Detection of norovirus epidemic genotypes in raw sewage using next generation sequencing

Tulio M. Fumian[a,b,*,1], Julia M. Fioretti[b,1], Jennifer H. Lun[a], Ingrid A.L. dos Santos[b], Peter A. White[a], Marize P. Miagostovich[b]

[a] Faculty of Science, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia
[b] Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil

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ABSTRACT

Noroviruses are a leading cause of epidemic and pandemic acute gastroenteritis (AGE) worldwide, and contaminated food and water are important routes for its transmission. Raw sewage has been used for viral surveillance to monitor the emergence of new norovirus strains with the potential to cause epidemics. In this study, we investigated norovirus occurrence and norovirus RNA levels in 156 samples collected from May 2013 to May 2014, across three different stages (52 samples each) of a wastewater treatment plant (WWTP) in Rio de Janeiro, Brazil. We also explored norovirus GII diversity in raw sewage samples by next-sequencing generation (NGS). In addition, we examined norovirus prevalence and molecular epidemiology from acute gastroenteritis cases. Using RT-qPCR, norovirus GI and GII was detected in 38.5% and 96.1% of raw sewage samples, 40.4% and 96.1% of primary effluent samples and 1.9% and 5.8% of final effluent samples, respectively. Norovirus RNA levels varied from 4 to 6.2 $\log_{10}$ genome copies per litre (gc L^{-1}) for GI and from 4.4 to 7.3 $\log_{10}$ gc L^{-1} for GII. Using MiSeq NGS, we identified 13 norovirus genotypes over the one-year period, with six dominant capsid genotypes, including GII.4, GII.17, GII.5, GII.2, GII.3 and GII.1. GII.4 noroviruses were the most prevalent in wastewater samples (68.5%), and a similar trend was observed in AGE cases (71%). The emergent GII.17 was the second most prevalent genotype (14.3%) identified in the raw sewage samples, however, it was not detected in clinical cases. Due to the high burden of norovirus outbreaks and the lack of vaccine and antiviral drugs, it is essential to understand the genotypic diversity of norovirus at the population level. Complementary data obtained from both clinical and environmental (sewage) samples proved to be an effective strategy to monitor the circulation and emergence of norovirus epidemic genotypes.

1. Introduction

Human noroviruses are the leading cause of epidemic and pandemic acute gastroenteritis (AGE) worldwide. Norovirus-associated outbreaks affect people of all age groups and are estimated to cause 212,000 deaths annually (Pires et al., 2015). Outbreaks are frequently reported in closed or semi-closed settings such as hospitals, cruise ships, long-term care facilities and schools (Hall et al., 2012).

Noroviruses belong to the family *Caliciviridae* (genus *Norovirus*) and are nonenveloped, positive-sense RNA viruses with a 7.5 kb genome that is organized into three open reading frames (ORFs 1–3) (Glass et al., 2000). ORF1 encodes a polyprotein that is post-translationally cleaved into non-structural proteins. ORF2 encodes the major capsid protein (VP1), with two domains (shell S and protruding P), whilst ORF3 encodes the minor capsid protein VP2 (Glass et al., 2000). Based on the amino acid sequence of VP1, norovirus can be classified into seven genogroups (GI–GVII), and further subdivided into more than 40 genotypes (Kroneman et al., 2013).

Noroviruses belonging to genogroups I (GI) and GII are the most common viruses that infect humans, however, a single genotype (GII.4) has been the most prevalent cause of infections worldwide (Eden et al., 2014; Hoa Tran et al., 2013). GII.4 noroviruses display a seasonal influenza-like epochal evolution, resulting in the replacement of the previous dominant strain every 2–5 years (Eden et al., 2014; Parra et al., 2017). So far, six pandemics GII.4 variants have emerged and have been associated with an increase of AGE outbreaks worldwide. The most recent pandemic variant (Sydney 2012) has been circulating since its emergence in 2012 (Bull et al., 2012; Eden et al., 2014; van

Beek et al., 2013). Recently, GII.17, an uncommon genotype, emerged in China and spread throughout other south-east Asian countries resulting in an increased number of AGE outbreaks reported within this region (Lu et al., 2015; Matsushima et al., 2015). This new GII.17 variant (strain Kawasaki 2014) has also been sporadically reported outside Asia, including countries of North and South America, Europe and Oceania (Andrade et al., 2017; Lun et al., 2018; Medici et al., 2015; Parra and Green, 2015).

Raw sewage represents a valuable source to characterize the genetic diversity of enteric viruses in a human population. As such, several studies have used raw sewage as a source of information on the viruses excreted within feces and urine for epidemiological analysis (La Rosa et al., 2017; Mabasa et al., 2018; Montazeri et al., 2015; Prado et al., 2018). Sewage also plays an important role in viral disease surveillance and has been used to monitor poliovirus circulation in communities worldwide as a part of the Global Polio Eradication Initiative by the World Health Organization (WHO) (Benschop et al., 2017). By combining clinical and environmental surveillance the WHO is able to effectively monitor poliovirus circulation in communities (Deshpande et al., 2003).

More recently, studies have employed next-generation sequencing (NGS) techniques to identify the viral diversity in urban sewage and shown that it closely reflects the pattern of infections in the population (Lun et al., 2018; Suffredini et al., 2018). One such study used NGS of raw sewage collected monthly from Australia to identify changing patterns in the prevalence of norovirus genotypes in 2016 (Lun et al., 2018). Interestingly, the changes observed in the sewage data largely matched those observed in clinical data. In addition, the authors saw a change in norovirus capsid genotype predominance, where GII.17 viruses dominated at the beginning of the year, followed by GII.4 and finally GII.2 viruses. Furthermore, an Italian study identified the circulation of eleven norovirus genotypes in raw sewage samples, using NGS technologies, over a 6-year period. This study demonstrated the early detection of the GII.17 Kawasaki 2014 strain and three uncommon genotypes (GII.5, GII.16, and GII.21) (Suffredini et al., 2018).

Additionally, an effective sewage treatment process is crucial to prevent environmental contamination since wastewater treatment plants (WWTP) are the last barrier before the discharge of effluents into water bodies. In the current study, we investigated the circulation of norovirus in sewage and clinical samples from Rio de Janeiro, Brazil. Firstly, we detected and quantified norovirus GI and GII RNA levels from three different types of sewage samples along the treatment process (raw, primary effluent and the final effluent) collected between May 2013 and May 2014. Secondly, we assessed the norovirus GII genotype diversity in raw sewage from Brazil using MiSeq. Thirdly, the GI and GII noroviruses from acute gastroenteritis (AGE) samples, obtained between 2012 and 2014 were characterized and quantified. Finally, the norovirus GII genotypic prevalence patterns found in raw sewage were compared with those found in clinical samples.

