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BRIEF REPORT

High genetic diversity of noroviruses in children from a community-based study in Rio de Janeiro, Brazil, 2014-2018

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Abstract

We report on the occurrence and diversity of noroviruses in children (younger than 5 years old of age) from a low-income urban area in Rio de Janeiro, Brazil. Sixty-one stool specimens collected from children between 1 and 4 years old with acute diarrhoeic episodes (ADE) and non-ADE were investigated. RT-qPCR and sequencing of PCR products after conventional RT-PCR analysis were performed. Noroviruses were detected in 29 (47.5%) samples: 21 (46.7%) from cases with ADE and 8 (50%) from non-ADE cases. Molecular characterization showed 10 different genotypes circulating in this community between November 2014 and April 2018.

Acute gastroenteritis (AGE) is the second leading cause of morbidity and mortality in children under 5 years old worldwide [1]. Rotavirus A (RVA) and norovirus are the most important viral pathogens in AGE [2]. After the introduction of RVA vaccines, noroviruses replaced RVA as the main cause of AGE affecting children [3], and GII.4 has been the predominant genotype for over 2 decades [4–6]. However, uncommon genotypes such as GII.17 and GII.2 have recently emerged and caused outbreaks in many countries worldwide [6, 7]. Currently, there are several norovirus vaccine candidates. It is important to understand the burden of norovirus-associated AGE as well as the genetic diversity of noroviruses prior to the introduction of an effective vaccine [8, 9]. In this study, we investigated the occurrence and genetic diversity of norovirus in stool samples from children with acute diarrhoeic episodes (ADE) and non-ADE from a low-income urban area, Manguinhos community, which is part of the metropolitan region of Rio de Janeiro, Brazil.

Stool specimens were obtained from children aged 1-4 years with diarrhoea (≥ 3 liquid or semi-liquid evacuations in a 24-h period) or who were asymptomatic (with no episodes of diarrhoea for at least 1 week before collection date) and were undergoing routine pediatric examinations at the Germano Sinval Faria Health Center (GSFHC), National School of Public Health, Oswaldo Cruz Foundation, between November 2014 and April 2018. Forty-nine children were enrolled in this study, and 61 stool samples were obtained. Ten children returned to the health unit during the study period, and a stool sample was collected each time. Thus, 2 or 3 stool samples were obtained from some of these children regardless of whether they had ADE. Samples were collected from children whose parents had formally agreed to take part in the research study, and none of the children

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had blood relatives (siblings). A new norovirus episode was defined when it occurred more than 2 weeks after the previous episode. Viral RNA was extracted from stool suspensions (10% w/v) using an automatic RNA extraction procedure according to the manufacturer's instructions (QIAcube® Automated System and QIAamp® Viral RNA Mini kit; QIAGEN, CA, USA). Norovirus screening was performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using primers, probes and conditions described previously by Kageyama et al. [10].

For norovirus genotyping, RT-PCR was performed using the primers Mon431/G2SKR (GII) and Mon432/G1SKR (GI) to generate 570-base-pair (bp) and 579-bp fragments, respectively [11]. The resulting amplicons were purified using Wizard® SV Gel and a PCR Clean-Up System kit (Promega, Madison, USA) following the manufacturer's instructions, and they were analysed by Sanger sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3500 Genetic Analyser® (Applied Biosystems, Foster City, CA, USA). Consensus sequences were obtained using the BioEdit 7.2.1 Sequence Alignment Editor [12]. Genotypes were assigned using the Norovirus Automated Genotyping Tool (https://www.rivm.nl/mpf/typingtool/norovirus/) [13], and nucleotide similarity was assessed using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained in the current study were deposited in the GenBank database under the accession numbers MH393565-MH393566, MH393568-MH393571, MH393574-MH393580, and MH393582-MH393588. Phylogenetic analysis were performed using the maximum-likelihood method, with the K2+G+I model applied for analysis of portions of the regions encoding the polymerase (RdRp/region B) and major capsid protein (VP1/region C). Phylogenetic trees were constructed with 2,000 bootstrap replicates in MEGA v.7.0.26 [14].

