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RESEARCH ARTICLE

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Detection and genotyping of enteric viruses in hospitalized children with acute gastroenteritis in Belém, Brazil: Occurrence of adenovirus viremia by species F, types 40/41

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Enteric adenovirus (AdV), sapovirus (SaV), and human astrovirus (HAstV) are important pathogens involved in the gastroenteritis etiology. In this study, a total of 219 fecal samples and sera were collected from children hospitalized for acute gastroenteritis (AGE) in two large pediatric hospitals in Belém, from March 2012 to April 2015. The samples were analyzed by polymerase chain reaction (PCR) for AdV and HAstV (astrovirus) detection, and Nested-PCR and qPCR for SaV detection. AdV was detected in 50.2% (110/219) of the cases, with 42.7% (47/110) being sequenced and classified as: species F (63.9% – 30/47), A (4.2% – 2/47), B (6.4% – 3/47), C (17.1% – 8/47), D (4.2% – 2/47), and E (4.2% – 2/47). Of the 110 AdV-positive feces samples, 80 paired sera presented sufficient amounts and were also tested for this virus, of which 51 (63.7%) showed positive results and 26 (70.3%) pairs (feces plus sera) presented concordant results after sequencing being classified as: species F (21/26; 80.8%), A (1/26; 3.8%), B (1/26; 3.8%), and C (3/26; 11.5%). Overall, HAstV rate in the feces samples was 1.8% (4/219), including both HAstV-1a (2/4; 50%) and HAstV-2c (2/4; 50%). SaV was detected in 4.6% (10/219) of the fecal samples, out of which 50% (5/10) of the positive samples were characterized into the genogroups GI.1 (1), GI.2 (2), and GII.4 (2). These findings highlighted the important contributions of AdV, HAstV, and SaV in the enteric virus spectrum in our region and showed the high genetic diversity of AdV. In addition, it demonstrated for the first time in Brazil, the circulation of AdV in the serum of hospitalized children with AGE.

KEYWORDS
adenovirus (AdV), astrovirus (AstV), children, diarrhea, hospital, sapovirus (SaV)

1 | BACKGROUND

Viruses are responsible for approximately 75% of all reported cases of acute gastroenteritis (AGE).[1] In this context, rotavirus and norovirus are recognized as the leading causes of AGE, followed by enteric adenovirus (AdV), sapovirus (SaV), and human astrovirus (HAstV).[2,3]

AdV are DNA viruses that are classified into seven species (A-G) and have more than 70 different types. AdV are associated with various morbid diseases, such as gastroenteric, respiratory, and ocular diseases (mainly conjunctivitis). Types 40 and 41, both from species F, are the main responsible for both isolated episodes and outbreaks of gastroenteritis, which predominantly affect children.[4] Types 40 and 41 have already been established as the primary pathogens responsible for nosocomial infection in both normal and immunosuppressed individuals, which could be caused by a clinical condition or acquired from organ transplantation.[5,6]

The role of AdV as the cause of viremia (detection of the virus or its genetic material in the blood) in transplanted patients, mainly of stem cells, is well defined.[7] Data involving viremia by AdV in immunocompetent children with AGE are very poor or inexistent.

The human SaV, a member of the *Caliciviridae* family, has been reported to cause outbreaks in indoor environments and has been associated with hospitalizations for AGE in various age groups worldwide.[2] The SaV are divided into five genotypes (GI-GV). Four of the genotypes, namely, GI and GII genogroups (with seven genotypes in each), GIV (with a single genotype, GIV.1), and GV (subdivided into four genotypes, of which GV.1 and GV.2 infect humans), are known to infect humans, whereas the GIII genotype only infects pigs.[4] These viruses account for approximately 8% of all AGE outbreaks and are detected in over 23% of samples testing negative for norovirus (NoV) and other pathogens.[2,8]

