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BRIEF REPORT

Molecular detection and genetic diversity of norovirus in hospitalized young adults with acute gastroenteritis in Bahia, Brazil

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Abstract The molecular epidemiology of a recent norovirus (NoV) outbreak in Brazil performed by comparative analysis with Genebank NoV sequences showed that the GII.4 strain was responsible for 72.5% of all NoV-positive cases (58/80). Other detected NoV strains included GII.3 (7/80; 8.8%) and GII.9 (8/80; 10%). This is the first outbreak reported in Bahia state, Brazil, during June–July of 2006, where NoV was identified as the principal etiologic agent in hospitalized young adults with acute gastroenteritis symptoms. These findings suggest that GII.4 is a predominant circulating genotype in NoV outbreaks in Brazil.

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Introduction

Norovirus (NoV), a member of the family *Caliciviridae*, is one of the main causes of endemic and epidemic gastrointestinal disease. Acute gastroenteritis affects up to 5 million children and adults worldwide [2, 12, 20]. NoV is commonly associated with large outbreaks in hospitals, cruise ships, schools, universities and restaurants. Transmission occurs by the fecal-oral route, predominantly through ingestion of contaminated water or food, person-to-person contact, airborne transmission, and contact with contaminated surfaces [14, 19]. Illness is characterized by a sudden onset of nausea, vomiting, abdominal pain, diarrhea, and occasionally, low-grade fever.

NoVs are small, icosahedral, single-stranded, positive-sense RNA viruses. The RNA genome encodes three open reading frames (ORFs), including ORF1, coding for the non-structural proteins RNA-dependent RNA polymerase (POL), helicase, and protease. ORF2 encodes a major capsid protein (VP1), and ORF3 encodes a minor capsid protein (VP2) [11]. NoVs are divided in five genogroups (GI to GV), based on sequence homologies in their RNAs. GI and GII are the most commonly described in human infections. GII strains have been the main cause for gastroenteritis outbreaks worldwide, including outbreaks in the United States of America [13], Europe [15, 18], and Brazil [2, 10, 21, 26]. However, Fankhauser et al. [8] reported that the numbers of GI strains detected in outbreaks were increasing from 4% in 1996 and 1997 to 26% between 1997 and 2000. Consistent with this apparent

trend, Chapin et al. [6] reported that a GI strain was found in all positive NoV samples from afflicted US tourists visiting Mexico and Guatemala between 1998 and 2002.

High recombination rates observed in NoVs make molecular characterization of viral genotypes a valuable approach for the surveillance of the variant strains associated with gastroenteritis outbreaks. NoV recombination usually occurs at the ORF1/ORF2 junction of the genome and has also been described within capsid genes [3, 17, 22, 27, 29]. However, it is necessary to study the implications of recombination between human noroviruses for gastroenteritis symptoms and the molecular basis of the selective advantage of some strains [1, 25].

NoV-associated gastroenteritis outbreaks in the Brazilian population have always been reported in children. This report represents the first study demonstrating an association of NoV GII.4 with a gastroenteritis outbreak among young adults in Bahia state, and it is only the second of this nature reported in Brazil in the 2000s. We have used molecular biology tools to characterize the epidemiological profile of this outbreak. Phylogenetic analyses were performed using sequence data obtained from a 298-bp fragment of the RNA polymerase gene (region between positions 4627 and 4925 relative to Norwalk-virus M87661) in NoV-positive samples, and the nucleotide sequences determined in this study were deposited in GenBank under the accession numbers EU494629–EU494708.

