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Research paper

Recombinant noroviruses detected in Mid-West region of Brazil in two different periods 2009–2011 and 2014–2015: Atypical breakpoints of recombination and detection of distinct GII.P7-GII.6 lineages

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ABSTRACT

Noroviruses are an important cause of acute gastroenteritis. The high incidence of norovirus is a reflection of its great genomic and antigenic variability resultant of evolutionary mechanisms, such as recombination. Herein, the main objective of this study was to characterize partially two regions of norovirus genome (RdRp and VP1) from fecal samples, collected in two different time periods (2009–2011 and 2014–2015) in the Mid-West region of Brazil. Twenty samples were sequenced and characterized (GI.P5-GI.5, GII.P16-GII.3, GI.P7-GI.7, GII.Pe-GII.4 and GII.P7-GII.6). Sequences of GII.Pe-GII.4 genotype were also characterized as Sydney 2012 variant. Genotypes GII.P7-GII.6, GII.P16-GII.3 and GII.Pe-GII.4 (16/20–80%) were identified as norovirus recombinants by phylogeny and bioinformatic analyzes. The GII.P7-GII.6 (62.5%) and GII.Pe-GII.4 (25%) genotypes had recombination point's upstream ORF1/2 overlapping region, whereas GII.P16-GII.3 (12.5%) genotype had the recombination point in the overlapping region. Furthermore, the GII.P7-GII.6, from samples collected in 2009–2011 had different recombinant points than the GII.P7-GII.6 from samples obtained in 2014–2015, forming two different clusters in the phylogenetic analysis. Our study brings information on the circulation of recombinant norovirus genotypes in Mid-West of Brazil, including recombinants with atypical recombination breakpoints, and provides evidence for the circulation of different lineages of the same recombinant genotype.

1. Introduction

Norovirus are important agents of acute gastroenteritis (AGE), being associated with 20% of all cases (Ahmed et al., 2014; Lopman et al., 2016). Norovirus particle has approximately 25 to 40 nm of diameter, is non-enveloped and its genome is composed by a 7.5 kb single-stranded RNA of positive polarity organized into three open-reading-frames (ORF). The first ORF encodes for the non-structural proteins and ORF2 and ORF3 encode the capsid proteins, VP1 and VP2, respectively. The *Norovirus* genus, which belongs to the *Caliciviridae* family, is further divided into genogroups and genotypes. So far, seven genogroups are recognized, with GI, GII and GIV being described causing infections in humans (Green, 2013). Genogroups are subdivided into genotypes based on the similarity of the sequences that encodes for the capsid protein (VP1) and RNA-dependent RNA-polymerase (RdRp). A new nomenclature system has been proposed for the differentiation of the polymerase and capsid genotypes consisting in addition of the letter P in the polymerase genotypes (e.g. GII.P1-GII.1). Currently, there are, at least, 29 capsid genotypes and 39 polymerase genotypes associated with infections in humans (Vinjé, 2015). One possible reason for the greater variety of genotypes for the polymerase region, in comparison with the capsid, is that some of those polymerases are considered “orphans”, since they were identified with an already established capsid genotype (Kroneman et al., 2013). A great variability is observed in genotype GII.4 that is characterized into variants (e.g. GII.4_Sydney_2012 and GII.4_New_Orleans) and point mutations or/and recombinations are considered to be responsible for the variation in these strains.

