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Research Article

Two-year monitoring of enterovirus and rotavirus A in recreational freshwater from an island region, Pará State, northern Brazil

Jainara Cristina dos Santos Alves[1], Dielle Monteiro Teixeira[1], Danielle Rodrigues de Deus[1]
Vanessa Cavaleiro Smith[1], Denise Suellen Amorim de Sousa Santos[2]
Renato da Silva Bandeira[1], Jones Anderson Monteiro Siqueira[1]
Lena Líllian Canto de Sá Morais[2], Jacqueline Cortinhas Monteiro[3]
Luana da Silva Soares[1], Fernando Neto Tavares[1] & Yvone Benchimol Gabbay[1]
[1]Virology Section, Evandro Chagas Institute, Brazilian Ministry of Health
Ananindeua, Pará, Brazil
[2]Environment Section, Evandro Chagas Institute, Brazilian Ministry of Health
Ananindeua, Pará, Brazil
[3]Virology laboratory, Institute of Biological Sciences, Federal University of Para
Brazilian Ministry of Education, Belem, Para, Brazil
Corresponding author: Yvone Benchimol Gabbay (yvonegabbay@iec.gov.br)

ABSTRACT. Enteric viruses are major causes of waterborne diseases and are present in large quantities in the stools of infected individuals. Its viability in the environment lasts for months, favoring the contamination of water used for consumption and recreation. The study aimed to monitor monthly the circulation of enterovirus (EV) and group A rotavirus (RVA) in recreational freshwater from an island region used as a bathhouse in northern Brazil, from January 2012 to December 2013. The viral RNA was obtained using guanidine isothiocyanate/silica after viral concentration by adsorption-elution method. The molecular detection was carried out by semi (EV) and nested-PCR (RVA) and the amplicons were sequenced on automated sequencer. At least one of these viruses was detected on 40.4% (42/104) of the samples. RVA was the most frequent (n = 32; 30.8%) when compared to EV (n = 20; 19.2%). Co-circulation between both was identified in 9.6% (n = 10). The highest viral positivity was found in SP02 (46.1%). The highest viral positivity was observed during high tides (57.7%; 60/104). Most EV samples were characterized as coxsackievirus (CV) A5 (85.7%, 12/14) and others as Sabin 1 poliovirus (14.2%, 2/14). The RVA positive samples were genotyped as G2, G3, G9, G12, P[8], P[4], and P[6]. These viruses were detected in 35.6% (37/104) of the samples with an acceptable concentration of fecal coliform bacteria. These results demonstrate the contamination of surface water intended for recreation by enteric viruses of Public Health concern even when bacterial indicators are within the tolerated limit, a factor that confirms the need for public policies aimed the sewage treatment before its release into water bodies.

Keywords: enterovirus; rotavirus; freshwater; beaches; northern Brazil

INTRODUCTION

Environmental monitoring is a useful tool for water quality control allowing the traceability of contamination sources to prevent possible waterborne diseases. It also provides information on potential exposures associated with recreational water use (Fumian et al. 2018, Girardi et al. 2019). Raw sewage is one of the main sources of contamination in these environments and if released without prior treatment, it can spread

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various pathogens associated with diseases such as gastroenteritis (Farkas et al. 2020).

Enteric viruses, such as enterovirus (EV) and rotavirus A (RVA), are excreted in high concentrations in the feces of infected individuals and are considered important causes of diarrheal disease and significant source of water contamination (Justino et al. 2019, Machado et al. 2020, Pérez-Martínez et al. 2021, Gholipour et al. 2022).

EV is non-enveloped single-stranded RNA-positive viruses with capsids measuring about 30 nm in diameter belonging to the Picornaviridae family (Zell et al. 2017). These viruses cause several clinical manifestations, ranging from mild (respiratory and gastrointestinal infections, herpangina and hand-foot-and-mouth disease) to severe diseases (pleurodynia, hepatitis, myopericarditis, pancreatitis, meningitis, encephalitis, paralysis, and neonatal sepsis) (De Crom et al. 2016). The EV is a known waterborne pathogen, with well documented spread in recreational water in both developed and developing countries and its use as a viral indicator of fecal contamination in these water matrices is also suggested (Allman et al. 2013, Lee et al. 2014, Maurer et al. 2015, Sarmirova et al. 2016, Aguirre et al. 2019, Masachessi et al. 2021).

