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Human norovirus detection in bivalve shellfish in Brazil and evaluation of viral infectivity using PMA treatment

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ABSTRACT

Noroviruses are the most common cause of gastroenteritis outbreaks in humans and bivalve shellfish consumption is a recognized route of infection. Our aim was to detect and characterize norovirus in bivalves from a coastal city of Brazil. Nucleic acid was extracted from the bivalve's digestive tissue concentrates using magnetic beads. From March 2018 to June 2019, 77 samples were screened using quantitative RT-PCR. Noroviruses were detected in 41.5%, with the GII being the most prevalent (37.7%). The highest viral load was 3.5 × 106 and 2.5 × 105 GC/g in oysters and mussels, respectively. PMA-treatment demonstrated that a large fraction of the detected norovirus corresponded to non-infectious particles. Genetic characterization showed the circulation of the GII.2[P16] and GII.4[P4] genotypes. Norovirus detection in bivalves reflects the anthropogenic impact on marine environment and serves as an early warning for the food-borne disease outbreaks resulting from the consumption of contaminated molluscs.

1. Introduction

Human enteric virus, particularly norovirus, is recognized as important causes of foodborne illness outbreaks globally (FAO/WHO, 2012; WHO, 2018). Proportion of norovirus-illnesses attributed to food ranges from 12 to 47% (FAO/WHO, 2012; Hardstaff et al., 2018). Worldwide, norovirus account for one-fifth of all cases of acute gastroenteritis (AGE) in children < 5 years of age, leading to an estimated US$ 4.2 billion in direct health system costs each year and over 200,000 childhood deaths mainly in low-income countries (Ahmed et al., 2014; Atmar et al., 2018; Bányai et al., 2018).

Norovirus belong to the genus *Norovirus* in the family *Caliciviridae*. Based on complete VP1 (capsid protein) amino acid sequence, norovirus is classified into ten genogroups (GI to GX), which can be further divided into 49 genotypes. Although viruses belonging to GI, GII, GIV, GVIII, and GIX can cause disease in humans, the majority of illnesses are associated to GI and GII genotypes (Vega et al., 2014; Chhabra et al., 2019). AGE outbreaks are frequently linked to the consumption of bivalves, especially those harvested in sewage-contaminated waters (Lees, 2000) and as filter-feeders, these animals may retain and concentrate bacteria and viruses. The consumption of raw or undercooked shellfish greatly increases the human health risk (El Moqri et al., 2019), and several human enteric viruses have been detected in bivalve mollusk species worldwide (Le Guyader et al., 2000; Benabbes et al., 2013; Souza et al., 2018; Meghnath et al., 2019). In Brazil, most of the studies have been carried out in southern region, specifically in Santa Catarina state, the largest national producer of bivalve molluscs (Corrêa et al., 2012; Souza et al., 2013, 2015; Leal et al., 2018). More recently, studies have demonstrated the detection of enteric viruses such as human adenoviruses (HAdV), rotavirus, and norovirus in high frequency and viral loads in water, mussels, and oysters (Keller et al., 2019; Pilloto et al., 2019; Souza et al., 2018).

Molecular methods, such as conventional and quantitative RT-PCR (RT-qPCR), are routinely used for the detection of norovirus in food matrices, including bivalve shellfish (Le Guyader et al., 2006; Uhrbrand et al., 2010; Polo et al., 2015; Anonymous, 2017). As molecular methodologies are able to detect both infectious and non-infectious viruses, limited conclusions can be drawn regarding the potential for health risk and disease through the consumption of contaminated shellfish (Karim et al., 2015; Hassard et al., 2017). Different strategies such as the use of Propidium monoazide (PMA) has been applied to predict viral particles infectivity (Coudray-Meunier et al., 2013; Kim and Ko, 2012; Sánchez et al., 2012). This approach is based on the ability of intercalating dyes (*e.g.* PMA) to penetrate only into damaged

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or altered capsids and intercalate covalently into a viral genome after exposure to strong visible light, thus interfering with PCR amplification (Nocker et al., 2006; Randazzo et al., 2018).

In the Southeast region of Brazil, only one study has investigated norovirus presence in bivalve molluscs (Keller et al., 2019), and from a virological standpoint, reveals the shellfish safety as a sanitary challenge in Brazil. Moreover, it has been demonstrated that bacteriological indicators are unreliable tools to indicate the viral presence in harvesting areas (Romalde et al., 2002; Richards et al., 2010).

The objective of our study was to evaluate norovirus GI and GII dissemination in bivalve shellfish during an ongoing monitoring at three different collecting points of Arraial do Cabo city, using real-time quantitative RT-PCR. We also evaluated norovirus infectivity by using PMAxx treatment. In addition, we also performed the molecular characterization using both direct sequencing and cloning to investigate norovirus genetic diversity in the bivalve positive samples.

