels to nylon membranes by the method of Southern (41), and the cDNA was bound to the membrane by UV cross-linking for 5 min. The bound DNA was examined by oligoprobe hybridization and immunological detection. Oligoprobes were 3′ end labeled with digoxigenin-dUTP by using terminal transferase and were purified by ethanol precipitation according to the instructions in a Genius nonradioactive end-labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Oligoprobe hybridization and immunological detection of positive samples were performed by following the instructions in the Boehringer Mannheim Biochemicals kit. Immunological detection of PCR product-oligoprobe hybrids was performed by using an anti-digoxigenin alkaline phosphatase antibody conjugate and an enzyme-catalyzed colorimetric reaction in which 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt were the substrates.

Sample processing for PCR amplification. (i) Virus concentration from beef extract by PEG precipitation. A model system consisting of 1 liter of a solution containing 1% beef extract powder (BBL, Becton Dickinson, Cockeysville, Md.) and 0.05 M glycine (BE/G) either with or without 0.15 mg of humic acid and seeded with either PV1 or HAV was used to investigate the RT-PCR compatibility of virus concentration by PEG precipitation (21). Humic acid was concentrated and purified from highly colored surface water as previously described (39). BE/G samples (1 liter) were supplemented with 13% PEG 8000 (Sigma Chemical Co., St. Louis, Mo.) and 0.2 M NaCl, and the preparations were mixed at 4°C for 2 to 15 h and then centrifuged at 7,000 × *g* and 4°C for 30 min. We observed no significant difference in the levels of recovery efficiency for viruses that were precipitated with PEG for 2 to 15 h; 2 h was the minimum time required, and 15 h was used for overnight precipitation, if it was convenient (data not shown). The resulting precipitates were resuspended in ca. 2 to 3 ml of 0.1× PCR buffer II (5 mM KCl, 1 mM Tris-HCl; pH 8) (Perkin Elmer, Norwalk, Conn.), and the Tris concentration was adjusted with 1 M Tris (pH 8) so that the final Tris concentration was 20 mM.

TABLE 1. Levels of recovery of PV1 and HAV from 1 liter of BE/G by PEG precipitation

Virus	% Initial viruses in supernatant	% Initial viruses in PEG concentrate
PV1	9 ± 9 (25)[a]	88 ± 33 (27)
HAV	6 ± 10 (16)	64 ± 27 (18)

[a] Mean ± standard deviation. The numbers in parentheses are the numbers of trials.

(ii) Purification of viruses in PEG precipitates. Viruses in PEG-precipitated beef extract eluates were further purified and concentrated by either chloroform extraction or Pro-Cipitate (Affinity Technology, New Brunswick, N.J.) purification. The former procedure consisted of emulsifying a sample with an equal volume of chloroform, separating the phases by centrifugation at 2,000 × *g* and 4°C for 15 min, extracting the interphase with 200 μl of resuspension buffer, and pooling this preparation with the aqueous phase of the sample. Pro-Cipitate, a commercially available protein purification and concentration agent, was also used for target virus purification and concentration from PEG precipitates. Equal volumes of Pro-Cipitate and a sample were combined, and the resulting preparation was mixed for 15 min and then centrifuged at 6,000 × *g* and 10°C for 15 min to precipitate the viruses. The virus-containing precipitate was resuspended in 0.1 M Tris-HCl (pH 9.0) at different sample-to-elution volume ratios, the preparation was mixed for 1 h and centrifuged at 6,000 × *g* and 18°C for 15 min to sediment the excess Pro-Cipitate, and the supernatant was retained. The viruses in the supernatant were then concentrated by a second PEG concentration step; 8% PEG 8000 and 0.2 M NaCl were added, and the preparation was mixed at 4°C for 2 to 15 h and then centrifuged at 7,000 × *g* and 4°C for 30 min. The resulting precipitate was resuspended in ca. 0.2 ml of 0.1× PCR buffer II containing 20 mM Tris (pH 8).

Samples were further purified by spin column gel chromatography. Sephadex G-200 (Pharmacia, Piscataway, N.J.) spin columns were prepared and centrifuged in a swinging bucket rotor as described by Maniatis et al. (23), except that silane-treated glass wool (Supelco, Bellefonte, Pa.) was used as the column support and the centrifugal force was decreased to 400 × *g* to avoid crushing the columns. Column volumes of 1 to 4 ml were used to process 0.2 to 0.8 ml of sample. The viruses present in the excluded volumes of spin column effluents were further concentrated by centrifugal ultrafiltration in a Centricon microconcentrator with a molecular weight cutoff of 100,000 (Amicon, Beverly, Mass.). The average retentate volumes were 20 to 60 μl, and 10-μl portions were used directly for the RT-PCR.

