

## Norovirus genogroups I and II in environmental water samples from Belém city, Northern Brazil

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### ABSTRACT

This study investigated the presence of norovirus (NoV) GI and GII in environmental samples from the northern region of Brazil. Water samples were collected monthly (November 2008/October 2010) from different sources and sewage and concentrated by the adsorption-elution method. The NoV investigation used molecular methods followed by sequencing reactions. The general positivity for NoV was 33.9% (57/168). Considering the results obtained only in the semi-nested RT-PCR (reverse transcription polymerase chain reaction) and only in the TaqMan<sup>®</sup> real-time PCR, the rates were 26.8% (45/168) and 27.4% (46/168), respectively, being for NoV GI 22.2% (10/45) and 19.6% (9/46); for GII 17.8% (8/45) and 15.2% (7/46); and for GI + GII 60% (27/45) and 65.2% (30/46), respectively. Different GI (GI.1, GI.4, GI.7 and GI.8) and GII (GII.4, GII.6, GII.9, GII.12 and GII.14) genotypes were detected. These results demonstrated the NoV was disseminated in the waters of Belém city due to a lack of sanitation that allowed the discharge of contaminated effluents into these aquatic ecosystems.

**Key words** | Belém city, genotypes, human norovirus, rainfall, sewage, surface water

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### INTRODUCTION

Poor sanitation conditions, lack of drinking water and inadequate sewage treatment increase the occurrence of several diseases in the population due to the input of pathogens

into the aquatic environment. Worldwide, approximately 2.4 million deaths could be prevented annually by improved sanitation, drinking water supply and hygiene promotion ([World](#)

Health Organization (WHO) & United Nations Children's Fund (UNICEF) 2005; Bartram & Cairncross 2010). Among water-related diseases, diarrhoea remains the primary cause of morbidity and mortality worldwide (Bartram & Cairncross 2010). In 2008, diarrhoea was responsible for 1.87 million deaths in children under 5 years old, mainly in developing countries (Boschi-Pinto *et al.* 2008).

Belém is located in the northern region of Brazil. This region has the lowest sanitation coverage (3.5%) among all Brazilian regions (Instituto Brasileiro de Geografia e Estatística (IBGE) 2011). In this city, 75% of houses are attended by water supply and 37% have sanitation (Prefeitura Municipal de Belém (PMB) 2014). These precarious sanitation conditions reflect on the health of the population. Diarrhoea is a leading cause of hospitalization and mortality in children under 1 year old (more than 60%), in the municipalities of the northern region (IBGE 2011; Buhler *et al.* 2014). In Belém, in the year of 2011, 1.4% of deaths in children under 5 years old were caused by diarrhoea (DATASUS 2011).

Enteric viruses possess specific characteristics that allow them to be easily found in aquatic ecosystems and wastewater, including high shedding in faeces and resistance to adverse environmental factors such as pH, temperature, and sunlight. Their persistence in different aquatic environments also depends on the viral composition, such as the protein capsid structure and the viral RNA or DNA genome, and the ability of the virus to attach to suspended solids (Fong & Lipp 2005).

Noroviruses (NoVs) are members of the *Caliciviridae* family and are recognized as a major cause of sporadic cases of acute gastroenteritis (AGE) and outbreaks (Patel *et al.* 2009). NoVs are excreted in high concentrations in the faeces from infected patients and are released into water bodies through sewage discharge. The viral particles can remain infectious for a long period of time enabling the infection of susceptible individuals (Bosch 1998). NoVs have been detected in many types of environmental samples, such as drinking water (Kukkula *et al.* 1999), seawater (Moresco *et al.* 2012; Victoria *et al.* 2014), sewage (La Rosa *et al.* 2010a, 2010b; Victoria *et al.* 2010a; Kitajima *et al.* 2012), rivers (Kitajima *et al.* 2010) and recreational waters (Félix *et al.* 2010; Wyn-Jones *et al.* 2011; Vieira *et al.* 2012).

NoVs are divided into seven genogroups based on VP1 amino acid identity (G); of these, GI, GII and GIV are associated with human infections (Vinjé 2015). NoV GII is the most

prevalent and is usually associated with AGE outbreaks (Matthews *et al.* 2012). However, GI has been detected with a higher positivity than GII in some environmental studies (Kitajima *et al.* 2010; La Rosa *et al.* 2010a; Moresco *et al.* 2012).

