

Low occurrence of Hepatitis A virus in water samples from an urban area of Southern Brazil

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ABSTRACT

Hepatitis A virus (HAV), a member of *Picornaviridae* family, is the main causative agent of acute viral hepatitis in the world, mainly in developing countries. HAV may be present in contaminated water and food and its presence is often associated to a lesser extent with socioeconomic factors and environmental quality. The main goals in the present study were to standardize a cell culture combined to a polymerase chain reaction protocol for the detection and quantification of viral viability and analyze whether the virus could be found in water samples collected in four urban streams of Sinos River watershed. Virus recovery was assayed from known virus concentrations measured in experimentally contaminated raw and ultrapure water (MilliQ®). Recovery rates ranged from 270% in raw water to 15,000% in ultrapure water. In a second step, a qPCR coupled to a previous passage in cells, demonstrated more analytical sensitivity when compared to samples assayed without a previous passage in cell cultures. HAV genome was detected in only 1 of 84 samples analyzed, pointing to a very low occurrence of HAV in water samples in the studied region. These findings are remarkable, since no more than 5% of the domestic sewage in this area is treated pointing to a low occurrence of HAV in the population living nearby during the study period.

KEYWORDS: HAV. Stream. ICC-PCR. RT-PCR. Hepatitis. Nested-RT-PCR. Enteric virus.

INTRODUCTION

Hepatitis A virus (HAV) belongs to the genus *Hepatovirus*, within the *Picornaviridae* family, order Picornavirales. The genome is composed of a positive single-stranded RNA, with approximately 7.5 Kb of length¹. Two noncoding regions are found in the genome flanking regions nominated P1 (comprising the ORF encoding the structural proteins VP1, VP2, VP3 and VP4) and P2 and P3, which encodes non-structural proteins related to viral replication¹. HAV capsids are icosahedral and the viral particle is non-enveloped, thus being resistant under environmental conditions, although the virus is transitionally enveloped during traffic from the liver to the intestinal epithelium^{1,2}. Three of the 6 HAV genotypes are associated with infections in humans (I-III)³.

HAV particles are highly stable in the environment and may be detected for long periods in various matrices such as surface and groundwater, sea waters and sediments⁴. Through the years, HAV outbreaks have been reported with increased frequency in large urban areas¹. HAV outbreaks are related mostly to low socioeconomic levels and low environmental sanitation¹. The spread of the virus through contaminated water and food increased the number of individuals who became not naturally immunized in so called developed regions. Actually,

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the absence of children vaccination in many places poses a challenge for the control of HAV infections nowadays⁵. As water and food brought up the nature of the infection, it is *a priori* advisable that studies on the environmental monitoring of HAV should be done, aiming to fulfill the knowledge about the epidemiology, whenever it is possible.

In Brazil, hepatitis A is a great matter of concern to public health, especially in the Northern part of the country. Children's immunization has been routinely implemented. Since 2014, the vaccine was included in the governmental calendar, increasing the access to immunization. In Rio Grande do Sul, the Southernmost Brazilian State, HAV cases decayed in the last 10 years. In 2014, there was a very low prevalence of the disease, with no more than 100 cases reported⁶. Many studies in other parts of the country showed a decrease of less than half that would be related to the viral prevalence in environmental samples^{7,8}.

Molecular-based methods are useful for the detection of HAV genomes in water samples⁹. Adoption of protocols integrating molecular detection with cell culture, namely ICC-PCR, may allow inferring about the viral viability, providing information on the infectivity and health risks¹⁰. Additional ICC-PCR benefits may also include removal of inhibitors and increasing of viral particles counting through a cell passage, thus enhancing the analytical sensitivity of the technique when compared to molecular or cell-based detections alone¹¹.

The main goal of the present study was to standardize and apply molecular techniques coupled to a cell culture, the polymerase chain reaction providing quantification and evaluation of HAV viral viability in water samples from streams in urban areas of the Sinos River watershed. This watershed is highly impacted by discharges of untreated domestic sewer in water bodies, and has been used as the main source of drinking water for nearly 1.500.000 inhabitants, and the waters were previously found to be contaminated by other enteric viruses^{12,13}.

