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Immunoaffinity Concentration and Purification of Waterborne Enteric Viruses for Detection by Reverse Transcriptase PCR

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To assess the risks from viral contamination of drinking-water supplies, there is a clear need for methods to directly detect viral pathogens. In this study, we developed a broad-spectrum immunocapture method for concentration and purification of enteric viruses. The method involved indirect antibody capture (AbCap) of intact viruses followed by release of virion genomic RNA and reverse transcriptase PCR for amplification and oligoprobe hybridization for detection. The procedure involved concentrating enteric viruses from large volumes of water by standard filtration-elution techniques with 1MDS filters and 1 liter of 1% beef extract–0.05 M glycine (BE/G) as an eluate. The BE/G eluate was concentrated and purified by polyethylene glycol (PEG) precipitation, ProCipitate (a commercially available protein precipitating reagent) precipitation, and a second PEG precipitation to a volume of approximately 500 μl. Aliquots of the second PEG precipitate were further processed by RNA extraction, AbCap, or cell culture analysis for infectious viruses. The AbCap method was applied to 11 field samples of fecally contaminated surface water. Of the 11 samples, 9 were positive for enteric viruses by the AbCap method; 4 of 11 samples were positive for enteric viruses by direct RNA extraction of a small aliquot of the second PEG concentrate; and 4 of 11 samples were positive for enteric viruses by measurement of cell culture infectivity. The results for enteric viruses were compared with those for standard bacterial and coliphage indicators of fecal contamination.

Enteric virus contamination of drinking-water sources is a public health concern. Wastewater discharges from sewage treatment plants and other point and nonpoint sources of fecal contamination may lead to viral contamination in watersheds. Current methods for assessing the microbial quality of drinking water and its sources are based on indicator bacteria, but studies have shown that indicator bacteria are inadequate at predicting the presence of enteric viruses in water (4, 8, 24, 25). There is a clear need for periodic or even routine monitoring of water supplies for possible viral contamination, at least to develop a database of virus occurrence for the purpose of risk assessment analysis (19, 20, 26). Unfortunately, conventional methods for enteric virus isolation from water are technically difficult, time-consuming, inefficient, and expensive, and they have relied on animal cell cultures to detect only the culturable viruses (10). Conventional cell culture assays do not detect some of the most important waterborne enteric viruses, including hepatitis A virus (HAV) and the Norwalk-type viruses.

The low level of viruses found in most environmental waters is a major analytical problem that necessitates the concentration of viruses from water. Typically, viruses in hundreds to thousands of liters of water are first concentrated by adsorption to a microporous filter. The adsorbed viruses are then eluted with approximately 1 liter of eluent and then further concentrated to a small volume for virus assay in cell cultures. Beef extract solution at pH 9.5 to 10 and a concentration of 1 to 3% is the most commonly used eluent. Beef extract is effective when used in conjunction with conventional viral assays in cell cultures, but it contains high concentrations of proteins, salts, and other solutes, which can interfere with virus detection by molecular techniques such as nucleic acid amplification and hybridization (gene probe) (21). Purification and concentration procedures are needed to effectively remove the interfering substances in beef extract and separate them from the viruses. The same procedures are likely to also remove the interfering substances found in environmental waters, such as humic acid and other organic compounds, thereby allowing the viruses to be detected via their nucleic acid.

Nucleic acid methods, especially in vitro enzymatic amplification of specific viral genomic nucleic acid sequences by PCR followed by detection of the amplified nucleic acid by hybridization, have emerged recently as sensitive and specific approaches for the detection of many viruses (1, 3, 6, 13, 14, 22, 26). A major obstacle to such nucleic acid detection is interference with the nucleic acid polymerase enzymes by inhibitory agents in the sample. Several techniques such as phenol-chloroform extraction, sodium dodecyl sulfate-proteinase K digestion, guanidinium thiocyanate extraction, and trichloroacetic acid precipitation, have been developed to purify viral nucleic acid in sample concentrates (13–15, 23, 32). Because these methods isolate viral nucleic acid from the sample, the infectivity of the viruses cannot be ascertained. In addition, these techniques expose labile viral nucleic acid, often single-stranded RNA, to conditions that could lead to degradation before analysis, thereby causing a loss of target and a decrease in sensitivity.

Alternative techniques that further concentrate and purify intact virions from the sample concentrate by physicochemical methods are less likely to cause degradation of viral nucleic acid than are processing methods that rely on release or extraction of the viral genomic RNA (1, 21). However, most virion concentration and purification methods, such as polyethylene glycol (PEG) precipitation, Sephadex gel chromatography, chelation of multivalent cation impurities, and ultrafil-

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tration, lack specificity; therefore, it is possible that inactivated viruses are being recovered and their nucleic acid is being amplified and detected (31). One approach to the nucleic acid-based detection of intact and hence potentially infectious viruses is virus capture mediated by antibody-antigen complexes. Immunoaffinity (antibody) capture of the virus particles onto a solid phase is a promising technique to remove enzyme inhibitors and will also exclude viral RNA not associated with the antibody-capturable virions.

Immunoaffinity capture in which monoclonal antibodies are used to isolate, concentrate, and purify specific virus particles for subsequent nucleic acid amplification and detection has been reported for viruses in clinical and environmental samples (7, 9, 11, 12, 18). The development of a polyclonal or multiclonal immunoaffinity technique would have the advantage of allowing a variety of enteric viruses to be isolated simultaneously from one sample for subsequent nucleic acid detection.

The objective of this study was to develop a broad-spectrum immunocapture method for concentration and purification of enteric viruses in partially concentrated and purified beef extract eluate from the filters used to adsorb and concentrate viruses from water. The method employed indirect antibody capture (AbCap) of intact viruses followed by release of virion genomic RNA and reverse transcriptase PCR (RT-PCR) for amplification and oligoprobe (OP) hybridization for detection. The method was applied to field samples of fecally contaminated surface water. The recovered viruses were assayed both by RT-PCR plus OP hybridization (RT-PCR–OP) and by cell culture infectivity. These results for enteric viruses were compared with those for standard bacterial and coliphage indicators of fecal contamination.