Concerning recombination events, there are currently two proposed models for norovirus strains: (1) The model proposed by Bull et al. (2007) postulates that the RdRp switches templates during replication mainly in the overlapping region of ORF1/2, which is considered a

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hotspot for recombination (replicative norovirus recombination) since in this region there is an RNA promoter. Also, the presence of an RNA stem loop, which contributes the RNA polymerase loss of processivity. However, “atypical” breakpoints have been described, and therefore a second model was proposed by Galli and Bukh (2014). It was developed targeting hepatitis C virus, but it has been also considered for norovirus (Begall et al., 2018). It hypothesizes that after a breakage in the genomic RNA strands from different norovirus strains, they are bound by the action of cellular enzymes or even by chemical repair (non-replicative norovirus recombination). This model can generate both homologous and non-homologous recombinants. The characterization of recombinants from different parts of the world and the determination of recombination points are important for a better understanding of norovirus variability and evolution. In this context, the main objective of this study was to characterize the two partial regions (ORF1 - RdRp and ORF2 - VP1) of noroviruses genome from fecal samples collected in two different periods of time in the Mid-West region of Brazil. Additionally, we have identified the recombination points of the recombinant variants.

2. Material and methods

2.1. Design of the study

In two previous studies (one prospective and one cross-sectional) conducted between 2009 and 2011 (Study 1 – S1) and between 2014 and 2015 (Study 2 – S2) in the Laboratory of Virology and Cell Culture of the Federal University of Goiás (LabVICC/UFG), fecal samples, from children with or without acute gastroenteritis symptoms, were screened for norovirus by conventional RT-PCR (de Oliveira et al., 2014) and by RT-PCR real time TaqMan (Dábilla et al., 2017) targeting the ORF1/2 junction (Table 1). For the present study, 59 norovirus positive samples (obtained in S1 and S2) from children were analyzed. Ethical approval was obtained for both studies by the Research Ethics Committee of the Federal University of Goiás/The Clinical Hospital (protocols: 087/2009 and 37,305,314.7.0000.5078), and samples were collected only after a consent form was signed by the parents or legal guardians of the child.

2.2. ORF1/2 amplification and characterization

2.2.1. RNA extraction and cDNA synthesis

Fecal suspensions (20% in PBS, pH 7.4) were used for viral RNA extraction using the isothiocyanate guanidine/silica method (Boom et al., 1990; das Dôres de Paula Cardoso et al., 2002). The complementary DNA (cDNA) was obtained using pd.(N)6 (Random Hexamer, Amersham Biosiences) and SuperScript III as Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

2.2.2. PCR amplification and genotyping

Duplex PCR was performed using primers Mon 431/432 (Beuret et al., 2002) and G1SKR/G2SKR (Kojima et al., 2002) targeting ORF1/2 junction region (Genome locations [Refs. - M87661/X86557] - ORF1: 5093–5112/4819–4839 and ORF2: 5652–5671/5366–5389). Positive samples were then submitted to a monoplex PCR using the same primer pairs above mentioned separately for norovirus GI and GII primer pairs (Mon 432/G1SKR and Mon 431/G2SKR). Reaction was performed in a 25 µL mixture of 5 µL cDNA, 5 U Platinum® Taq DNA Polymerase (Life Technologies™), and 500 nM of each primer. PCR amplification was performed with an initial denaturation at 95 °C for 7 min, followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Products were purified by using 65% isopropanol and 70% ethanol, and then submitted to a sequencing reaction [Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA)], in duplicate, in an automatic sequencer (DNA ABI PRISM 3130, Applied Biosystems). Sequences quality was determined by the phred score

Table 1
Summary of the origin of norovirus positive samples.

	Study design	Period of sample collection	Description of samples origin	References
1	Prospective	2009–2011	A prospective study that was conducted in a day-care center located in Goiânia, Goiás, Mid-West Brazil, in order to monitor the day-care center environment for calicivirus circulation. A total of 539 stool specimens were obtained from 56 children, 29 children had at least one calicivirus-positive sample, and 22 of them were positive for norovirus.	de Oliveira et al., 2014
2	Cross-sectional	2014–2015	A cross-sectional study conducted in samples from children up to six years of age hospitalized at the Hospital Materno Infantil, Goiânia, Goiás, Mid-West Brazil. The study evaluated the frequency, viral load and molecular profile of norovirus in samples with or without gastroenteritis symptoms. A total of 37 children were positive for norovirus in fecal samples, 19 in asymptomatic and 18 in the symptomatic group.	Dábilla et al., 2017

(Gordon et al., 2001) and consensus sequences were obtained using BioEdit 7.0.5.3 (Hall, 1999). Initially, norovirus genotypes were assigned using an online genotyping tool - Norovirus Typing Tool 2.0 (https://www.rivm.nl/mpf/typingtool/norovirus/) (Kroneman et al., 2011). The nucleotide sequences were deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers: MH271645 - MH271664.