MATERIALS E METHODS

Samples

From 2012 to 2014, 0.5 L of water samples were collected, aseptically bimonthly, in four streams crossing 4 cities of Sinos River watershed: Estancia Velha/Portao (cities of Estancia Velha and Portao, 73,494 habitants), Schmidt (Campo Bom city, 60,074 habitants), Pampa and Luiz Rau (both located in Novo Hamburgo city, 238,940 habitants, [Figure 1](#)); two samples per stream, near the source and the mouth, totaling 84 samples. Water samples were transported to the laboratory in freezer blocks.

Afterwards, samples were kept at 4 °C until processing. As mentioned before, this period was characterized by a very low prevalence of HAV-induced hepatitis in this region according to official reports.

Viral concentration

Samples were concentrated by an adapted adsorption-elution method¹⁴ described before¹⁵. Briefly, before filtration, pH was measured and adjusted to 4.5-5.5. Afterwards, 12.5 mL of MgCl₂ 1M were added to samples. Five hundred millilitres of samples were filtered with the aid of a vacuum pump (760 mm Hg), using a 0.45 µm nitrocellulose negatively charged membrane (HA 47 mm, Millipore®), for the adsorption of viral particles present in the sample; 87.5 mL of 0.5 mM H₂SO₄ was passed through the membrane to rinse out the cation. Then, 2.5 mL of solution 1 mM of NaOH were added to the membrane for elution of viral particles. In 2.5 mL of the final eluate, 12.5 µL of 50 mM H₂SO₄ solution and 12.5 µL TE 100X buffer were added for neutralization, then samples were aliquoted and stored at -80 °C until further processing.

Viral nucleic acids extraction

A volume of 400 µL of the final viral eluate was submitted to nucleic acid extraction using RTP® DNA/RNA Virus Mini Kit (Invitek®, Germany), following the manufacturer's instructions. The final volume of 60 µL containing putatively viral RNAs was stored at -80 °C.

Recovery of the viral concentration method

To assay the viral recovery rates of the filtration-elution method described, aliquots of 500 mL of ultrapure RNase/DNase free water (Direct 8®, MilliQ®) and of raw water of Pampa stream, previously tested negative for HAV, were artificially inoculated by spiking 1 mL of a viral suspension of 5.2 x 10⁷ HAV (HM 175) CG/ 5 -µL. Before the viral inoculation, 1 mL of each sample vial was used as the negative control. Borosilicate bottles containing 500 mL of water were left closed at room temperature (21 °C) for 30 min. Afterwards, 1 mL of each sample was aliquoted before the viral concentration by the method described above. Final eluates of 2.5 mL were stored at -80 °C until further analysis. Viral genome extraction was performed directly from samples or after inoculating them into cell monolayers (ICC-RT-qPCR) as described above and the following types of samples were analyzed: 1) water before virus inoculation as the negative control (NC); 2) HAV inoculated in water before concentration (BC); 3) virus

