# Genetic diversity of noroviruses in Brazil

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*Norovirus (NoV) infections are a major cause of acute gastroenteritis outbreaks around the world. In Brazil, the surveillance system for acute diarrhoea does not include the diagnosis of NoV, precluding the ability to assess its impact on public health. The present study assessed the circulation of NoV genotypes in different Brazilian states by partial nucleotide sequencing analysis of the genomic region coding for the major capsid viral protein. NoV genogroup II genotype 4 (GII.4) was the prevalent (78%) followed by GII.6, GII.7, GII.12, GII.16 and GII.17, demonstrating the great diversity of NoV genotypes circulating in Brazil. Thus, this paper highlights the importance of a virological surveillance system to detect and characterize emerging strains of NoV and their spreading potential.*

Key words: norovirus - genotypes - gastroenteritis - Brazil

Noroviruses (NoVs) are one of the major causes of acute gastroenteritis worldwide. In developing countries, these viruses are responsible for up to 1.1 million hospitalizations and have an estimated mortality rate of approximately 218,000 deaths per year (Patel et al. 2008). It is transmitted faeco-orally by ingesting contaminated food and water. However, infection can also occur through person-person contact, fomites or by aerosols produced during vomiting (Fankhauser et al. 2002, Koopmans et al. 2002, Lopman et al. 2002, Marks et al. 2003). These infections are often reported in nursing homes, kindergartens, hospitals, schools, cruise ships, restaurants, military installations and resorts (Leuenberger et al. 2007, Podewils et al. 2007, Rizzo et al. 2007, Verhoef et al. 2008).

NoVs are composed of a non-enveloped capsid with a single-stranded, positive-sense 7.7 kb RNA genome that encodes for three open reading frames (ORF). ORF-1 encodes a polyprotein that is cleaved post-translationally into non-structural proteins, including the RNA-dependent RNA polymerase (polymerase). The ORF-2 encodes viral protein (VP)1 that comprises the viral capsid and ORF-3 contains the VP2 gene, believed to be involved in genomic RNA packaging and virion assembly (Hardy 2005).

The *Norovirus* genus belongs to the *Caliciviridae* family and is divided into five genogroups (G); GI, GII and GIV have been shown to infect humans. Additionally, these genogroups can be further differentiated into more than 25 genotypes, based on the similarity between ORF-2 (Zheng et al. 2006).

NoV GII.4 is the most prevalent genotype and has been associated with global epidemics since the mid-1990s, mainly due to the emergence of new variants of GII.4 lineages that occurs in two-four-year intervals (Siebenga et al. 2007, 2009, Zheng et al. 2010).

Since the 1990s, the development of amplification protocols for different genomic regions, denominated regions A and B (ORF-1), C, D and E (ORF-2), has resulted in a NoV diagnostic breakthrough in several countries (Ando et al. 1995, Green et al. 1995, Noel et al. 1997, Fankhauser et al. 2002, Kojima et al. 2002, Vennema et al. 2002, Vinjé et al. 2004). A global electronic network surveillance of NoV, Noronet (noronet.nl), was established following an agreement between three networks involved in molecular surveillance of NoV: the Australian and New Zealand NoV Surveillance Network, the Foodborne Viruses in Europe Network and the CaliciNet in USA. This electronic surveillance system includes molecular and epidemiological data on NoV from different countries, as well as an automatic genotyping tool in which sequences of any region of the NoV genome can be entered and classified using phylogenetic methods. This approach enables the identification of the emergence of new epidemic strains around the world, demonstrating the importance of NoVs as agents of outbreaks and sporadic cases of acute gastroenteritis worldwide (Kroneman et al. 2011). In Brazil, the impact of NoVs is underestimated. Diagnostic and epidemiologic data are obtained mainly from research institutions, which demonstrates the importance of NoVs in communities and hospitalized children (Gabbay et al. 1994, Parks et al. 1999, Gallimore et al. 2004, Borges et al. 2006, Soares et al. 2007, Victoria et al. 2007, Ferreira et al. 2008, 2010, Xavier et al. 2009, Barreira et al. 2010, Luchs et al. 2011, Morillo et al. 2011). The aim of this study is to determine the NoV genotypes detected in different Brazilian states and to assess the genetic diversity of those viruses by raising NoV samples previously characterized. This study also highlights the importance of consolidating detection methods and molecular characterization to contribute to the establishment of a surveillance network that enhances the study of NoVs in Brazil and also worldwide.