2. Materials and methods

2.1. Site description and shellfish sampling

Bivalve shellfish samples (oyster and mussels) were collected at three areas from Arraial do Cabo city, a coastal and touristic spot located close to Rio de Janeiro, one of Brazil's major city. Arraial do Cabo covers an area of 152,305 km^2 that includes seven beaches and was established as an Extractive Marine Reserve in 1997. Due to its pristine marine waters, and considered one of the best spots for scuba diving in Brazil, Arraial do Cabo attracts large number of tourists from Brazil and Latin America countries, especially from Argentina and Uruguay. During summer seasons (particularly from December through February), population peaks from its 28 thousand inhabitants to > 300.000. This high-density population in a small-town coupled with the lack of appropriate urban infrastructure (*i.e.* sewage system network) lead to regularly discharge of untreated sewage in coastal waters, affecting the marine environment and human health.

Two species of molluscs bivalves were collected, *Pseudochama cristella* (popularly known oysters) and mussels *Perna perna*, at three shellfish growing areas in Arraial do Cabo city: Anjos beach (A1) located between the beaches of Forno and Atalaia; Farol beach (A2), located on the island of Cabo Frio, with controlled access by Brazilian Navy, and Forno beach (A3), between the beaches of Prainha and Anjos (Fig. 1). Samples were collected between March 2018 and June 2019, with bimonthly sampling during high summer season (December to March) and monthly during the rest of the year. A bivalve sample consisted of 12–15 individuals (oysters or mussels) collected at the same time and area.

2.2. Viral recovery method

Samples of bivalve molluscs were kept at 4 °C during the transport to the laboratory where they were processed immediately or stored at −80 °C. The digestive tissues of oysters and mussels samples were processed according to the method described at the revised ISO 15216-1:2017 (Anonymous, 2017). Briefly, homogenized digestive tissues (2 g ± 0.2) of each sample was inoculated with 10 μl of PP7 bacteriophage, used as a positive process control to monitor the extraction efficiency, and 2 ml of proteinase K solution (100 μg/ml, Invitrogen). Samples were vortexed for 5 min and incubated at 37 °C with shaking (320 rpm) for 60 min and then incubated at 60 °C for 15 min. After incubation, samples were centrifuged at 3000 ×*g* for 5 min and the soluble homogenate (~2.5 ml) was collected and stored at −80 °C until nucleic acid extraction.

2.3. Nucleic acid extraction

Two extractions methods were employed to recover norovirus RNA from the digestive tissue homogenate samples. For the silica/column method, a total of 140 μl of homogenate was used to extract RNA using the QIAamp® Viral RNA Mini Kit (QIAGEN, CA, USA) according to product manufacturer's instructions. In the second method, viral RNA was purified using the NucleoMag® RNA Virus Extraction Kit (Macherey-Nagel, Dueren, Germany) according to manufacturer's instructions. In brief, nucleic acid contained in 500 μl of viral extract was adsorbed onto magnetic beads, washed three times with different buffers, and released into 100 μl of elution buffer. For both methods, viral RNA was stored at −80 °C until use. Each batch of nucleic acid extractions included a negative extract control (sterile water), as well as in-house norovirus-positive control.

2.4. Norovirus and PP7 detection

A TaqMan®-based quantitative RT-PCR (RT-qPCR) was used for viral detection and quantification. Norovirus GI and GII were detected using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR kit (Invitrogen, CA, USA) with primers and probes as previously described (Kageyama et al., 2003). Assays were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA) using the following cycling conditions: reverse transcription at 50 °C for 60 min followed by a denaturation cycle at 95 °C for 5 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PP7 bacteriophage, used as an internal process control, was recovered using primers and probe described by Rajal et al. (2007), and same cycling conditions as described above for norovirus. To minimize inhibitors interference, undiluted and 1:10 diluted RNA samples were tested in duplicate. A non-template control and positive and negative controls (described above) were included in each RT-qPCR assay. Viruses concentrations were estimated based on synthetic DNA standard curves (gBlock® Gene Fragment, Integrated DNA Technologies, Iowa, USA) generated from ten-fold serially dilutions [10^6–10^0 genome copies (GC) per reaction] containing the target qPCR region for each virus (Fumian et al., 2016). Noroviruses recovered loads were expressed as GC per gram (GC/g) of digestive tissue. Forty cycles were used in RT-qPCR reactions and samples showing a characteristic sigmoidal curve with a cycle threshold (Ct) < 40 were regarded as positive.

To evaluate the PP7 bacteriophage recovery rates, experiments were carried out in triplicate on two different days. 50 μl of PP7 was spiked into 2 g of homogenized digestive tissue from three shellfish samples. These samples were subjected to RNA extraction and quantification by RT-qPCR as described above. The estimated PP7 recoveries rates were calculated in percentage according to the PP7 genome copies number previously inoculated and recovered from the samples.

2.5. Norovirus genetic characterization

Two approaches were used for norovirus GII molecular characterization: direct amplicon sequencing and cloning. For direct sequencing, norovirus-positive samples were subjected to conventional RT-PCR using the primers Mon 432/431 and G1SKR/G2SKR that target ORF1/2 junction region and generate amplicons of 543 and 557 base pairs for GI and GII, respectively (Beuret et al., 2002; Kojima et al., 2002). Amplicons obtained were purified using the ExoSAP-IT™ kit (Applied Biosystems) or QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's recommendations. The purified amplicons were submitted directly to DNA sequencing at the FIOCRUZ Institutional Platform using BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3730*xl* Genetic Analyzer (both from Applied Biosystems).

