Adenovirus, enterovirus and thermotolerant coliforms in recreational waters from Lake Guaíba beaches, Porto Alegre, Brazil

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ABSTRACT

In the present study, molecular detection of human adenoviruses (HAdV) and enteroviruses (EV) was performed in surface water samples collected from beaches Ipanema and Lami, located on the shores of Lake Guaíba, city of Porto Alegre, RS, southern Brazil. Furthermore, water safety was evaluated by counting thermotolerant coliforms (TC), following local government regulations. A total of 36 samples were collected monthly from six different sites along the beaches. Viral genomes were found in 30 (83.3%) samples. The higher detection rate was observed for HAdV (77.8%), followed by EV (22.2%). Although low concentrations of TC have been found, the occurrence of viral genomes in water samples was frequent and may pose a potential risk of infection for people bathing in these beaches.

Key words | adenovirus, enterovirus, lake beaches, viral analysis of water

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INTRODUCTION

Waterborne infectious diseases are a common cause of illness in humans worldwide, having a broader impact in developing countries. Water quality may be greatly impaired by the presence of pathogenic micro-organisms derived from fecal pollution. Currently, the microbiological monitoring of water safety is based on the detection of total and fecal coliforms in many countries (WHO 2008). The quality of surface water in Brazil is regulated by federal laws that define water classification and guidelines for different water uses. The Ordinance 357/2005 from the Brazilian National Environment Council (CONAMA 2005) establishes water microbiological standards for different purposes based on levels of thermotolerant coliforms (TC). Nevertheless, the lack or low concentrations of TC do not exclude the presence of different pathogens, especially enteric viruses, which may be spread by feces from both symptomatic and asymptomatic individuals (Fong *et al.* 2005).

The vast majority of enteric viruses are composed of a non-enveloped particle, which makes them highly resistant in the water environment and more tolerant to decontamination processes used in both drinking and wastewater treatments (Sobsey & Meschke 2003; Bosch *et al.* 2006). Human adenoviruses (HAdV) and enteroviruses (EV) have been suggested as reliable viral indicators for the monitoring

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of fecal contamination of water (Hot *et al.* 2003). HAdV are members of *Adenoviridae* family and can cause gastroenteritis, conjunctivitis, cystitis, as well as respiratory infections (ICTV 2009; Lenaerts *et al.* 2008). EV belongs to the *Enterovirus* genus of the *Picornaviridae* family, order *Picornavirales* (ICTV 2009). Most of these viruses cause asymptomatic infections. However, they may be also involved with gastroenteritis, mild respiratory illness, aseptic meningitis, acute flaccid paralysis, myocarditis and other clinical outcomes (Palacios & Oberste 2005).

Exposure to these viral agents during recreational activities is an important risk to public health. Many studies have reported the presence of enteric viruses in recreational waters in Europe and North America (McQuaig *et al.* 2012; Wyn-Jones *et al.* 2011). In Brazil, there are only few reports on the occurrence of enteric viruses in water from sea and lagoon beaches. Particularly regarding freshwater, a study demonstrated the presence of enteric viruses in recreational waters of the Rodrigo de Freitas lagoon in the city of Rio de Janeiro (Vieira *et al.* 2012). Sea water samples collected in beaches receiving water from the Rodrigo de Freitas lagoon were also heavily impacted by viral contamination (Victoria *et al.* 2014).

The Ipanema and Lami beaches are located on the shores of the Lake Guaíba, a freshwater lake that serves as a source of drinking water for the city of Porto Alegre, the larger city in the southern region of Brazil, with approximately 1,500,000 inhabitants. There is no information about the presence of enteric viruses in these waters, which are known to be highly polluted by non-treated sewage. The present study was designed to assess the viral contamination of surface water collected from beaches of Lake Guaíba.

MATERIALS AND METHODS

Study area and water sampling

The samples were collected from six points along the beaches of Ipanema and Lami. These waters are regularly used for bathing and a number of water sports, including water skiing, wakeboarding, jet skiing, sailing and parasailing. The lake receives great volumes of non-treated sewage from the Porto Alegre metropolitan area, which comprises a population of more than 3.7 million. The surface water quality in Lake Guaíba is monitored during the summer by the Waterworks Municipal Department (DMAE 2011). Ipanema beach is considered unfit for bathing and Lami beach is monitored weekly by examining three points along the waterfront by determining *E. coli* concentration (Bendati & Maizonave 1997). A previous study performed in samples collected from a water-stream from Porto Alegre city that drains directly into Lake Guaíba demonstrated the presence of enteric virus with the predominance of EV, followed by Torque teno virus and AdV (Vecchia *et al.* 2012).

