

Molecular Characterization of Astrovirus Infection in Children With Diarrhea in Beijing, 2005–2007

Li Guo,¹ Xiwei Xu,² Jingdong Song,³ Wei Wang,¹ Jianwei Wang,^{1*} and Tao Hung^{1,3}

¹State Key Laboratory of Molecular Virology and Genetic Engineering, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing, China

²Beijing Children's Hospital, Beijing, China

³National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

Human astroviruses (HAstVs) have been recognized as one of the major causes of acute gastroenteritis in children. To provide more insight into the prevalence of HAstV gastroenteritis in China, 664 fecal samples were collected from children affected with acute gastroenteritis in Beijing from March 2005 to November 2007. The samples were analyzed genetically. All eight serotypes (genotypes) of HAstVs were screened using RT-PCR assays targeting the ORF2 region in the study. The assays detected HAstVs in 52 (7.8%) of the patients, with HAstV-1 (50/52) being the dominant genotype during the study period. Two minor genotypes, HAstV-6 and HAstV-3, were also detected. Partial sequencing of the 50 HAstV-1 strains showed that the homology of the nucleotide sequence of the ORF1a region between these strains was 88.4–100%, whereas the homology of the amino acid sequences was 95.6–100%. In the ORF2 partial region, the nucleotide identities ranged from 91.5% to 100%, and amino acid identities ranged from 97.3% to 100%. The identity of the whole genome sequence between four randomly examined HAstV-1 strains was 91–99%. No recombination events were observed in HAstVs in this study. The findings of this study will provide baseline data for HAstVs surveillance and control. **J. Med. Virol.** 82:415–423, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: genetic analysis; human astrovirus; viral gastroenteritis

INTRODUCTION

Human astroviruses (HAstVs), members of the family *Astroviridae*, have been recognized as one of the most common causes of viral gastroenteritis in young children [Mendez and Arias, 2007; Nguyen et al., 2008]. HAstV infections mainly cause watery diarrhea, less commonly fever, vomiting, anorexia, and abdominal

pain in children, elderly adults, and immunocompromised individuals [Moser and Schultz-Cherry, 2005; Finkbeiner et al., 2008]. The incidence of HAstV-induced gastroenteritis is about 2–9% in children in both developed and developing countries [Mustafa et al., 2000; Guix et al., 2002; Chen et al., 2007; Liu et al., 2007], indicating the importance of HAstV gastroenteritis control for public health.

HAstVs are non-enveloped viruses of 28–30 nm in diameter and contain a 6–8 kb positive-sense, single-stranded RNA genome [Mendez and Arias, 2007]. Three open-reading frames (ORFs), that is, ORF1a, ORF1b, and ORF2, have been identified in the viral genome. ORF1a encodes a serine protease, ORF1b encodes an RNA-dependent polymerase, and ORF2 encodes a capsid precursor protein [Mendez and Arias, 2007]. HAstVs can be grouped into eight serotypes (HAstV-1 to HAstV-8), mainly based on the reactivities of the capsid proteins with polyclonal and monoclonal antibodies [Mustafa et al., 2000; Mendez and Arias, 2007; Resque et al., 2007; Finkbeiner et al., 2008]. HAstVs can also be grouped into genotypes based on the nucleotide sequence of the capsid region. A good correlation between serotype and genotype has been reported in previous studies, and genotyping based on molecular tests has been used to represent serotyping of the same virus strain [Noel et al., 1995; Mustafa et al., 2000].

HAstV-1 is the most common serotype of HAstV identified in children [Gabbay et al., 2007a; Soares et al., 2008]. However, predominant serotypes can vary with time and geographical location [Glass et al., 1996].

Li Guo and Xiwei Xu contributed equally to this work.

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*Correspondence to: Jianwei Wang, 9 # Dong Dan San Tiao, Beijing 100730, China. E-mail: wangjw28@163.com

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Data on prevalence, serotyping, and molecular characteristics of HAstV infections from different areas can provide insight into HAstV epidemics and lead to better surveillance and control of HAstV infections. Although two studies have reported the infection of HAstV in young children in Beijing [Liu et al., 2004, 2006], the molecular characteristics and circulation of HAstVs in the country have not been investigated completely.

To provide more insight into the prevalence of HAstV in China, the frequency and the principal circulating genotypes of HAstVs were examined in acute pediatric gastroenteritis cases in the city of Beijing for the time period between March 2005 and November 2007 using RT-PCR. HAstV-1 was identified as the predominant serotype throughout the study period. The genetic characteristics of the HAstV-1 strains were investigated by genome sequencing as well as by phylogenetic analysis.