To investigate norovirus GII genotype diversity, amplicons obtained with primers described above were cloned using the TOPO™ TA Cloning™ Kit (Invitrogen) into chemically competent *E. coli*. Around ten isolated clones were collected from each plate, grown overnight in liquid LB, submitted to RT-PCR amplification followed by DNA sequencing.

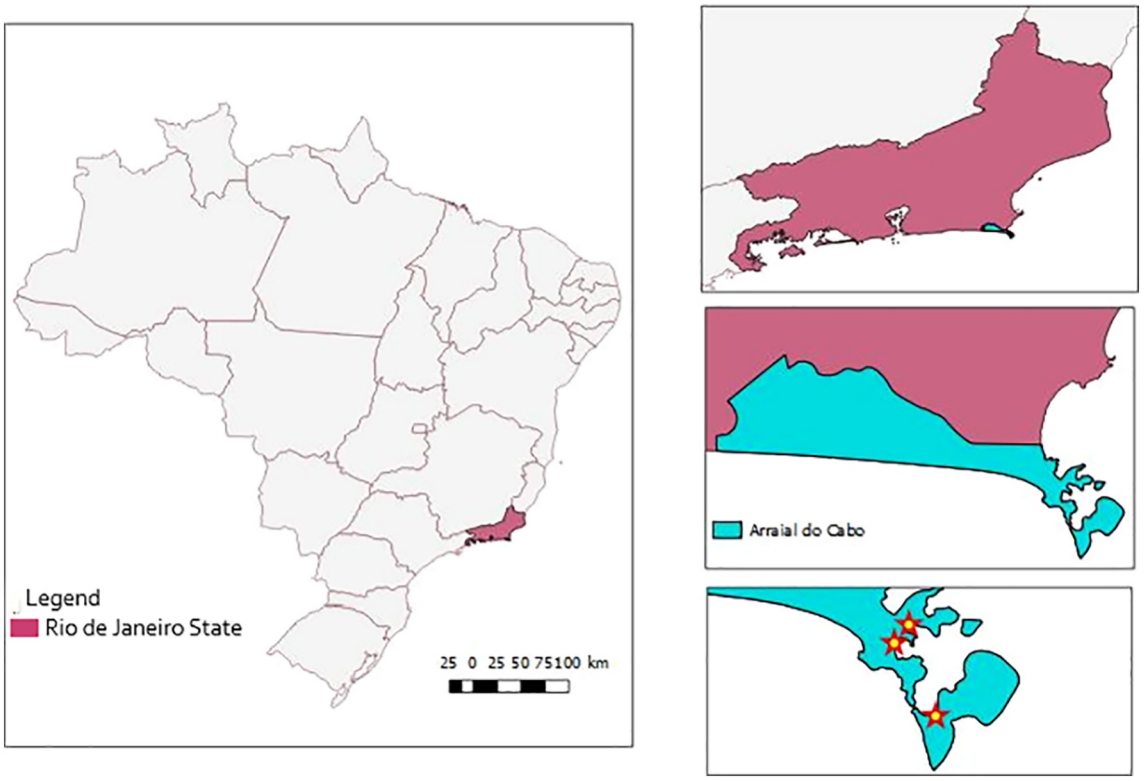

Fig. 1. Map of the collection sites. Shellfish sampling area at three beaches in Arraial do Cabo, Rio de Janeiro, Brazil. The sampling areas are indicated by yellow dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Phylogenetic analysis

Phylogenetic analyses of obtained sequences were performed based on the partial polymerase and capsid regions to determine norovirus genotypes. Consensus sequences were generated using Geneious Prime (Biomatters Ltd., Auckland, New Zealand) and norovirus genotypes were assigned based on the new nomenclature system using an online genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool). Phylogenetic trees were constructed using the neighbor-joining method (Kimura tow-parameter model, 2000 bootstrap replications for branch support) in MEGA 7 (Kumar et al., 2016) using norovirus reference sequences obtained from the GenBank database. Nucleotide sequences obtained from clinical samples were submitted to NCBI GenBank (accession numbers: MT269012 to MT269016 and MT269018 to MT269022).

2.7. PMAxx pretreatment in contaminated shellfish

To investigate norovirus capsid integrity, as an alternative method to evaluate viral infectivity, viral extract samples were aliquoted into two fractions of 100 µl and one fraction was treated with 100 µM of propidium monoazide (PMAxx) and 0.5% Triton 100-X (Fisher-Scientific), and the other fraction treated just with 0.5% Triton 100-X. Both fractions were incubated in the dark at room temperature for 30 min at 150 rpm and immediately exposed to 2 cycles of 15-min of photoactivation with a dark incubation of 15 min, at room temperature, between photoactivations using a photo-activation system (PMA-Lite LED Photolysis Device) (Randazzo et al., 2018). Viral RNA from both fractions were subsequently extracted with NucleoMag® RNA Virus Extraction Kit, as described above and RT-qPCR was performed for norovirus quantification. The efficacy of PMAxx pretreatment was estimated by comparing GC/g obtained from treated and untreated fractions.