RESULTS

Initial virus concentration by PEG precipitation. The levels of recovery efficiency for PV1 and HAV after PEG precipitation were determined by using 1-liter volumes of BE/G seeded with 100 to 1,000 PFU of test virus. The levels of virus recovery after PEG precipitation were based on the input virus titers seeded into beef extract just prior to processing. After overnight precipitation at 4°C, the samples were centrifuged, and each sediment was resuspended in 2 to 3 ml of 0.1× PCR buffer II containing 20 mM Tris (pH 8). As shown in Table 1, the average levels of virus recovery obtained with the cell culture infectivity procedure after direct PEG precipitation and resuspension of the PEG pellets were 88% for PV1 and 64% for HAV. After PEG precipitation, <10% of both viruses remained in the supernatants.

Sample cleanup and second PEG concentration step. Removal of nonpolar interfering substances and residual PEG by chloroform extraction resulted in low and highly variable levels of virus recovery (range, 4 to 68%). Therefore, virus adsorption to and elution from Pro-Cipitate was investigated as an alternative virus purification step. PEG-precipitated samples (ca. 3 ml) were first seeded with test viruses and then Pro-Cipitate purified by combining equal volumes of Pro-Cipitate and sample, mixing the preparations for 15 min, and then centrifuging them. The virus-containing sediments were resuspended by using different sample-to-elution volume ratios of 0.1 M Tris-HCl (pH 9.0), and each preparation was mixed for 1 h and then centrifuged to remove the excess Pro-Cipitate.

TABLE 2. Levels of recovery of PV1 and HAV with Pro-Cipitate

Elution ratio	PV1		HAV	
	No. of trials	% Recovery	No. of trials	% Recovery
4:1	8	30 (21)[a]	5	58 (22)
6:1	7	81 (28)	3	107 (10)
8:1	4	70 (23)	2	81 (18)

[a] The values in parentheses are standard deviations.

The resulting supernatant was assayed for viruses. Table 2 shows the levels of recovery of PV1 and HAV obtained with Pro-Cipitate when we used different ratios of elution buffer volume to Pro-Cipitate volume. The optimum ratio of elution buffer volume to Pro-Cipitate volume was at least 6:1; this ratio resulted in a level of PV1 recovery of 81% and a level of HAV recovery of 107%. At a ratio of 4:1, the levels of recovery for both PV1 (30%) and HAV (58%) were approximately one-half the levels of recovery observed at a ratio of 6:1. Pro-Cipitate elution at a ratio 6:1 resulted in sample volumes of 18 to 24 ml. These volumes were too large for subsequent processing by spin column chromatography and ultrafiltration, and therefore, a second PEG precipitation step was used. The levels of recovery of PV1 and HAV from the PEG-precipitated Pro-Cipitate eluents were 60 to 90% of the initial infectivity values (data not shown); these levels are similar to the levels of virus recovery from PEG-precipitated beef extract.

Removal of RT-PCR inhibitors and sample volume reduction. Although a second PEG precipitation step efficiently concentrated viruses from Pro-Cipitate eluates, as determined by the virus infectivity assay, these samples still contained enough impurities to make them incompatible with virus detection by RT-PCR amplification and oligoprobing. Therefore, we investigated additional virus purification by spin column chromatography. We found that spin column chromatography with Sephadex G-200 efficiently removed inhibitory solutes from beef extract after PEG precipitation, Pro-Cipitate purification, and a second PEG precipitation step. The column-to-sample volume ratios had to be high enough to effectively purify the sample but not so high that they reduced the levels of virus recovery. Column-to-sample ratios of 4:1 to 8:1 resulted in purification without large losses of virus. In experiments in which we determined levels of PV1 recovery at column-to-sample ratios of 4:1, 8:1, and 12:1, the levels of virus recovery were $\geq$100, 83, and 48%, respectively (data not shown).

The volumes of the samples used for spin column chromatography were about 500 μl. These samples were too large for practical analysis by RT-PCR, because for each RT-PCR a sample volume of only 10 μl is used. However, the samples were sufficiently pure to permit further concentration by ultrafiltration with small, commercially available 100,000-molecular-weight cutoff centrifugal ultrafiltration units. When 500-μl samples were concentrated 25-fold by this method, the levels of virus recovery were consistently >90% (data not shown), and the sample volume and purity were compatible with viral genomic amplification by RT-PCR.