RVA is a non-enveloped segmented linear double-stranded RNA virus with diameter of 60-100 nm, recently classified into Sedoreoviridae family (Matthijnssens et al. 2022). According the Rotavirus Classification Working Group (RCWG), this virus is classified into G and P genotypes based in VP7 and VP4 protein sequences respectively. The VP6 protein is also used on environmental and food samples genotyping (Assis et al. 2015, Marinho et al. 2018, El-Senousy et al. 2020). Most RVA infections occur primarily in children under five years and they are responsible for >500,000 deaths annually even in the global post-vaccination scenario, with main clinical manifestations ranging from diarrhea, vomiting, malaise and fever episodes to leading to rapid and intense dehydration, hindering the effectiveness of oral rehydration (Crawford et al. 2017). Several studies have already demonstrated not only the presence of this virus in water intended for recreation but also the risk assessment for RVA infection and illness (Vieira et al. 2012, Prez et al. 2015, Elmahdy et al. 2016, Bortagaray et al. 2020, Cadamuro et al. 2021).

In many countries, including Brazil, bathing water quality is still evaluated by the fecal indicator bacteria (FIB) index to prevent probable risks to human health (CONAMA 2000, Tiwari et al. 2021). However, the failure to identify sources of fecal contamination is a limitation concerning this microbiological quality assessment, in addition to not necessarily indicating the presence of other pathogens, such as viral ones (Tiwari et al. 2021).

In this study we conducted a two-year monitoring (2012 and 2013) of the presence of EV and RVA, two important enteric viruses of public health interest, in surface freshwater subjected to different water current flows (low and high tide) and rainfall regimes, obtained in an insular region of northern Brazil, which is very popular on weekends, holidays and vacation for recreational and bathing activities.

MATERIALS AND METHODS

Area of study

This study was carried out in an island region located in northern Brazil with humid tropical climate and direct influence of rivers that give rise to freshwater beaches, considered a tourist attraction for the region, mainly due to the proximity to the continent (about 70 km). This coastal zone is influenced by semidiurnal tides and precipitation throughout the year (Costa et al. 2022). In this area it is common to rain all year round, with periods when precipitation is lower (July to November) and more frequent (December to June). Disordered urbanization and the lack of basic sanitation have affected the quality of surface water in this region, which requires better public policies. Four sampling points were selected (SP01, SP02, SP03, SP04) around the island (Fig. 1).

Collection and viral concentration of the water samples

From January 2012 to December 2013, surface freshwater samples were collected monthly for each sampling point, with the exception of July, which was fortnightly (school vacation period), totaling 26 samples per point. The collections always took place on monday mornings, due to the large number of visitors during the weekends on the island. Two liters of water were collected and kept refrigerated (2-8°C) in sterilized polypropylene bottles and packed in isothermal boxes and send to the laboratory for immediately processing. The probable viral particles were concentrated from 2 L of water by adsorption-elution method in a filter membrane (Katayama et al. 2002), obtaining a final volume of 2 mL after a second concentration by filtration in Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore, County Cork, Ireland).

