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Reverse Transcription PCR To Detect Enteroviruses in Surface Water

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We have developed a simple, fast, and efficient procedure to detect enteroviruses in water samples. Aliquots of water are subjected to two-step filtration, with the second filter containing a positively charged nylon membrane that holds back virus particles. Viruses thus adsorbed are directly lysed, and RNA is isolated by hybridization to specific oligonucleotides bound to magnetic beads. The solution used contains guanidine thiocyanate, which lyses virus particles, inactivates enzymes, e.g., RNases, allows mild hybridization conditions, and does not influence biotin-streptavidin interaction on magnetic beads. Detection and specific identification are accomplished by reverse transcription PCR of the highly conserved noncoding region at the 5′ end of virus RNA combined with Southern hybridization. The system was tested with tap water artificially spiked with poliovirus vaccine and yielded a detection limit of 20 50% tissue culture infective doses per liter. We used the same procedure to investigate the water quality of surface water at public beaches by rivers and lakes. Of 40 samples tested, 7 were positive for enteroviruses. A comparison with enterobacterial contamination determined by PCR and classical microbiological methods in parallel showed that enteroviruses were found only in samples also positive for *Escherichia coli*. In conclusion, this procedure can easily be adapted to test large water samples and is simple enough to be used for routine determinations of water quality in terms of virus contamination.

Enteroviruses are a major cause of gastrointestinal symptoms, colds, and fever, mainly in young children (11, 17, 23). In addition, they are recognized as an important factor in acute infections especially of the central nervous system, i.e., meningitis and encephalitis (11), and in subacute and chronic infections of the cardiovascular system, i.e., pericarditis, myocarditis, and cardiomyopathy (10, 24, 30), and they can lead to postviral fatigue syndrome (9). No clear correlation between enterovirus serotypes and specific symptoms has been determined (23). Enteroviruses are found worldwide. Infections occur by the fecal-oral route, and in most cases, treated surface water acts as the carrier of pathogens. Enteroviruses are highly stable in water (12, 19) and are not completely eliminated by sewage treatment plants (2, 12). Thus, the increasing use of treated surface water for drinking purposes harbors a potential source of pathogens which cannot be screened in a satisfactory manner.

Classical methods of detection are based on inoculation of cell cultures, hatched eggs, or infant mice with test samples. Observations of cytopathic effect, hybridization with specific oligonucleotides, or reactions with antibodies indicate the presence of enteroviruses. These procedures are very laborious and time-consuming, though, and are not suitable for fast routine diagnosis. Besides, some serotypes either cannot be cultured or show no cell lysis. The use of DNA technologies has significantly improved the procedure to detect viruses. Reverse transcription followed by PCR (RT-PCR) can reduce the time for analysis to 2 days. The isolation and processing of samples for the detection reaction still require a great deal of time or rely on costly equipment (1, 15, 20). Other methods did not yield satisfactory results (16, 27). We aimed to develop a simple and efficient isolation and concentration procedure for enteroviruses in water to be followed by a detection reaction based on RT-PCR.

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MATERIALS AND METHODS

Virus samples. Vaccine "Poloral Berna," which contained 2×10^6 50% tissue culture infective doses ($TCID_{50}$) of poliovirus Sabin type 1 per ml, 2×10^5 $TCID_{50}$ of poliovirus Sabin type 2 per ml, and 6×10^5 $TCID_{50}$ of poliovirus Sabin type 3 per ml, was provided by R. Glück, Seruminstitut, Bern, Switzerland.

Bacterial strains. The strains used for determination of detection limits of bacterial PCR systems were *Escherichia coli* LMC 30 for *estA3* PCR, *E. coli* LMC 44 for *estA1* PCR, *E. coli* LMC 21 for *elt* PCR, and *Salmonella enteritidis* LMC 49 for *invA* PCR. *Listeria monocytogenes* LMC 72 was used to monitor successful isolation of bacterial pellets from water samples.

Water samples. Forty 1-liter samples of environmental surface water, collected by a state control laboratory on 9 and 16 August 1993 for routine determination of bacterial quality, were obtained to test the applicability of the developed system. Thirty-two samples were collected at public beaches by lakes, and 8 were collected from rivers near Bern, Switzerland, used for water sports. All samples were also tested for the presence of *Salmonella* spp. and *E. coli* by culture and for *Salmonella* spp. and enterotoxigenic *E. coli* by PCR.

