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Detection of Naturally Occurring Enteroviruses in Waters by Reverse Transcription, Polymerase Chain Reaction, and Hybridization

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Comparison in virus-seeded mineral water of three detection methods for enteroviruses, direct hybridization, cell culture, and reverse transcription into cDNA followed by polymerase chain reaction and hybridization, showed that the last procedure was 10 to 1,000 times more sensitive than detection by cell culture and 10^5 to 10^7 times more sensitive than direct hybridization. The presence of naturally occurring enteroviruses was also demonstrated in activated sludge and in concentrated and non-concentrated surface water samples by reverse transcription-polymerase chain reaction-hybridization. However, in activated sludge and in concentrated surface waters, enzymatic amplification was sometimes inhibited by contaminants.

Water is generally recognized as a vehicle for the transmission of diseases (7, 8), although the etiology of a number of waterborne outbreaks has remained unknown because not all infectious agents could be identified with the diagnostic procedures used at the time of the outbreak (see, for instance, references 11, 12, 27, and 37). The control of viruses is especially important in surface waters, because they not only are used frequently for bathing but also serve as resources for drinking water. Many surface waters, as receiving waters for sewage treatment plants, are fecally polluted; they contain viruses which develop in the gut, the enteric viruses, and are shed with the feces. Among them are representatives of the adeno-, entero-, and caliciviruses.

Current microbial standards used as safety criteria for water may not always be indicative of waterborne pathogens; in particular, the presence of enteric viruses is not always linked to the presence of *Escherichia coli* or other indicator organisms used in public health (3). Therefore, an additional indicator of the virological quality of water is required.

The monitoring of enteroviruses (an important group among the enteric viruses) in surface water might fill this gap. Enteroviruses are human pathogens with a wide spectrum of clinical manifestations. Their acid stability permits limited replication in the oropharynx, transit through the stomach, and implantation in the lower intestinal tract, where they undergo extensive replication. Close human contact appears to be the primary route of spread, the most frequent being the fecal-oral route. With the feces, enteroviruses are discharged into the sewage and from there partially into receiving waters.

The amount of enteroviruses present in waters is highly variable, depending on factors such as the hygienic level, the density of the population, the prevalence of infections in the community, and the season. At low, but still relevant, viral concentrations in water, their detection by cell culture inoculation is difficult, since large volumes have to be assayed. In those cases, viruses of large volumes of water are concentrated into smaller volumes, which are amenable to cell culture assays.

Concentrating viruses usually involves numerous steps and requires considerable manpower; in addition, cell culture facilities have to be available for the assay. In spite of these efforts, the efficiency of the method is poor, and viral assays in surface waters are carried out only occasionally.

Recent developments in molecular biology, i.e., direct hybridization with viral probes and enzymatic amplification (polymerase chain reaction [PCR]), suggest easier, sensitive, and specific approaches for the monitoring of viruses in environmental samples. Molecular hybridization and PCR have been used already for the detection and/or differentiation of enteroviruses (5, 18, 22) and for the detection of enteric and enteroviral RNA sequences in clinical specimens (14, 18, 21, 26, 27, 38) and in virus-seeded environmental samples (10). Other applications relevant for public health have also been achieved by PCR: detection of enterotoxigenic *E. coli* and virulent plasmids in stools (13) and in minced meat (36), detection of *Vibrio vulnificus* DNA (4) and *Vibrio vulnificus* in artificially contaminated oysters (16), and detection of *Legionella pneumophila* and other organisms relevant to public health in water (1, 2). Differentiation of active and inactive cysts of *Giardia lamblia* by PCR has been reported to be possible (25).

Enteroviruses have several genomic regions which are very conserved among the various virus types (19, 20, 23, 32). Thus, the choice of primers in the conserved sequences allows amplification of sequences of most viruses of this genus simultaneously (18, 28, 29), making the test more valid as a general viral indicator. Of course, for the detection of a particular enterovirus, e.g., a poliovirus, other virus-type specific sequences might be amplified. If the amount of foreign nontarget DNA is high, the specificity of the assay might be enhanced by carrying out a second, nested or "seminested" PCR by changing both or one of the primers. The second round of PCR essentially increases the sensitivity as well as the specificity of the test.

