Discrimination of adenovirus types circulating in urban sewage and surface polluted waters in São Paulo city, Brazil

Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Caixa Postal 66.208, CEP 05389-970, São Paulo, SP, Brazil
(E-mail: fabianamasantos@bol.com.br; mojuvi@ig.com.br; patthygarrafa@bol.com.br; telmaamo@usp.br; vhpelliz@usp.br; chmharsi@icb.usp.br; dumehner@icb.usp.br)

Abstract Owing to frequent overflow of sewage-polluted creeks and rivers in São Paulo city, the population that lives in those surrounding areas is constantly exposed to a high risk of infection by many pathogens. Our aim was to evaluate the occurrence of human adenovirus, especially F species (HAdV-40 and HAdV-41), in sewage and creek water in São Paulo city. A total of 95 water samples were collected from July 1998 to June 1999 and from September 2000 to December 2001 at Sewage Pumping Station Edu Chaves and Creek Pirajussara. Four-litre samples were concentrated by a two-step procedure based on filtration through electropositive filter membrane and ultracentrifugation. Adenoviruses were detected in 69 samples (72.6%) after amplification of a sequence of the hexon gene by PCR. The discrimination of human adenovirus F was done by RFLP. A first screening was performed by restriction of PCR product with TaqI, followed by the discrimination of HAdV-40 and HAdV-41 by RsaI restriction. Sixty-two (89.8%) samples showed F species viruses. Among them, human adenovirus, serotype 41, was the most frequently detected in 17 (62.9%) out of 27 samples and serotype 40 was identified only in 7 (25.9%) samples. No seasonal distribution of adenoviruses was observed.

Keywords Human adenovirus F; PCR; RFLP; sewage; surface water

Introduction
Many human enteric viruses have been detected in sewage, surface and drinking water, and the public health risks of such agents have been evaluated. On the other hand, the virus detection and identification methods that should be routinely applied for water treatment monitoring are still not feasible enough, especially for developing countries.

Among human enteric viruses, adenoviruses (HAdV) are a common cause of infantile gastroenteritis (Uhnoo et al., 1984). Many serotypes are difficult to cultivate in regular cell lines, being considered fastidious like the human adenovirus F, serotypes 40 and 41. For this reason their presence in polluted water and their role as originators of gastroenteritis have probably been underestimated (Irving & Smith, 1981; Krikelis et al., 1985).

The polymerase chain reaction (PCR) has become a rapid and sensitive alternative for identification of some HAdV serotypes in clinical samples (Allard et al., 1990, 1994; Pring-Åkerblom et al., 1997, 1999).

Recently, the detection of adenoviruses by PCR methodology has attracted much attention for the evaluation of water quality, because they are well characterized for nucleic-acid-based detection methods and are more resistant to inactivation than enteroviruses in various environments, including wastewater, seawater and tap water (Puig et al., 1994; Enriquez et al., 1995; Castignolles et al., 1998; Cho et al., 2000).

Adenoviruses have been detected worldwide in higher prevalence and greater numbers than enterovirus in raw sewage worldwide (Irving & Smith, 1981; Krikelis et al., 1985). Owing to the involvement of human adenovirus F in infantile gastroenteritis and to the risk
of waterborne transmission, the discrimination of enteric serotypes (HAdV40 and HAdV41) from all others is an essential requirement for water quality control, public health risk assessment and molecular epidemiological studies. However, the presence of human adenoviruses A to E should not be disregarded in water samples. Some authors reported the occurrence of conjunctivitis outbreaks after recreation in swimming pools and lagoons (D’Angelo et al., 1979; Martone et al., 1980; McMillan et al., 1992).

In Brazil, domestic sewage is commonly discharged without treatment into creeks and rivers, especially in the suburban areas. Owing to frequent overflow of such waters during the rainy season, the population is constantly exposed to a high risk of infection by many pathogens. Infectious rotavirus particles were detected year-round in such waters in São Paulo city (Mehnert & Stewien, 1993; Queiroz, 1999). Hepatitis A viruses, certainly infectious, were also detected at the same sites (Sassaroli et al., 1999). No data are available about the presence and the distribution of adenoviruses in sewage and surface polluted water in Brazil. Thus, the aim of the present research was to evaluate the occurrence of those viruses, especially the enteric HAdV-40 and HAdV-41, in raw sewage and creek water in São Paulo city.

Materials and methods
Water samples
A total of 95 samples of raw domestic sewage and sewage-polluted creek water was collected in the city of São Paulo during 1998–1999 and from September 2000 to December 2001 at two different sites: Sewage Pumping Station Edu Chaves (SPS Edu Chaves) and Pirajussara Creek, located on north and south districts of the city, respectively. Samples were collected in sterile 4-litre containers and transported back to the lab at room temperature for processing. To minimize the effects of diurnal variations, all samples were collected at the same place, between 8 and 9 a.m. on weekdays. Escherichia coli were determined by Multiple-Tube Fermentation Technique in 100 ml samples by using Colilert (IDEXX, Co., USA) as recommended by APHA (1998).

