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Presence of Human Immunodeficiency Virus Nucleic Acids in Wastewater and Their Detection by Polymerase Chain Reaction†

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The human immunodeficiency virus type 1 (HIV-1) released by infected individuals or present in human and hospital wastes can potentially cause contamination problems. The presence of HIV-1 was investigated in 16 environmental samples, including raw wastewater, sludge, final effluent, soil, and pond water, collected from different locations. A method was developed to extract total nucleic acids in intact form directly from the raw samples or from the viral concentrates of the raw samples. The isolated nucleic acids were analyzed for the presence of HIV-1 by using in vitro amplification of the target sequences by the polymerase chain reaction (PCR) method. HIV-1-specific proviral DNA and viral RNA were detected in the extracted nucleic acids obtained from three wastewater samples by this method. The specificity of the PCR-amplified products was determined by Southern blot hybridization with an HIV-1-specific oligonucleotide probe, SK19. The isolated nucleic acids from wastewater samples were also screened for the presence of poliovirus type 1, representing a commonly found enteric virus, and simian immunodeficiency virus, representing, presumably, rare viruses. While poliovirus type 1 viral RNA was found in all of the wastewater samples, none of the samples yielded a simian immunodeficiency virus-specific product. No PCR-amplified product was yielded when wastewater samples were directly used for the detection of HIV-1 and poliovirus type 1. The wastewater constituents appeared to be inhibitory to the enzymes reverse transcriptase and DNA polymerase. The method developed was highly sensitive and detected HIV-1 in amounts equivalent to as little as 0.04 and 0.4 pg of P24 antigen (0.7 and 7 cpm of reverse transcriptase activity, respectively) in sterile water and the viral concentrates, respectively. Although these findings suggest persistence of HIV-1 in wastewater, further investigation is warranted to determine the fate of HIV-1 in the environment.

Human immunodeficiency virus type 1 (HIV-1) has been detected in various body fluids, such as saliva (9, 10), tears (8), breast milk (27), semen (11, 17, 32), and cervical secretions (28, 30), and in feces (29, 31). However, transmission of HIV-1 is known to occur only through sexual contact, via parenteral exposure to blood or blood products, and perinatally from mother to fetus (14–17, 33).

Pathogenic viruses which are shed in feces by infected individuals have frequently been detected in water systems (6) and have been implicated in many waterborne viral diseases (12). Yolken and his coworkers (31) have suggested that fecal shedding of HIV-1 may play a role in HIV-1 transmission in environments prone to high levels of fecal and orally transmitted contamination. Transmission of an infectious agent through environmental routes requires that the agent be able to persist (1). Therefore, studies regarding the fate of HIV-1 in various environmental and engineering systems should help in determining the potential of HIV-1 transmission in the environment.

HIV-1 has been reported to survive on inanimate surfaces (3, 25) and remains infectious for more than 2 weeks at room temperature in cell culture suspensions (25). The survival capacity of HIV-1 in seeded sewage samples was reported to be fairly stable for up to 12 h at 25°C, and after 48 h, a 10^2- to 10^3-fold reduction in HIV-1 titer was observed (4). In another study, the seeded virus was detectable for up to 11 days in sewage samples (26). A recent report has indicated the presence of HIV-1 sequences in viral concentrates of wastewater (23). However, knowledge regarding the fate of HIV-1 in environmental systems remains limited. This is largely due to the lack of efficient methods for detection of HIV-1 in environmental samples. In this report we describe an efficient method for isolation of total nucleic acid from environmental samples and provide evidence for the presence of HIV-1-specific sequences in wastewater, using the polymerase chain reaction (PCR) method.

MATERIALS AND METHODS

Environmental samples. Samples of raw wastewater entering the treatment plant (10 to 20 liters), mixed liquor-suspended solids from an oxidation ditch (sludge) (5 liters), final effluent (5 to 10 liters), pond water (5 liters), and soil (1 kg) were collected in sterile containers from various locations as shown in Table 1. The average solid contents (wet) of wastewater, sludge, and pond water were 1.25, 2.6, and 0.2%, respectively. It was assumed that the composition of wastewater would vary from time to time. Therefore, samples were collected at different time periods to ensure random sampling. Samples were transported to the laboratory under refrigerated conditions, stored at 4°C, and used within 24 h.