In this paper, we report enterovirus detection in activated sludge and in surface waters by reverse transcription (RT), followed by two rounds (one of them seminested) of PCR.

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The results indicate that the assay is very sensitive, but considerably prone to inhibition. Positive results might not necessarily mean that the viruses are infectious.

MATERIALS AND METHODS

Cell cultures, viral strains, plasmids, and probes. Buffalo Green Monkey, HeLa, and Vero cells were kept in Eagle's minimal medium (MEM) supplemented with 5% fetal calf serum. Poliovirus type 3, Sabin strain (PV3); echovirus type 7 (E7); and coxsackievirus B type 5 (CB5) were water isolates from the Laboratory of Hygiene of the City of Paris, France. Viruses were multiplied in Vero cells (PV3, E7, and CB5), the 50% tissue culture infectious dose ($TCID_{50}$) per milliliter (calculated from the most probable number of infectious units as described in reference 15, adapted to cell cultures) in the supernatants was determined, and each supernatant was then adjusted to a titer of 10^9 $TCID_{50}$/ml. Poliovirus type 1, Mahoney strain, was provided by the Center for Enterovirus Detection in Lyon, France, and multiplied in HeLa cells, and the titer was adjusted similarly.

The poliovirus cRNA used in the experiment described in Fig. 2 was transcribed from plasmid pHK8, which carries the cDNA of the complete poliovirus 1 genome in the *Sac*I site of plasmid pGEM-2 (Promega, Madison, Wis.). The poliovirus cDNA in pHK8 is framed by a T7 and an SP6 promoter, so that viral RNA with either positive or negative polarity can be obtained by in vitro transcription with either SP6 or T7 RNA polymerase. Similarly, the radiolabeled cRNA probe used in all hybridization procedures was transcribed from the SP6 promoter of a pGEM-1 plasmid (Promega) containing a fragment of poliovirus 1 cDNA (bp 221 to 388) inserted in the *Bam*HI site. For cloning procedures, see reference 6. Transcription of the radiolabeled probe and the unlabeled poliovirus 1 cRNA used in the experiment of Fig. 2 was carried out by standard procedures (9).

Environmental samples. Ten grab samples of activated sludge were collected during May and June 1990 from a wastewater treatment plant located in Berlin, Germany, and subjected to chloroform extraction (see below). Five surface water samples were withdrawn from a fecally polluted lake in Berlin and concentrated by both flocculation and lyophilization. Finally, 10 surface water samples were collected during January and February 1991 from the river Seine (at Paris) and concentrated by adsorption-elution on membrane filters.

Treatment of environmental samples. (i) Chloroform extraction. Samples, 100 ml, of activated sludge were collected, kept refrigerated at 4°C, and processed on the day of collection or the day after. Ten milliliters of the sample (which had been homogenized by inverting the bottle several times) was placed in a 100-ml Erlenmeyer flask, 1 ml of chloroform was added, and the flask was shaken vigorously for 60 min at room temperature with the aid of a rocking machine. After shaking, the flask was left to stand at room temperature for 5 to 10 min until the chloroform settled; then the upper aqueous phase was centrifuged at 47,000 $\times g$ for 10 min (in the SS 34 rotor of a Sorvall centrifuge at 20,000 rpm), and the supernatant was saved for cell culture inoculation or, after its extraction, for RT-PCR.

(ii) Concentration by flocculation. Concentration by flocculation was carried out essentially as described in reference 35. Ten liters of surface water was mixed with 20 ml of 10% $Al_2(SO_4)_3$, and the pH was adjusted to 5.5 to 5.8 with HCl, after which a precipitate formed. When the precipitate had sedimented, after 2 to 12 h, we discarded the supernatant, centrifuged the sediment at 2,000 $\times g$ for 30 min, and resuspended the pellet in 10 ml of 0.1 M sodium citrate, pH 4.7. This suspension was shaken for 2 h at room temperature and overnight at 4°C. Afterwards, it was centrifuged at 2,000 $\times g$ for 30 min, the pellet was discarded, and the supernatant was centrifuged again at 100,000 $\times g$ to pellet the viruses. The pellet was resuspended in 2 ml of phosphate-buffered saline (PBS), and this suspension was treated with 0.2 ml of chloroform, as described for activated sludge. The suspension was used for RT-PCR after extraction, or mixed with MEM (1 ml of PBS to 9 ml of MEM) for inoculating Buffalo Green Monkey cell cultures.

