Assessment of burden of virus agents in an urban sewage treatment plant in Rio de Janeiro, Brazil

Tulio Machado Fumian, Carmen Baur Vieira, José Paulo Gagliardi Leite and Marize Pereira Miagostovich

ABSTRACT

Sewage discharge is considered to be the main source of virus contamination in aquatic environments. There is no correlation between the presence of viruses and the presence of fecal coliforms in water; therefore virological markers are needed when monitoring contamination. This study investigates DNA and RNA virus concentrations in wastewater and evaluates a potential virus marker of human contamination. Influent and effluent samples were collected twice a month throughout a 1-year period. Viruses were detected using quantitative polymerase chain reaction protocols; nucleotide sequencing was carried out for virus genotyping. Human adenovirus (HAdV) and polyomavirus JC (JCPyV) were the most prevalent viruses found in influent samples (100%) with a virus load that ranged from 10^6 to 10^5 genome copies per liter (gc l^{-1}). Norovirus genogroup II (NoV GII) and human astrovirus (HAstV) were less prevalent, and ranged from 10^4 to 10^3 gc l^{-1}. Quantitative data on virus profiles in wastewaters stress the high level of rotavirus species A environmental dissemination and address the potential of HAdV as a useful virological marker of virus contamination in aquatic environments. This study corroborates other studies performed in developed countries on DNA viruses as good markers of human fecal contamination.

Key words | Brazil, detection, environmental, genotyping, viruses, wastewater

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INTRODUCTION

Approximately 5,000 young children worldwide die every day from diarrheal diseases that have easily preventable causes. It is estimated that 88% of these diseases are attributed to unsafe water supply and inadequate sanitation and hygiene. Improved water supplies and basic sanitation could cut this toll dramatically and a simple, low-cost household water treatment could save further lives (WHO 2004, 2008; WHO & UNICEF 2005). The provision of clean drinking water and good sanitation coverage has progressed, but still represents a major challenge for health authorities, particularly in the developing world.

Raw sewage is a major carrier of disease-causing agents and the discharge of natural raw or inadequately treated sewage effluents is the most common source of enteric virus pathogens in aquatic environments (Bosch 1998). The safe treatment of sewage has been highlighted as a critical threat to the health issues of any community (Okoh *et al.* 2010).

Viruses are shed at high concentrations (up to 10^{11} copies g^{-1}) in the feces or urine of infected patients (symptomatic and asymptomatic), and are transmitted primarily through the fecal–oral route, either directly from person to person or by contact with contaminated water or food (Bosch *et al.* 2008). At this time, molecular methodologies such as quantitative polymerase chain reaction (qPCR) have been used widely to detect enteric viruses in aquatic environments such as sewage and river water samples (Pusch *et al.* 2005; da Silva *et al.* 2007; Fumian *et al.* 2010; Haramoto *et al.* 2010; Prado *et al.* 2011).

Currently, microbiological water quality control and the performance of wastewater treatment systems are monitored

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by the use of bacterial indicator organisms (Okoh *et al.* 2010). However, several reports have shown a lack of correlation between the presence or absence of bacterial indicators and virus contamination (Pusch *et al.* 2005; Carducci *et al.* 2008); some studies have aimed to identify a group of viruses that could be used as markers of human contamination in the environment (Pina *et al.* 1998; Jiang *et al.* 2001; Fong & Lipp 2005; Bofill-Mas *et al.* 2006).

DNA viruses, such as human adenovirus (HAdV) and polyomavirus JC (JCPyV), are usually more abundant than RNA viruses in water samples and have been recommended as potential biological indicators of human origin pollution and also as markers of virus contamination in water from developed countries (Bofill-Mas *et al.* 2000; Pina *et al.* 1998). However, there is a lack of data from developing countries on virus presence in wastewater or even for data that concerns a viral marker for human fecal contamination in the environment (Okoh *et al.* 2010). Therefore, characterization of wastewater from countries located in tropical regions is fundamental to the provision of data that will assist in evaluation of a single virus marker that could be used worldwide.

In the present study, data on DNA (HAdV and JCPyV) and RNA viruses such as rotavirus species A (RVA), norovirus (NoV) and human astrovirus (HAstV) are reported using samples from a wastewater treatment plant (WTP) located in Rio de Janeiro, Brazil. An ultracentrifugation protocol, qPCR and sequencing methodologies were used to detect, quantify and characterize viruses recovered from wastewater.