2.3. Recombination analyses

The recombination analyses were performed as described by Bull et al. (2007) by applying three different methods: phylogenetic analysis, SimPlot program 3.5 (Lole et al., 1999) and Recombination Detection Program (RDP4) program v4.95 (Martin et al., 2015) with algorithms implemented in the program (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq) using the standard settings.

2.3.1. Phylogenetic and molecular evolutionary analysis

Two data sets were constructed, considering ORF1 (RdRp region) and ORF2 (VP1 region) partial sequences, using the results obtained from the online genotyping tool. Comparable sequences for the same regions were retrieved from the NCBI, besides sequences obtained in S1 and S2. Viral phylogenies were estimated using the MEGA program 7.0 (Kumar et al., 2017). The analyses were performed by the Maximum Likelihood method, using nucleotide substitution model chosen by jModelTest program 2.1.9 (Darriba et al., 2012), 2000 replicates. Consensus sequences were constructed using the BioEdit program 7.0.5.3 (Hall, 1999) and each one is constituted by sequences of the same recombinant genotype characterized in this study, considering a nucleotide identity ≥99%.

2.3.2. Detection of recombinant breakpoints

To identify the recombination points, consensus sequences were used. These sequences were compared with wild type noroviruses' sequences, retrieved from the NCBI, aligned and recombination analyses were performed using SimPlot program 3.5, following the parameters: Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0. Different methods implemented in the RDP4 were also used to confirm the recombination events: RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq methods ($p < .01$).

3. Results

Twenty (33.9%) out of the 59 fecal samples, presented good quality to be sequenced and therefore were further characterized. Seven sequences from the S1 and thirteen sequences from the S2 were characterized as: one GI.P7-GI.7, six GII.P7-GII.6; one GI.P5-GI.5, two GI.P7-GI.7, two GII.P16-GII.3, four GII.P7-GII.6 and four GII.Pe-GII.4 (Table 2, Fig. 1). Sequences belonging to GII.Pe-GII.4 genotype were also identified as Sydney 2012 variant. Genotypes GII.P7-GII.6, GII.P16-GII.3 and GII.Pe-GII.4 (16/20–80%) were considered as having the potential to be norovirus recombinants based on the first screening performed by the Norovirus Typing Tool 2.0.

Those sequences were then submitted to SimPlot and RDP4 programs and recombination events were confirmed with the breakpoints determined (Fig. 2, Table 3). Strains characterized as GII.P7-GII.6 (62.5%) and GII.Pe-GII.4 (25%) had the recombination point upstream to the overlapping region of ORF1/2, whereas the GII.P16-GII.3 (12.5%) genotype had the recombination point in the overlapping region of ORF1/2.

The most frequent recombinant genotype was GII.P7-GII.6. While 10 sequences were characterized as the same RdRp and VP1 genotypes, SimPlot analyses showed different recombination points in relation to S1 (201 bp) when compared to those obtained in the S2 (221 bp). These recombination points correspond to norovirus genome positions 5011 and 5032, using KU870455 as a reference sequence. Further phylogenetic analysis using the complete sequence (RdRp and VP1) of both consensus sequences (S1 and S2), and other GII.P7-GII.6 sequences previously reported, showed two distinct clusters (Fig. 3). Strains in the first cluster, which include the GII.P7-GII.6_2010-consensus (S1), had an average of 97.9% nucleotide identity (SD – 0.9%) and strains in the second cluster, which includes the GII.P7-GII.6_2014–15-consensus (S2), an average of 96.8% (SD – 1.7%) nucleotide identity. In addition, a difference of 7% was observed between the two GII.P7-GII.6 consensus sequences.

