

First detection of a human astrovirus type 8 in a child with diarrhea in Belém, Brazil: comparison with other strains worldwide and identification of possible three lineages

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This study describes the genetic relationships of the first human astrovirus type-8 (HAstV-8) detected in Belém-Brazil, during a public hospital-based study. This strain was compared with other HAstV-8 strains identified elsewhere which have sequences available at GeneBank. The regions ORF1a (primers Mon348/Mon340) and ORF2 (primers Mon269/Mon270) were analyzed by nucleotide sequencing and a high similarity rate was observed among the Belém strain and other HAstV-8 strains. In ORF1a, homology values of 93-100% were detected, and in ORF2 96-99%. Considering the sequence variation (7%) observed in ORF2 region, it was suggested that HAstV-8 strains could be divided in three different lineages.

Key words: astrovirus type 8 - molecular characterization - Belém - Brazil

Human astrovirus (HAstVs) belongs to the *Astroviridae* family, and are classified in the genus *Mamastrovirus* (Monroe et al. 2005). They are small, round non-enveloped viruses, 28 nm in diameter and composed of a single-stranded positive-sense RNA genome (ssRNA). The genome contains three open reading frames (ORFs) designated ORF1a, ORF1b, and ORF2, as well as terminal non-coding regions (NCR). ORF1a and 1b encode non-structural proteins, including a serine protease (*Pro*) and RNA-dependent RNA polymerase (*Pol*), respectively, and these regions contain highly conserved sequences. ORF2 is located at the 3' end of the genome, encodes the capsid protein precursor, and is highly variable (Matsui & Greenberg 2001, Walter et al. 2001).

HAstVs were classified into eight genotypes based on the nucleotide sequence analysis of a 348-bp region of ORF2 (Mustafa et al. 2000). These eight genotypes showed good correlation with the eight serotypes determined according to the reactivity of the capsid proteins with type-specific monoclonal antibodies (Sakamoto et al. 2000, Taylor et al. 2001).

For the eight types of HAstV already reported, the HAstV-1 is considered the most prevalent and HAstV-6, 7, and 8 as the seldom detected (Mustafa et al. 2000, Guix et al. 2002, Méndez-Toss et al. 2004). After the description of the prototype HAstV-8 (Z66541) in United Kingdom in 1995, the first reports describing the detection of this type of virus relate to the occurrence of

single isolates like those occurring in Ghaza and Uganda (Monceyron et al. 1997), Pakistan (Sakamoto et al. 2000), Australia (Nadan et al. 2003) and, more recently, in the United States (acces. nos AY304469 and AY304470), Brazil (Silva et al. 2006, and acces. nos DQ381506 to DQ381508), and India (acces. nos AB116554, AB126670 to 126674, AB191789, AB194280, and AB211059). Studies conducted in Egypt, Spain, Korea, South Africa, Hungary, and Mexico demonstrated the presence of HAstV-8 in percentages that varied from 8 to 23%, considering the total of positive cases detected (Naficy et al. 2000, Guix et al. 2002, Kang et al. 2002, Nadan et al. 2003, Méndez-Toss et al. 2004, Jakab et al. 2004).

One strain of HAstV-8 (PA-NSC087-BR) was detected in Belém, North of Brazil, during a study conducted in a public hospital from November 1992 to November 1994 involving children aged 0-5 years, who were admitted with acute gastroenteritis (Gusmão et al. 1999). Such HAstV-8 isolate was obtained from a seven-month old female with acute diarrhea of four-day duration, which persisted for eight more days. Moderate dehydration was identified during the first 48 h of hospitalization. In addition, fever was recorded in the first day and acute respiratory infection was diagnosed on the second and third days of admission. A diarrheic stool sample was obtained four days after admission (March 1st, 1993), being stored at Instituto Evandro Chagas, at -20°C, until being processed.