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## SUBJECTS, MATERIALS AND METHODS

*Study area* - Brazil is a federation composed of 26 states and the Federal District (DF), Brasília. The states and the DF were grouped into regions that are merely geographical, with no political or administrative divisions, as follows: the North Region [states of Acre (AC), Amazonas, Amapá, Pará, Rondônia, Roraima and Tocantins], the Northeast Region [Alagoas (AL), Bahia (BA), Ceará (CE), Maranhão (MA), Paraíba, Pernambuco, Piauí, Rio Grande do Norte (RN) and Sergipe (SE)], the Central-West Region [Goiás, Mato Grosso, Mato Grosso do Sul (MS) and DF], the Southeast Region [Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP)] and the South Region [Paraná, Rio Grande do Sul (RS) and Santa Catarina].

*Stool samples* - A total of 90 stool samples positively diagnosed for NoV by reverse transcriptase-polymerase chain reaction (RT-PCR) of the B region (Fankhauser et al. 2002) was selected for this study in order to include samples that represent all regions of Brazil. Stool samples were obtained from acute gastroenteritis, sporadic or outbreak cases that occurred in different regions of the country between 2005-2009. Patients ranged in age from two months to 51 years old. All samples were processed at the Laboratory of Comparative and Environmental Virology and this study was approved by the Research Ethical Committee (311/2006) of Oswaldo Cruz Foundation.

*RNA extraction* - Viral RNA was extracted from 400 mL of a 10% (w/v) faecal suspension in Tris-$Ca^{2+}$ buffer, pH 7.2, by the guanidine isothiocyanate/silica method, as described by Boom et al. (1990), with modifications introduced by Cardoso et al. (2002), and/or with a QIAmp® viral RNA Mini kit (QIAGEN®, Valencia, CA, USA) according to the manufacturer's instructions. The synthesis of complementary DNA was carried out using a random primer, pd(N)6 (Amersham Biosciences, UK).

*Molecular characterization* - RT-PCR was performed in all 90 positive samples for NoV using a set of primers that target a partial region (D) of the genome that encodes the main capsid protein, VP1, which is situated in ORF-2 (Vinjé et al. 2004). First, PCR was performed for GII using a primer set including Cap C, Cap D1 and Cap D3. For negative samples, a new PCR for GI using the primer set including Cap A, Cap B1 and Cap B2 was performed. The amplicons obtained were purified with a QIAquick® PCR Purification Kit (QIAGEN®, Valencia, CA, USA) following the manufacturer's recommendations and later quantified on a Nanodrop spectrophotometer (Thermo Scientific®, USA) or by 1% agarose gel electrophoresis using the Low DNA Mass Ladder (Invitrogen®, Carlsbad, CA, USA) as a molecular marker. The set of primers used to obtain the PCR amplicon were employed individually for gene sequencing. DNA sequencing was performed by the dideoxynucleotide chain termination method, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 1, v. 3.1 and the ABI Prism 3730 Genetic Analyzer (both from Applied Biosystems®, Foster City, CA, USA) by the Genomic Platform of DNA sequencing PDTIS/Fiocruz.

*Phylogenetic analysis* - The nucleotide sequence data reported in this study were submitted to GenBank under accession: JN587115-JN587176. Sequence editing and multiple alignments were performed with the BioEdit Sequence Editor 7.0 software (Hall 1999). Phylogenetic trees were structured by the neighbour-joining method based on the Kimura two-parameter model (Kimura 1980) using the MEGA v.4.0 software package (Tamura et al. 2007). Confidence values of the internal nodes were calculated by bootstrap analyses using 1,000 replicates.

## RESULTS

Sixty-four (71%) out of 90 samples studied were positive for GII and 26 were negative for both the GI and GII. Fig. 1 shows the genotyping of NoV strains analysed in this study. Of 64 samples sequenced and genotyped, 29 were included in the phylogenetic tree, representing all identified genotypes according to their origin and year of collection. Samples presenting 100% nucleotide identity were represented by only one sample. The genotypes found were GII.4 (n = 50), GII.6 (n = 5), GII.7 (n = 1), GII.12 (n = 4), GII.16 (n = 1) and GII.17 (n = 2). One strain could not be genotyped.

Table shows the quantity, origin and genotypes of sequenced samples. NoV GII.4 strains were observed in nine out of 13 states investigated. Strains GII.16 and GII.17 were detected to circulate in August and September 2005 in AC (North Region). Another strain found circulating in 2005 was GII.7, from the DF (Central-West Region). GII.12 was detected in 2009 in SE (Northeast Region), MG (Southeast Region) and RS (South Region).