Table 2
Genotyping of ORF1/2 by the Norovirus Typing Tool.

Accession number	Collection year/ study period	BLAST score	ORF1 (Pol)	ORF2 (Cap)
MH271645	2010/S1	94.5	GII.P7	GII.6
MH271646	2010/S1	95.1	GII.P7	GII.6
MH271647	2010/S1	94.9	GII.P7	GII.6
MH271648	2010/S1	95.0	GII.P7	GII.6
MH271649	2010/S1	94.9	GII.P7	GII.6
MH271650	2010/S1	94.7	GII.P7	GII.6
MH271651	2010/S1	78.7	GI.P7	GI.7
MH271652	2010/S1	79.4	GI.P7	GI.7
MH271653	2010/S1	78.6	GI.P7	GI.7
MH271654	2014/S2	87.2	GII.P16	GII.3
MH271655	2014/S2	94.9	GII.Pe	GII.4
MH271656	2014/S2	94.9	GII.Pe	GII.4
MH271657	2014/S2	87.7	GII.P16	GII.3
MH271658	2014/S2	92.9	GII.P7	GII.6
MH271659	2014/S2	92.8	GII.P7	GII.6
MH271660	2014/S2	94.9	GII.Pe	GII.4
MH271661	2014/S2	94.9	GII.Pe	GII.4
MH271662	2014/S2	92.8	GII.P7	GII.6
MH271663	2015/S2	85.9	GI.P5	GI.5
MH271664	2015/S2	92.7	GII.P7	GII.6

4. Discussion

In this study three norovirus recombinants were first identified circulating in the Mid-West of Brazil: GII.P7-GII.6 (S1 and S2), GII.Pe-GII.4 (S2) and GII.P16-GII.3 (S2). Those recombinants had been detected worldwide, including in other Brazilian regions (da Silva et al., 2013; Eden et al., 2013; Fumian et al., 2016; Gondim et al., 2018; Huynen et al., 2013; Nahar et al., 2013). All recombinants were characterized as GII norovirus, whereas all samples characterized as GI norovirus had no evidence of recombination, corroborating with other studies showing that less GI norovirus recombinants have been described when compared to GII norovirus (Siqueira et al., 2017; Begall et al., 2018). However, this could be due to the fewer number of GI strains characterized, when compared to GII strains.

The most frequent recombinant genotype found was GII.P7-GII.6, being identified in the two sample collection periods. Detection of GII.6 capsid genotype was expected, since it was first reported in the region by de Oliveira et al. (2014) (S1) as the most predominant genotype and also detected in S2 (Dábilla et al., 2017). On the other hand, GII.P7-GII.6 was first detected in 2004 and since then it has been reported in several countries (e.g. Australia, China, South Africa, Uruguay) circulating between 2004 and 2014 (Arana et al., 2014; Lim et al., 2016; Mans et al., 2014; Yang et al., 2016; Yu et al., 2014). In Brazil, this recombinant has been reported in the North, Northeast and Southern regions, in association with AGE outbreaks or sporadic cases, between 2004 and 2015 (Fumian et al., 2016; Gondim et al., 2018; Hernandez et al., 2016; Reymão et al., 2018).

The GII.Pe-GII.4 recombinant was the second most frequently identified in this study, in samples obtained in 2014, and it was characterized as the Sydney 2012 variant. This variant was first identified in 2012 (Eden et al., 2013), and it predominated worldwide (Mans et al., 2014; Bruggink et al., 2017; Utsumi et al., 2017), in samples from