2.8. Data analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Box-and-whisker plots were produced to illustrate differences between medians. Norovirus RNA levels in bivalve samples and PMAxx treatment efficacy were analyzed for significant differences using the Independent-Samples Mann–Whitney *U* Test. Frequencies of norovirus detection from different sites and seasons were compared through Chi-square or Fisher's exact test. For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Norovirus detection and quantification in bivalve shellfish

Over a 16-month period, we collected and tested 77 bivalve shellfish samples using RT-qPCR, of these 19 oysters and 58 mussels samples. Norovirus was detected in 32 (41.5%) samples. Among these, norovirus GI and GII were detected in 9.4% and 87.5%, respectively (Table 1). All positive samples were obtained using the magnetic beads-based extraction method. Noroviruses RNA was not detected in any samples

Table 1
Norovirus GI and GII-positive samples detected from bivalve samples using RT-qPCR during the 16-month monitoring period.

Bivalves shellfish	Collected samples (n)	Norovirus detection n (%)			
		GI	GII	GI + GII	Total
Oysters (*Pseudochama cristella*)	19	0	7 (36.8)	0	7 (36.8)
Mussels (*Perna perna*)	58	3 (5.2)	21 (36.2)	1 (1.7)	25 (43.1)
Total	77	3 (3.9)	28 (36.4)	1 (1.3)	32 (41.5)

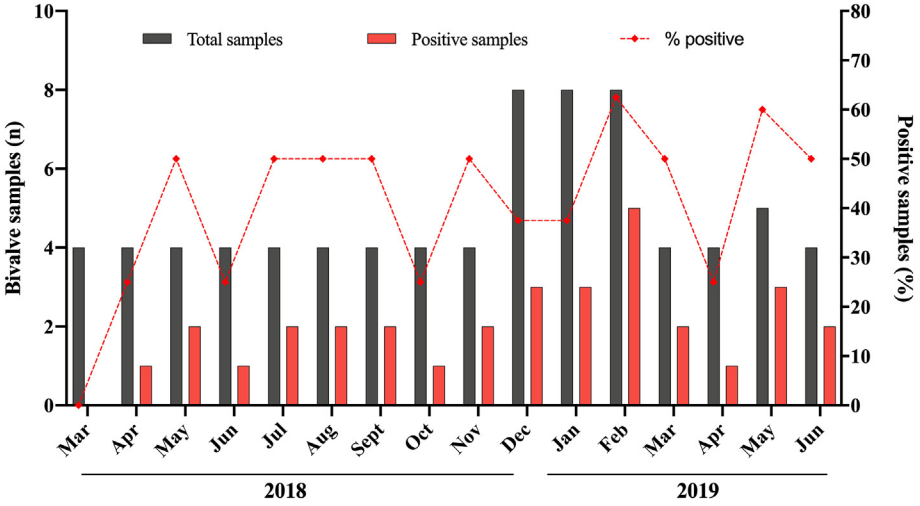

Fig. 2. Distribution and percentage norovirus-positive samples of bivalves collected over a 16-month period in three sampling sites from Arraial do Cabo, Brazil.

extracted with the silica/column-based method.

We detected norovirus year-round, with no markedly seasonality. At least one norovirus-positive sample was detected each month, except in March 2018, and detection rates varied from 25% to 60%. February and May 2019 showed the highest detection rate (approximately 60%) (Fig. 2). We also analyzed the frequency and viral load detection by seasons and sampling sites (Fig. 3). Higher norovirus detection rates were observed from samples collected in winter and summer, and from samples collected at Farol beach, however, no statistical difference was observed regarding virus detection ($p = 0.657$ and $p = 0.240$, Chi-square test) and concentrations according to seasons and sampling sites, respectively ($p > 0.05$, Mann-Whitney test).

Table 2 shows the number of samples analyzed and the detection rate of each genogroup by sampling site. Farol beach showed higher viral detection (57.9%) compared to the other sampling sites, and the detection of norovirus GII corresponded to 91% of these samples. Norovirus GI was detected in lower frequency compared to GII in all three sampling sites, with detection rates of 9.1% at Farol beach and 14.2% at Anjos and Forno beaches, among the positive samples.

Norovirus GI was detected only in mussels samples with median viral load of 2.1×10^2 GC/g (varying from 7.5×10^1 to 3.8×10^2 GC/g). Norovirus GII was detected in both oysters and mussels, with viral load varying from 2.9×10^4 to 3.2×10^7 GC/g and from 6.1×10^2 to 2.2×10^8 GC/g, respectively. The median values of GII from oysters and mussels were 3.6×10^6 and 2.9×10^5 GC/g, respectively. We found significant difference between GI and GII concentration in mussels samples ($p = 0.0005$) (Fig. 4A).

The internal process control (PP7 bacteriophage) was detected in 100% of bivalve shellfish samples (n = 77), and recovery rate was 23.4% ± 9.3 [mean ± standard deviation (sd)]. The viral load values of PP7 obtained for all samples ranged from 3.2×10^4 to 5×10^6 GC/g for oysters and 6×10^4 to 1×10^7 GC/g for mussels and the median values were 1.1×10^5 and 7×10^5 GC, respectively.