TABLE 3. Purification and concentration of viruses in 1-liter BE/G samples

Step	Vol	Concn factor (fold)
BE/G	1 liter	1
PEG precipitation 1	2-3 ml	500
Pro-Cipitate precipitation	15-20 ml	50
PEG precipitation 2	0.5 ml	2,000
Spin column chromatography	0.5 ml	2,000
Ultrafiltration	20-60 μl	50,000

RT-PCR detection of poliovirus in processed samples. On the basis of the levels of recovery efficiency for infectious viruses in each of the concentration and purification steps tested, the following sample treatment scheme was evaluated for virus detection and recovery by RT-PCR: (i) PEG precipitation and resuspension in 2 to 3 ml of 0.1 × PCR buffer II–20 mM Tris (pH 8), (ii) Pro-Cipitate precipitation (ratio of eluent volume to Pro-Cipitate volume, 6:1), (iii) a second PEG precipitation, (iv) Sephadex G-200 spin column chromatography, and (v) centrifugal ultrafiltration. This procedure is outlined in Table 3.

The sample-processing procedure described above was initially evaluated for its ability to remove inhibitors of RT-PCR amplification. After each treatment step a small aliquot of sample was obtained and diluted 10- and 100-fold. The undiluted and diluted aliquots were then supplemented with ca. 100 PFU of poliovirus; this was followed by RT-PCR amplification, agarose gel electrophoresis, and ethidium bromide staining of the gel for visualization of PCR products. As shown in Fig. 1, RT-PCR detection was prevented in the undiluted aliquots of the first and second PEG precipitates, indicating that RT-PCR inhibitors were present. However, viruses were detected in undiluted aliquots after they were supplemented with PEG-NaCl, after Pro-Cipitate treatment, after Sephadex G-200 spin

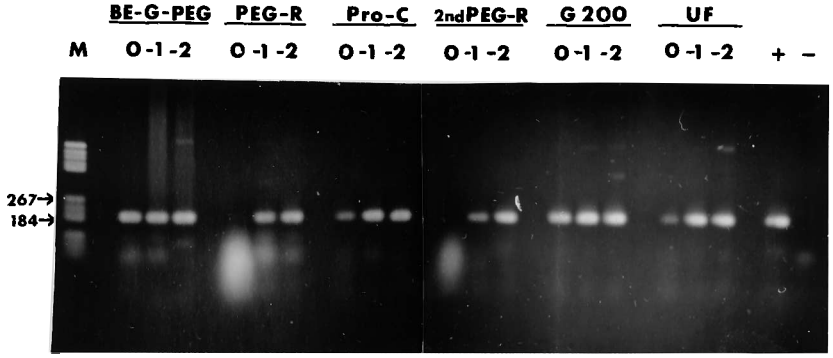

FIG. 1. RT-PCR detection after each treatment step. M, molecular weight markers; BE-G-PEG, beef extract-glycine containing 13% PEG and 0.3 M NaCl; PEG-R, resuspended PEG precipitate; Pro-C, resuspended sample after Pro-Cipitate precipitation; 2ndPEG-R, second resuspended PEG precipitate; G200, Sephadex G-200 spin column chromatography filtrate; UF, ultrafiltered concentrate; 0,-1,-2, undiluted, 10-fold dilution, and 100-fold dilution, respectively; +, PV1-positive control; −, complete reaction cocktail without virus.

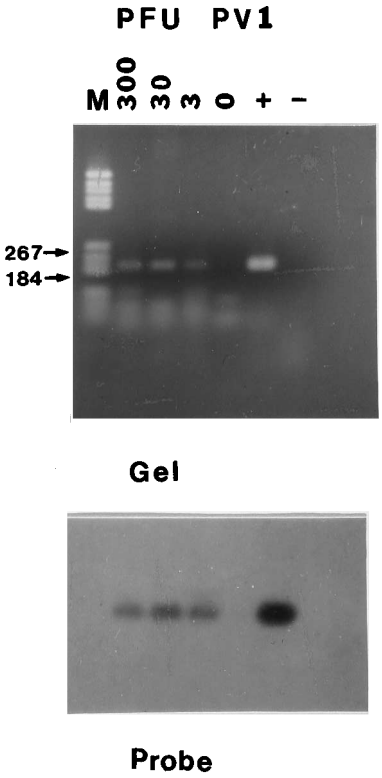

FIG. 2. Detection of poliovirus by RT-PCR and oligoprobe hybridization after concentration and purification from 1 liter of BE/G. PFU PV1, PFU of PV1; M, molecular weight markers; 300, 300 PV1 PFU seeded into 1 liter of BE/G mock eluate; 30, 30 PV1 PFU seeded into 1 liter of BE/G mock eluate; 3, 3 PV1 PFU seeded into 1 liter of BE/G mock eluate; 0, negative control; +, PV1-positive RT-PCR control; −, complete reaction cocktail without virus; Gel, agarose gel electrophoresis detection; Probe, nonradioactive oligoprobe detection of RT-PCR PV1 product.