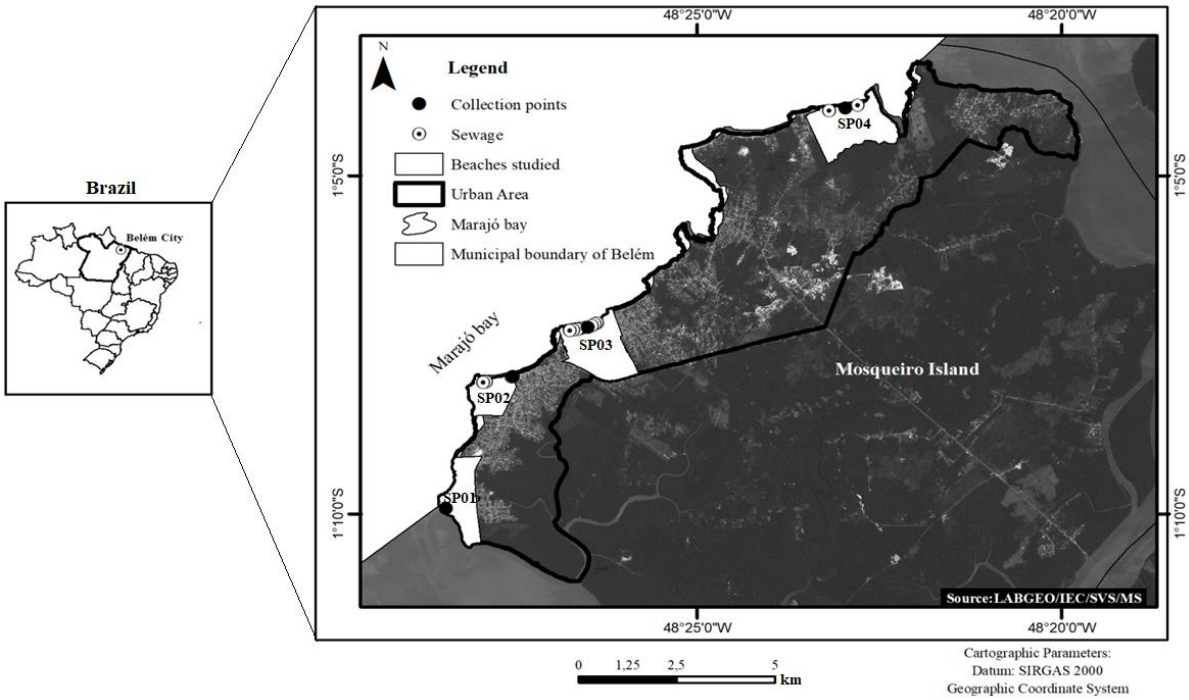

Figure 1. Map showing the four sampling points where the recreational surface freshwater samples were collected, in an island region of Pará State, northern Brazil, during a two-year monitoring (Janurary 2012 to December 2013).

Viral RNA extraction and reverse transcription for cDNA synthesis

Viral RNA was extracted from 400 µL of the previously concentrated sample using guanidine isothiocyanate/ silica method (Boom et al. 1990). The complementary DNA (cDNA) synthesis for EV and RVA was performed from 10 µL of extracted RNA added into 2 µL of dimethyl sulfoxide (DMSO) (SIGMA®), this mixture was incubated at 97°C for 7 min, followed by an ice bath for 2 min. Then, 38.0 µL of the reagent mixture (40 mM dNTP, 10X PCR Buffer, 50 mM $MgCl_2$, 3µg μL^{-1} random primer, SuperScript II Reverse Transcriptase, Nuclease Free Water) were added to the RNA+DMSO mixture and incubated at 42ºC for 60 min and 95ºC for 10 min. The product was stored at -20ºC until the use.

Enterovirus molecular detection

The semi nested-PCR was performed to detection a partial region of EV VP1 using a set of P2/P3 and P3/P10 genus-specific oligonucleotides in the first and second amplification steps, respectively (Zoll et al. 1992). In the PCR reaction, 5.0 µL of cDNA was used and, 2.0 µL of the PCR product generated on the nested-PCR. It was performed a reagent mixture containing 2.5 µL of 10X PCR Buffer; 0.3 µL of 50 mM $MgCl_2$; 2.0 µL of 10 mM dNTP; 0.9 and 0.6 µL of forward and reverse primers in the first and second amplifications round, respectively (P2/P3 and P3/P10); 0.3 µL of 5U μL^{-1} Taq DNA Polymerase; 13.1 and 16.0 µL of nuclease free water in the first and second reaction, respectively.

Rotavirus molecular detection and genotyping

The RVA detection was carried out by nested-PCR using specific primers targeting a partial region of VP6 protein (Gallimore et al. 2006). In the PCR was used 4.0 µL of cDNA and on the nested 2.0 µL of the PCR product. A 25.0 µL of PCR and nested reactions were performed containing 2.5 µL of 10X PCR Buffer; 1.5 µL of 50 mM $MgCl_2$; 2.0 µL of 10 mM dNTP; 1.0 µL of forward and reverse primers (VP6F/VP6R and VP6NF/VP6NR in the first and second step, respectively); 0.3 µL 5U μL^{-1} of Taq DNA Polymerase; 12.7 and 14.2 µL of nuclease free water in the first and second reaction, respectively.

A binary classification (G and P genotypes) for RVA was used, by the partial amplification of the segments that encode the VP4 (Gentsch et al. 1992) and VP7 protein (Gouvea et al. 1990, Das et al. 1994). VP4

semi nested-PCR reactions were carried out using 2.5 µL of 10X PCR Buffer; 1.5 µL of 50 mM $MgCl_2$; 2.0 µL of 10 mM dNTP; 0.5 µL of forward and reverse primers (4con3/4con2 and 4con3/Pool of P types, in the first and second step, respectively); 0.25 µL 5U µL of Taq DNA polymerase; 15.25 and 13.75 µL of nuclease free water in the first and second reaction, respectively. In the PCR was used 2.5 µL of cDNA and on the nested 1.0 µL of PCR product. For VP7 semi nested-PCR, 25 µL reactions were performed contai-ning 2.5 µL of 10X PCR Buffer; 1.5 µL of 50 mM $MgCl_2$; 2.0 µL of 10 mM dNTP; 0.5 µL of forward and reverse primers (end9/beg9 and end9/Pool of G types, in the first and second step, respectively); 0.3 µL 5U μL^{-1} of Taq DNA polymerase; 15.25 and 13.75 µL of nuclease free water in the first and second reaction, respectively.