MATERIALS AND METHODS

Clinical Specimens

Fecal samples were collected from pediatric patients who visited the outpatient clinic and the emergency room of the Beijing Children's Hospital from March 2005 to November 2007 [Guo et al., 2009]. Most samples were collected from October of a given year to March of the next year. Only a small portion of specimens were collected in other months during the study period. All selected cases had been diagnosed with acute gastroenteritis. Acute gastroenteritis was defined as having acute watery diarrhea, accompanied by other clinical signs and symptoms such as fever, nausea, vomiting, abdominal cramps, and normal or low leukocyte count. All patients were selected randomly by five clinicians whom the patients visited. The fecal samples were stored at -80°C prior to use.

Viral RNA Extraction

Viral nucleotides were extracted from the sample supernatants (10% in PBS, pH 7.2) using Trizol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's instructions. After extraction, nucleotides were dissolved in DEPC-treated H_2O and stored at -80°C prior to use.

Screening and Genotyping of HAstV Infections

HAstV strains were screened and genotyped by RT-PCR. A partial region of ORF2 was amplified with primers Mon269 and Mon270, generating a product with a predicted size of 449-nt as described elsewhere [Noel et al., 1995]. At the same time, Mon340 and Astman-2 primers [Rohayem et al., 2004] were used to amplify a partial region of ORF1a, producing a 345 bp amplicon. All RT-PCR amplicons were cloned into the pMD-18T vector (Takara, Dalian, China) and sequenced on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences of the PCR

amplicons were aligned with the corresponding HAstV sequences deposited in the GenBank database using BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Full-Length Genome Sequencing

To obtain the complete genomic sequence of identified HAstV-1 strains, random PCR was employed to recover cDNA fragments corresponding to viral genomes using primers FR26RV-N (GCCGGAGCTCTGCAGATATC-NNNNNN) and FR20RV (GCCGGAGCTCTGCAGATATC) as previously described [Allander et al., 2005]. The PCR products were separated on an agarose gel. Subsequently, fragments of 500–1,500 bp in length were excised and purified using the Agarose Gel DNA Purification Kit (TaKaRa). All purified products were cloned into the pMD18-T vector (TaKaRa) and transformed into chemically competent *E. coli* DH5 α . Transformants were randomly selected for DNA sequencing. The sequences were identified by blast analysis against the corresponding HAstV sequences from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Rapid amplification of cDNA ends (RACE) reactions were performed to obtain the actual 5'- and 3'-terminal sequences of the viral genome using the 5'- and 3'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's protocol.

Similarity Analysis of HAstV Genomes

Similarity plots showing the relationships among the aligned nucleotide sequences of viral genomes were created using SimPlot software version 3.5.1 (<http://sray.med.som.jhmi.edu/SCSoftware>) [Lole et al., 1999]. Similarity was calculated in each window of 200 bp using the Kimura two-parameter method with the concatenated genome sequence of the HAstV strains of Beijing as query, and with the concatenated viruses of HAstV-1 (GenBank accession numbers AY720892, L23513, and Z25771), HAstV-2 (GenBank accession number L13745), HAstV-3 (GenBank accession number AF141381), HAstV-4 (GenBank accession numbers AY720891, DQ070852, and DQ344027), HAstV-5 (GenBank accession number DQ028633), and HAstV-8 (GenBank accession number AF260508).

Phylogenetic Analysis

Multiple sequence alignments were created using the Clustal W and MegAlign programs in the DNASTar software package. HAstVs phylogenetic trees with 1,000 bootstrap replicates were created using the neighbor-joining method and the Kimura two-parameter model with MEGA software version 4.0 [Tamura et al., 2007].

Nucleotide Sequence Accession Numbers

Nucleotide sequences obtained in this study were deposited in GenBank (accession numbers FJ755364 through FJ755401 and GQ169027 through GQ169036 for the partial ORF2 genes, FJ755338 to FJ755363 for

the partial ORF1a gene region, and FJ755402 through FJ755405 for the complete genomes).

RESULTS

Prevalence of HAstV Infections in Children in Beijing

For this study, a total of 664 fecal samples were collected from pediatric patients (380 male and 284 female) suffering from acute gastroenteritis. Patients were aged from one month to 13 years (median age of 9.5 months, and average age of 11.9 month). The majority (95%) of patients were ≤ 2 years old. Out of the 664 fecal specimens, 52 (7.8%) tested positive for HAstV. This detection rate is comparable to that determined in previous years [Liu et al., 2004, 2006]. There was no significant difference ($P = 0.112$) in the detection rates between male (4.1%) and female (3.3%) of HAstV-positive patients, indicating that gender does not play a role in the susceptibility to HAstV infection. The ages of HAstV-positive patients ranged from 1 to 25 months (median age of 8.8 months, and average age of 11.3 months), and the majority (90%) of them were ≤ 2 years old.

