Journal of Medical Virology 80:306–315 (2008)

Genetic Analysis of the Hypervariable Region of the Human Astrovirus nsP1a Coding Region: Design of a New RFLP Typing Method

Susana Guix, Santiago Caballero, Cristina Fuentes, Albert Bosch,* and Rosa M. Pintó

Enteric Virus Laboratory, Department of Microbiology, University of Barcelona, Barcelona, Spain

Human astroviruses (HAstV) are causative agents of viral gastroenteritis worldwide. A hypervariable region (HVR) is located close to the C-terminus of the nsP1a, and recent data support the involvement of the HVR-containing nonstructural protein in viral RNA replication processes, suggesting a correlation between variability in this region and pathogenic properties. The HVR of the C-terminal nsP1a coding region of 104 wild-type and reference isolates of HAstV was sequenced. A phylogenetic analysis was performed to identify different genotypes, and a restriction fragment length polymorphism (RFLP) method was designed. An extensive nucleotide and deduced amino acid sequence variability was observed, as well as many insertions and deletions that retained the reading frame. The resultant phylogenetic tree supported the subdivision of HAstV into the two previously described major genetic groups, genogroup A and B, and the identification of 12 genotypes (9 within genogroup A, and 3 within genogroup B), which could be identified by RFLP. A correlation analysis was performed between genotype information and viral load using information from 35 clinical samples. Significant differences were observed between the viral load in clinical samples and certain HAstV genotypes that belonged to the same serotype, confirming the influence of C-terminal nsP1a variability on the viral replication phenotype. The use of the new RFLP typing method based on the HVR of the C-terminal nsP1a coding region by diagnosticians would help to understand the relationship between different genotypes and the severity of the gastroenteritis. ***J. Med. Virol. 80:306–315, 2008.***

KEY WORDS: astrovirus; hypervariable region; RFLP; genotyping

INTRODUCTION

Human astroviruses (HAstV) are causative agents of viral gastroenteritis worldwide mainly in children [Glass et al., 1996; Walter and Mitchell, 2003]. These nonenveloped positive-strand RNA viruses belong to the family *Astroviridae*, which includes both mammalian and avian astroviruses. The astrovirus genome consists of a 6.8-kb polyadenylated genome with three overlapping open reading frames (ORFs); ORF1a and ORF1b are linked by a translational ribosomal frameshifting and encode the viral protease and polymerase, respectively, and ORF2 encodes the structural proteins [Matsui and Greenberg, 2001].

On the basis of antigenic criteria, HAstV are divided into eight serotypes (HAstV-1 to HAstV-8). Phylogenetic analysis of different parts of the genome result in different genetic clustering [Belliot et al., 1997; Méndez-Toss et al., 2000; Taylor et al., 2001; Lukashov and Goudsmit, 2002; Silva et al., 2006]. Most studies based on the capsid region suggest a high correlation between serotypes and genotypes, a relationship which allows determining serotype information by sequence analysis. The comparison of sequences from the capsid region gives rise to radial tree topologies with equidistant clustering of serotypes. Interestingly however, phylogenetic analysis of the well-conserved partial sequence close to the protease motif coding region, results in only two clearly differentiated genogroups, called genogroup A (HAstV-1 to HAstV-5, and HAstV-8) and genogroup B (HAstV-6 and HAstV-7). This different phylogenetic clustering may be the result of recombination events between structural and nonstructural coding regions [Belliot et al., 1997].

Grant sponsor: European Union (partial support); Grant number: SP22-CT-2004-502571; Grant sponsor: Generalitat de Catalunya; Grant number: 2005SGR00966; Grant sponsor: Centre de Referència de Biotecnologia de Catalunya (CeRBa); Grant sponsor: Generalitat de Catalunya.

Santiago Caballero's present address is Department of Research and Development, Instituto Grifols, S.A., Parets del Vallés, Spain.

