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Detection of Enteroviruses in Groundwater with the Polymerase Chain Reaction

MORTEZA ABBASZADEGAN,[1]† * MARY S. HUBER,[2] CHARLES P. GERBA,[1,2] AND IAN L. PEPPER[1,2]

Department of Soil and Water Science[1] and Department of Microbiology and Immunology,[2] University of Arizona, Tucson, Arizona 85721

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Standard methods for the detection of enteroviruses in environmental samples involve the use of cell culture, which is expensive and time-consuming. The polymerase chain reaction (PCR) is an attractive method for the detection of enteroviruses in water because primary cell culture is not needed and the increased sensitivity of PCR allows detection of the low numbers of target DNAs and RNAs usually found in environmental samples. However, environmental samples often contain substances that inhibit PCR amplification of target DNA and RNA. Procedures that remove substances that interfere with the amplification process need to be developed if PCR is to be successfully applied to environmental samples. An RNA-PCR assay for the detection of enteroviruses in water was developed and used to test a variety of groundwater concentrates and humic acid solutions seeded with poliovirus type 1. The groundwater samples and humic acid solutions were treated with Sephadex G-50, Sephadex G-100, Sephadex G-200, Chelex-100 resin, and a mixed bed resin to remove PCR-inhibitory material from the samples. Sephadex G-100 in combination with Chelex-100 was found to be very effective in removing inhibitory factors for the detection of enteroviruses in groundwater concentrates by PCR. Viruses were detected in two of the groundwater concentrates by the RNA-PCR assay after treatment with Sephadex G-100 plus Chelex-100. This was confirmed by tissue culture, suggesting that the treatment protocol and, subsequently, the RNA-PCR assay are applicable for the detection of enteroviruses in environmental samples.

The enteroviruses (poliovirus, coxsackievirus types A and B, echovirus) can cause a variety of illnesses ranging from gastroenteritis to myocarditis and aseptic meningitis (16). Numerous studies have documented the presence of enteroviruses in raw and treated drinking water (13–15), wastewater (19, 31), and sludge (9). Enteroviruses in the environment pose a public health risk because these viruses can be transmitted via the fecal-oral route through contaminated water (3), and low numbers are able to initiate an infection in humans (32).

The standard method for the detection of enteroviruses in environmental samples involves cell culture, which is expensive and time-consuming (1, 2). An alternative method for the detection of enteroviruses in environmental samples is the polymerase chain reaction (PCR), which is the in vitro enzymatic amplification of target nucleic acids directed by a specific pair of oligonucleotide primers (24). By using repeated cycles of PCR, a 10^6-fold amplification of a single copy of target DNA can be completed within a few hours. The decreased time and cost and the increased sensitivity of PCR facilitate the detection of the low numbers of target DNAs and RNAs usually found in environmental samples.

PCR assays have been applied to the detection of enteroviruses in clinical (10, 23) and environmental (4, 20) samples. PCR assays must be able to detect viruses after concentration from large volumes (100 to 1,000 liters) of water (1). This is usually accomplished by a filter-adsorption elution method, resulting in a concentrate containing viruses as well as organic and dissolved solids. These other compounds, once concentrated, can interfere with the activity of the enzymes used in PCR.

Natural waters contain a wide variety of organic compounds that result from biosynthetic and biodegradative processes in the environment as well as organic compounds from material disposed in water (27). The majority of organic matter in water consists of humic substances which are divided into three groups on the basis of their solubilities in alkaline and acid solutions. Humic acid is stable in alkaline solution but precipitates in an acid solution. Fulvic acid is the humic acid that stays in the aqueous acidified solution. Humin is the fraction that cannot be extracted by either an acid or a base. Humic acid has a higher molecular weight, less oxygen, and more carbon side chains than fulvic acid (18).

Several studies have shown that they are dissolved and suspended matter, including humic substances, present in natural waters, which can interfere with the recovery of poliovirus from water with microporous membranes (5, 6, 26). It has also been suggested that the presence of humic substances in samples inhibits PCR (30).