Nucleotide sequencing of enterovirus positive samples

Semi nested-PCR EV positive amplicons were purified according to the manufacturer's recommendations using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified fragments were compared with those contained into Low DNA Mass Ladder (Invitrogen, USA) in a 2% agarose gel. For sequencing, the BigDye Terminator v3.1 kit (Life Technologies, Carlsbad, CA, USA) was used. Samples were submitted to 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min in a thermocycler (Eppendorf®, Hamburg, Germany). The DNA was precipitated with 70% isopropanol in a 96-well plate that was vortexed and incubated at room temperature for 15 min, followed by centrifugation for 45 min at 4000 g. Then, after removing the isopropanol, 70% ethanol was added and the plate was subjected to centrifugation for 10 min with the same rotation as before, but using 15 min. After ethanol disposal, the precipitated DNA was dried in a thermocycler at 60°C for 4 min, resuspended with 10 µL of Formamida Hi-Di™ (Applied Biosystems, Foster City, CA, USA), and subjected to denaturation at 95°C for 5 min and thermal shock on ice for 2 min, for subsequent sequencing on the 3130xL Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis of enterovirus sequences

The sequences obtained were edited and aligned in the BioEdit® v.7.2.0 and later compared to those already deposited on National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) or using the Basic Local Alignment Search Tool (BLAST tool). Maximum likelihood phylogenetic tree with 1000 bootstrap replicas were generated by the MEGA v 6.06 (Tamura et al. 2013), using the Kimura-2 parameters as substitution model (Kimura 1980). The nucleotide sequences of EV were deposited in GenBank under the accession numbers MT880856-MT880869.

Tide data and precipitation

The tide data were obtained from the website of Brazilian Navy Hydrography Center (https://www.marinha.mil.br/chm/) according to the available tide tables and considering the days and times of the collections conducted in the years of 2012 and 2013. Rainfall information was obtained from the website of the Brazilian National Institute of Meteorology (https://portal.inmet.gov.br/).

Statistical analysis

The G test was performed to assess the hypothesis that the occurrence of viruses was independent or it was associated with the sampling site and the association among virus contamination and bacterial concentrations. The Simple linear regression test was chosen to assess the hypothesis that viral positivity (dependent variable) did not change as a function of precipitation (independent variable). The chi square test was used to verify whether the viral positivity found varied between the first and second semesters of the years analyzed and between low and high tides. All tests were performed using BioEstat. 5.3 software (Ayres et al. 2007), considering P-values ≤ 0.05 statistically significant.

RESULTS

From January 2012 to December 2013 a total of 104 recreational surface freshwater were collected and analyzed and at least one of the enteric viruses (EV and RVA) investigated in the present study were detected in 40.4% (42/104) of these samples. RVA was the most frequent (n = 32; 30.8%) when compared to EV (n = 20; 19.2%). Co-circulation among both viruses was identified in 9.6% (N = 10) of the samples. The results of G-test showed that the virus occurrence (EV, RVA and co-circulation of both) is independent of the sampling point in the samples evaluated (Table 1).

In April 2012, RVA were detected at all sampling points, while EV was identified at all sites in the first collection of July 2013. In 2012, the co-circulation of both viruses was evidenced in the months of May to July and November, and in 2013 only in the months of March and July (second sampling). In some months of 2012 (March, August and December) and 2013 (January, May, June, September and December) there

Table 1. Detection of enterovirus (EV) and group A rotavirus (RVA) in 104 recreational surface freshwater samples from an island region, Pará State, northern Brazil, during a two-year monitoring (January 2012 to December 2013).