*Correspondence to: Albert Bosch, Department of Microbiology, University of Barcelona, Avda Diagonal 645, 08028 Barcelona, Spain. E-mail: abosch@ub.edu

Accepted 20 September 2007

DOI 10.1002/jmv.21058

Published online in Wiley InterScience (www.interscience.wiley.com)

None of the suggested cleavage sites within nonstructural polyprotein (nsPs) has been confirmed by N-terminal sequencing, and both cellular proteases and the viral encoded protease are responsible for the proteolytic maturation of nsP1a and nsP1a1b to their final products [Gibson et al., 1998; Willcocks et al., 1999; Geigenmüller et al., 2002; Kiang and Matsui, 2002; Méndez et al., 2003]. Apart from the protease and the RNA polymerase, the role of each of the other nonstructural mature proteins is still not fully understood. Close to the C-terminus of nsP1a coding region there is a hypervariable region (HVR) [Oh and Schreier, 2001; Méndez et al., 2003; Silva et al., 2006], and recent data support the involvement of this HVR-containing protein (hereafter referred to as C-terminal nsP1a protein) in viral RNA replication processes, as well as to different RNA loads in feces from children with gastroenteritis, suggesting a correlation between certain HAstV types and some viral properties related to its pathogenic phenotype [Guix et al., 2004, 2005]. In addition, an association between a selected type and cases of clinical persistent gastroenteritis has also been observed [Caballero et al., 2003; Guix et al., 2005].

While typing of HAstV has so far been mainly of epidemiological interest, the improved knowledge on differences in virulence among several types of viruses provides motivation for investigating new typing methods and increases the medical value of typing. In this study, the genetic diversity within the HVR of the C-terminal nsP1a coding region, which could modulate differences in viral RNA replication efficiency, and a new rapid astrovirus typing method by restriction fragment length polymorphism (RFLP) analysis are presented.

MATERIALS AND METHODS

HAstV Specimens

A panel of 80 positive stool samples and CaCo-2 cell-adapted strains was collected from different epidemiological studies. Wild-type HAstV from stools of children with gastroenteritis included 51 samples isolated in Spain [Guix et al., 2002], 7 samples isolated in the United Kingdom (kindly provided by W. D. Cubitt, Great Ormond Street Hospital for Children, London), 6 samples isolated in France (kindly provided by E. Kohli, Centre Hospitalier Universitaire, Dijon, France), and 1 sample isolated in Albania (kindly provided by M. Divizia, II Universitá di Roma Tor Vergata, Italy). Twenty-four CaCo-2 cell-adapted strains from The Netherlands, United Kingdom, and United States, as well as prototype strains belonging to all serotypes were employed. Finally, 10 HAstV complete genome sequences available at the Genbank were also included in the analysis (Acc. no.: L23513 for HAstV-1 Oxford reference strain; Z25771 for HAstV-1 Newcastle strain; AY720892 for HAstV-1 Dresden strain; L13745 for HAstV-2 Oxford reference strain; AF141381 for HAstV-3 Berlin strain; DQ070852 for HAstV-4 Goiana strain BrG4; DQ344027 for HAstV-4 Guangzhou strain; AY720891 for HAstV-4 Dresden strain; DQ028633 for HAstV-5 Goiana strain BrG5; and AF260508 for HAstV-8 Yuc-8 strain).

HAstV nsP1a HVR Amplification and Sequencing

Viral RNA was extracted from 50 μl of cell lysates or 10% (w/v) stool suspensions, using a previously described method [Boom et al., 1990], and the HVR region was amplified by RT-PCR using 5 μl of the extracted RNA. RT-PCR was carried out using primers A1 and A2 as described elsewhere [Willcocks et al., 1994; Guix et al., 2002]. Primers A1bis (5′-CCTGCCCC-CCGTATAATTAAAC-3′) and A2bis (5′-ATAGGACTCC-CATATAGGTGC-3′), which correspond to primers A1 and A2 with some modifications, were designed and used to allow specific amplification of isolates belonging to genogroup B. RT-PCR conditions for primers A1bis/A2bis were identical to primers A1/A2, but hybridization time was extended to 1 min at 55°C. All samples were independently analyzed using both pairs of primers. RT-PCR products of the expected size, ranging from 189 to 237 bp, were visualized by electrophoresis on a 1.5% agarose gel, and after purification of RT-PCR products using a commercial kit (High Pure PCR Product Purification Kit, Roche Diagnostics, Basel, Switzerland), they were directly sequenced in an ABI Prism 377 automated DNA sequencer using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA). Both forward and reverse primers were used for sequencing reactions.

