

ORIGINAL ARTICLE

SAPOVIRUSES IN CHILDREN WITH ACUTE GASTROENTERITIS FROM MANAUS, AMAZON REGION, BRAZIL, 2010-2011

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SUMMARY

Sapoviruses (SaVs) are responsible for acute gastroenteritis in humans, especially children and the elderly. In Brazil, data on SaVs infections are very limited, especially in Northern Brazil. Here, we investigated the occurrence of SaVs in samples from hospitalized children under ten years old that presented acute gastroenteritis. Positive samples were genotyped and phylogenetic analysis was performed using prototype strains sequences obtained from GenBank database. In total, 156 fecal samples were screened by RT-PCR for SaVs. A positivity rate of 3.8% (6/156) was found in children under three years of age. Four genotypes were detected: GI.1, GI.2 and GII.2?-GII.4?/GII.4, suggesting a possible inter-genotypes recombination. Most infections (83.3%) occurred between August and September. The positivity was similar to that found in other countries and genotyping demonstrated the presence of distinct genotypes. To our knowledge, this is the first study reporting the circulation of SaVs in *Manaus*, state of *Amazonas*, Amazon region, Brazil.

KEYWORDS: Sapovirus; Gastroenteritis; Amazon.

INTRODUCTION

Acute gastroenteritis (AGE) is a common disease worldwide, being a significant cause of morbidity and mortality. AGE manifested by vomiting and diarrhea is the second major cause of deaths among all infectious diseases in children under five years old, being responsible for 15% of the cases¹. Although it is a preventable disease, it is estimated that nearly 1.7 billion cases occur annually. The number of annual deaths is around 760,000 so that AGE is the second leading cause of deaths among children under five years of age². Several pathogens may lead to AGE, however, viruses currently account for about 70% of these cases³. Rotavirus (RV) and norovirus (NoVs) are considered the most frequent cause of acute childhood diarrhea, but the human astrovirus (HAstVs), sapovirus (SaVs) and enteric adenovirus (AdVs) are also important etiologic agents⁴.

Sapovirus belong to the genus *Sapovirus*, *Caliciviridae* family, which also includes the *Norovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus*, with the last three genera having exclusively veterinary importance⁵. SaVs were first described in Japan, after an outbreak in an orphanage in the city of Sapporo, being initially denominated Sapporo Virus⁶.

These viruses have a linear genome with 7.5 kilobases (Kb), single-stranded RNA and positive-sense, presenting a characteristic aspect of

“Star of David”, when observed in the electron microscope⁷. They are non-enveloped viruses and present two open reading frames (ORF's). The ORF 1 encodes non-structural proteins and the major structural protein (VP1); ORF 2 encodes the minor structural protein (VP2)^{8,9}. Some authors also describe the ORF 3, but with unknown function. SaVs have five officially described genogroups (GI-GV). Genogroups GI, GII, GIV and GV infect humans, whereas GIII infect only pigs⁵. Based on the classification system proposed by Oka *et al.*⁹, there are 20 genotypes within these five genogroups. Recently, a new genotype (GV.2) was described during a suspected foodborne gastroenteritis outbreak in Japan¹⁰.

SaVs are responsible for both sporadic cases and outbreaks of AGE, affecting mostly children and elderly people. Symptoms commonly observed in SaVs-infections are diarrhea, vomiting and abdominal pain, but most of the time these symptoms are less severe than the ones related to group A Rotavirus and NoVs¹¹. Transmission occurs mainly by the fecal-oral route, person-to-person contact and ingestion of contaminated food and water¹².

Studies involving the circulation of this virus have been conducted in several countries involving hospitalized patients with positivity of tests ranging from 1.2% to 15%^{13,14}, as well as sporadic cases in the community,

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with prevalence varying from 3.73% to 19%^{15,14}. Nevertheless, in Brazil, there are limited data, considering the small number of studies available^{16,17,18,19}. Thus, the present study aimed to investigate the SaV occurrence among hospitalized children with acute gastroenteritis from Manaus, Amazonas State, and also to improve the data on the molecular epidemiology of this virus in Brazil.

MATERIAL AND METHODS

Study design

The samples of this study were obtained through monitoring of AGE cases in the city of Manaus, capital of the Amazonas State, in the Amazon region, Northern Brazil. A National Network for the Surveillance of Acute Gastroenteritis caused by rotavirus was created in Brazil, since the introduction of the RV vaccine in 2006, involving three laboratories that are responsible for the detection and molecular characterization of this and other enteric viruses. The *Evandro Chagas* Institute (IEC) is one of these laboratories that receive samples from five states (Amazonas, Acre, Pará, Roraima, Amapá) located in the Amazon region.

From January 2010 to October 2011, 426 fecal samples were collected from hospitalized children ≤ 10 years of age that presented acute diarrhea, and other symptoms such as fever and vomiting. All samples were initially tested for RV and NoV, both by enzyme immunoassays (Ridascreen[®] Rotavirus enzyme-immunoassay EIA - R-Biopharm, Darmstadt, Germany; Ridascreen[®] Norovirus 3rd Generation EIA - R-Biopharm, Darmstadt, Germany) and reverse transcription-polymerase chain reaction (RT-PCR), and the samples with negative results were included in this study.