Sampling point	Viruses detection			
	Only EV % (n/total)	Only RVA % (n/total)	EV+RVA % (n/total)	% (n/total)
SP01	3.8 (1/26)	30.8 (8/26)	7.7 (2/26)	42.3 (11/26)
SP02	7.7 (2/26)	26.9 (7/26)	11.5 (3/26)	46.1 (12/26)
SP03	15.4 (4/26)	11.5 (3/26)	3.8 (1/26)	30.8 (8/26)
SP04	11.5 (3/26)	15.4 (4/26)	15.4 (4/26)	42.3 (11/26)
% (n/total)	9.6 (10/104)	21.1 (22/104)	9.6 (10/104)	40.4 (42/104)
G-test (*P*-value)	1.9425 (0.5844)	4.5376 (0.2090)	2.1599 (0.5399)	

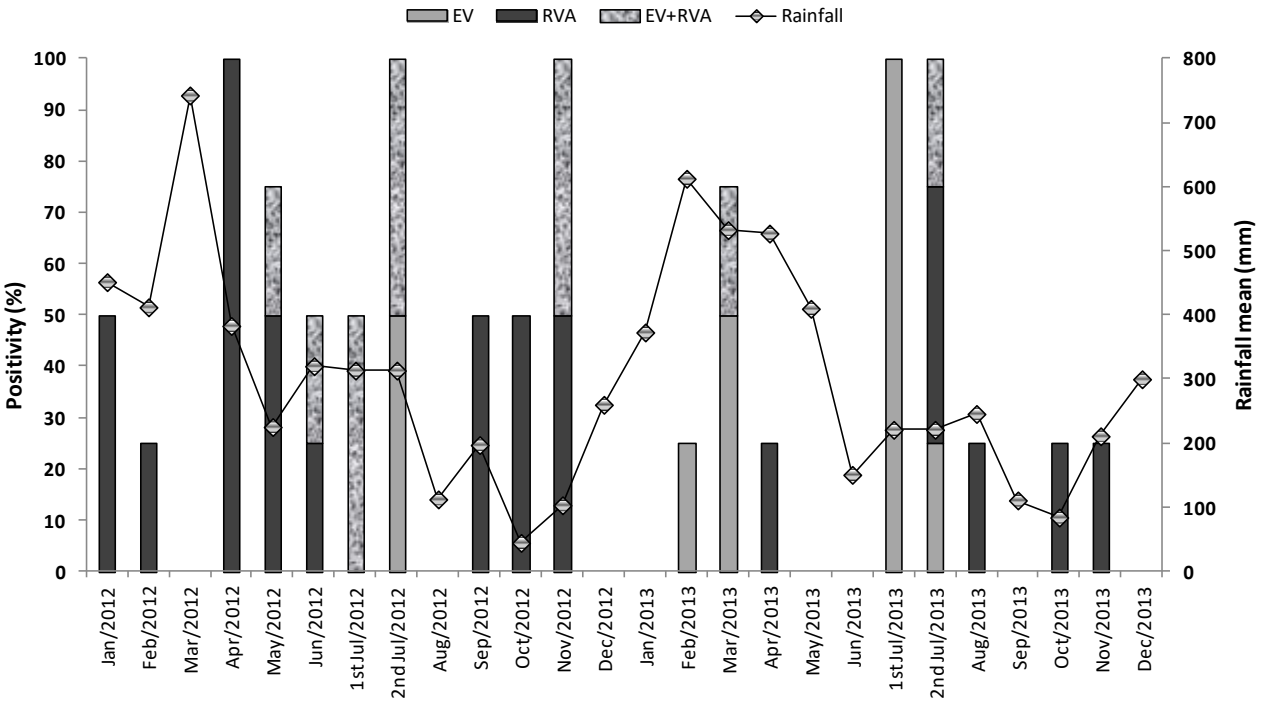

Figure 2. Rainfall and monthly distribution of enterovirus (EV) and rotavirus A (RVA) in 104 recreational surface freshwater samples from an island region, Pará State, northern Brazil, during a two-year monitoring (January 2012 to December 2013.

was no EV and RV positive sample (Fig. 2). It was not possible to establish a seasonal pattern for the circulation of viruses in the recreational freshwater assessed since positivity for EV (X^2 = 1.858; P = 0.1729) and RVA (X^2 = 0.013; P = 0.9087) did not vary between the first and second semesters of 2012 and 2013.

The average monthly rainfall obtained for the investigated period ranged from 44.30 to 742.50 mm in the two-years monitoring (Fig. 2). The simple linear regression test showed that the positivity obtained for the viruses did not change as a function of precipitation (F = 0.3185; P = 0.5841).