One hundred twenty-seven stool samples were collected from patients (children and adults) with symptoms of gastroenteritis. These patients were undergoing treatment at the Aliança Hospital in the city of Salvador, Bahia, Brazil, during a gastroenteritis outbreak from June–July, 2006. The stool samples were stored at −70°C until they were ready for processing (typically within 24 h after collection). Samples were screened for rotavirus (RTV) and adenovirus (AdV) using a commercial ELISA kit (EIARA Bio-Manguinhos, Rio de Janeiro, Brazil) using the procedure provided by the manufacturer. Total RNA was extracted from the stool samples using the QIAmp viral RNA kit (QIAGEN, Germany) and then subjected to a nested multiplex reverse transcription polymerase chain reaction (RT-PCR) assay designed for the detection of NoV. Nested RT-PCR was used for the detection of the NoV RNA polymerase gene. A first-round RT-PCR was performed using outer primers CAL-32 (5-ATGAATATGAATGAGGATGG-3) [24] and MO3-N (5-TCAGATGGGTCTTCATGATTGG-3) [16]. These primers span the nucleotide positions 4490–5127 of the reference sequence (M87661: Hu/NoV/GI.1/Norwalk virus/1968/US) and amplify a 638-bp outer product. The second round of PCR was done using inner primers JV-12 (5-ATACCACTATGATGCAGATTA-3) [29] and ACAL-36 (5-GACAAAACAGAAGGACCAAT-3) [24]. These inner primers generate a 428-bp final nested fragment. All primers were synthesized by Invitrogen Life Technologies. Amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer). The first-round RT-PCR cycling conditions were as follows: 48°C/45 min (RT-step), 94°C/2 min, followed by 35 cycles of 94°C/30 s, 37°C/30 s and 68°C/50 s and one extension step of 68°C/7 min. For the second-round PCR cycling, conditions were: 94°C/3 min followed by 30 cycles of 93°C/30 s, 37°C/15 s, 72°C/30 s, plus a final extension step of 72°C/5 min [16]. The inner PCR products were analyzed by electrophoresis in a 2% agarose gel and ethidium bromide staining. Sequencing was performed on the second-round PCR products using the inner primers and Big Dye Terminator chemistry V3.1. Multiple alignments of NoV RNA polymerase gene sequences were performed with Clustal W [7] using 10 and 0.1 for gap opening and gap extension penalties, respectively. Several RNA polymerase gene sequences from NoV genogroups I and II, collected in GeneBank (http://ncbi.nlm.nih.gov), were aligned with sequences from the samples we collected. The GeneBank sequences included the following strains: DSV-USA93, gi|454752; Hesse-DEU98, gi|3769664; NV-USA93, gi|106043086; SOV-GBR93, gi|1236787; Toronto-CAN93, gi|424053; Kobe-JAP06, gi/126471123 Melksham-GBR95, gi|976077; Hawaii-USA94, gi|9886086; and ARG320-USA, gi|6478609. The phylogenetic tree was calculated using the Kimura 2-parameter substitution model and 1,000 bootstrap replicates with the PHYLIP software [9]. For distance matrix calculations, we used *dnadist* and a neighbor-joining tree was built with *neighbor* using PHYLIP software.

The outbreak of acute gastroenteritis in June of 2006 resulted in a large number of patients being admitted for treatment at the Aliança Hospital. Approximately 42% of the patients were young adults with clinical symptoms of strong abdominal pain, vomiting, diarrhea and, occasionally, fever. These symptoms displayed a different pattern when compared with other outbreaks in previous years, in which RTV and AdV had been found to be the main etiologic agents [4, 23]. Out of the total of 127 samples analyzed in this study, 80 (63%) were positive for NoV, 32 (25.1%) were positive for RTV, 7 (5.1%) were positive for AdV, and the remaining eight samples (6.3 %) were negative. The patients who were positive for NoV were mainly in the age group ranging from 17 to 25 years old (66%), while 65.6% (21/32) of patients positive for RTV were children up to 3 years old. It is worth noting that coinfections with NoV/RTV and NoV/AdV were present in nine and three samples, respectively. Phylogenetic analysis of sequence data (Fig. 1) showed that all NoV infections were the GII.4 strain, which was responsible for 72.5% of all NoV-positive cases (58/80). GII.4 NoV strain sequences in this work had predominantly the AATTTG motif (54/58, 93.1% of total confirmed GII.4). We also detected three