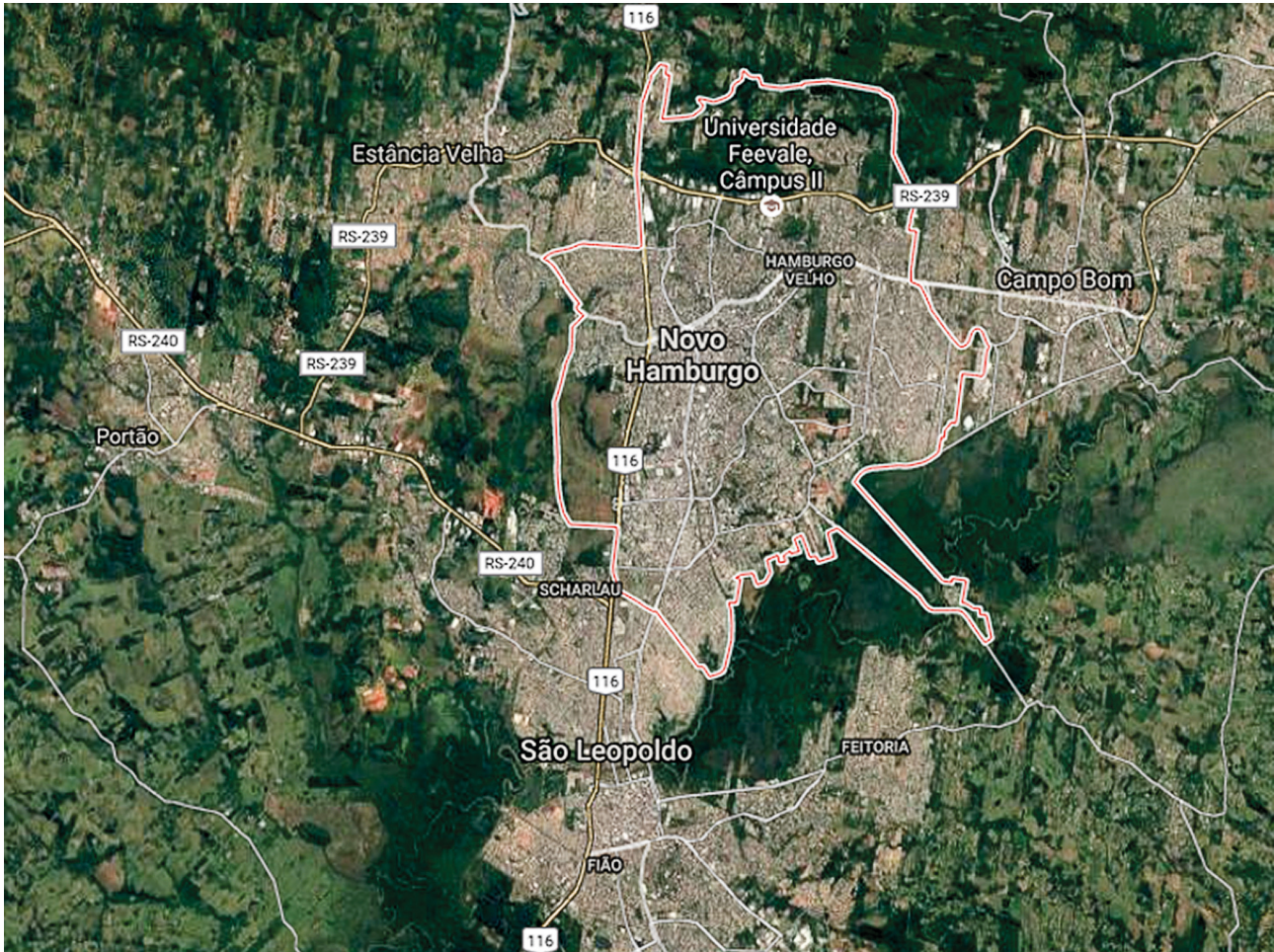


Figure 1 - Description of the counties Estancia Velha, Novo Hamburgo and Campo Bom.

inoculated in water after concentration, (AC); 4) virus that has a passageway by cell culture and analysis by ICC-RT-qPCR and Positive Control (PC).

Recovery rate calculation

Recovery rates were calculated using the values observed in the qPCR before the concentration process, defining as 100% of recovery, not evaluating losses in the processes of extraction and cDNA synthesis. Values obtained in 5 µL after the process of concentration were divided by the values obtained before the process and multiplied by 100, $X = b/a \times 100$, where X = recovery rate (%), a = quantification result for 5 µL by qPCR after concentration, and b = result for 5 µL by qPCR before concentration.

HAV genome detection

Complementary DNA synthesis (cDNA) using the RNA extracted from samples and controls was performed

using random primers of the High-Capacity cDNA Reverse Transcription kit™ (Applied Biosystems®, USA), following the manufacturer's instructions.