MATERIALS AND METHODS

Beef extract mock eluate RT-PCR compatibility. To investigate the production of virus concentrates free of enzyme inhibitors, a model system consisting of 1 liter of 1% beef extract (BBL, Becton Dickinson, Cockeysville, Md.) plus 0.05 M glycine (BE/G) was seeded with either poliovirus type 1 (PV1), HAV, or Norwalk virus and processed by PEG precipitation and AbCap. Briefly, 1-liter BE/G samples were supplemented with 13% PEG 8000 and 0.2 M NaCl, mixed at 4°C for 2 to 15 h, and then centrifuged at 7,000 × *g* and 4°C for 30 min. The resulting precipitates were resuspended in ca. 1 ml of 0.1× PCR buffer II (5 mM KCl, 1 mM Tris-HCl [pH 8.3]; Perkin-Elmer, Norwalk, Conn.). The Tris concentration was adjusted with 1 M Tris (pH 8.3) so that the final concentration was 20 mM. To better model actual filter eluates from field water samples, some BE/G samples were supplemented with 1.5 mg of humic acid (lyophilized, XAD-8 resin purified) per liter before the PEG precipitation.

Seeded beef extract eluates from environmental samples. Approximately 200-liter samples of raw surface source water for a North Carolina water treatment plant were filtered through positively charged pleated cartridge filters (Virosorb 1MDS; Cuno Inc., Meriden, Conn.). The watershed of this water source is highly protected from human fecal contamination, and enteric viruses have not been isolated in previous studies conducted by our laboratory (data not shown). The filters were then eluted with 1 liter of BE/G (pH 9.5). The eluate pH was adjusted to 7.2, viruses were added, and the samples were processed by the PEG procedure described above for subsequent AbCap processing followed by RT-PCR amplification and hybridization detection.

Field samples and processing for AbCap. The methods developed in this study were subsequently applied to the detection of human enteric viruses in field samples obtained from potentially or known fecally contaminated surface waters from a number of different sites in the United States, including the Piedmont region of North Carolina, southern Arizona, and the lower Great Lakes region. Viruses were concentrated from water by standard procedures with electropositive Virosorb 1MDS cartridge filters at ambient water pH levels. A 2- to 6-liter grab sample of water was also collected for bacterial and coliphage analysis. The 1MDS filter and grab samples were transported to the laboratory on chill packs by overnight air courier and processed within 24 h of collection. 1MDS filters were eluted with ca. 1 l of BE/G (pH 9.5). Following elution, BE/G eluates were adjusted to pH 7.2 and viruses were partially concentrated and purified by PEG precipitation, Pro-Cipitate precipitation, and a second PEG precipitation (21). Briefly, the BE/G eluates were supplemented with 10% PEG 8000 (Sigma Chemical Co., St. Louis, Mo.) and 0.2 M NaCl, mixed for 2 to 15 h at 4°C, and centrifuged at 7,000 × *g* and 4°C for 30 min, and the supernatant was discarded.

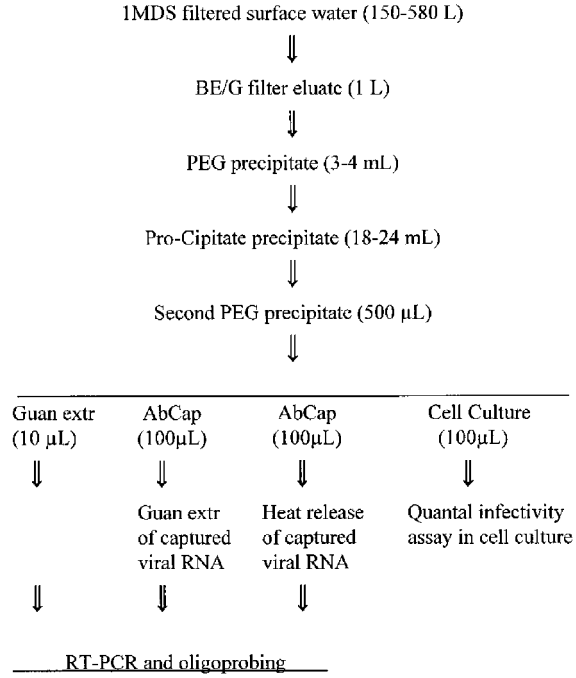

FIG. 1. Field sample-processing flow chart. Guan extr, guanidinium extraction.

The PEG pellet was resuspended with 4 ml of phosphate-buffered saline (PBS), an equal volume of Pro-Cipitate (Affinity Technology, Parsippany, N.J.) was added, the sample was mixed for 1 h at room temperature and centrifuged at 6,000 × *g* for 4°C for 15 min, and the supernatant was discarded. The Pro-Cipitate pellet containing viruses was eluted at a 6:1 elution buffer-to-sample volume ratio with 0.1 M Tris (pH 9) for 1 h at room temperature with mixing. The sample was centrifuged at 6,000 × *g* and 18°C for 15 min, and the supernatant was recovered and adjusted to pH 7.2. The Pro-Cipitate supernatant was then precipitated again with 8% PEG 8000–0.2 M NaCl by mixing for at least 2 to 15 h at 4°C, centrifuging at 7,000 × *g* and 4°C for 30 min, and discarding the supernatant. The second PEG pellet was resuspended with 0.5 ml of PBS, aliquoted into 0.1-ml volumes, and frozen at −80°C until analyzed or further processed. Aliquots of the second PEG precipitate were further processed by AbCap, RNA extraction, or cell culture analysis for infectious viruses (Fig. 1).

Bacterial analysis. Serial dilutions of the grab sample were analyzed by standard membrane filtration methods (2) for fecal coliforms, *Escherichia coli*, enterococci, and *Clostridium perfringens*, using mFC, mTEC, modified mE, and mCP media, respectively.

Coliphage analysis. Approximately 1 liter of grab sample was analyzed for both somatic and male-specific coliphages by a membrane filtration/elution technique and plaque assay with *E. coli* C and Famp and *Salmonella typhimurium* WG49 (27, 28).