The purpose of the study described here was to develop a PCR assay for the detection of enteroviruses and to use the PCR assay to evaluate the effectiveness of Sephadex G-50, Sephadex G-100, Sephadex G-200, Chelex-100 resin, a combinations of Sephadex and Chelex-100, and a mixed bed resin in removing inhibitory substances from groundwater concentrates and humic acid solutions and allowing the detection of enteroviruses in these samples.

MATERIALS AND METHODS

Viruses and cells. Poliovirus type 1 (LSc strain) was obtained from Charles P. Gerba at the University of Ari-

* Corresponding author.
† Present address: Quality Control and Research Laboratory, American Water Works Service Company, Inc., 1115 South Illinois Street, Belleville, IL 62220.

zona, Tucson. It was propagated in Buffalo Green Monkey kidney (BGM) cells, and virus infectivity was determined by a plaque assay (15). Other enteric viruses were obtained from the American Type Tissue Collection (Bethesda, Md.). Norwalk virus was obtained from University Medical Center, Tucson, Ariz.

Water sample collection. Groundwater samples were collected from eight different wells near Tucson, Ariz. Samples of 378 to 567 liters were collected by using an electropositive MK cartridge filter (AMF CUNO, Meriden, Conn.) placed within a plastic filter housing and connected to a flow meter. Following sampling, the filters were placed in Ziploc plastic bags and shipped on ice to the laboratory for processing. Separate filters, filter housings, and tubing were used for each well.

Filter elution and reconcentration. The viruses that adsorbed to the filter were eluted by passing 1 liter of 1.5% beef extract V (Becton Dickinson, Cockeysville, Md.) with 0.05 M glycine (pH 9.5), under pressure, through each filter. The eluates were immediately adjusted to neutral pH with 1 N HCl. The pH of the eluate must be adjusted immediately after elution to prevent virus inactivation because of the high pH of the eluent. The 1-liter volumes of eluate were reconcentrated by organic flocculation and were resuspended in 20 to 30 ml of buffer (12). The pH of the final sample was adjusted to 7.2. Bacteria were removed from the final samples by centrifugation at 15,000 × *g* and treatment with kanamycin, gentamicin sulfate, and penicillin G sodium (United States Biochemical Co., Cleveland, Ohio) and nystatin (Sigma) antibiotics, each at a final concentration of 100 U/ml.

Cell culture assay. The standard cell line used to assay environmental samples for enteroviruses is the BGM cell line. BGM cells were grown to confluent monolayers in 75-cm^2 plastic flasks. Before exposure to the sample, the growth medium was poured off and the cell monolayer was washed twice with Tris (Sigma Chemical Co., St. Louis, Mo.)-buffered saline solution (14). To prevent toxicity, growth medium with 8% fetal bovine serum was added to the flasks prior to inoculation. For each sample, a 3-ml volume of the final concentrate was inoculated into each of three flasks. The flasks were incubated at 37°C for 60 min and were rotated every 15 min to allow virus adsorption to the cells. Twenty milliliters of maintenance medium with 2% fetal bovine serum and 1 ml of gentamicin (50 μg/ml) was added to each flask. The flasks were incubated at 37°C and examined daily for 14 days for viral cytopathic effect. Any flask with a suspected viral cytopathic effect was confirmed by passage into a fresh monolayer of BGM cells and observed for a cytopathic effect. At least half of the reconcentrated sample was assayed.

Gene probe assay. The poliovirus cDNA clone PV104, consisting of base pairs 115 to 7440 inserted into the *PstI* site of the plasmid pBR322, was used for the poliovirus gene probe. The vector and cDNA insert were labeled with both [α-^{32}P]dATP and [α-^{32}P]dCTP (both with a specific activity of 3,000 Ci/mmol; New England Nuclear, Boston, Mass.) by nick translation (21). The specific activity of the probe was 8 × 10^8 cpm/μg of cDNA. The radiolabeled cDNA was separated from free nucleotides by chromatography through a Sephadex G-50 column (Pharmacia, Piscataway, N.J.) and was denatured by heating for 10 min in a boiling water bath.

