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Detection of caliciviruses associated with acute infantile gastroenteritis in Salvador, an urban center in Northeast Brazil

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Acute gastroenteritis caused by viruses is one of the leading causes of infantile morbidity. The aim of the present study was to investigate the presence of human caliciviruses of the genera norovirus and sapovirus in children up to 3 years of age with acute gastroenteritis from low-income communities in the city of Salvador, Brazil. This study is an extension of previous work carried out to establish the profile of the most prevalent enteric pathogens present in these communities. In this report, 139 fecal samples, collected from July 2001 to January 2002 were analyzed by RT-PCR and 13 (9%) were positive for human caliciviruses. By sequencing, seven isolates were characterized as norovirus genogroup GII and one as sapovirus genotype GII/1. Sequencing of the previously detected group-A rotaviruses and human astroviruses was also performed and revealed the circulation of rotavirus group A genotypes G1P[8] and G9P[8], and human astrovirus genotypes 6, 7, and 8. No mixed infection was observed. Community-based studies provide geographically representative information on disease burden. However, there are only a few reports in developing countries concerning the genotypes of the most important gastroenteric viruses detected in such communities. The present findings demonstrate the wide diversity of genotypes of the most important viruses responsible for acute gastroenteritis circulating in low-income communities.

Key words: Calicivirus; Sapovirus; Astrovirus; Rotavirus A; Gastroenteritis; Genotypes

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Introduction

Diarrheal diseases are a leading cause of morbidity and mortality worldwide and occur most often in children up to 5 years of age (1,2). Viral pathogens are the most common causes of gastroenteritis in communities and in other settings, including semi-closed institutions and hospitals. The viruses that have been associated with gastroenteritis are: group A rotavirus (RV-A), human caliciviruses (HuCaVs) (noroviruses [NoVs] and sapoviruses [SaVs]), human astroviruses (HAstVs), and human adenoviruses (HAdVs). These viruses mostly affect children in the first years of life and have been described as common causes of gastroenteritis especially in countries where there are large differences between the social classes in terms of hygiene and the quality of water, food, and sanitation (3).

Norovirus and sapovirus are two genera belonging to the *Caliciviridae* family, which infect humans. They are small non-enveloped viruses with icosahedral symmetry that contain a single-strand-positive sense RNA (4). The

NoV genome encodes three open reading frames (ORFs): ORF1 encodes non-structural proteins, ORF2 encodes a major capsid protein (VP1), and ORF3 encodes a minor capsid protein (VP2).

Based on extensive sequence analyses, NoVs have been divided into seven distinct genogroups (GI-GVII), of which GI, GIV, GVI, and GVII are found exclusively in humans and GII is associated with human and porcine infections. The genogroups GIII and GV are associated with bovine and murine infections, respectively (5).

SaVs have been classified into five well-established genogroups (GI-GV), among which GI, GII, GIV, and GV are known to infect humans and GIII to infect porcine species. Recent studies have reported the existence of two more genogroups, GVI and GVII, which are associated with swine infections (6). The SaV genome encodes two ORFs: ORF1 encodes one polypeptide that is cleaved in all non-structural proteins and one major capsid and ORF2 encodes the minor structural protein (6).

NoVs require special cell systems for *in vitro* replication (7,8) and so far human SaVs have not been isolated in any cell culture system. In the last decade, NoVs were recognized as one of the major cause of outbreaks and of sporadic cases of acute gastroenteritis throughout the world. NoVs and SaVs are clinically characterized by the acute onset of vomiting, diarrhea, or both, lasting 12-60 h. Both viruses are transmitted via many routes, such as fecally contaminated food and water, the environment and person-to-person contact (4,6). In clinical studies, approximately two thirds of persons infected with NoVs experienced symptoms of the disease (4,9).