Virus detection by AbCap. Solid-phase immunoaffinity virus capture by the AbCap method consisted of multiple adsorption and purification steps. The entire procedure was conducted at room temperature in 1.6-ml microcentrifuge tubes. Goat anti-human immunoglobulin G (IgG) antibodies covalently linked to paramagnetic beads (Bio Mag; Advanced Magnetics, Inc., Cambridge, Mass.) were used in 1-ml amounts (capable of binding >0.1 mg of human IgG) for each sample. The antibody-coated beads were magnetized, and the supernatant was removed. The beads were then combined with 1 ml of 1% blocking solution (Genius System blocking reagent; Boehringer Mannheim Corp., Indianapolis, Ind.) dissolved in TNT (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween 20) for 15 min with gentle mixing to minimize nonspecific binding. The antibody-coated beads were magnetized, and the supernatant was removed, with residual blocking reagent removed by washing the beads with 1 ml of TNT. The antibody-coated beads were magnetized, and the TNT was removed. To provide a solid phase containing a variety of antibodies against human enteric viruses, 1 ml of a pooled source of IgG as human serum immunoglobulin (HSIG) (GAMMAR; Armour Pharmaceutical Co., Kankakee, Ill.), diluted 1:100 in TNT, was added to the antibody-coated beads and allowed to bind for 30 min with gentle mixing. The antibody-coated beads were magnetized, the HSIG supernatant was removed, and the beads were washed with 1 ml of TNT. The antibody-bead complex was treated with an additional 1 ml of 1% blocking solution for 15 min with gentle mixing, and residual blocking reagent was removed by washing the beads with 1 ml of TNT. The antibody-coated bead complexes were magnetized, and the TNT

was removed. Partially purified samples containing viruses in volumes of 0.01 to 1 ml were then added to the antibody-bead complex, and the total volume was adjusted to ca. 1.4 ml with TNT. The virus-bead complex was gently mixed for 2 h, followed by four 1-ml TNT washes. Following the final wash, the beads were concentrated to the bottom of the 1.6-ml tubes with the magnet and the supernatant was discarded. The antigen-antibody-bead complex was resuspended in ca. 10 to 30 μl of 0.1× PCR buffer II plus 20 mM Tris (pH 8.3). Washing the magnetic bead complexes was essential to remove inhibitory substances present in the environmental concentrates as well as inhibitors present in the reagents, such as thimerosal (a preservative present in the HSIG solution). RNA from captured viral particles was then released by heating at 99°C for 5 min, the magnetic beads were removed by centrifugation at 8,000 × *g* for 2 min, and the supernatant containing released viral RNA was analyzed immediately by RT-PCR.

Extraction of viral RNA. For samples containing high concentrations of potential inhibitors, a final purification step of guanidinium extraction of viral RNA after antibody capture was used as an alternative to heat release of viral RNA (23). Viral RNA was extracted from AbCap final concentrates with a commercially available kit (ToTally RNA; Ambion Inc., Austin, Tex.). Briefly, the samples were mixed with guanidinium thiocyanate solution buffer to denature the virions and extract viral RNA. The RNA was further extracted twice with phenol-chloroform, sequentially precipitated with isopropanol and then lithium chloride, washed with ethanol, and dried by vacuum centrifugation. The RNA was resuspended with 10 μl of deionized water and analyzed immediately by RT-PCR.

Viruses and cells. PV1 (LSc) was propagated in BGMK (African green monkey kidney-derived) cells and assayed for infectivity by the plaque technique or by a quantal assay for cytopathic effects (CPE). HAV, cytopathic strain HM175 (5), 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 PEG precipitation. Norwalk virus (8FIIa) was processed from stool samples of infected human volunteers by preparing a 20% stool slurry in PBS followed by fluorocarbon extraction. All virus stocks were used for detection at concentrations that were not inhibitory to RT-PCR (data not shown).

The quantal assay for CPE of culturable enteric virus was performed on 100-μl aliquots of the second PEG precipitate by increasing the sample volume to 1 ml with PBS and then inoculating 0.2-ml volumes into each of five 25-cm^2 flasks containing newly confluent BGMK cell layers with their growth medium aspirated. After 90 min of adsorption and addition of maintenance medium, the cultures were observed daily for CPE over a period of 10 days. After day 10, the flasks were frozen and thawed, and 2 ml of the lysate was assayed as a second passage in each of five 25-cm^2 flasks containing newly confluent BGMK cell layers by the same methods as were used for the initial passage. Estimates of virus concentration were computed as the most probable number (MPN) of cytopathic units according to standard methods (2).

PCR primers and oligoprobes. The oligonucleotide primer and probe sequences for enteroviruses and hepatitis A virus used in this study were identical to those previously described (21). The highly conserved 5′ untranslated region of the enteroviruses was used as the target for the synthesis of a 197-bp panenterovirus cDNA (5′ primer, CCTCCGGCCCCTGAATG; 3′ primer, ACCG GATGGCCAATCCAA; internal oligoprobe, TACTTTGGGTGTCCGTGTT TC). For HAV, the genomic region corresponding to the interface of the VP1-VP3 capsid proteins was the target for a 192-bp cDNA (5′ primer, CAGCA CATCAGAAAGGTGAG; 3′ primer, CTCCAGAATCATCTCCAAC; internal oligoprobe, TGCTCCTCTTTATCATGCTATG). For Norwalk virus, the genomic region encoding the viral polymerase was the target for a 260-bp cDNA (6) (5′ primer, CAAATTATGACAGAATCCTTC; 3′ primer, GAGAAATATGA CATGGATTGC; internal oligoprobe, ATGTCATCAGGGTCAAAGAGG). The downstream or antisense 3′ primers are complementary to the plus-sense virion RNA, and the upstream 5′ primers are homologous to the plus-sense viral RNA. Internal oligoprobes were synthesized in the plus-sense orientation so that they hybridize only with cDNA or PCR products and not with viral genomic plus-sense RNA.

TABLE 1. End-point detection of PV1 RNA by AbCap–RT-PCR versus direct heat release–RT-PCR[a]

PV1 sample	AbCap–RT-PCR end point	Direct RT-PCR end point	Log_{10} difference in detectability
Purified RNA	10^{-2}	10^{-6}	4
Heat-inactivated virus capsids	10^{-3}	10^{-6}	3
Unmodified virus stock	10^{-6}	10^{-8}	2

[a] Aliquots (10 μl) of serial log_{10} dilutions of each sample were assayed by AbCap–RT-PCR and direct heat release–RT-PCR to obtain a detection end point. The detection end point is the last dilution yielding a PV1 RT-PCR product in a 10-μl volume.