Concentration of viruses from wastewater. Viruses were

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TABLE 1. Environmental samples

Sample: No.	Description	Location	HIV-1 result[a]
1	Wastewater	Belle Glade, Fla.	−
2	Sludge	Belle Glade, Fla.	−
3	Wastewater	Belle Glade, Fla.	+
4	Wastewater	Belle Glade, Fla.	+
5	Wastewater	Kanapaha, Fla.	−
6	Sludge	Kanapaha, Fla.	−
7	Wastewater	Vero Beach, Fla.	−
8	Sludge	Pontiac, Mich.	−
9	Wastewater	Pontiac, Mich.	+
10	Wastewater	Pontiac, Mich.	−
11	Sludge	Pontiac, Mich.	−
12	Final effluent	Pontiac, Mich.	−
13	Pond water	Rochester, Mich.	−
14	Pond water	Rochester, Mich.	−
15	Soil	Rochester, Mich.	−
16	Soil	Rochester, Mich.	−

[a] +, detected; −, not detected.

concentrated 100-fold from raw wastewater by the aluminum flocculation method as described previously (7). Briefly, wastewater samples were adjusted to 3 mM $AlCl_3$ with 1 M $AlCl_3$, and the pH was adjusted to 6.3 with 1 M sodium bicarbonate to allow flocculation of the viruses. The floc was then sedimented by centrifugation at 7,000 × *g* for 5 min and resuspended in 3% beef extract containing 0.5% Tween 80, and the viruses were eluted by adjusting the pH to 9 and shaking the mixture at 60 rpm for 15 min at room temperature. The beef extract was centrifuged at 14,000 × *g* for 10 min, and the supernatant containing the viruses was collected and neutralized with 2 N HCl. This method was also used to recover viruses from pond water.

Isolation of total nucleic acid. A modified method (2) was used to isolate total nucleic acid in intact form from the environmental samples. Briefly, a 100-ml sample was mixed thoroughly in a final concentration of 1% of sodium dodecyl sulfate (SDS)–20 mM EDTA–1 M NaCl, and a 10% CTAB-NaCl (hexadecyltrimethyl ammonium bromide-sodium chloride) solution was added to a final concentration of 1%. The mixture was then extracted by using 15 ml of phenol-chloroform-isoamyl alcohol (25:24:1) at 65°C. The extraction with phenol-chloroform-isoamyl alcohol was repeated once, and the aqueous phase was saved. The DNA from the organic phase was separately extracted with an equal volume of 1 M uncalibrated Tris. Nucleic acids from both aqueous and organic phases were pooled, extracted by chloroform-isoamyl alcohol (24:1), and precipitated by using a 0.7 volume of isopropanol. The pellet was washed with 70% ethanol and dried under vacuum (SpeedVac SVC100; Savant Scientific Instruments, Farmingdale, N.Y.). The dried nucleic acids were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). All of the reagents used for isolation of total nucleic acid were prepared in diethyl pyrocarbonate (DEPC)-treated, autoclaved, deionized water. The glassware was treated overnight in DEPC-treated water and baked at 180°C for 12 h. This practice ensured the isolation of RNA without noticeable degradation.

Soil and sludge samples were first suspended in TE buffer and urea-lysine (4 M urea, 0.5 M lysine), respectively, stirred constantly for 10 min, and left on ice for 10 min for sedimentation of soil particles. The nucleic acid was isolated from the supernatant by using the procedure described above.

Synthesis of oligonucleotides. The primers and probe used in this study are given in Table 2. The primer pairs SK38-SK39 and SK68-SK69 and a probe, SK19, specific to HIV-1 have been used in several studies (22). The primers specific to simian immunodeficiency virus macaque 239 (SIV_{mac239}) and poliovirus type 1 were chosen from the published nucleotide sequences of these viruses (21, 24). The primers and probe were chemically synthesized on a DNA synthesizer (Cyclon Plus; Milligen/Bioresearch, Burlington, Mass.) or purchased commercially (Keystone Laboratories Inc., Menlo Park, Calif.).