Three points were selected in each beach, corresponding to the points for discharge of storm waters. Although there are no official numbers regarding the water volumes discharged at these points, it is known by the water companies and city authorities that many illegal connections from household tanks were built by local populations in to these pipes, which were designed originally only as a defense against rainwater. At Ipanema beach, all points presented same level of urbanization, and were named Point 1 (−30° 8′ 30.85″; −51° 13′ 38.13″), Point 2 (−30° 8′ 27.07″, −51° 13′ 39.40″), and Point 3 (−30° 8′ 22.73″, −51° 13′ 42.32″); for the Lami beach, Point 1 (South, −30° 14′ 34.04″, −51° 4′ 44.55″) has the lowest level of urbanization, compared to Point 2 (Middle, −30° 14′ 30.15″, −51° 5′ 0.07″) and Point 3 (North, −30°14′ 29.35″, −51° 5′ 7.40″). Sampling was carried out on the banks of the Lake Guaíba.

Water samples (500 mL) were collected aseptically, taken directly at a depth of approximately 30 cm, in sterilized glass bottles. The samples were transported to the laboratory and kept under refrigeration (4 °C), until sample concentration. The sampling was carried out on a monthly basis for 6 months, from November 2011 to April 2012.

Sample concentration

Putative viral particles present on the samples were concentrated using an adsorption-elution method with negatively charged membranes (HA, Millipore, USA), as described previously by Katayama *et al.* (2002) with modifications. Briefly, 500 mL of each water sample was mixed with 0.3 g $MgCl_2$ and pH adjusted to 5.0 with 10% HCl. Subsequently, the resulting mixture was filtered through a type HA negatively charged sterile membrane (0.45 μm pore size; 47 mm

diameter). After, the membrane was rinsed with 87.5 mL of 0.5 mM H_2SO_4 (pH 3.0), followed by elution of putative viral particles adsorbed with 2.5 mL of 1 mM NaOH (pH 10.5). The filtrate was then neutralized with 12.5 μL of 50 mM H_2SO_4 and 12.5 μL of 100x Tris-EDTA (TE) buffer. The resulting mixture was aliquoted and stored at –80 °C until further processing. The recovery efficiency for HAdV and EV of the method were 96% and 50%, respectively.

Viral nucleic acid extraction

Viral nucleic acids (RNA, EV and DNA, HAdV) were extracted from 400 μL of the concentrated sample using the RTP® DNA/RNA Virus Mini Kit (Invitek™, Berlin, Germany) according to the manufacturer's instructions. The viral RNA or DNA so obtained was kept at –80 °C until analysis.

Detection of viral genomes

A previous step of cDNA synthesis was conducted for amplification of EV genomes. High Capacity cDNA Reverse Transcription commercial kit (Applied Biosystems, USA) was used for this purpose, with the aid of random primers following the manufacturer's instructions. RNAse inhibitor (Applied Biosystems™, USA) was added as recommended.

All quantitative polymerase chain reactions (qPCRs) were performed using Platinum® SYBR® Green qPCR Super Mix-UDG (Invitrogen, USA), following the methodology recommended by the manufacturer and carried out as follows: 25 μL reaction mixtures consisting of 12.5 μL Supermix (Invitrogen, USA), 5.5 μL nuclease-free water, 1 μL of each nucleotide (20 pM) and 5 μL of DNA. To generate standard curves, 10-fold serial dilutions of standard controls from 10^{-1} to 10^{-5} were prepared, starting at 6.01×10^7 genome copies per reaction (HAdV-5) and 3.77×10^6 (EV). All standard controls and samples were run in triplicates. DNAse/RNAse free water was used as a negative control during all PCR reactions. Standard samples of Human adenovirus 5 (HAdV-5, prototype strain Ad5) and EV (Poliovirus 1, Sabin strain) cultivated in A549 and VERO cells, respectively, were used throughout as positive controls.

