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Presence of Torque Teno Virus (TTV) in Tap Water in Public Schools from Southern Brazil

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Abstract Torque teno virus (TTV) was surveyed in tap water collected in schools from three municipalities located in the south of Brazil. TTV genomes were found in 11.7 % (4/34) of the samples. TTV DNA was detected in 10.5 % (2/19) of the samples collected at the city of Caxias do Sul and in 25 % (2/8) of the samples from Pelotas. Those cities have a low rate of sewage treatment. All samples from Santa Cruz do Sul, which has nearly 92 % of its sewage treated, were negative. These results suggest that the amount of sewage treated may have an effect on the detection rates of TTV DNA in drinking water in a given urban area, showing a mild negative correlation ($r = -0.76$), when comparing the percentage of sewage treatment to the detection of TTV genomes. The detection rate of TTV was also compared with *Escherichia coli*, showing a strong correlation ($r = 0.97$), indicating that TTV may be a suitable marker of fecal contamination.

Keywords Torque teno virus · TTV · Tap water

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Introduction

Enteric viruses, which are transmitted by the fecal–oral route, are some of the most important causes of diarrhea in infants and children especially in developing countries (Boschi-Pinto et al. 2008; Cheng et al. 2005; Hamza et al. 2011). The group of enteric viruses includes rotavirus, adenovirus, enterovirus, norovirus, and hepatitis A and E viruses (Fong and Lipp 2005). All these virus species share common structural features, mainly the absence of an envelope, which makes them more resistant to the common water treatment processes (Fong and Lipp 2005). There are several studies on the molecular detection of different species of enteric viruses in drinking water (Carter 2005; Haramoto et al. 2005; He et al. 2009; Kittigul et al. 2001; Li et al. 2011). Recently, Torque teno virus (TTV), particles of which are also non-enveloped and highly stable in the environment, has been proposed as a potential viral marker of water contamination (Diniz-Mendes et al. 2008; Griffin et al. 2008; Hamza et al. 2011; Haramoto et al. 2010; Verani et al. 2006). It is believed that the fecal–oral route is one of the modes of transmission of TTV, along with blood transfusion, organ transplant, sexual intercourse, and mother to child transmission (Hino and Miyata 2007).

TTV was the first discovered member of the *Anelloviridae* family, which is presently divided into 9 genera (Biagini 2009). The genome is composed by single-stranded negative sense DNA, of 3.4–3.9 Kb in length (Hino and Miyata 2007). The virus was first described in Japanese patients suffering from post-transfusion non-A–G hepatitis (22). Although first described as a possible cause for liver disease, the association of TTV with disease in human beings is also uncertain since the virus seems to be ubiquitous in the population and more than 90 % of healthy

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individuals have been found to be infected in a study in Russia (Vasilyev et al. 2009).

As mentioned before, a number of studies have been conducted on the detection and quantification of TTV genomes in water collected from rivers, streams, and wastewater (Diniz-Mendes et al. 2008; Hamza et al. 2011; Haramoto et al. 2005; Haramoto et al. 2008). The rate of detection is highly variable and seems to be dependent on the type of water, geographic region, and the laboratory methods applied for detection (Diniz-Mendes et al. 2008; Hamza et al. 2011). TTV DNA was detected in 96.8 % of influent samples collected from activated sludge wastewater treatment plants in Japan (13). In another study, TTV was found in only 12.6 % of the samples from a sewage treatment plant located in Pune, India (Vaidya et al. 2002). TTV DNA was detected in 25 % of the samples during the 1-year monitoring of a river contaminated with wastewater in Italy simultaneously with rotavirus and other viral agents (Verani et al. 2006).