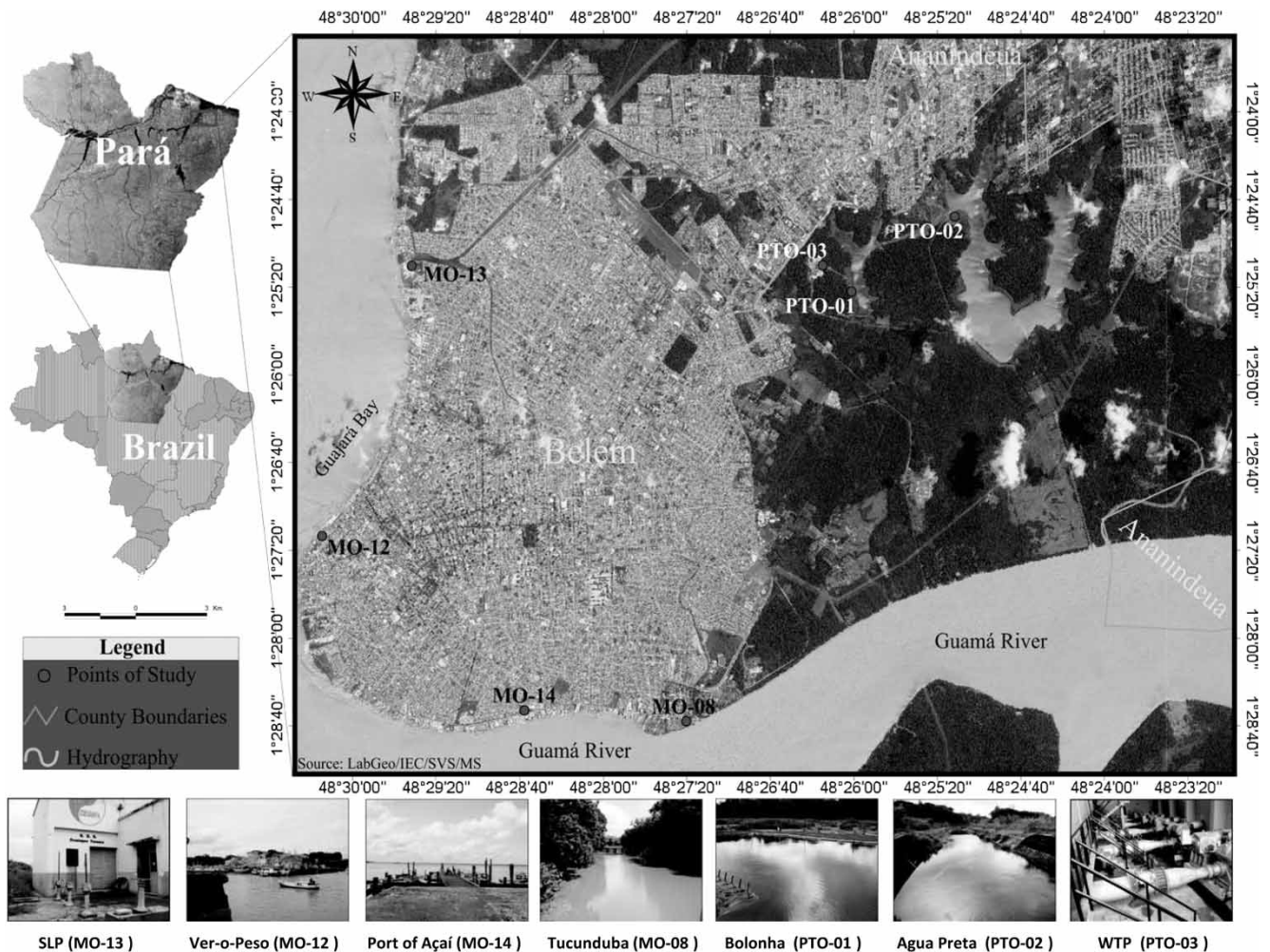
In the Amazon region, northern Brazil, data concerning NoV circulation in aquatic environments are limited to one study conducted in the state of Amazonas by Miagostovich *et al.* (2008). The present study investigated the dissemination of NoV GI and GII in environmental samples from Belém city, Pará state, during a 2-year monitoring period. Furthermore, NoV-positive samples were characterized using molecular methods, and the positivity rate was correlated with rainfall data to verify possible NoV seasonality during the study period.

## MATERIAL AND METHODS

### Study area

Surface water ( $n = 120$ ), untreated sewage ( $n = 24$ ) and treated water ( $n = 24$ ) samples were collected once a month from November 2008 to October 2010 from seven collection points in Belém city (Figure 1), capital of Pará state, located in the Amazon region, northern Brazil. Belém presents a well-defined tropical rainforest climate that is divided into two annual seasons: an intensive rainy season (December to June) with the average rainfall ranging from 145 to 379 mm (average 283.7 mm, with peaks of 500 mm in some months), and a season with a lower intensity of rain (July to November) with rainfall values of 89–136 mm (average 110.2 mm). Both humidity (average 83.2%, minimum of 75% and maximum of 93%) and temperature (average 26.8 °C, minimum of 26.4 °C and maximum of 27.2 °C) are normally high and vary little throughout the year.

The seven collection points include: Port of Açaí, located on the Guamá River, which daily receives people from the surrounding islands that arrive in small boats to trade local products and fruits; Ver-o-Peso, located in Guajará Bay, which is an important and famous Brazilian outdoor fair frequented by a large numbers of visitors; Tucunduba, an impacted stream affected by the discharge of untreated sewage of the population living around this aquatic ecosystem; sewage lift plant (SLP), which receives influents from approximately 20 neighbourhoods of Belém, serves only 29%



**Figure 1** | Map of Belém city, Pará state, showing the water and sewage sampling sites. Source: Geoprocessing Laboratory (LABGEO) Evandro Chagas Institute, Secretariat of Health Surveillance, Brazilian Ministry of Health.