METHODS

Sewage samples

Sewage samples were collected from an urban WTP located in the metropolitan area of Rio de Janeiro, Brazil. This plant receives sewage from around 1.5 million people living in both the Central and North zone of the city. This sewage treatment employs a secondary treatment (aerobic process: activated sludge) with an inflow mean of 2,500 l s^{-1}. Initial sewage treatment is composed of a grid separation and a primary sedimentation. There are four aeration tanks placed in parallel that offer the capacity to treat 625 l s^{-1} of effluents. A secondary sedimentation is performed in four secondary settling tanks without chlorination before effluents are discharged into the water environment.

In total, 48 sewage samples were collected bi-monthly (at a 15-day interval) from August 2009 to July 2010: 24 samples were collected from raw sewage (influent) and 24 were collected from the final treated sewage (effluent). At each sampling point, 50 ml of sewage was collected in sterile plastic bottles, kept at 4 °C and transported to the Laboratory of Comparative and Environmental Virology (LVCA), Fiocruz, Brazil, for immediate analysis.

Virus concentration and nucleic acid extraction

Virus particles were concentrated using an ultracentrifugation method as described previously (Pina *et al.* 1998). Briefly, 42 ml of sewage was ultracentrifuged at 100,000 g for 1 h at 4 °C using a Beckman ultracentrifuge equipped with a type 35 rotor. Virus particles were resuspended in 3.5 ml of 0.25 mol l^{-1} glycine buffer (pH 9.5) and incubated on ice for 30 min. The solution was neutralized by the addition of 3.5 ml of 2× phosphate-buffered saline (PBS) (pH 7.2). The supernatant was clarified by centrifugation (12,000 g for 15 min), and viruses were recovered by ultracentrifugation at 100,000 g for 1 h at 4 °C using a SW41 rotor. Virus particles were resuspended in 400 µl of 1× PBS pH 7.2 and this volume was processed immediately for nucleic acid extraction or stored at –80 °C until virus DNA/RNA extraction.

Viral nucleic acid was extracted by the glass powder method (Boom *et al.* 1990); for NoV and HAstV, cDNA synthesis was performed by reverse transcription using a random primer (PdN6-50 A260 units–Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK). To avoid false-negative results, sewage samples were inoculated with 500 µl of internal control (bacteriophage PP7) before the concentration assay, and the extracted nucleic acid was diluted 10-fold (Fumian *et al.* 2011). For all molecular procedures, four separate rooms were used to avoid cross contamination of samples. The samples were analyzed in duplicate and specific positive and negative controls (positive clinical samples previously sequenced for each virus type and RNA/DNA-free water) were used in all procedures.

Virus detection and quantification

Detection of viruses was performed using TaqMan® qPCR. Primer and probe sequences and final concentrations, annealing temperature conditions and references for quantification and characterization of each virus group are shown in Table 1. Quantification was carried out in a final volume of 25 μl that contained 5 μl of the DNA extract or prepared cDNA mixed with 12.5 μl of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA). Optical plates that contained the mixtures were placed into an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) under the following conditions: incubation at 50 °C for 2 min to activate uracil-N-glycosylase, initial denaturation at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and at 56–60 °C, dependent upon the virus type, for 1 min. A 10-fold serial dilution of plasmid DNA that contained the target sequences (specific to each virus group) was used to generate a standard curve between log standard concentrations (10^6–10^1 copies per reaction) and threshold (C_t) value. Recombinant plasmids used as standard for quantification of HAdV, NoV GI, NoV GII and HAstV were constructed at the LVCA using pCR2.1 vectors (Invitrogen, USA) that contained the insert generated with specific primers used in the qPCR reaction (Table 1). The lengths of the insert and target regions were 69 bp (hexon gene),

Table 1 | Oligonucleotide primer and probe sequences, and amplification conditions used for detection and molecular characterization of enteric viruses