Infection by TTV has been described in Brazilian transfusion donors and the virus was also found in 92.3 % of water samples collected from streams in Manaus, in the north of the country (Diniz-Mendes et al. 2008). Since TTV may indicate the presence of other viruses related to children's diarrhea, it may be of interest to assess whether the virus is present in drinking water (Griffin et al. 2008; Hamza et al. 2011). In the present study, the occurrence of TTV genomes was surveyed in tap water samples from schools located in three municipalities in Rio Grande do Sul, the southernmost state of Brazil. All these samples of water were collected in the schools' kitchens, and were used for cooking meals, preparing fruit juices, and washing raw vegetables to be served to children at lunch.

Materials and Methods

Collection of Samples

Thirty-four (34) tap water samples (500 mL) were collected in sterile plastic bottles, the faucets having been sterilized with ethanol before collecting the samples. The collections were made from July to September 2009, once per school, from taps in the kitchens of 19 schools in the city of Caxias do Sul (430,000 inhabitants), 8 in Pelotas (347,000 inhabitants), and another 7 in the municipality of Santa Cruz do Sul (120,000 inhabitants) (IBGE 2011). All schools received water treated at conventional water treatment plants, monitored in accordance with the Brazilian guidelines for the quality of drinking water (Brazil 2004). The samples were transported to the laboratory on ice and were kept in this condition until virus concentration.

The detection of TTV was compared to the percentage of sewage treatment in each municipality, as well as to the counting of thermotolerant coliforms for each sample. The secondary data regarding the percentages of sewage treatment were collected from the Brazilian National Sanitation Information System (SNIS, http://www.snis.gov.br/PaginaCarrega.php?EWRErterterTERTer=29), which is maintained by the federal government. Correlation coefficients were calculated using the CORREL function on the Microsoft Excel 2010™ spreadsheet software (Fig. 1).

Concentration of Virus from Tap Water Samples

Putative viral particles present in the samples were concentrated using an adsorption–elution method with negatively charged membranes (HA, Millipore, USA), as described previously with minor modifications (Katayama et al. 2002). 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). 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 (TE) buffer. The resulting mixture was aliquoted and stored at −80 °C until further processing.

Viral Nucleic Acid Extraction

Viral nucleic acids were extracted using 400 µl of the 2.5 mlconcentrated sample using the RTP® DNA/RNA Virus Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The viral DNA so obtained was kept at −80 °C until analysis. In order to verify the presence of possible inhibitors in the concentrated water used for the molecular detection of enteric viruses, 5 µl of each concentrated water sample was used in an additional PCR reaction using CytB gene as a target. In this additional PCR reaction, the concentrated water was used as part of the reaction, along with nuclease-free water, in order to verify whether it would inhibit the amplification of CytB gene in a DNA cell extract using universal CytB gene primers. The PCR reactions for the amplification of CytB gene were carried out in a final volume of 50 ul with the LGC Easy Taq Polymerase® kit as follows: 5 µl of 10x PCR Buffer, 1 µl of dNTP mix (10 mM), 1.5 µl of $MgCl_2$ (50 mM), 0.5 µl of Taq polymerase, 0.5 µl of each primer (20 pmol), 5 µl of extracted DNA, and 36 µl of water. Of the total water volume, 31 µl was RNase/DNase-free and 5 µl came from the wastewaters previously concentrated; this represents 10 % of the final volume of the reaction and