The molecular characterization shows GII.4 strains grouping into three variants: 2004, 2006a and 2006b (Fig. 1). The 2006a and 2006b variants were represented by two large clusters: (i) the first composed with samples from MA, RN, AL, CE, BA, SE (Northeast Region) and MG (Southeast Region) during 2007-2009 (ii) and the second represented the 2006b variant grouped samples from AL, BA (Northeast Region), MS (Central-West Region) and MG (Southeast Region).

To assess the diversity of NoV circulating in the country, a survey of NoV nucleotide sequences available in GenBank was conducted. Fig. 2 illustrates a summary of all NoVs genotyped by region D from Brazil between 1995-1999 and 2004-2009, including results from this study and others previously published.

## DISCUSSION

The characterization of NoVs into genogroups and genotypes was established by sequencing the complete genomic region coding for the VP1 capsid protein. To be classified into a genogroup or a genotype, the lineages must have a 55% and 85% similarity in amino acid sequence of the entire VP1 protein, respectively (Zheng et al. 2006). Since a correlation was established between the complete nucleotide sequence of VP1 and a partial nt sequence (region D) (Vinjé et al. 2004), this region has been used to genotype NoV. The comparison of different strains has been hampered by the use of different approaches for molecular characterization of NoV (Victoria et al. 2007, Ferreira et al. 2008, 2010, Bruggink & Marshall 2009, Nataraju et al. 2010).

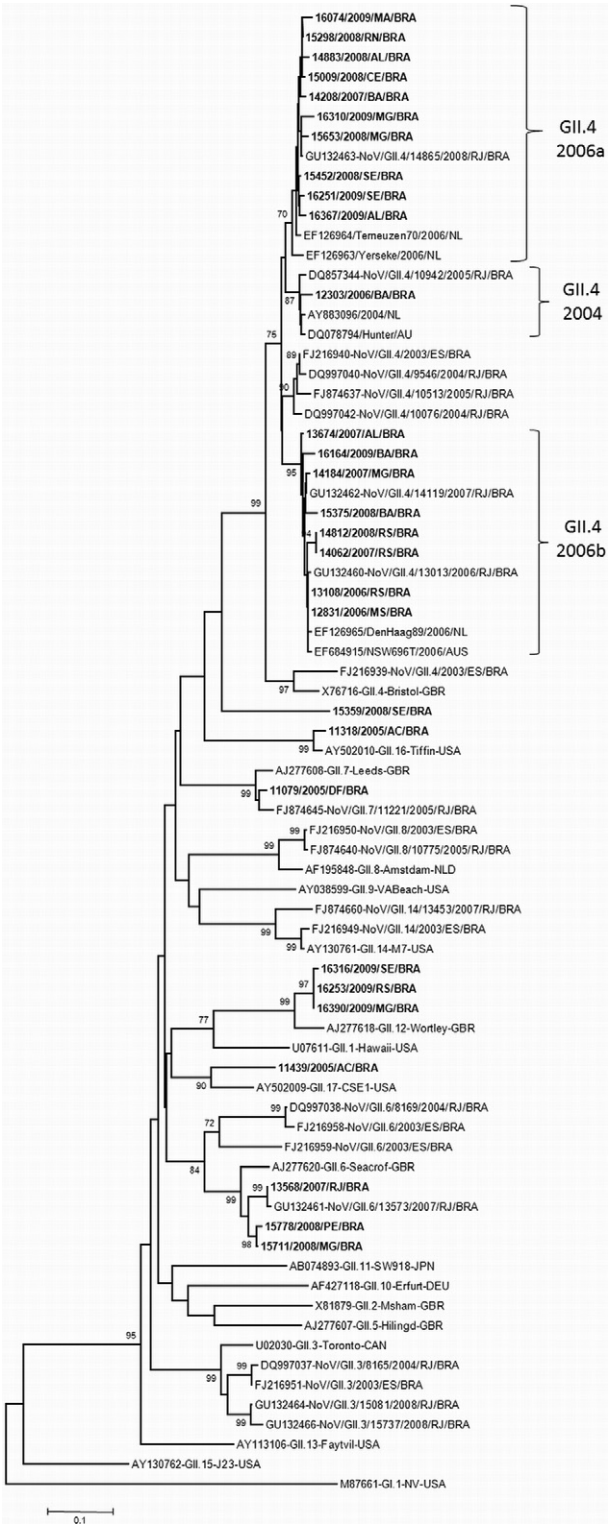


Fig. 1: phylogenetic tree based on a partial fragment (221 nt) of the norovirus (NoV) capsid (region D) from samples obtained in different Brazilian states. Internal node numbers represent the bootstrap value obtained from 1,000 replicates. Genotypes reference strains and Brazilian NoV strains identified by sequencing region D and retrieved from GenBank (ncbi.nlm.nih.gov) are listed with their GenBank accessions. The strains detected in this study are presented in bold.