RT-PCR. An RNA-PCR kit (Perkin Elmer-Roche, Alameda, Calif.) was used throughout this study. The manufacturer's instructions were followed, except that the reaction volume for reverse transcription was increased from 20 to 30 μl to accommodate a 10-μl virus sample. Viral RNA was released from virions by heating the reaction mixtures at 99°C for 5 min or by guanidinium extraction followed by addition of RT (50 U) and RNase inhibitor (20 U) in a total volume of 30 μl of RT-PCR buffer. Reverse transcription was carried out at 42°C for 1 h with downstream (3′) primers, after which the tubes were heated to 99°C for 5 min to inactivate the enzyme. After being chilled, the tubes were supplemented with 2.5 U of *Taq* polymerase and the upstream (5′) primer for either panenterovirus, HAV capsid, or Norwalk virus polymerase in a total volume of 100 μl of RT-PCR buffer. PCR amplification was performed for 40 cycles, 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 volume of PCR product was analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Southern transfer and nonradioactive oligoprobe detection. The PCR product was transferred from the electrophoresed agarose gels to nylon membranes by the method of Southern (29), and the cDNA was bound to the membranes by UV cross-linking for 5 min. 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 as specified by the instructions in the Genius nonradioactive end-labeling kit (Boehringer Mannheim Biochemicals). Oligoprobe hybridization and immunological detection of positive samples were performed as specified in the instructions in the Boehringer Mannheim Biochemicals kit. Immunological detection of PCR product-oligoprobe hybrids was performed with an anti-digoxigenin–alkaline phosphatase antibody conjugate and an enzyme-catalyzed colorimetric reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium as substrates.

RESULTS

Comparison of AbCap–RT-PCR with direct RT-PCR after heat treatment. Direct RT-PCR after heat treatment at 99°C for 5 min to release virion RNA is a simple and sensitive method for detection of low levels of viruses. However, a potential disadvantage of this method is the possibility of detecting RNA that is not associated with intact and potentially infectious viruses. In contrast, AbCap of viruses prior to RT-PCR should exclude the recovery and detection of free RNA or RNA associated with antigenically denatured, noninfectious viruses. To test this hypothesis, we compared the end-point titers of direct RT-PCR (after heat release of viral RNA) and AbCap–RT-PCR for diluted PV1 stocks receiving the following treatments: (i) none (stock virus, untreated), (ii) heat inactivation of stock virus (56°C for 50 min), and (iii) RNA extraction from stock virus, yielding purified RNA. The virus stocks receiving these treatments did not necessarily have identical titers, because the main goal of the experiment was to determine the relative RT-PCR detectability of the samples by the two preparation methods: AbCap and heating at 99°C. As shown by the results summarized in Table 1, the difference between heat release plus RT-PCR and AbCap–RT-PCR was smallest for untreated virus stock (2 log_{10} units), greatest for purified RNA (4 log_{10} units), and intermediate for heat-inactivated virus stock (3 log_{10} units).

Detection of viruses in mock eluates by AbCap–RT-PCR. The AbCap method was tested in a series of experiments on progressively more realistic and inhibitory mock filter eluates. The least inhibitory sample consisted of an RT-PCR-compatible buffer seeded with low levels of virus. The results of experiments with this mock eluate showed that the AbCap method could effectively isolate and retain viruses during repeated washes and concentration steps when 1-ml volumes of 0.1× PCR buffer were seeded with 200, 20, and 2 PFU of PV1 and processed by the AbCap method. When these samples were analyzed by RT-PCR amplification followed by gel electrophoresis and filter hybridization of the Southern blot-transferred DNA, as little as 2 PFU of PV1 was successfully recovered and detected (Fig. 2). The AbCap procedure was then tested on a more realistic sample of PEG-precipitated beef extract. BE/G mock eluates (1 liter) were PEG precipitated,

and the PEG pellets were resuspended with 3 ml of phosphate buffer. Volumes (1 ml) of the resuspended PEG precipitate were seeded with either 200, 20, or 2 PFU of PV1 and processed by the developed AbCap method. The RT-PCR-amplified product was analyzed by gel electrophoresis followed by Southern transfer onto membranes and oligoprobe detection. As shown by the results in Fig. 3, The AbCap method was effective in reducing the PEG-concentrated inhibitory components present in beef extract eluates, with successful detection of as little as 2 PFU of PV1. The AbCap method was tested with an even more inhibitory mock sample consisting of PEG precipitate from beef extract that had been supplemented with 1.5 mg of purified humic acid. Humic acid is a naturally occurring organic material that is commonly found in surface waters; it can interfere with nucleic acid polymerase function. Volumes (1 liter) of BE/G mock eluates with or without 1.5 mg of purified humic acid were PEG precipitated, and the PEG pellet was resuspended with 1 ml of phosphate buffer. Then 400 PFU of PV1 was seeded into the 1-ml volumes of resuspended PEG precipitate and processed by the developed AbCap method. RT-PCR amplified product was analyzed by gel electrophoresis followed by Southern transfer and oligoprobe detection. As shown in Fig. 4, positive results were obtained from processed mock eluate samples with and without added humic acid.

Detection of enteric viruses in seeded environmental filter eluates by AbCap–RT-PCR. Seeded environmental samples consisting of beef extract eluates of 1MDS filters that were used to process 200 liters of surface water were also analyzed by the AbCap method. The surface waters filtered for these trials were collected during the summer from a shallow oligotrophic reservoir in central North Carolina. This water contains high levels of dissolved and particulate organic material, including algae, thus presenting a complex mixture of material that can interfere with the polymerase enzymes used for nucleic acid amplification. Surface water filtered through a Virosorb 1MDS filter was eluted with BE/G, seeded with 10^2 PFU of PV1 and HAV and 10^3 PCR units of Norwalk virus, PEG precipitated, and then assayed by the AbCap method. RT-PCR-amplified product was analyzed by gel electrophoresis followed by a Southern transfer and oligoprobe detection. As shown by the positive, confirmed detection of these viruses in Fig. 5, the AbCap–RT-PCR method can successfully remove inhibitors from large volumes of surface waters and detect different enteric viruses in the same sample.