HAstV, which belongs to the *Astroviridae* family, genus *Mamastrovirus* (MAstV), is a common cause of AGE in children, the elderly, and immunocompromised individuals. In particular, HAstV has been reported to account for up to 10% of sporadic cases of nonbacterial diarrhea.[9] On the basis of the complete sequence of the capsid or open reading frame 2 (ORF2) region, HAstV are classified into eight classic types (HAstV-1 to HAstV-8), the MAstV-1 genotypes, and the nonclassic MAstV-6 (including Melbourne-1 [MLB-1] to MLB-3), MAstV-8 (HAstV Virginia2 [VA2], VA4, and human-mink-ovine-like-A [HMO-A]) and MAstV-9 (HAstV VA1, VA3, and HMO-B) genotypes.[4] Recently, HAstV-MLB2 viral RNA was detected for the first time in the plasma of a child with severe AGE and high fever, suggesting the potential of the HAstV to spread beyond the gastrointestinal tract.[10]

Recent studies have reported the presence of rotavirus (RV), NoV, and HAstV in the blood, suggesting the possibility of the extraintestinal spread of these viruses, which in turn causes clinical manifestations such as seizures and disseminated intravascular coagulation.[10-12] To SaV, no report has been observed until the present date. In the present research, the detection of AdV-, SaV-, and HAstV was done in the feces of hospitalized children with AGE in Belém, Brazil, as well as in the blood only for AdV, indicating a potential viremia among these children. In addition, genotyping was realized in some viral isolates from feces and serum.

2 | METHODOLOGY

2.1 | Study design

2.1.1 | Patients

A total of 482 paired stool and serum samples from the same individuals were obtained from eutrophic children (that presents good nutrition) aged less than nine years old and who were hospitalized for AGE in two large pediatric hospitals in Belém, from March 2012 to April 2015. Notably, these children were not diagnosed with other clinical conditions at the time of hospitalization. All samples (stools and sera) were collected as soon as possible after hospitalization and sent to the Evandro Chagas Institute, where they were processed and kept frozen (−20°C) until use. Initially, they were tested for RV and NoV[13,14] by enzyme immunoassay, and only samples with negative results (N = 219 paired samples) were selected for current study. Serum and fecal samples were analyzed following the same methodology of detection, as described below.

2.1.2 | Molecular detection

All the samples were submitted to nucleic acid extraction by QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. The reverse transcription (RT) to SaV and HAstV was performed to obtain complementary DNA using a random primer. Specimens were screened for HAstV by polymerase chain reaction (PCR) using the Mon269/Mon270 primer pair.[15] AdV was detected by nested PCR using the primers Hex1Deg and Hex2Deg in the first step, followed by the NeHex3Deg and NeHex4Deg primers in the second step.[16] SaV was initially detected by TaqMan-based RT-qPCR using the primers SaV124F/1F/5F and SaV1245R and MGB probes SaV124TP/5TP, which were used to amplify human SaV genogroups (GI, GII, GIV, and GV).[17] SaV detection was also conducted by nested PCR using the primers SV-F13/F14 and SV-R13/R14 in the first step and the primers SV-F22 and SV-R2 in the second step.[18]

2.1.3 | Purification and sequencing of PCR amplicons

All positive samples were purified using the QIAquick® PCR Purification or the QIAquick® Gel Extraction commercial kits according to the manufacturer's instructions and then sequenced using the Big Dye Terminator kit (v.3.1) and the same primers, on an automated sequencer.

The obtained sequences were compared with those available from GenBank using the BLAST program. Phylogenetic analysis for SaV and HAstV were performed by the neighbor-joining method, Kimura 2-parameter, and 1000 bootstrap replicates. The AdV phylogenetic clustering was performed using the RaXML program by maximum likelihood method, and 1000 bootstrap replicates.[19] All the sequences obtained in this study were deposited in the GenBank database with the numbers MH289542-MH289627.

2.1.4 | Statistical analysis

Statistical analysis was performed using the BioEstat 5.0 software.[20] Fisher's exact test was conducted to determine any association between the viral infection and age groups.

2.1.5 | Ethical considerations

This study was approved by the Evandro Chagas Institute Ethical Committee (CEP/IEC) under registry number 284.852 – Registration No. 0039/2011.