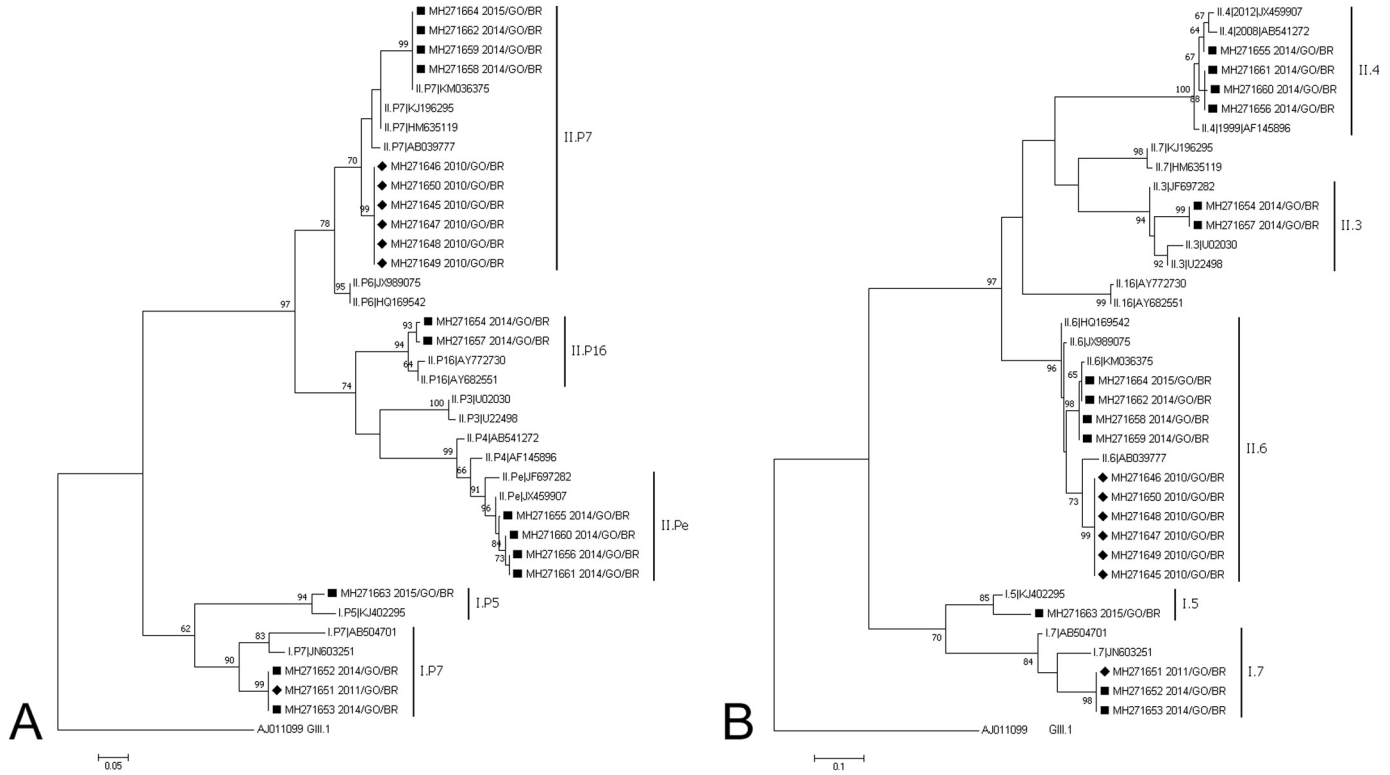

Fig. 1. Phylogenetic analyses of norovirus sequences based on the polymerase (ORF1) and capsid genes (ORF2). (A) Phylogenetic tree of 220 bp within the polymerase region (ORF1) using the Kimura 2-parameter model. (B) Phylogenetic tree of 280 bp within the capsid region (ORF2) using Tamura-Nei model. References strains of norovirus genotypes are named with the genotype (ORF 1 or ORF2)|accession numbers. Samples characterized in this study are marked ◆ (S1) and ■ (S2). Bootstraps values were obtained in 2000 replicates and values higher than 60% are shown near to the nodes.