Genetic Data Analysis of the nsP1a HVR Region

Sequence alignments were performed using CLUSTAL W [Higgins et al., 1994]. In nucleotide alignments, positions of insertions and deletions (*indels*) were corrected manually according to their position in amino acid alignments. Nucleotide and amino acid distances were calculated as the number of substitutions per site (*p-dist*) using pairwise deletion for treating *indels* with the MEGA2.1 program [Kumar et al., 1993], as well as Ks and Ka values according to the Nei–Gojobori method, where Ks is the frequency of synonymous substitutions per synonymous site and Ka is the frequency of nonsynonymous substitutions per nonsynonymous site [Nei and Gojobori, 1986]. Phylogenetic analysis were carried out with the MEGA2.1 program, after generating distance matrixes using the Kimura 2-parameters method [Kimura, 1980], and treating *indels* with the pairwise deletion option in both cases. Phylogenetic trees were constructed using the UPGMA and neighbor joining methods after performing a bootstrap of 100 replicates. HAstV isolates were classified into genotypes, using the following four criteria to define a genotype. First, samples were divided according to their pattern of *indels*. Second, after constructing a phylogenetic tree, genotypes were established so that mean nucleotide distances within groups were lower than 10%, and mean nucleotide

distances between groups were higher than 10%. Third, all identified genotypes should provide a bootstrap value >50, and fourth, they should include more than one isolate to be considered significant.

HAstV RFLP Typing Assay

Following the purification of the RT-PCR product obtained with primers A1/A2 or A1bis/A2bis, approximately 200 ng of DNA were digested overnight at 37°C with 1 U of *Dde*I restriction enzyme. Digested products were analyzed on an 8% acrylamide gel after ethidium bromide staining.

HAstV Protease Coding Region Sequences From Genbank

Besides the complete genome sequences available at the Genbank, a collection of sequences from the well-conserved protease coding region from different epidemiological studies in Korea [Kang et al., 2002], and South Africa [Taylor et al., 2001], as well as the Oxford prototype strains [Walter et al., 2001] were used in this study (Accession numbers: AF361028, AF361029, AF361030, AF361031, AF361032, AF361033, AF361034, AF361035, AF361036, AF290504, AF290505, AF290506, AF290507, AF290508, AF290509, AY027809, AB242158, AB242159, AB211059, DQ139812, DQ139813, DQ139814, DQ139815, DQ139816, DQ139817, DQ139818, AY962540, AY962541, AY962542, AY962543, AY962544, AY962545, AY962546, AY962547, AY962548, AY962549, AY962550, AY962551, AB194280, AB191789, AB126670, AB126671, AB126672, AB126673, and AB126674).

HAstV 5′ ORF2 Sequencing and Serotyping

Serotype information of isolates was determined by sequencing 348-bp of the 413-bp RT-PCR amplicon obtained after amplification with primers Mon244 and Mon245, according to the procedure previously described [Noel et al., 1995].

Statistical Analysis

Statistical comparisons between means from different groups were performed using the ANOVA analysis.

Accession Numbers

Nucleotide sequences determined in this study have been deposited in GenBank under the accession numbers EF195385 to EF195478.

RESULTS

Sensitivity and Specificity of HVR RT-PCR

The specificity of primers A1/A2 and A1bis/A2bis was determined by using RNA extracted from HAstV-1 to HAstV-7 prototype strains and a HAstV-8 strain isolated in The Netherlands, as well as nucleic acid from uninfected CaCo-2 cells, human rotavirus type 3, strain Ito^r and human enteric adenovirus type 40. Primers A1/A2 amplified only serotypes HAstV-1 to HAstV-5, and HAstV-8 (genogroup A), while primers A1bis/A2bis amplified only serotypes HAstV-6 and HAstV-7 (genogroup B). No amplicons were obtained with the heterologous templates. The estimated detection limit of both RT-PCR reactions was 15 RNA molecules [Caballero et al., 2003].