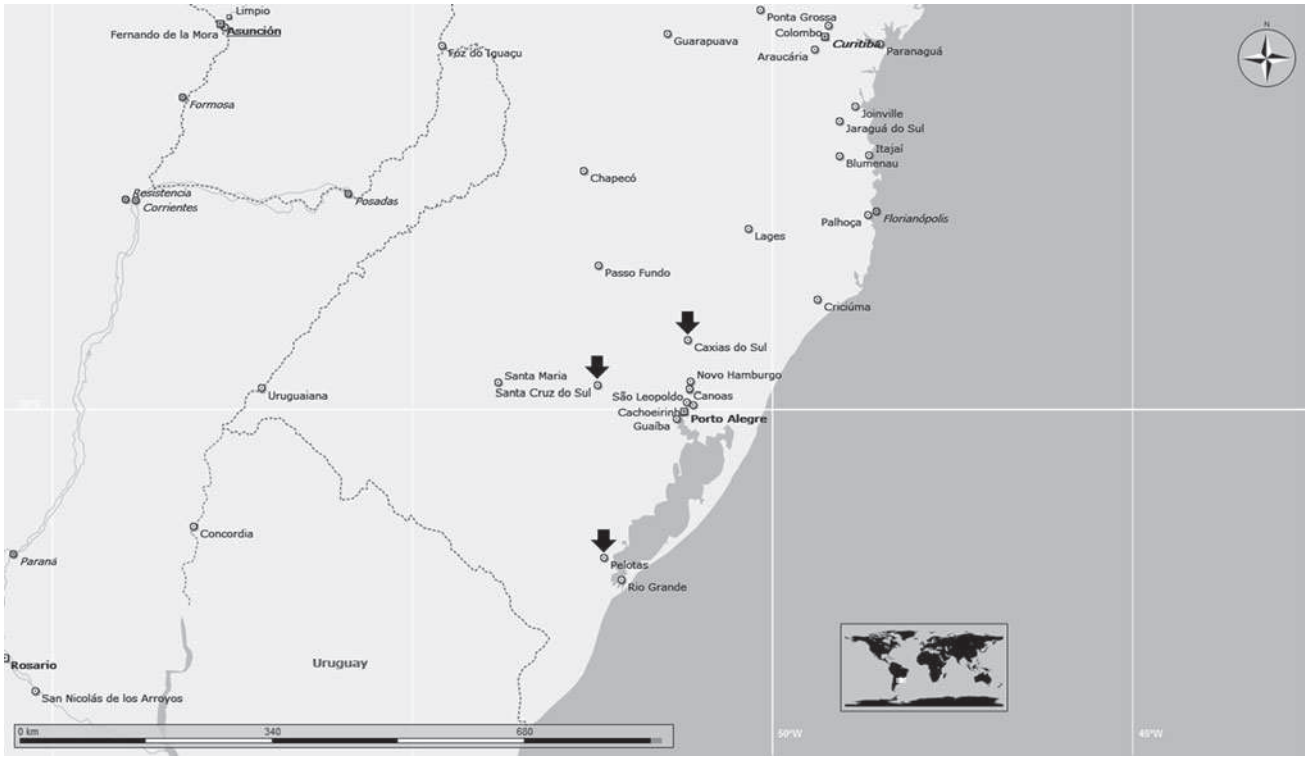

Fig. 1 Locations of the municipalities where the tap water samples were collected (*arrows*)

is equivalent to the volume of DNA commonly added in the PCR reactions for routine viral detection in our laboratory (approximately 20 ng, as compared on gel electrophoresis with Low Mass DNA Ladder, Invitrogen®, Carlsbad, CA). A touchdown PCR was performed with the following amplification conditions: an initial step of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C for denaturation, an initial annealing temperature of 57 °C which was decreased by 0.5 °C at each of the 29 subsequent cycles, and 30 s at 72 °C for extension, with a final step for prolongation of the chain for 10 min at 72 °C.

Polymerase Chain Reaction for TTV

A conventional polymerase chain reaction (TTV-PCR) was applied for the detection of TTV DNA. Two forward primers and one common reverse primer were used (F1—5′-GGG AGC TCA AGT CCT CAT TTG-3′; F2—5′-GGG CCW GAA GTC CTC ATT AG-3′) and a common reverse primer (5′-GCG GCA TAA ACT CAG CCA TTC-3′) targeted the ORF2 of the genome, with amplicons of an expected size of 103–107 bp. Although originally designed for the detection of Torque teno sus virus (Teixeira et al. 2011), TTV from human sera was efficiently amplified by those primers in previous assays and its identity was confirmed by sequencing (data not shown). To a final volume of 50, 25 µl of 2× PCR Master Mix^{tm} (LGCbio, Brazil), 22,5 µl of nuclease-free water, 0.5 µl of each primer (20 pM), and 1.0 µl of the DNA extracted from the concentrated water sample were added. Afterward, PCR products were stained with a non-toxic fluorescent dye, Blue Green (LGCBio, Brazil), analyzed by electrophoresis on 2 % (w/v) agarose gel, and visualized under ultraviolet (UV) light. The analytical sensitivity of the TTV-PCR was calculated on 10 genome copies per 500 mL, based on a previous assay using tenfold dilutions of a TTSV-cloned genome diluted in mock-contaminated ultrapure water samples.