Fivefold dilutions of each Freon-extracted water sample were made (0.2 ml of sample to 0.8 ml in distilled water). One milliliter of the original sample was thus diluted to 1:15,625. Proteinase K (Sigma) was added to the original sample and to each dilution at a final concentration of 150 μg/ml in the original sample and 100 μg/ml in each dilution. Each tube was incubated for 30 min at 65°C in a water bath. After incubation, the samples were placed on ice and then centrifuged in a Microspin 24S centrifuge (Sorval Instruments, Boston, Mass.). The original sample was centrifuged for 2 min at 12,250 × *g* in the centrifuge (Microspin 24S), while the dilutions were centrifuged for 1 min, and then both were spotted under vacuum onto a nylon membrane (Gene Screen Plus, DuPont, Boston, Mass.) by using a dot blot apparatus (MilliBlot-D; Millipore). The membrane was air dried and baked in an oven (Napco, Portland, Oreg.) for 2 h at 80°C.

Each membrane was placed in a Seal-a-Meal bag (Dazey Corp., Industrial Park, Kans.) for prehybridization, which was done at 42°C with constant agitation for 4 h. Prehybridization was done in a solution of 50% deionized formamide–1% sodium dodecyl sulfate (SDS)–5% dextran sulfate–5× SSPE buffer (0.75 M NaCl, 0.05 M NaH_2PO_4, 5 mM EDTA–45 μg of sheared, denatured salmon sperm DNA per ml (all from Sigma), with a final pH of 7.4. The hybridization solution was the same as the prehybridization solution, except that the concentration of deionized formamide was reduced to 45% and the salmon sperm DNA concentration was lowered to 2 μg/ml. Between 10 and 20 ng of ^{32}P-labeled probe was added to the sealable bag and was hybridized for 36 h at 42°C (28).

After hybridization, the membranes were washed in 200-ml volumes of buffers as follows: 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min with constant agitation, 2× SSC–1.0% SDS at 50°C for 30 min with constant agitation, and 0.1× SSC at room temperature for 30 min with constant agitation (recommended washing procedure; Gene Screen Plus; Dupont). The membranes were air dried and put on Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a Lightning-Plus (DuPont; Wilmington, Del.) intensifying screen for an exposure period of 48 h.

Controls for gene-specific probe. Dilutions of the poliovirus cDNA fragment within the vector (10, 1, and 0.1 ng) were used as a positive control on each membrane. High-pressure liquid chromatography-grade water (1 ml, 100 μl, and 10 μl) was used as the negative control. A blank sample was processed and was used as a negative control. A 1-ml volume of all solutions used during the processing of the samples (distilled water, Tris buffer, 1.5% beef extract, 0.15 M Na_2HPO_4, Freon) was tested for possible nonspecific binding of the cDNA probes.

Primers for enteroviruses. The selection of the primers and the probe was based on alignments of poliovirus types 1, 2 and 3 and coxsackievirus group B types 1, 3, and 4 by a multiple alignment computer program (7) and computer-assisted analysis of the genomic RNA sequences of the six enterovirus serotypes. Three 17- to 20-base regions were derived from conserved sequences in the 5′ end of the noncoding region of the enteroviruses, within a 149-base segment. These three oligomeric strands were synthesized as single-stranded DNA by using an automated synthesizer (Applied Biosystems, Foster City, Calif.). The downstream primer, base pairs 577 to 594 (5′-TGTCACCATAAGC AGCC-3′), and the internal probe, base pairs 531 to 550 (5′-CCCAAAGTAGTCGGTTCCGC-3′), were synthesized antisense to genomic viral RNA, and the upstream primer, base pairs 445 to 465 (5′-TCCGGCCCCTGAATGCGGCT-3′), was synthesized sense to genomic RNA. All the map positions refer to poliovirus type 1 (Mahoney strain).