HuCaVs have been described as a common cause of epidemic gastroenteritis in children and adults mostly in developed countries and there is a lack of information concerning the prevalence of these viruses in developing countries, including Brazil. Modest information is available concerning the role of these viruses in endemic pediatric gastroenteritis. The knowledge of the genetic diversity of these viruses is limited to studies in children from day-care centers and hospitals (10-14). The prevalence and genotype diversity of calicivirus strains circulating in low-income communities is rarely reported.

The main objective of the present report was to detect and characterize HuCaVs in children up to 3 years of age with acute gastroenteritis in communities from the city of Salvador, in the Northeast region of Brazil. In addition, RV-A and HAstV were characterized to complement information from a previous study of the most common enteric pathogens present in children with acute gastroenteritis (2) and in order to determine the diversity of enteric viruses in these communities.

Material and Methods

Study population and sample collection

The field studies have been described in detail elsewhere (2). In summary, participants were patients with diarrhea from a large longitudinal study in 21 areas of the city of Salvador, Northeast of Brazil, representing the city's poorer socioeconomic and sanitary conditions. From July 24, 2001 to January 31, 2002, 139 diarrheic stool samples were collected in the patient's household. All samples were previously tested for RV-A, HAstV and HAdV as described by Barreto et al. (2). The present study was approved by the Ethics Research Committee of Instituto de Saúde Coletiva, Universidade Federal da Bahia, and all parents or persons responsible for the children gave written informed consent to participate.

RNA extraction and reverse transcription

Viral RNA was extracted from clarified stool samples by the glass powder method (15) with modifications (16) and complementary DNA (cDNA) reaction was carried out using a random initiator (hexamer $pd(N)_6$ 50 A_{260} units (Amersham Biosciences, UK) as described (14). The synthesized cDNA was submitted to nucleic acid amplification by polymerase chain reaction (PCR) for HuCaV detection and/or RV-A and HAstV genotyping.

Human calicivirus detection and nucleotide sequencing

The 139 fecal samples were tested by PCR using two different sets of primers: i) 289/290, which detects both NoVs (319-bp amplicons) and SaVs (331-bp amplicons) (17) and ii) a generic NoV PCR system based on the degenerate primers Mon 431/Mon 433 and Mon 432/Mon 434 of region B within 3'-end of ORF1 (RNA polymerase) (18). To avoid false-positive and -negative results, previously typed NoV GI/2a and NoV GII/4 strains and Milli-Q water were used as positive and negative controls, respectively. These controls were used in all PCR steps, including RNA extraction. Four different rooms were used for these steps and for all procedures to avoid cross-contamination in PCR amplification. The PCR products were resolved by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

The amplicons obtained by PCR were purified, quantified, sequenced, and analyzed as recently described (13). Phylogenetic trees were constructed by the neighbor-joining method using the Mega (version 3.1) software, supported by a bootstrap using 2000 replicates. The nucleotide sequences for region B determined in this study were deposited in the GenBank database and assigned the accession numbers EU155112 to EU125116, EU259613, and EU259614.

Rotavirus A and astrovirus genotyping

The 11 RV-A-positive samples (2) were characterized as genotypes G and P by using primers targeting the VP7 and VP4 genes. The products of the first PCR amplification were used as template for a hemi-nested multiplex PCR as described (19,20), with modifications (21).

The 7 samples positive for HAstVs (2) were submitted to hemi-nested multiplex PCR for genotyping using the primers previously described (22).

Results

A total of 139 fecal samples were collected and examined from children up to 3 years of age with acute gastroenteritis and HuCaVs were detected in 13 (9%) of them. Initially, HuCaVs were submitted to RT-PCR using primers 289/290, resulting in seven (5%) positive samples. To broaden the detection of NoV strains, all samples were also tested with primers Mon 431/Mon 433 and Mon 432/Mon 434, targeting region B and a more conserved region of the genome, and NoVs were detected in 11 (8%) of them. Five samples were positive when tested with all primer sets (289/290 and Mon 431/Mon 433 and Mon 432/Mon 434), whereas 2 samples were positive only with the 289/290 primer set.