Twenty-nine (47.5%) out of the 61 stool specimens collected were positive for norovirus, representing 53.1% (26/49) of the children who were examined at the GSFHC (19 out of 35 with ADE, 6 out of 9 without ADE and 1 out of 5 who provided samples during both ADE and non-ADE). Norovirus was detected in 46.7% (21/45) and 50% (8/16) of ADE and non-ADE cases, respectively. The B-C typing region of 21 (72.4%) out of 29 norovirus-positive samples was sequenced in order to determine the norovirus genotype (Fig. 1). Ten different genotypes were detected, and the most frequent were GII.P4-GII.4 (4/21), GII.P7-GII.6 (4/21), and GI.7-GI.7 (4/21), followed by GII.P16-GII.2 (2/21), GII.P17-GII.17 (2/21), GII.P16-GII.4 Sydney (1/21), GII.Pe-GII.4 Sydney (1/21), GII.P7-GII.7 (1/20), GI.Pd-GI.3 (1/21), and GI.P1-GI.1 (1/21) (Table 1). Of the positive samples, 21 (72.4%) were identified as GII, seven (24.1%) as GI, and one (3.5%) as a GI/GII mixed infection (GI.P7-GI.7 and GII.P17-GII.17). Norovirus GII was observed in 14 ADE and seven non-ADE cases, while GI was present in seven ADE cases. Twenty-four (92.3%) out of 26 positive children had one norovirus episode, and 7.7% (2/26) had two. Two children, aged 15 and 20 months (both non-ADE), were positive for norovirus GII in the first episode (both untypable), and in the second episode, one 19-month-old child with ADE shed norovirus GII.P4-GII.4 and the other, a 27-month-old child (non-ADE), shed norovirus GII.P7-GII.6. One 12-month-old child who presented with ADE was observed to shed norovirus GI.P7-GI.7 genotype after an interval of 7 days. Analysis according to age group showed that the highest norovirus detection rate was observed in children aged 12-23 months (48.7%, 19/39).

The frequent detection and genetic diversity of noroviruses in non-ADE children observed in this community may be a consequence of frequent exposure to these viruses. This can result in asymptomatic episodes due to some degree of acquired mucosal immunity by children who are constantly challenged by noroviruses [3, 15]. Individuals living in this community have limited access to public services, especially sanitation; as such, it is considered a precarious area of the city in which to live. Presumably, such an environment, together with the environmental stability of noroviruses and the low infective dose, would drive viral transmission, causing norovirus-associated ADE in susceptible children [16, 17]. Post-symptomatic norovirus shedding can be detected after resolution of symptoms for several weeks or months [18, 19], and several studies focusing on non-ADE children have demonstrated that asymptomatic excretion of norovirus in stool samples is common, particularly in low income/hygiene settings and does not necessarily reflect a pre- or post-symptomatic event [16, 18, 20]. Norovirus detection rates ranging between 0% and 49% have been reported worldwide [19, 21, 22]. In developing countries, studies have addressed the prevalence of noroviruses in hospitalized children, an approach that causes the prevalence of viral infection to be underestimated, because it does not include genotypes circulating in asymptomatic individuals, particularly those living in communities [3, 23, 24].

The highest norovirus rate detected in children aged 12-23 months (48.7%, 19/39) was consistent with other studies of outpatient children from developing countries [25, 26]. Here, noroviruses were detected during all seasons, similar to observations in Cochabamba, Bolivia [24], and in rural communities in the Vhembe district of South Africa [26]. Xavier et al. [27] studied ADE in a community of the city of Salvador in northeastern Brazil, in the pre-RVA vaccination era and found 9.0% human caliciviruses in children up to 3