3 | RESULTS

At least one of the viruses AdV, SaV, and HAstV were detected in 56.6% (124/219) of the stool samples from children hospitalized for AGE in Belém, Brazil, from March 2012 to April 2015. The positivity rates of each enteropathogen were as follows: AdV – 50.2% (110/219), SaV – 4.6% (10/219), and HAstV – 1.8% (4/219). Two different methodologies were used for the detection of SaV, Nested RT-PCR detected five (2.3%) positive samples and the RT-qPCR ten (4.6%), including those previously described.

Negative results were obtained in the sera of children whose stools were either SaV- or HAstV-positive. Of the 110 AdV-positive stool samples, 80 sera with sufficient amounts of sample were tested, of which 51 (63.7%) were positive.

AdV infection was observed in all age groups, with the highest positivity rates (61.9%) observed in children aged 12 to 24 months old. SaV-related gastroenteritis was also detected in all age groups, with the maximum positivity (23.5%) found in children aged 48 to 60 months old. HAstV was detected only in children between 6 and 24 months (Table 1).

Out of 110 AdV-positive stool samples, 47 samples (42.7%) had the sufficient amount for genetic characterization. Species F, A, B, C, D, and E were identified in 30 (63.9%), 2 (4.2%), 3 (6.4%), 8 (17.1%), 2 (4.2%), and 2 (4.2%) samples, respectively (Figure 1). A total of 40 (78.4%) of the 51 AdV-positive serum samples were sequenced. The species F, A, B, and C, were characterized in 32 (80.0%), two (5%), one (2.5%), and five (12.5%), serum samples respectively (Figure 1). In 26 (70.3%) paired samples, similar results were obtained, which were classified as species F, A, B, and C in 21 (80.8%), one (3.8%), one (3.8%), and three (11.5%) pairs, respectively.

The four HAstV-positive samples were sequenced and classified as HAstV-1a (50% of samples) and HAstV-2c (50%). Partial sequencing of the capsid gene regions of five SaV-positive samples yielded the genotypes GI.1 (one sample), GI.2 (two samples), and GII.4 (two samples).

4 | DISCUSSION

This study demonstrated the presence of AdV, SaV, and HAstV in clinical specimens of hospitalized children with AGE. Out of the 219 fecal samples, 50.2%, 4.6%, and 1.8% tested positive for AdV, SaV, and HAstV, respectively, thereby suggesting their importance in the pathogenesis of childhood diarrhea.

In a previous study conducted in Belém, Brazil,[21] enzyme-linked immunoassay and immunochromatographic tests detected AdV at a rate of 9.1% (15/164), which was lower than the 50.2% positive rate reported in the current study, which could be attributed to differences in the sensitivities of the techniques used for AdV detection. The PCR/nested-PCR method used in the current study was demonstrated to be sensitive for AdV detection. However, the positivity rate may be overestimated, because only RV- and NoV-negative samples were tested. In addition, the primers used detected all human AdV types and not only the enteric ones.

Nested PCR yielded a 63.7% AdV positivity rate in the sera of patients whose stools also tested positive for AdV. To our knowledge, our study is the first in Brazil to detect AdV in the sera of eutrophic children with no other clinical condition than AGE at the time of

TABLE 1 Age-group distribution of AdV-, SaV-, and HAstV-related gastroenteritis in children in Belém, Pará, Brazil from March 2012 to April 2015

Age (mo)	AdV/total (%)	SaV/total (%)	HAstV/total (%)	Pos/total (%)
0-6 mo	11/21 (52.4)	0/21 (0)	0/21 (0)	11/21 (52.4)
>6-12 mo	22/38 (57.9)	2/38 (5.3)	1/38 (2.6)	25/38 (65.8)
>12-24 mo	52/84 (61.9)*	3/84 (3.6)	3/84 (3.6)***	58/84 (69.0)
>24-36 mo	9/26 (34.6)	1/26 (3.8)	0/26 (0)	10/26 (38.5)
>36-48 mo	7/14 (50)	0/14 (0)	0/14 (0)	7/14 (50)
>48–60 mo	4/17 (23.5)	4/17 (23.5)**	0/17 (0)	8/17 (47.1)
>60 mo	5/19 (26.3)	0/19 (0)	0/19 (0)	5/19 (26.3)
Total	110/219 (50.2)	10/219 (4.6)	4/219 (1.8)	124/219 (56.6)