Genotyping of HAstVs

Phylogenetic analysis based on a partial ORF2 nucleotide sequence revealed that 50 out of the 52 HAstV strains detected in this study were HAstV-1. The remaining two HAstV strains were identified as belonging to HAstV-6 and HAstV-3 (Fig. 1).

The HAstV-1 viruses detected in this study could be divided into a major viral strain cluster (comprising 48 strains) and a minor viral strain cluster (comprising 2 strains; Fig. 1). The major viral cluster strains exhibit 95.3–98.7% nucleotide identity to a Japanese strain (GenBank accession number AB009985, isolated in 1997) [Wang et al., 2001], and are also similar (95.4–98.3% nucleotide identity) to the strains found in Wuhan [Liu et al., 2007] and Shanghai [Shan et al., 2009]. In contrast, the minor viral cluster strains display 98.7% identity to a German strain (GenBank accession number AY720892, isolated in 2004). The nucleotide identity among the HAstV-1 strains identified in this study and that of the references, which include a UK strain (the earliest isolate of HAstV-1, GenBank accession number Z25771, isolated in 1990) [Willcocks et al., 1990], a US strain (GenBank accession number L23513, isolated in 1994) [Lewis et al., 1994], the Japanese strain, and the German strain was shown in Table I. Because the years the UK strain and the US strain were isolated are significant earlier than strains isolated for this study, and because the genetic distance between the UK/US strains and the Japanese strain is similar to that between the UK/US strains and the German strain, the UK/US strains may be the ancestor of the Chinese strains identified in this study, the Japanese strain (1997), and the German strain (2004).

Variation of HAstV-1

HAstV-1 strains were analyzed based on the sequences of two partial genes, ORF1a and ORF2. The results showed that, among the strains detected in this study, the nucleotide identity for the ORF1a region ranged from 88.4% to 100%, with an amino acid identity of 95.6–100%; whereas the nucleotide identity for the ORF2 ranged from 91.5% to 100%, with an amino acid identity of 97.3–100%.

To characterize the variation of identified HAstV-1 more precisely, the full-length genome sequences were analyzed from four strains (termed 128-BJ05, 176-BJ06, 291-BJ07, and 293-BJ07) randomly selected from the major (128-BJ05, 291-BJ07, and 293-BJ07) or minor (176-BJ06) viral strain clusters. The complete genome sequences of three viral ORF regions (ORF1a, ORF1b, and ORF2), the 5'-NTR, and the 3'-NTR were then evaluated by the Clustal W and MegAlign programs in the DNASTar software package using the full-length genome sequences deposited in GenBank, that is, the UK strain (GenBank accession number Z25771), the German strain (GenBank accession number AY720892,) and the US strain (GenBank accession number L23513) as references (Fig. 2 and Table II). The homologies of the complete nucleotide sequences among the four strains were 91–99%. The 5'-NTR (85 nt) sequences had the highest nucleotide variation, with homologies of 84.2–98.8%.

It has been recognized that there is a variable region downstream of the conserved region (the N-terminal 415 amino acids) of the ORF2 capsid polypeptide sequences among different serotype HAstV strains [Méndez-Toss et al., 2000]. To evaluate the variability of HAstV-1 strains further, the consensus of the full-length ORF2 genes were analyzed between the four identified strains. Pairwise amino acid comparisons revealed that the identities of the N-terminal 415 amino acid sequences were as high as 97.3–99.8% among the four HAstV-1 strains, whereas sequences encoding the downstream amino acid 416–788 region showed higher variation, with amino acid sequence identities of 93.1–98.6%. The eight carboxy-terminal amino acids were highly conserved for all four strains, consistent with a previous report [Willcocks et al., 1995]. For the two short variable regions, VR1 (aa 292–319) and VR2 (aa 387–399) [Méndez-Toss et al., 2000], variations ($V_{301} \rightarrow A_{301}$, $T_{307} \rightarrow V_{307}$) were observed only in the VR1 domain of 176-BJ06 and 291-BJ07. The same variations were also found in a Germany HAstV-1 strain (GenBank accession number AY720892) (Fig. 3A). No variations were observed in the VR2 region (Fig. 3B).