(iii) Concentration by lyophilization. Surface water, 10 ml, was collected in Falcon tubes, frozen to −80°C, and freeze-dried. The lyophilizate was resuspended in 100 μl of sterile distilled water and used for RT-PCR after extraction.

(iv) Concentration by adsorption-elution. Ten liters of surface water was filtered through a 1-MDS filter (AMF/Cuno, Meriden, Conn.), and the filter was eluted with 50 ml of 3% beef extract. The concentration factor achieved was 200. The solution was used for RT-PCR after extraction.

Detection of enteroviruses by inoculation of cell cultures. For the experiment in Fig. 1, 10-fold dilutions of the three viruses used, ranging from 10^9 to 10^{-2} $TCID_{50}$/ml, were prepared in mineral water. One hundred microliters of each dilution was mixed with 100 μl of 2× MEM, and the mixture was delivered to the corresponding well of a 96-well microtiter plate which had been seeded the day before with 5×10^4 cells per well suspended in 100 μl of MEM containing 2% fetal calf serum. The plates were incubated at 37°C for 6 days, the supernatants were collected, and 200 μl of each supernatant was processed for hybridization or RT-PCR as described below.

For inoculating cell cultures with extracts of activated sludge, we took 5 ml of the supernatant obtained by chloroform extraction, mixed it with 5 ml of 2× MEM (2% fetal calf serum), and added 2,000 IU of penicillin, 2 mg of streptomycin, 0.5 mg of kanamycin, and 250 IU of nystatin (Moronal). We distributed 100 μl of this mixture to each well of a 96-well microtiter plate which had been seeded the previous day with ca. 5×10^4 Buffalo Green Monkey or Vero cells per well in 100 μl of MEM (2% fetal calf serum). This medium was not removed before the addition of the sample, so the total final volume per well was 200 μl, which included 1.5% serum. After 7 days of incubation, the wells were examined for the presence of a cytopathic effect. The supernatants were then extracted for hybridization or RT-PCR.

Extraction for hybridization or RT, gel electrophoresis and blotting of the agarose gels. A 200-μl portion of the viral dilutions (see Fig. 1A), the culture supernatants (see Fig. 1B), the activated sludge samples after the chloroform step, or the surface water concentrates was incubated with proteinase K (100 μg/ml) for 60 min at 37°C, and the reaction was stopped by incubating the samples at 95°C for 10 min. For RT, 5 μl was taken. For hybridization, 50 μl of each sample was mixed with 50 μl of a mixture of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and formaldehyde (3:2, vol/vol); the mixture was heated for 15 min at 60°C and applied to a hybridization membrane (Hybond N; Amersham) with a slot blot manifold. The membrane was dried at room temperature and fixed for 3 min under a shortwave UV lamp.

Gel electrophoresis in 1% agarose and blotting were carried out by standard procedures (10).

Hybridization. All filters were hybridized with the same

radiolabeled RNA probe described above. Prehybridization of filters was carried out under shaking at 42°C for 2 h in 50% formamide, 5× SSC, and 3× Denhardt solution, and 100 μg of yeast RNA per ml. Hybridization was carried out overnight under the same conditions in the same fresh mix without yeast RNA and in the presence of ^{32}P-labeled probe (10^6 cpm/ml). Four successive 30-min washings of the filters at 65°C were done in 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC, always in the presence of 0.1% sodium dodecyl sulfate. X-ray films (Kodak) were exposed to the dried filters for 1 to 3 days at −70°C in the presence of intensifying screens.