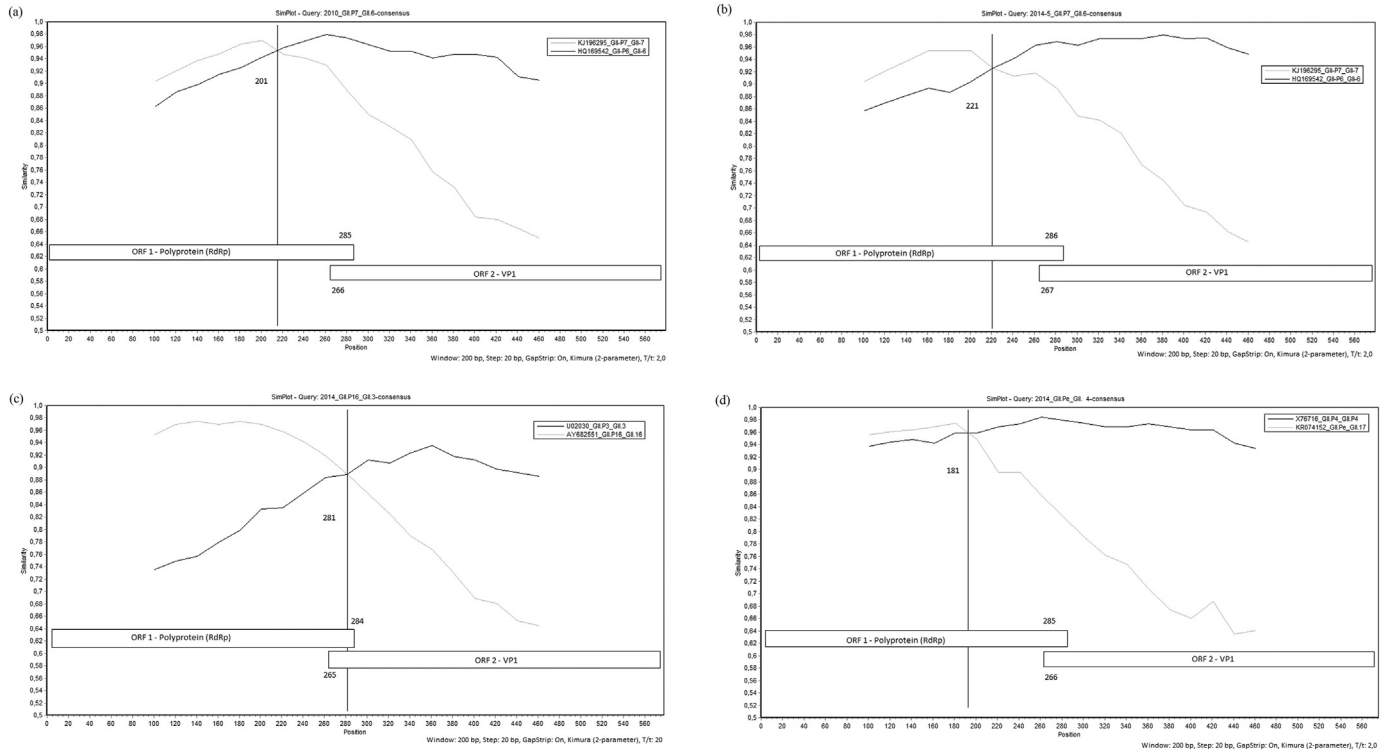

Fig. 2. SimPlot analyses of the norovirus recombinant detected in this study. (a) 2010_GII.P7-GII.6-consensus, (b) 2014–15_GII.P7-GII-consensus, (c) 2014_GII.P16_GII.3-consensus and (d) 2014_GII.Pe_GII.4-consensus. The y-axis gives the percentage of identity with norovirus wild type used in the analysis.

Table 3
Analyses of consensus sequences of each recombinant genotype by RDP4.