column chromatography, and after ultrafiltration. Thus, RT-PCR inhibitors were successfully removed as viruses were concentrated by the overall procedure.

The concentration and purification procedure was then used to recover and detect progressively lower input levels of viruses in seeded 1-liter samples of BE/G mock eluate. One-liter samples of BE/G were seeded with 300, 30, and 3 PFU of poliovirus and then processed by using the following procedure: precipitation with 13% PEG–0.2 M NaCl, resuspension in 1 ml of 0.1 × PCR buffer II–20 mM Tris (pH 8), Pro-Cipitate precipitation (Pro-Cipitate elution ratio, 6:1), concentration by a second PEG precipitation, purification by Sephadex G-200 spin column chromatography (column-to-sample volume ratio, 6:1), and concentration to ca. 30 μl by centrifugal ultrafiltration. A 10-μl portion of the final concentrate was assayed by the RT-PCR and was analyzed by agarose gel electrophoresis. The identity of the PCR product was confirmed by Southern transfer and nonradioactive oligoprobe hybridization. As shown in Fig. 2, a poliovirus PCR product was detected at an input level as low as 3 PFU per 1-liter sample. Because only 10 μl of the final sample (volume, 20 to 60 μl) was analyzed, the recovery method may be even more sensitive (at least two- to sixfold more sensitive).

Recovery and RT-PCR oligoprobe detection of poliovirus in 1-liter BE/G mock eluates. A total of 50 1-liter samples of BE/G were seeded with levels of PV1 ranging from <10 to >100 PFU prior to the initial PEG precipitation step. The samples were processed by using the method outlined in Table 3 and Fig. 1, and the final concentrates were analyzed by the RT-PCR. As shown in Table 4, the average level of poliovirus detection was 50%; this value did not decrease significantly when lower virus input levels were used ($P = 0.837$ [Fisher exact test, two-tailed]).

TABLE 4. RT-PCR detection with 1-liter BE/G samples

Input PFU per sample	No. of trials	RT-PCR detection		% of positive samples
		No. negative	No. positive	
10^2	7	3	4	57
10^1	33	16	17	52
$<10^1$	10	6	4	40
Total	50	25	25	50

Recovery and RT-PCR oligoprobe detection of enteric viruses in 1-liter BE/G mock eluates supplemented with humic acid. To better represent environmental filter eluates, 1-liter BE/G mock eluates were supplemented with 0.15 mg of humic acid. Then 1-liter mock eluates with and without added humic acid were seeded with poliovirus (10^2 PFU), HAV (10^2 PFU), and NV (10^3 amplifiable genomic units) and processed by using the method described above. As shown in Fig. 3, PCR products for all three viruses were detected in samples with or without humic acid. PCR amplicon bands were less intense in the samples supplemented with humic acid, suggesting that some interference still occurred or virus recovery was less efficient in humic acid-supplemented beef extract.