Genetic Characterization of the HVR Variability

Sequence heterogeneity of the HVR within the C-terminal nsP1a gene was analyzed in a panel of 104 HAstV strains, and compared to the variability found in other regions of the genome employed by two widely used HAstV detection methods [Noel et al., 1995; Belliot et al., 1997]. Genetic variability parameters calculated for the data sets corresponding to the three genomic regions are summarized in Table I. Although sample size (number of isolates and sequence length) was not homogenous between groups, remarkable differences could be detected for most parameters. The nucleotide genetic distance (p-dist) within the HVR was higher than within the protease coding region and comparable to the capsid region. In contrast, amino acid distance within HVR was more than three times higher than that in the ORF2. Regarding synonym and non-synonym substitutions, the HVR displayed a relatively low Ks but an extremely high Ka, resulting in a Ks/Ka ratio of 3.93, which was fourfold lower than that of the capsid coding region. Finally, it is interesting to note the high number of insertions and deletions (*indels*) found within the HVR, which resulted in variability on the RT-PCR product size ranging from 192 to 237 bp with primers A1/A2, and 189 bp with primers A1bis/A2bis. A deduced amino acid alignment of the 56 different sequences observed among the 104 isolates analyzed during the study is depicted in Figure 1, showing the extremely high degree of variability as well as the different *indels* variable in both size and position. All *indels* were multiple of three nucleotides, confirming the location of a coding sequence.

TABLE I. Genetic Analysis of Three Different Genomic Regions Used in Molecular Typing Methods

	Genomic region		
	Protease[a]	HVR C-term nsP1a[b]	N-term ORF2[c]
n	55	104	98
Length (bp)	198	146–194	348
nt position L23513	1,229–1,426	2,385–2,533	4,574–4,921
No. of *indels*	0	7	0
p-dist (nt)	0.117	0.196	0.201
p-dist (aa)	0.039	0.175	0.042
Ks	0.374	0.377	0.560
Ka	0.018	0.096	0.031
Ks/Ka	20.77	3.93	18.06

HVR, hypervariable region.
[a]Belliot et al. [1997].
[b]Present work.
[c]Noel et al. [1995].