Recombination Analysis

To clarify whether recombination is a mechanism responsible for HAstV-1 variation, the similarities between the four HAstV-1 Beijing strains identified in this study and HAstV-1, HAstV-2, HAstV-3, HAstV-4, HAstV-5, and HAstV-8 references were analyzed at the genome level by similarity plotting (Fig. 4). The plots

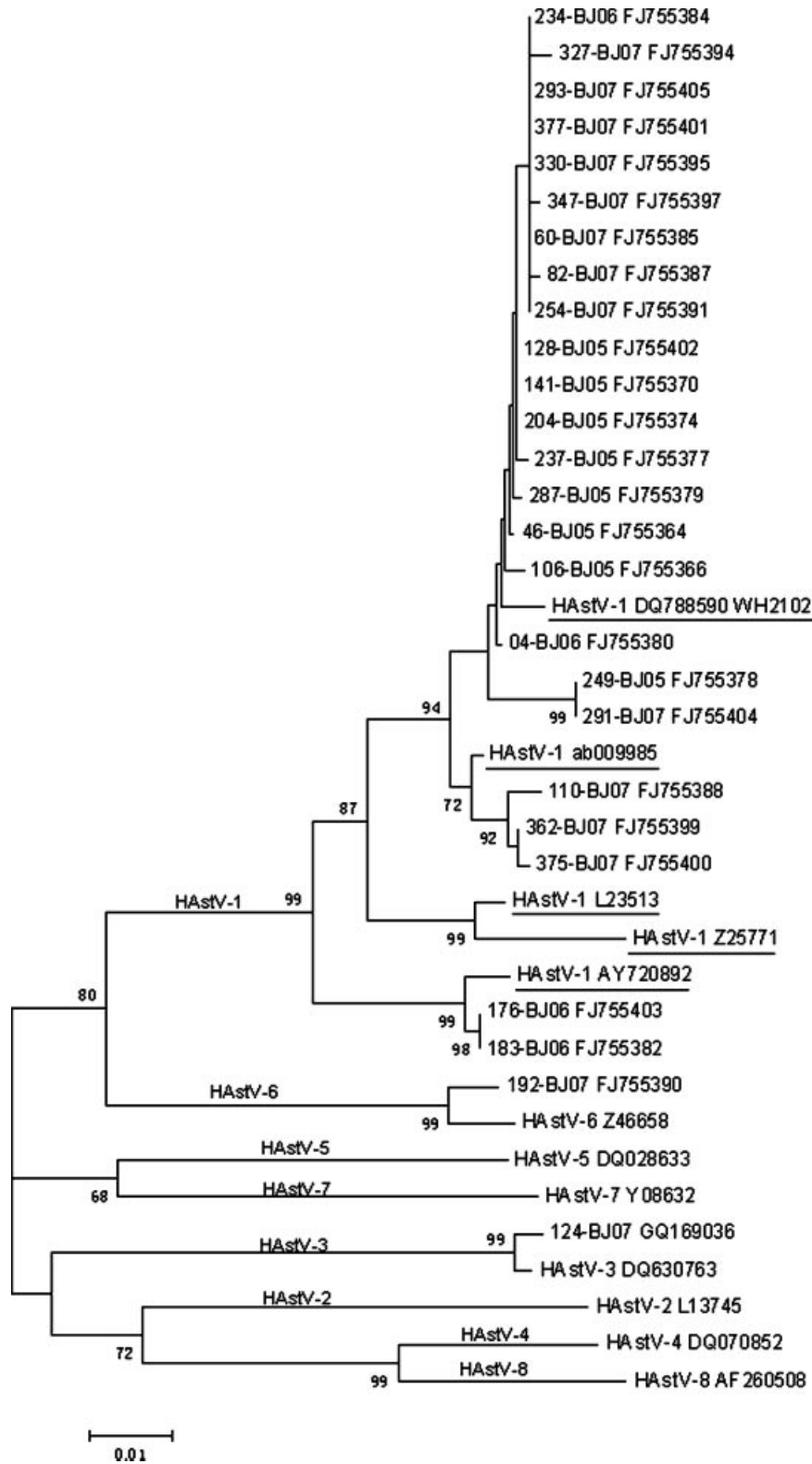


Fig. 1. Phylogenetic analysis of HAstVs based on sequence analysis of the ORF2 regions. The phylogenetic tree with 1,000 bootstrap replicates was generated by using the neighbor-joining method and the Kimura two-parameter model with MEGA software version 4.0 based on nucleotide sequences from 24 randomly selected representatives of the 50 HAstV-1 strains as well as two minor strains, HAstV-3 and HAstV-6, identified in this study. The geographic region and year of the isolated HAstV strains are indicated in the strain name. BJ, Beijing.

TABLE I. Pairwise Nucleotide Sequence Comparisons of Partial ORF2 Between HAstV-1 Strains

Strains	Nucleotide sequence identity (%)					
	Major cluster in this study	Minor cluster in this study	UK strain (Z25771)	US strain (L23513)	Japanese strain (AB009985)	German strain (AY720892)
Major cluster in this study ^a	94.2–100	91.5–92.2	91.1–92.9	92.2–95.1	95.3–98.7	90.4–92.9
Minor cluster in this study ^b		100	90	90.4	92.2	98.7
UK strain (Z25771)			100	97.1	92.4	90.4
USA strain (L23513)				100	95.1	90.9
Japanese strain (AB009985)					100	91.3
German strain (AY720892)						100

^aThe major cluster comprises 48 strains.