RT. Five microliters of each extract was used for cDNA synthesis in a reaction containing the following, in 20 μl: 50 mM Tris-HCl (pH 8.3); 4.6 mM $MgCl_2$; 10 mM dithiothreitol; 40 mM KCl; 17 U of RNase inhibitor (RNA Guard; Pharmacia); 200 mM each dATP, dCTP, dGTP, and dTTP; 2 μM downstream primer or 2 μM oligo$(dT)_{15}$ primer; and 12 U of reverse transcriptase (avian myeloblastosis virus; Boehringer). Synthesis was carried out at 42°C for 1 h. The reaction was then stopped by heating for 5 min at 65°C.

Enzymatic amplification of the cDNA (PCR). (i) Primers. The primers used were selected in the 5′ noncoding region of poliovirus type 1. They represent conserved sequences shared with several other enteroviruses, as follows: downstream primer (primer 3), 5′ TGG CTG CTT ATG GTG ACA AT 3′ (577 to 596); downstream primer (primer F2), 5′ GTC GTA ACG CGC AAG 3′ (510 to 524); upstream primer (primer 2), 5′ CAA GCA CTT CTG TTT CCC CGG 3′ (162 to 182). The sequences of the primers are expressed in the same sense as the viral genome. The numbers in parentheses after the sequences represent the locations of their first and last nucleotides in the genome of poliovirus 1 (Mahoney).

The RNA probe used for the detection of the PCR products did not overlap with the primers, since the probe was transcribed from bp 221 to 388 of poliovirus 1 cDNA.

(ii) Reaction conditions. For enzymatic amplification, 5 μl of the RT reaction was added to 95 μl of the reaction mix containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 1 μl of each upstream and downstream primer (10 μM). The reaction mix was heated for 5 min at 94°C and cooled to the annealing temperature (50°C), and 0.5 μl of *Taq* polymerase (2.5 U; Cetus) plus 2 drops of paraffin oil were added. Thirty thermic cycles were applied (72°C for 1.2 min, 94°C for 1 min, and 50°C for 1.2 min); the final elongation at 72°C continued for 10 min.

For seminested PCR, which was introduced in the experiments shown in Fig. 4 and 5, the template was the product from the first amplification, and the downstream primer was primer F2. The conditions of amplification were the same as for the first PCR. The product from the first PCR had a length of 435 bp (bp 162 to 596 of poliovirus 1 cDNA), whereas that from the second seminested PCR was 363 bp (bp 162 to 524 of poliovirus 1 cDNA).

For slot blots of the PCR products, aliquots of each PCR reaction were mixed with equal volumes of 20× SSC–formaldehyde (3:2, vol/vol); the mixtures were heated for 15 min at 60°C, applied to the hybridization membrane, and fixed under UV for 3 to 4 min.

RESULTS

Sensitivity of different procedures for the detection of enteroviruses or enteroviral RNA. (i) Sensitivity of cell culture, molecular hybridization, and RT-PCR for the detection of enteroviruses. Dilutions of PV3, CB5, or E7 were used to compare the detection sensitivity of either direct hybridization, cell culture, or RT-PCR followed by hybridization. The following three procedures were carried out in parallel with viral suspensions containing decreasing amounts of viruses: (i) extraction of the RNA as described in Materials and Methods followed by application of 50 μl on a hybridization membrane; (ii) inoculation of Vero cell cultures, collection and extraction of the culture supernatants 6 days postinfection, and application of 50 μl of each supernatant on a hybridization membrane; (iii) extraction of the RNA and performance of RT-PCR with 5 μl of each extract and application of 10 μl of the PCR products on a hybridization membrane. All membranes were hybridized with the same ^{32}P-labeled riboprobe.

Figure 1 shows the results of the three procedures. By direct hybridization, PV3 caused positive signals with 10^4 to 10^7, CB5 caused positive signals with 10^5 to 10^7, and E7 caused positive signals with 10^6 to 10^7 $TCID_{50}$ (panel A). The different sensitivities for each virus probably reflected different sequence homologies to the probe used. Supernatants of Vero cells which had been infected with as little as 1 to 10 $TCID_{50}$ of PV3, CB5, or E7 yielded a positive signal (panel B). After RT-PCR, positive hybridization signals were observed in all cases including the PCR tubes which had received cDNA of 10^{-1} to 10^{-2} $TCID_{50}$.