2. Materials and methods

2.1. Waste water treatment plant (WWTP)

The studied WWTP is located in the metropolitan area of Rio de Janeiro, a major city of Brazil. It is the largest WWTP in Rio de Janeiro and receives urban sewage from approximately 1.5 million inhabitants (approximately 25% of the city's population), with the catchment area spanning both the central and peripheral regions of the city. The plant employs a primary treatment (physical) followed by a secondary (biological) treatment process (aerobic process using activated sludge), with a mean inflow of $2500\,L\,s^{-1}$. There are four aeration tanks in parallel ($11,500\,m^3$ each) with the capacity to treat $625\,L\,s^{-1}$ of effluent and secondary sedimentation is performed in four additional secondary settling tanks ($8800\,m^3$ each). No chlorination is used in the final effluent before discharge into Guanabara Bay ($22°40'–23°00'$ S and

$43°00'–43°20'$ W; total area of $380\,km^2$), which is an ecosystem that surrounds a large part of the city, including the ports.

2.2. Sample collection and virus concentration

Between May 2013 and May 2014, three types of sewage samples (100 mL of grab samples) along the treatment process were collected weekly from a WWTP in Rio de Janeiro, the largest facility in Brazil. These sample types included: i) influent, which consists of raw sewage; ii) primary effluent, which has undergone mechanical treatment and primary sedimentation; iii) secondary effluent, which is the final effluent obtained after the activated sludge process. A low-cost methodology based on the adsorption of viruses to pre-flocculated skimmed-milk proteins was used for virus concentration (Calgua et al., 2013). Briefly, 50 mL of sewage sample were mixed with 100 mL of glycine buffer 0.25 M, pH 9.5. The mixture was stirred for 30 min on ice and then centrifuged at $8000 \times g$ for 30 min at 4 °C. The supernatant (150 mL) was adjusted to a pH of 3.5 using 1 M HCl and then 1.5 mL of pre-flocculated skimmed-milk solution (1%, w/v) were added. The sample was then stirred for 8 h at room temperature and flocs were sedimented by centrifugation at $8000 \times g$ for 30 min at 4 °C. The pellet was resuspended in 500 μL of phosphate buffered saline (pH 7.5). Before concentration, all samples were spiked with bacteriophage PP7 [$6.8\log_{10}$ genome copies (gc) μL^{-1}], which was used as an internal process control. Additional samples were collected to determine the recovery efficiency of the method. A sample from each treatment stage was divided into two parts and known concentrations of norovirus GII fecal suspension [$6.5\log_{10}$ gc μL^{-1}] were spiked into one part, and the non-spiked half was used as a negative control. Viral recovery assays were performed in triplicate for all samples.

2.3. Clinical samples

Fecal samples from AGE patients of all ages were obtained from public health centers, including hospitals and the central laboratory of Rio de Janeiro, Brazil between January 2012 and December 2014. This study was part of the national viral AGE surveillance program and was approved by the ethics committee of Fiocruz (CEP No. 311/06). No patient information was used, and all data were maintained anonymously and securely.

2.4. RNA extraction and cDNA synthesis

A volume of 140 μL of concentrated wastewater samples or fecal suspensions (10% w/v) was used for viral RNA extraction using a QIAamp Viral RNA Mini kit (QIAGEN, CA, USA), according to the manufacturer's instructions. Extracted RNA was diluted 10-fold before it was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies™, NY, USA), and an aliquot was immediately stored at −80 °C. In each extraction procedure, RNAse/DNAse-free water was used as negative control.

2.5. Viral detection and quantification

A TaqMan®-based quantitative PCR (qPCR) was used for the detection and quantification of GI and GII norovirus and bacteriophage PP7, using primers and probes previously described (Kageyama et al., 2003; Rajal et al., 2007). All qPCR assays were performed with TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA) using the ABI 7500 Real-Time PCR System (Applied Biosystems). Undiluted and ten-fold dilutions of the nucleic acid extract of sewage samples were analyzed in duplicate (4 runs/sample). A non-template control and a positive control were included in each run. Standard curves generated from ten-fold serially diluted plasmids (5×10^6–5×10^0 plasmid copies/reaction) containing the target qPCR region for each virus were used to calculate the viral load of norovirus GI, GII and bacteriophage PP7 in each sample.

2.6. Norovirus molecular characterization by Sanger sequencing

Norovirus detected from clinical samples were genotyped using a reverse-transcription PCR (RT-PCR) with primers targeting the ORF1/2 junction region. The primers used to amplify regions B and C of norovirus were Mon432 and G1SKR for GI, and Mon431 and G2SKR for GII, generating amplicons of 543 and 557 base pairs (bp), respectively (Beuret et al., 2002; Kojima et al., 2002). Amplicons obtained were purified using a QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's recommendations. Purified products were sent to the FIOCRUZ Institutional Platform for DNA sequencing (PDTIS) for DNA sequencing, which was performed using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems).

2.7. Norovirus phylogenetic analysis

Phylogenetic analyses of norovirus-positive clinical samples were performed on the partial polymerase and capsid regions to determine norovirus genotypes. Consensus sequences were generated using Geneious® version 11.0.2 (Biomatters Ltd., Auckland, New Zealand) and phylogenetic trees were constructed using the maximum likelihood method (2000 bootstrap replicates for branch support) in MEGA 7 (Kumar et al., 2018), with norovirus reference sequences obtained from the National Center for Biotechnology Information (NCBI) database. Norovirus genotypes were confirmed with online genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool) (Kroneman et al., 2011). Nucleotide sequences obtained from clinical samples were submitted to NCBI GenBank (accession numbers: MH393638 to MH393675).

2.8. MiSeq NGS and data analysis

NGS was used to characterize norovirus GII diversity within raw sewage samples collected between May 2013 and April 2014. Firstly, RNA extracted from 12 sewage samples (one per month) was used for reverse-transcription PCR (RT-PCR) targeting the 5′-end of capsid gene (region C) of norovirus GII (Lun et al., 2018), that allows for the differentiation among genotypes. A second-round PCR was performed to attach sequencing adapters using KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Cape Town, South Africa) generating a final amplicon size of 373 bp. All PCR amplicons were cleaned up using Agencourt AMPure XP beads (Beckman Coulter, California, USA) and quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, CA, USA). Nextera XT indexes (Illumina) were attached using KAPA HiFi HotStart ReadyMix and the library was quantified using KAPA Library Quantification Kit (Kapa Biosystems, Cape Town, South Africa). The library was then normalized, pooled and the fragment sizes were evaluated using Tape Station D1000 (Agilent Technologies, California, USA). Libraries were submitted to Ramaciotti Centre for Genomics (UNSW, Sydney, Australia) for paired-end sequencing on the MiSeq platform using a v2 300 cycle kit (2 × 150 bp) (Illumina). NGS data analysis was performed using Geneious as previously described (Lun et al., 2018). One sample (May 2014) had to be excluded due to the number of available reactions.

