Human adenovirus (HAdV), human enterovirus (hEV), and genogroup A rotavirus (GARV) in tap water in southern Brazil

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ABSTRACT

The effects of viral gastroenteritis are more devastating in children than in any other age category. Thus, children exposed to the consumption of low quality water are at an increased risk of infection, especially in regions where sanitation is inadequate. The present study aimed to provide a survey of the occurrence of representative enteric viruses: human adenovirus (HAdV), human enteroviruses (hEV), and genogroup A rotavirus (GARV) in tap water samples collected in public schools located at six municipalities of Rio Grande do Sul, southern Brazil. Seventy-three schools were included in the study and tap water samples were analyzed by conventional PCR for the presence of HAdV, hEV, and GARV genomes. hEV showed the highest detection rate (27.4%), followed by HAdV (23.3%), and GARV (16.4%). New approaches to water monitoring should be considered to promote a better water quality and reduce the risk of waterborne diseases, especially considering drinking water to be served to vulnerable individuals.

Key words | GARV, HAdV, hEV, tap water, virological analysis of water

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INTRODUCTION

The transmission of viruses through consumption of contaminated water and food is well recognized. Gastroenteritis associated with water ingestion is commonly reported and is an important health concern that can be caused by a wide array of viruses (Gallimore *et al.* 2004; Fabiana *et al.* 2007; Grant *et al.* 2012). Water quality may be greatly affected by the presence of pathogenic microorganisms derived from fecal pollution. Enteric viruses, which are transmitted by the fecal–oral route, are one of the most important causes of diarrhea in infants and children (Cheng *et al.* 2005; Boschi-Pinto *et al.* 2008; Hamza *et al.* 2011).

The most commonly studied enteric viruses on environmental matrices are human adenovirus (HAdV) and human enteroviruses (hEV), genogroup A rotaviruses (GARV), norovirus and hepatitis A and E viruses (Leclerc *et al.* 2002; Fong *et al.* 2005; Abdel-Moety *et al.* 2008; Gibson *et al.* 2011). Most of these viruses are non-enveloped, which makes them highly resistant in the environment as well as more resistant than bacteria to decontamination processes used in drinking and wastewater treatments (Sobsey & Meschke 2003; Van Heerden *et al.* 2003; Bofill-Mas *et al.* 2006; Bosch *et al.* 2006).

The HAdV is a virus with double-stranded DNA virus genome 28–45 kilobases (kb) long, belonging to genus *Mastadenovirus*, family *Adenoviridae* (ICTV 2009). At least 57 serotypes of HAdV in six subgroups (A–F) have been

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described (Buckwalter *et al.* 2012); HAdV serotypes 40/41, included in group F, are the major causes of gastroenteritis in young children and are readily spread by the fecal–oral route. The HAdV can cause respiratory infections, gastroenteritis, conjunctivitis and cystitis in humans (Uhnoo *et al.* 1984; Lenaerts *et al.* 2008; ICTV 2009). hEVs are positive-sense RNA viruses, with a genome of 7.2–8.5 kb, belonging to *Enterovirus* genus of the *Picornaviridae* family which comprises, among others, polioviruses, coxsackieviruses, and echoviruses. These viruses may replicate in the respiratory tract and the gut and can be transmitted through aerosols and by the respiratory route or via the fecal–oral route (Rodríguez-Lázaro *et al.* 2012). Infections by hEVs are usually asymptomatic or cause mild illness (Pallansch & Roos 2001; ICTV 2009; Lee *et al.* 2009). Rotaviruses (RV) are segmented double-stranded RNA viruses with genome of approximately 18.5 kb. These viruses belong to the *Reoviridae* family, and there are five major groups (A–E); group A RV (GARV) is associated with a large majority of human RV infections and represents the major cause of child mortality (Rodríguez-Lázaro *et al.* 2012) because of severe gastroenteritis in infants (Caprioli *et al.* 1996; Midthun & Kapikian 1996). Approximately 2 million deaths related to diarrhea are reported each year globally; GARV is responsible for almost 440,000 deaths per year among children under 5 years old, and is considered as the most important viral agent in severe cases of gastroenteritis in overcrowded hospitals (WHO 2009).

Given that these non-enveloped viruses are more resistant to decontamination processes used in both drinking and wastewater treatment, the present study aimed to provide a survey of the occurrence of HAdV, hEV, and GARV genomes in tap water samples from schools located at six municipalities of Rio Grande do Sul, southern Brazil.