Under our experimental conditions, no quantitative results for the RNA present in the samples can be expected, since the conditions were chosen to maximize sensitivity rather than to obtain quantitative results. Accordingly, the intensities of the slots in Fig. 1C do not show a decreasing pattern, but rather represent yes-or-no information. No radioactive signals were present in the negative controls carried out with plain mineral water.

(ii) Sensitivity of hybridization or RT-PCR followed by hybridization for detecting enteroviral RNA. The assays were carried out with viral RNA extracted from HeLa cells infected with poliovirus 1 and with poliovirus cRNA synthesized in vitro from plasmid pHK8. Serial 10-fold dilutions of both viral (Fig. 2A) and in vitro transcribed RNAs (Fig. 2C) were prepared and applied to a hybridization membrane starting with 100 ng/spot and decreasing to 0.1 fg/spot. For RT, the amounts of RNA given on the right margin of Fig. 2 were used, and a 5-μl aliquot of the RT mixture was included in the PCR. Ten microliters of the PCR products obtained was applied to the membrane (panel B), which was then hybridized with the ^{32}P-labeled riboprobe. Figure 2 shows that both viral and cRNAs were detected directly down to 1 ng/spot. One femtogram of RNA, which corresponds to ca. 200 genomic copies, was still detected by RT-PCR-hybridization.

Detection of naturally occurring enteroviruses in activated sludge by cell culture and RT-PCR. Ten samples of activated sludge were analyzed for the presence of enteroviruses by cell culture and by RT-PCR followed by electrophoresis of the PCR product, blotting of the gel, and hybridization of the membrane with the ^{32}P-labeled cRNA probe used in the preceding experiment. All samples were found positive by cell culture when 5 ml was assayed: the highest amount was 15 microtiter wells displaying a cytopathic effect, and the lowest was 3. On average, eight wells displaying cytopathic effect were found per 5 ml. Figure 3 shows that of 10 samples analyzed, only six were found positive by RT-PCR-hybridization. Negative controls with distilled water did not show any signal (results not shown).

Detection of naturally occurring enteroviruses in surface waters. Samples of the river Seine, both native and concentrated as described in Materials and Methods, were used for

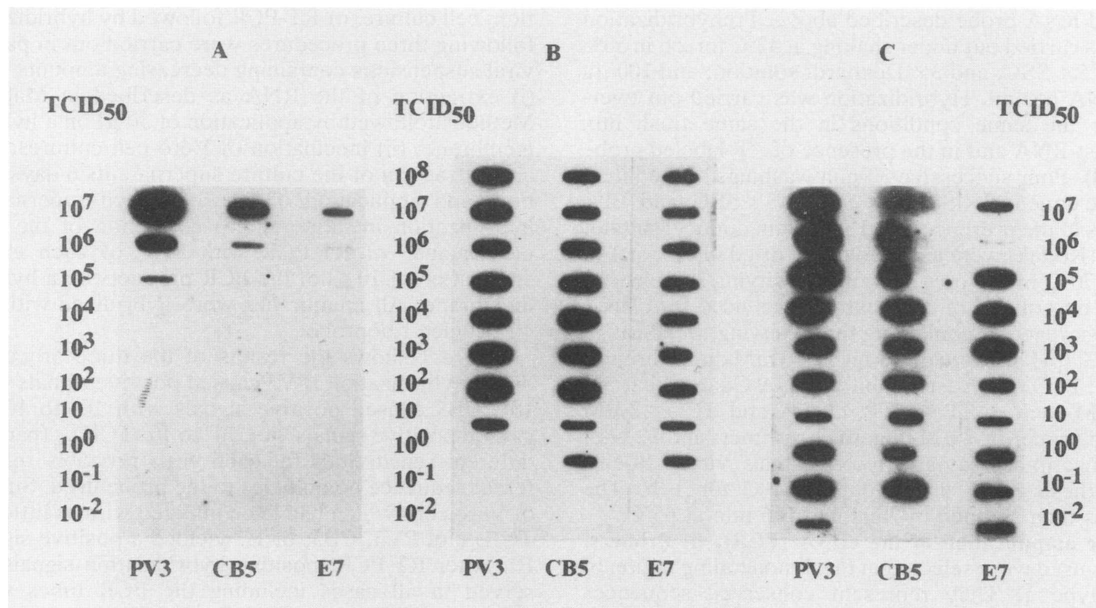

FIG. 1. Detection of PV3, CB5, and E7 by direct hybridization (A), cell culture plus hybridization (B), and RT-PCR plus hybridization (C). The $TCID_{50}$ given in the margins correspond to the viral amounts taken for direct blotting, for inoculating the cultures, and for carrying out the RT.