^bThe minor cluster comprises 2 strains.

showed high identities of ORF1a and ORF1b between the four strains as well as between the HAstV-1, HAstV-2, HAstV-3, HAstV-4, HAstV-5, HAstV-8 strains. The highest sequence identities were observed in ORF2 between the Beijing strains and the HAstV-1 strains, in contrast to the great differences observed between HAstV-1 strains and other serotypes. There was no clear evidence of genetic recombination between the

four strains selected in the study and other genotypes of HAstV strains.

DISCUSSION

To provide a more comprehensive overview of the molecular characterization of the HAstVs currently circulating in Beijing, a larger scale (664 samples)

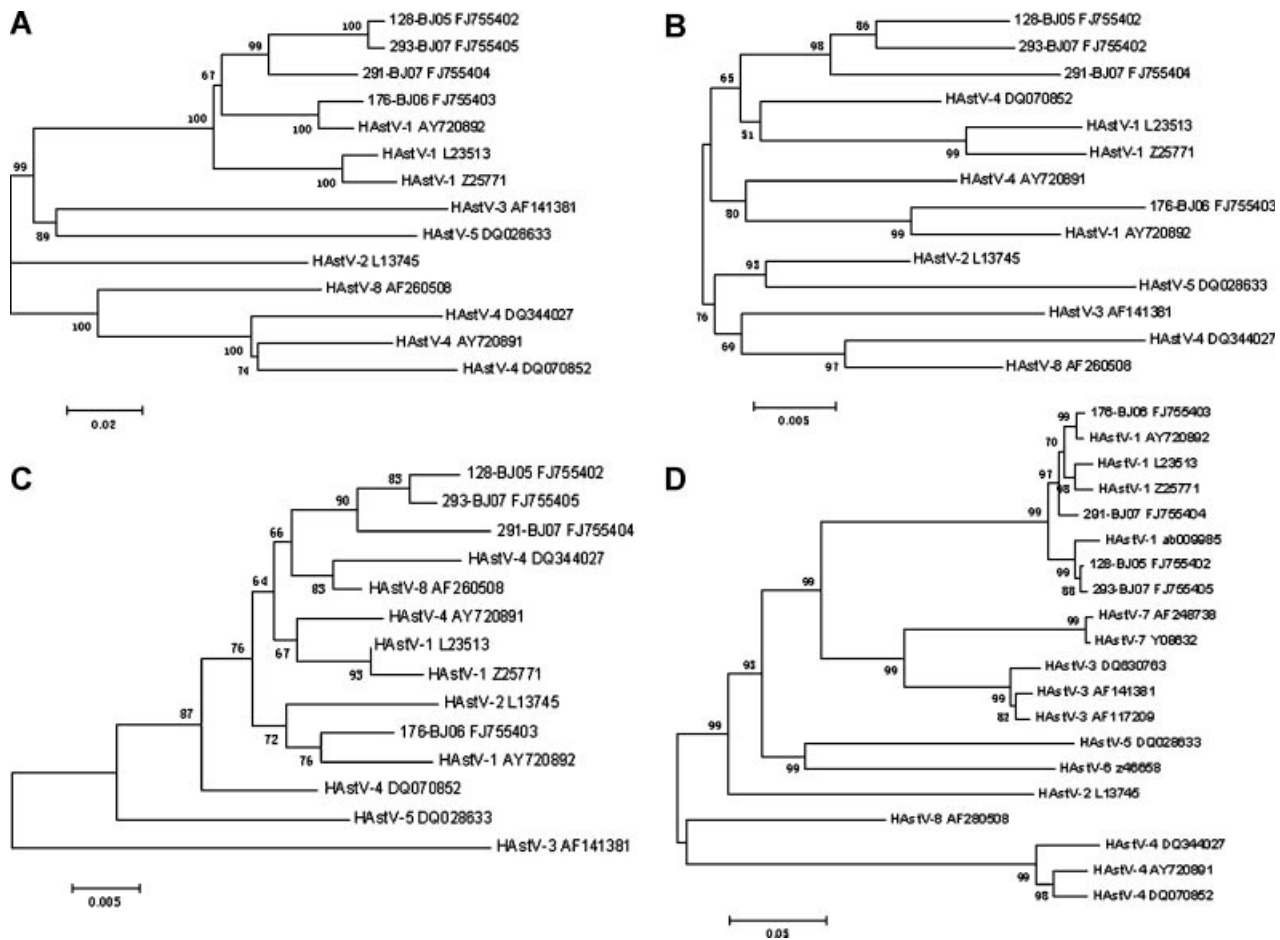


Fig. 2. Phylogenetic analysis of HAstV-1 strains based on full-length genome sequencing. The phylogenetic trees with 1,000 bootstrap replicates were generated using the neighbor-joining method and the Kimura two-parameter model with MEGA software version 4.0 based on nucleotide sequences of full-length genomes (A) and on amino acid sequences of the ORF1a (B), ORF1b (C), and ORF2 (D) regions.