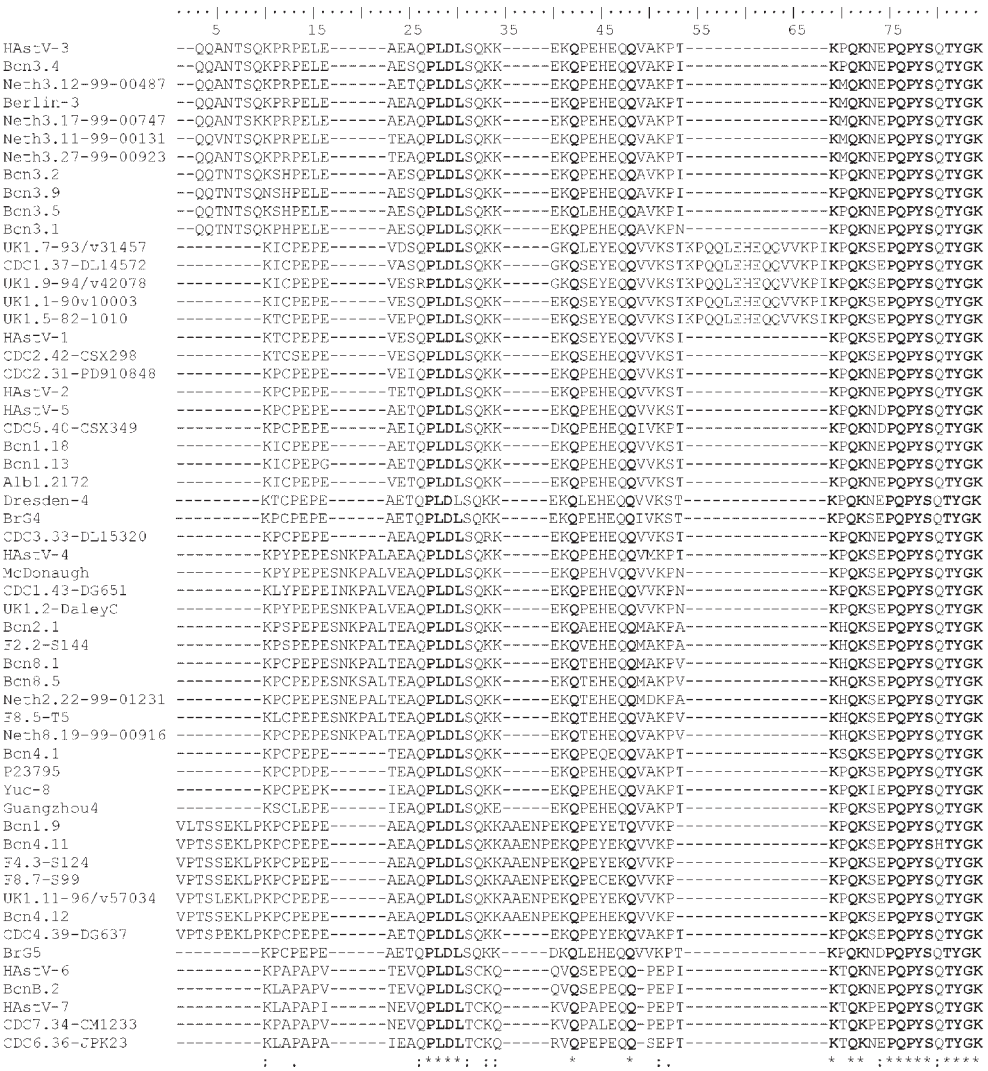

Fig. 1. Deduced amino acid alignment of the HVR region, including all 56 different sequences found during the study (- indicates a missing amino acid, * indicates a conserved amino acid,: indicates a conservative substitution, and . indicates a semi-conservative substitution). Completely conserved amino acids are depicted in bold.

Phylogenetic Analysis of HVR, Genotype Classification, and Design of a RFLP Typing Method

A neighbor joining phylogenetic analysis of 104 sequences generated two clearly differentiated genogroups, as previously described based on the well-conserved protease coding region [Belliot et al., 1997]: genogroup A included serotypes HAstV-1 to HAstV-5 and HAstV-8, while genogroup B included serotypes HAstV-6 and HAstV-7 (data not shown). Mean genetic distances (p-dist) between the two genogroups were 0.32 and 0.35 for nucleotides and amino acids, respectively.

However, since one of the main features of the HVR genetic variability was the presence of many *indels*, and since gap sites are ignored in most distance estimation methods, HVR-derived genotypes were first established according to the size of the obtained RT-PCR product. Within each of these groups, UPGMA trees were generated, and different genotypes were identified following the criteria described in the Materials and Methods Section, resulting in a total of 12 genotypes, which were named using roman numerals (Fig. 2). While in most cases, all samples belonging to the same genotype also shared the same serotype, there were six genotypes that included isolates belonging to more than one serotype, as for example genotype HAstV-II, which included samples of serotypes 2 and 8, or HAstV-VI, which included samples of serotypes 1, 4, and 8. These results indicate that recombination events could have occurred between different types, and suggest that recombination between particular serotypes may be more common than between others.

At the same time, the sequences obtained were used to select a specific restriction enzyme for discrimination purposes in a diagnostic assay. A total of 24 distinct