3.2. Efficiency of PMAxx pretreatment on shellfish

Sixteen norovirus-positive samples (six oysters and ten mussels) were used to evaluate the treatment with PMAxx in discriminating between infectious and non-infectious particles. The results, based on norovirus viral load (GC/g) of digestive tissue, showed a reduction of up to 3 logs in norovirus concentration comparing PMAxx-treated and untreated samples. Overall, PMAxx-treated samples had statistically lower viral load compared to untreated samples ($p = 0.0064$), with median values of 2.5×10^4 GC/g and 3×10^5 GC/g, respectively (Fig. 4B).

3.3. Norovirus characterization

Norovirus genotyping was performed using two different approaches. By using the direct sequencing, we were able to characterize norovirus genotypes in 31% (10/32) of positive samples. After constructing phylogenetic trees of the polymerase (Pol) and capsid genes, two different norovirus genotypes were identified. Four strains were characterized as GII.4[P4 New Orleans] and six as GII.2[P16]. We also performed molecular cloning of three samples in order to investigate norovirus genotype diversity. A total of nine clones were isolated and sequenced, and the genotype GII.2[P16] was identified.

Phylogenetic analysis showed that all the GII.4[P4 New Orleans] strains found in our study were more closely related to the strains GII.4[P4 New Orleans] isolated in 2018 and 2019 in the US (GenBank MK762561and MT026582) and also with norovirus strains from Rio de Janeiro, Brazil, isolated in 2017 and 2018 (GenBank MH393572;

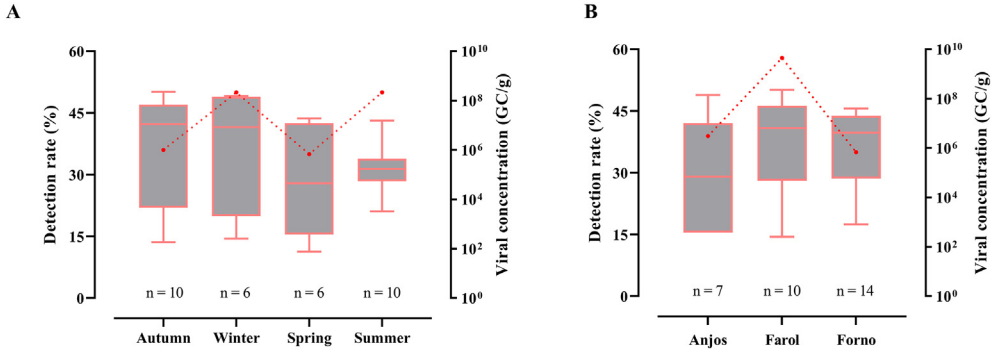

Fig. 3. Detection rates and concentration of norovirus in bivalve samples by season (A) and sampling site (B). Box and whisker plots show the first and third quartiles (equivalent to the 5th and 95th percentiles), the median (horizontal red line in the box) and range concentrations [genome copies per gram (GC/g) of digestive tissue]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Norovirus frequency of detection according to different sampling areas.

Collecting sites	Samples analyzed	Norovirus detection n (%)	Genogroups identified	*p*-Value
Anjos beach	18	7 (38.9)	GI, GII	Site 1 *vs* site 2 ($p > 0.05$)
Forno beach	40	14 (35)	GI, GII	Site 2 *vs* site 3 ($p > 0.05$)
Farol beach	19	11 (57.9)	GII, GI + GII	Site 3 *vs* site 1 ($p > 0.05$)

MH393575 – MH393577), in both the polymerase and capsid regions. The recombinant noroviruses GII.2[P16] found in the bivalves clustered with emergent GII.2[P16] viruses recently detected in Japan, US, France and Germany (Fig. 5A and B).

4. Discussion

In the present study, we used RT-qPCR and sequencing to assess norovirus GI and GII dissemination and diversity in shellfish samples in Brazil. We tested 77 bivalve shellfish samples collected between March 2018 and June 2019, and norovirus was detected in 41.5% of samples and two GII genotypes were identified – GII.4[P4 New Orleans] and GII.2[P16]. We also demonstrated, by using PMAxx treatment, that a significant portion of noroviral RNA detected from the bivalves came from damaged-capsid viruses.

Noroviruses are a major cause of foodborne AGE outbreaks in humans worldwide, and shellfish consumption is a common source of norovirus infection (Le Guyader et al., 2009; Smith et al., 2012; Campos and Lees, 2014; Le Mennec et al., 2017). Here, we used a standard method based on the proteinase K digestion (ISO 15216-1:2017) to assess norovirus contamination of shellfish (oysters and mussels) harvested in Arraial do Cabo, Southeast Brazil. This method has been extensively evaluated, with recovery rates varying from 21 ± 15% to 34 ± 5% (Polo et al., 2015; Lowther et al., 2018; Purpari et al., 2019). The recovery efficiency found in our study (23.4%) was similar to recovery rates previously found using the proteinase K digestion method (Le Guyader et al., 2009; Comelli et al., 2008; Gyawali et al., 2019; Lowther et al., 2019).