Abbreviations: AdV, adenovirus; HAstV, human astrovirus; SaV, sapovirus.
Exact fisher test, BioEstat 5.0. For each virus individually:
*$P < 0.008$ AdV > 12-24M.
**$P < 0.004$ SaV > 48-60M.
***$P < 0.300$ HAstV > 12-24M.

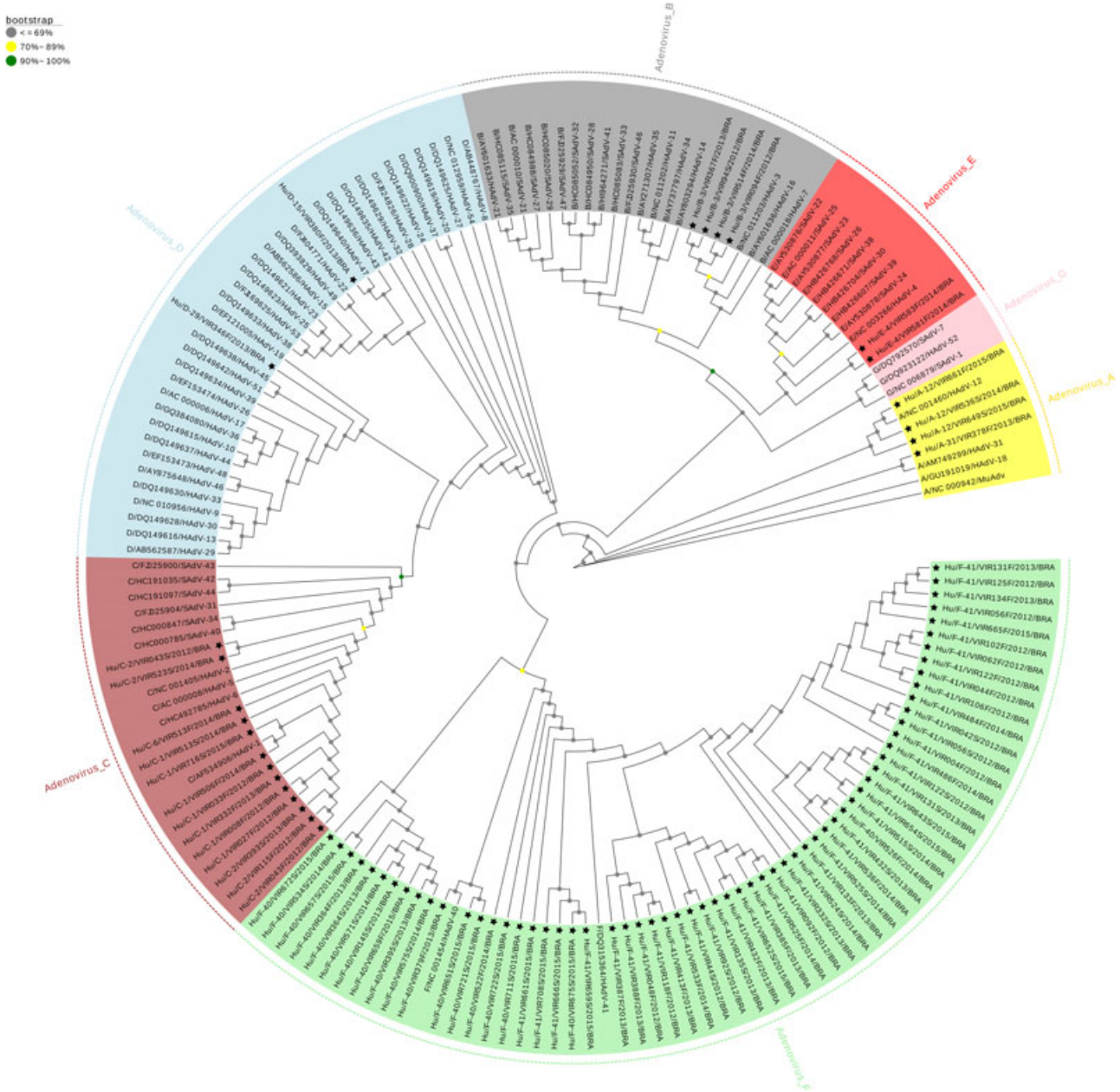

FIGURE 1 Dendrogram of adenovirus positive fecal and sera samples from children hospitalized for acute gastroenteritis in Belém, Brazil, from March 2012 to August 2015. The samples sequenced in this project are indicated by the abbreviation VIR, followed by the number of the sample and then F to indicate fecal and S to serum samples. All were grouped by species

hospitalization. However, studies have indicated that the gastrointestinal tract acts as a reservoir for the persistence of AdV infection, due to the high rates of replication that occur in the gastrointestinal tract, which facilitate it escape to blood systems.

AdV viremia has been predominantly detected in immunodeficient patients who underwent solid organ transplantation or allogeneic stem cell transplantation.[22-24] Lion et al[25] analyzed feces and blood samples of 304 children before being submitted to allogeneic human stem cell transplantation, also found that those with the pre-transplant shedding of AdV had a high risk of invasive infection—characterized by viremia—than the children without this agent.

In this context, AdV is recognized as a major cause of morbidity and mortality after allogeneic stem cell transplantation. So, patients with diarrhea and increased viral load in stool may be at risk of adenoviremia, as demonstrated in a study conducted in Goiás, Brazil, with transplant individuals.[26]

Consistent with previous reports, the highest proportion of AdV-related hospitalizations in our study was identified among children aged 12 to 24 months.[27-29] Similar to a previous study in Tanzania,[28] we observed no seasonal pattern for AdV, which may be explained by the lack of well-defined seasons in Belém, Pará, wherein the seasons are only divided into more rainy and less rainy periods. On the other hand, the peak of AdV incidence in Istanbul, Turkey occurred during