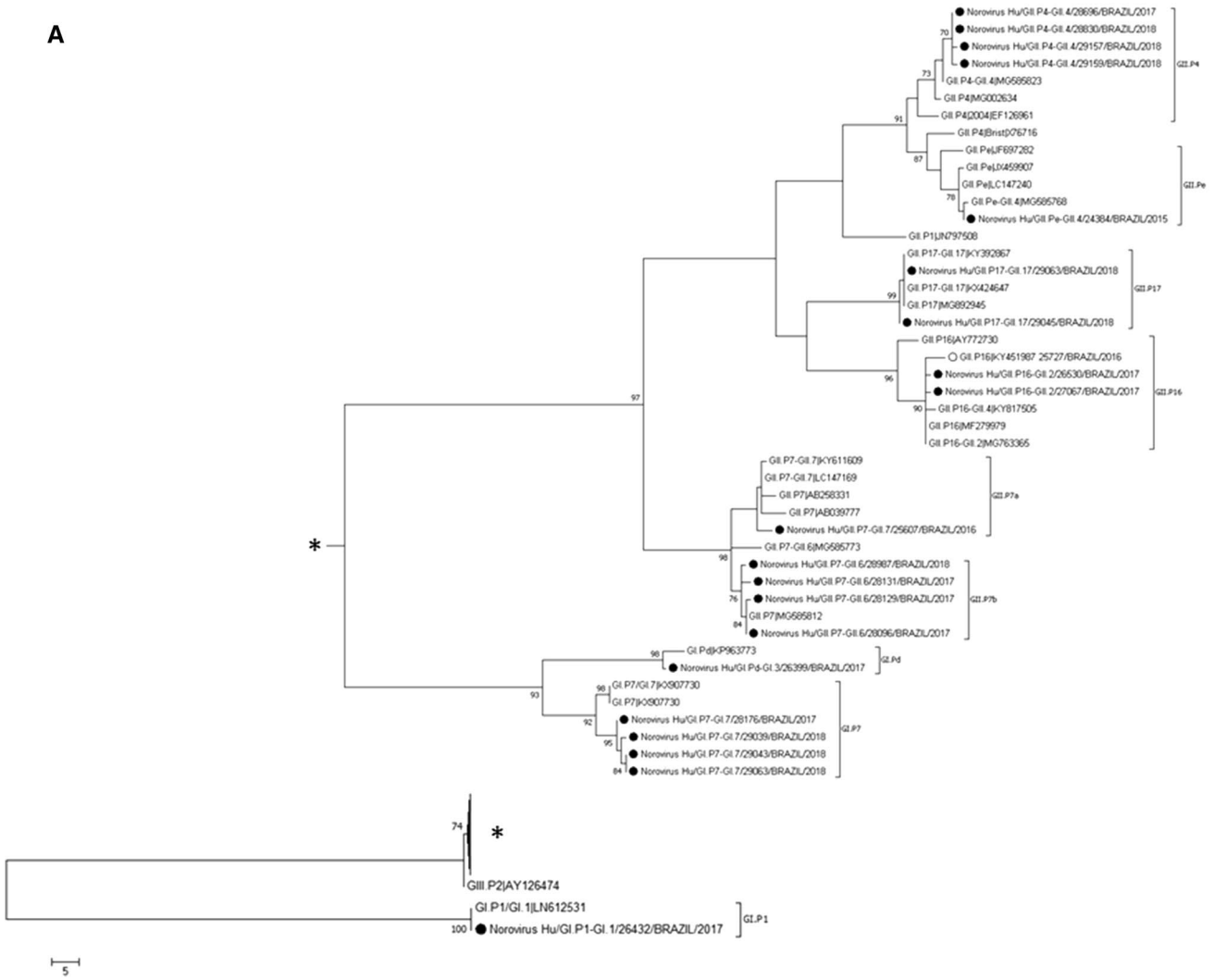

Fig. 1 Phylogenetic analysis of GI/GII noroviruses based on partial nucleotide sequences of the polymerase and capsid regions from children exhibiting acute diarrhoeic episodes (ADE) or those without ADE in the Manguinhos community, Rio de Janeiro, Brazil, from 2015 to 2018. (A) Phylogenetic tree of a 179-bp portion of the polymerase gene (RdRp/region B). (B) Phylogenetic tree of a 215-bp portion of the gene encoding the major capsid protein (VP1/region C). The names of the reference strains of norovirus genotypes are shown with their respective GenBank accession numbers. Nucleotide sequences were analysed using the maximum-likelihood method with the K2+G+I nucleotide substitution model, and bootstrap values > 70% are shown at the nodes of the tree as percentages based on 2,000 replicates. The norovirus strains reported in this study are indicated by filled black circles The strain GII.P16-GII.4 reported by Barreira et al. [34] (KY451987) is indicated by an empty circle