Amplification of DNA and RNA. To amplify DNA, a typical PCR mixture included 50 mM KCl, 10 mM Tris-HCl (pH 8.13), 1.5 mM $MgCl_2$, 0.001% gelatin, 25 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 25 ng of each primer, 2.5 U of *Taq* polymerase, and 1 to 2 μg of the isolated nucleic acid from the environmental samples or 2 ng of the plasmid pBH10-R3 DNA in a final volume of 100 μl, unless otherwise stated. The PCR mixture was then overlaid with 100 μl of mineral oil (Fisher Scientific, Fair Lawn, N.J.). The PCR mixture was heated to 94°C for 4 min and then amplified for 35 cycles at 94°C (1 min), 50°C (1 min), and 72°C (2 min) in a thermal cycler (thermal cycler, GTC2; Precision Scientific, Chicago, Ill.). The final chain elongation step was extended to 7 min. PCR was also routinely performed with no template in the reaction mixture to check for contamination.

To detect the presence of viral RNA, samples were reverse transcribed in 24 μl of the following reverse transcription (RT) assay mixture: 100 mM Tris-HCl (pH 8.4), 130

TABLE 2. Oligonucleotides used in this study

Primer	Target virus	Sequence (5′–3′)	Position	Reference
SK38	HIV-1	ATAATCCACCTATCCCAGTAGGAGAAAT	1551–1578	22
SK39	HIV-1	TTTGGTCCTTGTCTTATGTCCAGAATGC	1638–1665	22
SK19 (probe)	HIV-1	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC	1595–1635	22
SK68	HIV-1	AGCAGCAGGAAGCACTATGG	7801–7820	22
SK69	HIV-1	CCAGACTGTGAGTTGCAACAG	7922–7942	22
S3	SIV_{mac239}	CCTGGAGGAGGAGATCCGGAAGT	7744–7766	24
S4	SIV_{mac239}	AGGTCTCCCTCTCTTAGGGGC	7953–7973	24
P1	Poliovirus type 1	ACACATCAGAGTCGTGT	3271–3287	21
P2	Poliovirus type 1	TGCACCTTGCGATTGAATCGGT	3531–3551	21

mM KCl, 10 mM $MgCl_2$, 100 mM dithioerythreitol, 50 μM deoxynucleoside triphosphates, 25 ng of negative-sense primer (SK39 or SK69 for HIV-1, S2 for SIV_{mac239}, and P2 for poliovirus type 1), 20 U of ribonuclease inhibitor (RNasin; Promega Corp., Madison, Wis.), and 10 U of reverse transcriptase from avian myeloblastosis virus (Promega Corp). The RT mixture was heated at 65°C for 2 min before the enzymes were added and incubated at 42°C for 1 h. The reverse-transcribed material was then supplemented with 3.5 μl of 5 M KCl, 1 μl of 50 μM deoxynucleoside triphosphates, 25 ng of the positive-sense primer (SK38 or SK68 for HIV-1, S3 for SIV_{mac239}, and P1 for poliovirus type 1), and 0.5 μl (2.5 U) of *Taq* DNA polymerase, and the volume was adjusted to 100 μl and subjected to PCR amplification as described above. To check for contamination, an RT-PCR mixture without template was simultaneously run under the same conditions. Poliovirus type 1, grown in BGMK cells, was used to standardize the RT-PCR amplification protocol.

To determine the specificity and sensitivity of the RT-PCR amplification procedures, pBH10-R3 containing HIV-1 proviral DNA and cell-free HIV-1 ($HIV\text{-}1_{SF2}$) were obtained from Robert C. Gallo, National Institutes of Health Bethesda, Md., and from Jay Levy (18), AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, Md., respectively. SIV_{mac239} was kindly supplied by Ronald C. Desrosiers (New England Primate Research Center, Southborough, Mass.).