This research is in accordance with the ethical standards and was approved by the Ethics Committee on Human Research of the *Evandro Chagas* Institute under the registration number 0002/2012/IEC/SVS/MS-N^o0049/2011.

RNA extraction and reverse transcription

A total of 300 μ L of a fecal suspension (10% w/v) prepared in Tris/HCl/Ca²⁺ buffer was used for the nucleic acid extraction as described by Boom *et al.*²⁰, modified by Cardoso *et al.*²¹. The reverse transcription (RT) was performed using the pd(N)₆ random primerTM (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for the complementary DNA synthesis (cDNA).

SaV detection

For the amplification of SaV genomes, the polymerase chain reaction (PCR) was performed using the primers p289/290 that target the RNA polymerase region of NoVs and SaVs²². The PCR product was visualized on agarose gels (1%). Amplicons that showed size of 319 bp and 331 bp were considered positive for NoVs and SaVs, respectively. Additionally, in order to evaluate another partial region of the genome, an additional PCR was performed using the primers SLV 5317/5749, which are specific for the viral capsid region of SaVs²³, considering that this region is more variable, when compared with the polymerase one. Furthermore, one third of the samples that yielded a negative PCR result were submitted to a second round of amplification (nested-PCR), using in the first round the

primers SV-F13/SV-R13, SV-F14/SV-R14 (polymerase-capsid junction), and in the second round the primers SV-F22/ SV-R2 (capsid region)²⁴.

Sequencing and phylogenetic analysis

Positive samples were purified from the gel or from the PCR product, according to the manufacturer's instructions using a purification kit (QIAquick[®] Gel Extraction Kit or QIAquick[®] PCR Purification Kit, QIAGEN Inc., Hilden, Germany). Purified DNA was sequenced using the Big Dye Kit (v. 3.1) (Applied Biosystems, Foster City, CA, USA) on an ABI Prism sequencer 3130XL DNA Sequencer (Applied Biosystems, Foster City, USA). The oligonucleotides used in the sequencing reaction were the same previously described in the amplification protocols.

The sequences obtained by both polymerase and capsid region, were edited using the BioEdit Sequence Alignment Editor program (v.7.1.3.0) available from: www.mbio.ncsu.edu/bioedit/bioedit.html and compared with prototype sequences from GenBank database (National Center for Biotechnology Information, U.S. available from: www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed on the MEGA 5.2 program²⁵ (www.megasoftware.net) using the Kimura 2-parameter method with 2,000 bootstrap replicates. The sequences of this work were also deposited in the same database with the accession numbers: KF924388-KF924393. None of the samples were identified as NoVs by genomic DNA sequencing.

Statistical analysis

Statistical analysis involving correlation of the epidemiological data with the frequency of SaVs was performed by simple logistic regression using the BioEstat 5.3 software²⁶ (www.mamiraua.org.br/pt-br/downloads/programas/bioestat-versao-5.3).

RESULTS AND DISCUSSION

Of the 156 fecal specimens analyzed, six presented the SaVs amplicons of 331 bp, corresponding to 3.8% of the total. All the samples tested by nested-PCR showed negative results.

In comparison with other studies, the positivity rate was higher than that observed in Thailand (1.2%)¹³ and China (0.5%)²⁷, but similar (3.9%) to the one found in children with AGE of five locations from Japan during 2007 and 2008²⁸ and lower than the positivity in Philippines (7.0%), where the virus was detected in hospitalized children with AGE²⁹.

In Brazil, few studies have described the circulation of this virus among children. A research conducted in the state of Pará which is also located in the Amazon region, found a frequency a little higher (4.9%) among diarrheic children¹⁷. A recent study conducted in a day-care in Midwest Brazil detected SaVs in 4.6% of children, and the circulating genotypes were GI.1 and GI.3¹⁹.

All of these infections occurred in infants < 3 years of age (4.7%, $p = 0.5925$), but the p -value showed no correlation between age and the presence of infection, which may be justified by the small number of positive cases. No children under six months of age had the infection. Most infections ($n = 05$; 83.3%) occurred between August and

September, which are less rainy months in the Amazon. Diarrhea was reported in all infected children and the absence of precise information about fever and vomiting prevented the analysis of these signs and symptoms.

Despite several attempts to sequence all the positive samples, it was possible to genetically characterize only half of the samples. This limitation may have occurred due to the low concentration of viral genomic DNA in the specimens. Phylogenetic analysis allowed to characterize three of the six positive samples, being one only possible through the capsid region analysis (GI.1) and other two samples by both polymerase and capsid region analysis (GI.2/GI.2 and GII.2?-GII.4?/GII.4) (Fig. 1 and 2). The GI.1 strain is commonly found worldwide, in studies conducted in Brazil, Thailand and China^{17,13,30}. One sample presented the same genotype (GI.2) in both regions showing high similarity (99%) with the strain detected in midwest Brazil¹⁸. Genotype GI.2 is more associated with outbreaks and sporadic cases of AGE³¹. In a study conducted in Europe, this genotype was responsible for 36% of sporadic cases, furthermore, caused 58% of outbreaks in the Netherlands, 27% in Sweden, 40% in Slovenia and 100% in Hungary³².