Virus	Names	Sequence (5′ → 3′)	Annealing temperature (°C)	Final concentration (nM)	References
HAdV[a]	HAdF	CWTACATGCACATCKCSGG	60	900	Hernroth *et al.* (2002)
	HAdR	CRCGGGCRAAYTGCACCAG		900	
	HAdP1[c]	CCGGGCTCAGGTACTCCGAGGCGTCCT		225	
JCPyV[a]	JE3F	ATGTTTGCCAGTGATGATGAAAA	60	500	Pal *et al.* (2006)
	JE3R	GGAAAGTCTTTAGGGTCTTCTACCTTT		500	
	JE3P[c]	AGGATCCCAACACTCTACCCCACCTAAAAAGA		200	
NoV GI[a]	COG1F	CGYTGGATGCGNTTYCATGA	56	600	Kageyama *et al.* (2003)
	COG1R	CTTAGACGCCATCATCATTYAC		600	
	RING1a[c]	AGATYGCGATCYCCTGTCCA		250	
	RING1b[c]	AGATCGCGGTCTCCTGTCCA		250	
NoV GII[a]	COG2F	CARGARBCNATGTTYAGRTGGATGAG	56	600	Kageyama *et al.* (2003)
	COG2R	TCGACGCCATCTTCATTCACA		600	
	RING2[c]	TGGGAGGGCGATCGCAATCT		250	
HAstV[a]	Av1	CCGAGTAGGATCGAGGGT	55	120	Le Cann *et al.* (2004)
	Av2	GCTTCTGATTAAATCAATTTTAA		120	
	AvP[c]	CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC		100	
HAdV[b]	Hex1deg	GCCSCARTGGKCWTACATGCACATC	55	500	Allard *et al.* (2001)
	Hex2deg	CAGCACSCCICGRATGTCAAA		500	
	Hex3deg	GCCCGYGCMACIGAIACSTACTTC	55	500	
	Hex4deg	CCYACRGCCAGIGTRWAICGMRCYTTGTA		500	
JCPyV[b]	EP1A	TGAATGTTGGGTTCCTGATCCCACC	59	500	Bofill-Mas *et al.* (2000)
	EP2A	ACCCATTCTTGACTTTCCTAGAGAG		500	
	P1A	CAAGATATTTTGGGACACTAACAGG	59	500	
	P2A	CCATGTCCAGAGTCTTCTGCTTCAG		500	
NoV[b]	JV13I	TCATCATCACCATAGAAIGAG	45	440	Vennema *et al.* (2002)
	JV12Y	ATACCACTATGATGCAGAYTA		440	
HAstV[b]	Mon269	CAACTCAGGAAACAGGGTGT	50	400	Noel *et al.* (1995)
	Mon270	TCAGATGCATTGTCATTGGT		400	

[a]Primers and probes used for detection (qPCR).

[b]Primers used for molecular characterization.

[c]6-Carboxyfluorescein (FAM) as reporter dye is coupled in the 5′ end of the oligonucleotide, and 6-carboxy-tetramethylrhodamine (TAMRA) as the quencher dye is coupled in the 3′ end of the oligonucleotide.

85 bp (ORF-1/2 junction), 98 bp (ORF-1/2 junction) and 90 bp (3′ end of the genome) for HAdV, NoV GI, NoV GII and HAstV, respectively. The plasmid used as standard for JCPyV quantification and that contained the whole JCPyV genome strain Mad-1 in pBR322 was kindly donated by Dr Rosina Girones of the Department of Microbiology, University of Barcelona, Barcelona, Spain. Amplification data were collected and analyzed with Applied Biosystems 7500 Software® v2.0 (Applied Biosystems). Sample signals that crossed the threshold line, and presented a characteristic sigmoid curve, were regarded as positive. Virus concentration results are presented as genome copies per liter (gc l^{-1}) of sewage samples.

Nucleotide sequencing and phylogenetic analysis

The molecular characterization and phylogenetic analysis of HAdV, JCPyV, NoV and HAstV was performed by direct PCR amplicon sequencing using the amplicons generated from the hexon gene, an intergenic region fragment, the polymerase gene and the VP1 protein, respectively (Table 1).

The amplicons generated by reverse transcription (RT)-PCR and nested-PCR (nPCR) were purified using a QIAquick PCR Purification Kit® or QIAquick Gel Extraction Kit® (Qiagen, CA, USA), in accordance with the manufacturer's instructions. PCR products were sequenced using an ABI Prism® 3100 Genetic Analyzer and Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) in both directions.