The internal probe was 5′ end-labeled (NEN Research

Products, Boston, Mass.) with [α-^{32}P]ATP (Amersham, Arlington Heights, Ill.). The PCR products were transferred from an agarose gel to a GeneScreen Plus hybridization membrane (NEN Research Products) for Southern hybridization.

RT and enzymatic amplification. Procedures were based on single-tube reverse transcription (RT) and PCRs. The RT reaction volume was increased to 30 μl to accommodate a larger sample volume. Ten microliters of sample plus 7.5 mM $MgCl_2$, 1× PCR amplification buffer (10× buffer containing 500 mM KCl and 100 mM Tris-HCl [pH 8.3]), and deoxynucleoside triphosphates at 200 μM each were added to a 0.5-ml tube. One hundred microliters of mineral oil was added on top of the mixture to prevent any sample loss during heating. The tube was heated at 99°C for 5 min to liberate the genomic viral RNA from the viral protein coat, and then 50 U of reverse transcriptase, 20 U of RNase inhibitor, and 50 μM random hexamers (GeneAmp RNA PCR kit; Perkin-Elmer Cetus, Norwalk, Conn.) were added to the tube. Samples were placed in a DNA thermal cycler (Perkin-Elmer Cetus) for the reverse transcriptase reaction with a temperature profile of 25°C for 10 min, 42°C for 45 to 60 min, and 99°C for 5 min to completely denature the reverse transcriptase. After the RT reaction, the PCR cocktail containing 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus), 1× amplification buffer, 2 mM $MgCl_2$, primers at 0.5 μM each, and double-distilled water was prepared and added underneath the mineral oil. PCR amplification was performed by using a DNA thermal cycler (Perkin-Elmer Cetus) with a temperature profile of 94°C for 45 s, 55°C for 30 s, and 72°C for 45 s for a total of 30 cycles.

The PCR products were separated on a 1.6% agarose gel (FMC, Rockland, Maine) and visualized by ethidium bromide staining.

Sensitivity of RT and enzymatic amplification (RT-PCR). The sensitivity of the RT-PCR procedure was determined by amplifying the 149-base region of poliovirus type 1. A 10-fold dilution of the virus was prepared, and each dilution was subjected to RNA-PCR. The RNA-PCR procedure resulted in detection of 0.1 PFU of poliovirus, as determined by ethidium bromide staining.

Specificities of the enterovirus primers. The specificities of the primers within the enteroviruses and other enteric viruses were examined. We screened the following viruses: poliovirus types 1, 2, and 3; coxsackievirus group A type 1 and group B types 1, 2, 3, 5, and 6; echovirus types 7, 17, 19, 21, 23, 24, 27, and 29; bovine enterovirus type 1; porcine enterovirus; calicivirus (feline picornavirus); human rotavirus (WA strain, tissue culture adapted; ATCC 2018-VR); rotavirus SA-11; Norwalk virus; adenovirus 41; and hepatitis A virus (HM 175). The viruses were purified or treated with Sephadex G-200, and the concentration was adjusted to a minimum of 10^3 PFU per reaction before RNA-PCR amplification. All entroviruses tested were detected by the appearance of the expected 149-bp fragment upon electrophoretic analysis. In some instances, a double PCR was performed to ensure the detection of minimally amplified PCR product. However, the double PCR did not change any of the results obtained during the first attempt.