Culture of bacteria. Classical detection of *E. coli* and *Salmonella* spp. was performed as previously described (26). For the detection of *E. coli*, 100-ml water samples were filtered through membrane filters (pore size, 0.45 μm) and filters were preincubated on tryptone soy agar at 37°C for 4 h. Filters were transferred to *E. coli* direct agar (20 g of tryptic casein peptone, 5 g of yeast extract, 1.5 g of bile salts, 5 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 5 g of NaCl, 20 g of agar per liter [pH 7.2]) and incubated at 44°C for 10 to 20 h. Then membrane filters were soaked with 1 ml of indole reagent (dimethylaminobenzaldehyde; 50 mg/ml) for 5 min and checked for pink-colored colonies under 254-nm UV light. Pink-colored colonies with rings of the same color were considered to be *E. coli* colonies and were enumerated. For classical detection of *Salmonella* spp., 1-liter water samples were filtered through membrane filters (pore size, 0.45 μm). Filters were incubated in 20 ml of Rappaport broth (4.5 g of peptone, 7.2 g of NaCl, 1.4 g of KH_2PO_4, 36 g of $MgCl_2 \cdot 6H_2O$, 36 mg of malachite green oxalate per liter) at 43°C for 24 h. *Salmonella* spp. were isolated from this enrichment broth by plating onto brillant green agar (10.0 g of peptone, 3.0 g of yeast extract, 10.0 g of lactose, 10.0 g of saccharose, 5.0 g of NaCl, 0.08 g of phenol red, 0.0125 g

TABLE 1. Oligonucleotides used for bacterial PCR systems

Microorganism	Target gene	Oligonucleotide (5′–3′ sequence)[a]
Salmonella spp.	*invA*	SA07 (TGAATATCGTACTGGCGATATTGGTGTTTA)
		SA08 (GGACAAATCCATACCATGGCGAGTCAT)
		SA09 (GAAATTATCGCCACGTTCGGGC)
		SA10 (TCATCGCACCGTCAAAGGAACC)
L. monocytogenes	*hlyA*	L01 (CGGAGGTTCCGCAAAAGATG)
		L04 (CCTCCAGAGTGATCGATGTT)
Enterotoxigenic *E. coli*		
Heat-labile toxin type I	*elt*	LT01 (TTACGGCGTTACTATCCTCTCTA)
		LT02 (CATTTCAGGTCGAAGTCCCG)
		LT03 (GGTCTCGGTCAGATATGTGATTC)
Heat-stable toxin type I	*est*	EC01 (TTITTTCTGTATTITCTTTIICIICTTTIIITCAG)
		EC02 (GCAGGATTACAACAIAITTCACAGC)
		EC05 (CATTAGAGACTAAAAAGTGTGAT)
		EC06 (GCTACTATTCATGCTTTCAGGA)

[a] I, inosine.

of brillant green, 15 g of agar per liter [pH 6.9]) and xylose lysine desoxycholate agar (3.0 g of yeast extract, 5.0 g of L-lysine hydrochloride, 3.5 g of xylose, 7.5 g of lactose, 7.5 g of saccharose, 5.0 g of NaCl, 0.08 g of phenol red, 6.8 g of $Na_2S_2O_3$, 2.5 g of sodium desoxycholate, 0.8 g of $Fe(III)NH_4$ citrate, 15 g of agar per liter [pH 7.4]). Suspect colonies were confirmed by group-specific serotyping.

Oligonucleotides for enteroviruses. Capture and primer oligonucleotides were based on those previously described by others (5, 14, 22, 31). All oligonucleotides were synthesized by ANAWA Laboratories (Wangen, Switzerland) with an automated synthesizer (Applied Biosystems, Foster City, Calif.) and stored freeze-dried at −20°C. Capture primer EV04, bases 359 to 374 (5′-GGCCGCCAACG CAGCC-biotin-3′), and downstream RT-PCR primer EV03, bases 584 to 603 (5′-ATTGTCACCATAAGCAGCCA-3′), were synthesized antisense to genomic virus RNA. Upstream PCR primer EV02, bases 449 to 468 (5′-TCCTCCGGC CCCTGAATGCG-3′), was synthesized sense to genomic virus RNA. Map positions refer to poliovirus Sabin type 2 (28).

Oligonucleotides for bacterial PCRs. Oligonucleotides were synthesized by ANAWA Laboratories with an automated synthesizer (Applied Biosystems) and stored freeze-dried at −20°C. All primers have been extensively tested in previously published (7, 8) or unpublished work done by our group. The primer sequences used for various bacterial PCR systems are listed in Table 1.

Isolation of enteroviruses. One-liter surface water samples were prefiltered through a borosilicate glass filter (AP 2504700; Millipore, Molsheim, France). A positively charged nylon filter with a pore size of 0.2 μm (Zetapore; AMF-CUNO, Meriden, Conn.) was used to adsorb viruses. Samples of tap water from our laboratory were spiked with serial dilutions of virus vaccine and used to optimize the isolation procedure and determine detection limits.