MATERIALS AND METHODS

Water samples

Seventy-three tap water samples, one for each school, were collected aseptically in 500 mL sterile plastic bottles. The tap water samples were collected from the kitchen faucet of the schools, which used the tap water for the cooking of meals, preparation of fruit juices and washing of raw vegetables served to children for lunch. The sampling was performed from July to November 2009, in 19 schools from the city of Caxias do Sul (435,564 inhabitants), 16 from Pelotas (328,275 inhabitants), 12 from Santa Maria (261,031 inhabitants), 10 from Passo Fundo (184,826 inhabitants), nine from Bagé (116,764 inhabitants), and seven from Santa Cruz do Sul (118,374 inhabitants) (IBGE 2010). Figure 1 shows the location of the municipalities within the so-called mesoregions of the State of Rio Grande do Sul (geographic economic clusters within this state). All schools received water from conventional water treatment plants and were monitored following the Brazilian guidelines for drinking water quality (Ministry of Health 2011). The conventional water treatment includes the processes of coagulation, flocculation, decantation, filtration, chlorination, and fluoridation. The samples were transported to the laboratory under refrigeration (4 °C), and were kept in this condition until sample concentration.

Sample concentration

Tap water samples were concentrated using an adsorption-elution method with negatively charged membranes (HA, Millipore, USA), based on the method proposed by Katayama *et al.* (2002) with few modifications, as described by Vecchia *et al.* (2012). 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 vacuum filtered through a type HA negatively charged sterile membrane (0.45 μm pore size; 47 mm diameter). The membrane was rinsed with 87.5 mL of 0.5 mM H_2SO_4 (pH 3.0), followed by elution of viral particles adsorbed to the membrane 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 100× Tris–EDTA buffer. All procedures were made on biosafety cabinets to avoid sample contamination. The resulting mixture was aliquoted and stored at −80 °C until further processing.

Viral nucleic acid extraction

The viral nucleic acids (DNA of HAdV; and RNA of hEV and GARV) were extracted from 400 μL of the concentrated

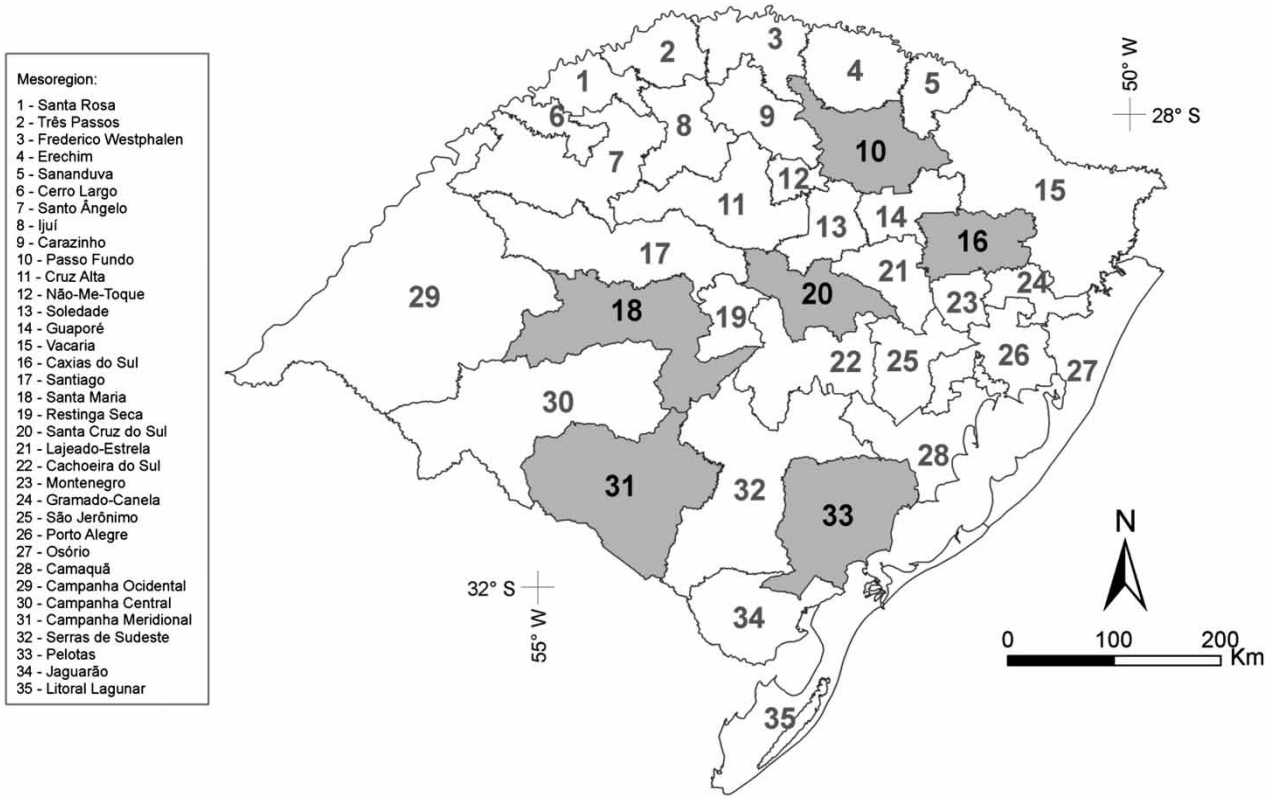

Figure 1 | Mesoregions of the State of Rio Grande do Sul. Each region hachured (in gray) on the map represents one of the municipalities in which sampling was performed.

sample using the RTP® DNA/RNA Virus Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The obtained viral DNA and RNA were kept at −80 °C until further analysis.