TABLE II. Sequence Comparisons of Genome Regions Among HAstV-1 Strains

HAstV-1 strains	Source	Percent identity											
		5'-NTR		ORF1a		ORF1b		ORF2		3'-NTR			
		Nucleotide	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide
Viruses detected in this study	Beijing, China	84.2-98.8	90.7-98.9	95.2-98.3	92.4-99.0	97.1-99.0	90.3-99.3	95.4-99.1	90.7-98.9				
Z25771	UK	58.8-80.0	89.9-90.6	95.1-96.4	92.7-93.6	97.3-97.7	91.1-93.0	95.3-97.6	98.8-100				
AY720892	Germany	84.7-98.8	91.2-97.4	95.1-96.2	92.5-98.0	96.9-98.4	90.3-98.3	95.4-99.0	98.8-100				
L23513	USA	67.1-82.4	90.4-90.7	95.8-97.6	93.2-94.5	97.5-97.9	91.5-93.4	94.4-96.7	98.8-100				
AB009985	Japan	/	/	/	/	/	91.2-95.1	95.2-98.1	/				

screening of HAstV infection were conducted in children suffering from acute gastroenteritis. Furthermore, the genetic characteristics of multiple HAstV strains by both partial and complete viral genome sequencing were analyzed. The results showed that HAstVs were frequently associated with pediatric cases of gastroenteritis in Beijing during the study period, with a detection rate of 7.8%. HAstV-1 was the predominant serotype, consistent with reports from most parts of the world at present [Mendez and Arias, 2007], and with a previous report from Beijing [Liu et al., 2004] as well as with data from other areas in China [Liu et al., 2007; Shan et al., 2009]. The percentage of HAstV-1 in this assay (7.5%) is higher than that found in Brazil [Gabbay et al., 2007a], but lower than that found in Vietnam [Nguyen et al., 2008].

Nucleotide variations among the HAstV-1 strains identified in Beijing over a nearly 3-year period varied up to a maximum of 8.5% in the sequenced 449-nt region of the ORF2. Nucleotide sequence divergence in the sequenced 449-nt region between the HAstV-1 strains detected in Beijing and the UK strain (GenBank accession number Z25771) varied up to 10% (Table I). However, the variations of the amino acid sequences for the same region is lower (1.3-4%). Similar to previous findings from other countries [Mustafa et al., 2000; Schnagl et al., 2002; Gabbay et al., 2007a], 8 of the 50 HAstV-1 strains identified in this study differed by only one amino acid residue and 3 strains differed by only two amino acid residues from the US strains (GenBank accession number L23513). None of the nucleotide differences were reflected in amino acid changes in the remaining 37 identified HAstV-1 strains. Among four randomly selected HAstV-1 isolates, the difference of the complete nucleotide sequences were up to maximum of 9%, whereas amino acid differences of the ORF1a, ORF1b, and ORF2 region were 4.8%, 2.9%, and 4.6%, respectively. These data suggest that HAstV-1 was undergoing variation over the period of sample collection in Beijing.

Two variable regions in the ORF2 of HAstV, VR1 and VR2, have been used to evaluate the variation of HAstVs. When the documented sequences of HAstV-1 were compared to the VR1 and VR2 amino acid sequences of four HAstV-1 strains of Beijing, changes were only found in the VR1 region. These data suggest that variations in VR1 and VR2 exist among different serotypes [Méndez-Toss et al., 2000], and the variations within the same serotypes may be little [Liu et al., 2008].

Recombination plays an important role in the evolution of RNA viruses as it generates genetic variation and ultimately produces new virus strains and types [Worobey and Holmes, 1999]. Recombination is frequently observed in many RNA viruses, such as in coronaviruses [Woo et al., 2005], influenza A viruses [He et al., 2009], enteroviruses [Smura et al., 2007], and noroviruses [Bull et al., 2007; Guo et al., 2009]. Recombination between HAstV strains has not been seen except in one study that reported a potential recombination site at the ORF1b/ORF2 junction in a