RNA extraction and hybridization. Extraction and specific isolation of enterovirus RNA was achieved by using streptavidin-linked magnetic beads (Dynabeads; Dynal, Oslo, Norway) coated with biotinylated oligonucleotide EV04 in an extraction solution with an empirically optimal guanidine thiocyanate (GuSCN) concentration (50 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2% (wt/vol) *N*-lauroylsarcosin, 2 M GuSCN). Beads for specific hybridizations were prepared as follows: 5 μl (capacity of binding 10 pmol of biotin) of Dynabeads was washed with 0.5 ml of phosphate-buffered saline (PBS) which contained 0.1% bovine serum albumin (BSA) three times on the magnetic concentrator before being used. Beads, 150 μl of PBS which contained 0.1% BSA, and 1.4 μl (28 pmol) of EV04 oligonucleotide were mixed in a 1.5-ml Eppendorf tube and incubated on a shaker at room temperature for 30 min. Excess oligonucleotide was removed by washing beads on the magnetic concentrator with low-stringency hybridization solution (Promega, Madison, Wis.) three times, and 30 μl of extraction solution was added to beads. Nylon filters with adsorbed viruses were incubated in a petri dish (diameter, 50 mm) with 1.8 ml of extraction solution at room temperature for 30 min to extract virus RNA. Part (1.2 ml) of this solution and 30 μl of extraction solution which contained oligonucleotide-labeled Dynabeads were incubated in a 1.5-ml Eppendorf tube on a shaker at 30°C for 30 min, and tubes were centrifuged at 800 × *g* for 15 s. Beads were washed with 0.5 ml of 5× RT-buffer (Gibco BRL, Life Technologies, Gaithersburg, Md.) three times on the magnetic concentrator and diluted with 5 μl of sterile deionized water.

Preparation of bacterial DNA samples. An adaption of a method previously described was used (18). Fifty milliliters of water was prefiltered through a borosilicate glass filter (AP 2504700; Millipore). Three hundred microliters of *L. monocytogenes* organisms, cultured overnight in tryptone soy broth at 37°C, was added to guarantee a visible bacterial pellet for the isolation procedure. Bacterial cells were pelleted by centrifugation at 2,500 × *g* at 4°C for 30 min. Water was discarded, and bacterial pellets were washed three times with 1 ml of PCR buffer (10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$ [pH 8.4]) and repelleted at 8,000 × *g* for 10 min after each washing step. Washed pellets were suspended in 100 μl of 2-mg/ml lysozyme (Sigma, St. Louis, Mo.) in PCR buffer and incubated at room temperature for 15 min. Proteinase K (Merck, Darmstadt, Germany) was added to 20 μg/ml and incubated at 60°C for 60 min and at 95°C for 15 min. Cell debris was removed by centrifugation at 14,500 × *g* at 4°C for 3 min, and lysates (10-μl aliquots) were subjected to PCR without further purification. All samples were tested for the presence of *Salmonella* spp., enterotoxigenic *E. coli*, and *L. monocytogenes*. Detection limits for PCR systems after sample preparation were determined as follows: serial dilutions of *E. coli* and *Salmonella* spp. in 50-ml volumes were processed as described above, and 1/10 of lysed bacteria were subjected to PCR.

RT-PCR of virus samples. Optimal conditions for RT-PCR were determined empirically. RT was performed with Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and the first-strand RT buffer supplied with the enzyme. Optimized final concentrations in a total volume of 20 μl were 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol; 0.5 mM (each) dATP, dCTP, dGTP, and TTP (Promega); 1.25 μM primer EV03; 20 U of RNasin (Promega); 100 U of Moloney murine leukemia virus reverse transcriptase; and 5 μl of magnetic bead-bound RNA suspension. The reaction mixture was incubated in a thermal cycler (Hybaid Ltd., Teddington, Middlesex, United Kingdom) at 37°C for 60 min and then at 95°C for 5 min to inactivate reverse transcriptase. Ten microliters of cDNA product was added to 90 μl of a PCR reaction mixture which contained (in a final volume of 100 μl) 10 mM Tris-HCl (pH 9.0); 50 mM KCl; 0.1% Triton X-100; 2.5 mM $MgCl_2$; 0.01% gelatin, 2 μg of BSA (Sigma) per ml; 200 μM (each) dATP, dCTP, dGTP, and TTP (Promega); 0.5 μM (each) oligonucleotides EV02 and EV03; and 2 U of *Taq* DNA polymerase (ANAWA Laboratories). Reaction tubes were covered with 80 μl of mineral oil (Sigma) and incubated in a thermal cycler (Hybaid) with the following temperature cycling program: 94°C for 1 min, 50 cycles at 94°C for 5 s, at 55°C for 30 s, and at 72°C for 40 s, and a final extension step at 72°C for 3 min. PCR products were separated on a 1.5% agarose gel (Boehringer) and visualized by ethidium bromide staining. Tenfold serial dilutions of vaccine Poloral Berna in distilled water were used to determine the sensitivity of RT-PCR. Virus particles were lysed with proteinase K (2 mg/ml) at 60°C for 30 min, and the enzyme was subsequently inactivated at 90°C for 15 min. Without further purification, the lysate was subjected to RT and amplification.