For quantitative PCR (qPCR), primers and a TaqMan® HAV-specific probe were used aiming at amplifying the conserved noncoding region 5'-UTR of the HAV genome, described before¹⁶ (sense 5'-GGTAGGCTACGGGTGAAAC-3'; reverse 5'-AACAACTCACCAATATCCGC-3'; probe FAM5'-CTTAGGCTAATACTTCTATGAAGAGATGC-3'BHQ). All qPCR assays were performed using TaqMan® Universal PCR Master Mix (Applied Biosystems®). For a final reaction volume of 20 µL, the reagents mixture contained 100 nM of each primer and the Taqman® probe, and 5 µL of cDNA. Each run was composed of 1 cycle of 60 min, denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 15 s. All reactions performed in 48 wells microplates (MicroAmp Applied Biosystems) in a StepOne real-time PCR system (Applied Biosystems®, USA).

Positive (plasmids) and negative (RNase/DNase free water) controls were included, and standard curves were

made using decimal serial dilutions from 10^{-1} to 10^{-10} genome copies of a plasmid containing the amplicon, kindly provided by Dr. Celia Barardi (Federal University of Santa Catarina, Brazil). Following the addition of 15 μL of the reaction mixture and 5 μL of cDNA, the microplate was sealed. All the samples and controls were tested in duplicate, to determine the efficiency and the limit of analytical sensitivity, values were calculated from a standard curve with dilution series of controls containing known concentrations of the target. Typical values obtained for efficiency were 103% and $R^2 = 0.99$.

Viral viability assay by ICC-RT-qPCR

Viral viability evaluation was performed using RT-PCR real time assay coupled to cell culture (ICC-RT-qPCR - *integrated cell culture qPCR*). This assay aims to detect possible infectious viral particles in environmental or biological samples. For the ICC-RT-qPCR, FRKH-4 cells (*Rhesus* Monkey Kidney Fibroblasts) were cultivated in 24-well plates (2.5×10^5 cells/well) at 37 °C and 5% CO_2 atmosphere until cellular confluence. Afterwards, 500 μL of the following were added to each well: concentrated water samples diluted 1:2 (non-cytotoxic dilution) and sterilized in 0.22 μm membranes positive control (HAV HM175 5.2×10^7 CG/ 5 μL) and negative control (Eagle's minimum essential medium – E-MEM). After 1 h of viral adsorption and uniform shaking, every 15 min, the initial inoculum was removed. In each plate cavity, 1 mL of E-MEM containing 1% of antibiotics (penicillin and streptomycin) and 2% of fetal bovine serum (FBS, Cultilab®) was added. Cell monolayers were incubated for 72 h at 37 °C in atmosphere with 5% of CO_2 . Afterwards, the supernatant of each cavity was removed and the lysis buffer was added directly to the monolayer cell. Cell lysates were transferred to 1.5 mL microtubes and the nucleic acids extraction, cDNA synthesis and qPCRs were performed previously described protocols.

Table 2 - Recovery rates of Hepatitis A (HAV) virus before and after the adsorption-elution concentration method in ultrapure and raw experimentally contaminated water.

Samples	Quantification (CG/5 μL)	Recovery rate (%)
Ultrapure water before inoculation	Neg	Neg
Ultrapure water + HAV before concentration	4.2×10^2	100
Ultrapure water + HAV after concentration	6.3×10^4	15000
Raw water before inoculation	Neg	Neg
Raw water + HAV before concentration	1.04×10^3	100
Raw water + HAV after concentration	2.7×10^3	270

RESULTS

The analytical sensitivity of qPCR fell under 100 genomic copies in serial dilution curves performed with plasmid dilutions. During viral recovery assays using HAV-spiked in ultrapure water, the minimum viral detection limit was 3 genomic copies, when PCR was coupled to cell culture (Table 1).