(157,607/543,543) of the inhabitants from these neighbourhoods, and only 8% of these influents are sedimented in the plant before their prior launch into Guajará Bay (PMB 2014); and two lakes (Bolonha and Água Preta) whose waters are responsible for providing 65% of the water supply consumed in Belém after a previous treatment processes in a water treatment plant (WTP). These lakes and the WTP are located in a state unit of environmental conservation.

### Viral concentration, nucleic acid extraction and cDNA synthesis

Viral particles were concentrated by the adsorption-elution method as previously described by Katayama *et al.* (2002)

using two litres of water. The eluate (15 mL) was reconcentrated by filtration using an Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore, Ireland) to obtain a final volume of 2 mL. Nucleic acids were extracted using the isothiocyanate guanidine method (Boom *et al.* 1990), and the complementary DNA (cDNA) was obtained after reverse transcription using a pd(N)<sup>6</sup>™ random primer (Amershan Biosciences, UK) and the Superscript™ II Reverse Transcriptase (Invitrogen, USA).

### NoV detection

The semi-nested RT-PCR (reverse transcription polymerase chain reaction) (Boxman *et al.* 2006) and TaqMan® real-time PCR (rtPCR) (Kageyama *et al.* 2003) were performed

for NoV detection using specific primers and probes for GI and GII in separate reactions. The RT-PCR was performed in a final volume of 10  $\mu\text{L}$  containing 2  $\mu\text{L}$  of RNA template, 40 mM of deoxynucleotides, 10X of PCR buffer, 50 mM of  $\text{MgCl}_2$ , 20  $\mu\text{M}$  of each primer (JV13I and JV12Y), 20 U/ $\mu\text{L}$  of SuperScript II Reverse Transcriptase (Invitrogen, USA) and 5 U/ $\mu\text{L}$  of Taq DNA polymerase (Invitrogen, USA). The semi-nested PCR was carried out in a final volume of 25  $\mu\text{L}$  containing 2  $\mu\text{L}$  of RT-PCR products, 40 mM of deoxynucleotides, 10X of PCR buffer, 50 mM of  $\text{MgCl}_2$ , 20  $\mu\text{M}$  of each primer (JV13I/G1 for GI detection and JV12Y/NoroII-R for GII detection) and 5 U/ $\mu\text{L}$  of Taq DNA polymerase (Invitrogen, USA). Amplicons of 187 base pairs (bp) and 236 bp were considered positive for NoV GI and GII, respectively, after semi-nested RT-PCR.

For the rtPCR, the TaqMan<sup>®</sup> Universal PCR Master Mix was used with the pairs of primers and probes previously described (Kageyama *et al.* 2003). The reactions were performed in duplicate in a final volume of 15  $\mu\text{L}$ , containing 3  $\mu\text{L}$  of cDNA, 20  $\mu\text{M}$  of each primer and 10  $\mu\text{M}$  of probes. All samples that crossed the threshold within 40 cycles and had a characteristic sigmoid curve were considered positive. All tests were conducted in different rooms to avoid cross-contamination. In each experiment, we used UltraPure<sup>™</sup> DNase/RNase-Free Distilled Water (Invitrogen, USA) as a negative control and a NoV-positive stool sample previously genotyped by sequencing as a positive control.

### NoV molecular characterization

NoV-positive samples were directly sequenced using primers targeting the 5' ORF2 region (COG1F/G1SKR and COG2F/G2SKR) as previously described (Kojima *et al.* 2002; Kageyama *et al.* 2003). Amplicons were purified with the commercial QIAquick<sup>®</sup> PCR Purification and QIAquick<sup>®</sup> Gel Extraction kits (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. DNA sequencing was performed in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were edited with the BioEdit<sup>®</sup> Sequence Alignment Editor software, aligned using the Clustal W package and compared with other sequences from the GenBank database and the online NoV genotyping tool

(Kroneman *et al.* 2011). Phylogenetic trees were constructed with the MEGA 6.0 program (Tamura *et al.* 2013) using the neighbour-joining algorithm with the method of Kimura-2 parameters and employing the non-parametric bootstrap reliability test using 2000 replicates. The nucleotide sequences of NoV GI and GII were deposited in GenBank under the accession numbers KR053441-KR053451 and KP893924-KP893938, respectively.

### Statistical analysis

All statistical analyses were performed in BioEstat 5.0 (Ayres *et al.* 2007) using Pearson's linear correlation and the Chi-square test to evaluate the relationship among NoV frequencies and precipitation data and the G-Test to verify differences in NoV detection among the study sites. Statistically significant values were expressed as  $p \leq 0.05$ .

## RESULTS

Over a 2-year period (November 2008 to October 2010), a total of 168 environmental samples collected from seven sampling points in Belém city were tested for NoV. A total of 33.9% (57/168) of the samples were positive regardless of the technique used (Table 1). Considering only the semi-nested RT-PCR, NoV was detected in 26.8% (45/168) of these samples, with 22.2% (10/45) positive only for GI, 17.8% (8/45) positive only for GII, and 60% (27/45) positive for both. Considering only rtPCR, the NoV positivity rate was 27.4% (46/168); of this total, 19.6% (9/46), 15.2% (7/46), and 65.2% (30/46) were specific for GI, GII, and GI plus GII, respectively.

