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Analysis of Viral RNA Persistence in Seawater by Reverse Transcriptase-PCR

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It is important to determine the stability of naked viral RNA in seawater, since false-positive results can occur when reverse transcriptase-PCR (RT-PCR) is used to detect viruses if the RT-PCR amplifies free RNA instead of RNA from intact viruses. An acid guanidinium thiocyanate-phenol-chloroform method was used to extract total RNA from a filtered poliovirus cell culture suspension. The sensitivity of detection in this viral RNA study was 600 fg when RT-PCR was used. The extracted total RNA was seeded into filtered and unfiltered seawater, and the resulting preparations were incubated at 4°C and at room temperature (23 ± 1°C). Our results showed that the seeded RNA was more stable in filtered seawater than in unfiltered seawater at both temperatures. The viral RNA could not be detected by the RT-PCR after 2 days of incubation in unfiltered seawater and after 28 days of incubation in filter-sterilized seawater. Therefore, because of the relatively short life of viral RNA in natural water, the detection of virus in environmental samples by the RT-PCR was mainly due to the presence of well-protected viral particles and not due to the presence of naked viral RNA.

Reverse transcriptase-PCR (RT-PCR) has been widely used to detect RNA viruses in different aquatic environments. For example, enteroviruses, hepatitis A virus, rotavirus, poliovirus, human immunodeficiency virus, and Norwalk virus have been detected in sewage influent and effluent, sewage sludge, groundwater, river water, and ice by RT-PCR (1, 2, 7, 10, 11, 12, 17, 18). Because the viral RNA in each virion is protected by a protein coat or an envelope, this RNA is able to persist longer than naked RNA and can be detected by RT-PCR. Using the hexadecyltrimethylammonium bromide-3,5-diamidinobenzoic acid method, other researchers have found dissolved DNA consisting of soluble DNA, viral DNA, and bound DNA in marine water and other waters (10a, 19). In addition, these workers reported that the turnover of soluble DNA was much faster than the turnover of viral DNA and bound DNA in aquatic environments. In this study, total RNA was extracted from poliovirus and seeded into seawater to determine RNA stability by an RT-PCR. Because the RT-PCR is based on amplification of nucleic acids, it was important to determine whether the positive RT-PCR products were amplified from the RNA of whole viruses or from RNA released into the environment from lysed viruses.

Poliovirus type 1 strain LSc was maintained in Buffalo green monkey kidney cells, and viral RNA was extracted from poliovirus type 1 strain LSc by an acid guanidinium thiocyanate-phenol-chloroform method (6). An RNAgents total-RNA isolation kit (Promega Corp., Madison, Wis.) with a slight modification was used to extract the RNA. Briefly, 0.5 ml of a filtered (pore size, 0.2 μm) poliovirus suspension (5×10^6 PFU/ml) was concentrated to 150 μl with a Centricon-100 microconcentrator (Amicon, Inc., Beverly, Mass.). The concentrated poliovirus preparation was mixed with 3 ml of a denaturing solution and 0.24 ml of 2 M sodium acetate (pH 4.0). Then 2.4 ml of a phenol-chloroform-isoamyl alcohol mixture was immediately added to the virus homogenate, and the preparation was shaken. A biphasic state was obtained after centrifugation at $10,000 \times g$ for 20 min at 4°C, and the upper aqueous phase was mixed with an equal volume of cold isopropanol to precipitate the RNA. The RNA pellet obtained by centrifugation at $10,000 \times g$ for 15 min at 4°C was dissolved in 0.5 ml of the denaturing solution, and the RNA was reprecipitated with an equal volume of cold isopropanol. The resulting RNA pellet was washed with 75% ethanol, resuspended in 200 μl of diethylpyrocarbonate-treated deionized water, and stored at −20°C before analysis. In the extraction process, only RNase-free disposable plasticware and diethylpyrocarbonate-treated water were used in order to avoid RNA degradation. The extracted viral RNA was quantified with a UV spectrophotometer (model DU7400; Beckman Instruments, Inc., Fullerton, Calif.) by using a previously described protocol (14).