Most samples were collected at high tide period (57.7%; n = 60) than at low tide (42.3%; n = 44). At low tide 36.4% (16/44) were positive for these viruses, of which 62.5% (10/16) were RVA, 18.75% (3/16) were EV and EV+RVA each. At high tide, 43.3% (26/60) were positive with 46.1% (12/26) for RVA, 26.9% (7/26) for EV and EV+RVA, each. The qui-square showed that both tidal regime did not influence in the positivity obtained for EV (X^2 = 0.976; P = 0.3232) and RVA (X^2 = 0.000; P = 0.9868).

Table 2. Relationship among viruses detection and concentration of bacteria (*Escherichia coli*) used as indicator of fecal contamination according current resolution to concentration (<2000 most probable number per 100 mL) in 104 recreational surface freshwater samples from an island region, Pará State, northern Brazil, during a two-year monitoring (January 2012 to December 2013).

Escherichia coli	Virus detection	
	% (N positive/total)	% (N negative/total)
N concentration above	4.8 (5/104)	2.9 (3/104)
N concentration according	35.6 (37/104)	56.7 (59/104)
Number of samples	40.4 (42/104)	59.6 (62/104)

The samples showed concordance of 61.5% (64/104) concerning results between viral and bacterial detection. Of the total samples that presented acceptable concentration of *E. coli* (>2000 most probable number per 100 mL) according the current resolution (CONAMA 2000), 35.6% (37/104) exhibited the EV and/or RVA presence (Table 2). The G-test was performed in order to assess a possible relationship between virus contamination and bacterial concentrations in recreational freshwater samples, showing that viral contamination is independent of bacterial contamination (G = 0.8862; P = 0.3465).

Of the 18 EV positive samples submitted to nucleotide sequencing, 66.7% (12/18) were classified as non-poliovirus EV, 11.1% (2/18) as human poliovirus 1 (Fig. 3), and 22.2% (4/18) were not analyzed due to their low quality. All non-polio EV were closely related to the human EV species A and showed 100% of similarity among them and 99% of nucleotide identity in relation to the coxsackieviruses (CV) A5 Swartz strain (AY421763). These samples were detected at sampling points SP01 (1st collection July 2013), SP02 (1st collection July 2012; March, 1st and 2nd collection July 2013), SP03 (2nd collection July and November 2012, 1st and 2nd collection July 2013) and SP04 (May and November 2012, 1st collection July 2013). The two poliovirus 1 samples showed 98% nucleotide identity among them and in relation to the Sabin 1C prototype (AY184219) and were detected at sampling point SP01 and SP02 in the 1st and 2nd July collection of 2012, respectively (Fig. 3).

Genomic sequencing could not be performed for rotavirus due to the small amount of product verified in these samples. However, nested-PCR genotyping was performed in 28.1% (9/32) of the VP6 RVA positive samples; of these seven could be genotyped for the P (VP4) and G (VP7) genotypes. The P genotypes found were P[8], P[4] and P[6] and the G genotypes were G1, G2, G3, G9 and G12 (Table 3).

DISCUSSION

Viruses are known etiologic agents of disease outbreaks associated with recreational waters. Many bathing areas used for recreation are located close to urban regions and are, therefore, subject to contamination by human waste cause by the intense urban occupation, lack of adequate infrastructure and sewage services added to population increase on vacation and holidays (Sinclair et al. 2009).

In the present study, it was evaluated the EV and RVA presence in recreational surface freshwater during a two-year monitoring using a conventional molecular method. In the same years (2012-2013) the EV were detected in percentages that ranged from 15-25% from natural bathing sites in Slovak (Sarmirova et al. 2016). In the USA, 17% of the samples from three inland freshwater beaches were EV positive (Lee et al. 2014). These percentages are similar to that found in the present study (19.2%). The EV was also the most common virus detected in 56% (14/25) of the recreational sites collected from lakes in China, surpassing even the NoV positivity (Allmann et al. 2013). In midwest and south regions of Brazil, the EV was also detected in lake water used for recreations (Maurer et al. 2015, Cadamuro et al. 2021). These data show that EV circulation is common and well reported in recreational waters.

The nucleotide sequencing of the EV samples revealed the presence of species A and C circulating on these freshwater beaches, such as coxsackieviruses (CV) A5 and polioviruses (PV) 1 Sabin. The CVA5 strain has already been the cause of three recreational waterborne disease outbreaks, one in the USA, one in France and another one in Mexico (Sinclair et al. 2009). This strain has been linked to hand, foot and mouth disease (HFMD), exanthema, herpangina, onychomadesis, stomatitis, and gastroenteritis, in addition to severe cases such as acute encephalopathy when there is co-infection with norovirus (Boros et al. 2017).