2.9. Norovirus GII.17 specific sequencing

Raw sewage samples that tested positive for GII.17 by NGS were subjected to nested RT-PCR using specific primers to for this genotype, previously described by La Rosa et al. (2017) targeting region C in ORF2 and producing a 310 bp amplicon. Samples were submitted to the Ramaciotti Centre for Genomics (UNSW) for Sanger sequencing, and phylogenetic analyses were performed as described in Section 2.7. GII.17 sequences obtained were deposited to the GenBank Database under the accession numbers: MH785022 to MH785026.

2.10. Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Box-and-whisker plots were produced to illustrate the differences between medians. Norovirus GI and GII RNA levels in wastewater and clinical samples were analyzed for significant differences using the Independent-Samples Mann–Whitney U Test. Frequencies of norovirus detection in wastewater and clinical samples were compared through the Chi-square or Fisher's exact test. For all analyses the level of significance is indicated as: not significant if $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. Norovirus GI and GII $\log_{10}$ reduction was estimated using values found in raw sewage and final effluent samples. For final effluent samples, concentrations below the limits of detection (LOD) were assigned a value equal to the GI and GII LOD (3.9 $\log_{10}$) divided by square root of 2 (Croghan and Egeghy, 2003), in order to calculate the reduction rates of the activated sludge process.

3. Results

3.1. Norovirus detection and quantification in sewage samples

Throughout the study period (May 2013 to May 2014), a total of 156 wastewater samples were collected in three different stages of the WWTP. In total, we detected norovirus GI and GII in 42 (26.9%) and 103 (66%) samples, respectively. The internal process control (PP7 bacteriophage) was detected in 100% of sewage samples (n = 156), and PP7 recovery rate was 53.2% ± 43.9 [mean ± standard deviation (sd)]. Recovery rates of norovirus GII obtained for each treatment stage were (mean ± sd): (i) raw sewage, 118.7% ± 92.5; (ii) primary treatment, 135.9% ± 129.3; and (iii) final effluent, 68.5% ± 38.

Monthly distribution of norovirus GI and GII are shown in Fig. 1. Norovirus GI was detected in eight of 13 months and within 38.5% (n = 20), 40.4% (n = 21) and 1.9% (n = 1) of samples from raw sewage, primary effluent and final effluent, respectively. Over the study period, norovirus GII was detected in 96.1% (n = 50) of both raw sewage and primary effluent samples, and in 5.8% (n = 3) of final effluent samples (Fig. 1). We observed a significant reduction of GI and GII detection rates ($p \leq 0.05$) when the raw sewage and primary effluent were compared to the final effluent samples. The frequency of GI (38.5 and 40.4%) and GII (96.1% in both) noroviruses detected in raw sewage and primary effluent samples were significantly different ($p \leq 0.05$).

Norovirus GI concentrations ranged from 4.2 to 6.2 $\log_{10}$ gc L^{-1} (median of 5.4 $\log_{10}$) in raw sewage and from 4.1 to 5.7 $\log_{10}$ gc L^{-1} (median of 5 $\log_{10}$) in primary effluent samples (Fig. 2A). Norovirus GII concentrations ranged from 4.4 to 7.1 $\log_{10}$ gc L^{-1} (median of 6.4 $\log_{10}$) and from 4.5 to 7.3 $\log_{10}$ gc L^{-1} (median of 6.3 $\log_{10}$) in raw sewage and primary effluent samples, respectively (Fig. 2A). In the final effluent, GI was detected in one of 52 samples (4.7 $\log_{10}$ gc L^{-1}) and GII in three samples (from 4.9 to 6.1 $\log_{10}$ gc L^{-1}). We observed a significant difference ($p \leq 0.001$) between norovirus GI and GII RNA concentrations in raw sewage and primary effluent samples, as well as comparing GI concentrations detected in raw sewage and primary effluent samples ($p \leq 0.05$). The median $\log_{10}$ reduction values of the activated sludge process were 0.3 and 1.2 $\log_{10}$ for norovirus GI and GII, respectively.

We also analyzed norovirus detection rates and viral loads in the raw sewage samples among the seasons. A higher detection rate of norovirus GI was observed in samples collected during the colder months (autumn and winter) compared to the warmer months ($p \leq 0.05$), and the median viral levels ranged from 4.2 to 5.3 $\log_{10}$ gc L^{-1}. Norovirus GII was detected at higher rates (> 92%) in all four seasons compared to GI, and the median concentrations ranged from 5.6 to 6.5 $\log_{10}$ gc L^{-1} (Fig. 2B).
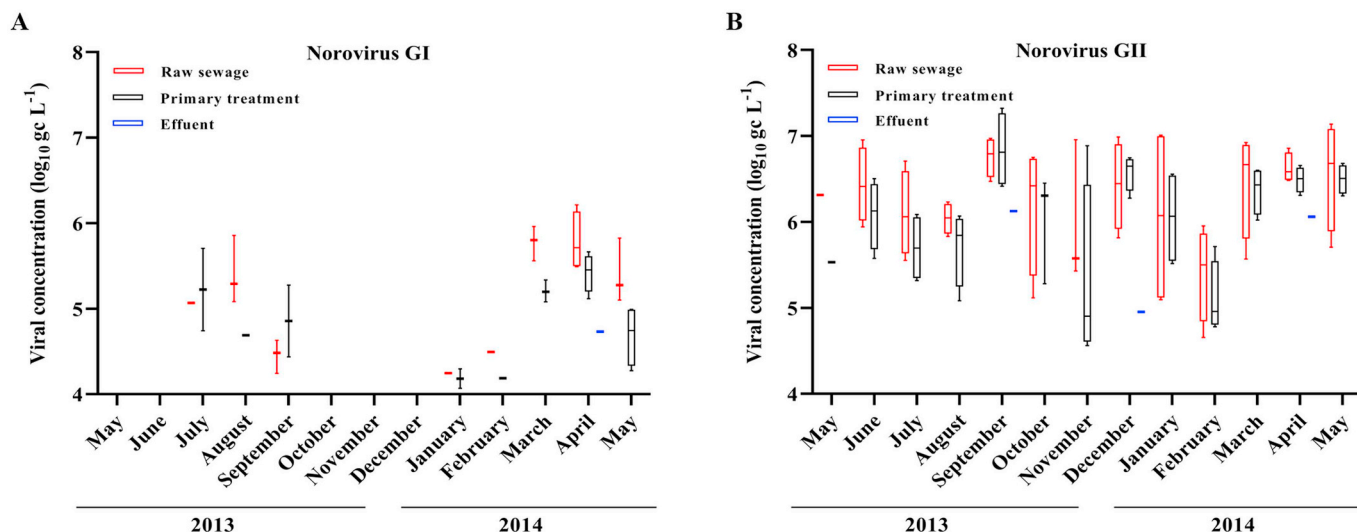
Fig. 1. Box-and-whisker plots showing the monthly profile of norovirus GI and GII in wastewater samples. Norovirus GI (A) and GII (B) distribution and viral load in raw sewage, primary treatment and final effluent samples weekly collected over a 13-month period of a wastewater treatment plant in Rio de Janeiro, Brazil. Box and whisker plots show the first and third quartiles (equivalent to the 5th and 95th percentiles), the median (the horizontal line in the box) and range concentrations [($\log_{10}$ genome copies per litre (gc L^{-1})].