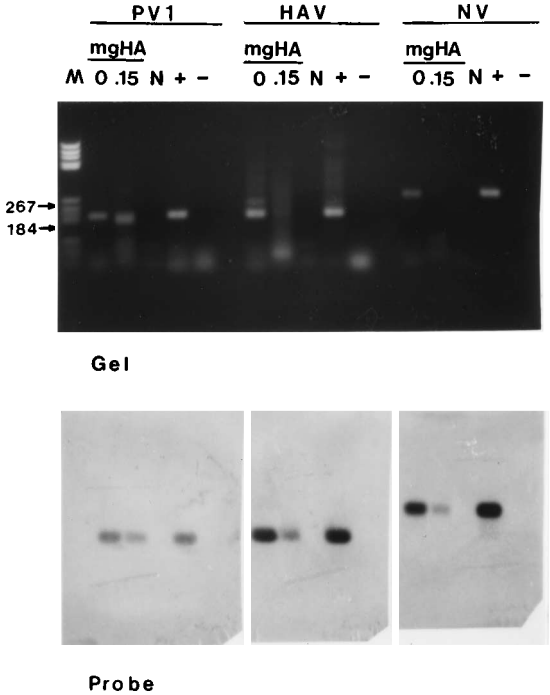

FIG. 3. Detection of PV1, HAV, and NV seeded into 1 liter of BE/G containing humic acid by RT-PCR and oligoprobe hybridization. mgHA, milligrams of humic acid added to 1-liter BE/G sample; M, molecular weight markers; 0, no humic acid added to 1 liter of BE/G; .15, 0.15 mg of humic acid added to 1 liter of BE/G; N, negative control (no virus added); +, positive virus RT-PCR control; −, complete reaction cocktail without virus; Gel, agarose gel electrophoresis detection; Probe, nonradioactive oligoprobe detection of RT-PCR product.

TABLE 5. Poliovirus detection in 1-liter BE/G mock eluate samples by RT-PCR and quantal infectivity assay

RT-PCR assay results	Infectivity assay results		Total no. of samples
	No. of samples positive	No. of samples negative	
Positive	4	6	10
Negative	2	10	12
Total	6	16	22

Comparison of levels of virus recovery and detection by molecular techniques and the cell culture infectivity procedure. A total of 22 experiments were performed to determine if comparable results would be obtained when processed samples were analyzed by the cell culture infectivity method and by RT-PCR amplification. One-liter BE/G mock eluate samples seeded with about 10^1 infectious units of poliovirus were processed, and the final concentrates were analyzed by both methods. Following sample processing, 10 μl of the ultrafilter retentate (an average of 31% of the total volume) was used in the RT-PCR amplification assay. The remaining ultrafilter retentate volume (an average of 69% of the sample) was increased to 1 ml, and the resulting preparation was assayed for infectivity as determined by the presence of CPE by using 10 inoculated cell culture wells and quantal analysis. There were 16 samples that exhibited no CPE, and these samples were given less-than values for level of recovery. These values were based on the input virus titer from quantal data calculated by using the Thomas formula and the percentage of the sample volume analyzed; we assumed that 1 of 10 wells was positive for CPE to compute the virus titer in the concentrate. As shown in Table 5, both detection methods gave positive results: 6 samples were positive as determined by the infectivity method, and 10 samples were positive as determined by the RT-PCR. Although samples were sometimes found to be positive by one method but not by the other, four samples were found to be positive by both methods. For 14 of the 22 samples, the results obtained with the two detection methods were in agreement. However, two samples that were found to be negative by the RT-PCR were found to be positive by the infectivity method, and six samples that were found to be positive by the RT-PCR were found to be negative by the infectivity method. As determined by chi-square analysis, there was no difference in virus detection by the two assay methods ($P = 0.34$ [Fisher exact test, two-tailed]). The grand total level of infectivity recovery efficiency for all 22 samples was estimated to be ≤17%. The level of infectivity recovery efficiency for the six samples which exhibited CPE was 16%. A comparison of the RT-PCR results in Tables 4 and 5 showed that there was no significant difference in levels of recovery efficiency between the two sample sets ($P = 0.78$ [Fisher exact test, two-tailed]).

DISCUSSION

RT-PCR followed by nonradioactive oligoprobe hybridization is a rapid and sensitive molecular method for detecting viruses. However, environmental samples must be sufficiently concentrated to allow efficient detection of very few viruses in large samples and the samples must be purified to reduce the levels of inhibitors of the molecular enzymes used in the RT-PCR (35). In this study we developed a multistep procedure to concentrate and purify intact viruses from beef extract mock eluates obtained from adsorbent filters so that they were compatible with in vitro, enzymatic amplification and detection of viral genomic RNA. With this procedure the viral genome remains protected by the viral capsid until immediately before the RT-PCR, thus limiting viral RNA degradation during concentration and purification steps. Other researchers have used techniques such as proteinase K digestion (20), guanidine thiocyanate extraction (34, 46), and trichloroacetic acid extraction (50) to purify samples and remove inhibitors. Although these procedures can improve sample quality, they may reduce the sensitivity of the assay as a result of viral RNA degradation prior to conversion of RNA to cDNA and subsequent amplification and detection.