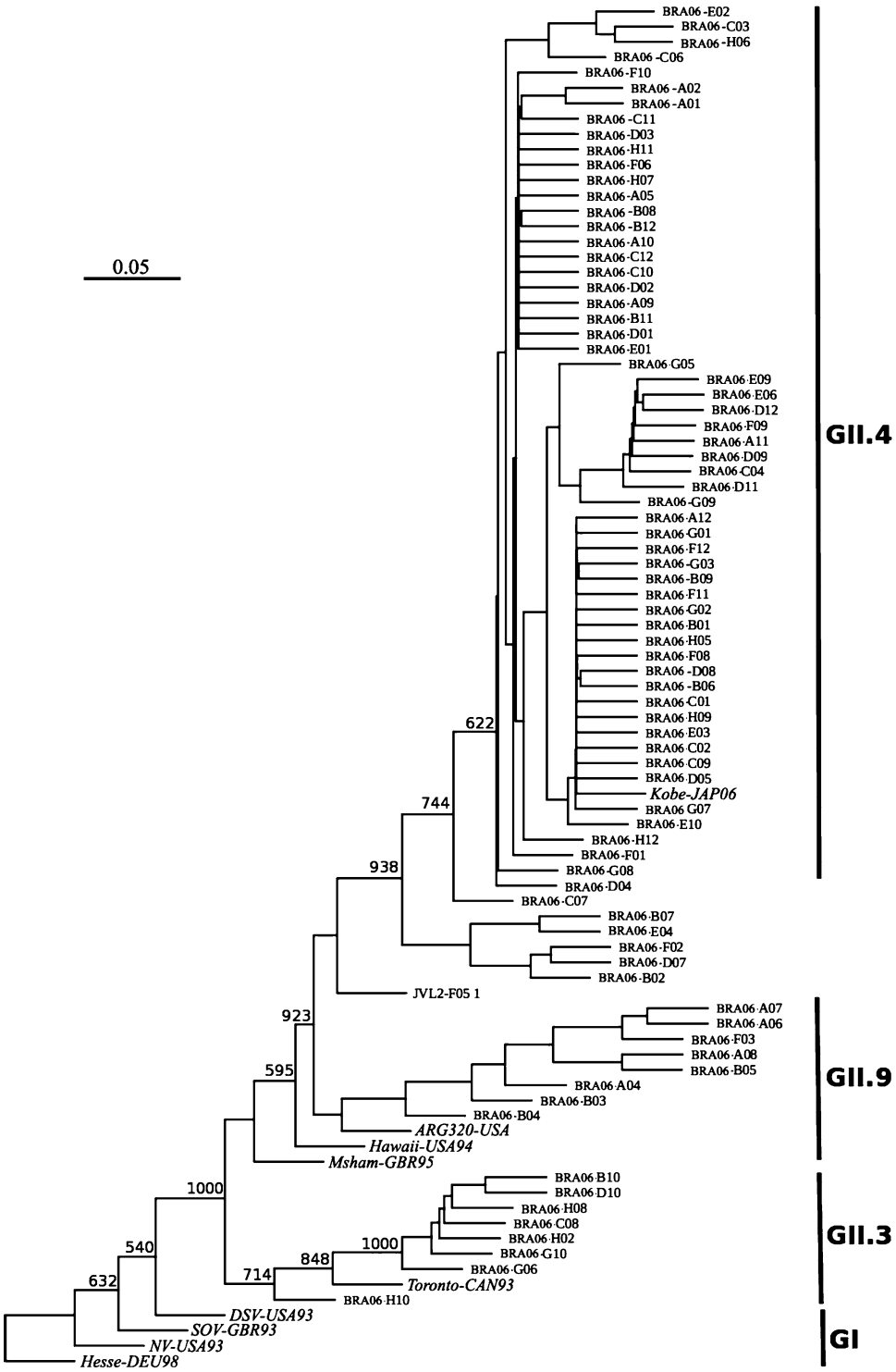

Fig. 1 Phylogenetic analysis of the RNA polymerase gene (ORF 1) of NoV strains isolated in Bahia, Brazil. The sequences of the tree were calculated using Kimura 2-parameter substitution model using PHYLIP software [9] and 1000 bootstrap replicates. *Dnadist* was used to calculated distance matrix and the neighbor-joining tree was built with *neighbor* (9). Numbers at the branches are percentages obtained after 1000 replications of bootstrap sampling. Only bootstrap values higher than 500 are shown. The *bar* denotes phylogenetic distances between strains. The sequences in the tree show, successively: JVL-2 (code) and patient involved. Reference strains derived from Genbank (http://www.ncbi.nlm.nih.gov) used in this study were: DSV-USA93, gil454752; Hesse-DEU98, gil3769664; NV-USA93, gil106043086; SOV-GBR93, gil1236787; Toronto-CAN93, gil424053; Kobe-JAP06, gi:126471123 Melksham-GBR95, gil976077; Hawaii-USA94, gil9886086 and ARG320-USA, gil6478609

samples with the AGTTTG motif, and one with the AACTTG motif (Table 1). Other detected NoV strains included GII.3 (7/80; 8,8%) and GII.9 (8/80; 10%). The sequences from seven samples could not be clearly correlated to any of the reference strains used in the analysis. GII.7 was excluded from the list of reference strains since it did not align well with the sample sequences (data not shown). Interestingly, the GI strain was not detected in any of the stool samples (Fig. 1).

Here, we have described a gastroenteritis outbreak in Bahia, Brazil (June–July of 2006), that affected mainly young adults. This study represents a molecular

Table 1 Detection of norovirus in stool samples of patients with acute gastroenteritis

Genogroup/Genotype[a]	NoV positive samples[b] (n = 80)	Motif[c] (n)
GII.4	58	AATTTG (54)
		AGTTTG (3)[d]
		AACTTG (1)
GII.3	7	
GII.9	8	
N.d.[e]	7	

[a,c] Phylogenetic analyses were performed using sequence data obtained from a 298-bp fragment of the RNA polymerase gene (region between positions 4627 and 4925 relative to Norwalk-virus M87661) in NoV-positive samples

[b] All of these samples were positive in nested multiplex RT-PCR

[d] Motif detected for the first time in GII.4 strains

[e] Not determined. It could not be correlated with any reference strain