Seawater samples were collected from a coastal area in southern California. Filtered and unfiltered samples were used for the viral seeding experiment. The filtered sample was obtained by using a 0.2-μm-pore-size Nalgene disposable filter (Nalge Co., Rochester, N.Y.). Both filtered and unfiltered samples (25 ml each) were seeded with 750 ng of total viral RNA, and the preparations were incubated at 4°C and at room temperature (23 ± 1°C) for 35 days. Similar experiments with nonseeded controls were also performed in parallel at these two temperatures. Aliquots (2 ml) were collected from each of the samples at time zero, after 4 h, and after 1, 2, 3, 7, 14, 21, 28, and 35 days and were concentrated to 100 μl with Centricon-100 microconcentrators (Amicon, Inc.) at 4°C.

The RT-PCR was used to detect viral RNA in seeded and unseeded concentrates. All of the RT-PCR reagents were provided in a PCR RNA kit (Perkin-Elmer, Norwalk, Conn.), and the reactions were carried out in a GeneAmp PCR System

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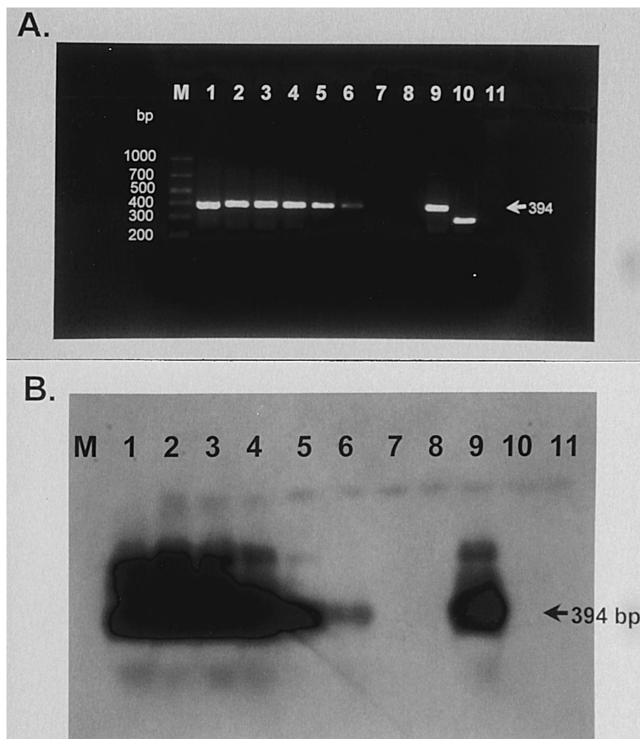

FIG. 1. Sensitivity of detection of total RNA extracted from a poliovirus filtered culture by the RT-PCR. Lane 1, 60 ng (2.5×10^4 PFU) of total RNA was used as the RT-PCR template; lane 2, 6 ng (2.5×10^3 PFU); lane 3, 600 pg (2.5×10^2 PFU); lane 4, 60 pg (2.5×10^1 PFU); lane 5, 6 pg (2.5 PFU); lane 6, 600 fg (2.5×10^{-1} PFU); lane 7, 60 fg (2.5×10^{-2} PFU); lane 8, 6 fg (2.5×10^{-3} PFU); lane 9, poliovirus (200 PFU); lane 10, RNA control from an RNA PCR kit (Perkin-Elmer); lane 11, negative control containing no template; lane M, molecular size standards (BioMarker Low; BioVentures, Inc., Murfreesboro, Tenn.). The PFU values given above for lanes 1 through 8 are the estimated PFU values before RNA extraction (assuming that the final RNA extract [6,000 ng/200 μl] was extracted from 2.5×10^6 PFU of poliovirus and only 2 μl was used for the RT-PCR). (A) Amplified RT-PCR products (394 bp) on an ethidium bromide-stained 2% SeaKem agarose gel. (B) Autoradiogram of the panel A gel produced by Southern blotting with a poliovirus RT-PCR internal probe and subsequent detection by chemiluminescence.