Nucleotide sequences were edited and aligned using BioEdit® Sequence Alignment Editor software (Hall 1999). The sequences were compared with their prototypes as well as with other sequences deposited at the National Center for Biotechnology Information (NCBI/GenBank) (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) v.4.0 software (Tamura *et al.* 2007) with the neighbor-joining method, using genetic distance corrected by the Kimura two-parameter model with 2,000 pseudoreplicates.

Statistical analysis

The frequency of detection of each tested virus obtained in 48 samples of wastewater using qPCR assay was compared using both a chi-squared test and Fisher's exact test at a significance level of 0.05. The occurrences of HAdV, JCPyV and RVA in the effluent were analyzed for significant differences. Statistical analyses were performed using GraphPad Prism® software version 5.

RESULTS

Virus detection and concentration in influent and effluent samples

Table 2 shows results of virus detection from influent and effluent samples obtained in the WTP located in an urban area of the city of Rio de Janeiro, Brazil. Results showed that there was a higher detection level of HAdV and JCPyV when compared with NoV and HAstV ($p < 0.05$, chi-squared test). The detection level of HAdV was significantly higher when compared with that for JCPyV ($p = 0.02$, Fisher exact text). There was no difference in levels of NoV GII and HAstV detection ($p = 0.08$, Fisher exact test).

Monthly mean concentrations of HAdV, JCPyV, NoV GII, and HAstV detected in wastewater samples are illustrated in Figure 1. HAdV concentration ranged between 5.23×10^5 and 7.04×10^6 gc l^{-1} (mean of 2.97×10^6 gc l^{-1}) and between 7.40×10^3 and 6.48×10^4 gc l^{-1} (mean of 2.55×10^4 gc l^{-1}) in influent and effluent samples, respectively.

JCPyV concentration in influent samples ranged between 3.26×10^4 and 2.11×10^6 gc l^{-1} (mean of 5.98×10^5 gc l^{-1}). In effluent samples, JCPyV DNA genome could be detected in four samples during the study period. Concentration ranged between 2.54×10^3 and 4.28×10^3 gc l^{-1} (mean of 3.31×10^3 gc l^{-1}).

Table 2 | Percentage of positive samples detected by quantitative PCR in influent and effluent from the wastewater treatment plant located in Rio de Janeiro, Brazil

	Viruses – number of positive samples (%)			
Waste samples (*n*)	HAdV	JCPyV	NoV GII	HAstV
Influent (24)	24 (100)	24 (100)	13 (55)	7 (29)
Effluent (24)	15 (75)	4 (17)	2 (8%)	0 (0)
Total (48)	39 (81.6)	28 (58.3)	15 (31.3)	7 (14.6)