RDP4 analyses							
Sequences	Methods						
	RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3Seq
2010_GII.P7-GII.6-consensus	ns	ns	ns	8.97×10^{-05}	2.96×10^{-02}	5.39×10^{-04}	7.44×10^{-03}
2014-5_GII.P7-GII.6-consensus	3.85×10^{-02}	3.03×10^{-03}	ns	1.35×10^{-08}	3.81×10^{-03}	7.28×10^{-04}	4.32×10^{-03}
2014_GII.Pe-GII.4-consensus	ns	3.36×10^{-02}	1.65×10^{-03}	6.31×10^{-05}	4.92×10^{-02}	2.58×10^{-20}	2.28×10^{-02}
2014_GII.P16-GII.3-consensus	2.65×10^{-06}	2.62×10^{-07}	4.33×10^{-08}	1.95×10^{-10}	3.68×10^{-11}	2.61×10^{-14}	2.73×10^{-11}

ns – Non statistical significance.

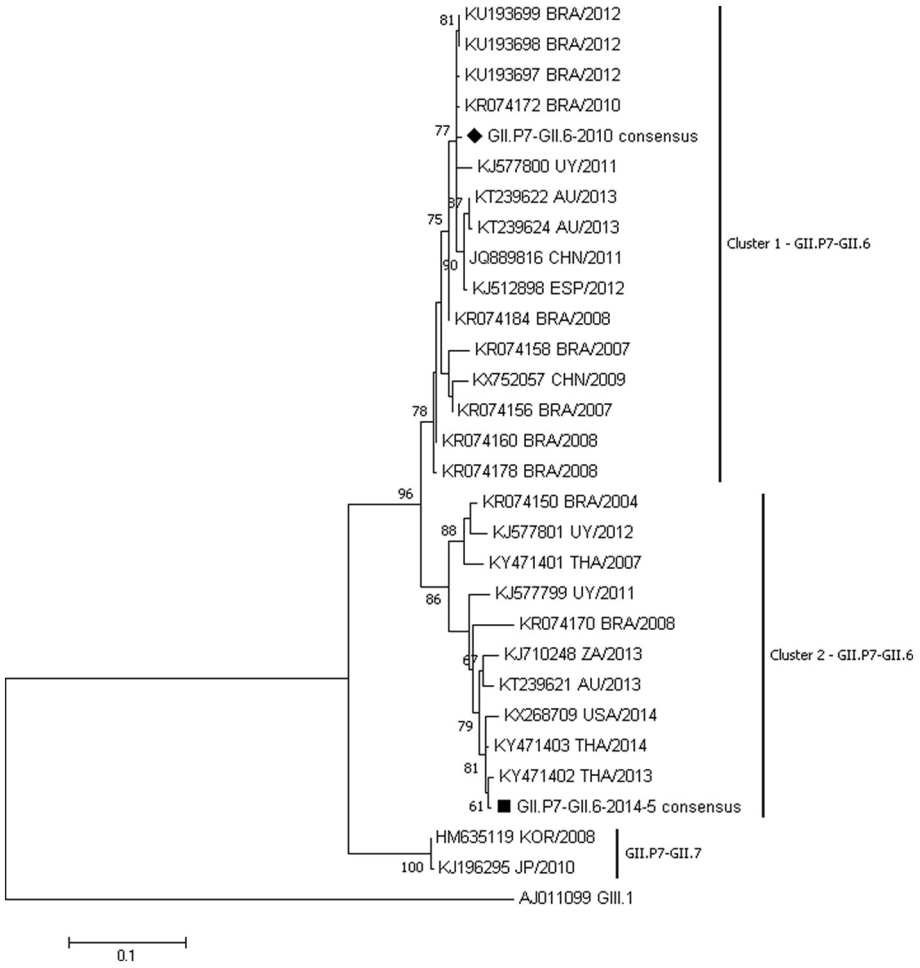

Fig. 3. Phylogenetic analysis of GII.P7-GII.6 norovirus consensus sequences based on the partial region of the polymerase (ORF1) and capsid gene (ORF2) sequences. Phylogenetic tree of 480 bp was constructed using Kimura 2-parameter model. References strains and other recombinants sequences of norovirus are designated by their accession numbers followed by country/year that they were collected. Samples characterized in this study are marked ♦ (S1) and ■ (S2). Bootstraps values were obtained in 2000 replicates and values higher than 60% were considered.