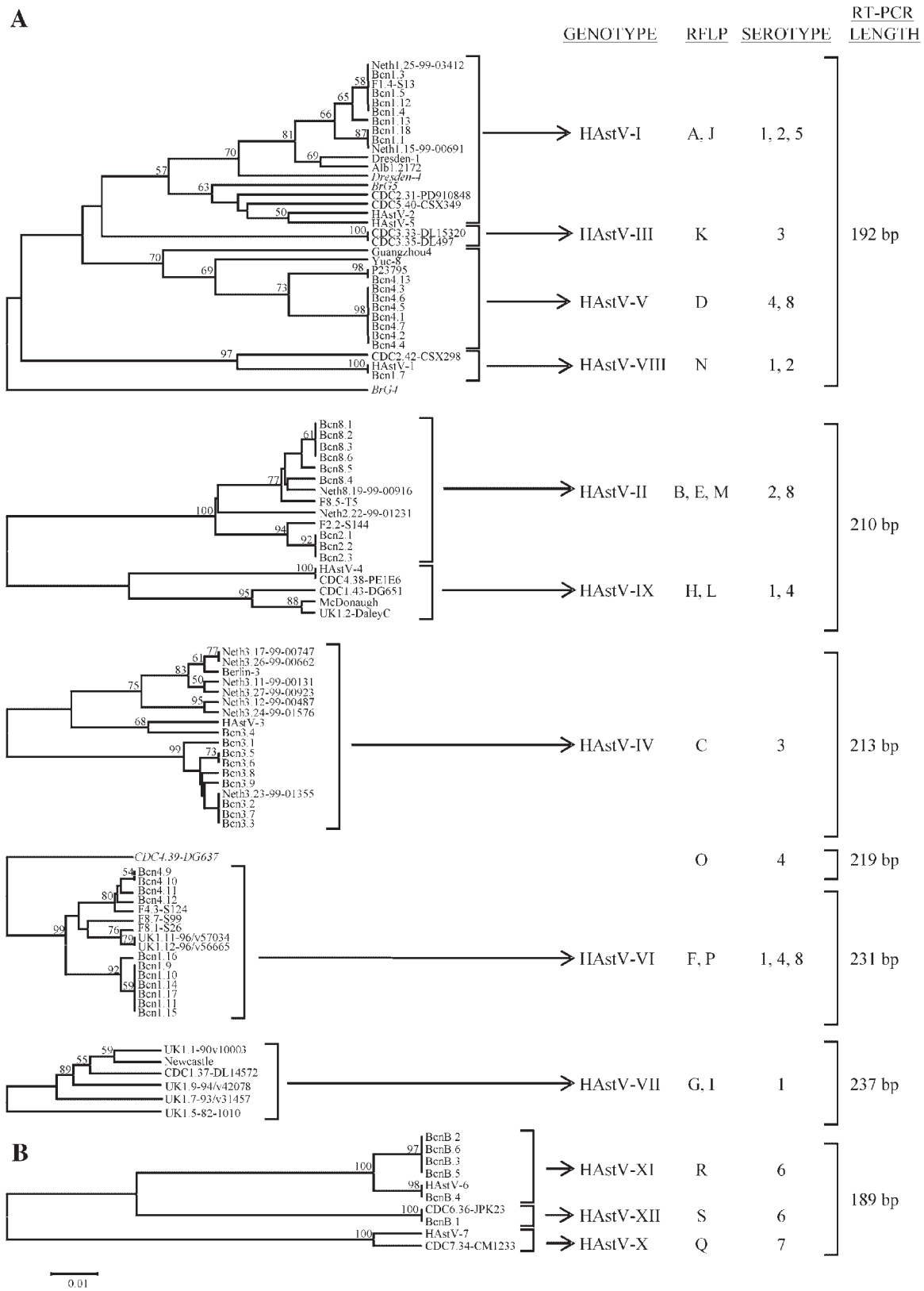

Fig. 2. UPGMA phylogenetic analysis of the HVR of HAstV. Established HVR-derived genotypes within genogroup A (**A**) and B (**B**) are shown, as well as the different RFLP patterns found within each genotype and the molecular size of the RT-PCR product. Unclassified sequences are shown in italics. The genetic cluster containing isolate *CDC4.39-DG637*, which produced an RT-PCR product of 219 bp was not considered a genotype because only one sequence was available. Numbers at each node of the tree show bootstrap percentages obtained after 100 replicates, which are higher than 50. Scale bar is shown as the number of substitutions per site.

RFLP patterns were identified, and capital letters were assigned to them. The use of the *Dde*I restriction endonuclease allowed the differentiation of all HVR-derived genotypes. Figure 3A shows the main characteristics of each pattern, as well as the serotypes contained within each pattern and its frequency in the representative HAstV collection used in the study. The most prevalent patterns corresponded to C, F and A (16%, 15%, and 11%, respectively). Results of RFLP analysis of six isolates are shown in Figure 3B, as an example.