In the present study, initially we used the silica column-based method to extract viral RNA of the bivalve samples. From 40 samples tested, we did not detect norovirus in none of the samples. So, all norovirus-positive results reported here were obtained using the magnetic silica-based RNA extraction method. We believe that the poor norovirus RNA recovery using the QIAamp® Viral RNA Mini kit was due to the smaller volume of bivalve homogenates input (140 µl *vs* 500 µl) and mostly to column clogging during the procedure. Similar observations were also reported by Tunyakittaveeward et al. (2019).

Noroviruses were detected at high positivity rates, similar to the detection rates found in shellfish samples (34.4% to 51.5%) collected from different areas in Italy and France (Le Guyader et al., 2009; Pepe et al., 2012; Suffredini et al., 2011, 2012). In Brazil, Keller et al. (2019) also found high rates of noroviruses detection in bivalve samples collected from a mangrove forest in the Vitória Bay, state of Espírito Santo, Brazil. In that study, noroviruses were detected in 53% (9/17) and 85% (11/13) from mussel and oyster samples, respectively.

Regarding the genogroups, we found a higher detection of GII compared to GI (37.7% *vs* 5.2%). Our results are in agreement with other studies demonstrating a higher detection of GII over GI. Das et al. (2020) have detected only GII in 41 of 104 seafood samples obtained from retail markets in Mumbai, India. In Galicia, Spain, Vilariño et al. (2009), have detected GII in 53.7% of bivalve molluscs, whilst GI and other enteric viruses at lower percentages. Another study conducted with bivalve molluscs from coastal areas of southeastern Italy showed similar results for GII detection (39.7%) (Fusco et al., 2019). However, several studies have demonstrated a higher detection of norovirus GI compared to GII (Le Guyader et al., 2012; Kittigul et al., 2016; Tunyakittaveeward et al., 2019). Divergent findings between GI and GII detection rates may be explained by local epidemiology, seasonality and viral load excreted by the infected population (Le Guyader et al., 2012; Atmar et al., 2018; Farkas et al., 2018). The higher detection rate of GII over GI observed in our study could be related to higher GII prevalence in clinical stool samples from AGE cases, as demonstrated by many studies conducted in Brazil (Fioretti et al., 2011; Fumian et al., 2013, 2016; Andrade et al., 2014; Reymão et al., 2018; Cantelli et al., 2019).

Our results showed a median viral load of norovirus GII for oysters

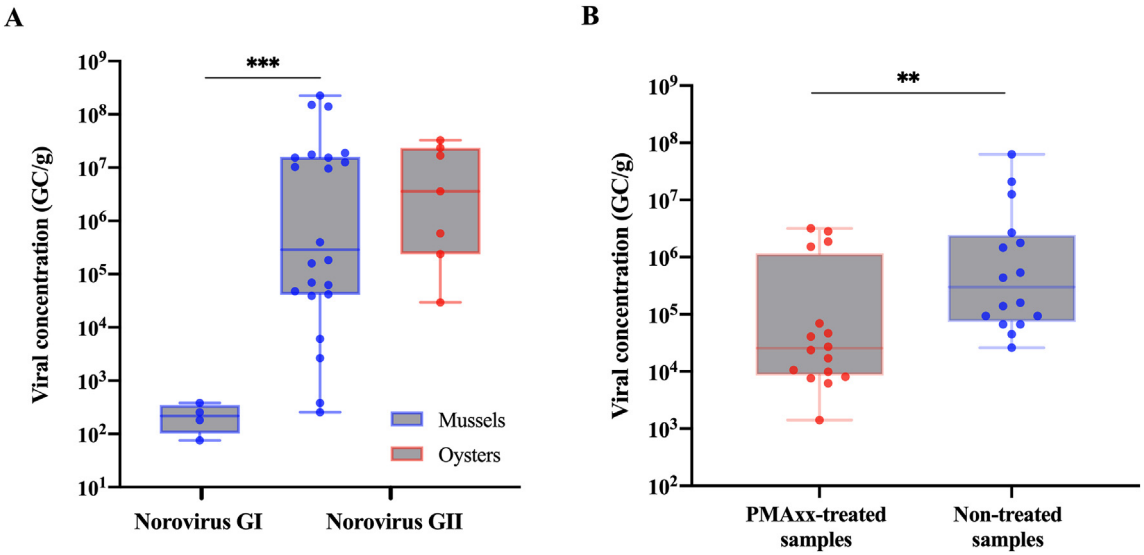

Fig. 4. Norovirus GI and GII viral load by oyster and mussel samples (A) and among PMAxx-treated samples and non-treated samples (B). Box and whisker plots showing all the values and distributed within the first and third quartiles (equivalent to the 5th and 95th percentiles), the median (horizontal line in the box) and range concentrations [genome copies per gram (GC/g) of digestive tissue].