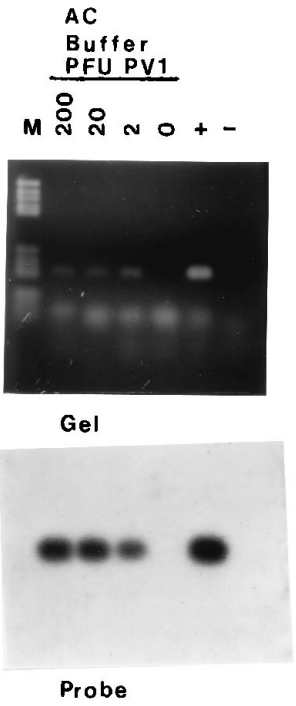

FIG. 2. Detection by the AbCap (AC) method of PV1 seeded into 0.1× PCR buffer. Lanes: M, markers; 200, 200 PFU of PV1 in 1 ml of buffer; 20, 20 PFU of PV1 in 1 ml of buffer; 2, 2 PFU of PV1 in 1 ml of buffer; 0, negative sample control; +, RT-PCR PV1-positive control; −, complete RT-PCR cocktail without virus.

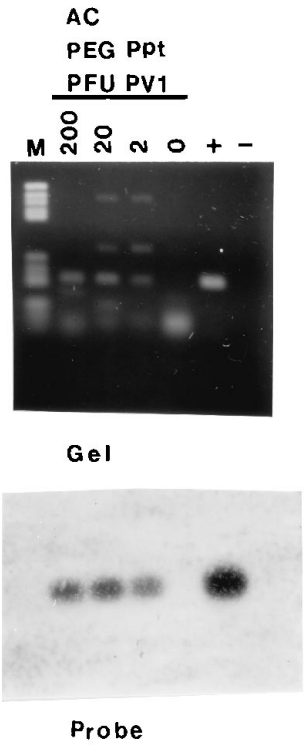

FIG. 3. Detection by the AbCap (AC) method of PV1 seeded into PEG precipitate. Lanes as in Fig. 2 with PEG precipitate instead of buffer.

Field samples. Eleven samples of surface water obtained from sites potentially affected by human fecal contamination were filtered through Virosorb 1MDS cartridge filters (Table 2). The 1MDS filters were eluted with BE/G, and the eluates were adjusted to pH 7.2 and then PEG precipitated. Viruses in the resuspended PEG precipitate were further purified by ProCipitate treatment and then further concentrated and purified by a second PEG precipitation. The second PEG pellet was resuspended with 0.5 ml of PBS and divided into aliquots that were either analyzed directly for viruses or additionally processed by one or more steps of further virus concentration and purification before being subjected to viral analyses. The equivalent volumes of initial surface water assayed when 100-μl volumes of second PEG precipitate were analyzed are shown in Table 2.

RNA extraction of 10 μl of the second PEG precipitate. Experiments in our laboratory have determined that 10 μl of a resuspended second PEG precipitate from BE/G filter eluate is the maximum volume that guanidinium extraction can successfully purify for virus detection by RT-PCR–OP (data not shown). Therefore, 10-μl volumes of the second PEG precip-

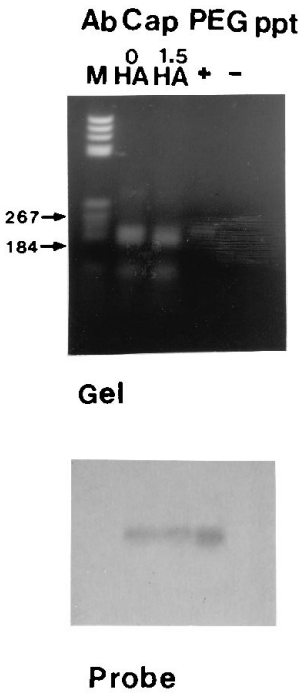

FIG. 4. Detection by the AbCap method of PV1 seeded into PEG plus humic acid precipitate. Lanes: M, markers; 0HA, 1 liter of BE/G, without humic acid, PEG precipitated, seeded with PV1, and analyzed by Ab/Cap; 1.5HA, as for 0HA but with 1.5 mg of humic acid; +, RT-PCR PV1-positive control; −, complete RT-PCR cocktail without virus.

itate for each sample were purified with the Ambion ToTally RNA extraction kit and analyzed by RT-PCR–OP. As shown in Fig. 6, 4 of 11 samples were positive for enteroviruses. The positive 10-μl samples represent initial water volumes of 6.1 to 9.2 liters.

Antibody capture of 100 μl of the second PEG precipitate. Volumes (100 μl) of resuspended second PEG precipitates for each sample were further concentrated and purified by the AbCap method. For enterovirus detection, viral RNA was extracted from the final AbCap sample concentrate by either heat release or guanidinium extraction and analyzed by RT-PCR–OP. As shown in Fig. 7, 8 of 11 samples were positive by the AbCap–heat release method, and as shown in Fig. 8, 7 of 11 samples were positive by the AbCap/guanidinium extraction method. When samples processed by AbCap and heat release of viral RNA were analyzed by RT-PCR–OP for HAV, 1 of the 11 samples was positive (sample number 7; Santa Cruz River, Rio Rico, Ariz.), as shown in Fig. 9.

BGMK cell culture infectivity of 100 μl of the second PEG precipitate. Volumes (100 μl) of resuspended second PEG precipitate for each sample were subjected to quantal assay for viral CPE by the cell culture methods described above. As shown in Table 2, 4 of 11 samples were positive for CPE on BGMK cells. The infectivity titers of the sample concentrates were 1.1 MPN units per 100 μl of the second PEG concentrate for samples 3 and 5 and 4.7 MPN units per 100 μl of the second PEG concentrate for samples 8 and 9. On the basis of the original water samples, the infectivity titers are 1.5, 1.8, 19, and 12 MPN units per 100 liters for samples 3, 5, 8, and 9, respectively.

