Molecular detection of hepatitis A virus in urban sewage in Rio de Janeiro, Brazil

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Introduction

Hepatitis A virus (HAV) has been associated with many outbreaks of waterborne origin in communities and schools (De Serres et al. 1999; Villar et al. 2004). The traditional detection of viruses in water, including monitoring treatment removal efficiency, is carried out by a labour-intensive tissue culture methodology (American Public Health Association 1992) that shows lack of sensitivity, lengthy analysis time (up to 6 weeks) and problems in detecting low virus numbers, which is the typical situation in environmental water samples (Tsai et al. 1994). Moreover, tissue culture is not applicable for primary isolation of wild-type HAV which makes this methodology not available for HAV detection in environment.

The use of molecular techniques, such as polymerase chain reaction (PCR), that is faster and more sensitive for the detection of viruses has been well documented in recent years (Tsai et al. 1993; Morace et al. 2002; Kittigul et al. 2006; Myrmel et al. 2006; Villar et al. 2006; De Paula et al. 2007). More recently, real-time PCR was also applied to quantify the presence of HAV in complex environmental matrices (Abd el-Galil et al. 2005; Brooks et al. 2005; Jothikumar et al. 2005; Villar et al. 2006; Rose et al.

Abstract

Aims: A one-year survey was conducted to examine hepatitis A virus (HAV) prevalence, distribution of genotypes and their relationship to bacterial indicators in raw and treated sewage samples.

Methods and Results: Fifty sewage samples (raw = 25 and treated = 25) were collected twice monthly from one sewage treatment plant in Rio de Janeiro. Virus concentration was performed by adsorption to an electronegative membrane followed by ultrafiltration. Viral RNA was detected by nested reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR and positive products were directly sequenced. Total and faecal coliform concentrations were also determined. By nested RT-PCR, HAV RNA was detected in 16/50 (32%) and eight (16%) of them were found in treated sewage samples. By real-time PCR, HAV RNA was detected in 46/50 (92%) samples and 24 were from treated sewage. Phylogenetic analyses classified nine isolates (56%) as subgenotype IA and seven (44%) as IB.

Conclusions: Real-time PCR was more sensitive than nested RT-PCR; the presence of subgenotypes IA and IB was described and bacterial indicators cannot be used to predict HAV presence in sewage.

Significance and Impact of the Study: These results demonstrated that HAV still remains in the environment after sewage treatment and could play an important role in maintaining the endemicity of HAV infection.
et al. (2006; De Paula et al. 2007). This method improves the accuracy and sensitivity of traditional PCR by adding a fluorescently labelled probe so that the target gene can be detected and quantified without subsequent verification. Among the various quantitative PCR strategies available, those based on real-time monitoring of the amplification reaction are the most accurate (Kalinina et al. 1997).

These new methods offer several advantages such as rapid turnaround time and a high degree of sensitivity. Through enzymatic amplification, PCR is capable of detecting the viral genomic RNA in diluents containing less than 1 PFU (Tsai et al. 1993). Because the PCR does not require cell cultivation, it has been effectively used to detect viruses which are difficult to cultivate (Tsai et al. 1993; Morace et al. 2002; Kittigul et al. 2006; Myrmel et al. 2006) such as HAV from sewage samples (Vaidya et al. 2002; Pinto et al. 2007).

Therefore, the presence of HAV was assayed in raw and treated sewage samples at one treatment plant in Rio de Janeiro (Brazil) by nested and real-time PCR, and the possible HAV reduction by treatment was calculated throughout the year 2005. Furthermore, the genetic diversity of HAV present in sewage was investigated by nucleotide sequencing. The concentration of faecal and total coliform was also tested and the relationship between coliforms and HAV was determined.

Materials and methods

Water samples

From January to December 2005, 50 raw and treated sewage composite samples were collected biweekly from one activated sludge plant responsible for the urban sewage treatment of the city of Rio de Janeiro, Brazil. For each one, eight 250 ml deal were collected until form 2 l of sample stored in glass bottles.

The samples were delivered to the laboratory at the same day of collection and immediately analysed. Total and faecal coliforms were assayed by Colilert (IDEXX Laboratories, Westbrook, ME) according to the protocols described by the manufacturer. Temperature and pH of the samples were determined on site upon collection.