By sequencing, 1 of the 2 positive strains with primer 289/290 was characterized as SaV genogroup GII genotype 1 (GII/1; Figure 1). This strain showed 94% nucleotide sequence identity when compared to the Bristol/UK/98-GII/1 prototype strain (data not shown).

The phylogenetic analysis performed with sequences of region B of NoV genome revealed that 7 of 11 positive samples clustered within genogroup GII. Four strains that clustered with NoV genotype 4 (GII/4) displayed a maximum of 2.4% nucleotide sequence divergence and a maximum and minimum nucleotide identity of 88.7 and 87.3%

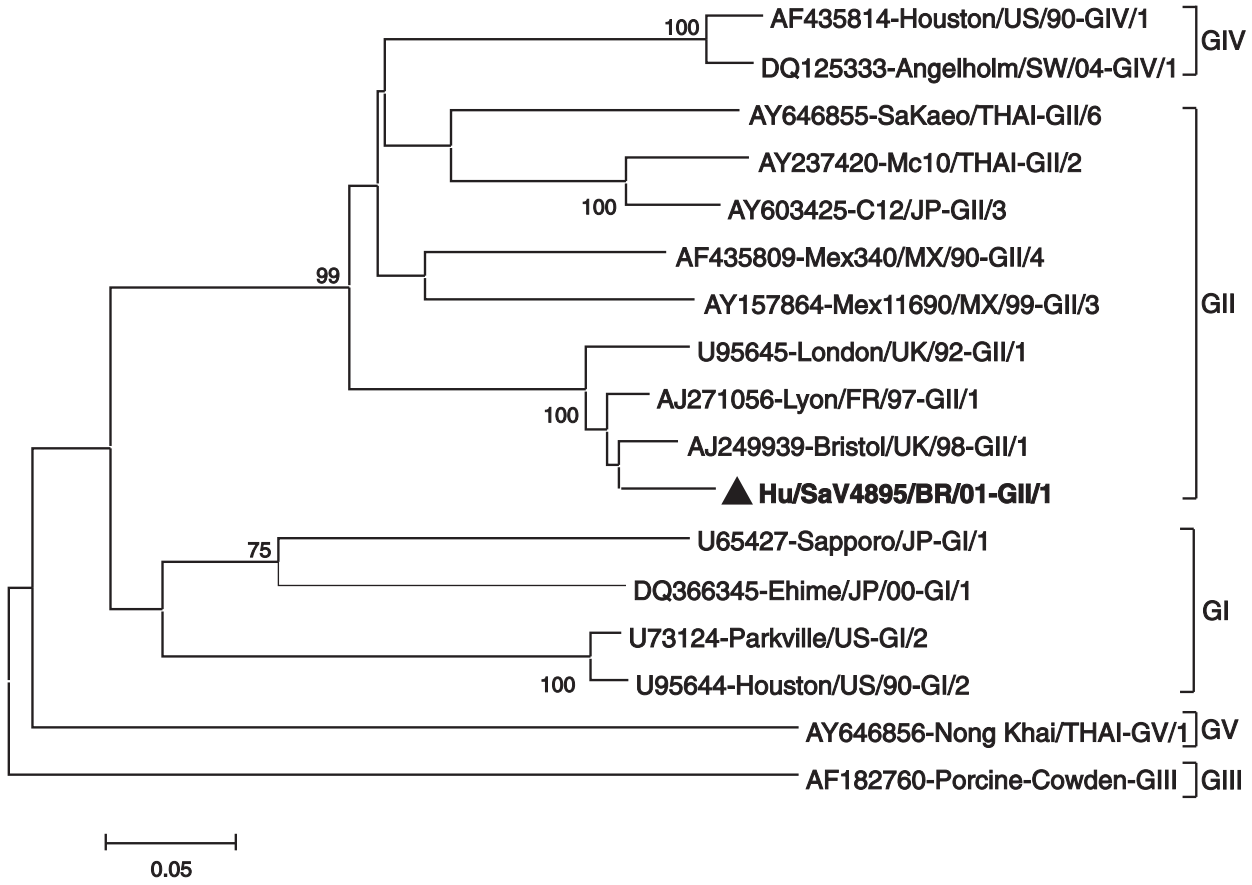

Figure 1. Phylogenetic tree of human sapovirus reference strains and Brazilian samples. Outgroup: SaV genogroup GIII (porcine enteric calicivirus). Strain denomination: GenBank accession number, place, and strain numbers followed by genogroup/genotype. The scale bar indicates nucleotide substitutions per position. The human sapovirus reference strain is reported in bold type.