PCRs of bacterial DNA samples. Reaction conditions were optimized for each assay, and reactions took place in 100-μl reaction mixtures which contained 10 μl of lysate in 1× *Taq* DNA polymerase buffer; $MgCl_2$ as specified for each assay (see below); 0.2 mM (each) dATP, dCTP, dGTP, and TTP; 0.5 μM (each) primer; 2 μg of BSA per ml; and 2 U of *Taq* DNA polymerase. *Taq* DNA polymerase with buffer and $MgCl_2$ stock solution (PHC-1 or PHC-3) were purchased (Techne, Princeton, N.J.). Segment temperatures were as follows: 94°C, annealing temperature as specified for each assay (see below), and 72°C. Reaction times were 30, 60, and 60 s (PHC-1) or 5, 30, and 40 s (PHC-3). These parameters gave almost identical temperature profiles for both units as determined by thermocouple within separate tubes. Seminested and nested PCRs were used for in-process product identification; they consisted of 25 cycles of original PCR and then 40 cycles of a second PCR, in which one or both of the original primers were replaced by internal oligonucleotides. Two microliters of the original PCR product was used as a template.

For *L. monocytogenes hlyA* PCR, the assay has previously been described (7). The optimal magnesium concentration in the amplification reaction is 2.0 mM for primers L01 and L04. The optimal annealing temperature is 55°C.

For *Salmonella* sp. *invA* PCR, the optimal magnesium concentration in the amplification reaction is 1.5 mM for all primer combinations. Optimal annealing occurs at 65°C for outer primers SA07 and SA08 and at 60°C for internal nested oligonucleotides SA09 and SA10.

For enterotoxigenic *E. coli* heat-labile toxin type I *elt* PCR, the assay has previously been described (8). The optimal magnesium concentration in the

amplification reaction is 1.5 mM for all primer combinations. The optimal annealing temperature is 55°C. This assay detects two highly homologous *elt* alleles originally found in one human *E. coli* isolate and one animal *E. coli* isolate. Oligonucleotides LT01 and LT03 were used in the original PCR. In seminested PCR, the LT02-LT03 combination was used.

For enterotoxigenic *E. coli* heat-stable toxin type I *est* PCR, the assay has previously been described (4). The optimal magnesium concentration in the amplification reaction is 4.0 mM for all primer combinations. The optimal annealing temperature is 50°C. This assay detects three *est* alleles. *estA1*, detected in an animal isolate, is about 70% homologous to *estA2* and *estA3*, found in human *E. coli* isolates. *estA2* and *estA3* are highly homologous. The original PCR with EC01 and EC02 amplifies all three alleles. Discrimination between *estA1* and *estA2* or *estA3* is achieved by seminested PCR (EC05-EC02 and EC01-EC06, respectively).

Agarose gel electrophoresis of amplification products. Twenty microliters of PCR product was separated by gel electrophoresis through 1.5% agarose (Boehringer). DNA was stained with ethidium bromide and made visible by UV transillumination (254 nm).

Southern blot hybridization. To improve the sensitivity and specificity of enterovirus PCR, Southern blot hybridization was performed with a PCR product of vaccine Poloral Berna that was randomly prime labeled with digoxigenin-11-dUTP (Boehringer). PCR products, separated by agarose gel electrophoresis, were transferred to positively charged nylon membranes (Boehringer) according to the instructions of the manufacturer. Membranes were incubated in a prehybridization solution which contained 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% blocking reagent (Boehringer), 0.1% *N*-lauroylsarcosin sodium salt (Sigma), and 0.02% sodium dodecyl sulfate (SDS) at 42°C for 2 h. Hybridization was performed in 50% prehybridization solution–50% formamide–digoxigenin-labeled probe at a final concentration of 25 ng/ml at 42°C overnight. Membranes were washed twice with 2× SSC which contained 0.1% SDS at room temperature for 10 min and four times with 0.5× SSC–0.1% SDS at 55°C for 10 min. Color reactions were carried out with anti-digoxigenin alkaline phosphatase conjugate Fab fragments (Boehringer) according to the manufacturer's protocol. Only bands of correct length were considered to be positive.