Concentration methods

Each 2 l of sewage sample was concentrated to detect HAV using the method described by Katayama et al. (2002) with the exception that we added 1:5 mmol of MgCl₂ and adjusted the pH to 5:0 with HCl. This method was previously evaluated to concentrate HAV (Villard et al. 2006) and it uses a HA-negatively charged membrane (Millipore, Burlington, MA, USA) and the eluate (10 ml) was reconcentrated using a Centriprep YM-50 Concentrator (Millipore) to obtain a final volume of 2 ml.

Qualitative nested reverse transcription PCR and sequencing

Viral RNA was extracted from 140 µl of the eluate using the Qiagen Viral RNA KIT (Qiagen, Valencia, Spain). Reverse transcription was carried out at 37°C for 1 h using 10 µl of RNA, random primer (Invitrogen, Rockville, MD, USA) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Then, VP1/2A region was amplified using nested reverse transcriptase (RT)-PCR as described elsewhere (De Paula et al. 2002). The sensitivity and specificity of primers to detect HAV RNA in water samples were previously demonstrated (Villard et al. 2006). The PCR products were loaded onto a 2% agarose gel, electrophoresed and stained with ethidium bromide to visualize bands (expected length, 247 bp).

Amplicons were purified using the QiAquick Gel extraction kit (Qiagen) according to the manufacturer’s recommendations. Direct nucleotide sequencing reaction was performed in both directions of HAV RNA-positive samples with a Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) and an automatic DNA sequencer (ABI Prism 310; Applied Biosystems). The sequences reported in this paper have been deposited in the GenBank sequence database under the following accession numbers: EF204116 to EF204131.

Sequence analysis

The GCG software package, version 10-1 (Wisconsin Sequence Analysis Package; Genetic Computer Group, Madison, WI, USA), was used for editing, aligning of nucleotides and translation of nucleotides into amino acid sequences. Multiple alignments were initially performed with the Clustal X program (Thompson et al. 1997). A matrix was then generated to construct the Kimura two-parameter model (Felsenstein 1993). Using this matrix and the neighbour-joining method (Saitou and Nei 1987), a phylogenetic tree was generated, the reliability of which was assessed by bootstrap resampling (1000 pseudoreplicates). These methods were implemented using the MEGA 2-1 program (Kumar et al. 2001).

Quantitative PCR standard curve

The 5’ noncoding region of the HAV genome was chosen to design the TaqMan assay. The primers, probe and reaction conditions were previously described (Villard et al. 2006; De Paula et al. 2007). All PCR reactions were carried out using the 7500 Real-Time PCR System (Applied
HAV in sewage in Rio de Janeiro

L.M. Villar et al.

Biosystems). The RT step for the real-time assay was performed as described before for qualitative PCR.

Calculation of HAV and coliforms removal

The HAV removal was determined by dividing the result of the subtraction among HAV concentration found by TaqMan Assay in raw and treated sewage samples by the concentration found in the raw sewage samples. Total coliforms (TC) and faecal coliforms (FC) removal was determined the same way, by dividing the result of the subtraction value of coliforms found in raw and treated sewage samples by the concentration found in the raw sewage samples as was determined by Colilert.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using the chi-square ($\chi^2$) test for independence or with Yate’s continuity correction. Differences between groups were considered to be statistically significant at $P < 0.05$. All calculations were undertaken by using SPSS version 8.0.

Results

HAV detection in sewage

Table 1 shows the water quality parameters of raw and treated sewage samples. For raw sewage samples, TC yielded a population density from $6.3 \times 10^7$ to $2 \times 10^8$ most probable number (MPN) ml$^{-1}$, the density of FC ranged from $5.5 \times 10^6$ to $8.2 \times 10^7$ MPN ml$^{-1}$, but TC and FC mean values did not show any seasonal variation. HAV was detected by nested PCR in 8 of 25 raw samples, and a tiny high positivity was observed during autumn (4/7) and spring (3/6). By real-time PCR, viral load ranged from $1.2 \times 10^5$ to $8.9 \times 10^7$ copies ml$^{-1}$, summer presented the highest mean value ($8.9 \times 10^7$ copies ml$^{-1}$) and HAV RNA was detected in 22 samples (88%).

For treated sewage samples, TC concentration ranged from $1 \times 10^5$ to $2 \times 10^6$ MPN ml$^{-1}$, and FC concentration ranged from $1.1 \times 10^4$ to $4.5 \times 10^4$ MPN ml$^{-1}$, but the mean values of TC and FC did not show any seasonal variation. HAV was detected by nested PCR in 8 of 25 treated samples, the highest positivity was observed during spring (3/6) and by real-time PCR the viral load ranged from $1.7 \times 10^5$ to $3.8 \times 10^5$ copies ml$^{-1}$, 24 samples were positive (96%) and a higher mean value was observed in autumn ($3.8 \times 10^7$ copies ml$^{-1}$). None of these variables were statistically significant in relation to positivity to nested and real-time PCR.