Amplification was performed using a thermal cycler (iQ5™ Real-Time PCR Detection System, Bio-Rad Laboratories). The PCR conditions were the same for HAdV and EV, and performed as follows: a denaturation step of 95 °C for 5 min followed by a two-step cycling protocol including a denaturation step at 95 °C for 15 s and an annealing/extension 60 s step at 55 °C for HAdV and 56 °C for EV. This step was repeated for 40 cycles in both cases, a melting-curve analyses were performed to verify product specificity (from 55 to 95 °C, 15 s each step). The sequences of the primers, their location in the viruses' genomes and the conditions used for amplification are given in Table 1.

The sensitivity of the assays was determined using 10-fold serial dilutions of each standard DNA or RNA, the analytical sensitivity of qPCR was of 10 genome equivalent copies/5 μL for both viruses. Only the results from assays within the range of $E = 90$–110%, slope in the range of 3.2 and $R^2 = 0.98$–0.99 were considered. All results were confirmed by checking of the peaks obtained during the high resolution melting curve.

TC

The most probable number (MPN/100 mL) of TC was determined in all samples. We used the method of multiple tubes

Table 1 | Oligonucleotides and conditions used for amplification of AdV and EV in qPCR

		Oligonucleotide					
Virus	**Target gene**	**Name**	**Sequence**	**Polarity**	**Position**	**Annealing Temp. (°C)**	**Amplicon (bp)**
AdV[a]	Hexon	VTB2-HadVCr	5'-GAGACGTACTTCAGCCTGAAT-3'	Forward	106–126	55°C	102
		VTB2-HAdVCr	5'-GATGAACCGCAGCGTCAA-3'	Reverse	190–207		
EV[b]	5'UTR	ENT-F1	5'-CCTCCGGCCCCTGAATG-3'	Forward	443–459	56°C	117
		ENT-R2	5'-ACACGGACACCCAAAGTAG-3'	Reverse	541–559		

[a]Primer sequences reported by Wolf *et al.* (2010).
[b]Primer sequences modified from those described by Tsai *et al.* (1993).

with culture medium A-1, according to the procedure described by Rice *et al.* (2012). Thermotolerant coliform counts were converted to log 10 values to ensure data normality.

Meteorological data

Meteorological data were recorded for the 2 weeks preceding the sample collections during the period of November 2011 to April 2012, using as source the publicly available data from the National Institute of Meteorology (INMET-8 District of Meteorology, Porto Alegre – RS, Brazil). Meteorological data such as precipitation, maximum and minimum temperatures and relative humidity were recorded for the 2 weeks preceding the sampling days.

RESULTS

Thirty-six samples in total were collected and analyzed by qPCR for viral presence. Viral genomes were detected in 30 samples (83.3%). The higher detection rate was observed for HAdV in 28 samples (77.8%); viral loads ranged from 1.35×10^2 to 4.08×10^4 genome equivalent copies per liter. EV was detected in only 8 samples (22.2%) in viral loads from 5.92×10^3 to 4.93×10^4 genome copies per liter. Thermotolerant coliform counting showed relatively low numbers in all samples with maximum of 240 MPN/100 mL. Results are summarized in Tables 2 and 3.

Comparing data from both beaches, the prevalence of HAdV was similar whereas EV was more frequently found in Lami (38.9%), with only one positive sample in Ipanema. In six samples (33%) from Lami, both viruses were detected. The highest viral load was found in November for EV in Lami beach (4.08×10^4 genomic copies/L) and in December for HAdV in Ipanema beach (4.08×10^4 genomic copies/L).