years old. The MAL-ED group detected norovirus in 23.5% of ADE (range, 7.1-32.8%) and 19.0% of non-ADE (range, 2.2-30.4%) in children up to 2 years old between November 2009 and February 2012 [28].

Norovirus GII was the most prevalent genogroup (73.3%), with circulation of 7 genotypes, which was consistent with studies in Brazil [23] and worldwide [29], showing the co-circulation of different GII genotypes in Manguinhos between 2014 and 2018. The predominant genotype in non-ADE children was GII.P7-GII.6 (3 cases), while GII. P4-GII.4 was predominant in those with ADE (2 cases). According to partial RdRp analysis (Fig. 1A), GII.P7 formed two different clusters (GII.P7a and GII.P7b), suggesting some degree of variability in this genotype. The GII. P7-GII.7 strain was detected in a child with ADE in May 2016, while GII.P7-GII.6 viruses were detected in September 2017 in three non-ADE children and one ADE child in February 2018 at different sites in Manguinhos.

Norovirus GII.4 was detected in association with multiple polymerase genotypes, including GII.P4, GII.Pe and

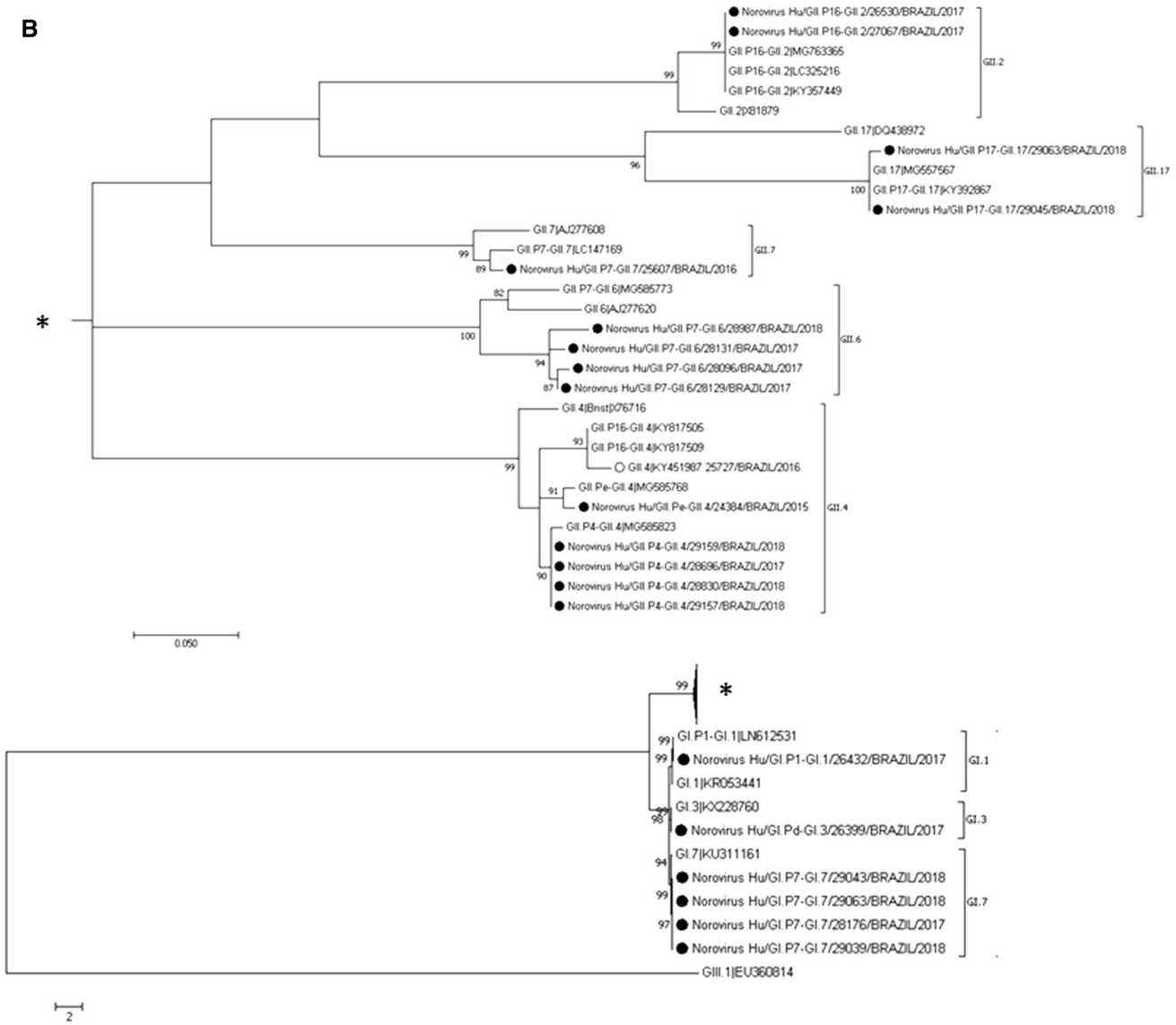

Fig. 1 (continued)

GII.P16. Furthermore, the GII.P16 genotype was detected in association with two GII variants, GII.4 and GII.2, both of which emerged as a major cause of ADE outbreaks in different countries during 2016-2017 [30–34]. The GII.P16-GII.2 viruses exhibited significant similarity (99% identical nucleotides) to strains described in 2016 in Germany [30] and subsequently detected in Italy, China and Japan [31–33]. This study shows original data on detection