Analyses of PCR products. Ten microliters of the PCR product was fractionated on 2% agarose gel (Fisher Scientific, Springfield, N.J.) by electrophoresis and visualized under UV light after being stained with ethidium bromide.

For Southern blot hybridization, the gel-fractionated PCR product was transferred onto a GeneScreen Plus membrane (DuPont Biotechnology Systems, Boston, Mass.) following the procedures described by the manufacturer. The blot was baked at 80°C for 2 h and kept in the prehybridization solution containing 1% SDS, 1 M NaCl, 10% dextran sulfate, and 200 μg of fish sperm DNA (United States Biochemicals, Cleveland, Ohio) per ml at 42°C with constant, gentle agitation. After 6 h, ^{32}P-labeled oligonucleotide probe (SK19) was added to the mixture, and the mixture was further incubated for 10 h. The blot was then washed once with 100 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature with constant agitation, twice with 100 ml of 2× SSC with 1% SDS at 65°C for 30 min, and twice with 100 ml of 2× SSC at room temperature for 15 min. The washed blot was kept at room temperature for 2 min to dry, wrapped with Saran Wrap, and autoradiographed on Kodak XAR-5 X-ray film. The probe was prepared by 5′-end labeling of SK19 oligonucleotide with [^{32}P]ATP (NEN, DuPont, Boston, Mass.) and T4 polynucleotide kinase (Promega Corp.).

Sensitivity of PCR method. To determine the sensitivity of the RT-PCR method, viral concentrate of wastewater and raw wastewater samples with no detectable amount of HIV-1 were inoculated with known concentrations (10-fold serial dilutions) of HIV-1 (ranging from 0.0004 to 400 pg of P24 antigen [0.007 to 7×10^3 cpm of equivalent reverse transcriptase activity]). Aliquots of the spiked samples were analyzed either directly or after extraction of nucleic acid. Similarly, HIV-1 was also spiked into sterile water to compare the detection efficiency of the method for the viral concentrate and wastewater samples.

To analyze the sample directly, aliquots (1 to 10 μl) of the seeded samples were treated with 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) for 5 min at room temperature and then subjected to RT-PCR amplification as described above. For the indirect method, the nucleic acid extracted from the samples was used for RT-PCR amplification.

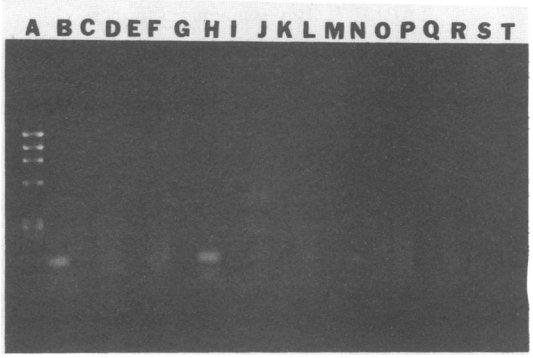

FIG. 1. Agarose gel (2%) electrophoresis of the PCR-amplified products of environmental samples with HIV-1-specific primer pair SK38-SK39. The isolated nucleic acid was denatured at 94°C for 4 min and used for PCR amplification at a 37°C annealing temperature, as described in Materials and Methods, and 10 μl of the amplified product was analyzed. Shown are size marker ϕ-X174 digested with *Hae*III (lane A); amplified product (115 bp) of plasmid pBH10-R3, positive control, (lane B); unamplified template of samples 1, 2, 3, 4, 7, and 9, (lanes C, E, G, I, K, and M, respectively); amplified samples 1, 2, 3, 4, 7, 9, 10, 11, 12, 13, and 15 (lanes D, F, H, J, L, N, O, P, Q, R, and S, respectively); and the negative control without template (lane T). The numbers represent the samples given in Table 1.