Taking into account the three points of sampling in Ipanema, Point 1 showed slight variations in the number of HAdV viral copies over the 6 months, except in April when values were a little higher and no detection in January. Point 2 displayed slight differences in the concentrations of HAdV, except for the months of November, January and February, when no virus material was revealed. In relation to EV, only in February was its presence observed. At

Table 2 | Detection of AdV and EV (genomic copies/L) and thermotolerant coliforms (TC; MPN/100 mL) in water samples collected from the Ipanema beach, Porto Alegre, Brazil, during the period of November 2011 to April 2012

		Ipanema		
Harvesting points		**1**	**2**	**3**
November	AdV	2.53×10^2	ND	1.39×10^3
	EV	ND	ND	ND
	CT	110	22	220
December	AdV	7.75×10^2	4.08×10^4	1.51×10^3
	EV	ND	ND	ND
	CT	23	13	23
January	AdV	ND	ND	1.35×10^2
	EV	ND	ND	ND
	CT	70	50	23
February	AdV	3.23×10^3	ND	1.62×10^3
	EV	ND	1.21×10^4	ND
	CT	13	2	4
March	AdV	3.06×10^3	1.44×10^4	2.03×10^4
	EV	ND	ND	ND
	CT	170	2	2
April	AdV	2.05×10^4	1.82×10^4	1.61×10^4
	EV	ND	ND	ND
	CT	8	4	240

ND = not detected.

Table 3 | Detection of AdV and EV (genomic copies/L) and thermotolerant coliforms (TC; MPN/100 mL) in water samples collected from the Lami beach, Porto Alegre, Brazil, during the period of November 2011 to April 2012

		Lami		
Harvesting points		**1**	**2**	**3**
November	AdV	ND	3.9×10^3	5.31×10^2
	EV	ND	4.93×10^4	ND
	CT	50	33	13
December	AdV	ND	9.04×10^3	3.65×10^2
	EV	ND	ND	$5{,}92 \times 10^3$
	CT	8	2	2
January	AdV	ND	8.72×10^3	3.75×10^3
	EV	ND	ND	1.35×10^4
	CT	8	23	13
February	AdV	5.46×10^3	4.44×10^3	2.16×10^3
	EV	1.81×10^4	1.03×10^4	ND
	CT	2	23	2
March	AdV	1.36×10^3	1.58×10^3	7.18×10^2
	EV	1.93×10^4	ND	ND
	CT	2	2	2
April	AdV	6.94×10^3	3.54×10^3	ND
	EV	ND	ND	1.91×10^4
	CT	170	23	30

ND = not detected.

Point 3, HAdV was detected in all samples analyzed while EV was not detected at all.

In Lami beach, at Point 1 no HAdV genomes were detected in the first 3 months, unlike what happened in the coming months where the number of viral copies was approximately 10^3 genomic copies/L. Regarding the EV, a similar number was observed in February and March. At Point 2, HAdV was detected in samples of every month, with an average of 10^3 genomic copies/L. On the other hand, EV was detected only in November and February (10^4 genomic copies/L). At Point 3, HAdV was present in all months except in April and EV appeared in December, January and April with titers ranging from 10^3 to 10^4 genomic copies/L.

All sampling points showed the presence of relatively low numbers of TC with higher concentrations in Ipanema beach, notably in November. Meteorological data are presented in Table 4.

DISCUSSION

The consumption of and recreation in contaminated water may pose serious risks to public health (Pond 2005; Fong & Lipp 2005; Carducci *et al.* 2006). In Porto Alegre city, the southernmost large urban area in Brazil, is Lake Guaíba, the main source of water supply of this same municipality. This lake receives untreated sewage of at least one-third of the population of the city.

In the present study, fecal contamination in surface water was evaluated by detection of TC and the presence of HAdV and EV. HAdV genomes were prevalent in both beaches (77.8%), whereas EV was more frequently detected in Lami beach (38.9%), with only one positive sample in Ipanema. The number of genome copies per liter ranged from 10^2 to 10^4, similar to a mean value of over 3,000 gc/L reported by Wyn-Jones *et al.* (2011) in 60% of 132 nested-PCR HAdV-positive water samples analyzed by qPCR. HAdV and EV are among the most commonly found viruses in waters as reported by several publications in Brazil and abroad. Aslan *et al.* (2011) using reverse transcription (RT)-PCR for analysis of water samples from the Great Lakes region in Chicago (USA) reported that HAdV was predominant (36%) compared with EV (20%).