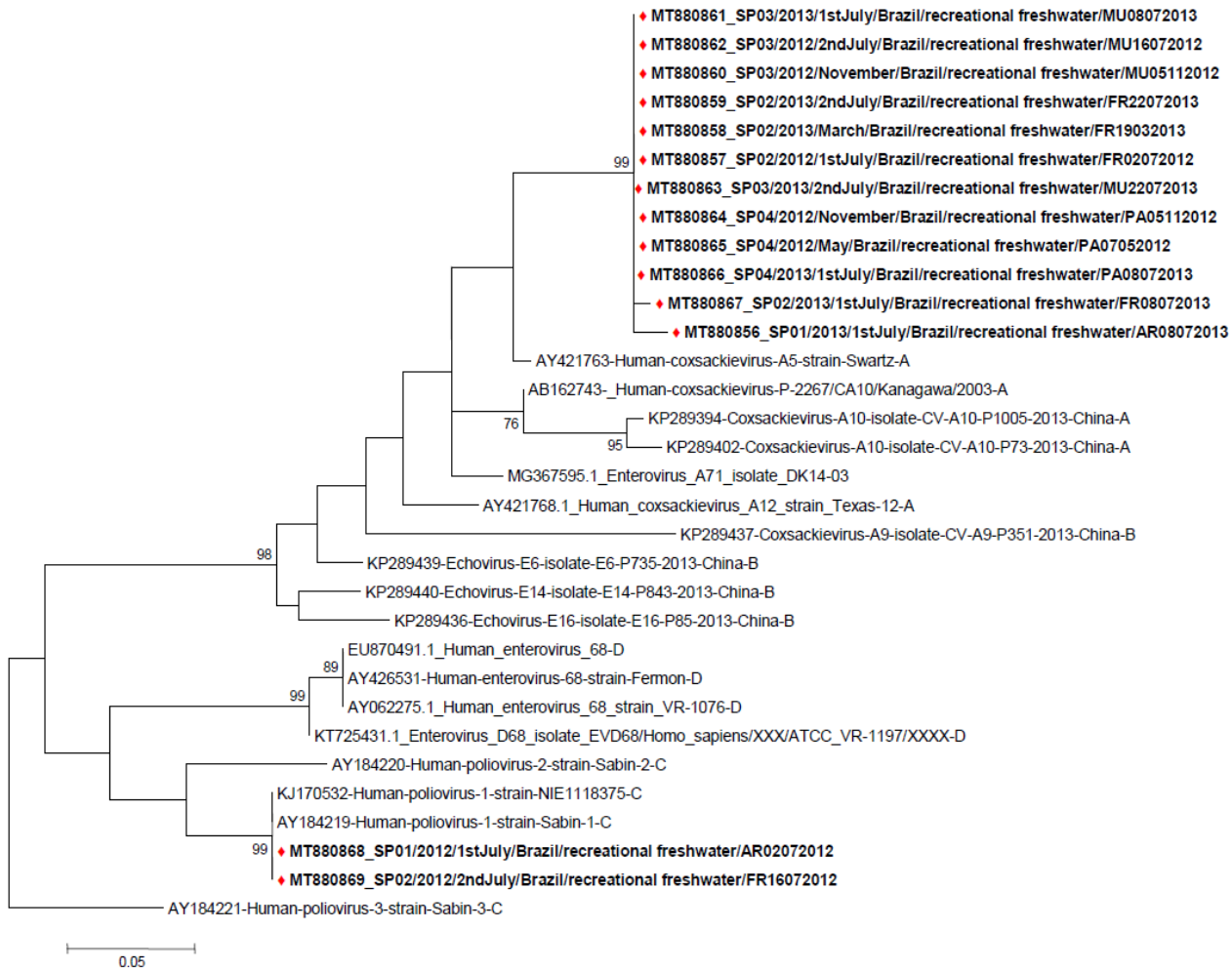

Figure 3. Phylogenetic analysis by maximum likelihood method in fourteen enterovirus positive recreational freshwater samples obtained in an island region, Pará State, northern Brazil, during a two-year monitoring (January 2012 to December 2013). The samples of this study are in bold and marked with a red filled diamond. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model. The analysis involved 32 nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

It is noteworthy that in 5 of the 12 positive samples for EV classified as CVA5, the presence of norovirus was also observed, one of them confirmed as GII.4 genotype (De Deus et al. 2019). In the years of 2010 and 2011, the CVA5 strain was responsible for 5.5% of acute gastroenteritis in children hospitalized in the same location where the present study was carried out (Machado et al. 2020).