people of all ages causing sporadic cases and outbreaks until 2015, when GII.P16-GII.4 Sydney 2015 variant increased in prevalence (Cannon et al., 2017). In Brazil, sporadic cases were reported, among children, in North and Southeast regions from 2012 to 2015 (Gonçalves Barreira et al., 2017; Gondim et al., 2018).

The third recombinant genotype observed was GII.P16-GII.3, in samples collected in 2014. It was first characterized by Nahar et al. (2013) in Asia, in a sample collected in 2011. However, Fumian et al. (2016) analyzing samples from Southern Brazil, obtained in 2010, also detected this recombinant, showing that it was already circulating in Brazil since at least 2010. It was first detected in the Southern and Northeast regions of Brazil (Fumian et al., 2016; Gondim et al., 2018), in samples obtained in 2010. Curiously, in the present study this recombinant was only detected in samples from S2 collected in 2014. Even though a relatively small number of samples were sequenced, we believe that the data reinforces the importance of continuous genomic norovirus monitoring in all regions of Brazil since the country has large territorial dimensions, and the genotypes circulating in one region may not be the same as those that circulate in other regions at the same period of time. The recombination point analysis by the SimPlot and RDP4 showed that GII·P16-GII.3 presented the recombination at the hotspot region, in the overlapping of ORF1/2 (Bull et al., 2007). However, GII.P7-GII.6 and GII.Pe-GII.4 had atypical recombination points, located upstream the overlap of ORF1/2 region. Atypical

recombination points had already been identified in GII.4 variants and in GII.P7-GII.6 (Waters et al., 2007; Fumian et al., 2016; Siqueira et al., 2016; Cai et al., 2017).

Although, the recombinants sequences presenting atypical breakpoints appear to be homologous to wild type strains, and there was no evidence of deletion or duplication in these sequences, a larger fragment of the genome should be analyzed in order to confirm the statement that homologous non-replicative recombination events have occurred (Begall et al., 2018; Galli and Bukh, 2014). Besides understanding the recombination model that gave rise to those atypical points of recombination, evaluating how much deeper the gene is affected is also important. Because the point of recombination occurs at the 3′ end of the region that translates to viral polymerase, this may impact the activity of the enzyme during the replication process or even the enzyme binding with the template strand, since in that final region is the region encoding the RNA binding site (Deval et al., 2017).

Interestingly, two distinct recombination points were identified in the sequences of GII.P7-GII.6 recombinants, considering the two periods of sample collection (S1 and S2), and phylogenetic analysis revealed two distinct clusters of the same recombinant. GII.P7-GII.6 strains with distinct recombination breakpoints have also been described by Fumian et al. (2016) and by Cai et al. (2017) in samples collected in Southern Brazil and in China, respectively. This finding may suggest the circulation of two lineages of the same recombinant genotype overtime. Furthermore, nucleotide identity analysis of the two consensus sequences generated showed 7% of divergence between them. The circulation of different lineages of the same recombinant genotype reinforces the great norovirus variability.

5. Conclusions

This study provides information on the circulation of recombinant norovirus genotypes in the pediatric population in Mid-West Brazil considering two sample collection periods (2009–2011 and 2014–2015). Characterization of such strains contributes to a better understanding of the molecular epidemiology of norovirus. The identification of atypical recombination points may contribute to help characterize the mechanisms of norovirus recombination. It also raises questions about the functionality of the affected gene, considering viral fitness and higher potential for dissemination. Furthermore, the circulation of different lineages of the same recombinant genotype may demonstrate an ever higher norovirus genetic variability than previously expected, which is extremely relevant in a pre-vaccine scenario.

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