RESULTS

Extraction of nucleic acids. Initially, several methods were tested to extract total nucleic acids from the environmental samples (2, 23). These methods were found inefficient; either the nucleic acid was degraded or the yield was poor, especially in the case of raw wastewater and sludge samples. Therefore, a modified method (2) which consistently yielded large amounts of intact nucleic acid (1 to 2 mg/liter) was developed. The extraction of nucleic acid with hot phenol-chloroform-isoamyl alcohol immediately following SDS-EDTA-NaCl-CTAB treatment was found to be a crucial and important step of this procedure. This step presumably inactivated nucleases in the samples and thus protected the nucleic acids during the extraction.

Proviral DNA. To determine the presence of HIV-1-specific sequences, first, PCR experiments were carried out at a low annealing temperature (37°C) and with larger amounts of sample with primer pair SK38-SK39 (Table 2) in order to maximize the chances of detection of the target nucleic acid. These results (Fig. 1) showed the presence of a single band of the PCR-amplified products in two of the samples (samples 3 and 9, lanes H and N, respectively) and one of the samples had three bands (lane J). A 115-bp band was commonly found in three of the wastewater samples and in the pBH10-R3 control. The primer pair SK38-SK39 has been reported to amplify the 115-bp target of the *gag* region of HIV-1 (22). Conceivably, >115-bp bands found in sample 4 were the product of nonspecific amplification. All other samples showing no amplified product had smearing, resulting from the dimer and oligomer formation of the primers. Primer dimerization and nonspecific amplification have been reported in PCR with a high concentration of primer, template, and low annealing temperature (13). When the anneal-

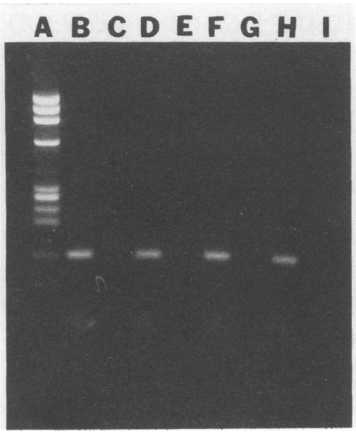

FIG. 2. Agarose gel (2%) electrophoresis of the PCR-amplified product representing wastewater samples with primer pair SK38-SK39 as described in the legend to Fig. 1. Shown are size marker φX174 digested with *Hae*III (lane A); amplified product of plasmid pBH10-R3, positive control (lane B); amplified plasmid pBH10-R3, positive control (lanes B and H); primer pair SK38-SK39 (lane C); unamplified template of samples 4 and 9 (lanes E and G, respectively); amplified samples 3, 4, and 9 (lane D, F, and H, respectively); and the negative control without template (lane I). The numbers represent the samples given in Table 1.

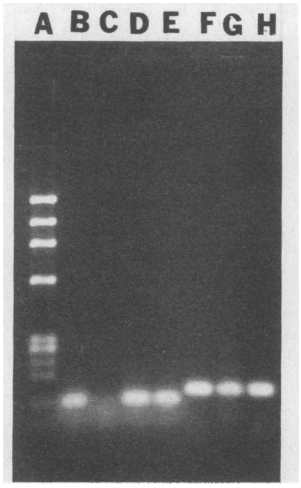

FIG. 3. Agarose gel (2%) electrophoresis of the PCR-amplified product of wastewater samples with primer pairs SK38-SK39 and SK68-SK69 specific for the *gag* and *env* regions of HIV-1, as shown in Fig. 2. Shown are size marker φX174 digested with *Hae*III (lane A); amplified products (115 and 142 bp) of positive control plasmid pBH10-R3, with use of primer pairs SK38-SK39 and SK68-SK69, respectively (lanes B and F, respectively); PCR without template, negative control (lane C); amplified products (115 bp) of samples 3 and 9 with use of primer pair SK38-SK39 (lanes D and E, respectively); and amplified products (142 bp) of samples 3 and 9 with primer pair SK68-SK69 (lanes G and H, respectively). The numbers represent the samples given in Table 1.

ing temperature and Mg^{+2} concentration were increased to 50°C and 3.0 mM, respectively, and the primer concentration was reduced to 25 ng, not only the nonspecific bands disappeared but also the PCR product was increased significantly (Fig. 2). The primer pair SK38-SK39, however, failed to amplify any DNA from the other 13 samples under these conditions. These results suggest that the proviral DNA complementary to the *gag* region was present in wastewater samples 3, 4, and 9 (Fig. 2, lanes D, F, and H, respectively).

