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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.I, 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 cases[1]. 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 age[2]. Several pathogens may lead to AGE, however, viruses currently account for about 70% of these cases[3]. 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 agents[4].

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 importance[5]. SaVs were first described in Japan, after an outbreak in an orphanage in the city of Sapporo, being initially denominated Sapporo Virus[6].

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 microscope[7]. 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 pigs[5]. 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 Japan[10].

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 NoVs[11]. Transmission occurs mainly by the fecal-oral route, person-to-person contact and ingestion of contaminated food and water[12].

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-Nº0049/2011.

RNA extraction and reverse transcription

A total of 300 µL of a fecal suspension (10% w/v) prepared in Tris/HCl/Ca^{2+} 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 primer™ (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 SaVs[22]. 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 SaVs[23], 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 program[25] (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 software[26] (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 2008[28] and lower than the positivity in Philippines (7.0%), where the virus was detected in hospitalized children with AGE[29].

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 children[17]. 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.3[19].

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 Brazil[18]. Genotype GI.2 is more associated with outbreaks and sporadic cases of AGE[31]. 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 Hungary[32].

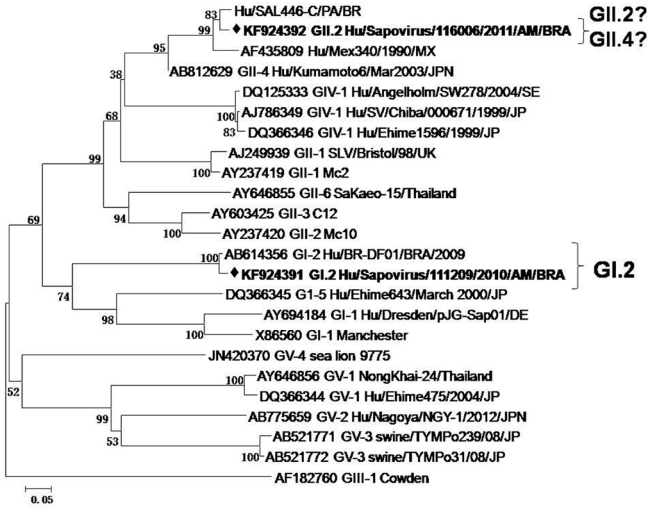

Fig. 1 - Dendrogram constructed using partial sequences of the amplified polymerase region from positive sapovirus samples recovered from diarrheic children of *Manaus* City, state of *Amazonas*, Brazil. Study samples were marked in bold. The genotype classification system followed previously established criteria[9]. 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 caracterized 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.

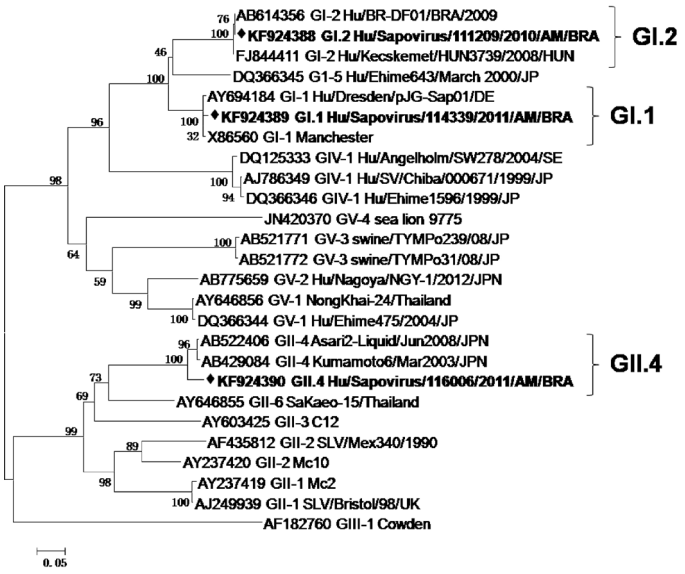

Fig. 2 - Dendrogram constructed using partial sequences of the amplified capsid region from positive sapovirus samples recovered from diarrheic children of *Manaus* City, state of *Amazonas*, Brazil. Study samples were marked in bold. The genotype classification system followed previously established criteria[9]. 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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REFERENCES

1. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet. 2010;375:1969-87.

2. World Health Organization. Diarrhoeal disease. Geneva: WHO; 2013. [cited 2015 Aug 18]. Available from: http://www.who.int/mediacentre/factsheets/fs330/en/

3. Chow CM, Leung AK, Hon KL. Acute gastroenteritis: from guidelines to real life. Clin Exp Gastroenterol. 2010;3:97-112.

4. Ramani S, Kang G. Viruses causing childhood diarrhoea in the developing world. Curr Opin Infect Dis. 2009;22:477-82.

5. Clark IN, Estes MK, Green KY, Hansman GS, Knowles NJ, Koopmans MK, et al. Family caliciviridae. In: King AM, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. London: Elsevier; 2012. p.977-86.

6. Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, et al. An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol. 1979;4:249-54.

7. Hansman GS, Oka T, Katayama K, Takeda N. Human sapoviruses: genetic diversity, recombination, and classification. Rev Med Virol. 2007;17:133-41.

8. Green KY. Caliciviridae: the noroviruses. In: Knipe DM, Howley PM, editors-in-chief. Fields' virology. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013. p.582-608.