A recent clinical study developed in the same city demonstrated the participation of CVA4, CVA6 and CVB5 in cases of HFMD, however no episodes due to CVA5 infection were described (Justino et al. 2020). Another interesting fact is that most of the CVA5 samples were obtained in the month of July, when the places are very frequented by bathers, due to the school vacation. This finding demonstrates the potential risk of serious illness due to contact with these contaminated water if viruses are still viable, which unfortunately was not possible to assess in the present study.

Unlike the EV, there are no reports on the RVA participation as a cause of waterborne disease outbreaks in recreational environments (Sinclair et al. 2009). However, its circulation in this type of sample has already been evidenced in lakes (Cadamuro et al. 2021) and rivers (Bortagaray et al. 2020). Besides that, the quantitative microbial risk assessment has been carried out as a way of assessing the risk associated with exposure to RVA by the river water used for recreation and consumption (Bortagaray et al. 2020).

Table 3. Detection of group A rotavirus genotypes G and P in recreational surface freshwater samples from an island region, Pará State, northern Brazil, during a two-year monitoring (January 2012 to December 2013). *NG: not genotyped.

Sampling point	Month/Year	RVA genotypes	
		VP7 (G)	VP4 (P)
SP02	April/2012	G1	P[4] and P[8]
SP04	April/2012	NG*	P[8]
SP01	May/2012	G12	NG*
SP02	1st July/2012	G1, G2 and G3	NG*
SP02	2nd July/2012	NG*	P[8]
SP01	November/2012	G3	P[6]
SP02	November/2012	G3 and G9	P[8]
SP03	November/2012	G3 and G9	P[8]
SP02	April/2013	G1	P[4] and P[8]

It was not possible to establish a seasonal pattern for the circulation of EV and RVA in the recreational freshwater assessed, similar to what was found in another study carried out in the midwest and south regions of Brazil, where there was also no difference in viral concentrations found comparing the dry and rainy seasons in a large freshwater reservoir with lakes that are used for recreation (Cadamuro et al. 2021). Besides, study with clinical samples collected in the same region, shows that RVA infections tend to occur throughout the year, showing a "see-saw effect" between infections of RVA and norovirus, another important etiological agent involved in cases of gastroenteritis (Siqueira et al. 2013). In Uruguay, RVA gastroenteritis occurs more in winter with simultaneous viral detection in the environment (Bortagaray et al. 2020).

The tidal status at the time of water collection in relation to viral positivity was evaluated, showing no influence on the results obtained. However, the effect of tides on virus richness and diversity has already been evidenced in other studies, mainly due to the mixture of river and marine waters (Robins et al. 2019, Adriaenssens et al. 2021). In addition, tidal movements disperse the viruses to other locations, showing that the intensity and timing of water flows must be taken into account when assessing the microbiological water quality and the risk to the pathogen exposure (Robins et al. 2019).

The microbiological quality of these recreational surface waters was monitored by the determination of fecal indicator bacteria (FIB). Our results did not show a relationship between EV and RVA detection and bacterial contamination, in line with what has been previously proposed (Aguirre et al. 2019, Masachessi et al. 2021). Masachessi et al. (2021) even proposed a pipeline involving the detection of picobirnavirus, human adenovirus and infectious EV in samples with FIB within acceptable limits by the guidelines as a way to improve the assessment of microbiological quality in freshwater, in addition to reporting the presence of other enteric viruses, such as RVA.

This study focused on the molecular RNA detection of EV and RVA and particle viability was not evaluated. In this sense, future studies aiming to analyze the infectivity of viral particles detected should be carried out as a complement to molecular methods and to confirm the health risks caused by water contact. In addition, it is essential to assess the sources of faecal contamination in recreational areas through microbial source tracking, to mitigate future infections and improve the water quality management.

CONCLUSION

It was demonstrated the circulation of EV and RVA in recreational surface water in an island region, Pará state, northern Brazil, showing the presence of faecal contamination sources of unknown origin in these environments. In addition, the results showed that viral contamination by EV and RVA can exist even in samples with a bacteria concentration as recommended by the guidelines, reinforcing the importance of analyzing the presence and viability of viruses in water, especially those intended for consumption and recreational activities.

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