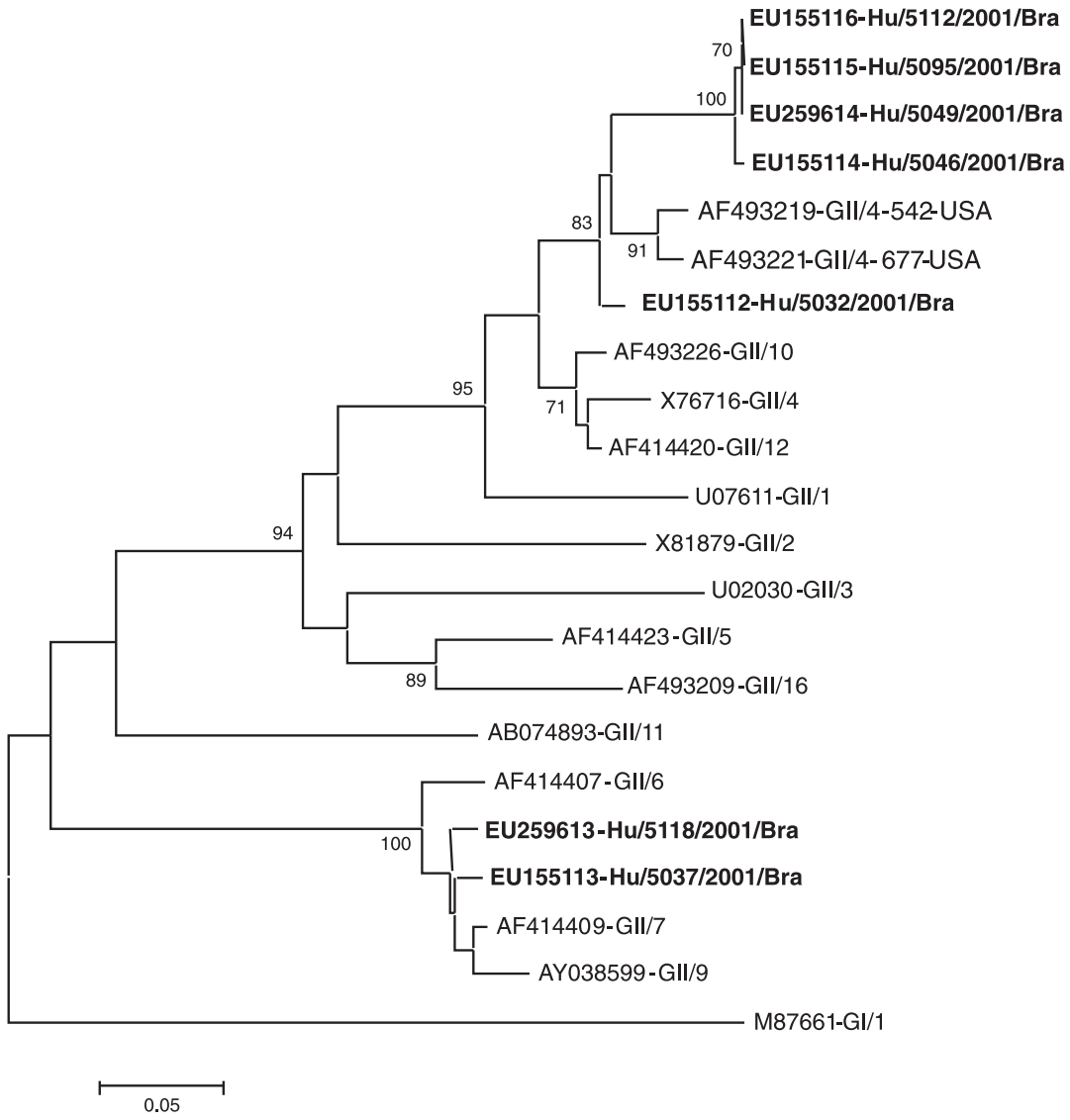

Figure 2. Phylogenetic tree of 172 nucleotides within the polymerase region (region B) of norovirus (NoV) strains collected in Salvador, BA, in 2001 and NoV genogroup GII reference strains (in bold). Outgroup: NoV genogroup GI (M87661). Our strain denominations: GenBank accession number followed by isolation, year and place strains. Prototype strains: GenBank accession number followed by genogroup/genotype. The scale bar indicates nucleotide substitutions per position.

with the Bristol GII/4 prototype, respectively. It was not possible to determine the genotypes of the 3 remaining strains based on region B analysis (Figure 2).

Of the 11 RV-A-positive samples, 3 were characterized as genotype G1P[8], 7 as genotype G9P[8], and 1 sample could not be typed. Of the 7 positive HAstV samples, three were genotyped as HAstV-6, 1 as genotype HAstV-7, and 3 as HAstV-8. No mixed infection was observed concerning RV-A, HAstVs and/or HuCaVs.

Discussion

Viral gastroenteritis is a common disease in humans and involves children and elderly people worldwide. In infancy, RV-A are considered to be the most important etiological agents of acute non-bacterial gastroenteritis, including outbreaks and sporadic cases, independent of improvements in basic sanitation and hygiene procedures. With improved molecular diagnostic techniques, the im-

portance of HuCaVs, mainly NoVs, and other previously under-reported pathogens is becoming more apparent and the number of viruses associated with gastroenteritis continues to increase (3,9).