This was the first time that a HAstV-8 was detected in Belém. Furthermore, this type has rarely been detected worldwide (Guix et al. 2002, Méndez-Toss et al. 2004), and up to now, only two studies described molecular analysis of the HAstV-8 strains (Méndez-Toss et al. 2000, Taylor et al. 2001). The aim of this report is therefore to describe the antigenic and genetic relationships of this HAstV-8 strain (PA-NSC087-BR), in light of compari-

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son with other HAstV-8 strains identified elsewhere, to gathering more complete characterization data on this type. This is the first approach to compare different strains of HAstV-8 already described in relation to the ORF1a and 5' end ORF2 region of the HAstV-8.

The specimens collected in this study were screened for the presence of HAstV antigen by a commercial qualitative enzyme immunoassay (EIA) kit (IDEIA™ Dako Cytomation, Ely, UK), with subsequent confirmation by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers Mon 269 (4526-4545 nt) and Mon 270 (4955-4974 nt), yielding an expected product size of 449 nt in the 5' end of ORF2, as described previously by Noel et al. (1995) with few modifications (Gabbay et al. 2005). The positive samples by RT-PCR were genotyped by two methodologies: the type-specific RT-PCR using primers described by Sakamoto et al. (2000), that amplified the 3' end region of ORF2, and by the nucleotide sequencing of RT-PCR astrovirus amplicons that was carried out under the conditions described by Gabbay et al. (2005), with the primers Mon 269 / Mon 270. Another pair of primers (Mon 348 / Mon 340), directed towards the ORF1a (Belliot et al. 1997), was also used with the HAstV-8 sample, to obtain data from another region of the genome, therefore broadening the phylogenetic analysis. Sequence data obtained with sample PA-NSC087-BR with primers Mon 269 / Mon 270 were aligned and edited using BioEdit Sequence Alignment Editor (v.7.0.5.2) program and compared with the eight prototype sequences (HAstV1 [L23513], HAstV2 [L13745], HAstV3 [L38505], HAstV4 [L38506], HAstV5 [U15136], HAstV6 [L38507] and HAstV7 [L38508], Oxford reference strains and HAstV-8 [Z66541], United Kingdom reference) and with the strains Yuc-8 (AF260508), AS20 (AF292079), Melb8A (AF175261), RJ8398/BR/04 (DQ381508), Bcn8-5 (AF34880), BrG8 (DQ139832) and RJ8163/BR/04 (DQ381507) obtained from GenBank (www.ncbi.nlm.nih.gov), using the BLAST program. The sequence data obtained with sample PA-NSC087-BR with primers Mon 340 / Mon 348 were aligned with the sequences AS20 (AF290509), KS106205 (AF361030), KS106207 (AF361032), HAstV-2 (L13745), BrG5 (DQ028633), HAstV-3 (AF141381), HAstV-1 (L23513), BrG4 (DQ070852), V770/2002 (AB126670), I/V813/2002 (AB126672), Yuc-8 (AF260508), HAstV-6 (AF290507), HAstV-7 (AF290508). Phylogenetic trees were constructed by neighbor-joining method using the Mega (v. 3.1) software, supported by bootstrap using 2000 replicates. The nucleotide sequences determined in this study have been deposited in the GenBank database and assigned the accession numbers DQ990460 and DQ990461.

All the specimens of this study were tested for the presence of bacteria and parasites following the specifications of the "WHO Manual for Laboratory Investigation of Acute Enteric Infections, Programme for Control of Diarrhoeal Diseases". Samples were also tested for rotavirus antigen using a commercial EIA (DAKO-PATTS EIA kits-Copenhagen, Denmark), and for calicivirus using the RT-PCR technique including a pair of primers p289 and p290 as described by Jiang et al. (1992).

After the detection of HAstV by both EIA and RT-

PCR, the sample PA-NSC087-BR was tested by the type-specific PCR that assigned this sample as a HAstV-8. Nucleotide sequence analyses using the pair of primers Mon 269/Mon 270 and Mon 340/Mon 348 confirmed this strain as belonging to genotype 8. A good correlation between these two methodologies was also demonstrated in studies conducted in a periurban community of Mexico City and Hungary for all genotypes (Walter et al. 2001, Jakab et al. 2004).