In order to determine whether other regions of proviral DNA are also present in these samples, the samples were subjected to PCR amplification with a second primer pair, SK68-SK69, targeting the *env* region of HIV-1. This primer pair also yielded a product of 142 bp only in those samples which had shown 115-bp bands with primer pair SK38-SK39 (Fig. 3).

To test whether the amplified products were from the DNA in the samples and not an artifact of PCR, both known and unknown samples showing the specific PCR-amplified products were treated with DNase. The DNase was inactivated by heating at 90°C for 15 min or extraction with phenol-chloroform-isoamyl alcohol (25:24:1), and the samples were subjected to PCR amplification. No amplified products were found in DNase-treated samples, as expected (data not shown).

Viral RNA. The wastewater samples were also analyzed for the presence of viral RNA. The samples were first treated with DNase, reverse transcribed, and then subjected to PCR amplification as described above. The results shown in Fig. 4 revealed that samples 3, 4, and 9 (lanes E, H, and K, respectively) contained HIV-1 RNA as well. The amplified products in these samples were similar in size (142 bp), as resulted from the authentic HIV-1 (Fig. 4, lane B); then, primer pair SK68-SK69 was used for PCR amplification. Similarly, RT-PCR amplification with primer pair SK38-SK39 resulted in a product of the desired 115-bp size from samples 3, 4, and 9 (data not shown). When the samples including HIV-1 control were treated with both DNase and RNase, no amplified target product was observed. Enteric virus such as poliovirus type 1 is commonly found in wastewater and the method developed here detected a poliovirus-specific PCR product (281 bp) in all the wastewa-

FIG. 4. Agarose gel (2%) electrophoresis of the RT-PCR-amplified products of HIV-1, poliovirus type 1, and SIV_{mac239}. The isolated nucleic acid was reverse transcribed with the negative-sense primers SK69, P2, and S4 of HIV-1, poliovirus type 1, and SIV_{mac239}, respectively, and amplified by PCR as described in Materials and Methods. Shown are size marker φX174 DNA digested with endonuclease *Hae*III (lane A); HIV-1 (lane B); unamplified samples 3, 4, and 9 (lanes C, F, and I, respectively); samples 3, 4, and 9 amplified after DNase and RNase treatment (lanes D, G, and J, respectively) and after DNase treatment only (lanes E, H, and K, respectively); poliovirus type 1 (lane L); samples 3, 4, and 9 with poliovirus-specific primer pair P1-P2 (lanes M, N, and O, respectively); SIV_{mac239} RNA (lane P); samples 3, 4, and 9 with primer pair S3-S4 (lanes O, R, and S, respectively); and negative control without template (lane T). The numbers represent the samples given in Table 1.

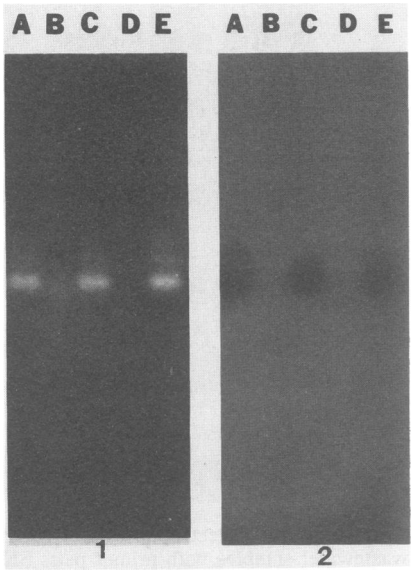

FIG. 5. Southern blot analysis of the PCR-amplified products of samples 3 and 9 with ^{32}P-labeled probe SK19. Panel 1 is an agarose gel (2%) stained with ethidium bromide following electrophoresis; panel 2 is a Southern blot. Shown are the 115-bp PCR product of the plasmid pBH10-R3 (lane A); primer pair SK38-SK39 only (lane B), unamplified template of sample 9 (lane D), and PCR-amplified products of samples 3 and 9 (lane C and E, respectively). The numbers represent the samples given in Table 1.