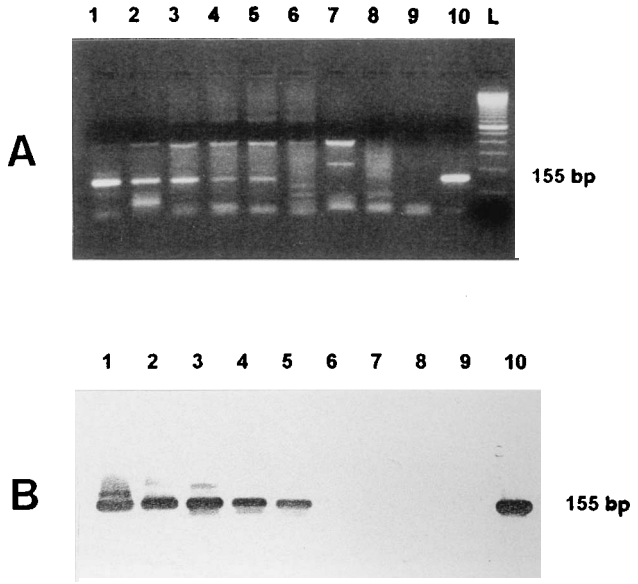

FIG. 1. Sensitivity of enterovirus-specific RT-PCR for tap water samples. Tenfold serial dilutions of vaccine Poloral Berna were added to 1 liter of tap water and subjected to the isolation and detection protocol described in Materials and Methods. (A) Ethidium bromide-stained agarose gel; (B) stained filter after Southern blot hybridization with digoxigenin-labeled enterovirus PCR product (poliovirus Sabin types 1, 2, and 3). Lanes: 1, 200,000 $TCID_{50}$; 2, 20,000 $TCID_{50}$; 3, 2,000 $TCID_{50}$; 4, 200 $TCID_{50}$; 5, 20 $TCID_{50}$; 6, 2 $TCID_{50}$; 7, no enterovirus RNA; 8, RT negative control; 9, PCR negative control; 10, PCR positive control (cDNA of 1,500 $TCID_{50}$); L, 100-bp DNA ladder (Gibco BRL).

RESULTS

Sensitivity of RT-PCR for enterovirus RNA. In analyses of 10-fold serial dilutions of vaccine Poloral Berna in distilled water, amplification reactions produced 155-bp fragments as expected. The detection limit for this method was 0.03 $TCID_{50}$, which corresponds to 1 to 5 virus particles (2, 25). After Southern blot hybridization, detection limits remained the same but signal intensities were higher. Experiments with other viruses were not performed as the primers used in our assay have been extensively tested by other groups and found to be very specific for enteroviruses (5, 14, 22, 31).

Optimization of GuSCN concentration. Several concentrations of GuSCN in the extraction mixture were tested for optimal lysis of viruses and hybridization of enterovirus RNA to oligonucleotide-labeled magnetic beads. At 2 M GuSCN, products of RT-PCR revealed their best sensitivities, while 1 and 3 M GuSCN concentrations in the extraction mixture resulted in significantly smaller amounts of PCR product (data not shown).

Detection of enterovirus RNA in samples of artificially spiked tap water. In these experiments, vaccine Poloral Berna was serially diluted in 1-liter samples of tap water. Following RNA isolation and RT-PCR amplification, products separated by agarose gel electrophoresis were usually detected by Southern blot hybridization to guarantee high specificity and sensitivity. The agarose gel and corresponding Southern blot of one amplification experiment are shown in Fig. 1. With fewer poliovirus particles per sample, agarose gels revealed increasing amounts of false-length fragments after PCR. These fragments produced no signal after Southern blot hybridization, though. The detection limit was 20 $TCID_{50}$, equal to about 1,000 virus particles in the original 1-liter sample.

RT-PCR detection of enteroviruses in surface water from lakes and rivers. Forty 1-liter samples were treated as described above to isolate enterovirus RNA. After RT-PCR, weak unspecific band patterns were visible on ethidium bromide-stained agarose gels for most samples. After Southern blot hybridization and stringent washing, seven samples (17.5%) showed bands of the correct size for the enterovirus PCR product and were considered to be positive. No unspecific hybridization was observed. The agarose gel and Southern blot of some samples are shown in Fig. 2, and the results are summarized in Tables 2 and 3.

Detection of enterobacteria in water from lakes and rivers. Colony numbers of *E. coli* in 100-ml aliquots and of *Salmonella* spp. in 1-liter aliquots were determined by culture (Table 3). In nine samples (22.5%), no *E. coli* colonies were detected, while two samples (5%) contained more than 100 CFU in the volume tested. One sample (no. 17) was positive for *Salmonella* spp. Analyzed by enzymatic DNA amplification, six samples (15%) yielded positive results for *elt* PCR specific for the gene that produces heat-labile toxin in *E. coli*; three of them were not positive for *E. coli* by culture (Table 3). No PCR-positive results were obtained for *E. coli* that contained heat-stable enterotoxin genes (*estA1* or *estA3*). *Salmonella* spp. were detected in two samples by PCR (no. 17 and 37). All samples positive by PCR are shown in Fig. 3. With *elt* PCR, each negative sample shows a strong unspecific band caused by listerial DNA in the reaction (Fig. 3, lanes 7 through 9). The nature of this band was not investigated further. All samples yielded strong bands with the *L. monocytogenes*-specific amplification reaction, indicating that the isolation procedure was properly performed and that the samples contained no substances to strongly inhibit PCR (data not shown). The detection limits obtained were less than 1 CFU/ml of original sample for *E. coli estA3* PCR and *elt* PCR and *Salmonella* sp. *inv* PCR and less than 10 CFU/ml for *E. coli estA1* PCR, as determined by agarose gel electrophoresis. Comparison of enterovirus contamination with enterobacterial contamination showed that all water samples positive for enterovirus RNA contained *E. coli*