3.2. Norovirus GII genetic diversity in raw sewage samples by NGS

Using NGS, we investigated norovirus GII diversity in raw sewage samples collected monthly from a WWTP in Rio de Janeiro between May 2013 and April 2014. An average of 410,514 reads (range 113,374–1,086,726) was generated from each monthly sample. NGS reads were mapped to norovirus reference sequences to determine the relative abundance of each genotype present within each sample. Thirteen norovirus capsid genotypes were identified over the one-year period, with six dominant genotypes; GII.4, GII.17, GII.5, GII.2, GII.3 and GII.1 (Fig. 3A and B).

Among the major genotypes identified, the pandemic GII.4 was the most prevalent throughout the studied period (Fig. 3A). Except for October 2013, February and April 2014, GII.4 represented > 50% of the monthly viral population, and for three months it was in absolute abundance representing > 99.9% of the assembled sequences (Fig. 3B). Of all the total reads assembled to GII.4, the pandemic Sydney 2012 variant represented > 99% of the circulating viruses.

In late 2013, an increase in GII.17 prevalence was observed from

October onwards, and reached a proportion of 62% of all genotypes identified by February 2014 (Fig. 3B). All the reads assembled to GII.17 belonged to the recently emerged epidemic Kawasaki variant (cluster C) (Fig. 4). Phylogenetic analysis of selected sequences obtained by NGS, showed that the GII.17 strains isolated here clustered with the emergent GII.17, clusters C-I and C-II (Fig. 4). Moreover, we confirmed these results by RT-PCR amplification and direct sequencing using specific primers targeting the new emergent GII.17 variant, as previously described (La Rosa et al., 2017). All five samples sequenced belonged to the C-I cluster and showed 100% of nt identity with the prototype GII.P17-GII.17/Kawasaki 323 in the analyzed region (273 bp of the 5′ end of ORF2).

GII.2 noroviruses were detected in May, June and August of 2013, and represented 13%, 36% and 11% of the monthly norovirus population, respectively. GII.1 and GII.3 noroviruses were detected at similar levels, responsible for 10 to 18% of the genotype distribution. Interestingly, a less common genotype, GII.5, was detected at high percentages in two months, October 2013 (43%) and April 2014 (58%) (Fig. 3B). Other genotypes such as GII.6, GII.12, GII.13, GII.15, GII.16,
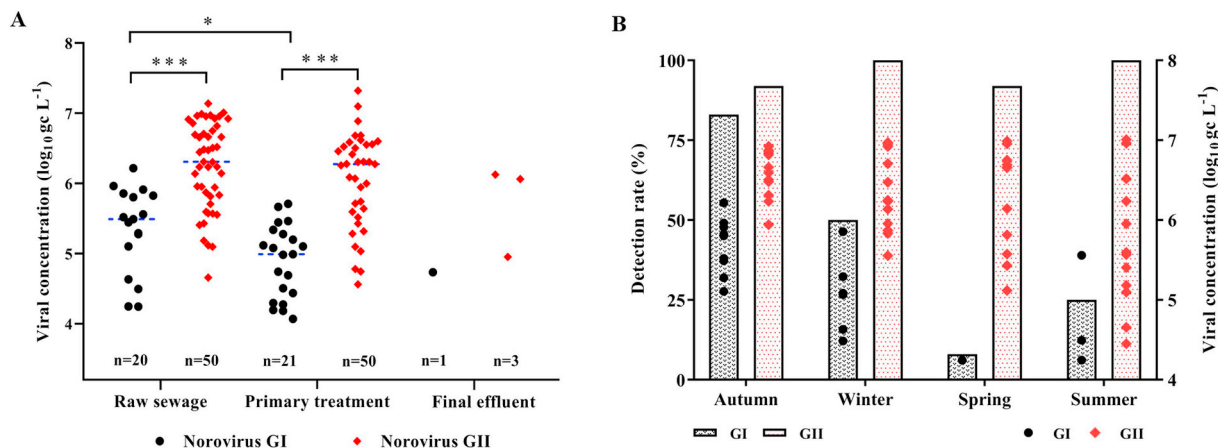

Fig. 2. Norovirus viral load and seasonal distribution in wastewater samples. (A) Norovirus GI and GII viral loads in raw sewage, primary treatment and final effluent samples weekly collected over a 13-month period of a wastewater treatment plant in Rio de Janeiro, Brazil. The plots show the values of viral concentrations [($\log_{10}$ genome copies per litre (gc L^{-1})] and median values are indicated by the blue trashed line. (B) Norovirus GI and GII detection rates and viral loads in raw sewage samples by season. *$p \leq 0.05$; ***$p \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
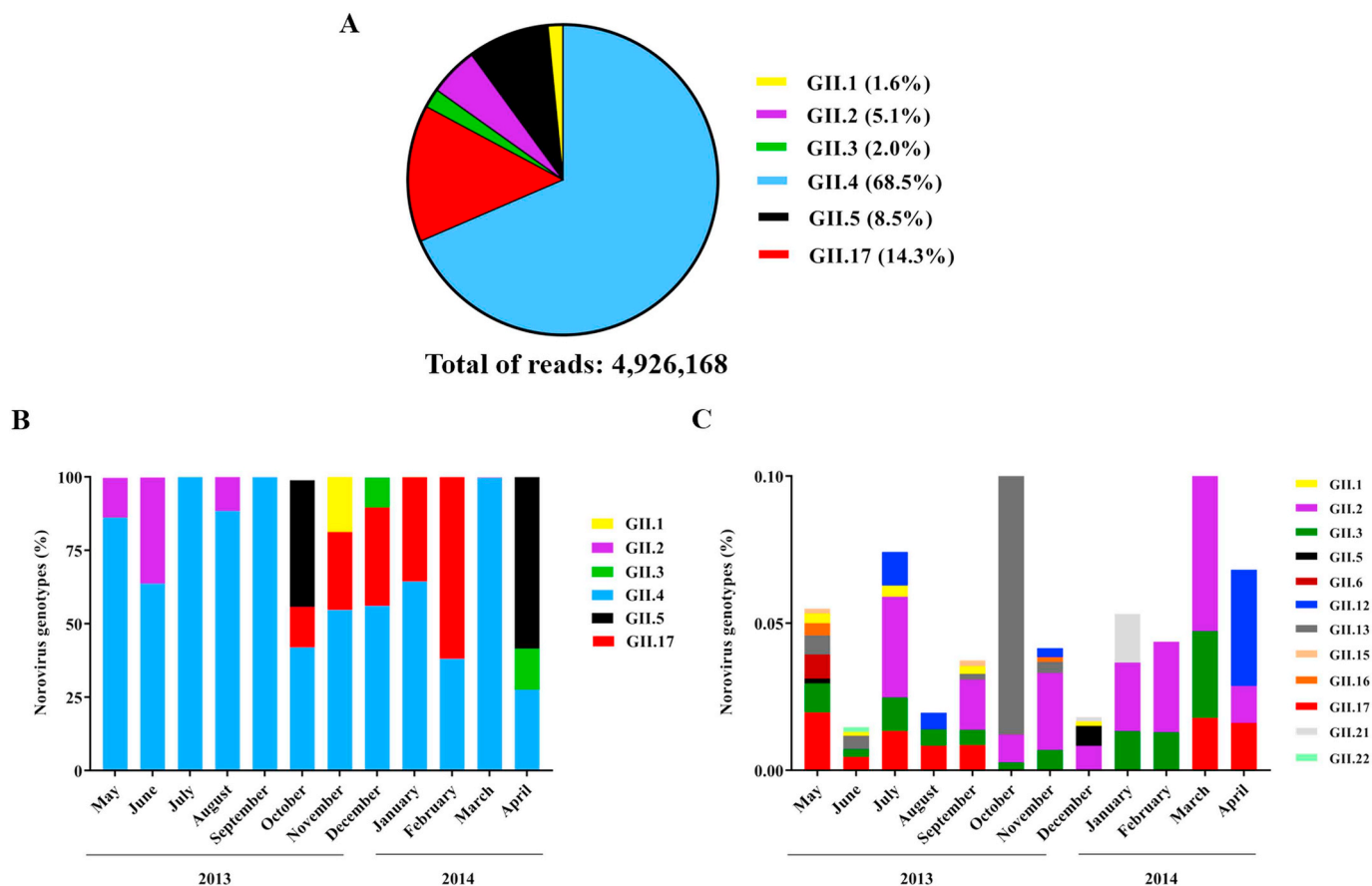
Fig. 3. Norovirus genotype distribution in raw sewage samples. (A) Total distribution of norovirus GII major capsid genotypes identified by MiSeq next-generation sequencing (NGS) technology over a one-year period (May 2013–April 2014) in raw sewage samples in Rio de Janeiro, Brazil. The total number of reads is listed below the pie chart, as well as the relative prevalence of each major norovirus capsid genotype. Norovirus GII monthly distribution of major genotypes (B) (> 10% of monthly identified sequences) and minor genotypes (C) (< 0.1% of monthly identified sequences). The Y-axis represents the percentage norovirus distribution in each sample and the time is indicated on the X-axis.