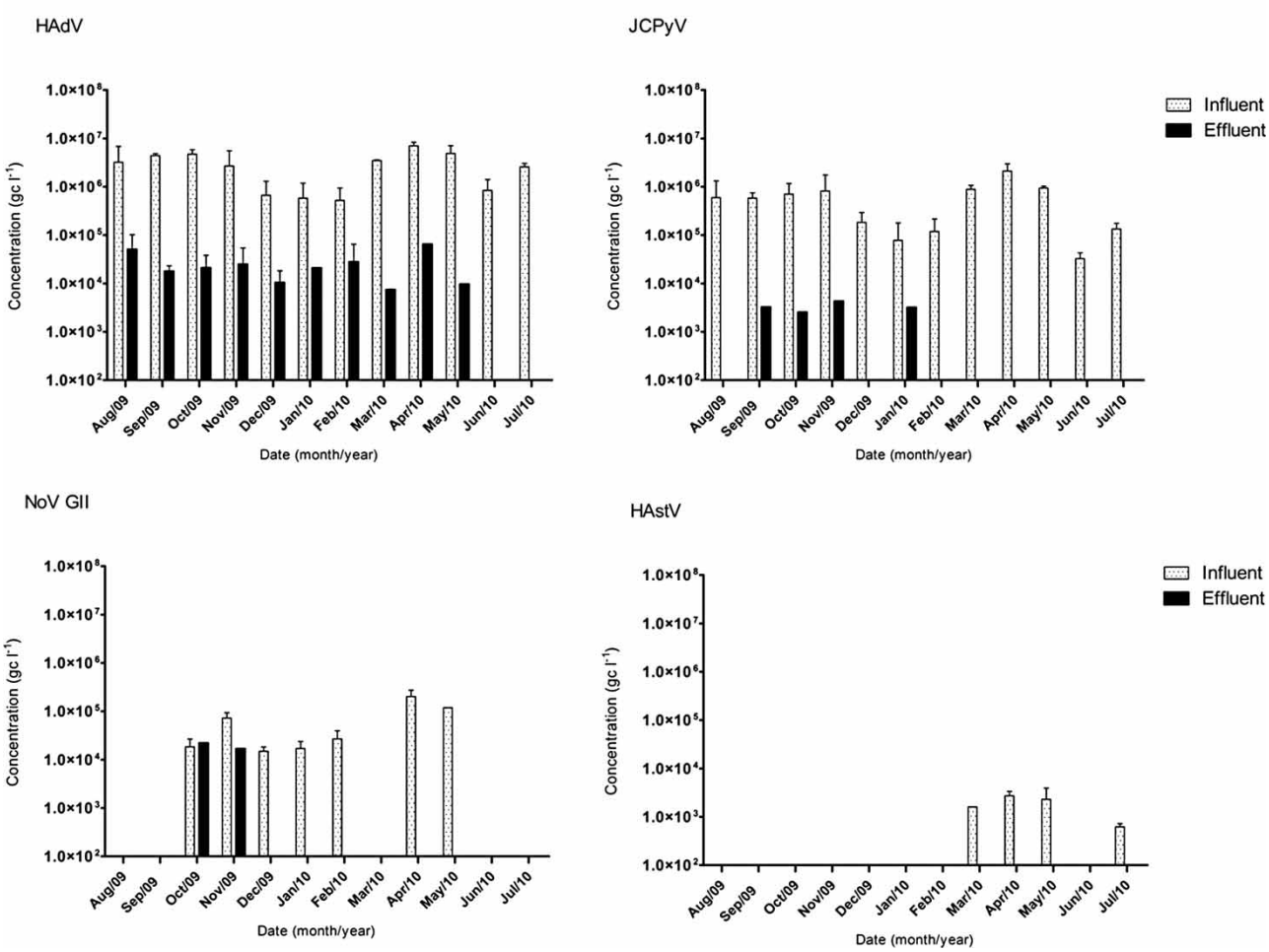

Figure 1 | Monthly mean concentration [genome copies per liter (gc l^{-1})] of human adenovirus (HAdV), polyomavirus (JCPyV), norovirus genegroup II (NoV GII) and human astrovirus (HAstV) in influent and effluent samples collected over a 1-year period in a wastewater treatment plant (WTP) located in Rio de Janeiro, Brazil.

NoV GII concentrations ranged between 1.50×10^4 and 2.03×10^5 gc l^{-1} (mean of 6.75×10^4 gc l^{-1}) in influent samples. In effluent samples NoV GII concentrations ranged between 2.19×10^4 and 1.66×10^4 gc l^{-1}, respectively. NoV GI was not detected in the tested samples.

HAstV mean virus concentrations ranged between 6.23×10^2 and 2.71×10^3 gc l^{-1} (mean of 1.81×10^3 gc l^{-1}) and were not detected in effluent samples.

Results obtained by qPCR analysis demonstrated that HAdV was found in both raw and treated sewage all year round at high concentrations. In contrast, JCPyV, detected throughout the year in raw sewage, was detected in four effluent samples only. JCPyV frequency detection in effluent samples was significantly lower when compared with HAdV ($p = 0.001$, chi-squared test) and RVA ($p = 0.002$, chi-squared test).

Molecular characterization

HAdV, JCPyV, NoV and HAstV nucleic acids from the amplicons obtained by nPCR were sequenced and confirmed by nucleotide sequence search using the Basic Local Alignment Tool (BLAST) tool.