Analyses of water samples for indicator bacteria and coliphages. The results of indicator bacteria and coliphage analyses of the above-mentioned 11 water samples are summarized in Table 3. Water samples from all sites were positive for all of the indicator bacteria, with concentrations ranging from about 10^2 to 10^4/100 ml. The two samples that were negative for enteroviruses by the AbCap and other processing methods contained small numbers of bacteria (28 to 230 CFU/100 ml). However, the levels of indicator bacteria in the samples that were positive for enteroviruses by one or more processing methods were highly variable, ranging from the lowest to the highest levels detected. Hence, there was no clear relationship between the levels of indicator bacteria in the samples and the positivity of the samples for enteroviruses.

The grab samples of water from all 11 sites were also positive for all of the coliphage indicators tested, at concentrations ranging from 3 to over 800/100 ml. As with the bacterial analyses, the two samples that were negative for enteroviruses when processed and analyzed by any method contained small numbers of coliphages (7 to 51 PFU/100 ml). However, the coliphage levels of the samples that were positive for enteroviruses by one or more processing methods were highly variable, ranging from the lowest to the highest levels detected. Hence, as for the bacterial indicators, there was no clear relationship between the levels of coliphage indicators in the samples and the positivity of the samples for enteroviruses.

Statistical comparison of virus detection by different processing and analysis methods. Statistical comparisons of the three sample concentration and purification methods for the molecular detection of enteric viruses (guanidinium extraction, AbCap-RNA extraction, and AbCap-heat release) revealed no significant difference between the methods (two-tailed Fisher exact test). Statistical comparison of virus detection by all of

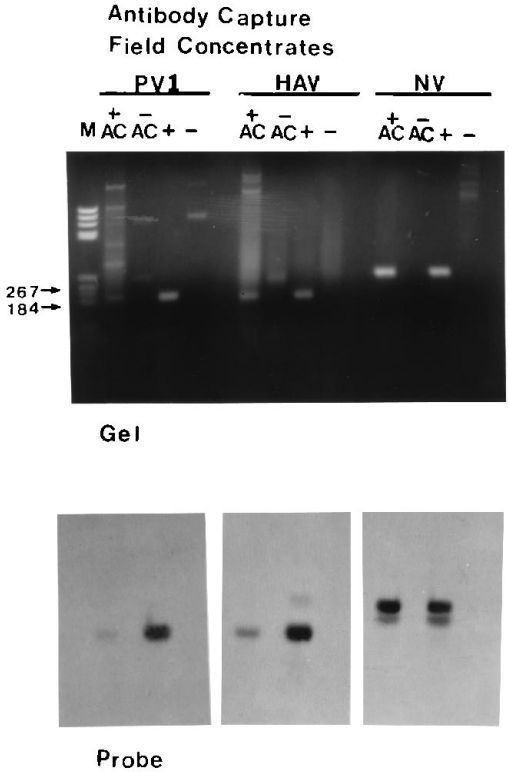

FIG. 5. Virus detection by the AbCap method in seeded filter eluate of environmental water. Lanes: M, markers; NV, Norwalk virus; +AC, seeded environmental PEG precipitated eluate detection by AbCap; −AC, negative AbCap sample control; +, RT-PCR PV1, HAV, or NV positive control; −, complete RT-PCR cocktail without virus.

the nucleic acid methods combined (9 of 11 positives) with cell culture infectivity (4 of 11 positives) also revealed no significant difference between the two different classes of virus detection techniques ($P = 0.08$, two-tailed Fisher exact test). It should be noted that the sample volumes subjected to the two methods of analysis were not the same (100 μl for cell culture infectivity and a total of 310 μl for RT-PCR–OP). Therefore, definitive comparisons of virus recovery and detection by the different methods would require the analysis of equal sample volumes as well as more field samples.

DISCUSSION

Current research is focusing on the use of molecular techniques targeting nucleic acids to detect enteric viruses in environmental samples (3, 16, 30, 32). There are several reports of application of the antigen capture techniques, developed to detect HAV in clinical specimens by Jansen et al. (12), to the detection of HAV in seeded environmental samples (7, 9). These methods are effective, but they have been limited to small sample volumes and they use monoclonal HAV antibodies, which isolate only this virus.

The use of a goat anti-human IgG antibody on paramagnetic beads to capture pooled human immunoglobulins (HSIG) provided an effective method to capture and concentrate different enteric viruses in samples and separate them from interfering substances for subsequent RT-PCR detection. Because the captured viruses are associated with a solid surface (the magnetic beads), which can be held in place by a magnetic field, the surrounding supernatant matrix of inhibitory and interfering materials can be removed and the solid-phase virus-antibody-bead complex can be repeatedly washed without virus loss. This technique effectively reduces potential inhibitory substances originating from the reagents and from the environmental samples. The GAMMAR HSIG that is used for virus capture consists primarily of IgG. It effectively binds to the anti-human IgG-coated magnetic beads and provides antibodies to many of the human enteric viruses, including many viruses undetectable or difficult to detect by cell culture techniques (e.g., HAV and the Norwalk group of viruses).