Treatment of water samples for removal of inhibitory substances. Eight methods were tested to determine the most efficient procedure for removing inhibitory substances from groundwater samples prior to PCR. We purified the samples by chromatography through Sephadex G-50, Sephadex G-100, or Sephadex G-200 (Pharmacia) spun columns, Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.), which is a chelating ion-exchange resin, or a mixed bed resin (Bio-Rad), which is an analytical-grade exchange resin. In addition, we combined Sephadex with Chelex-100 in a spun column procedure. First, the bottom of a 1-ml disposable syringe was plugged with a small amount of sterile silane-treated glass wool (Supelco, Bellefonte, Pa.). The syringe was filled with 0.4 ml of Chelex-100, and then 0.4 ml of Sephadex G-100 or Sephadex G-200 was added on top of the Chelex-100. The syringe was inserted into a 15-ml disposable polypropylene graduated conical tube (Becton Dickinson, Lincoln Park, N.J.) and was centrifuged at 1,600 × *g* for 3 min at room temperature in a swinging-bucket rotor in a bench-top centrifuge (International Equipment Co., Needham, Mass.). Additional Sephadex was added, and the column was recentrifuged until the volume of the packed column was 0.8 to 0.9 ml. A 300-μl sample was layered on top of the packed column, and the column was centrifuged at 1,600 × *g* for 4 min. The sample was recovered in a 1.5-ml microcentrifuge tube without a cap; the microcentrifuge tube was placed in the bottom of the 15-ml tube.

For the Chelex-100 and mixed bed resin treatments, a 1-ml sample was mixed with 200 μl of Chelex-100 or the resin, vortexed for 5 min, and centrifuged for 1 min in a microcentrifuge. The supernatant was then removed and assayed by PCR.

To determine the maximum concentration of organic matter with inhibitory effects on PCR, 10^3 PFU of poliovirus type 1 (LSc strain) was added to distilled water with humic acid (Sigma) at concentrations ranging from 1 mg/ml to 10 ng/ml. Each concentration of humic acid was treated as described earlier, before the poliovirus was added and RNA-PCR was performed.

RESULTS

Specificities of the primers. To evaluate the specificities of the enterovirus primers, a group of enteroviruses, as well as other enteric viruses, were subjected to PCR by using primers flanking a sequence in the conserved 5′-noncoding region of poliovirus specific to the enteroviruses. Amplification produced a 149-bp DNA fragment for all strains of human enteroviruses tested except for echoviruses (Table 1). The 149-bp fragment in the other group of viruses was not amplified. However, in some of the nonenterovirus group as well as in five of the echoviruses, nonspecific amplification was observed by ethidium bromide staining. The nonspecific amplification was minimized by increasing the annealing temperature to 55°C during PCR amplification.

Sensitivities of the primers. To determine the sensitivities of the primers for the detection of enteroviruses, PCR was performed on poliovirus type 1 serially diluted in distilled water (Table 2 and Fig. 1) as well as in a groundwater concentrate sample (Table 3). Both tests are necessary to determine the applicability of the system under optimal conditions as well as in an actual environmental sample. Similar sensitivity limits (0.1 PFU) were achieved in seeded distilled water or groundwater concentrate after treatment with Sephadex G-200 or Chelex-100. Poliovirus type 1 was repeatedly detected in a 10-fold higher diluted sample by the PCR assay but not by tissue culture method. This suggests that the sensitivity of the PCR assay is 1 order of magnitude higher than that of the tissue culture technique (data not shown). All PCR analyses were run at least twice.

Detection of enteroviruses. The enteroviruses have single-stranded RNA genomes, so reverse transcription of viral RNA to cDNA is required before PCR can be performed.

TABLE 1. Specificities of the primers for detection of enteroviruses

Virus[a]	Enterovirus-specific amplification	Southern hybridization
Poliovirus 1	+	+
Poliovirus 2	+	+
Poliovirus 3	+	+
Coxsackie A1	+	+
Coxsackie B1	+	+
Coxsackie B2	+	+
Coxsackie B3	+	+
Coxsackie B5	+	+
Coxsackie B6	+	+
Echovirus 7	+	+
Echovirus 17	+	+
Echovirus 19	+	+
Echovirus 21	+	+
Echovirus 23	−	−
Echovirus 24	+	+
Echovirus 27	+	+
Echovirus 29	+	+
Bovine enterovirus 1	−	−
Porcine enterovirus	−	−
Calicivirus[b]	−	−
Human rotavirus[c]	−	−
Rotavirus SA-11	−	−
Norwalk virus	−	−
Adenovirus 41	−	−
Hepatitis A virus (HM 175)	−	−

[a] Virus concentrations were approximately 10^3 per reaction. The viruses were purified or treated with Sephadex G-200 or Sephadex G-100 plus Chelex-100.
[b] Feline picornavirus.
[c] Tissue culture adapted (ATCC 2018-VR).