JCPyV strains showed a high nucleotide identity with each other, and ranged from 98.9 to 100%. The phylogenetic tree obtained with JCPyV sequences and JCPyV prototypes available from the GenBank database is shown in Figure 2. All sequences detected in this study

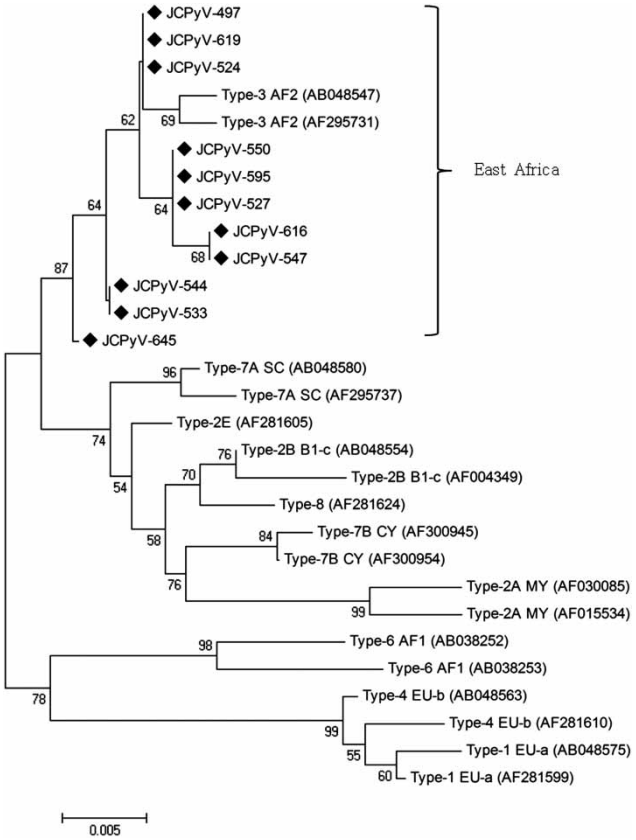

Figure 2 | Phylogenetic dendrogram based on partial intergenic region of 11 sequenced polyomavirus (JCPyV) strains. All sequences obtained from the GenBank database are named in accordance with Pavesi (2003); the corresponding accession numbers are indicated on the right hand side of the figure. Brazilian environmental samples are marked with a solid diamond. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes and values lower than 50% are not shown.

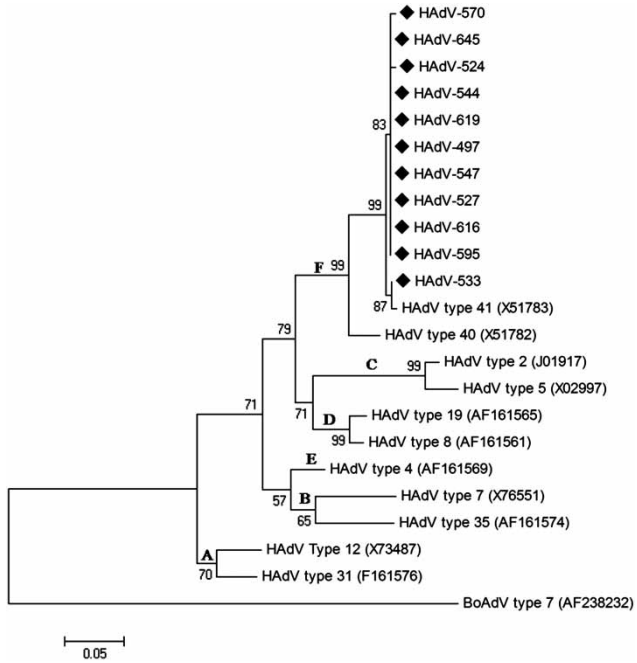

Figure 3 | Phylogenetic tree of 11 human adenovirus (HAdV) (marked with a solid diamond) isolated from influent samples with prototype sequences between 253-bp sequences within the hexon gene. The six human adenovirus species (A–F) are indicated with letters. The sequence of bovine adenovirus type 7 available in GenBank was used as out group. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes and values lower than 50% are not shown.

clustered with genotype type-3 (AF2), which was of East African origins.

The phylogenetic tree generated with HAdV sequences revealed that all sequences clustered with HAdV type 41, and showed a nucleotide homology of between 95.3 and 98.9% with prototype HAdV41 sequence (GenBank accession number X51783; Figure 3).