the summer (June-September).[27] In Japan, whose seasons are more defined, there was a higher positivity of AdV-positive cases during the winter (December) and spring (March).[29]

The most frequently detected genotype in our study was genotype 41, species F, which accounted for 63.9% of all isolates from stool samples and 80.0% of paired fecal and serum samples. AdV type 41 species F has been recognized as the predominant viral type responsible for gastroenteritis outbreaks or sporadic cases of gastroenteritis requiring hospitalization. A country-wide high prevalence of AdV type 41-related gastroenteritis in Japan has also been reported among Japanese children with diarrhea from 1995 to 2009.[29] Interestingly, one AdV A12 sample was found to be associated with a case of gastroenteritis in our study. In Brazil, this AdV type was found to cause an AGE outbreak in Rio de Janeiro, Brazil, with a positivity rate of 44% (4/9).[30] We also detected non-enteric AdV types, including species B, C, and D, which are generally associated with respiratory and ocular diseases.[31-33] However, these types of AdV could be excreted in the feces, without any association with the gastroenteritis cases verified in the current study.

Despite the high genetic diversity observed in AdV, additional analysis are required to establish a well-defined characterization approach that incorporates a larger AdV sequence region, considering that the one used in this study contemplated only ~171 bp.

Epidemiological studies involving HAstV and SaV pathogens remain scarce in Brazil, particularly in the northern region. The low positivity for HAstV (1.8%) found in our study, was a bit bigger than the rates previously reported in other parts of Brazil, such as Rondônia (0.8%)[34] and in Brasília (0.5%).[35] However, the detection rates in the current study were lower than those previously reported in Belém (3.9%)[36] and in São Luís (8.0%).[37] Given the similarity in clinical and epidemiological characteristics of the cohorts from the comparative studies, the differences in results are probably due to methodological inconsistencies, as well as the fact that we only searched for HAstV, SaV and AdV in rotavirus and norovirus negative samples.