Phylogenetic trees based on the 348-bp fragment of the ORF2 region (Fig. 1A) and on the 246-bp fragment of the ORF1a region (Fig. 1B) were constructed to assess the genetic relatedness between the PA-NSC087-BR strain and other available sequences of HAstV-8 obtained from different countries.

Analysis of the ORF1a region showed that PA-NSC087-BR group together with the other HAstV-8 genotypes as well as with genotypes HAstV-1 to HAstV-5 in a genogroup A, as defined by Belliot et al. (1997) and Taylor et al. (2001). Comparing the strain PA-NSC087-BR with the strain Yuc-8, a 97% nt and a 100% aa similarities were observed. With the other HAstV-8 (AS20, KS106205, KS106207, and V813/2002) the similarities were 93-94% at nt level and 96-99% at aa level. In one HAstV-8 sample from India (V770/2000), the sequence described in GeneBank for ORF1a region was a little small (220 bp), and when it was compared with the strain PA-NSC087-BR a similarity of 93% in nt and of 98% in aa was seen. The comparison of the strain PA-NSC087-BR with the strain classified as HAstV-1, 2, 3, 4, and 5 showed significant homology (90-93% in nt and 96-97% in aa), in contrast with HAstV types 6 and 7 where similarity was lower (80% nt and 92-95% aa).

As previously demonstrated (Belliot et al. 1997, Taylor et al. 2001), the nucleotide sequence analysis of ORF1a region from HAstV-1 to HAstV-8 indicate that HAstVs fall into two distinct genogroups. This fact was also observed in this study however, we did not verify a high similarity between HAstV-8 and HAstV-4 strains, as reported by Taylor et al. (2001). This may be explained by the fact that HAstV-4 strain used in our analysis was different from that utilized in that research.

Considering the amino acid sequence of the ORF1a region, the strain PA-NSC087-BR demonstrated a pattern similar to that of strain Yuc-8, and a difference of one (V770/2002 and V834/2000), two (AS20 and KS106207), and three (KS106205) amino acids as compared to other strains sequenced (Fig. 2A).

Analysis of the ORF2 region indicates that PA-NSC087-BR strain has a higher pairwise similarity with the strain SXPAS706 (99% in nt), and with the other strains (Yuc-8, AS20, RJ8398, Bcn8-5, BrG8, Melb8A), of 96-98% in nt. A divergence of 6% in nt sequence was noted in relation to the HAstV-8 prototype, and of 7% with the HAstV-8 strain RJ8163. Based on the sequence variations observed among strains of a same type, some authors (Medina et al. 2000, Guix et al. 2002) have proposed that strains showing a sequence diversity of at least 7% could be classified as new lineages. Using this same criterion, we observed, as for the ORF2 region, HAstV-8 could be divided in three different lineages and two of these were detected in Brazil (Fig. 1A).

acids was observed (Fig. 2B). The strains Melb8A and RJ8163 showed a difference to the prototype of 9 and 19 amino acids, respectively. This clearly demonstrated the great difference between the prototype and the other type 8 strains circulating worldwide and sustains the classification of this type into three lineages. The significant correlation (97 to 99%) observed between the strain detected in Belém and other from various countries (Pakistan, Mexico, South Africa, Spain, and Brazil), suggests that a genetically similar HAstV-8 strain was circulating in these several countries.

No other enteropathogen was detected in the PANSOC087-BR sample, suggesting that astrovirus had a role in the etiology of diarrhea. Of note, the clinical course appeared to be more severe than it has previously been appreciated (Nadan et al. 2003).

We cannot rule out the possibility of a nosocomial infection since the fecal sample of this child was obtained four days after admission; however, there is a strong evidence of community-acquired infection because we had a single HAstV-8 detection into the ward environment. It is important to mention that during this study a total of 372 specimens were tested yielding an astrovirus positivity of 8.9% (33/372), and with the detection of three HAstV-1 nosocomial infections.

In summary, we characterized the first case of HAstV-8 isolate in Belém, Brazil, emphasizing that comparison of sequences obtained from the ORF2 region allowed us to sustain the existence of three distinct lineages within HAstV-8.

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