Initially, we used an RNA-PCR kit (Perkin-Elmer Cetus) and the recommended 15-min incubation period for the RT reaction. Even though the reaction worked in the majority of cases, in many instances amplification did not occur or the fragment could not be visualized by ethidium bromide staining until a second PCR was performed on the products of the first PCR. This phenomenon was observed in groundwater concentrates and distilled water seeded with poliovirus type 1. The problem was solved by increasing the reverse transcriptase incubation time from 15 to 60 min.

Treatment of groundwater samples for PCR assay. Ten groundwater samples were collected and assayed for the presence of enteroviruses by using tissue culture and gene probe hybridization methods. Viruses were detected in two samples by tissue culture assays and in one sample by hybridization assays. To further evaluate the samples for the presence of enteroviruses, we used PCR because of its high sensitivity. The 149-bp sequence was not amplified in any of the samples. As a control, we seeded the 10 groundwater samples with poliovirus type 1 (10^3 PFU per reaction tube) and performed RNA-PCR. We observed amplification of the 149-bp fragment in only 1 of the 10 seeded samples (Table 4). We concluded that the water samples contained some material, possibly humic acid or other organic substances, which may have inhibited the reverse transcriptase or *Taq* polymerase enzymes, resulting in no PCR amplification. A general protocol was needed to remove inhibitory factors while retaining the maximum number of viruses in the water samples for PCR assay, because of the variability in composition from one environmental sample to another. Five methods were tested to determine the most efficient procedure for removal of inhibitory factors (Table 4). The best results were obtained when Sephadex G-100 in combination with Chelex-100 was used for the treatment of the seeded samples. The treatment was sufficient for successful RNA-PCR amplification for all 10 groundwater concentrates. RNA-PCR was performed on the concentrates after they were treated with Sephadex G-100 plus Chelex-100; the 149-bp sequence was amplified in two of the samples that were positive by tissue culture (Table 5).

To determine whether *Taq* polymerase or reverse transcriptase enzymes were inhibited, we seeded the water concentrates with poliovirus cDNA and performed PCR. The results of the experiment indicated that inhibition can occur during the RT or PCR step. However, in repeated experiments we observed that the reverse transcriptase is more sensitive to inhibition than the *Taq* polymerase (data not shown). This might be due to concentration of some inhibitory substances as well as the beef extract in water concentrates which may interfere with amplification. However, the treatment of groundwater concentrates with Sephadex G-100 plus Chelex-100 allowed for the successful detec-

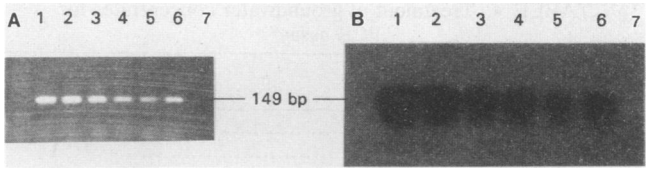

FIG. 1. (A) Ethidium bromide-stained agarose gel electrophoresis analysis of RNA-PCR amplification product of serial dilutions of poliovirus at concentrations ranging from 10^4 to 10^{-2} PFU (lanes 1 through 7) by using 30 cycles of PCR. (B) Southern hybridization analysis of the agarose gel shown in panel A.