PEG precipitation was a reliable method for initial reconcentration and partial purification of viruses in adsorbent filter mock eluates obtained from environmental water samples. Our method can be used with a variety of aqueous samples (21, 22, 30), the procedure is simple, and the reagents are inexpensive. Sample volume is reduced 250- to 300-fold, and levels of virus recovery efficiency are >50%. The levels of viral recovery efficiency which we obtained by using PEG precipitation from beef extract eluates are similar to or greater than the levels obtained by other researchers who analyzed beef extract eluates by using acid precipitation (25, 36, 38). After modifications in the process used to produce beef extract in the 1970s decreased the ability of beef extract to flocculate, research focused on procedural modifications, such as the addition of a flocculating and precipitating supplement like $FeCl_3$ or diatomaceous earth to improve virus precipitation (4, 28, 29). Such supplements can be highly inhibitory to the RT-PCR (32) and should be avoided. The use of 1.0% beef extract instead of 1.5 or 3.0% beef extract in the initial 1-liter mock eluate reduced the level of molecular inhibitors which were present in the beef extract when it was concentrated, and this value is well within the range of beef extract concentrations typically used for virus elution from filters (36, 40).

The use of a commercially available protein-adsorptive reagent, Pro-Cipitate, for purification of PEG precipitates resulted in effective virus recovery and sample purification and dramatically improved virus detection by the RT-PCR. The required Pro-Cipitate elution ratio of ≥6:1 increased the sample volume, making samples unmanageable for low-volume ultrafiltration. Therefore, a second PEG precipitation step was used to reduce the sample volume to <0.5 ml. This allowed us to perform the final purification and concentration steps of spin column chromatography and ultrafiltration with minimal virus loss and improved the compatibility of samples with RT-PCR amplification.

The overall procedure used to concentrate and purify viruses from beef extract filter eluates consisted of PEG precipitation, Pro-Cipitate precipitation, a second PEG precipitation, Sephadex G-200 spin column chromatography, and ultrafiltration. This sample-processing technique can be used to concentrate and purify viruses in a 1-liter beef extract-glycine mock sample, reducing the volume to 20 to 60 μl (equivalent to 16,000- to 50,000-fold concentration). The PCR product obtained from genomic amplification was successfully detected in concentrated samples that were seeded initially with as little as 3 PFU of poliovirus in a 1-liter sample of BE/G. For some samples a distinct PCR product band was not detected by ethidium bromide staining of the agarose gels, but PCR amplification of the target viral genome was verified by Southern transfer and nonradioactive oligoprobing. Such hybridization assays are essential to increase amplicon detection and to verify PCR amplification.

In experiments to determine levels of virus recovery efficiency and detection in seeded samples by using both infectiv-

ity and RT-PCR assays, only a portion of the final sample concentrate volume (average, 31%) was subjected to RT-PCR analysis. The remaining concentrate volume was used to determine virus infectivity. Despite the fact that only about one-third of each total sample concentrate was analyzed by RT-PCR, 50% of the samples were virus positive. If the entire sample volume had been analyzed by the RT-PCR, the percentage of positive samples may have been even higher. For those samples that were also assayed for the presence of viruses by the infectivity procedure, the estimated level of infectivity recovery efficiency was about 16%, which also may have been an underestimate because only some of the final concentrate was analyzed.

One-liter BE/G mock eluates were supplemented with aquatic humic acid, seeded with virus, and then analyzed by the method described above to determine if the procedure could contend with this interfering substance. Humic acid is a commonly occurring organic compound found in environmental samples and can interfere with conventional as well as molecular detection techniques (14, 39, 43). Using our technique, we successfully recovered and detected PV1, HAV, and NV in 1-liter BE/G eluates containing humic acid. Some humic acid may have remained in the final sample concentrates, as suggested by the RT-PCR product bands having reduced intensities. Other concentration and purification techniques, such as antibody (immunoaffinity) capture or guanidinium extraction of viral RNA in the final concentrate, are procedural modifications that are being investigated in our laboratory with the hope that they will further improve viral detection by the RT-PCR.

The procedure developed in this study is a step toward the goal of a rapid, simple, and economical method for detecting low levels of enteric viruses in water and other environmental samples. With this procedure it is possible to detect less than 10 PFU of a target virus in a mock beef extract filter eluate sample in less than 3 days by relatively simple processing steps. Hence, routine viral monitoring of environmental samples with molecular biological methods may be possible. Because the use of beef extract as an elution medium is a potentially limiting factor in the detection of viruses by molecular biological methods, further research should focus on substitutes for beef extract or alternative methods that do not rely on this eluate.

ACKNOWLEDGMENTS

We thank Douglas A. Wait for technical assistance.

This research was supported by contract 612-90 from the American Water Works Association Research Foundation, by grant R817153 from the United States Environmental Protection Agency, by National Water Research Institute Project HR-92-01 and Project HR-92-06, by Perkin Elmer Corp., and by Roche Molecular Systems.