RT, which was followed by seminested PCR and agarose electrophoresis of 10 μl of the second PCR.

Figure 4 shows that all nonconcentrated samples except one were readily found positive for enteroviral sequences (panel A). After concentration, only four samples were positive (panel B).

Similar results were obtained by analyzing, in the same manner, native and concentrated specimens collected in a lake located in Berlin. All five native samples were positive (Fig. 5A), with one sample (panel A, sample 2) yielding a smear of nondefined fragments that contained enteroviral sequences as shown by hybridization. After 5,000-fold concentration from 10 liters, two samples (samples 3 and 4 in panel B) became negative. The samples concentrated by lyophilization (panel C) were all positive. None of the 5,000-fold-concentrated samples were positive by cell culture. The concentrated samples 3, 4, and 6 in Fig. 4 yielded positive results in cell cultures. The isolates were identified as Coxsackie B4 in samples 3 and 4 and as Coxsackie B5 in sample 6.

DISCUSSION

Comparison of three different detection procedures for enteroviruses as shown in Fig. 1, cell culture inoculations, slot blotting of viral suspensions plus hybridization with specific probes, and amplification of subgenomic sequences by RT-PCR followed by hybridization, clearly pointed to RT-PCR as the most sensitive procedure. Direct slot blot hybridization was approximately 10^5 times less sensitive than cell culture inoculation, which itself proved to be 10 to 1,000 times less sensitive than RT-PCR. The differences between cell culture inoculation and PCR probably reflect

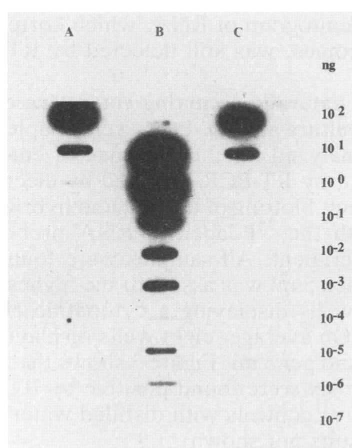

FIG. 2. Detection of poliovirus 1 RNA by direct hybridization (A and C) and RT-PCR plus hybridization (B). Fifty microliters of either extract or the PCR mixture was used for blotting.

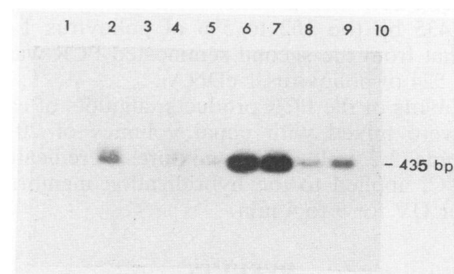

FIG. 3. Detection of naturally occurring enteroviruses in activated sludge by PCR plus hybridization. RNA extracts from 10 samples of activated sludge were subjected to RT-PCR, and 10 μl of the reaction mixture was separated by gel electrophoresis, transferred to a hybridization membrane, and hybridized with a cRNA probe.