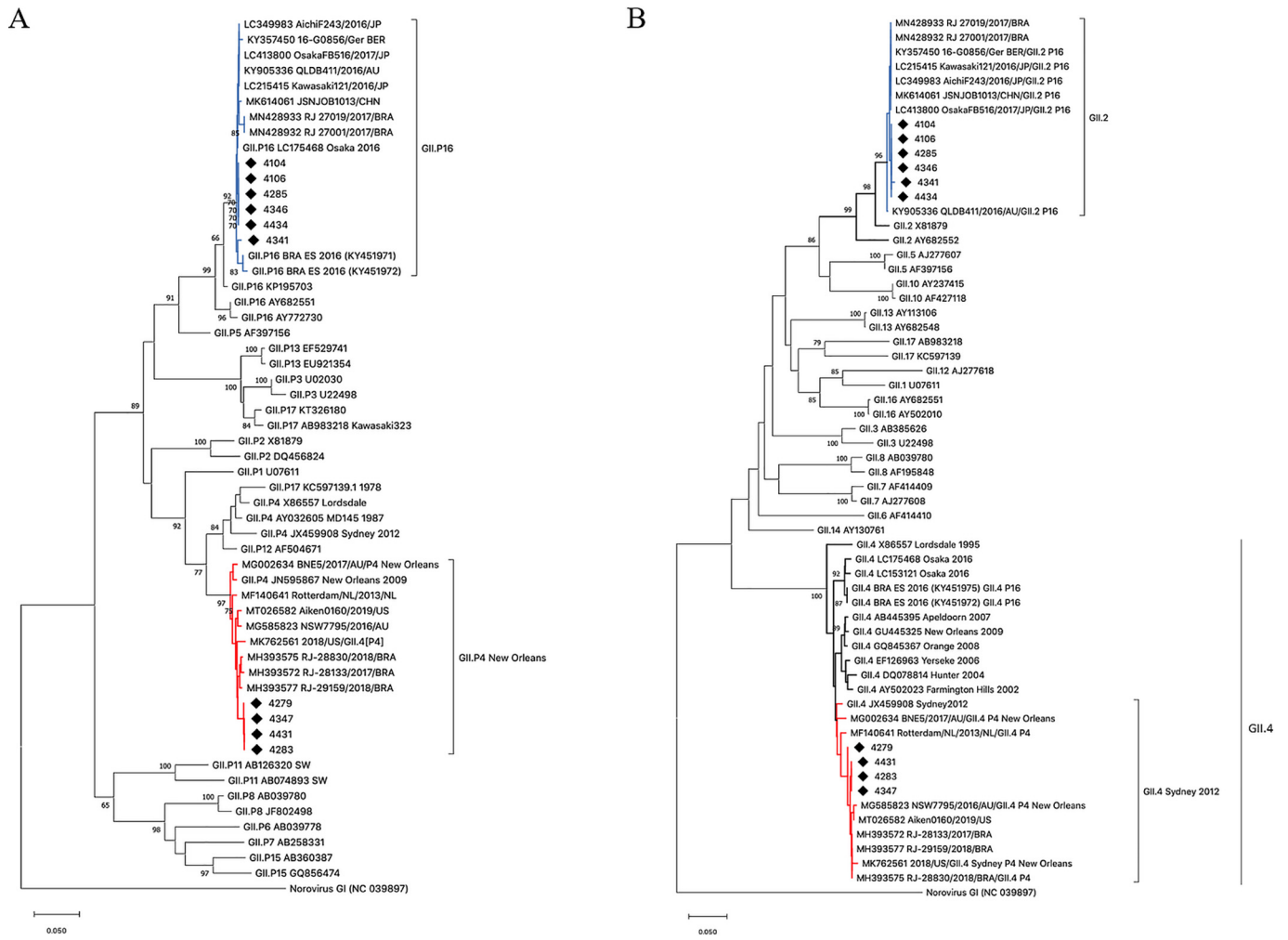

Fig. 5. Phylogenetic trees based on polymerase (A) and capsid (B) regions of GII norovirus. Norovirus GII strains (n = 10) isolated from bivalve samples in this study are shown in the phylogenetic analysis and are marked with a filled diamond. Reference strains were downloaded from GenBank and labelled with their accession number followed by genotype, year and country of isolation. Maximum likelihood phylogenetic trees were constructed with MEGA X software and bootstrap tests (2000 replicates) based on the Kimura two-parameter model. The bootstrap percentage values of ≥70% are shown at each branch point.

(3.6×10^6 GC/g) and mussels (2.9×10^5 GC/g) samples, higher than GI (2.1×10^2 GC/g). Fusco et al. (2017) also reported high norovirus GII concentrations (up to 1.1×10^6 GC/g) in bivalves samples collected during a three-year study in southern Italy. A study carried out in Bangkok, Thailand, with 70 oyster samples collected at a local market has found norovirus in 28.6% (n = 20). GI and GII were detected in 20% and 4.3% of samples with viral load varying from 3.4×10^1 to 1.1×10^5 GC/g and from 1.3×10^3 to 1.6×10^4 GC/g, respectively (Tunyakittaveeward et al., 2019). In Brazil, Keller et al. (2019) reported lower levels of norovirus GII (5.4×10^1 GC/g) compared to our findings. Also, in Brazil Leal et al. (2018) detected high concentrations of human adenovirus (up to 1.3×10^8 GC/g) in oyster samples collected from Cananeia estuary, considered the main producer of oysters on the coast of Sao Paulo state.