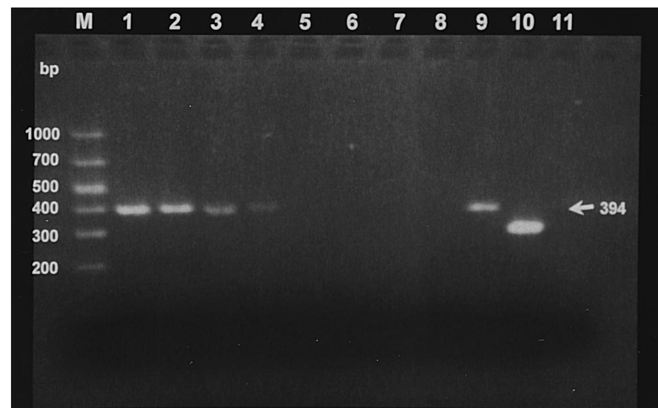

FIG. 2. RT-PCR and PCR of total RNA extracted from a poliovirus filtered culture. The amplified RT-PCR products (lanes 1 through 4) and the PCR products (lanes 5 through 8) were analyzed on an ethidium bromide-stained 2% SeaKem agarose gel. Lanes 1 and 5, 6 ng of total RNA was used as the RT-PCR template; lanes 2 and 6, 600 pg; lanes 3 and 7, 60 pg; lanes 4 and 8, 6 pg; lane 9, poliovirus (200 PFU); lane 10, RNA control from an RNA PCR kit (Perkin-Elmer); lane 11, negative control containing no template; lane M, molecular size standards (BioMarker Low; BioVentures, Inc.).

9600 thermocycler (Perkin-Elmer). Duplicate 2-μl samples of concentrate were used as the templates in each reaction, and random primers were used to initiate reverse transcription. The RT-PCR was performed as described previously (18), except that only one set of primers was used. Two primers for the poliovirus 5′ noncoding region (4, 15), primer Polio-R (5′-ACGGACACCCAAAGTA-3′) and primer Polio-L (5′-A GCACTTCTGTTTCCC-3′), were used to amplify a 394-bp sequence. An internal oligonucleotide probe (18) (POLIO-IN; 5′-ACATAAGAATCCTCCGGCCCCTGA-3′) was used to verify the amplified PCR products by DNA hybridization.

Both Southern blotting and dot blotting were used to transfer amplified DNA onto Hybond-N+ positively charged nylon membranes (Amersham, Arlington Heights, Ill.) with a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) and a Minifold I dot blotter (Schleicher & Schuell, Keene, N.H.), respectively. The blotted membranes were used for DNA hybridization, and chemiluminescent detection was performed as previously described (17, 18).

Figure 1 shows the detection sensitivity of the RT-PCR when poliovirus genomic RNA was used as the template. The results of both gel electrophoresis and Southern analysis demonstrated that the detection limit was 600 fg of viral RNA. Because the poliovirus which we used was propagated in Buffalo green monkey kidney cells, the extracted total RNA could have contained RNA released from these cells. The actual detection limit when the RT-PCR is used for viral RNA detection should be less than 600 fg. On the basis of the expected number of PFU in each serially diluted virus sample, the level of viral RNA detection sensitivity was 0.25 PFU (assuming that the final RNA extract [6,000 ng/200 μl] was extracted from 2.5×10^6 PFU of poliovirus and only 2 μl was used for the RT-PCR). Because we used reverse transcription of RNA, the sensitivity of detection by the RT-PCR was expected to be less than the sensitivity of detection by the PCR alone when other extracted genomic DNA was used (10 fg) (5).

Figure 2 shows the amplification products of viral RNA obtained with both the RT-PCR and the PCR. The amplified 394-bp DNA fragments were observed when the RT-PCR was used (Fig. 2, lanes 1 through 4) but not when the PCR was used (lanes 5 through 8). This indicated that the viral RNA was obtained during the extraction process and that the target noncoding region of the viral genomic RNA could be amplified only by RT-PCR. Therefore, reverse transcription of the genomic RNA into cDNA was required to obtain successful amplification of the target sequence by the PCR. No PCR products were found with RNase-digested templates (data not shown). Our results support the hypothesis that the poliovirus primer set is specific, because no cross-reactivity between viral RNA and possible contaminant Buffalo green monkey kidney cell RNA was observed in the RT-PCR.

A preliminary test revealed that viral RNA persisted longer in filtered seawater than in unfiltered seawater, as determined by the RT-PCR (data not shown). This finding was confirmed by the results of a detailed time course study. Figure 3 shows the stability of viral RNA in both filtered seawater and unfiltered seawater. The viral RNA was detected in filtered seawater at both room temperature (23 ± 1°C) and 4°C for up to 21 days by the RT-PCR but degraded after 28 days of incubation (Fig. 3A). In unfiltered seawater, RNA degradation was apparent after 2 days at both 23 and 4°C (Fig. 3B). Similar results were obtained when we used duplicate concentrates as templates for the RT-PCR. Although the initial RNA seeding concentration was 30 ng/ml, the amplified viral RNA was