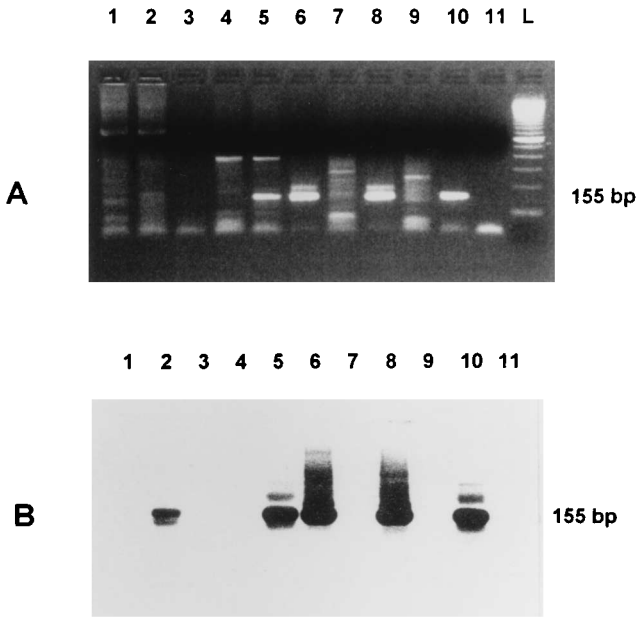

FIG. 2. Detection of enteroviruses in surface water samples. One-liter samples of surface water were treated as described in Materials and Methods and subjected to RT-PCR. Several independent positive and negative control samples were included to monitor each processing step. (A) Ethidium bromide-stained agarose gel; (B) stained filter after Southern blot hybridization with digoxigenin-labeled enterovirus PCR product (poliovirus Sabin types 1, 2, and 3). Lanes: 1, sample no. 38, negative; 2, sample no. 39, positive; 3, sample no. 40, negative (Table 3); 4, negative control (total isolation and detection procedure; no vaccine added); 5, positive control (total isolation and detection procedure; 5,000 $TCID_{50}$); 6, positive control for RT (3,000 $TCID_{50}$); 7, negative control for RT; 8, positive control for RT (3,000 $TCID_{50}$); 9, negative control for RT; 10, positive control for PCR (cDNA of 500 $TCID_{50}$); 11, negative control for PCR (no DNA); L, 100-bp DNA ladder (Gibco BRL).

also. A summary of all enterovirus RT-PCR, bacterial culture, and bacterial PCR results is shown in Tables 2 and 3.

DISCUSSION

The main goal of this project was the development of a rapid, simple, and efficient procedure to isolate and detect enteroviruses from environmental water samples. The primer oligonucleotides used for subsequent detection of enterovirus RNA have been successfully applied by other groups and have shown high-level specificities and sensitivities for enterovirus species (5, 14, 22, 31). We were able to confirm these findings with our RT-PCR system. Our protocol has a detection limit of less than 10 virus particles when applied to pure Poloral Berna vaccine. This sensitivity is similar to those of systems developed by other groups (1, 21) and of bacterial PCR systems developed by our group (8, 18, 29).

Isolation of virus particles from water samples was achieved by a two-step procedure. First, water was filtered through a borosilicate glass filter to hold back large, potentially inhibiting particles and then through a positively charged nylon membrane which adsorbed virus particles. In the second step, viruses were lysed directly on the membrane and RNA was specifically isolated by hybridization to a DNA probe bound to magnetic beads. For this simple lysis and isolation procedure, an extraction solution which contained GuSCN was prepared. GuSCN has several properties that render it well suited for this purpose: first, it destroys the envelope of virus by denaturing proteins and thus releasing RNA (3); second, RNases and other enzymes are inactivated (6); third, the interaction be-

TABLE 2. Results of various analyses of lake and river bathing waters

Microorganism(s) (assay)	No. of positive samples/ no. of samples tested	% Positive
Enteroviruses (EV PCR)[a]	7/40	17.5
E. coli (culture)	31/40	77.5
E. coli heat-labile toxin type I (*elt* PCR)	6/40	15.0
E. coli heat-stable toxin type I (*est* PCR)	0/40	0.0
Salmonella spp. (culture)	1/40	2.5
Salmonella spp. (*invA* PCR)	2/40	5.0

[a] EV PCR, RT-PCR assay developed in this study.