NoV RNA was found in all sampling points, with the exception of WTP. The SLP (79.2%,  $p < 0.0001$ ) and the Tucunduba stream (66.7%,  $p = 0.0009$ ) were the most contaminated sites, with the highest number of NoV-positive samples for both GI and GII (Table 1). NoV was also detected in a lower percentage in the water samples from the Bolonha and Água Preta lakes, which are responsible for the city's water supply (Table 1).

NoV GI and GII were detected simultaneously during the study period with the exception of December 2008, when no positive samples were detected, and in January and February 2009, when only GII was detected. In July



**Table 1** | NoV GI and GII detection in 168 environmental samples from Belém, Pará, collected from November 2008 to October 2010 by semi-nested RT-PCR and real-time PCR (rtPCR)

Combined results (semi nested and/or rtPCR)					
Environmental samples	Sampling points	NoV GI only positive/total n (%)	NoV GII only positive/total n (%)	NoV GI + GII positive/total n (%)	Total
Surface water	Bolonha lake	3/24 (12.5)	4/24 (16.7)	0/24 (0)	7/24 (29.2)
	Água Preta lake	2/24 (8.3)	2/24 (8.3)	0/24 (0)	4/24 (16.7)
	Port of Açai	4/24 (16.7)	1/24 (4.2)	1/24 (4.2)	6/24 (25.0)
	Ver-o-Peso	1/24 (4.2)	2/24 (8.3)	2/24 (8.3)	5/24 (20.8)
	Tucunduba stream	0/24 (0)	1/24 (4.2)	15/24 (62.5)	16/24 (66.7)
Untreated sewage	SLP	2/24 (8.3)	0/24 (0)	17/24 (70.8)	19/24 (79.2)
Treated water	WTP	0/24 (0)	0/24 (0)	0/24 (0)	0/24 (0)
Total		12/168 (7.1) <sup>a</sup>	10/168 (6.0) <sup>b</sup>	35/168 (20.8) <sup>c</sup>	57/168 (33.9)

<sup>a</sup>Positive by both methods ( $n = 2$ ), only by semi-nested ( $n = 5$ ) and only by rtPCR ( $n = 5$ ).

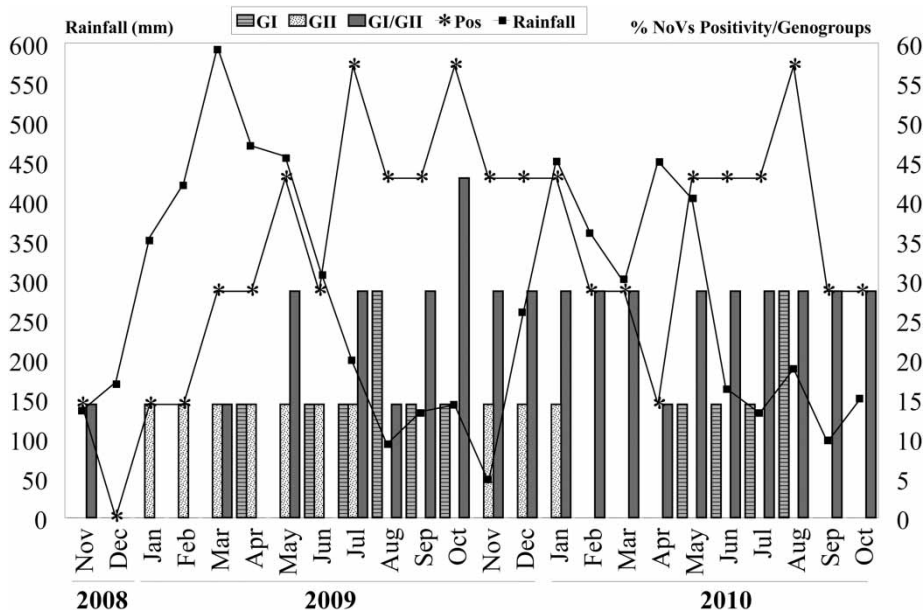
<sup>b</sup>Positive samples by both methods ( $n = 3$ ), only by semi-nested ( $n = 5$ ) and only by rtPCR ( $n = 2$ ).

<sup>c</sup>GI + GII positive samples by both methods ( $n = 22$ ), GI + GII only by semi-nested ( $n = 1$ ), GI + GII only by rtPCR ( $n = 5$ ), GI positive samples by semi-nested and GI + GII in rtPCR ( $n = 3$ ), GI + GII positive samples by semi-nested and only GI by rtPCR ( $n = 2$ ), GI + GII positive samples by semi-nested and only GII by rtPCR ( $n = 2$ ).

and October 2009 and August 2010, a greater number of NoV-positive samples (57.1%, 4/7) was observed (Figure 2). Moreover, NoV positivity was compared during the highest (December to June) and lowest (July to November) rainfall periods. Although a trend in increased positivity for NoV was observed during the less rainy period (Figure 2), the statistical analysis showed no correlation among these variables

(Pearson's linear correlation test [ $r = -0.2560$ ,  $p = 0.2271$ ]; Chi-square [ $p = 0.3831$ ]).