Only four HAstV-positive gastroenteritis cases were identified in our samples, all of which were identified among children between the ages of 6 and 24 months. Although the number of HAstV-related gastroenteritis cases was low, our results were in accordance with those of previous studies, in which the peak of positivity was observed among children younger than two years old.[9,36,38,39] Notably, previous surveys conducted in Belém, Brazil reported a higher rate (3.9%) in a similar pediatric population hospitalized for gastroenteritis.[36] Our findings were also consistent with the ones obtained in Spain, in which 80% of HAstV-related gastroenteritis cases were detected in children aged less than three years.[40]

Given the scarcity of data from Brazil, our study also improved current knowledge on the occurrence of SaV in cases of infantile gastroenteritis in our region. The rate obtained for SaV-related gastroenteritis in our study (4.6%) is similar to previously reported rates in Brazil. For instance, a positivity of 3.8% (6/156) was reported among children admitted for AGE in Manaus, Amazonas state, Northern Brazil.[41] A similar (4.9%) rate of SaV-related gastroenteritis was reported in Belém, Brazil in 2003 based on a cohort of children who sought treatment from a public outpatient unit.[42] Furthermore, a lower rate of 2.5% was reported among diarrheic children of African-descendant who lived in a semi-closed community on the outskirts of Belém (the "Quilombola") from 2008 to 2010.[43]

SaV-related gastroenteritis was observed in children from all age groups, except for those aged 0 to 6 months and 36 to 48 months, with a slightly higher positivity in the ages between 48 and 60 months (23.5%; $P > 0.004$). The above results were partially consistent with those reported in Japan, wherein SaV was more strongly associated with older children (91%).[44]

The types identified in the four HAstV positive samples (HAstV-1a and HAstV-2c) were consistent with those reported in other studies conducted elsewhere, in which HAstV-1a was found to be the predominant type among classic HAstV types, followed by type HAstV-2.[9,39,40,45,46] In Belém, HAstV-1a has been previously detected at the rates of 24.3% and 66.6% among children hospitalized due to AGE.[42,47]

Notably, the occurrence of HAstV-2c was reported previously in Belém, Brazil (23.9%).[44] Moreover, HAstV-2c has been reported to be associated with an extensive gastroenteritis outbreak among Maxakali Indians, from the Macuri Valley in northeastern Minas Gerais in January 2004.[48]

The SaV genotype GI.1 accounted for 20% of the isolates in our study. The GI.1 lineage was previously reported with a positivity of 16.6% in Manaus, Brazil,[36] 75% in Japan from 2000 to 2007[46] and 66.7% in China.[49] GI.2 was identified in two samples in our study. The GI.2 genotype is strongly associated with outbreaks and sporadic cases of AGE worldwide.[36,50,51]

Children whose fecal samples tested positive to one of these three viruses showed the typical clinical symptoms associated with the viral infection, including diarrhea, vomiting, and fever. However, we found no statistically significant differences in the clinical parameters in the positive cases, because the symptoms observed in patients with and without these viruses were highly similar. The aforementioned results suggested the possible involvement of other pathogens, such as other viruses, bacteria, and parasites, in these episodes. Thus, the results obtained in this study, together with previous findings from Brazil, have demonstrated the need to improve monitoring of the circulation of these viruses in the country to serve as a basis for possible control measures.

Studies investigating the positivity of AdV, HAstV, and SaV and sequence analyses of samples obtained from hospitalized children with AGE are scarce in Brazil. To our knowledge, the current study is the first to report AdV detection from serum samples of children with AGE. Our findings highlighted the importance of identifying etiological agents that spread beyond the intestines and enter the bloodstream, thereby affecting other organs. Our results also demonstrated that different genotypes of these viruses circulate in the Brazilian population.

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CONFLICTS OF INTEREST

The authors have declared that there are no conflicts of interest.

AUTHORS' CONTRIBUTION

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