Sequences of NoV amplified from the polymerase region (ORF-1) were classified as genogroup II (GII), genotypes GII.4 (4/5) and GII.g (1/5) based on the NoV genotyping tool available online (Kroneman *et al.* 2011). HAstV sequences amplified from VP1 protein were classified as genotype 1 (HAstV-1).

All sequences obtained in the study have been deposited at the NCBI (GenBank database, http://www.ncbi.nlm.nih.gov/) under the following accession numbers: HAdV: JN654703–JN654713; JCPyV: JN644470–JN644480; NoV: JN654714–JN654718; and HAstV: JN799266–JN799271.

DISCUSSION

Sewage discharge is considered the main source of microbiological contamination in several aquatic environments and enteric viruses, highly stable and present in high concentration in that type of water, become a source of human diseases through exposure to these contaminated waters (Okoh *et al.* 2010). In South American countries, where in general there is no adequate sewage network, common pollution problems associated with urban sewage discharges (Rodríguez-Díaz *et al.* 2009) have contributed to morbidity and mortality from acute diarrhea. In these regions, however, the association between virus agents and water-related diseases is still difficult to infer.

In the present study DNA viruses HAdV and JCPyV were detected frequently and at high concentrations in raw sewage samples throughout the year. These viruses followed a similar pattern to DNA viruses worldwide and were corroborated to be useful virus markers of human fecal contamination (Bofill-Mas *et al.* 2006; Carducci *et al.* 2008; Katayama *et al.* 2008; Fong *et al.* 2010; Fumian *et al.* 2010; La Rosa *et al.* 2010; Schlindwein *et al.* 2010; Kokkinos *et al.* 2011).

A lower JCPyV (17%) detection level in treated sewage samples compared with HAdV detection levels (75%) could be explained by a 1-log lower JCPyV concentration in influent samples. In this study virus-negative samples were classified as containing virus genomes with a concentration of less than 2.0×10^3 and 5.8×10^3 gc l^{-1} for DNA and RNA virus, respectively, when considering the detection limit of 5 gc per reaction for qPCR. A physical removal process, such as activated sludge used in the WTP studied, is able to remove about 90–99% of virus load (between 1 and 2 logs) in the wastewater (Ueda & Horan 2004), and reduce JCPyV concentrations to non-detectable levels in effluent samples. Similar findings regarding JCPyV reduction in concentration were demonstrated in a previous study (Fumian *et al.* 2010). Therefore, JCPyV detection during environmental surveillance studies is especially important to corroborate the human origin of the contamination, as it is exclusive to the human host. Other studies presented preferable features for HAdV as an indicator of virus contamination in water, such as high stability in the environment, high resistant to ultraviolet (UV) light disinfection, and worldwide occurrence throughout the year, with fecal excretion of gastroenteric and non-gastroenteric serotypes (Thompson *et al.* 2003; Flomenberg 2005; Bofill-Mas *et al.* 2006; Girones *et al.* 2010).

In this study the importance of a thorough characterization of the local wastewater was also demonstrated. RVA concentration in raw sewage was similar to the HAdV and JCPyV concentrations and highlighted these viruses as important environmental contaminants in this region; similar findings to those observed in other developing countries such as Venezuela and China (Rodríguez-Díaz *et al.* 2009; He *et al.* 2011). The high RVA concentration (mean of 10^6 gc l^{-1}) in the environment is remarkable; especially as this study was conducted after the introduction of the RVA vaccine (Rotarix®, GlaxoSmithKline, Rixensart, Belgium) that became available to the whole birth cohort from 2006 onwards in Brazil. The high concentration of RVA detected during the year, coupled with a prolonged survival in the environment, t99 (time required to observe a reduction of 99% in the initial viral concentration) of 32 and 10 days in river waters at 4 and 20 °C, respectively (Rzezutka & Cook 2004), highlight and suggest the potential of these viruses as virological markers in some geographical areas.