Cell cultures and viruses
Adenovirus prototypes of human adenovirus A (HAdV-31), B (HAdV-3), C (HAdV-5), D (HAdV-9) were originally obtained from National Public Health Laboratory, London, UK and the F prototypes (HAdV-40 and HAdV-41) from C.D.C., Atlanta, USA.

All prototypes were maintained in HEp-2 cells, but HAdV40, that was cultivated in PLC/PRF/5 cells. Both cell lines were grown in Eagle’s minimum essential medium with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 100 U of penicillin G/ml and 100 µg/ml of streptomycin. Virus seeds were inoculated onto 75% confluent monolayers of either cell line and incubated for 48 to 72 hours on 5% CO₂ atmosphere.

Sample processing
For virus concentration, a two-step procedure described by Mehnert et al. (1997) was used with minor modifications (Queiroz et al., 2000). Detoxification of concentrates was done by organic extraction using Vertrel-XF (1,1,1,2,3,4,4,5,5,5,5,5-decafluoropentane, catalog no. 138495-42-8; Du Pont Fluoroproducts, Wilmington, Del.) as previously described (Queiroz et al., 2001). Afterwards, viral DNA was extracted by Trizol® reagent (catalog no. 15596-018; Gibco-BRL/Life Technologies, Gaithersburg, Md.), recovering the DNA from the aqueous phase, as recommended by the manufacturer and directly used for PCR.

For viral infectivity assays, aliquots of 500 ml were decontaminated with antibiotics (1000 U of penicillin G/ml and 1,000 µg/ml of streptomycin) and stored at −20°C until use.
PCR

Enzymatic reactions were performed in 0.2 ml thin-walled tubes with specific primers as described by Allard et al. (1990) for the amplification of a 301 bp fragment, corresponding to the hexon protein gene, with minor modifications.

Amplification of viral DNA was carried out in 50-ml volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 mM deoxynucleoside triphosphates, 2.5 U of Taq polymerase (Gibco-BRL/Life Technologies) and 82 pmoles of the primer hexAA1885 and 85 pmoles of the primer hexAA1913. The cycling conditions were as follows: initial heat denaturation at 94 °C for 4 min; 40 cycles of template denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, and sequence extension at 72 °C for 45 seg; final extension at 72 °C for 7 min. Milli-Q water aliquots were included as negative control and HAdV5 infected tissue culture fluids as positive control. Amplification products were detected by 1.5% agarose gel electrophoresis in TAE buffer and ethidium bromide (0.5 µg/ml) staining. Detection limits of PCR were determined by subjecting ten-fold serial dilutions of HAdV-5 virus suspension to the enzymatic reactions. Water samples that did not show amplification of naturally occurring adenoviruses were inoculated with 5 ml of viral DNA, corresponding to $10^4$ DICT₅₀ and reamplified by PCR to exclude the interference of unspecific inhibitors.

All precautions were taken to avoid false positive results due to cross contamination with previous amplified DNA. Thus, nucleic acid extractions, master mix preparations, DNA amplifications and product analyses were conducted in different areas.

Infectivity assays

Infectivity of the detected adenoviruses was assayed in cell cultures followed by PCR. Twenty-two water samples were passaged three times in HEp-2 and PLC/PRF/5 cell cultures for virus isolation. Cell cultures were cultivated in tubes and after confluence inoculated with 200 ml of each water sample. Cultures were kept at 37°C for 6 to 12 days and daily observed for CPE. The presence of adenovirus was confirmed in infected cell culture fluid by PCR performed as described above.

RFLP for adenovirus characterization

For discrimination of human adenovirus F from the other species that could be present in sewage and surface water, the 301 bp amplimers were removed from agarose gels, purified by using the Ultrafree-Da purification kit (Millipore) and subjected to TaqI and Rsal (BRL/Life Technologies) endonucleases digestions accordingly to Allard et al. (1994). Restriction profiles were analyzed by 1.5% agarose gel electrophoresis in TAE buffer and ethidium bromide (0.5 µg/ml) staining.

Results and discussion

In Brazil, viral gastroenteritis and hepatitis A outbreaks are frequently associated with contaminated water ingestion, but there is little or no information about the viruses that are circulating in sewage and sewage-polluted waters. In the past, the detection and identification of different members of the genus Enterovirus in water samples were performed (Christovão et al., 1967; Homma et al., 1975). More recently, the detection and genotyping of other viruses, like rotaviruses and hepatitis A virus, present in polluted water samples in São Paulo city were possible due to an efficient concentration method applied in association to direct immunoperoxidase method and RT–PCR (Mehnert & Stewien, 1993; Queiroz, 1999; Sassaroli, 2002).

The establishment of fast, simple and efficient virus detection methodologies is very important for developing countries, where sanitary conditions have to be improved and
the water quality adequately monitored. Recently, the Brazilian Ministry of Health recommended the inclusion of enteric viruses assays for assessment of water quality, but the virological parameters to be considered were still not defined. The public health importance of human adenoviruses, besides their high resistance to environmental conditions and to water treatment processes strongly suggests that they should be considered as new parameters.