GII.21 and GII.22 were also detected but represented < 0.1% of the genotypes identified (Fig. 3C).

3.3. Clinical samples

Between January 2012 and December 2014, a total of 316 fecal samples were collected from AGE-associated inpatients (n = 192) and outpatients (n = 124) of all ages. Norovirus was identified in 87 (27.5%) samples, with infection rates of 32.5% in 2012, 19.2% in 2013 and 34.8% in 2014. Among the positive samples, 75 (86.2%) belonged to the GII genogroup, 9 (10.3%) belonged to the GI genogroup and three samples showed GI and GII co-infection (3.5%) (Table 1). Norovirus was detected at higher infection rates in children under 11 months (30.1%, 25/83) and in patients over 12 years (37.6%, 32/85, $p \le 0.05$) compared to infection rates found in age groups between 12 and 23 months (21.8%), 2 to 4 years (19.6%) and > 4 to 12 years (18.9%).

Norovirus viral load in fecal samples for GI and GII ranged from 3.8 to 9 $\log_{10}$ genome copies per gram (gc g^{-1}), and 4.2 to 12 $\log_{10}$ gc g^{-1}, respectively (Table 1). A lower viral concentration was observed in GI-positive samples compared to GII-positive samples ($p \le 0.05$). Overall, we found a higher norovirus detection rate in samples collected during the winter season [56.9% (37/65)] compared to those collected in autumn [16.5% (19/115)], spring [13.8% (5/58)] and summer [29.5% (23/78)] ($p \le 0.05$).

A total of 38 norovirus sequences were obtained from the 87 norovirus positive samples (GI - 6 and GII - 32) (Fig. 5). Norovirus GII.4 was detected in 71% of the genotyped samples, and the percentage of

detection in each year was 81% (2012), 30% (2013) and 100% (2014). We also identified circulation of other low prevalence genotypes, such as: GI.3, GI.4, GI.7, GII.2, and GII.7 (Table 1).

4. Discussion

In the present study, we used MiSeq NGS to assess norovirus GII diversity in raw sewage samples in Brazil. Over a one-year period (May 2013 to April 2014), we identified 13 GII genotypes circulating in raw sewage, and from those, six (GII.1, GII.2, GII.3, GII.4, GII.5 and GII.17) were detected as major genotypes (> 10% of monthly viral population).