of recombinant GII.P16-GII.2 in Brazil. The finding of the GII.P17-GII.17 genotype in both ADE and non-ADE cases suggests that this genotype had continued to circulate in the Brazilian population since 2015, when it was first described [35].

In conclusion, this study highlights the diversity of noroviruses co-circulating in the low-income community of Manguinhos, Rio de Janeiro, Brazil. These results contribute to our understanding of norovirus strain diversity and emphasize the importance of surveillance studies in communities both in the norovirus pre-vaccination period and as a follow-up in the post-RVA vaccination era.

Table 1 Norovirus genotypes of 21 strains detected in children with acute diarrhoeic episodes (ADE) or non-ADE in the Manguinhos community, Rio de Janeiro, Brazil, from 2015 to 2018

Year	Norovirus genotype	Number of cases (%)	ADE	Age (months)	Non-ADE	Age (months)
2015	GII.Pe-GII.4	1 (4.8)	1	13	-	-
2016	GII.P16-GII.4	1 (4.8)	-	-	1	17
	GII.P7-GII.7	1 (4.8)	1	16	-	-
2017	GII.P16-GII.2	2 (9.5)	2	22, 48	-	-
	GI.Pd-GI.3	1 (4.8)	1	19	-	-
	GII.P7-GII.6	3 (14.2)	-	-	3	23, 27, 29
	GI.P7-GI.7	1 (4.8)	1	23	-	-
	GII.P4-GII.4	1 (4.8)	1	29	-	-
	GI.P1-GI.1	1 (4.8)	1	21	-	-
2018	GII.P7-GII.6	1 (4.8)	1	12	-	-
	GII.P4-GII.4	3 (14.2)	2	19, 30	1	26
	GI.P7-GI.7	3 (14.2)	2	12, 13	1	30
	GII.P17-GII.17	2 (9.5)	1	14	1	30

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of Fiocruz (CEP 311/06; CEP 688.566/14).

Informed consent Informed consent was obtained from the parent or guardian of each child included in this study.

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