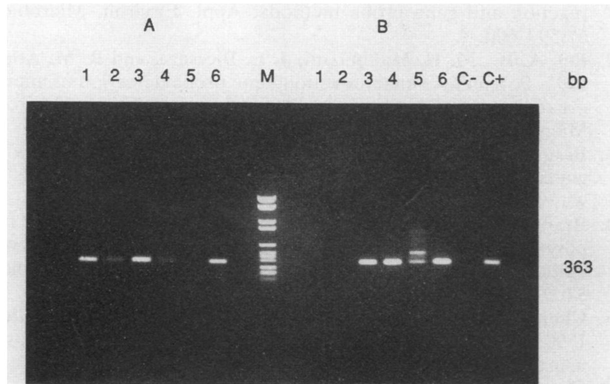

FIG. 4. Detection of enteroviruses in unconcentrated (A) and 200×-concentrated (B) surface waters by RT followed by two successive PCR amplifications. After RT-PCR was carried out, 10 μl of the PCR products was analyzed by gel electrophoresis. C−, negative control; C+, positive control, consisting of cDNA from poliovirus 1; M, molecular weight marker (marker VI; Boehringer Mannheim).

the fact that in culture supernatants the ratio of viral infectious units to physical particles is around 0.01 and might be much less, depending on the virus and the cell culture system used. As Fig. 2 shows, a similar relationship between the sensitivity of hybridization and that of RT-PCR can be estimated from the amounts of viral RNA needed to obtain positive results. Both authentic and in vitro synthesized RNAs yielded similar results.

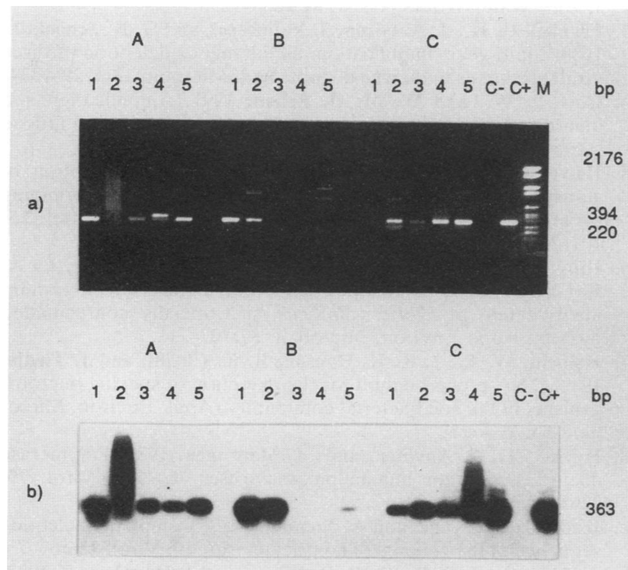

FIG. 5. Detection of enteroviruses in unconcentrated (A), 5,000×-concentrated (B), and 20×-concentrated (lyophilized) (C) surface waters by RT followed by two successive PCR amplifications and hybridization. After RT-PCR was carried out, 10 μl of the PCR products was analyzed by gel electrophoresis and blotted, and the membrane was hybridized with a radiolabeled cRNA probe. C−, negative control (tap water); C+, positive control, consisting of cDNA from poliovirus 1; M, molecular weight marker (marker VI; Boehringer Mannheim). Panels: a, ethidium bromide-stained gel; b, autoradiography.

To establish the suitability of RT-PCR for practical applications in public health monitoring, we used RT-PCR for the detection of enteroviral sequences in two areas of considerable environmental interest: activated sludge and polluted surface waters.

The activated sludge samples analyzed generated, on average, eight wells showing cytopathic effects per 5 ml. Because of the low ratio of infectious units to physical particles mentioned above, and also the high amount of proteases and other inactivating agents present in activated sludge, the number of virions (active or inactive) was probably much higher.

In spite of this, PCR was not positive in all samples (Fig. 3). In samples of sludge which yielded negative RT-PCR results, the addition of 10^3 $TCID_{50}$ of poliovirus did not lead to positive results (not shown). These results clearly point to a critical issue in the interpretation of PCR findings: the presence of enteroviruses does not necessarily generate positive PCR results. The negative results probably are a consequence of the presence of compounds that inhibit RT or PCR.