ter samples (samples 1, 3, 4, 5, 7, 9, and 10). The poliovirus-specific PCR product was not found in other environmental samples (data not shown), whereas SIV_{mac239} representing rare viruses in the environmental samples was not detected in any of the environmental samples when the samples were screened with the SIV-specific primer pair (Fig. 4; only samples 3, 4, and 9 are shown).

Southern blot hybridization. To verify the specificity of the PCR-amplified product from the wastewater samples, the product generated by primer pair SK38-SK39 was analyzed by Southern hybridization with the ^{32}P-labeled probe (SK19). The results of this experiment (Fig. 5) showed hybridization of the HIV-1-specific amplified product with the probe SK19.

Sensitivity of the assay. In this study HIV-1 sequences were found only in wastewater and not in other environmental samples (Fig. 1). Therefore, the sensitivity of the method developed for the detection of HIV-1 by PCR was investigated by seeding wastewater samples and viral concentrates with a known amount of HIV-1. Sterile water samples were also spiked for comparison. The seeded samples were analyzed by RT-PCR as such (direct method) or after extraction of total nucleic acid (indirect method). The results of the direct method show that an amount of HIV-1 equivalent to 0.04, 0.4, and 400 pg of P24 antigen (0.7, 7, and 7×10^3 cpm of equivalent reverse transcriptase activity, respectively) can be detected in the sterile water, in viral concentrates of wastewater, and in raw wastewater samples, respectively (Fig. 6, lanes F, L, and M, respectively). The poor detection in wastewater and viral concentrate could be attributed to the inhibition of RT and DNA polymerase activity by the components of these samples.

However, the sensitivity of the detection method was improved 10^2-fold when the seeded wastewater samples were first extracted with the hot phenol-chloroform-isoamyl

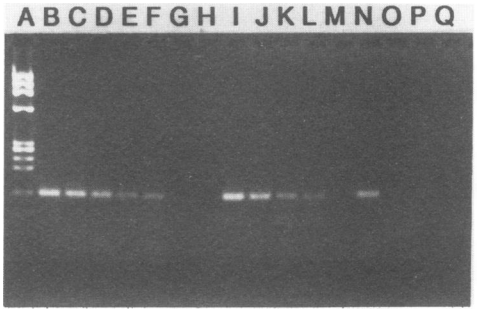

FIG. 6. Agarose gel (2%) electrophoresis of the RT-PCR-amplified products of HIV-1-spiked sterile water, viral concentrate of wastewater and raw wastewater with primer pair SK38-SK39. A 10-fold dilution of a known amount of HIV-1 was prepared in sterile water, viral concentrate of wastewater, and raw wastewater. An aliquot (1 to 10 μl) of the sample was subjected to RT-PCR amplification, as described in Materials and Methods. Shown are size marker φX174 DNA digested with endonuclease *Hae*III (lane A); HIV-1 in amounts equivalent to 400, 40, 4, 0.4, 0.04, 0.004, and 0.0004 pg of P24 antigen either in sterile water (lanes B, C, D, E, F, and G, and H, respectively) or in viral concentrate of wastewater (lanes I, J, K, L, and M, respectively); HIV-1 in amounts equivalent to 400, 40, and 4 pg of P24 antigen in raw wastewater (lanes N, O, and P, respectively); and RT-PCR mixture without template (lane Q).

alcohol for the isolation of nucleic acid. HIV-1 equivalent to 4 pg of P24 antigen was detected in the spiked wastewater when the RT-PCR amplification was carried out with nucleic acid extracts. This increased sensitivity may be because of the removal of inhibitory components from wastewater during nucleic acid extraction steps.