The pandemic GII.4 Sydney 2012 was the most predominant norovirus, being detected as the single genotype in three months, and in the other months its proportions varied from 27% to 88%. We also confirmed the high circulation of GII.4 Sydney 2012 from AGE cases from 2012 to 2014 (71%). The detection rates of GII.4 in raw sewage reflect the high circulation of this genotype during the study period. After its first identification in Australia 2012 (Eden et al., 2014), GII.4 Sydney 2012 was the most prevalent genotype worldwide in the following years (van Beek et al., 2018; White, 2014) and studies from Brazil have also demonstrated a high prevalence of GII.4 Sydney 2012 (Fioretti et al., 2014; Reymao et al., 2018; Santos et al., 2017). In Northeast Brazil, Santos et al. (2017) detected GII.4 in 79.3% of norovirus-positive fecal samples (n = 280) collected between 2006 and 2013, and after its first detection (March 2012), the Sydney 2012 variant accounted for 100% of samples genotyped in that year. Similar results were observed in samples collected from North Brazil during
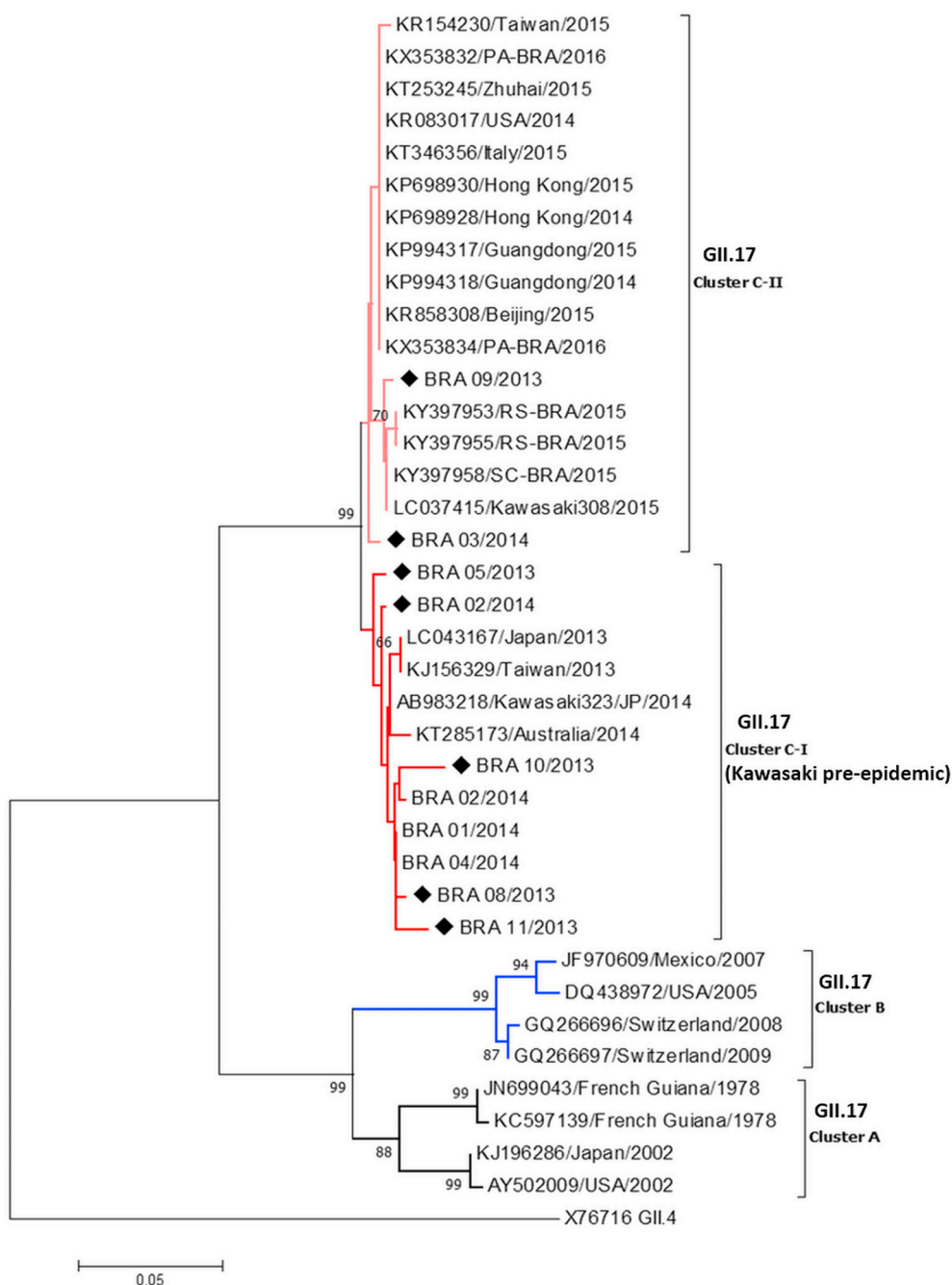
Fig. 4. Phylogenetic tree based on capsid region of GII.17 norovirus. Representative (n = 7) norovirus GII.17 strains isolated in raw sewage samples are shown in this phylogenetic analysis. Strains obtained in this study are marked with a black filled diamond and names contain the collection date (M/Y). Reference strains were downloaded from GenBank and labelled with their accession number followed by genotype, country and year. Phylogenetic analysis was based on 223 bp of the 5′-end of the capsid gene of norovirus GII.17 viruses. Sequence alignments were performed using the MUSCLE algorithm. Neighbor-joining phylogenetic trees were constructed with MEGA 7 software and bootstrap tests (2000 replicates) based on the Kimura two-parameter model. The bootstrap percentage values are shown at each branch point for values ≥60%.

Table 1
Norovirus detection and viral load in clinical samples tested during 2012–2014.

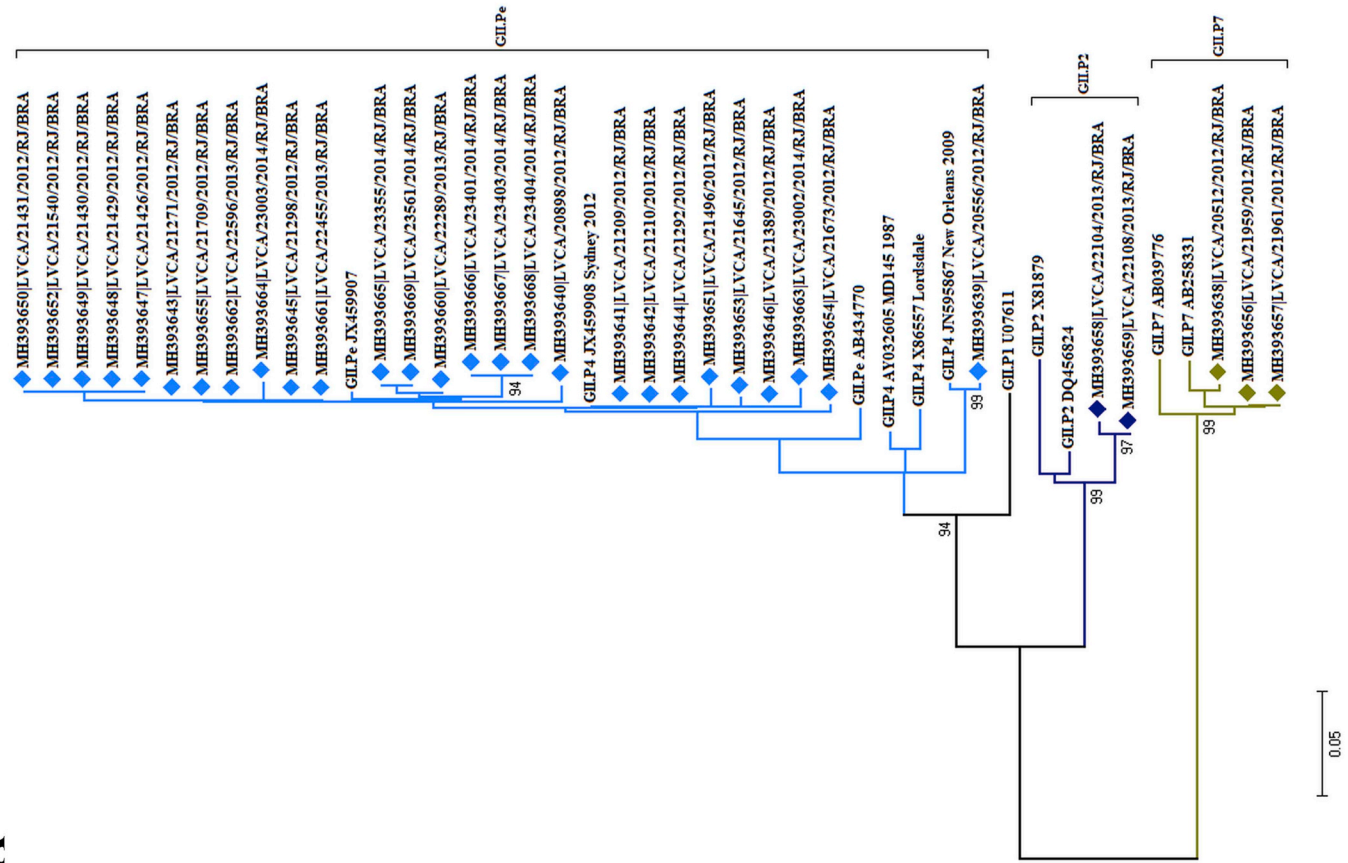|	Norovirus GI			Norovirus GII			
Year	Viral load range (log$_{10}$ gc g^{-1})	Pos/tested (%)	Genotypes (n of sequences)	Viral load range (log$_{10}$ gc g^{-1})	Pos/tested (%)	Genotypes (n of sequences)	Total pos/tested (%)
2012	6.4–8.8	2/120 (1.7)	GI.P7-GI.7 (1)	4.2–12	39/120 (32.5)	GII.Pe-GII.4 (17) GII.P7-GII.7 (3)	39/120 (32.5)
2013	3.8–9	9/130 (6.9)	GI.P4-GI.4 (3) GI.Pd-GI.3 (2)	4.5–11	17/130 (13.1)	GII.P2-GII.2 (2) GII.Pe-GII.4 (3)	25/130 (19.2)
2014 Total	4	1/66 (1.5) 12/316 (3.8)	ND	4.2–10.4	22/66 (33.3) 78/316 (24.7)	GII.Pe-GII.4 (7)	23/66 (34.8) 87[a]/316 (27.5)