AbCap provides an approach for the detection of intact and hence potentially infectious viruses because it captures only those virus particles with functional antigenic epitopes on the virus surface. This reduces the potential of detecting free viral RNA or RNA associated with uncapturable, damaged virions. The results summarized in Table 1 indicate that direct RT-PCR (after heat release of virion RNA) may result in detection of RNA that is not associated with intact virus particles. Purified viral RNA contains no capsid proteins and hence no antigenic epitopes, thereby precluding the formation of antigenic complexes with the antibody-coated magnetic beads. High levels of viral RNA free of virions must be present before a positive signal can be detected by AbCap–RT-PCR, as indicated by the 4 $\log_{10}$ unit difference in detectability compared with heat release–RT-PCR (Table 1). Hence, free viral RNA present in a sample is not likely to be detected by the AbCap method. Viruses exposed to heat inactivation show a 3 $\log_{10}$ unit difference between AbCap–RT-PCR and heat release–RT-PCR. The results suggest that heat inactivation causes RNA loss from capsids or alters antibody capturable epitopes located on the capsid surface. The reduced sensitivity of the AbCap method for detection of heat-inactivated virions reduces the potential of isolating and detecting the RNA of noninfectious virus particles. The 2 $\log_{10}$ unit difference between heat release–RT-PCR and AbCap–RT-PCR observed in untreated virus stock could be due to the presence in the virus stock of viral RNA that was not associated with antibody-capturable virus particles. Other studies in our laboratory have determined that for cell culture-adapted strains of enteroviruses, HAV, and rotaviruses, direct RT-PCR after heat treatment is 1 to 2 $\log_{10}$ units more sensitive than is virus infectivity with an end-point detection of 0.1 to 0.01 infectious units. This RT-PCR end-point titer could be detecting viral RNA that is not associated with intact and potentially infectious virus particles. It could also be due to a reduced efficiency of viral RNA recovery and RT-PCR detection by the AbCap method. In other experiments, however, AbCap–RT-PCR has detected between 0.1 and 1 PFU of PV (data not shown). The results of this study indicate that the use of an AbCap method with broadly reactive polyclonal antisera makes it possible to isolate

TABLE 2. Field sample data and enterovirus detection by different sample processing and virus assay methods

Site no.	State	Location of site	Filtered vol (liters)	2nd PEG vol (μl)	vol of RW per 100 μl of 2nd PEG[a] (liters)	Guanid. extraction[b]	AbCap RNA extraction[c]	AbCap–heat[d]	No. of cell cultures positive/total no.[e]
1	N.C.	Haw R.[f] near Pittsboro	341	580	58.8	–[g]	–	–	0/5
2	N.C.	Morgan Ck.[f] near Chapel Hill	378	650	58.1	–	+	–	0/5
3	N.C.	Morgan Ck. near Chapel Hill	416	560	74.3	+	–	+	1/5
4	N.C.	Meeting of the Waters Ck. near UNC[h]	454	660	68.8	+	+	+	0/5
5	N.C.	Meeting of the Waters Ck. near UNC	378	620	61.0	+	–	+	1/5
6	Ariz.	Santa Cruz R. near Nogales	583	630	92.5	+	+	+	0/5
7	Ariz.	Santa Cruz R. near Rio Rico	458	670	68.4	–	+	+	0/5
8	Ill.	Des Planes R. near Chicago	151	610	24.8	–	+	+	3/5
9	Ill.	San Ship Canal near Chicago	227	590	38.5	–	+	+	3/5
10	N.C.	French Broad R. near Ashville	284	630	45.1	–	+	+	0/5
11	N.C.	Mud Ck. near Hendersonville	303	570	53.2	–	–	–	0/5

[a] Calculated volume of raw water (RW) examined when 100 μl of a second PEG precipitate was further processed and assayed for enteric viruses.
[b] Guanidinium (Guanid.) extraction of 10 μl of the second PEG precipitate followed by RT-PCR–OP detection of enteric viruses.
[c] AbCap RNA extraction of 100 μl of the second PEG precipitate followed by RT-PCR–OP detection of enteric viruses.
[d] AbCap–heat release of 100 μl of the second PEG precipitate followed by RT-PCR–OP detection of enteric viruses.
[e] BGMK cell culture; number of flasks positive for CPE out of five flasks, testing a total of 100 μl of the second PEG precipitate.
[f] R., river; Ck., creek.
[g] –, negative for enteroviruses; +, positive for enteroviruses.
[h] UNC, University of North Carolina.

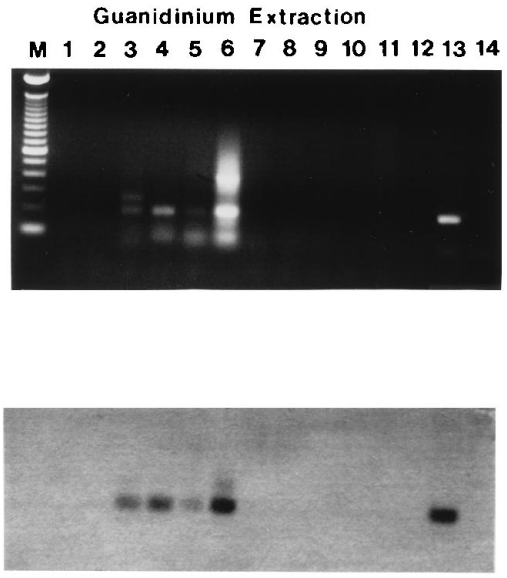

FIG. 6. Guanidinium extraction of 10 μl of the second PEG concentrate for RT-PCR–OP detection with enterovirus primers and probe. Lanes: M, 100-bp molecular size marker; 1 to 11, samples from sites 1 to 11, respectively (see Table 2); 12, negative sample control; 13, positive RT-PCR control; 14, negative RT-PCR control.

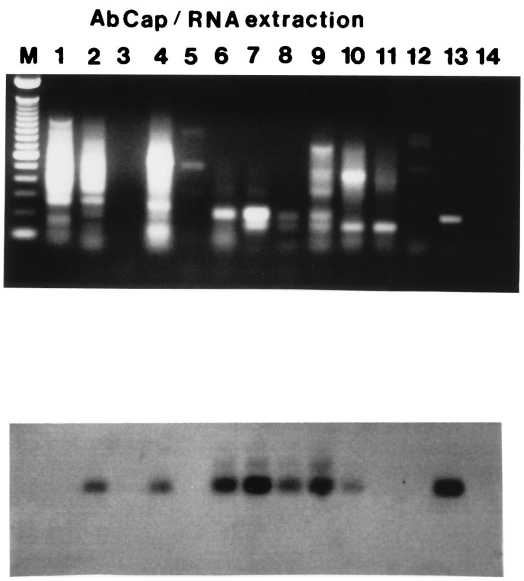

FIG. 8. AbCap RNA extraction of 100 μl of the second PEG concentrate for RT-PCR detection with enterovirus primers and probe. Lanes as in Fig. 6.

a number of different human enteric viruses simultaneously for subsequent detection by RT-PCR and nucleic acid hybridization. By using the developed AbCap method with subsequent heat release or guanidinium extraction of viral RNA, 9 of 11 samples were positive for enteroviruses in this study.