Polymerase chain reaction (PCR) assays for the detection of viral genomes

In order to achieve amplification of hEV and GARV genomes, a previous step of cDNA synthesis was carried out before amplification, using the High Capacity cDNA Reverse Transcription™ commercial kit (Applied Biosciences™, USA), with the aid of random primers and RNAse Inhibitor (Applied Biosciences), following the manufacturer's instructions.

Conventional polymerase chain reactions were optimized for the detection of HAdV, hEV, and GARV genomes. Reactions were standardized as follows: (1) HAdV and GARV: 50 μL reaction mixtures consisting of 25 μL of GoTaq® Green Master Mix (Promega, USA), 18 μL of nuclease-free water, 1 μL of each primer (20 pmole), and 5 μL of nucleic acid; (2) hEV: 25 μL final volume containing 12.5 μL of 2× PCR Master Mix™ (LGCbio, Brazil), 7.5 μL of nuclease-free water, 1 μL of each primer (20 pmole), and 3 μL of cDNA product. DNase/RNase free water was used as a negative control during all PCR assays. The positive controls used in amplifications were Poliovirus-1 (Sabin strain), kindly provided by Dr Carlos Nozawa; HAdV types 2 and 5, kindly provided by Dr Célia Barardi; and Human-GARV (VP6 I-2 Genotype), isolated from a clinical sample collected from a child with diarrhea at the nearby municipality of Porto Alegre, diagnosed and isolated in our laboratory.

All PCR reactions were performed as described previously (Vecchia *et al.* 2012) using primers designed to anneal in highly conserved regions of each viral genome. The primers used for HAdV amplification targeted the hexon protein gene, VTB2-HAdVCf (5′-GAGACGTACTTCAGCCTGAAT-3′) and VTB2-HAdVCr (5′-GATGAACCGCAGCGTCAA-3′) (Wolf *et al.* 2010). The primers used in hEV PCR reactions targeted

the 5-UTR region, ENT-F1 (5′-CCTCCGGCCCCTGAATG-3′) and ENT-R2 (5′-ACACGGACACCCAAAGTAG-3′) (Tsai *et al.* 1993; Vecchia *et al.* 2012). Finally, the primers used to amplify GARV genome targeted the VP6 protein gene, ROTAFEEVALE-FW (5′-GATGTCCTGTACTCCTTGT-3′) and ROTAFEEVALE-REV (5′-GGTAGATTACCAATTCCTCC-3′) (Vecchia *et al.* 2012).

Amplification of the target genomic fragments was performed using a thermal cycler (MultiGene®; Labnet International, USA). The PCR conditions were optimized for each virus group and were as follows: (1) HAdV: 98 °C for 7 min, 40 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; (2) hEV: 98 °C for 5 min, 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min; and (3) GARV: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 54 °C for 1 min (which was decreased by 0.5 °C at each of the 39 subsequent cycles, i.e., touchdown PCR), 72 °C for 1 min. Then, all reactions were left at 72 °C for 7 min for final elongation and submitted to an infinite cycle at 4 °C.

After the reactions, PCR products were stained with a nontoxic fluorescent dye, SYBR® Safe DNA Gel Stain (Invitrogen), analyzed by electrophoresis on 2% (w/v) agarose gel and visualized under ultraviolet (UV) light. The analytical sensitivity of each PCR has been described elsewhere (Vecchia *et al.* 2012). The identity of the amplicons was confirmed by nucleotide sequencing and all the positive samples were confirmed as expected viral targets.

Data analyses

The viral detection rate was compared with the percentage of sewage treatment on each municipality. The secondary data regarding the percentages of sewage treatment were collected from the Brazilian Sanitation National Information System (SNIS 2010). Correlation coefficients were calculated using the CORREL function on Microsoft Excel 2010™ spreadsheet software.

RESULTS

Overall, hEV were present in the majority of samples analyzed, achieving the highest detection rate (27.4%), followed by HAdV (23.3%) and GARV (16.4%). Results are summarized in Table 1. More than one type of the tested viruses was detected in 10 (13.7%) of the 73 samples analyzed. Two samples showed the simultaneous detection of HAdV, hEV, and GARV and eight samples showed the simultaneous detection of two viral species (four samples with HAdV and hEV genomes, three with HAdV and GARV, and one with hEV and GARV genomes). The three groups of viruses investigated in this study were detected in all cities, except for hEV, which was not found in the Santa Cruz do Sul samples.