gc g^{-1}: genome copies per gram; ND: not determined.
[a] Three samples (two from 2012 and one from 2013) were positive for both norovirus GI and GII.
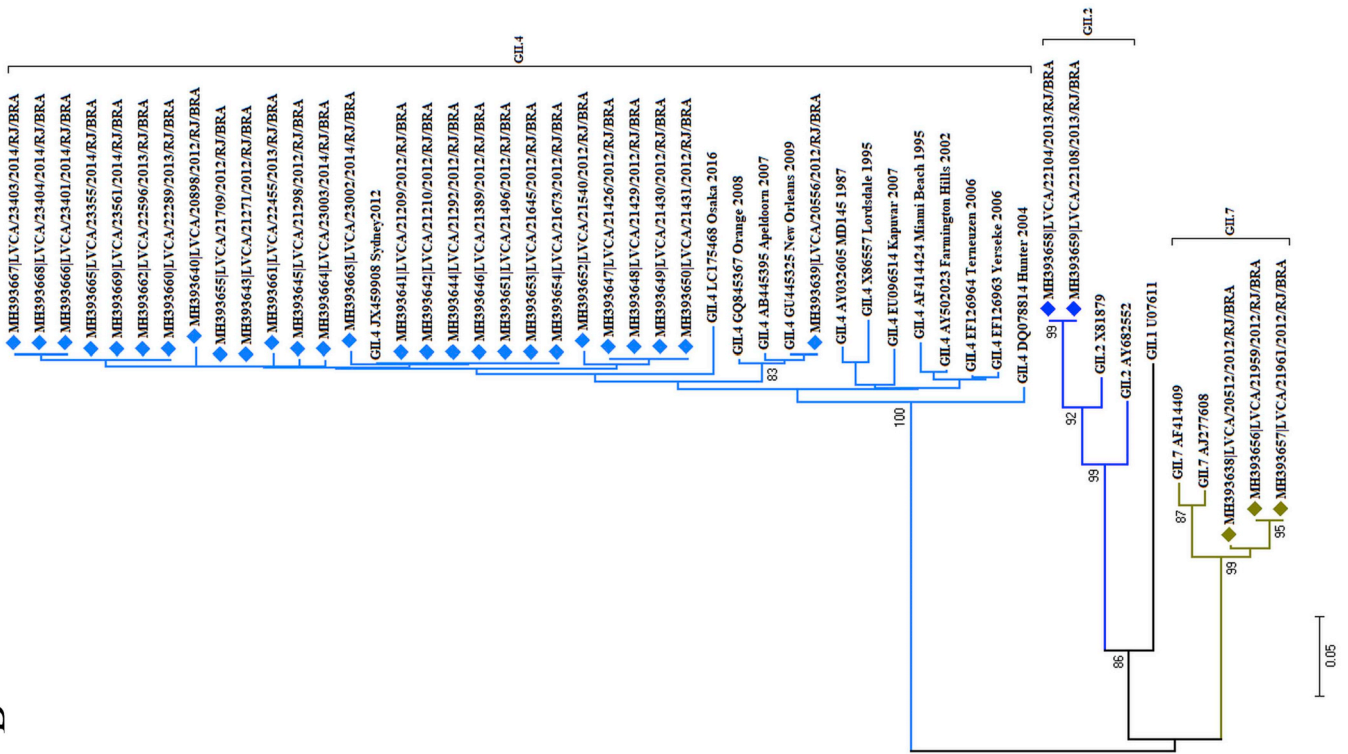
(caption on next page)

B

A

Fig. 5. Phylogenetic trees based on polymerase (A) and capsid (B) regions of GII norovirus. Norovirus GII strains (n = 32) isolated in clinical samples from acute gastroenteritis cases are shown in the phylogenetic analysis. Strains obtained in this study are marked with a filled diamond. Reference strains were downloaded from GenBank and labelled by genotype with their accession number. (A) Phylogenetic analysis of 172 bp of the 3′-end of the polymerase gene of GII norovirus. (B) Phylogenetic analysis of 223 bp of the 5′-end of the capsid gene of GII norovirus. Sequence alignments were performed using the MUSCLE algorithm. Maximum likelihood phylogenetic trees were constructed with MEGA 7 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.

2012 and 2015, where GII.4 Sydney 2012 was the most prevalent variant detected in 93% of cases in 2012 to 55% of cases in 2015 (Reymao et al., 2018).

Another major genotype detected in raw sewage was the new emergent GII.17 Kawasaki virus. This strain emerged in the winter of 2014–15 and became the predominant genotype in China and Japan (Lu et al., 2015; Matsushima et al., 2015). After its emergence in Asia, GII.17 Kawasaki was also detected sporadically in several countries worldwide (Chan et al., 2017). This GII.17 variant was first identified in AGE cases in 2015 in North Brazil (Silva et al., 2017), followed by other studies reporting its detection in different regions of the country (Andrade et al., 2017; Barreira et al., 2017). We detected a high prevalence of GII.17 Kawasaki in raw sewage collected between October 2013 and February 2014, approximately one and a half years before its first detection in clinical samples. The detection of GII.17 was confirmed by RT-PCR using specific primers followed by Sanger sequencing. Our results are similar to those found in Italy (Suffredini et al., 2018), Japan (Kazama et al., 2017) and South Africa (Mabasa et al., 2018), where GII.17 Kawasaki was detected in sewage samples at least one year prior its detection in AGE patients.