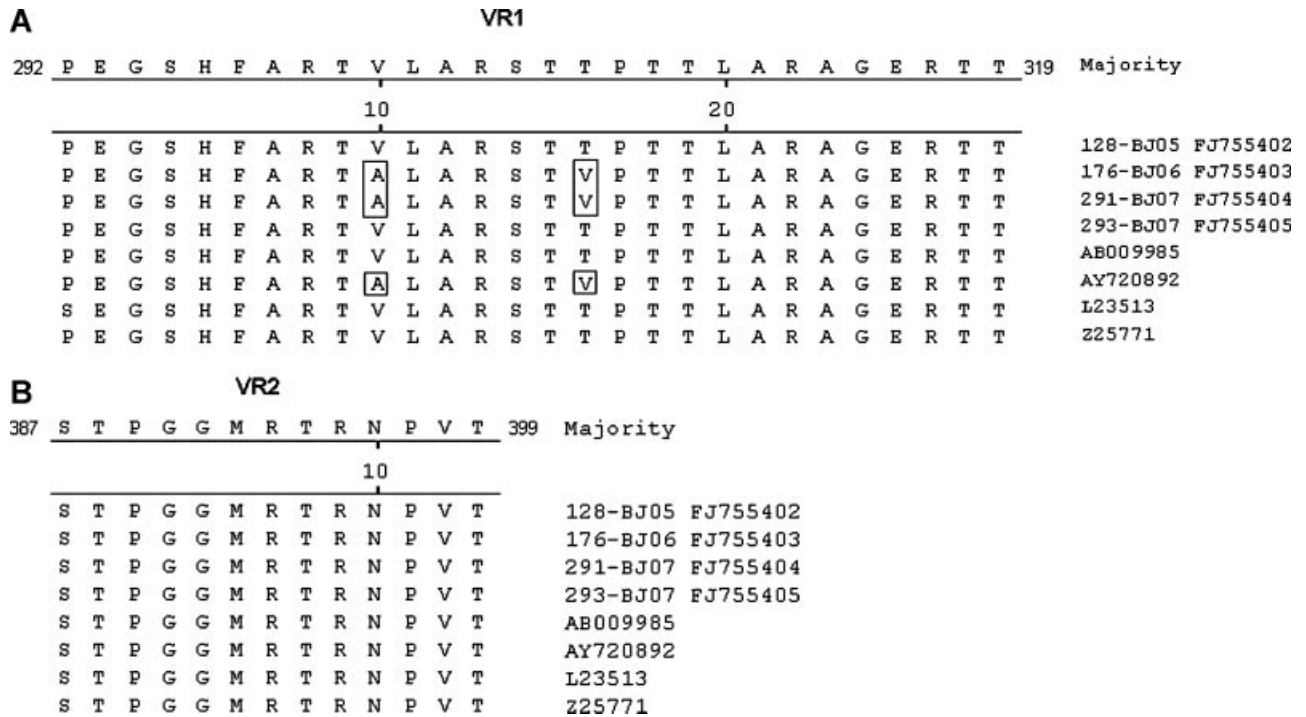


Fig. 3. Alignment analysis among ORF2 variable regions (VRs) of HAstV-1 strains. The alignment of VR1 (A) and VR2 (B) among Beijing HAstV-1 strains was generated by DNASTar software using known sequences of HAstV-1 strains as references (GenBank accession numbers AB009985, AY720892, L23513, and Z25771).