REFERENCES

1. **Abbaszadegan, M., M. S. Huber, C. P. Gerba, and I. L. Pepper.** 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl. Environ. Microbiol. **59:**1213–1219.
2. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
3. **Cromeans, T., M. D. Sobsey, and H. A. Fields.** 1987. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. J. Med. Virol. **22:**45–56.
4. **Dahling, D. R., and B. A. Wright.** 1986. Optimization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. Appl. Environ. Microbiol. **51:**790–812.
5. **Dahling, D. R., and B. A. Wright.** 1988. A comparison of recovery of virus from wastewaters by beef extract-celite, ferric chloride, and filter concentration procedures. J. Virol. Methods **22:**337–346.
6. **Deetz, T. R., E. M. Smith, S. M. Goyal, C. P. Gerba, J. J. Vollet, Y. L. Tsai, H. L. Du Pont, and B. H. Keswick.** 1984. Occurrence of rota- and enterovirus in drinking water and environmental water in a developing nation. Water Res. **18:**567–571.
7. **De Flora, S., G. P. De Renzi, and G. Badolati.** 1975. Detection of animal viruses in coastal seawater and sediments. Appl. Microbiol. **30:**472–475.
8. **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.
9. **De Leon, R., Y. S. 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. Am. Water Works Assoc. Proc. **1989:** 833–853.
10. **De Leon, R., and M. D. Sobsey.** 1991. New methods for virus detection in water, p. 400–421. *In* J. R. Hall and G. D. Glysson (ed.), Monitoring water in the 1990's: meeting new challenges. Publication ASTM STP 1102. American Society for Testing and Materials, Philadelphia.
11. **Demeke, T., and R. P. Adams.** 1992. The effects of plant polysaccharides and buffer additives on PCR. BioTechniques **12:**332–333.
12. **Gerba, C. P., A. B. Margolin, and M. J. Hewlett.** 1989. Application of gene probes to virus detection in water. Water Sci. Technol. **21:**147–154.
13. **Gerba, C. P., and J. B. Rose.** 1990. Viruses in source and drinking water, p. 380–395. *In* G. A. McFeters (ed.), Drinking water microbiology. Springer-Verlag, New York.
14. **Gurrman-Bass, N., and J. Catalano-Sherman.** 1985. Effects of humic materials on virus recovery from water. Appl. Environ. Microbiol. **49:**1260–1264.
15. **Hurst, C. J., D. R. Dahling, R. S. Safferman, and T. Goyke.** 1984. Comparison of commercial beef extracts and similar materials for recovering viruses from environmental samples. Can. J. Microbiol. **30:**1253–1263.
16. **Imai, H., O. Yamada, S. Morita, S. Suehiro, and T. Kurimura.** 1992. Detection of HIV-1 RNA in heparinized plasma of HIV-1 seropositive individuals. J. Virol. Methods **36:**181–184.
17. **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.
18. **Jiang, X., M. K. Estes, T. G. Metcalf, and J. L. Melnick.** 1986. Detection of hepatitis A virus in seeded estuarine samples by hybridization with cDNA probes. Appl. Environ. Microbiol. **52:**711–717.
19. **Kolk, A. H. J., A. R. J. Schuitema, S. Kuijper, J. vanLeeuwen, P. W. M. Hermans, J. D. A. van Embden, and R. A. Hartskeerl.** 1992. Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. J. Clin. Microbiol. **30:**2567–2575.
20. **Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J. M. Lopez-Pila.** 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol. **59:**1213–1219.
21. **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.
22. **Lund, E., and C. Hedstrom.** 1966. The use of an aqueous polymer phase system for enterovirus isolations from sewage. Am. J. Epidemiol. **84:**287–291.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Margolin, A. B., K. J. Richardson, R. De Leon, and C. P. Gerba.** 1989. Application of gene probes to the detection of enteroviruses in water, p. 265–270. *In* R. A. Larson (ed.), Biohazards of drinking water treatment. Lewis Publishers, Chelsea, Mich.
25. **Melnick, J. L., R. Safferman, V. C. Rao, S. Goyal, G. Berg, D. R. Dahling, B. A. Wright, E. Akin, R. Stetler, C. Sorber, B. Moore, M. D. Sobsey, R. More, A. L. Lewis, and F. M. Wellings.** 1984. Round robin investigation of methods for the recovery of poliovirus from drinking water. Appl. Environ. Microbiol. **47:**144–150.
26. **Morris, R., and W. M. Waite.** 1980. Evaluation of procedures for recovery of viruses from water. II. Detection systems. Water Res. **14:**795–798.
27. **Muscillo, M., F. A. Aulicino, A. M. Patti, P. Orsini, L. Volterra, and G. M. Fara.** 1994. Molecular techniques for the identification of enteric viruses in marine waters. Water Res. **28:**1–7.
28. **Payment, P., S. Fortin, and M. Trudel.** 1984. Ferric chloride flocculation for nonflocculating beef extract preparations. Appl. Environ. Microbiol. **47:**591–592.
29. **Payment, P., and M. Trudel.** 1987. Second-step reconcentration of environmental samples by ammonium sulfate flocculation of beef extract. Can. J. Microbiol. **33:**571–572.
30. **Philipson, L., P. A. Albertsson, and G. Frick.** 1960. The purification and concentration of viruses by aqueous polymer phase systems. Virology **11:** 553–571.
31. **Rotbart, H. A., M. J. Levin, and L. P. Villarreal.** 1984. Use of subgenomic poliovirus DNA hybridization probes to detect the major subgroups of enteroviruses. J. Clin. Microbiol. **20:**1105–1108.