TABLE 3. Enterovirus contamination and *E. coli* contamination in bathing water samples

Body of water and sample no.	Sampling site	Culture of *E. coli* (CFU/100 ml)	PCR result	
			E. coli LTI[a]	Entero-viruses
Lake of Biel				
1	Biel	0	−	−
2	Vingelz	3	−	−
3	Tüscherz	0	+	−
4	Twann	0	+	−
5	Erlach	1	−	+
6	Vinelz	0	−	−
7	Lüscherz	1	−	−
8	Täuffelen	13	−	+
9	Mörigen	1	−	−
10	Sutz-Lattrigen	12	−	−
11	Ipsach	2	−	−
12	Nidau	1	−	−
Lake of Thun				
13	Thun	3	−	−
14	Hilterfingen	10	−	−
15	Oberhofen	0	+	−
16	Gunten	0	−	−
17	Merligen	0	−	−
18	Unterseen	1	−	+
19	Leissigen	17	−	−
20	Faulensee	5	−	−
21	Spiez	3	−	+
22	Krattigen	4	−	−
23	Einigen	2	−	−
24	Du Lac Därligen	5	−	−
Lake of Brienz				
25	Brienz	0	−	−
26	Iseltwald	0	−	−
27	Bönigen	2	−	−
28	Niederried	4	+	−
29	Oberried	3	+	−
Lake Neuchâtel, 30	Gampelen	34	−	−
Moossee, 31	Moossee	12	−	−
Burgseeli, 32	Burgseeli	2	−	−
Aare River				
33	Thun	7	−	+
34	Münsingen	51	−	−
35	Muri	22	−	+
36	Eichholz Bern	86	−	−
37	Marzili Bern	44	+	−
38	Lorraine Bern	22	−	−
Gürbe River, 39	Mühlethurnen	147	−	+
Giesse River, 40	Belp	112	−	−

[a] *E. coli* LTI, *E. coli* strains that produce heat-labile toxin type I.

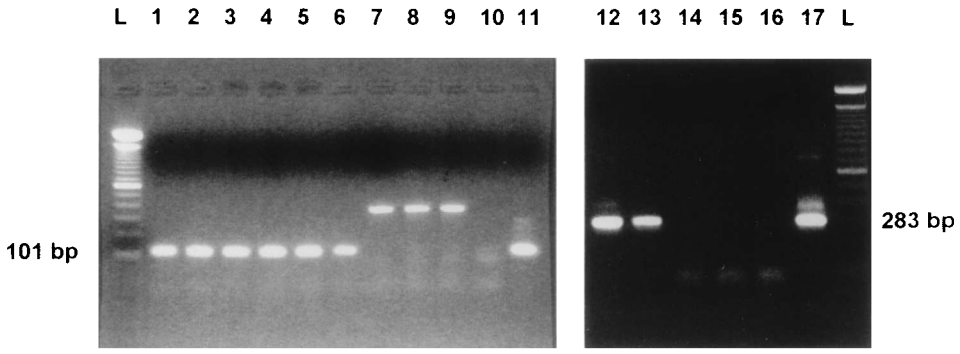

FIG. 3. Detection of bacteria in surface water by PCR. Fifty-milliliter samples of surface water were treated as described in Materials and Methods and subjected to PCR. An ethidium bromide-stained agarose gel of PCR products from *E. coli elt* PCR (lanes 1 through 11) and *Salmonella* sp. *invA* PCR (lanes 12 through 17) is shown. Lanes: 1, sample no. 3, positive; 2, sample no. 4, positive; 3, sample no. 16, positive; 4, sample no. 28, positive; 5, sample no. 29, positive; 6, sample no. 37, positive; 7, sample no. 38, negative; 8, sample no. 39, negative; 9, sample no. 40, negative; 10, PCR negative control; 11, PCR positive control (50,000 cells of a heat-labile toxin type I-producing *E. coli* strain); 12, sample no. 37, positive; 13, sample no. 17, positive; 14, sample no. 30, negative; 15, sample no. 24, negative; 16, PCR negative control; 17, PCR positive control (5,000 cells of *S. enteritidis*); L, 100-bp DNA ladder (Gibco BRL). With *elt* PCR, each negative sample (lanes 7 through 9) yields a strong unspecific band caused by listerial DNA in the reaction. The nature of this band was not investigated further.

tween biotin and streptavidin is not inhibited, as was observed in our experiments; and fourth, gentle conditions with respect to hybridization temperature and pH are possible (28). The amount of GuSCN in the extraction mixture was empirically optimized, and after RT-PCR, the best results were obtained with a concentration of 2 M. A 1 M concentration probably didn't sufficiently lyse virus particles or inactivate RNases, while 3 M amounts might have hampered the binding of biotin to streptavidin and thus reduced the number of template molecules for RT-PCR.