DISCUSSION

In the present study, we were able to detect enteric viruses in tap water from public schools located at six cities of Rio Grande do Sul, southern Brazil. Our group recently described the presence of Torque teno virus (TTV) at these same sampling sites in a minor study made in parallel to the present one (Vecchia *et al.* 2013). Several studies have reported the presence of enteric viruses in tap water treated by conventional processes, which usually includes

Table 1 | Frequency of detection of the HAdV, hEV, and GARV viruses for each municipality

City	Number of samples	HAdV	hEV	GARV
Bagé	9	1/9 (11.1%)	1/9 (11.1%)	1/9 (11.1%)
Caxias do Sul	19	4/19 (21%)	10/19 (52.6%)	3/19 (15.8%)
Passo Fundo	10	4/10 (40%)	6/10 (60%)	3/10 (30%)
Pelotas	16	5/16 (31.2%)	1/16 (6.2%)	3/16 (18.8%)
Santa Cruz do Sul	7	1/7 (14.3%)	0/7 (0%)	1/7 (14.3%)
Santa Maria	12	2/12 (16.7%)	2/12 (16.7%)	1/12 (8.3%)
Total	73	17/73 (23.3%)	20/73 (27.4%)	12/73 (16.4%)

coagulation, flocculation, sedimentation, filtration, and chlorination (Ehlers *et al.* 2005; Heerden *et al.* 2005; Ye *et al.* 2012). The presence of GARV in drinking water was previously reported by He *et al.* (2009) in Beijing, China, who found GARV genome in 22.4% (32/143) of tap water samples, while in our study GARV genome was present in 16.4% (12/73) of samples. HAdV and hEVs were detected in drinking water of urban areas in South Korea by ICC-PCR in 39.1% (9/23) and 47.8% (11/23) of the tap water samples, respectively (Lee & Kim 2002), while our results showed a detection rate of 23.3% (17/73) for HAdV and 27.7% (20/73) for hEV. In our previous published results for TTV, only 11.6% of the samples were positive (in a limited number of samples analyzed) (Vecchia *et al.* 2013). In another study conducted by Lee *et al.* (2005), HAdV and hEVs were detected in tap water samples in Seoul, South Korea, using ICC-multiplex-nested-PCR assay. Fifty samples were tested and 58% (29/50) were positive for at least one virus, whereas we detected HAdV and/or hEV in 42.74% (31/73) of the total samples. These tap water samples were treated with conventional flocculation, sedimentation, filtration, and chlorination, which is similar to the treatment applied to water samples analyzed in this study. Nevertheless, it is important to keep in mind that the sample volume analyzed in these studies are different from the volume used in our study, thus making difficult the comparison of the results.

The percentage of treated sewage of each municipality was compared with the viral detection rates, since enteric viruses can be introduced in the environment through untreated or inadequately treated wastewater (Bosch *et al.* 2006; Okoh *et al.* 2010). As mentioned before, the sewage treatment rate of each municipality was taken from Brazilian SNIS, which is maintained by the Brazilian Government and provides data on the current situation of sanitation and wastewater treatment in Brazil. However, these data must be used cautiously since there is a lack of studies and supportive data about how the sewage discharge may impact the water quality in these areas. The municipality of Santa Maria has the highest sewage treatment rate (64.7%), followed by Passo Fundo (21.5%), Pelotas (19.2%), Bagé (16.8%), Santa Cruz do Sul (16.1%), and Caxias do Sul (12.1%) (SNIS 2010). Correlation analysis performed between the percentage of treated sewage and the detection of viruses showed, as expected, a negative relationship ($r = -0.16$). An additional analysis was performed between the percentage of treated sewage and each virus individually, which showed a negative correlation for HAdV ($r = -0.14$), hEVs ($r = -0.14$), and GARV ($r = -0.41$). Nevertheless, these correlation analyses were based upon a small sample size and an increase in the number of samples is required to better evaluate the relationship between the percentage of treated sewage and the viral detection rates.

CONCLUSIONS

The viral monitoring of drinking water is not mandatory according to Brazilian legislation (Ministry of Health 2011); however, virological analyses of water are important for risk assessment studies in order to reduce the health and economic impacts caused by gastroenteritis outbreaks. This is especially important when it comes to environments frequented by children, which are the most vulnerable population to viral gastroenteritis.

Although viral viability was not assayed in this study, the presence of enteric viruses' genomes in drinking water samples represents a risk to public health and may indicate the inefficiency of conventional water treatment to promote the total disinfection of viral agents. Therefore, these analyses must be taken into consideration for a new monitoring approach to promote a better water quality and reduce the risk of waterborne diseases, especially when it comes to susceptible groups such as children.

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