Only a small number of studies have demonstrated progress of *in vitro* cell culture and animal models to better understand NoV infection. In the last decade, with improvements of the amplification protocols for the diagnosis of NoVs, the real importance of these viruses as etiological agents of acute gastroenteritis in outbreaks as well as in sporadic cases has been well established (23). Due to the genetic diversity of NoVs, molecular characterization of different genotypes has been used to better understand the epidemiology of strains associated with this syndrome. NoV prevalence studies have been conducted on different continents; however, the available data are mainly from countries in the developed world. In developing countries NoV prevalence is underestimated or even unknown, particularly in very low-income communities (9,10). Parks et al. (10) reported for the first time NoV infection in children living in shantytown communities of Fortaleza, in Northeast Brazil.

There are few studies evaluating the prevalence of NoVs in Brazil and most concern outbreaks at day-care centers or among hospitalized children, showing a prevalence of 8.6 to 33.3% (10-13,24). The present report determined the frequency and the genetic diversity of NoVs in children up to three years of age, with acute gastroenteritis, living in the shantytowns of the city of Salvador, BA, in Northeast Brazil, and also determined RV-A and HAstV genotypes previously identified in these same stool samples (2).

In the present report, the use of specific primers for NoVs (Mon 431-434, targeting region B) increased the detection of these viruses compared with 289/290 primers (to detect HuCaVs). Our results agree with a previous report showing that region B primers are able to amplify more than 90% of the NoVs GI and GII (18).