Efficiency of the treatment

After primary (sedimentation) and biological (activated sludge) secondary treatment, 99.9% of TC and 99.9% of FC were removed, but only 42.3% of HAV. These results demonstrated that sewage treatment was efficient to reduce a high concentration of TC and FC but it was not efficient to reduce HAV particles. The mean concentration of TC was $5.7 \times 10^7$ MPN ml$^{-1}$ in HAV-positive raw sewage samples, while it was $4.3 \times 10^7$ MPN ml$^{-1}$ in HAV-negative raw sewage samples. The mean concentration of FC was $3.2 \times 10^4$ MPN ml$^{-1}$ for HAV-positive treated sewage samples while it was $3.7 \times 10^4$ MPN ml$^{-1}$ for HAV-negative treated sewage samples. These results showed that there was no association between bacterial indicators concentration and HAV detection showing that these indicators cannot predict the presence of HAV.

Phylogenetic analysis of HAV in sewage water samples

A total of 16 out of 50 samples were HAV RNA-positive by nested RT-PCR and were submitted to nucleotide sequencing in order to confirm the presence of HAV.

### Table 1 Water quality parameters and HAV detection by nested and real-time PCR in raw and treated sewage

<table>
<thead>
<tr>
<th>Season</th>
<th>Type</th>
<th>pH</th>
<th>Temperature (°C)*</th>
<th>Total coliforms (MPN ml$^{-1}$)*</th>
<th>Faecal coliforms (MPN ml$^{-1}$)*</th>
<th>Real-time PCR (copies ml$^{-1}$)*</th>
<th>Real-time PCR (n total$^{-1}$)</th>
<th>Nested PCR (n total$^{-1}$)</th>
<th>Genotypes (n type$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>Raw sewage</td>
<td>6</td>
<td>27.7</td>
<td>$6.3 \times 10^7$</td>
<td>$5.5 \times 10^6$</td>
<td>$8.9 \times 10^4$</td>
<td>6/6</td>
<td>1/6</td>
<td>1(A)</td>
</tr>
<tr>
<td></td>
<td>Treated sewage</td>
<td>6</td>
<td>28.5</td>
<td>$1.8 \times 10^5$</td>
<td>$4.1 \times 10^4$</td>
<td>$2.7 \times 10^2$</td>
<td>6/6</td>
<td>2/6</td>
<td>2(B)</td>
</tr>
<tr>
<td>Autumn</td>
<td>Raw sewage</td>
<td>6.2</td>
<td>25</td>
<td>$1.8 \times 10^5$</td>
<td>$7.8 \times 10^4$</td>
<td>$6.9 \times 10^2$</td>
<td>7/7</td>
<td>4/7</td>
<td>2(A)/2(B)</td>
</tr>
<tr>
<td></td>
<td>Treated sewage</td>
<td>6</td>
<td>27.8</td>
<td>$1 \times 10^5$</td>
<td>$1.1 \times 10^4$</td>
<td>$3.8 \times 10^2$</td>
<td>7/7</td>
<td>1/7</td>
<td>1(B)</td>
</tr>
<tr>
<td>Winter</td>
<td>Raw sewage</td>
<td>6</td>
<td>25.5</td>
<td>$2 \times 10^5$</td>
<td>$2.7 \times 10^4$</td>
<td>$3.3 \times 10^2$</td>
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</tr>
<tr>
<td></td>
<td>Treated sewage</td>
<td>5.7</td>
<td>25.1</td>
<td>$2 \times 10^5$</td>
<td>$4.2 \times 10^4$</td>
<td>$3.6 \times 10^2$</td>
<td>6/6</td>
<td>2/6</td>
<td>2(B)</td>
</tr>
<tr>
<td>Spring</td>
<td>Raw sewage</td>
<td>6.5</td>
<td>25.3</td>
<td>$1 \times 10^5$</td>
<td>$8.2 \times 10^4$</td>
<td>$1.2 \times 10^2$</td>
<td>4/6</td>
<td>3/6</td>
<td>3(A)</td>
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<tr>
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<td>$1.7 \times 10^2$</td>
<td>5/6</td>
<td>3/6</td>
<td>3(A)</td>
</tr>
</tbody>
</table>

*Mean values are shown.