Results

Overall, TTV genomes were found in 11.7 % (4/34) of the samples. TTV DNA was detected in 10.5 % (2/19) of the samples from the city of Caxias do Sul and 25 % (2/8) of the samples from Pelotas. All samples from Santa Cruz do Sul were negative. The detection rate of TTV was compared to the detection rates of *Escherichia coli* in the same samples (22), which presented a strong correlation ($r = 0.97$). The detection rate of TTV was also compared to the level of sewage treated in each municipality (IBGE, 2010) and a mild negative correlation ($r = -0.76$) was found. Results are summarized in Table 1.

Table 1 Rates of TTV DNA and *E. coli* detection in tap water samples collected from public schools in Southern Brazil and percentage of treated sewage of each municipality

Municipality	TTV (%)	*E. coli* (%)	Treated sewage (%)
Caxias do Sul	10.5 (2/19)	5.2 (1/19)	12.1
Pelotas	25 (2/8)	25 (2/8)	19.2
Santa Cruz	0 (0/7)	0 (0/7)	16.1
Total	11.7 (4/34)	8.8 (3/34)	

Discussion

The detection of TTV DNA in water samples may indicate the presence of other enteric viruses and fecal contaminants (Ahmed et al. 2009; Hamza et al. 2011). In the present study, the most populated municipalities, Caxias do Sul and Pelotas, treated their sewage very poorly at the time of sampling. On the other hand, the Santa Cruz do Sul samples were negative for TTV genomes; the population is smaller and 91.46 % of the sewage produced in the city is treated by conventional processes. Correlation analysis showed a mild negative correlation ($r = -0.76$) between the percentage of sewage treatment and TTV % of each municipality, which suggests that the level of sewage treated might have an effect on the detection rates of TTV DNA on those tap water samples. Other studies showed that a significant reduction in the number of genome copies in influent to effluent samples may be achieved in sewage treatment plants (Haramoto et al. 2005; Haramoto et al. 2008).

The detection rates of TTV genomes found in the present study (11.7 %) were within the range of detection presented in other studies. The levels of detection varied from 5.6 to 92 % in different types of water (Diniz-Mendes et al. 2008; Hamza et al. 2011; Haramoto et al. 2005; Verani et al. 2006). In this study, 500 ml of water samples were used to concentrate TTV, which is a lower volume when compared to other studies. Nevertheless, it has been demonstrated that no higher volumes are required when highly sensitive techniques are performed for viral genome detection, and larger volumes may concentrate more PCR inhibitors (1, 28).

The correlation between the presence of TTV (%) and *E. coli* (%) was strong ($r = 0.97$), although other enteric viruses may also be present in those tap water samples (Fong and Lipp 2005; Hamza et al. 2011; Haramoto et al. 2010). Besides, the correlation analyses were based upon a small sample size, and further assays may include a higher sample size to better evaluate the relationship between the presence of TTV and *E. coli* as well as the sewage treatment rate. All samples were tested after DNA extraction for the presence of PCR inhibitors, and no inhibitions were observed for the amplification of a control DNA fragment.

The presence of these viral genomes and the risk of gastroenteritis associated with its consumption indicate that Brazilian guidelines for the quality of drinking water should be revised to include viruses, as an auxiliary indicator of fecal contamination. Initiatives to improve sewage treatment in many Brazilian cities and to minimize the discharge of wastewater must be prioritized to avoid the burden of gastroenteritis in Brazil.

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