We detected norovirus along the year without seasonality. These data are in agreement with studies of noroviruses detection in stool samples from Brazil, reporting their circulation year-round without marked seasonal peak (Victoria et al., 2007; Andrade et al., 2014; Santos et al., 2017; Reymão et al., 2018). Fumian et al. (2019) studying wastewater samples from a treatment plant in Rio de Janeiro, demonstrated the detection of norovirus GII at high concentrations (up to 7.3 $\log_{10}$ GC/L) and without any seasonality. In contrast, norovirus shows a marked winter seasonality in temperate-zone countries (Lopman et al., 2012; Siebenga et al., 2009) supporting their higher detection in environmental samples, including bivalve samples, in those countries during winter seasons (Le Guyader et al., 2000; Kitajima et al., 2010; Mans et al., 2013; Lowther et al., 2012; Suffredini et al., 2012; Kauppinen and Miettinen, 2017).

It is worth mentioning that in January 2019, a sewer pipe ruptured in the city of Arraial do Cabo causing a large disposal of storm water and sewage towards the beaches. At that time, city's beaches were declared unsuitable for bathing according to the Rio de Janeiro State Environment Institute (INEA). According to the INEA weekly monitoring, Anjos beach continued unsuitable (*E. coli* values > 2000 MPN/100 ml) until mid-March. This was a bold event, but other issues concerning the massive unregulated tourism, especially in high season between December and February, and the lack of proper infrastructure of the city may justify the high concentration and dissemination of noroviruses found in the bivalve samples from our study. And due to the proximity of the three sampling sites, there were no differences in norovirus detection among the sites.

Concerning norovirus characterization, the genotype GII.2[P16], detected in the bivalve samples in our study by direct sequencing and cloning, belong to an emerging recombinant genotype that has recently emerged and spread worldwide (Ao et al., 2017; Bidalot et al., 2017). Cheung et al. (2019) demonstrated higher viral load and shedding of the GII.2[P16] compared to other pandemic genotypes in different age groups patients showing acute diarrhea symptoms. This could explain

its high detection and abundance in bivalve molluscs samples. The other strain detected in our study, GII.4[P4 New Orleans] was identified as the predominant strain in France during the 2016–2017 season (Bidalot et al., 2017), as well as in Australia in 2015–2016, where authors emphasized its pandemic potential (Bruggink et al., 2016). In addition, these two strains were recently detected as the most prevalent genotypes detected in stool samples from children living in Rio de Janeiro, Brazil (Cantelli et al., 2019), demonstrating their local circulation.

Considering the health risk trough shellfish consumption, it is important to minimize overestimation of viral particles detected by quantitative molecular methods that do not distinguish between infectious and non-infectious viruses. Therefore, using PMAxx treatment, previously optimized for shellfish samples (Randazzo et al., 2018), we demonstrated a significantly reduction of norovirus load in PMAxx-treated samples compared to untreated. Many studies have used this method to evaluate the portion of potential infectious viruses recovered from environmental waters when using quantitative molecular methods (Leifels et al., 2015; Randazzo et al., 2016; Blanco et al., 2017). In agreement with our results, Randazzo et al. (2018) observed a reduction in viral concentrations in the PMAxx-treated bivalve samples. In addition, by using the PMA-qPCR/RT-qPCR, Quijada et al. (2016) demonstrated significant reductions of > 2.7 log in the reduction of HAdV-2 and mengovirus titers from spiked clam and sausage samples. Here, we successfully used for the first time in Brazil the PMAxx treatment in shellfish samples, reinforcing its usefulness in discriminating between intact and damaged capsids. However, considering that norovirus capsids could be damaged during viral extraction process from digestive tissues and its low infectious dose, low levels of norovirus detection are still of a concern for potential infection for shellfish consumers.

In summary, our study demonstrated the dissemination of norovirus GI and GII in bivalve shellfish samples collected from Arraial do Cabo, Brazil. The increased rate of contaminated bivalves with high viral concentrations reveals the health risk for consumers, especially as they are usually eaten raw or undercooked. In developing countries, where a large fraction of sewage is discharged without any treatment, raw sewage serves as the main source of contamination of shellfish production waters, representing a challenge to their harvesting. Monitoring and characterization of enteric viruses in bivalve samples is necessary to implement effective depuration process according to specific pathogen (Pilloto et al., 2019). The norovirus genotypes characterized in bivalve samples reflect the circulating strains in local population that reach the coastal waters trough the discharge of raw sewage. Arraial do Cabo is a touristic spot for Brazilians and South Americans. Tourism represents the main economic activity and is totally dependent on the city's natural oceanic beauties. The adoption of public policies in order to control and regulate massive tourism and the adoption of proper sanitation measures to reduce marine pollution, especially at high season, is vital to preserve the environmental balance of this marine reserve.

CRediT authorship contribution statement

Sylvia Kahwage Sarmento: Investigation, Methodology, Validation, Writing - original draft.**Caroline Rezende Guerra:**Investigation, Methodology, Validation, Resources, Writing - review & editing.**Fábio Correia Malta:**Investigation, Methodology, Writing - review & editing.**Ricardo Coutinho:**Resources, Conceptualization, Writing - review & editing.**Marize Pereira Miagostovich:**Resources, Funding acquisition, Writing - review & editing.**Tulio Machado Fumian:**Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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