Guanidinium RNA extraction is effective for purifying viral RNA in environmental samples for subsequent RT-PCR–OP detection of enteric viruses (23). Using this technique, 4 of the 11 surface water samples were positive for enteroviruses in this study. However, the method is limited by the small environmental concentrate sample volume (10 μl) that can be successfully examined per RNA extraction, because larger sample volumes give inadequate RNA purification from RT-PCR inhibitors. Perhaps larger sample volumes could be processed by using larger volumes of RNA extraction reagents, but these increased volumes would be unwieldy and would preclude facile processing of the samples. However, for surface waters containing moderate levels of viruses (the equivalent of about 1 infectious unit per 10 liters), the use of RNA extraction of a second PEG resuspension from beef extract filter eluate provides a simple and rapid method of virus detection by RT-PCR–OP.

When aliquots of second PEG concentrates of beef extract filter eluates from field water samples were assayed for CPE by serial passages in BGMK cells, 4 of 11 samples were positive for culturable enteric viruses suspected to be enteroviruses. Because each field sample was divided into several aliquots for enteric virus detection by several methods, only a small portion

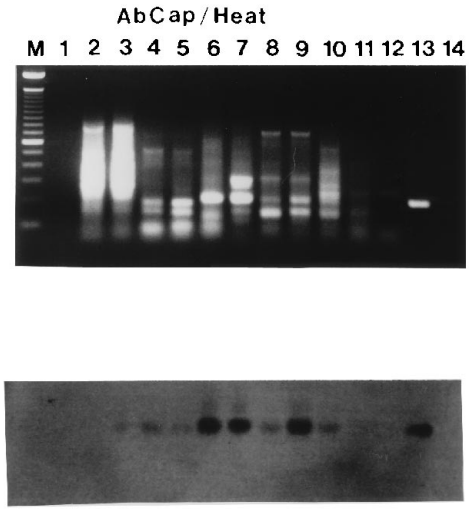

FIG. 7. AbCap-heat release of 100 μl of the second PEG concentrate for RT-PCR–OP detection with enterovirus primers and probe. Lanes as in Fig. 6.

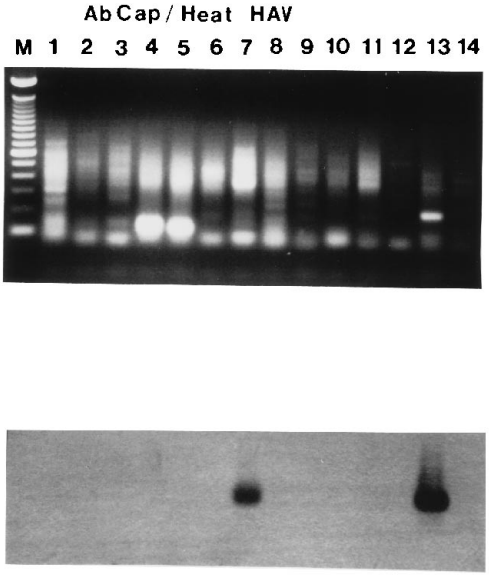

FIG. 9. AbCap-heat release of 100 μl of the second PEG concentrate for RT-PCR–OP detection with HAV primers and probe. Lanes as in Fig. 6.

TABLE 3. Indicator bacteria and coliphages in field water

Site[a]	Presence (no./100 ml) of[b]:						
	FC	EC	Enter	Cperf	C	Famp	WG49
1	230	200	28	47	51	7	14
2	300	100	200	100	14	8	53
3	300	100	200	100	14	8	53
4	45,000	29,000	3,800	900	228	120	40
5	45,000	29,000	3,800	900	228	120	40
6	120	120	100	710	33	4	5
7	80	60	20	740	41	15	27
8	13,000	6,000	3,100	550	564	203	86
9	20,000	15,000	3,900	1,600	808	250	194
10	195	80	10	250	24	8	9
11	130	80	50	140	18	4	3

[a] Site numbers are as in Table 2.
[b] FC, fecal coliforms; EC, *E. coli*; Enter, enterococci; Cperf, *Clostridium perfringens*; C, coliphages on somatic host strain *E. coli* C; Famp, coliphages on male-specific host strain *E. coli* Famp; WG49, coliphages on male-specific host strain *Salmonella typhimurium* WG49.

(100 μl) of the second PEG suspension (the equivalent of about 25 to 92 liters of water) was assayed by cell culture infectivity or further processed by separate methods for detection by RT-PCR–OP. The number of positive samples found by cell culture infectivity was less than that found by RT-PCR–OP on AbCap samples (100 μl) and the same as for guanidinium extracted samples (10 μl). However, more field samples would have to be analyzed to determine if there is significantly better virus recovery and detection by AbCap and RT-PCR–OP.

There is a great need for information on the presence of enteric viruses in surface waters that may be used as drinking-water sources and for other beneficial purposes. Studies have attempted to quantitatively assess the risks for enteric virus levels in drinking water (17, 19, 20). These quantitative risk assessments for enteric viruses have been limited by the lack of reliable data on the presence of viruses in drinking water and its sources. To verify an acceptable risk of 1 in 10,000, it has been calculated that 100 liters of raw water must be analyzed for enteric viruses (20). By using the methods described in this study, a variety of enteric viruses in water samples can be sufficiently concentrated and purified to enable 100 liters of surface water to be assayed by AbCap and RT-PCR–OP when processing only 100 to 300 μl of a concentrate from the successive steps of PEG precipitation, Pro-Cipitate precipitation, and a second PEG precipitation of beef extract eluate from conventional electropositive adsorbent filters.

The presence of bacterial and coliphage indicator organisms at each sampling site indicated that all of the field surface waters in this study were potentially affected by human fecal waste. Waters that contained larger numbers of indicators were positive for enteric viruses by AbCap. However, the indicator bacteria and coliphage levels of the samples that were positive for enteroviruses by one or more processing methods were highly variable, ranging from the lowest to the highest levels detected. More field samples would have to be analyzed to ascertain the existence of a clear relationship between the levels of indicators in the samples and their positivity for enteric viruses.

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