Our results and those reported in the literature show variations in the frequencies of HAdV and EV. Factors such as depth and distance of sampling points, seasons when samples were collected, level of precipitation, the type of methodology utilized and regional features of viral distribution may have influences. It is important to emphasize that the detection of high or low levels of virus in a specific geographic area depends not only on virus excretion patterns in the population, but also the sample volume and protocol that is used for virus detection (Girones *et al.* 2010). It is also known that the doubled-stranded DNA of HAdV genome gives greater stability under adverse conditions (Wyn-Jones *et al.* 2011), which could allow it to stay for longer in the environment. A Europe-wide surveillance study was carried out to determine the frequency of occurrence of two human enteric viruses in recreational waters. From 1,410 samples, nearly 40% were positive for one or more of the target viruses. HAdV, detected in 36.4% of samples, was more prevalent than norovirus (9.4%) mainly in freshwater samples (Wyn-Jones *et al.* 2011).

In the present study, the frequency of positivity for HAdV and EV was irregular in both beaches, depending on the month and the sampling point. For Ipanema beach, at Point 3, detection of HAdV-positive samples occurred in all samples. Near this collection point is located the outflow of the Arroyo Capybara, one of the most polluted streams

Table 4 | Meteorological data recorded for the 2 weeks preceding the sample collections during the period of November 2011 to April 2012. *Source:* 8° Meteorology District – Inmet, Porto Alegre, Brazil

	November	December	January	February	March	April
Precipitation (mm)	6.5	18.7	110.2	27.70	4.8	25.10
Maximum temperature (°C)	29.7	30.8	25.1	31.9	31.1	26.5
Minimum temperature (°C)	17.8	18.2	22.2	18.1	17.2	18.3
Relative humidity (%)	72	86	79	68	78	92

flowing into Lake Guaíba (Bendati *et al.* 2000). On the other hand, on Lami beach HAdV-positive samples were detected mostly at Points 2 and 3. At Point 1 the lake is wider, consequently the water volume is bigger which could explain the lower counts observed. A survey conducted by Vieira *et al.* (2012) in which 144 water samples from Rodrigo de Freitas Lagoon in Rio de Janeiro, Brazil, were analyzed by qualitative and quantitative polymerase chain reaction assays, HAdV virus was less prevalent (16.7%), while group A rotavirus was the most prevalent (24.3%) followed by norovirus (18.8%). Other factors, including water volume of the samples, viral recovery efficiency, qPCR analytical sensitivity and the presence of putative inhibitors may influence the detection rates of viral genomes in water and of course this may also have to be taken into account in the present study (Girones *et al.* 2010).

It is known that excessive rainfall may cause an increase on the transport of pathogens, augmenting penetration into the soil and transport to rivers, surface water and wells. Furthermore, long periods of dry weather may reduce the volume of the flowing rivers, which can increase the concentration of pathogens (Cann *et al.* 2013). A 2-year study using samples from deep well and surface waters demonstrated that the HAdV and EV levels in the wells were associated with precipitation events and overall detection percentages varied through time from zero in early June 2008 to 100% in March and May of 2009 (Bradbury *et al.* 2013). Thus, meteorological data were evaluated in order to verify the relationship between the amount of rain and microbiological results obtained (Table 4). However, except for January, when the precipitation was approximately 110 mm, rain levels were very low during the sampling period making it difficult to assess to what extent the rain would have influence on the sites evaluated.

A study performed by Bendati *et al.* (2000) demonstrated that the upstream water of Lake Guaíba is the most polluted, as it receives water highly contaminated from rivers that flow into the water body and downstream the level of contamination reduces drastically. Thus, one expects a good water quality on Ipanema and Lami beaches. The analysis of water collected from both beaches showed that all samples were within the bacterial parameters for bathing (<1,000 MPN/100 mL) that have been established by the Brazilian regulation, although viruses were detected in 84% of those samples. These data corroborate previous studies that have shown no association between bacterial indicators and viral contamination (Carducci *et al.* 2006; Vieira *et al.* 2012). Because recreational waters are not subjected to any treatment and are considered suitable for swimming at certain bacterial levels, the presence of viruses presents a potential risk to public health that cannot be disregarded.

In conclusion, to our knowledge, this is the first description of viral genomes in water samples taken from the Lake Guaíba, Porto Alegre, Brazil. It has demonstrated the presence of enteric viruses in recreational waters that are considered suitable for bathing. Future studies may be carried out to assess whether or not these viruses are infectious and risk analysis is also necessary. Present results emphasize the need to include virological parameters when determining water quality to reduce the potential exposure of population to these pathogens.

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