Previously, our group has estimated that GII.17 Kawasaki variant was introduced in Brazil in 2014, possibly during the highly touristic 2014 FIFA World Cup event (Andrade et al., 2017). In this previous study, we used sequences of GII.17-positive cases firstly identified in 2015 for phylogeographic analysis using BEAST (Andrade et al., 2017). However, our new findings showed that this variant has been circulating in Brazil since mid-2013. So, the unmatched results are likely a reflection of underreported cases or low-profile circulation of the new variant. This demonstrates the usefulness of sewage data to enhance the generation of a complete picture of norovirus genotypes within a population.

More recently in Brazil, Prado et al. (2018) have detected GII.17 Kawasaki variant from wastewater collected between 2015 and 2016 from São Paulo city. The authors used direct amplicon sequencing and detected the GII.17 Kawasaki in 50% of the GII-genotyped samples. GII.4 Sydney was also detected, but in lower percentage (23%). Other studies elsewhere have also detected the GII.17 Kawasaki from environmental samples, such as river and estuary waters, seawater, bivalve shellfish, wastewaters and surface water (Boonchan et al., 2017; Kim et al., 2016; Kiulia et al., 2014; La Rosa et al., 2017; Lun et al., 2018; Pu et al., 2016).

In our study, four other major capsid genotypes (GII.1, GII.2, GII.3 and GII.5) were detected in sewage samples. These genotypes are frequently reported from clinical cases and environmental samples in Brazil and elsewhere (Barreira et al., 2017; Fumian et al., 2016; Lun et al., 2018; Santos et al., 2017; Siqueira et al., 2017; Tao et al., 2015). Interestingly, we detected an uncommon genotype, GII.5 in October 2013 and April 2014 circulating at high levels (43% and 58% of the circulating viruses, respectively). Using the same approach, Suffredini et al. (2018) and Kazama et al. (2017) detected this genotype in raw sewage collected in the same year (2013) in Italy and Japan. Globally, only a few studies have identified GII.5 in clinical samples (Alam et al., 2016; Cannon et al., 2017; Fukuda et al., 2010). Norovirus surveillance data from the NoroNet network between 2005 and 2016 in 19 countries and four continents demonstrated that GII.5 circulated in four years (2006, 2012–2014) at low prevalence rates (< 1.7%), and samples obtained in 2013 and 2014 accounted for 72% of its total detection (van Beek et al., 2018). In Brazil, GII.5 was identified in a single sewage

sample from São Paulo in 2015 (Prado et al., 2018), and to our knowledge there is no report of this genotype in clinical samples. Our findings matched with data showing the global circulation of this uncommon genotype (GII.5) in specific years.

Interestingly, during the study GII.5 and GII.17 noroviruses were only detected in sewage samples. This could be related to asymptomatic or mild infections caused by these genotypes that do not lead to clinic or hospital visits. For example, Chan et al. (2015) demonstrated GII.17 infections occurred in older individuals compared to GII.4 infections (median age of 49 years and 1 year, respectively), and are less likely to infect the at-risk groups. This demonstrates the importance of using wastewater samples as a molecular surveillance tool.

In this study, norovirus was detected at high levels across the year in the raw and primary treated sewage. In contrast, a significant reduction of both genogroups in the final effluent was observed, with positivity rates of 1.9% and 5.8% for GI and GII, respectively. Similar findings were reported by previous studies showing the removal of norovirus in final effluent samples (Haramoto et al., 2006; Katayama et al., 2008). We also identified the removal efficiencies for norovirus GI and GII (0.3 to 1.2 log reduction), similar to other studies (Montazeri et al., 2015; Prado et al., 2018). Studies have reported values of norovirus $\log_{10}$ removal by the activated sludge process varying from 0.3 to 2.1 for GI and 0.3 to 2.5 for GII viruses (da Silva et al., 2007; Flannery et al., 2012; Francy et al., 2012; Katayama et al., 2008; Nordgren et al., 2009).

Norovirus GII concentrations were significantly higher than GI in both raw sewage and primary effluent ($p \leq 0.001$). This result reflects the pattern observed with clinical data, where a higher GII prevalence (24.7% *vs* 3.8%) and viral load ($p \leq 0.05$) were found. Similar to our study, Chan et al. (2006) have shown a GII viral load > 100-fold higher than GI in fecal samples of patients with norovirus-associated gastroenteritis. In relation to wastewater samples, many studies worldwide have reported a higher concentration of norovirus GII compared to GI (Campos et al., 2016; da Silva et al., 2007; Eftim et al., 2017; Prado et al., 2018).

We observed an Autumn/Winter seasonality of norovirus GI, demonstrated by significantly higher detection rates and viral concentrations compared to samples collected in Spring and Summer. Despite having a higher prevalence throughout the study period, no seasonality was observed for GII noroviruses. In agreement with our results, Mounts et al. (2000) demonstrated the cold weather seasonality of GI infections in 12 studies conducted during a 21-year period. In general, norovirus infections display a winter seasonality in temperate-zone countries (Lopman et al., 2009; Siebenga et al., 2009). A meta-analysis study of norovirus in raw sewage from different temperate-climate countries showed a large degree of seasonality and a winter peak for both genogroups (Eftim et al., 2017). In tropical zones, such as Brazil, norovirus usually circulates throughout year, without a marked seasonal peak (Reymao et al., 2018; Santos et al., 2017). However, Prado et al. (2018) demonstrated that both genogroups peak during the winter months (July/August) in raw sewage of São Paulo city, Brazil. In comparison, continental-area countries show remarkable differences in weather patterns, therefore displaying different norovirus seasonality. Zeng et al. (2012) found seasonal peaks of norovirus between late summer and fall in the southeastern areas of China (Shanghai and Hangzhou) and norovirus winter peak was only seen in northern China, characterized by a longer and colder winter. Therefore, differences in norovirus seasonal profiles and study locations could explain divergent results of GI and GII prevalence obtained from sewage samples.

The reduction of viral loads by effective treatment of wastewater is crucial to minimize contamination of waterbodies used by the public thus mitigating the risk of viral transmission to the population. Our study focused on norovirus detection and quantification from wastewater and clinical samples, and the use of NGS to explore GII genetic diversity in the raw sewage. We demonstrated a reduction in norovirus detection and viral load throughout the stages in the conventional activated sludge process. Moreover, we successfully identified the circulation of six major genotypes in raw sewage samples over a one-year period. Among those, GII.4 Sydney and GII.17 Kawasaki, were considered emergent epidemic variants with widespread circulation. In summary, we successfully demonstrated the use of raw sewage samples as a surveillance tool to detect norovirus strains responsible for both symptomatic and asymptomatic infections within the population.

5. Conclusion

Our study demonstrated the use of NGS to study genetic diversity of virus populations overcame sensitivity and scalability issues compared to the labor- and time- intensive cloning, usually performed to obtain a more detailed picture of the circulating genotypes within an environmental sample. We observed a high genetic diversity of norovirus in raw sewage, represented by the circulation of major and minor genotypes. The combination of clinical and environmental (sewage) data enhanced norovirus surveillance strategy and revealed the circulation of pandemic and epidemic strains, as well as minor norovirus genotypes likely associated with asymptomatic or underreported infections in the human population.

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