A

RFLP	RT-PCR Length (bp)	*Dde*I restriction sites	Fragments (bp)	Serotype	Frequency (%)
A	192	3	35/36/57/64	1	11
B	210	5	35/30/24/42/9/70	2	4
C	213	1	92/121	3	16
D	192	2	71/57/64	4, 8	10
E	210	4	35/30/24/42/79	8	8
F	231	4	98/15/48/6/65	1, 4, 8	15
G	237	1	173/64	1	5
H	210	1	89/121	1	3
I	237	2	128/45/64	1	1
J	192	3	71/57/15/49	2, 5	5
K	192	2	35/36/121	3	2
L	210	1	146/64	4	2
M	210	3	35/30/75/70	2	1
N	192	1	128/64	1, 2	4
O	219	2	98/57/64	4	1
P	231	3	113/48/6/65	1	1
Q	189	1	119/70	7	2
R	189	2	47/63/79	6	6
S	189	1	110/79	6	2

B

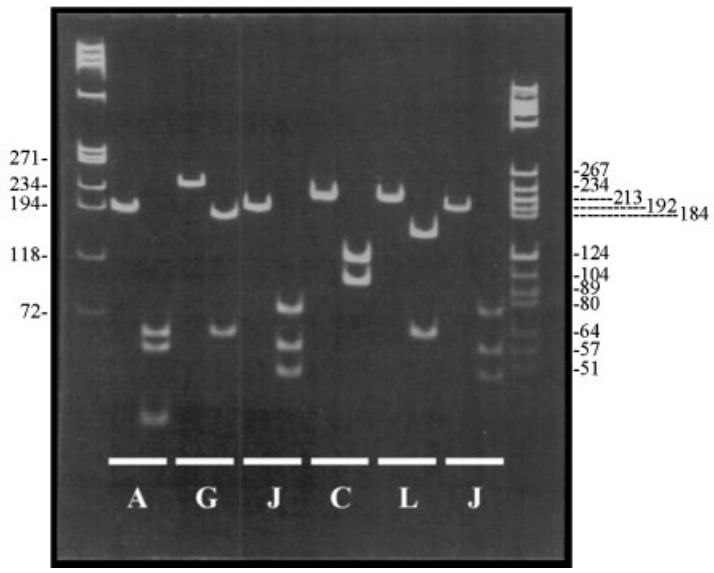

Fig. 3. RFLP analysis after *Dde*I digestion of the RT-PCR amplicon corresponding to the HVR of HAstV. **A**: Characterization of RFLP patterns found during the study: length of the amplicon, number of restriction sites, resulting digestion products and frequency according to the collection analyzed. **B**: Example of RFLP analysis of six samples on 8% TBE-acrylamide gel. For each sample, the first lane corresponds to nondigested RT-PCR product, and the second to digested DNA. Molecular size DNA markers in basepairs (bp) are shown on both sides of the gel.

Relationship Between HVR-Derived Genotypes, Serotypes, and Viral Load in Stool Specimens

An unrooted phylogenetic tree was constructed using the 348-bp sequences from the ORF2 region used to infer the serotype information (Fig. 4). Within a particular serotype, samples belonging to different HVR-derived genotypes clustered together with a high bootstrap value, indicating that within serotypes, different genetic lineages correlate with variability on the HVR contained in the genome region coding for one of the viral nonstructural proteins. Unfortunately, it was not possible to amplify the 5′ ORF2 region of samples belonging to genogroup B following the procedure described by Noel et al. [1995].