9. Oka T, Wang Q, Katayama K, Saif LJ. Comprehensive review of human sapoviruses. Clin Microbiol Rev. 2015;28:32-53.

10. Shibata S, Sekizuka T, Kodaira A, Kuroda M, Haga K, Doan YH, et al. Complete genome sequence of a novel GV.2 sapovirus strain, NGY-1, detected from a suspected foodborne gastroenteritis outbreak. Genome Announc. 2015;3:e01553-14.

11. Sakai Y, Nakata S, Honma S, Tatsumi M, Numata-Kinoshita K, Chiba S. Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan. Pediatr Infect Dis J. 2001;20:849-53.

12. Mikula C, Springer B, Reichart S, Bierbacher K, Lichtenschopf A, Hoehne M. Sapovirus in adults in rehabilitation center, upper Austria. Emerg Infect Dis. 2010;16:1186-7.

13. Chaimongkol N, Khamrin P, Malasao R, Thongprachum A, Kongsricharoern T, Ukarapol N, et al. Molecular characterization of norovirus variants and genetic diversity of noroviruses and sapoviruses in Thailand. J Med Virol. 2014;86:1210-8.

14. Bucardo F, Reyes Y, Svensson L, Nordgren J. Predominance of norovirus and sapovirus in Nicaragua after implementation of universal rotavirus vaccination. PLoS One. 2014;9:e98201.

15. Wang G, Shen Z, Qian F, Li Y, Yuan Z, Zhang J. Genetic diversity of sapovirus in non-hospitalized adults with sporadic cases of acute gastroenteritis in Shanghai, China. J Clin Virol. 2014;59:250-4.

16. Xavier MP, Oliveira SA, Ferreira MS, Victoria M, Miranda V, Silva MF, et al. Detection of caliciviruses associated with acute infantile gastroenteritis in Salvador, an urban center in Northeast Brazil. Braz J Med Biol Res. 2009;42:438-44.

17. Aragão GC, Oliveira DS, Santos MC, Mascarenhas JP, Oliveira CS, Linhares AC, et al. Molecular characterization of norovirus, sapovirus and astrovirus in children with acute gastroenteritis from Belém, Pará, Brazil. Rev Pan-Amaz Saúde. 2010;1:149-58.

18. Dos Anjos K, Lima LM, Silva PA, Inoue-Nagata AK, Nagata T. The possible molecular evolution of sapoviruses by inter- and intra-genogroup recombination. Arch Virol. 2011;156:1953-9.

19. Oliveira DM, Souza M, Fiaccadori FS, Santos HC, Cardoso DD. Monitoring of Calicivirus among day-care children: evidence of asymptomatic viral excretion and first report of GI.7 Norovirus and GI.3 Sapovirus in Brazil. J Med Virol. 2014;86:1569-75.

20. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495-503.

21. Cardoso DD, Fiaccadori FS, Souza MB, Martins RM, Leite JP. Detection and genotyping of astroviruses from children with acute gastroenteritis from Goiânia, Goiás, Brazil. Med Sci Monit. 2002;8:CR624-8.

22. Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods. 1999;83:145-54.

23. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods. 2003;114:37-44.

24. Okada M, Yamashita Y, Oseto M, Shinozaki K. The detection of human sapoviruses with universal and genogroup-specific primers. Arch Virol. 2006;151:2503-9.

25. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-9.

26. Ayres M, Ayres M Jr, Ayres DL, dos Santos ASS. BioEstat 5.3: aplicações estatísticas nas áreas das ciências biológicas e médicas. Belém: Instituto de Desenvolvimento Sustentável Mamirauá; 2007.

27. Lu L, Jia R, Zhong H, Xu M, Su L, Cao L, et al. Molecular characterization and multiple infections of rotavirus, norovirus, sapovirus, astrovirus and adenovirus in outpatients with sporadic gastroenteritis in Shanghai, China, 2010-2011. Arch Virol. 2015;160:1229-38.

28. Chanit W, Thongprachum A, Khamrin P, Okitsu S, Mizuguchi M, Ushijima H. Intergenogroup recombinant sapovirus in Japan, 2007-2008. Emerg Infect Dis. 2009;15:1084-7.

29. Liu X, Yamamoto D, Saito M, Imagawa T, Ablola A, Tandoc AO 3rd, et al. Molecular detection and characterization of sapovirus in hospitalized children with acute gastroenteritis in the Philippines. J Clin Virol. 2015;68:83-8.

30. Ren Z, Kong Y, Wang J, Wang Q, Huang A, Xu H. Etiological study of enteric viruses and the genetic diversity of norovirus, sapovirus, adenovirus, and astrovirus in children with diarrhea in Chongqing, China. BMC Infect Dis. 2013;13:412.

31. Mans J, Murray TY, Kiulia NM, Mwenda JM, Musoke RN, Taylor MB. Human caliciviruses detected in HIV-seropositive children in Kenya. J Med Virol. 2014;86:75-81.

32. Svraka S, Vennema H, van der Veer B, Hedlund KO, Thorhagen M, Siebenga J, et al. Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe. J Clin Microbiol. 2010;48:2191-8.

33. Hansman GS, Takeda N, Oka T, Oseto M, Hedlund KO, Katayama K. Intergenogroup recombination in sapoviruses. Emerg Infect Dis. 2005;11:1916-20.

34. Katayama K, Miyoshi T, Uchino K, Oka T, Tanaka T, Takeda N, et al. Novel recombinant sapovirus. Emerg Infect Dis. 2004;10:1874-6.

35. Phan TG, Okitsu S, Müller WE, Kohno H, Ushijima H. Novel recombinant sapovirus, Japan. Emerg Infect Dis. 2006;12:865-7.

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