The NoV strains could be genotyped (5' ORF2 region) from 25 of 57 (43.9%) of the NoV-positive samples, with 12% (3/25) GI, 24% (6/25) GII and 64% (16/25) GI + GII. Only 11 of 19 and 14 of 22 positive samples provided reliable sequences and could be genotyped for GI and GII,

**Figure 2** | Monthly distribution of norovirus GI and GII positivity in environmental samples and average values of rainfall in Belém city, Northern Brazil, collected from November 2008 to October 2010.

respectively. Among the GI strains, GI.8 was the most frequent ( $n=7$ ), followed by GI.4 ( $n=2$ ), GI.1 ( $n=1$ ) and GI.7 ( $n=1$ ) (Table 2, Figure 3). Five genotypes were detected among the NoV GII strains, with GII.4 ( $n=8$ ) the most prevalent, followed by GII.6 ( $n=2$ ), GII.9 ( $n=2$ ), GII.12 ( $n=1$ ) and GII.14 ( $n=1$ ) (Table 2, Figure 4).

## DISCUSSION

The present study investigated the dissemination of NoV GI and GII in different water bodies in the Amazon region, Belém city, Northern Brazil, during a 2-year period. We found widespread NoV dissemination and detected both genogroups in the water samples analysed. This finding diverges from the clinical data obtained in recent years in Belém city, where NoV GII was almost exclusively associated with AGE cases among hospitalized children (Aragão *et al.* 2010; Siqueira *et al.* 2011, 2013a).

Our results demonstrated that NoV GI circulated at a similar frequency to GII in the Belém population. The low incidence of GI among hospitalized children could be explained by the fact that strains belonging to this

genogroup were probably more often associated with asymptomatic, mild or sporadic infections (Kitajima *et al.* 2012). Thus, the burden of NoV GI causing AGE infections may be underestimated, leading to GII being well defined as the main viral agent of AGE outbreaks and sporadic cases worldwide (Matthews *et al.* 2012).

Our data are in agreement with other studies, where similar and even higher detection of NoV GI was observed in coastal waters in southern Brazil (Moresco *et al.* 2012), in river water and wastewater samples from countries such as Japan (Kitajima *et al.* 2010, 2012), and influent/effluent samples from a sewage treatment plant in Italy (La Rosa *et al.* 2010a).

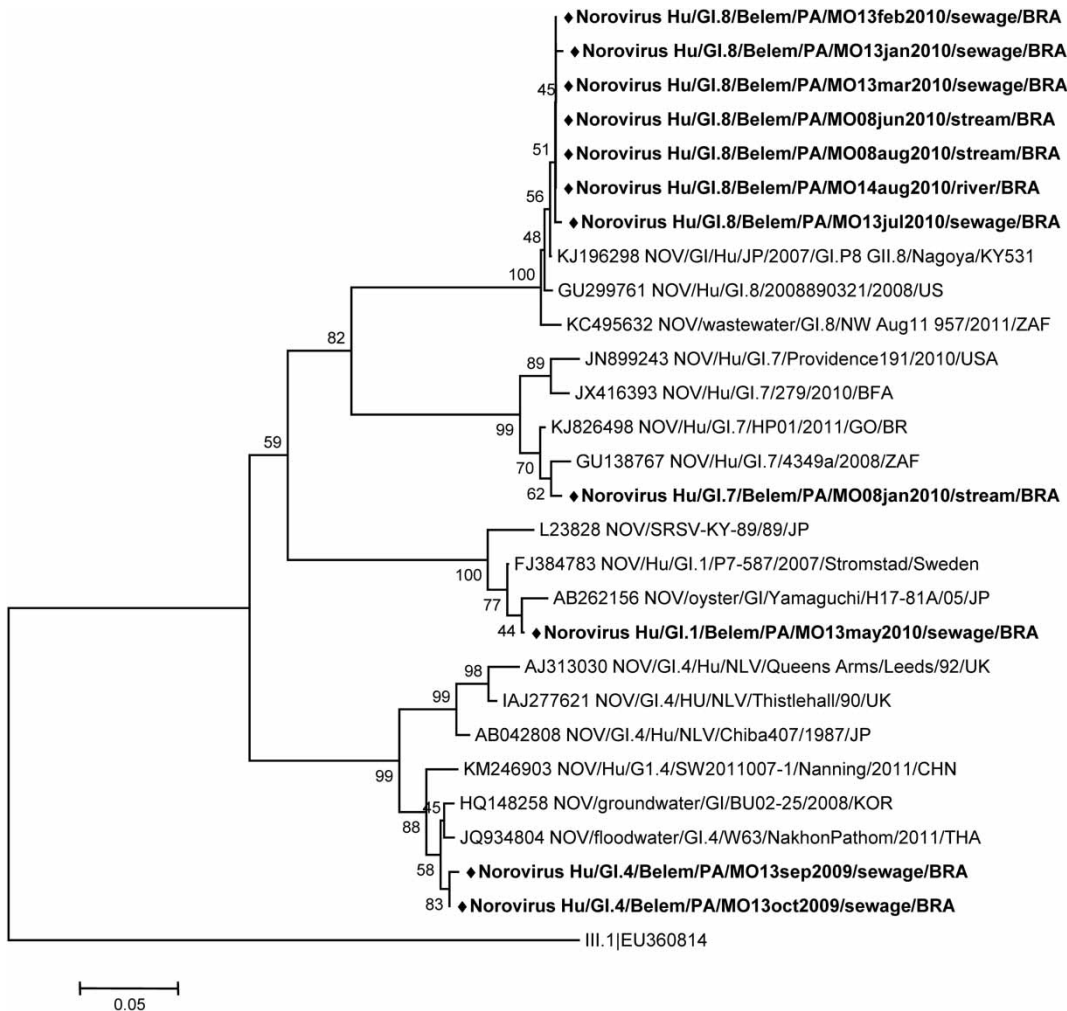
Only the water samples from the WTP demonstrated negative results for NoV. Maybe the volume used for concentration (2 litres) was inappropriate since in treated water samples the viral particles are much diluted. However, to process large volumes specific instrumental apparatus is necessary which is not cost-effective for viral detection and monitoring. The treatment process applied on the WTP is composed for fast mixture, flocculation, sedimentation, filtration, disinfection, pH correction and fluoridation. Unfortunately, the interference from inhibitors was not evaluated in this study and this can be considered