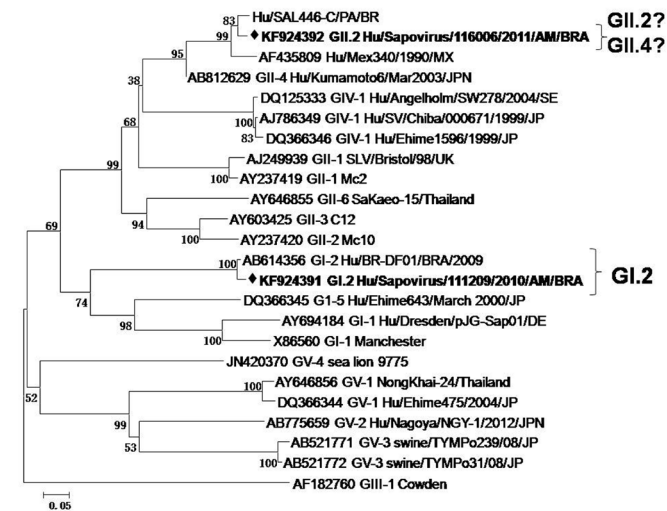


Fig. 1 - Dendrogram constructed using partial sequences of the amplified polymerase region from positive sapovirus samples recovered from diarrheic children of Manaus City, state of Amazonas, Brazil. Study samples were marked in bold. The genotype classification system followed previously established criteria⁹. The number above each branch corresponds to the bootstrap value (2,000 replicates). The scale bar is proportional to the genetic distance.

The other sample (ID 116006) demonstrated, in the first analysis of the polymerase region, a great similarity with the sample Mex 340, that was classified as GII.2, and regarding the capsid region, with Kumamoto6 characterized as GII.4 (Fig. 1 and 2). However when the polymerase sequence of Kumamoto6 strain was included and compared with the present sample, a 95% of identity was observed, but only 46% of coverage. The complexity of the sequences analyses was related to the fact that only a few polymerase sequences are available in GenBank and none of the propotype GII.4. Therefore, other tests such as cloning (possibility of co-infection) and sequencing of a larger fragment of the genome are necessary to confirm if this strain is really a complete GII.4 sequence or a possible recombination of GII.2/GII.4.

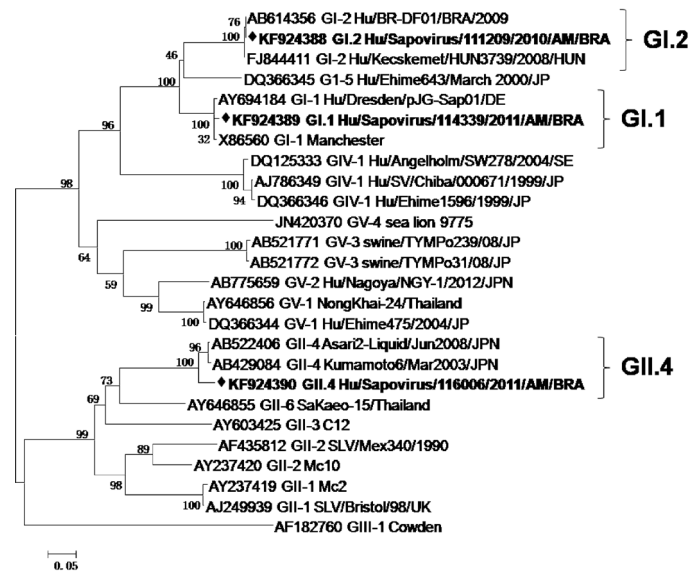


Fig. 2 - Dendrogram constructed using partial sequences of the amplified capsid region from positive sapovirus samples recovered from diarrheic children of Manaus City, state of Amazonas, Brazil. Study samples were marked in bold. The genotype classification system followed previously established criteria⁹. The number above each branch corresponds to the bootstrap value (2,000 replicates). The scale bar is proportional to the genetic distance.

Genetic recombinations involving SaVs have been described previously and the genogroup II is the most associated with these events^{33,34,35}. As far as we know, this is the first time that SaVs - GII.4 was detected in Brazil.

This study is the first to evidence the circulation of sapoviruses in children with gastroenteritis in the city of Manaus, Amazonas. In addition, although the frequency of SaVs was low (3.8%- 6/156), the molecular characterization data demonstrated the circulation of different genotypes, that are commonly found elsewhere, and also found one case of GII.4 or a possible inter-genotype recombination that needs complementary studies using a larger fragment of the viral genome. Although, the primer pair used for the screening of SaV polymerase region (p289/290) are not specific primers, additional specific primers were used to amplify the capsid region.

A continued surveillance of these pathogens is important to monitor their impact on the population and the emergence of new strains, as well as to provide more subsidies on the epidemiology of SaVs in Brazil.

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