ND, not detected; HAV, hepatitis A virus; PCR, polymerase chain reaction; MPN, most probable number.
RNA and to carry out the genotyping. A BLAST software evaluation of the 218-bp fragment (positions 3024–3191) indicated that HAV RNA-positive samples showed homologies: 85–96% to the HAV strain HAS-15 and 88.5–100% to strain HM-175. Figure 1 shows the phylogenetic tree of nucleotide sequences from the 16 positive samples, together with 13 HAV strains selected randomly from environment and Brazilian patients, as well as six reference sequences from HAV subgenotypes IA, IB, IIA, IIB, IIIA and IIIIB. Nine samples belong to subgenotype IA and seven samples to subgenotype IB. In the raw sewage samples, subgenotype IA was found in 6/8 samples while in the treated sewage samples subgenotype IB was found in 5/8 samples.

Identity among nucleotide sequence of sewage isolates and the river water isolate previously in Rio de Janeiro (Villar et al. 2006) varied from 84.9% to 95%. Identity among sewage isolates and the tap water isolate from a day care centre experienced an outbreak in the year 2004 (Rio de Janeiro) (Villar et al. 2006) and it varied from 88.1% to 99.5%. A comparison of the predicted amino acid sequences of the VP1/2A region from sewage isolates demonstrated an identity to HAS-15 from 86.1% to 100% and related to HM-175 from 87.5% to 100%. Identity among aminoacid sequences from sewage samples was from 87.5% to 100% (data not shown).

**Discussion**

HAV has also been detected in raw urban sewage in other countries, such as India and Italy (Morace et al. 2002; Vaidya et al. 2002). In the present study, HAV was detected by nested RT-PCR in 16/50 (32%) and by real-time PCR in 46/50 (92%) sewage samples. This high rate of HAV positivity provides convincing evidence that HAV infection is widespread in the Brazilian environment. Since, the prevalence of infection has diminished (Vitral et al. 2006), outbreaks of hepatitis A still occur in Brazil, as documented in the last years (Villar et al. 2004; Morais et al. 2006). HAV prevalence determined by nested RT-PCR in this study (32%) was high compared with the study conducted by Vaidya et al. (2002) in India.
(24-42%), but it was low compared with data obtained in sewage samples from Cairo city (71%) (Pinto et al. 2007). These differences in HAV prevalence could be related to the different epidemiology of HAV infection in these different geographical areas, as well as to the methods used to concentrate and detect HAV in the sewage samples.

In this study, we showed the applicability of molecular methods to detect HAV RNA in sewage samples, especially TaqMan PCR system which is highly sensitive to detect and enumerate HAV RNA as it detects HAV in 46 samples showing 92% prevalence. Moreover, the present study showed no correlation between viral contamination and bacterial indicators using PCR as it was demonstrated by other researchers (Skraber et al. 2004; Choi and Jiang 2005) and that sewage treatment was efficient to reduce coliforms, but not to reduce virus that can persist in the environment longer than bacteria (Lucena et al. 2004). So risks based on bacterial standards may seriously underestimate the risk of virus-associated waterborne illness (Noble and Fuhrman 2001; Jiang and Chu 2004).

The presence of subgenotypes IA and IB was observed in Brazilian sewage as it was previously demonstrated among sera collected from patients with acute hepatitis (De Paula et al. 2002, 2004; Villar et al. 2004) and from water collected from river and tap water in Brazil (Villar et al. 2006; De Paula et al. 2007). Furthermore, close genetic relationship was observed among sewage and clinical and environmental isolates showing that HAV strains have been disseminated into the environment in our region. The presence of subgenotypes IA, IB and IIIA was demonstrated in Spain sewage reflecting the diversity of HAV in the environment such as in clinical patients (Pina et al. 2001).

Conclusion

The present study describes the first detection of HAV in sewage samples in Rio de Janeiro, Brazil, showing that, although the efficiency of sewage treatment was good to eliminate bacterial indicators, it was not efficient to eliminate HAV. As the virus still persists in the sewage it probably constitutes one important source of HAV dissemination in the environment. No correlation was observed among coliforms and HAV presence showing that these indicators could not be used to indicate HAV presence. The presence of two subgenotypes IA and IB was demonstrated in the sewage samples. Real-time PCR and nested RT-PCR were suitable to detect HAV in raw and treated sewage samples, however real-time PCR was more sensitive and it could enumerate HAV RNA showing in this way the efficiency of sewage treatment. Monitoring the presence of virus is a critical component of the evaluation of the quality of sewage in the environment.

Acknowledgements

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References