**Table 2** | Partial molecular characterization of the 5'-end ORF2 region in 25 norovirus positive environmental samples from Belém city, Northern Brazil, collected from November 2008 to October 2010

Sampling point	N positive semi-nested and/or rtPCR	N positive 5'-end ORF2	GI only Genotypes (N)	GII only Genotypes (N)	GI + GII Genotypes (N)
WTP	0	–	–	–	–
Bolonha lake	7	1	–	GII.4 (1)	–
Água Preta lake	4 <sup>a</sup>	–	–	–	–
Port of Açaí	6 <sup>a</sup>	1	GI.8 (1)	–	–
Ver-o-Peso	5	1	–	–	NT/NT <sup>b</sup> (1)
Tucunduba stream	16 <sup>a</sup>	11	NT <sup>b</sup> (1)	GII.4 (2) GII.9 (1) NT <sup>b</sup> (1)	NT <sup>b</sup> /GII.4 (2) GI.8/GII.4 (1) GI.8/GII.9 (1) GI.7/NT <sup>b</sup> (1) NT <sup>b</sup> /GII.14 (1)
SLP	19 <sup>a</sup>	11	GI.4 (1)	GII.4 (1)	GI.8/NT <sup>b</sup> (3) NT <sup>b</sup> /GII.6 (2) GI.8/GII.4 (1) GI.4/GII.12 (1) GI.1/NT <sup>b</sup> (1) NT/NT <sup>b</sup> (1)
Total	57	25	12,0% (3/25)	24,0% (6/25)	64,0% (16/25)

<sup>a</sup>One sample for each place could not be tested for the 5'-end ORF2 region due to insufficient sample volume.

<sup>b</sup>Not typed due to low quality of sequence.



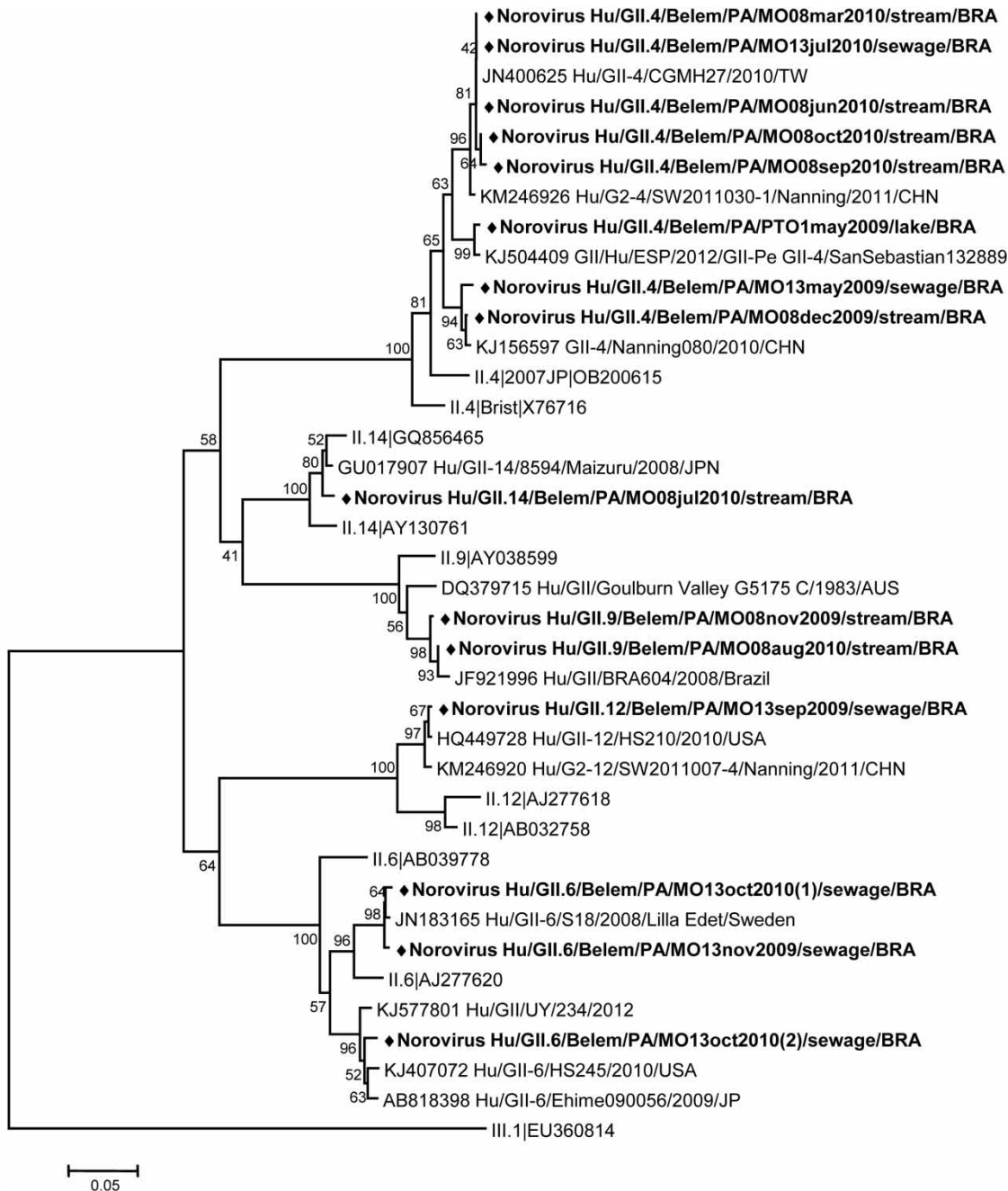
**Figure 3** | Phylogenetic tree based on the 5' ORF2 region of norovirus GI with amplicons of 380 base pairs identified from environmental samples from Belém city, Northern Brazil. The tree was constructed using the neighbour-joining method by Kimura-2 parameter analysis. The test was performed with 2000 bootstrap replicates. Study samples are marked with bold and filled diamonds. The scale bar is proportional to the genetic distance.