characterization of the second NoV outbreak reported in Brazil between 2002 and 2006. Phylogenetic analysis identified GII.4 as the predominant strain in fecal samples from infected individuals. While earlier outbreaks caused by noroviruses in Brazil were mostly in children [5, 10, 21, 28] this one largely affected young adults. We have no data that would indicate that this difference in the affected population group is due to particular characteristics associated with the viral strain. GII.4 is one of the most common strains among NoV infections, as indicated by reports from many countries [13, 24]. Lopman et al. [18] described the emergence of a new NoV GII.4 variant in Europe, which had a consistent mutation in the polymerase gene (AACTTG to AATCTG starting at position 4820 relative to Norwalk-virus M87661) as compared with the previously identified GII.4. It was suggested that such a variant might be more virulent or more environmentally stable than the earlier GII.4 strain. The NoV GII.4 AATCTG variant was not detected in any of the samples in this study, nor has it been detected anywhere in Brazil. Indeed, we confirmed that NoV GII.4 with the AATTTG motif was most frequent (54/58, 93.1%). Lopman and coworkers [18] also reported the presence of this NoV GII.4 strain variant in European surveys carried out in outbreaks before or during 2002. Although these authors did not seem to ascribe any particular importance to the appearance of this strain, it is worth noting that the incidence of this variant has increased in Europe since 2002. The presence of the AATTTG NoV GII.4 variant has been commonly observed in infections contracted by travelers to developing countries [6].

In contrast to report from surveillance work in Europe [18] in which the AACTTG motif was found to be the most common variant sequence observed, we found only one sample in our study that displayed this motif. Unexpectedly, we also detected three sequences with the AGTTTG motif in the samples. To our knowledge, this is the first time the AGTTTG motif has been reported in GII.4 NoV strains. Future studies should be done taking into consideration these results in order to evaluate the epidemiological impact of the appearance of this new variant motif.

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