Global NoV analysis was performed by grouping the nucleotide sequences obtained in this study with all previous NoV VP1 region D sequences from Brazilian strains for which data was available on GenBank (Castilho et al. 2006, Victoria et al. 2007, Ferreira et al. 2008, 2010, Barreira et al. 2010). The data analysis showed a great diversity of NoV strains circulating in the country, including GI.2, GI.3, GI.6, GII.2, GII.3, GII.4, GII.6, GII.7, GII.8, GII.12, GII.14, GII.16 and GII.17.

NoV GII.4 was the most prevalent genotype detected and was identified in 50 out of 64 sequenced strains and in nine out of 13 states evaluated. These results are in agreement with prior studies published in other countries (Iritani et al. 2010, Pang et al. 2010) and Brazil (Barreira et al. 2010, Ferreira et al. 2010). Three GII.4 variants were found circulating in Brazil, showing its breadth of diversity and distribution. The high prevalence of GII.4 worldwide has been explained to be a result of point mutations that originated as new variants and replaced previous variants (Siebenga et al. 2009). This is probably due to the absence of protective immunity in the susceptible community, causing new outbreaks of acute gastroenteritis; this allows the GII.4 genotype to remain a prevalent strain. The GII.4 variant substitution phenomenon was observed in all continents throughout the investigation period (Siebenga et al. 2009, Motomura et al. 2010, Zheng et al. 2010).

This analysis was the first to identify the genotypes GII.12, GII.16 and GII.17 in Brazil. The GII.16 and GII.17 strains were found circulating simultaneously during a Rotavirus-A G9 gastroenteritis outbreak that occurred in 2005 in AC (North Region) (Siqueira et al. 2010, Tort et al. 2010). These genotypes were also found circulating in South Korea, 2007/2008 (GII.16), Argentina, 2005/2006, and Thailand, 2006/2007 (GII.17) (Gomes et al. 2008, Chung et al. 2010, Kittigul et al. 2010). In Brazil, GII.12 was observed circulating in three different regions: Northeast, Southeast and South. However, there are a few reports of NoV GII.12 worldwide, including Japan in 2001/2002 and 2004/2005 (Fukuda et al. 2010). Further, in the United States this genotype was responsible for 16% of all reported NoV outbreaks during the winter of 2009/2010 (Vega & Vinjé 2011) and, in Vietnam, GII.12 was found in hospitalized children at a higher prevalence than GII.4 (Tamura et al. 2010).

Regarding other NoV genotypes, GII.7 was detected in 2005 in the DF and RJ (Ferreira et al. 2010). GII.6 circulated in PE (Northeast Region), ES, MG, RJ and SP (Southeast Region) from 2003-2004 and 2007-2008 (Castilho et al. 2006, Barreira et al. 2010, Ferreira et al. 2010).

GI.2, 3 and 6 and GII.2, 3, 8 and 14 NoV characterized by region D were previously described in other Brazilian studies; however, these genotypes were not found in the present study. GI has been described as a genogroup that circulates to a lesser extent than GII (Castilho et al. 2006, Victoria et al. 2007, Barreira et al. 2010, Ferreira et al. 2010). The evaluation of outbreaks that occurred in Japan during 2002-2006 revealed that only one out of 12 outbreaks was caused by GI (Shinkawa et al. 2008).

In Brazil, other NoV molecular characterization studies have been carried out. However, they could not be included in the phylogenetic analysis because