Throughout the period of study, adenoviruses were detected in 69 samples (72.6%) out of the 95 submitted to PCR (Table 1). The detection limit of PCR showed to be $10^{-4}$ DICT$_{50}$ or equivalent to 1 infectious dose. Most of the samples were subjected to a reamplification reaction, suggesting that these viruses are present at low concentrations in environmental water samples (Figure 1). Unspecific inhibitors were efficiently removed and did not interfere in the reactions. E. coli numbers ranged from $2.4 \times 10^7$ to $1.6 \times 10^8$ MPN/100 ml in sewage and $8.0 \times 10^5$ to $1.6 \times 10^8$ MPN/100 ml in Pirajussara creek, showing the high level of fecal contamination of the surface water. The infectivity of 22 of the detected viruses were assayed in cell culture and the virus presence confirmed by typical CPE observation and PCR. Nineteen (86.4%) samples passaged in PLC/PRF/5 and 16 (72.7%) cultivated in HEp-2 cells were positive. The PLC/PRF/5 cells should be preferred because they are more permissive for adenovirus, especially for specie F (Grabow et al., 1990).

No seasonal variation of HAdV could be detected at both collection sites in São Paulo city throughout the period of study. Similar results were reported by Tani et al. (1995) after a five-year longitudinal study developed in Japan.

Adenoviruses detected in all 69 samples were subjected to specie characterization by RFLP. The discrimination between human adenovirus F and all other species is based on a restriction site for the endonuclease TaqI localized in the hexon gene sequence. Therefore, the presence of two fragments, showing 191 bp and 110 bp, is suggestive for the presence of enteric adenovirus (serotypes 40 and 41). All other species missed this restriction site, except for HAdV-21, but in this case the site is localized at a different position (Allard et al., 1994). By using TaqI endonuclease, a first screening for specie F viruses was performed and they were detected in 62 (89.8%) samples. Five (7.2%) samples did not show human adenovirus F viruses and 2 (2.9%) were not characterized due to low DNA concentration (Table 1). Non-restricted fragments of 301 bp could be observed in 65 (94.2%) samples, even after prolonged enzymatic digestion, possibly due to the presence of other species of adenovirus than F (Figure 1).

Discrimination of both enteric serotypes of adenoviruses, was possible by restriction of PCR products with RsaI, resulting in fragments of 256 bp and 45 bp for HAdV-40 and 211 bp and 90 bp for HAdV-41 (Figure 2).

**Table 1** Detection and identification of human adenovirus F in raw sewage and sewage-polluted creek water samples in São Paulo city, Brazil

<table>
<thead>
<tr>
<th>Site</th>
<th>Samples</th>
<th>Positive (%)</th>
<th>Species$^a$(%)</th>
<th>RFLP</th>
<th>HAdV Serotypes$^b$(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>by PCR (%)</td>
<td>F only</td>
<td>Other only</td>
<td>NC$^c$</td>
</tr>
<tr>
<td>Pirajussara Creek</td>
<td>49</td>
<td>36 (73.4)</td>
<td>28 (55.5)</td>
<td>2 (11.1)</td>
<td>2 (5.5)</td>
</tr>
<tr>
<td>Pirajussara Creek</td>
<td>46</td>
<td>33 (71.7)</td>
<td>28 (56.1)</td>
<td>1 (2.1)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>69 (72.6)</td>
<td>60 (65.2)</td>
<td>2 (2.1)</td>
<td>5 (5.5)</td>
</tr>
</tbody>
</table>

$^a$– after Taq1 restriction

$^b$– after Rsa1 restriction

$^c$– species could not be characterized
Only 27 samples could be analysed by this method. The DNA concentration of the other 42 samples, obtained after amplification and/or purification, was insufficient for restriction analyses. Adenovirus serotype 41 was the most frequent, being detected in 17 (62.9%) samples out of 27 samples. Serotype 40 was identified only in 7 (25.9%) samples. Human adenovirus F was not detected in two samples (Table 1).

A seasonal occurrence of HAdV-41 in the examined water samples could not be observed during the whole period of study. The predominately detection of HAdV-41 and the restricted occurrence of HAdV-40 year-round in sewage and polluted surface water in São Paulo is in accordance to clinical epidemiological findings reported by Hársi et al. (1995) in the same city. A tentative of subgenus-specific characterization of the other species was performed using a VA-RNA PCR described by Kidd et al. (1996). Human adenovirus A, B:1, B:2, C and D were presumptively identified, but results should be confirmed by another method (Santos, 2003).

Conclusions
The detection of infectious human adenovirus F in sewage and sewage polluted surface water in São Paulo city revealed that a real risk of infection must be considered during recreational activities in polluted waters or overflow situations, especially for children younger than 5 years of age, elderly or immunodepressed. The concentration and detection methods adopted at present are feasible to be routinely applied for assessment of water quality and epidemiological studies.

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**References**