Based on the sequencing of region B amplicons and comparing these results to those obtained by Fankhauser et al. (25), four strains (5046, 5049, 5095, and 5112) were suggested to have the GII/4 genotype (Figure 2). This is the predominant genotype associated with NoV gastroenteritis worldwide, including Brazil (13,26). Two strains (5037 and 5118) were grouped with reference strains belonging to GII/6, GII/7 and GII/9, whereas strain 5032 formed an independent cluster (Figure 2). These results agree with a report that has proposed that region B is an appropriate region for NoV detection but is not an appropriate region for strain discrimination at the genotype level (27). Phylogenetic analysis within the capsid region is needed to ascertain the genotypes of these strains. A recent study has suggested that the capsid region is the most appropriate for phylogenetic analysis in order to identify NoV genotypes (27).

A strain (4895) was detected by RT-PCR assay (primers 289/290) and sequenced. In order to genotype the sample, phylogenetic analysis was carried out and the strain (4895) was genotyped as SaV GII/1 (Figure 1). This study represents one of the first molecular characterizations of a SaV strain in Brazil. Further studies are needed to understand the epidemiological role of SaVs in human diarrhea in the country.

NoV prevalence (7.9%) was similar to that observed for RV-A (7.9%) (3) and highlights the importance of these viruses in children's communities, especially because an increasing number of studies have observed the prevalence and clinical impact of NoVs in the same range as observed for RV-A (13,28,29).

Studies of RV-A infections in humans have identified distinct G and P genotypes circulating simultaneously in different parts of the world (1). Our data show the presence of RV-A G1P[8] and G9P[8], confirming that these two genotypes are those more frequently associated with RV-A illnesses worldwide (30-33). In the city of Salvador, Serravalle et al. (34) also detected RV-A genotype G9 in hospitalized children during the period from 1999 to 2000 and in 2002, highlighting the importance of this genotype in children's communities.

Most worldwide studies have shown that genotype HAstV-1 is the most prevalent, followed by HAstV genotype-2, -3, -4, and -5 (35-38). Interestingly, in our study HAstV-1 was not detected, but HAstV-6, -7, and -8, reported to be rare, were the only genotypes observed. HAstV-6 and -7 were first reported in Brazil by Gabbay et al. (38). The detection of HAstV-8 in 3 samples agrees with previous findings, suggesting that this is an emergent genotype (38,39).

Mixed infections involving NoVs, RV-A and HAstVs are common in developing countries and may be associated with poor environmental and sanitary conditions (32,34,36). Besides the diversity of viruses and genotypes circulating in these communities, the presence of mixed infections should be expected but this was not observed in the present study.

Community-based studies provide geographically representative information on disease burden. Our data indicate that NoV and other different genotypes of enteric viral pathogens can be detected in communities by using molecular approaches. This is of considerable significance to understand the natural course of infections as well as to clarify the importance of the epidemiology of gastroenteric

viral infections. In the communities under study, gastroenteric viruses were responsible for 26.6% of the total enteric pathogens present (2). Most of the studies concerning seasonality have reported winter peaks for both outbreaks and sporadic cases of acute gastroenteritis (1,3). In our study, it was not possible to determine the seasonality of these viruses due to the limited period of study (6 months). In the present study, the correlation between enteric virus genotype and illness severity was not demonstrated, probably due to the limited number of samples studied.

It is known that the frequency of RV-A, NoV, and HAstV infections is similar in developing and developed countries (9,36) and an alternative always considered to prevent these infections has been the development and introduction of new vaccines, mainly targeted at the pediatric population. However, recently, a study conducted in Salvador has demonstrated that a city-wide sanitation intervention, i.e., reducing direct exposure to unfavorable sanitation conditions, was a highly effective and sustainable measure to reduce the burden of gastroenteric infections (40). Consequently, in any decision-making process concerning the development and introduction of new vaccines, the contribution played by sanitation and other environmental improvements should be considered carefully.

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