TABLE

Assessment of distribution of Norovirus strains according to the Brazilian Regions and year

| Regions | States | Samples sequenced (n) | Year/genotypes | | | | |
|---|---|---|---|---|---|---|---|
| | | | 2005 | 2006 | 2007 | 2008 | 2009 |
| Northern | Acre | 3 | GII.16<br>GII.17 | - | - | - | - |
| Northeast | Alagoas | 7 | - | - | GII.4 | GII.4 | GII.4 |
| | Bahia | 18 | - | GII.4 | GII.4 | GII.4 | GII.4 |
| | Ceará | 1 | - | - | - | GII.4 | - |
| | Maranhão | 1 | - | - | - | - | GII.4 |
| | Sergipe | 5 | - | - | - | GII.4<br>GII.[a] | GII.4<br>GII.12 |
| Southeast | Rio Grande do Norte | 3 | - | - | - | GII.4 | - |
| | Pernambuco | 1 | - | - | - | GII.6 | - |
| | Minas Gerais | 6 | - | - | GII.4 | GII.4<br>GII.6 | GII.4<br>GII.12 |
| Southern | Rio de Janeiro | 3 | - | - | - | GII.6 | - |
| | Rio Grande do Sul | 14 | - | GII.4 | GII.4<br>GII.6 | GII.4 | GII.4<br>GII.12 |
| Midwest | Mato Grosso do Sul | 1 | - | GII.4 | - | - | - |
| | Distrito Federal | 1 | GII.7 | - | - | - | - |

*a*: this strains could not be genotyped.

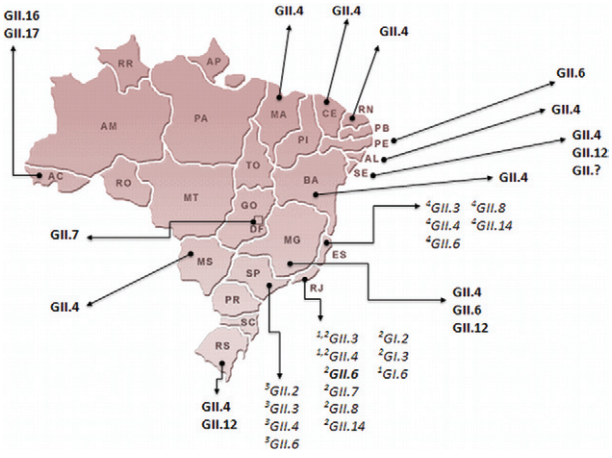


Fig. 2: distribution of genotypes found in the states of Brazil according to region D classification. The genotypes in bold are the ones obtained in this study. Numbers in italic were obtained from Victoria et al. (2007) (1), Ferreira et al. (2010) (2), Castilho et al. (2006) (3) and Barreira et al. (2010) (4), respectively. AC: Acre; AL: Alagoas; AM: Amazonas; AP: Amapá; BA: Bahia; CE: Ceará; DF: Federal District; ES: Espírito Santo; GO: Goiás; MA: Maranhão; MG: Minas Gerais; MS: Mato Grosso do Sul; MT: Mato Grosso; PA: Pará; PB: Paraíba; PE: Pernambuco; PI: Piauí; PR: Paraná; RJ: Rio de Janeiro; RN: Rio Grande do Norte; RO: Rondônia; RR: Roraima; RS: Rio Grande do Sul; SC: Santa Catarina; SE: Sergipe; SP: São Paulo; TO: Tocantins.

nucleotide sequencing and genotyping was performed in regions that encode for RNA dependent-RNA polymerase, known as region A and B of the genome (Parks et al. 1999, Gallimore et al. 2004, Borges et al. 2006, Campos et al. 2008, Luchs et al. 2011, Morillo et al. 2011).

The impact of NoV infections in outbreaks and sporadic cases of acute gastroenteritis has been characterized by the emergence of novel strains, which have been identified using new protocols that allow for the rapid diagnosis and molecular characterization of those viruses. Protocols using primers that amplify regions A and B have been widely used for viral detection because this is a highly conserved region (Ando et al. 1995, Jiang et al. 1999, Fankhauser et al. 2002). Still, regions C and D, which comprise different partial sequences of the gene encoding for VP1 protein, are recommended for the characterization of different genotypes (Mattison et al. 2009). Region D, used in this study, was previously described to efficiently genotype NoV for characterization and has been used as an alternative to full sequencing of VP1 that relies on about 1,600 nucleotides (Vinjé et al. 2004). The high genetic variability of region D may explain why we failed to amplify all previously detected strains by the polymerase conserved region. Recently, Mattison et al. (2009) suggested the use of region C for genotype characterization and region D for characterizing variants of GII.4 samples. Certainly, new protocols for NoV characterization will emerge in order to increase the number of genotyped samples that encompass more variants of NoV. Further studies using different set of primers or complete VP1 sequencing must be performed in order to achieve this goal.

In conclusion, new studies should be conducted to standardize and validate protocols for NoV diagnosis that allow for the establishment of NoV networks, which will demonstrate the true impact of these infections in developing countries, as well as facilitate future molecular epidemiology and viral evolution studies.

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