Attempts to detect enteroviruses by RT-PCR in polluted surface waters produced unexpected results. All but one sample yielded positive results without previous concentration; similarly, the PCR-hybridization results obtained with surface water samples concentrated (20-fold) by lyophilization were all positive. It was surprising to find positive results with as little as 5 μl of unconcentrated surface water. Since we have included negative controls in our assays, we think that our results are not false positives. The high amounts of (apparently noninfectious) virions in polluted surface waters might be explained if one considers that virions are shed in high amounts but that the vast majority are noninfectious. From results obtained with dilutions of activated sludge, we have observed that the rate of total virions (as determined by RT-PCR) to infectious units can be as high as 10^6, i.e., 10^3 to 10^4 higher than found in culture supernatants. This high ratio might be due to inactivation by, e.g., proteases present in sewage. If one considers that wastewater treatment plants typically remove viruses only partially (90 to 95%), then finding a high amount of virions in receiving waters is not that surprising after all.

After concentration by adsorption-elution or by flocculation, two of the initially positive samples became negative, in spite of the much larger concentration factor. Possibly, the concentration steps led not only to the enrichment of viruses, but also to that of impurities and humic acids. As in the activated sludge, these impurities might then have inhibited the amplification reaction.

The inhibition of PCR by humic acids and other compounds expected to be in environmental extracts has been reported previously (31, 33), and the processing of water or soil extracts with reverse transcriptase or restriction enzymes was reported to be possible only after extensive purification of the nucleic acids (17, 30, 31). The purification sometimes included density gradient centrifugation or other time-consuming procedures.

To be of practical, generalized use in public health, procedures must not be too expensive, cumbersome, or time-consuming, since, as a rule, the resources of public health laboratories are limited. Therefore, simple purification procedures are needed. It has been reported that gel filtration through Sephadex G200 separated enteroviruses from PCR-inhibiting compounds in sewage (10) or DNA of other microorganisms present in soil extract (34). This kind of approach promises a more adequate solution to the large

number of routine determinations required for use in public health.

As long as inhibiting impurities might be suspected in the extracts, both negative and positive controls should always be included: a known number of the organisms to be detected should be mixed with an aliquot of the sample, and the extraction and PCR procedures should be carried out in parallel with the other samples examined.

Sometimes we observed that the PCR products were not homogeneous, but ran in electrophoresis as a smear, as in panel A, track 2, or panel C, track 5, of Fig. 5. We presume that this is due to the formation of polymers of the PCR products. During optimization of the PCR conditions, we have frequently observed the formation of such high-molecular-weight products when the concentration of magnesium was suboptimal. It is conceivable that impurities present in the extract interfere with the magnesium, partially chelating it and lowering its actual concentration.

The inoculation of cell cultures with the concentrate of surface waters yielded negative results in spite of the apparently high amount of virions present in those waters. The waters in question serve as sites of discharge for the effluents of wastewater treatment plants, and the concentrates of samples taken on other occasions have been repeatedly positive by culture inoculation. We assume that the disinfection of the wastewater effluent prior to its discharge into the surface waters inactivates the major part of the viruses, rendering them still able to produce positive RT-PCR results but not to successfully infect cell cultures.

What, then, is the significance of finding a water sample which is positive for enteroviruses by PCR? Presumably a positive result proves the presence of not enteroviral RNA but rather encapsidated enteroviruses in the water, since free RNA is rapidly degraded in sewage (24). Nothing can be stated about the infectivity of the nucleocapsids, however, since viruses inactivated by chemical disinfection, heat inactivation, or proteases present in the water presumably are still able to yield a positive PCR signal. A positive PCR signal obtained with environmental waters should therefore not be considered equal to the detection of infectious viruses. Rather, PCR might serve as an indicator, signaling that enteroviruses have been or are present in the analyzed waters, but not necessarily in an infectious form.

This interpretation of positive PCR signals in environmental waters does not diminish the potential value of PCR for monitoring the environment for viruses. One can envisage situations in which PCR is used to screen environmental samples before other, more cumbersome procedures are carried out. Alternatively, PCR might be used for obtaining epidemiological data rather than for monitoring purposes. The knowledge that, for instance, sequences of virulent poliomyelitis viruses are present in water is very valuable because it means that such strains are circulating in the population, regardless of whether the strains have been inactivated before their discharge.

We regard RT-PCR as a tool which will take an important place in public health monitoring. Before this can happen, simple and inexpensive procedures must be developed for freeing the viruses and microorganisms, or their DNA, from impurities which inhibit the enzymes involved in the reaction.

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