TABLE 2. Sensitivity of PCR assay for detecting poliovirus type 1 in distilled water[a]

Treatment	Detection at viral concn (PFU) of:						
	10^4	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
Sephadex G-100	+	+	+	+	+	+	−
Sephadex G-200	+	+	+	+	+	+	−
Chelex-100	+	+	+	+	+	+	−
Mixed bed resin	+	+	+	+	+	−	−
No treatment	+	+	+	+	+	+	−

[a] Viruses were diluted in distilled water. Ten microliters of the seeded sample was used for RNA-PCR assay.

TABLE 3. Sensitivity of PCR assay for detecting poliovirus type 1 in a groundwater sample[a]

Treatment	Detection at viral concn (PFU) of:						
	10^4	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
Sephadex G-100	+	+	+	+	+	+	−
Sephadex G-200	+	+	+	+	+	+	−
Chelex-100	+	+	+	+	+	+	−
Mixed bed resin	+	+	+	+	+	−	−
No treatment	+	+	+	+	+	+	−

[a] The sample was used as a control because the amplification was observed in only 1 of the 10 groundwater concentrates without any treatment. Three hundred microliters of a groundwater concentrate was processed for each of the treatments. Viruses were diluted in the treated groundwater sample. Ten microliters of the seeded sample was used for RNA-PCR assay.

TABLE 4. Treatment of groundwater concentrates for PCR assay[a]

Treatment	PCR result (no. positive)
No treatment	1
EGTA[b]	1
Sephadex G-200	8
Sephadex G-100 + Chelex-100	10
Chelex-100	8
Mixed bed resin	5

[a] A total of 10 groundwater concentrates were evaluated. The samples were seeded with 10^3 PFU of poliovirus per reaction. Ten microliters of the seeded sample was used for RNA-PCR assay.
[b] EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

tion of enteroviruses in groundwater samples by PCR (Table 5).

To determine the effect of organic matter on PCR, we looked at amplification of poliovirus in humic acid solutions because humic acid makes up the majority of organic matter in water and can be concentrated on the filters used to collect the virus (26). Six concentrations of humic acid were separately treated eight different ways, as described earlier. Each one of the treated samples was seeded with poliovirus and then RNA-PCR was performed. The data outlined in Table 6 show that the humic acid inhibition on PCR was reduced the most by Sephadex G-100, Sephadex G-100 plus Chelex-100, and Sephadex G-200 plus Chelex-100. However, when Sephadex G-100 was combined with Chelex-100, the intensity of the band on agarose gels was greater, and in some instances, the removal of inhibitory substances was 1 log unit greater than that seen with Sephadex G-100 alone.

DISCUSSION

PCR is an attractive method for the routine monitoring of human enteroviruses in water samples because PCR is faster, simpler, and less expensive than the standard cell culture methods for the detection of the enteroviruses in water samples. Currently, the standard methods for the detection of enteroviruses in water samples involve cell culture assays, which are expensive and time-consuming (1). Results may take 2 weeks to be known. BGM cells are commonly used for the enterovirus assays, but not all human enteroviruses grow in this cell line. To be able to identify all the human enteroviruses in a sample, more than one cell line is required (25). Another problem with cell culture assays is that environmental samples may contain organic and inorganic materials that are toxic to the cells.

The primers designed for the present study were selected from the 5′-noncoding region of the poliovirus viral genome, which is a conserved region in the enteroviruses (11, 22, 29). RNA-PCR amplification of the 149-bp region was diagnostic for enteroviruses in water, because our results indicate that the primers are likely unique to human enteroviruses and the 149-bp band was produced only when human enteroviruses were present in a sample. The specificity and sensitivity of the primers selected for the present study appear to be sufficient for use in the routine monitoring of environmental samples for the presence of human enteroviruses.

The sensitivity of PCR is also 1 log unit greater than that of tissue culture (PFU), which is an important issue because of the low number of viruses usually found in water concentrates. PCR cannot be performed on most of the concentrated water samples unless some of the organic material is removed prior to the PCR. The selection of the removal treatment must be based on the applicability and high efficiency of the protocol.