DISCUSSION

The results of this study clearly demonstrate the presence of both proviral and viral nucleic acids of HIV-1 in three wastewater samples collected from two different locations. The viral nucleic acid was not detected in 13 other environmental samples (Fig. 1). Since enteric viruses are commonly found in wastewater, we used poliovirus type 1 as a representative to test our method. All of the wastewater and sludge samples, but not the final effluent, pond water, and soil samples, were found to be positive for poliovirus type 1 both by the RT-PCR (Fig. 4) and by tissue culture assay (data not shown). However, SIV_{mac239} used as a representative of rare viruses was not detected in any of the samples (Fig. 4). The PCR-amplified products from samples 3, 4, and 9 were found to be specific to HIV-1, as judged by Southern hybridization (Fig. 5). These results suggest that this method was highly specific and efficient for the detection of HIV-1 and poliovirus type 1 in wastewater samples.

This is the first instance where HIV-1 was detected in wastewater by the PCR method. An earlier report also suggested the presence of homologous sequences of HIV-1 in wastewater samples by hybridization (23). However, in that study the hybridization signals obtained were weak, and a large amount of wastewater sample was required to be processed. In contrast, the method described here with the PCR is more sensitive, specific, and reproducible in detecting HIV-1 and poliovirus type 1 in the samples. The RT-PCR protocol developed during this study could detect as little as 0.04, 0.4, and 400 pg of P24 antigen (0.7, 7, and 7×10^3 cpm of equivalent reverse transcriptase activity) of HIV-1 when

the spiked samples of sterile water, viral concentrate, and raw wastewater, respectively, were used directly for the amplification of the target sequences. Amplification of the target sequences at a very high concentration (400 pg) of the virus in raw wastewater was presumably possible because of the slight activity of reverse transcriptase and DNA polymerase in spite of the inhibitory elements present in the samples. However, such large amounts of HIV-1 are unlikely to be present in even highly polluted wastewater. Therefore, direct detection of HIV-1 sequence in wastewater is not feasible yet. The method developed in this study for the extraction of nucleic acids from environmental samples eliminated inhibitory elements for RT-PCR quite effectively and allowed the detection of HIV-1 in amounts comparable to picogram quantities of P24 antigen of HIV-1 in the samples (Fig. 6). It was observed that isolation of nucleic acid in intact form was very important for the detection of the target sequences by RT-PCR. If the nucleic acids were degraded during the isolation, no amplification of the target sequences was achieved by PCR. More recently, this method was also found to be equally efficient in detecting HIV-1 sequences in clinical samples, which were kindly provided by D. Wiedbrauk, William Beaumont Hospital, Royal Oak, Mich. (data not shown). Therefore, the methods developed in this study for the detection of HIV-1 in wastewater samples can also be potentially applied to investigate other human pathogenic viruses in various environmental systems. Since the PCR technique utilizes only the nucleic acids for detection, it is a relatively safe method for such investigations.

Although the presence of HIV-1 and poliovirus type 1 nucleic acids in wastewater does not necessarily indicate the presence of infectious viral particles, cell culture assays using BGMK cells detected poliovirus plaques in viral concentrates of wastewater and sludge samples (data not shown). Because HIV-1 is a highly infectious agent, the presence of viable HIV-1 in wastewater even in small amounts may not be desirable. The inability to detect HIV-1-specific sequences in the environmental samples may be due to the low numbers of the viruses not detectable by this method.

The relative significance of this study should be considered in the light of increasing cases of HIV-1 infection in the population. Shedding of HIV-1 nucleic acids in stools (31), urine (19), and menstrual blood of symptomatic and asymptomatic patients (28, 30) could be the major sources of HIV-1 nucleic acids in wastewater. Hospital wastes could be another important source of HIV-1 in the environment. More importantly, with the increase in the number of AIDS patients, the burden of HIV-1 in environmental systems is likely to increase, and recent reports have suggested that HIV-1 remains infectious at room temperature either in dry form or in liquid medium (3) and persists in the wastewater for several days (4, 26).

We hope these findings will provide an impetus for further investigations on the fate of HIV-1 not only in wastewater but also in other environmental systems.

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