The viral concentration protocol was efficient, as inferred from the results of recovery assays, because quantification was 150 X higher in ultrapure concentrated water with 6.3×10^4 GC/ 5 μL (genomic copies/ 5 μL) than in non-concentrated water samples with 4.2×10^2 GC /5 μL (Table 2). In assays performed with ultrapure water samples spiked with HAV after the described cell culture (Table 1), it was possible to observe an increase of approximately 1 log in the quantification when compared to samples assayed without a cell culture step (Table 2). Before concentration, in samples assayed without a cell culture step, the value was 4.2×10^2 GC/ 5 μL and after cell culture, the value was 1.1×10^3 GC/ 5 μL . After concentration, in samples assayed without a cell culture step, the value was 6.3×10^4 GC/5 μL and after cell culture, the value was 1.3×10^5 . From the environmental samples analyzed, only one was HAV-positive (1.5×10^4 cg/L) coming from point 4 of the Pampa stream, which crosses

Table 1 - Analytical sensitivity limit of ICC-qPCR reaction to Hepatitis A virus artificially inoculated in ultrapure water before and after the viral concentration process by the adsorption-elution method.

Sample dilutions	Quantification before concentration ^a	Quantification after concentration ^a
Non-diluted	1.1×10^3	1.3×10^5
10^{-1}	1.1×10^2	1.6×10^5
10^{-2}	10	1.8×10^4
10^{-3}	3	1.5×10^3
10^{-4}	3	3.1×10^2

^a values in genomic copies/5 μL .

the larger city included in this study, namely Novo Hamburgo (July/2013).

DISCUSSION

ICC-PCR assays have been used for detecting several other enteric viruses in environmental samples^{10,17,18}. The results have shown that the qPCR using TaqMan[®] was adapted to the integrated use proposed in the cell culture protocol, increases the technique sensitivity possibly by viral growth that may enhance the number of genome copies and decrease amplification inhibitors.

The recovery percentage in raw water was lower than in ultrapure water as expected, probably due to the presence of inhibitors. An increase in the quantification of raw samples was also observed. In other studies, the best recovery was found in distilled water and treated wastewater effluents (100%), and the lowest recovery (10%) in seawater¹⁹. In addition, like in this study, no detection of HAV was found in coastal water²⁰ and for both, Norovirus (NoV) and Human astrovirus (HAstV), the recovery percentage from mineral and river water samples was between 18% and 64%²¹.

These results show that the use of integrated cell culture to ensure the detection of HAV in water may favor analytical sensitivity, as it was observed in the past for other enteric viruses²². Another advantage is the inference of measures to control infectivity and consequently the health risk posed by the presence of HAV particles in water¹⁸.

The Pampa stream is highly impacted and urbanized, receiving industrial garbage discharges and untreated sewer²³. Other studies reported poor water quality conditions in this particular stream by monitoring of physical and chemical parameters¹². Although the detection of a solely positive sample for HAV shows that no matter this stream has been hugely contaminated by domestic sewage, the detection of this virus under environmental matrices is often sporadic. No other samples were positive for HAV gene sequences, even after cell passages.

In another study conducted in the North region of Brazil, 23% of HAV positive samples were detected by *nested*-RT-PCR and 92% using RT-qPCR, but it may be speculated that these samples were collected during or after HAV outbreaks⁷. The same may have happened in another survey on recreational seawater in Florianópolis, in which 46% of the samples were found to be contaminated by HAV²⁴. On the other hand, many studies in Brazil showed low occurrence or absence of HAV in water, as has also been reported for the area of the study, regarding acute viral hepatitis of official government reports throughout the sampling period. In 2009, HAV was detected in 8.3% of freshwater samples from Florianópolis¹⁹ and no positive

results for HAV were found in 108 samples analyzed in Rio de Janeiro²⁵. Other studies have also shown a low incidence of HAV in environmental matrices, ranging from 20% to 32% positive samples, respectively, reinforcing the sporadic occurrence of HAV in surface waters in Brazil^{26,27}.

However, since HAV is an endemic condition in Brazil, with the improvement of sanitation in recent decades, a decrease in the seroprevalence has been observed in several regions of the country. Unlike low endemic countries, the vaccine is only recommended depending on epidemiological studies of the region and risk groups involved, especially for children under five years old²⁸.

CONCLUSION

Based on viral recovery assays and qPCR efficiency evaluation performed in this study and in the environmental samples analysis, we can conclude that there was a low virus occurrence in superficial waters in the region studied.

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