a limitation of our study. So the absence of positive samples in the treated water samples also may have been the result of treatment efficiency applied in the plant or a reflection of the inhibitor action in these samples.

When these samples were excluded, the rate of NoV positivity increased from 33.9% (57/168) to 39.6% (57/144). The high contamination of the SLP was expected because it was characterized as raw sewage that did not receive any disinfection treatment. The results in the Tucunduba stream showed its impacted nature due to the direct discharge of effluents from the domestic sewage produced by the population living in the urban settlement around this aquatic environment.

In the present study, the NoV positivities obtained by semi-nested (26.8%) and rtPCR (27.4%) were similar, possibly because both methods used a highly conserved region of the genome as their target and thus increased their sensitivity. The rtPCR was used in a qualitative manner to evaluate the presence or absence of NoV because a standard curve was not yet available during the study period, which prevented the quantification of the genogroup data. Although the analysis complemented and provided more robustness to the results (even though it was qualitative), this fact could represent a limitation of this research.

The viral concentration recovery rates are very important because they allow the evaluation of efficiency of the



**Figure 4** | Phylogenetic tree based on the 5' ORF2 region of norovirus GII with amplicons of 390 base pairs from environmental samples from Belém city, Northern Brazil. The tree was constructed using the neighbour-joining method by Kimura-2 parameter analysis. The test was performed with 2000 bootstrap replicates. Study samples are marked with bold and filled diamonds. The scale bar is proportional to the genetic distance.

method used. However, since it was a pilot study, NoV recovery was not evaluated locally and this can be considered as a limitation of our research. On the other hand, the adsorption-elution method has been already employed for NoV concentration, in studies conducted in other

Brazilian regions. In the southern region, *Victoria et al. (2010b)* demonstrated NoV recovery rates which ranged from 1.6 to 6.0% in surface water and sewage, and in the southeast region the NoV GII recovery in river water ranged from 3.3 to 7.7% (*Miagostovich et al. 2014*) but in



another study evaluating different MgCl<sub>2</sub> concentrations the rates were 17.8%, 9.1% and 3.7% when 5, 25 and 50 mM were used, respectively (Victoria *et al.* 2009).

Molecular techniques are typically employed in virus research in aquatic environments. However, the presence of inhibitory compounds in water samples may influence the molecular detection and possibly the rtPCR results (Rodríguez *et al.* 2012). In a study performed in Rio de Janeiro, southeast Brazil, NoV GII was detected more frequently in recreational waters by conventional PCR than quantitative PCR (qPCR) (16% versus 4.2%); the authors suggested that this finding might be related to the presence of inhibitors in these waters (Vieira *et al.* 2012). Despite this limitation, rtPCR has been widely used for NoV detection and quantification in different environmental samples worldwide (La Rosa *et al.* 2010b; Victoria *et al.* 2010a; Kitajima *et al.* 2012; Moresco *et al.* 2012).

We performed direct sequencing of the nested/semi-nested PCR amplicons instead of cloning them because demonstrating the genetic diversity of NoV in the environmental samples was not the main objective of our study. Although this step compromised our understanding of the circulation of less prevalent genotypes, it was a time and cost effective alternative (La Rosa *et al.* 2010a; Fumian *et al.* 2013) that allowed us to assess the prevalence of the NoV GI and GII strains circulating in the surface water and sewage of Belém city.