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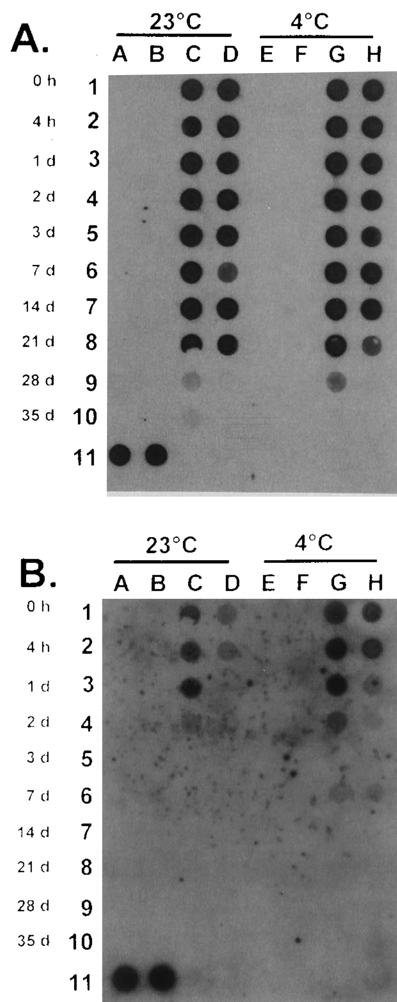

FIG. 3. Stability of viral RNA in filtered seawater and unfiltered seawater. Viral RNA was amplified by the RT-PCR and was subsequently analyzed by dot blotting with a poliovirus RT-PCR internal probe. The viral RNA was seeded into filtered seawater (A) and unfiltered seawater (B) and incubated at room temperature (23°C) (columns A through D) and 4°C (columns E through H) for up to 35 days. The initial seeding concentration was 30 ng of total RNA per ml of seawater. Columns A and E, unseeded water concentrate; columns B and F, 10-fold-diluted unseeded water concentrate; lanes C and G, RNA-seeded water concentrate; columns D and H, 10-fold-diluted RNA-seeded water concentrate. The dots in column A, row 11 show the results obtained with poliovirus RNA, and the dots in column B, row 11 show the results obtained with poliovirus.

found at higher concentrations at 4°C than at 23°C. This suggested that the higher temperature facilitated the degradation of naked RNA in seawater. We previously reported that poliovirus virions could not be detected after 7 days of incubation at 25°C, nor were they detected after 21 days at 4°C by the RT-PCR (17). Because the extracted viral RNA was not protected by a protein coat, it was more easily degraded by RNases in the environment than intact virus. The detection limit for total RNA was 600 fg, and the seeding results indicated that after 2 days at 23°C the RNA could not be detected (concentration, <600 fg). This conclusion assumes that the initial seeded RNA concentration was 60 ng per 2-ml sample and that only 2% of the resulting concentrate (100 μl) was tested by the RT-PCR. Therefore, there was a 2×10^3-fold reduction in the target viral RNA concentration within 2 days in unfiltered nonsterile seawater. However, it took 28 days to obtain a similar reduction in the target viral RNA concentration in filter-sterilized seawater. It has been reported by several investigators that the rate of turnover of mRNA in prokaryotes is high (3, 8, 13, 16). Although the genomic RNA of poliovirus could be more stable than mRNA, it was still subjected to degradation by endonucleases and exonucleases of different microfloras in the seawater. This finding is supported by the results of Fujioka et al. (9), who reported that antiviral activity of seawater was related to the growth activities of microorganisms and that this activity was lost after the seawater samples were subjected to autoclaving or filter sterilization.

In conclusion, in this study we used the RT-PCR to show that poliovirus genomic RNA was not stable in nonsterilized seawater. Because poliovirus is a single-stranded RNA virus, this conclusion should be applied only to single-stranded viral RNAs, such as RNAs from hepatitis A virus, Norwalk virus, human immunodeficiency virus, etc. Viral RNA could be detected by the RT-PCR in filter-sterilized seawater for a longer period of time. This observation indicated that the major cause of viral RNA degradation in the seawater was indigenous microorganisms. However, the single-stranded RNA of poliovirus may not reflect the stability of rotavirus double-stranded RNA. The positive RT-PCR results obtained with samples collected from seawater or other environmental sources indicated the presence of protein-coated virions but not free single-stranded viral RNA.

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