Single-stranded RNA viruses, such as NoV and HAstV, were detected at lower concentrations in sewage samples, as observed in a previous survey carried out in a smaller WTP in the city of Rio de Janeiro, Brazil (Guimarães *et al.* 2008; Victoria *et al.* 2010), as well as in other similar studies performed in Greece and China (He *et al.* 2011; Kokkinos *et al.* 2011). Arraj *et al.* (2008) did not detect NoV and enteroviruses for the 18-month period of their study in a WTP, and related negative results to the small sample volume, to the sensitivity of the detection methods or to local epidemiological circumstances. However, results obtained from five WTP in Italy showed a high NoV prevalence in wastewater samples and showed a different pattern of waterborne virus circulation in accordance with the profile of the population studied (La Rosa *et al.* 2010). In Venezuela, a high detection rate of NoV (75%) was demonstrated in urban sewage, followed by HAstV and RVA (67%) and HAdV (50%) (Rodríguez-Díaz *et al.* 2009). Together, these results reinforce the importance that studies should be carried out in different cities and countries worldwide to obtain an epidemiology profile of viruses in wastewater samples. Prado *et al.* (2011) reported a 58% hepatitis A virus (HAV) detection with mean concentrations of 6.5×10^5 gc l^{-1} in raw sewage samples obtained in this same WTP.

RVA, NoV and HAstV excretion patterns and their dissemination as environmental contaminants in water was described as dependent on seasonality, specific geographic area of study (high or low prevalence of specific pathogens) and also the potential outbreaks in the population (Girones *et al.* 2010). The use of different protocols could also cause mismatch results between unrelated studies. Bofill-Mas *et al.* (2006) compared two qPCR methods for their suitability in the detection and quantification of HAdV in waste samples and concluded that results differed significantly between both methods.

The environmental approach for detection and molecular characterization of virus in influent samples from the WTP contributes to epidemiological surveillance studies, which can provide information on genotypes that circulate in a particular geographic region. The direct sequencing of PCR amplicons used in the present study implies some loss of information on less prevalent genotypes, but this approach is time and cost effective compared with cloning and sequencing. With regard to the molecular characterization of NoV GII and HAstV from the sewage samples analyzed, it was found that the sequences belonged to the most globally prevalent genotypes, NoV GII.4 and HAstV type 1, and that these results are in agreement with data from the LVCA (the Regional Reference Laboratory for virus gastroenteritis) obtained from clinical samples from the same geographical area (Filho *et al.* 2007; Victoria *et al.* 2007; Ferreira *et al.* 2010; Carvalho-Costa *et al.* 2011) and also with previous studies performed in a small WTP in the same geographical region (Guimarães *et al.* 2008; Ferreira *et al.* 2009; Victoria *et al.* 2010). Therefore, particularly in relation to HAstV characterization from sewage samples, similar studies have found an unexpected prevalence of genotypes types 3 and 8 (Arraj *et al.* 2008; Rodríguez-Díaz *et al.* 2009).

For DNA virus characterization, data for HAdV corroborated previous studies that demonstrated that species F of HAdV (types 40 and 41) is the most prevalent DNA virus in aquatic environments (Ko *et al.* 2005; Haramoto *et al.* 2010; Fong *et al.* 2010; Cantalupo *et al.* 2011; Kokkinos *et al.* 2011); for JCPyV these results agreed with those observed in a study performed in a smaller WTP in Rio de Janeiro (Fumian *et al.* 2010). Brazil is a country the size of a continent; further studies with samples from other states should be carried out to clarify if the African-origin pattern for JCPyV is a strict characteristic of Rio de Janeiro's population only or if it reflects the overall population of Brazil. In a study performed on southern Californian wastewater, Rafique & Jiang (2008) found a higher diversity of JCPyV genotypes. These genotypes were characterized using PCR amplicon cloning and demonstrated the effective use of this approach to study virus diversity in sewage samples.

The results obtained in the present study show that the WTP could be efficient at removal of approximately 2-log units of viruses present in raw sewage. Quantitative data showed high levels of HAdV, JCPyV and RVA environmental dissemination, and addressed the potential of HAdV as a useful virological marker of virus contamination. In contrast, because of the sampling procedure used (grab sample) we could not properly infer the real removal efficiency of the WTP studied. The virus load reduction performed by the WTP should be highlighted, as sewage treatment is one of the most effective systems by which to reduce the presence and concentration of viruses and of other microbiological agents that circulate in aquatic environments and that are generated by human sewage discharge. However, our data, together with previous data from previous studies that detected infectious HAdV in a WTP after sewage treatment with activated sludge and final chlorination (Carducci *et al.* 2008), stressed the need for use of a virological marker as a parameter for water quality control.

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