Primers targeting the 5' ORF2 region of the NoV genome were used to genotype the positive samples in this study, with genotypes GI.8 ( $n = 7$ ) and GII.4 ( $n = 8$ ) being the most prevalent. GI.8 was detected in three of the seven collection points: river ( $n = 1$ ), impacted stream ( $n = 2$ ) and untreated sewage ( $n = 4$ ). GI.8 was identified in sporadic human cases that occurred in 1995 in a day care centre in Rio de Janeiro, southeast Brazil (Ferreira *et al.* 2012). In Japan, this genotype was detected in sewage 1 year after an outbreak, demonstrating its circulation in the community over a long period of time (Iwai *et al.* 2009). The GI.4 strain was previously detected in Brazil in a faecal sample obtained in 2003 from a diarrhoeic child in Belém city (Aragão *et al.* 2010). Similarly, GI.1 and GI.4 were also isolated from children who lived near the Quilombola communities located in a semi-isolated rural area in Espírito Santo State, southeastern Brazil (Vicentini *et al.* 2013). GI.7 was detected in a sample from the Tucunduba stream (January 2010); in Brazil, this genotype was recently

described for the first time in the central west region of the country in a study involving children attending a day care centre (Oliveira *et al.* 2014).

The GII.4 genotype was the most frequently detected genotype in these samples (57.1%, 8/14). Moreover, this strain was the most prevalent among hospitalized children, corroborating its high level of dissemination. This finding is in accordance with many reports created in Brazil (Aragão *et al.* 2010, 2013; Fioretti *et al.* 2011; Siqueira *et al.* 2011, 2013a; da Silva *et al.* 2013; Vicentini *et al.* 2013) and worldwide (Bull *et al.* 2006; Siebenga *et al.* 2009). Although our samples showed similarity with some GII.4 variants (Den Haag 2006b, New Orleans 2009 and Sydney 2012), the region tested did not allow us to define them as variants. For this purpose, complementary tests to analyse the P2 region of the gene encoding the VP1 protein would be necessary.

NoV GII.6 (14.3%, 2/14), GII.9 (14.3%, 2/14), GII.12 and GII.14 (7.1% each, 1/14) were also observed with a low incidence. During our study, GII.6 was detected in sewage samples collected in November 2009 and October 2010. In Brazil, this genotype was also described in 2008, when it was detected in a stool sample from a child from a Quilombola community located in the metropolitan region of Belém (Aragão *et al.* 2013) and in clinical samples from Rio de Janeiro (Ferreira *et al.* 2012) and Espírito Santo (Vicentini *et al.* 2013). GII.9 was detected in the Tucunduba stream in November 2009 and August 2010 and was also responsible for 10% of the AGE cases during a 2006 outbreak in Bahia city, northeastern Brazil (Campos *et al.* 2008). We detected GII.12 in a sewage sample (September 2009). Although this genotype was detected at a low prevalence, it was detected in the same year in three Brazilian states (Sergipe, Minas Gerais and Rio Grande do Sul) in clinical specimens obtained from AGE cases and outbreaks (Fioretti *et al.* 2011). GII.14 was found in July 2010 in the Tucunduba stream and was responsible for a nosocomial outbreak in a hospital in Rio de Janeiro in 2007 (Ferreira *et al.* 2010).

Clinical data obtained from hospitalized children in Belém city revealed that NoV infections occurred throughout the year without a defined seasonal pattern (Siqueira *et al.* 2013b). Recent studies have evaluated the influence of rains on the occurrence of enteric viruses in water (Hata *et al.* 2014; Victoria *et al.* 2014). In Rio de Janeiro city, southeast Brazil, the concentrations of enteric viruses did not decrease in water samples

from urban beaches even after rainfall events (Victoria *et al.* 2014). However, during a study conducted in Japan with river water samples impacted by combined sewer overflows, a higher NoV prevalence was found in samples collected during the rainfall-affected periods compared with samples obtained during dry weather (Hata *et al.* 2014). In our study, in agreement with the results of Moresco *et al.* (2012) for coastal waters in Florianópolis (Southern Brazil), it was not possible to establish a NoV seasonality pattern in the environmental samples evaluated, although it was possible to observe that NoV was detected more frequently during less rainy weather.

In conclusion, this study demonstrated for the first time the NoV dissemination and the presence of different genotypes in environmental matrices from Belém city, principally during low-rainfall periods. Molecular characterization of NoV in environmental samples is an important approach to provide information about the epidemiology of these viruses and to demonstrate that other strains in addition to GII.4 also circulate in the population. Despite its fastidious nature that hampers our understanding of its viability and infectivity, the detection of the NoV genome in the studied aquatic ecosystems demonstrates the high level of faecal contamination at these sites. Further studies concerning the monitoring and circulation of NoV and other enteric viruses are necessary in the aquatic environments used for the public water supply to avoid possible water-related infections and even outbreaks.

## CONCLUSIONS

The results obtained in this study are important due to the lack of research on the detection of enteric viruses in aquatic environments in the Amazon region. Our results demonstrated the widespread dissemination of several NoV genotypes in surface water and raw sewage. Furthermore, this study highlights the need to investigate viral indicators to assess the microbiological safety of water and the potential risk for the population.

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## CONFLICT OF INTEREST

No conflict of interest declared.

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