Tap water samples artificially spiked with serial dilutions of vaccine were subjected to this isolation procedure. The detection limit for virus RNA after RT-PCR, agarose gel electrophoresis, and Southern blot hybridization was 20 $TCID_{50}$, corresponding to up to 1,000 virus particles in the original 1-liter sample. Hence, the detection limit after isolation from tap water is approximately 500-fold higher than that for RT-PCR with pure virus RNA (see above). Several factors may be responsible for this increase. Loss of sensitivity may be due to virus or RNA degradation in unsterile water before sample preparation, and some virus adsorption to the glass prefilter or RNA adsorption to the nylon membrane during isolation is unavoidable. In addition, virus particles associated with solids in water samples may be removed by filtration through the prefilter. These problems have to be addressed in future work. Further, linear dilutions down to low virus concentrations may not be accurate as aggregates of virus particles are common. Still, a detection limit of a few hundred virus particles in the original 1-liter sample is sufficient when the fact that the volumes of test samples can be significantly increased, if appropriate filtration equipment is available, is taken into account. An accumulation of ions or humic acids which may alter reaction conditions and impair successful RT of RNA (1, 16) shouldn't occur with our protocol, as RNA is specifically extracted with beads and thoroughly washed before enzymatic reactions.

The increasing number of false-length fragments with decreasing concentrations of virus RNA in test samples is most likely caused by coisolation of other DNA or RNA molecules in tap water that can unspecifically adsorb to these beads. These molecules are reverse transcribed under the nonstringent conditions of this reaction and can be amplified if test samples contain little or no perfectly matching template (Fig. 1A). These bands were not seen after Southern blot hybridization (Fig. 1B), though.

The application of this isolation and detection system to naturally contaminated surface water samples again yielded significant amounts of incorrect amplification products after RT-PCR which were visible on stained agarose gels (Fig. 2A). Subsequent Southern blot hybridization revealed seven (17.5%) positive samples (Fig. 2B) and didn't show any unspecific cross-reactions. All signals were rather weak, suggesting only a low level of virus contamination. The incidence of positives is quite low, compared with the results published by Northern Irish and French groups (13, 16), which found contamination in up to 80% of the samples tested. It must be noted that sewage treatment in Switzerland is more widespread and that our test samples were collected after 3 days with no rain, which reduces the chance of contamination by untreated effluents from treatment plants or fields. The bacterial quality of water samples was also very good, and much lower *E. coli* colony numbers were detected by classical culture from these samples than in previous determinations in the same year.

Virus contamination was found only in samples that were positive for *E. coli* also (Table 3). This finding suggests an identical source and indicates that bacteriological water quality with *E. coli* as the indicator organism may also serve for monitoring virological water quality. However, statistical analysis using binomial distribution showed that the number of samples investigated was too small to support this assumption at the 95% confidence level usually applied. In other studies, researchers have found no association between enterovirus contamination and enterobacterial contamination in water so far (2, 12, 13). Six of the seven samples contaminated by enteroviruses had very low colony numbers of *E. coli* only and received the best rating according to Swiss quality standards for bathing water, which consider only bacterial contamination. The detection of virus RNA strongly indicates the presence of intact, encapsulated viruses, as free RNA is quickly degraded in the environment. It is not known whether these viruses are in an infectious state, but as infections with enteroviruses occur with as few as 100 to 1,000 virus particles (2, 12), positive findings by RT-PCR still suggest a serious health risk, especially for children. The determination of enterovirus contamination is indispensable to properly determining water quality and the health risk associated with possibly contaminated drinking water. At this time, however, we cannot exclude detection of poliovirus Sabin serotypes that originated from vac-

cination campaigns. These attenuated virus strains do not pose a hazard to human health.

With a simple procedure to isolate bacteria, further interesting observations were obtained. Six samples yielded positive PCR results for enterotoxigenic *E. coli* that produce heat-labile toxin, but only three of these samples contained culturable *E. coli* organisms. Likewise, two samples were PCR positive for *Salmonella* spp., but only one of them was positive by culture. Whether the discrepancy is caused by dead bacteria detected by PCR or the nonculturability of viable but weakened bacterial strains on artificial culture media cannot be deduced from these experiments. Positive PCR results indicate that these bacteria were present at one time and might help to track down potential sources. As several amplification reactions are possible with each sample and PCR generally seems to be more sensitive than culture, it can be used as a fast and simple screening method and, in combination with culture, also give more detailed information on the pathogenicity of bacteria, e.g., enterotoxigenic or verotoxigenic *E. coli*.

In conclusion, our experiments show that this sample preparation protocol for enteroviruses in combination with RT-PCR is very easy to perform, gives fast, sensitive, and specific results, and can easily be adapted for routine use in control laboratories. No sophisticated apparatus or handling procedures are necessary, in contrast to classical virus detection or protocols described up to now, and the time for analysis can be significantly reduced. The applicability of this system was tested with artificially spiked tap water and 40 naturally contaminated bathing water samples. Parallel detection of enterobacteria in surface water samples showed that determination of both bacterial contamination and virus contamination is necessary to obtain sufficient information on water quality.

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