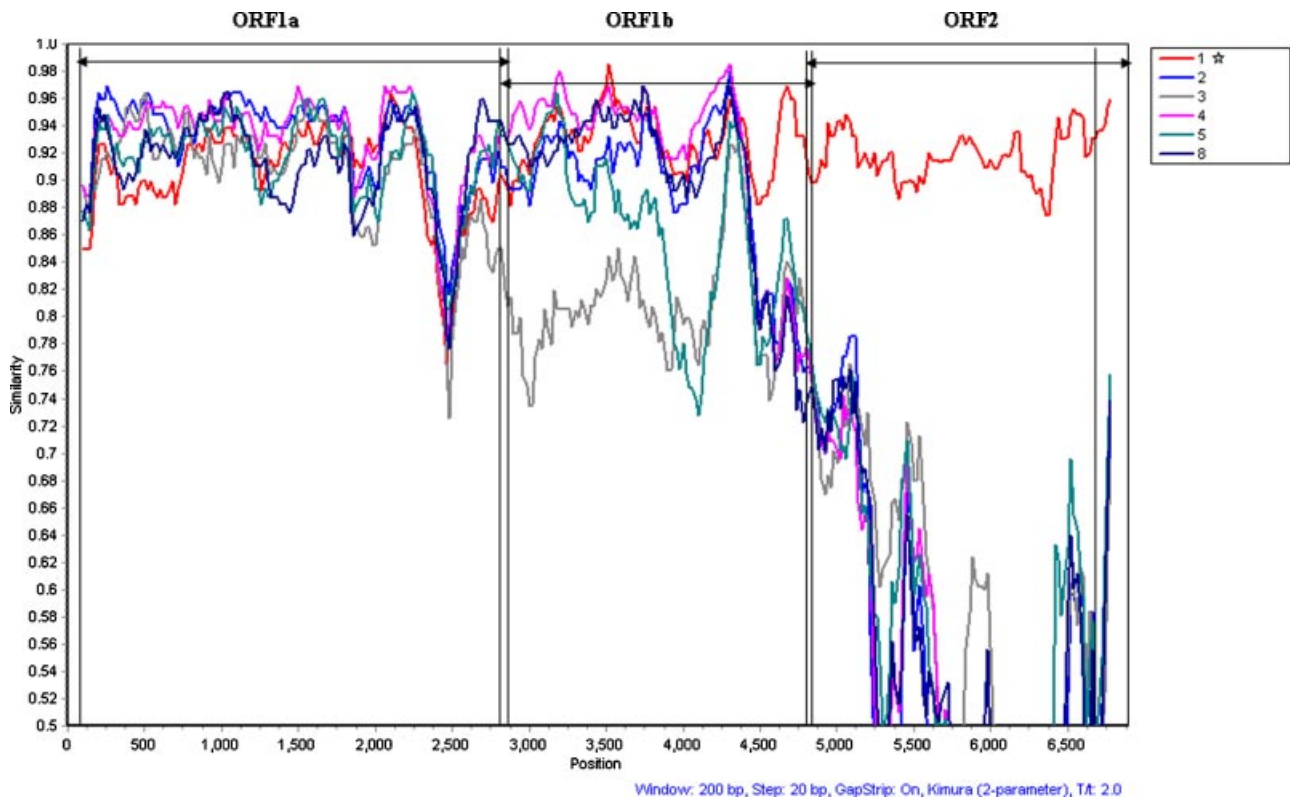


Fig. 4. Similarity analysis of HAstVs based on complete genome sequences. Similarity plots were created using SimPlot software version 3.5.1. Data are represented as a percentage of the identity to the putative parental strains. 1, 2, 3, 4, 5, and 8 represent all obtained, complete genomes of HAstV-1 (GenBank accession numbers AY720892, L23513, and Z25771), HAstV-2 (GenBank accession number L13745), HAstV-3 (GenBank accession number AF141381), HAstV-4 (GenBank accession numbers AY720891, DQ070852, and DQ344027), HAstV-5 (GenBank accession number DQ028633), and HAstV-8 (GenBank accession number AF260508), respectively.

novel HAstV strain [Walter et al., 2001]. However, when the HAstV-1 strains isolated in this study were analyzed for recombination using similarity plots, no recombination events were observed. Therefore, similar to other reports, these data suggest that recombination may not be a major mechanism responsible for HAstV variation.

Aside from HAstV-1, which is the most frequently observed HAstV strain in most parts of the world, two minor genotypes (HAstV-6 and HAstV-3) were also detected in this study. HAstV-6 infections have been identified previously in the United Kingdom, Brazil, and Germany [Lee and Kurtz, 1994; Noel et al., 1995; Gabbay et al., 2007b; Silva et al., 2008], but no HAstV-6 infection has been reported in China before this study. A HAstV-3 infection had previously been identified in Wuhan, China [Liu et al., 2007], but not yet in Beijing. The emergence of the HAstV-6 and HAstV-3 in Beijing indicates the need to monitor HAstV gastroenteritis epidemics caused by other serotypes in addition to the overwhelmingly dominant HAstV-1. To provide further insight into the genetics and molecular evolution of astroviruses, a full-length genome sequence and corresponding analysis for HAstV-3 and HAstV-6 would need to be performed.

Although no large HAstV epidemic has been reported in Mainland China, sporadic infections are common. This study shows that the HAstV-1 dominant strain in Beijing is similar to those in Wuhan [Liu et al., 2007] and Shanghai [Shan et al., 2009]. Nevertheless, because these observations are derived from research based on a limited sample number and geographical scale, they may not completely reflect the features of HAstVs circulating in China. A nationwide, large-scale investigation over a longer time span would be necessary to characterize HAstV infections accurately throughout the country.

In summary, we have investigated the prevalence, predominant genotype and molecular features of HAstVs associated with acute gastroenteritis in Beijing over a nearly 3-year period. These findings not only demonstrate a predominance of HAstV-1 infections, but also reveal the presence of minor genotypes, HAstV-6 and HAstV-3, in Beijing. Genetic analysis of multiple HAstV genome sequences shows that nucleotide variations exist among the HAstV-1 strains and provides a basis to build on in the fight against HAstV gastroenteritis.

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