32. **Schwab, K. J., R. De Leon, R. Baric, and M. D. Sobsey.** 1991. Detection of rotaviruses, enteroviruses and hepatitis A virus by reverse transcriptase-polymerase chain reaction. Am. Water Works Assoc. Proc. **1990:**475–495.
33. **Shieh, Y. S. C., R. S. Baric, M. D. Sobsey, J. Ticehurst, R. De Leon, and R. Walter.** 1991. Detection of hepatitis A virus and other enteroviruses in water by ssRNA probes. J. Virol. Methods **31:**1119–1126.
34. **Shieh, Y. S. C., C. Nguyen, M. V. Vocal, and H. W. Chu.** 1993. Tumor-suppressor p53 gene in hepatitis C and B virus-associated human hepatocellular carcinoma. Int. J. Cancer **54:**558–562.
35. **Sobsey, M. D.** 1994. Molecular methods to detect viruses in environmental samples, p. 387–400. *In* R. C. Spencer, E. P. Wright, and S. W. B. Newsome (ed.), Rapid methods and automation in microbiology and immunology. Intercept, Ltd., Andover, England.
36. **Sobsey, M. D., and S. Glass.** 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl. Environ. Microbiol. **40:**201–210.
37. **Sobsey, M. D., and S. Glass.** 1984. Influence of water quality on enteric virus concentration by microporous filter methods. Appl. Environ. Microbiol. **47:** 956–960.
38. **Sobsey, M. D., S. Glass, R. R. Jacobs, and W. A. Rutala.** 1980. Modifications of the tentative standard method for improved virus recovery efficiency. Am. Water Works Assoc. J. **72:**350–355.
39. **Sobsey, M. D., and A. R. Hickey.** 1985. Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. Appl. Environ. Microbiol. **49:**259–264.
40. **Sobsey, M. D., R. S. Moore, and S. Glass.** 1981. Evaluating adsorbent filter performance for enteric virus concentrations in tap water. Am. Water Works Assoc. J. **73:**542–548.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
42. **Stetler, R. E., M. E. Morris, and R. S. Safferman.** 1992. Processing procedures for recovering enteric viruses from wastewater sludges. J. Virol. Methods **40:**67–76.
43. **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.
44. **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.
45. **Tsai, Y. L., C. J. Palmer, and R. L. Sangermano.** 1993. Detection of *Escherichia coli* in sewage and sludge by polymerase chain reaction. Appl. Environ. Microbiol. **59:**353–357.
46. **Tsai, Y. L., M. J. Park, and B. H. Olson.** 1991. Rapid method for direct extraction of mRNA from seeded soils. Appl. Environ. Microbiol. **57:**765–768.
47. **Tsai, Y. L., M. D. Sobsey, L. R. Sangermano, and C. J. Palmer.** 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. Appl. Environ. Microbiol. **59:**3488–3491.
48. **Wilde, J., J. Eiden, and R. Yolken.** 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reaction. J. Clin. Microbiol. **28:**1300–1307.
49. **Xu, L., D. Harbour, and M. A. McCrae.** 1990. The application of polymerase chain reaction to the detection of rotaviruses in faeces. J. Virol. Methods **27:**29–38.
50. **Yang, F., and X. Xu.** 1993. A new method of RNA preparation for detection of hepatitis A virus in environmental samples by the polymerase chain reaction. J. Virol. Methods **43:**77–84.