We believe that for each environmental sample both a positive sample seeded with virus as well as a negative control sample need to be run simultaneously with the environmental sample to allow reasonable interpretation of the data that are obtained. Negative amplification from a sample does not necessarily mean that no human enteroviruses are present in the sample. Controls must be run through the same procedures as the samples to ensure that PCR inhibition is not occurring.

Humic substances adsorb to other organic substances, including soil particles, and are polyphenolic molecules that act as weak acids and that tend to form complexes with metal ions, such as Ca^{2+} and Mg^{2+}, causing them to precipitate (17). The enzymes used in RT and PCR are sensitive to the contaminants and inhibitory substances present in environmental samples (8), such as organic material and metal ions. Organic substances, especially humic acid, can adsorb proteins or enzymes and interfere chemically or sterically with their active sites, and they can also bind divalent cations such as Ca^{2+} and Mg^{2+}, preventing them from being used as cofactors for the enzymes involved in PCR (17). Metal ions also reduce the specificity of PCR primers, resulting in nonspecific amplification in a sample

TABLE 5. Detection of enteroviruses in groundwater concentrates

Sample	Tissue culture result[a]	Gene probe	RNA-PCR amplification			
			Not seeded or treated	Seeded but not treated[b]	Seeded and treated[c]	Treated[c]
SAWP 40	+	+	−	−	+	+
SAWP 51	+	−	−	−	+	+
SC 1	−	−	−	−	+	−
SC 2	−	−	−	−	+	−
SC 3	−	−	−	−	+	−
SC 4	−	−	−	−	+	−
SC 5	−	−	−	−	+	−
SC 6	−	−	−	−	+	−
SC 7	−	−	−	−	+	−
SC 8	−	−	−	+	+	−

[a] Samples SAWP 40 and SAWP 51 contained most probable numbers of 1.2 and 0.23 virus per ml of concentrate, respectively.
[b] Seeded with 10^3 PFU of poliovirus.
[c] Treated with Sephadex G-100 plus Chelex-100.

TABLE 6. Removal of inhibitory effects of humic acid from water samples by different treatments for RNA-PCR assay[a]

Treatment	PCR results at humic acid concn of:					
	1 mg	100 μg	10 μg	1 μg	100 pg	10 pg
Sephadex G-50	−	−	−	+	+	+
Sephadex G-100	−	−	+	+	+	+
Sephadex G-200	−	−	−	+	+	+
Chelex-100	−	−	−	+	+	+
Chelex-100, boiled	−	−	−	+	+	+
Sephadex G-100 + chelex-100	−	−	+	+	+	+
Sephadex G-200 + chelex-100	−	−	+	+	+	+
Mixed bed resin	−	−	−	+	+	+
No treatment	−	−	−	+	+	+

[a] Poliovirus type 1 (10^3 PFU per reaction) was added to distilled water with humic acid at concentrations ranging from 1 mg/ml to 10 ng/ml. Ten microliters of the seeded sample was used for the RNA-PCR assay.

(24). In order to allow PCR to be used for the detection of enteroviruses in groundwater samples, we recommend treatment of the samples with Sephadex G-100 combined with Chelex-100 to remove organic substances as well as metal ions prior to the PCR assay. With the seeded samples treated in this way, the sensitivity of the assay was not significantly reduced by resin treatment. The amplification of the 149-bp sequence of enteroviruses in two of the groundwater samples and confirmation of the results by tissue culture suggest that the treatment protocol and, subsequently, the RNA-PCR assay are applicable for the detection of enteroviruses in environmental samples.

In conclusion, the use of RNA-PCR for the detection of enteroviruses in groundwater samples is feasible, provided that the samples are first treated with Sephadex G-100 and Chelex-100 to remove any inhibitory factors from the water samples to facilitate RT-PCR analysis. The specificity and sensitivity of the primers designed for the present study were sufficient for use in the routine testing of environmental samples for enteroviruses.

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