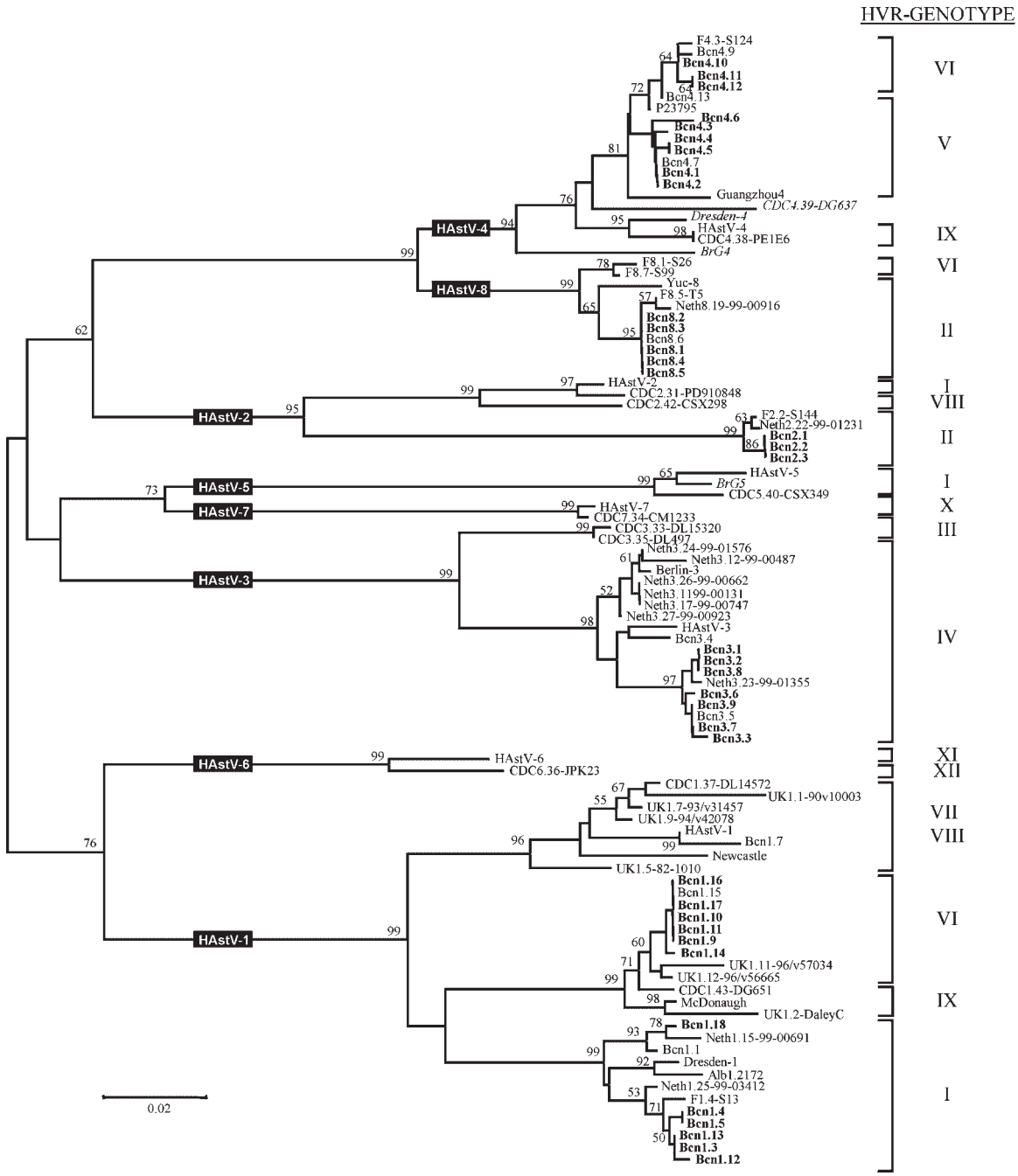

Fig. 4. Phylogenetic analysis based on the 348-bp sequence from the 5′ end of ORF2 [Noel et al., 1995], using a neighbor joining Kimura 2-parameters and a bootstrap of 100 replicates. Within each serotype, HVR-genotypes are indicated. Isolates in bold correspond to stool samples which viral load was quantified. Unclassified sequences are shown in italics. Numbers at each node of the tree show bootstrap percentages higher than 50. Scale bar is shown as number of substitutions per site.

Information on the virus load present in stool suspensions could be obtained for a subset of 35 clinical specimens and has been published elsewhere [Caballero et al., 2003]. These studies concluded that the mean viral titer in serotype 3-containing feces was higher than in any of the other serotype-containing samples, and reported that 42.9% of astrovirus 3 isolates were implicated with cases of persistent diarrhea, some of them lasting for 3 months. The time of stool collection did not influence the observed differences regarding viral titer, since all samples were collected between days 1 and 3 after the onset of disease. In the present study, within each serotype, differences in viral load in fecal samples were further investigated depending on the HVR genotype information, and a clear association between these two parameters was found. The subset of 35 samples for which viral load was examined (depicted in bold in Fig. 4) contained samples belonging to serotypes 1, 2, 3, 4, and 8. Serotypes 1 and 4 contained samples belonging to more than one genotype. Figure 5 shows the mean viral load observed for each HVR genotype. While within serotype 1 no differences in titer were observed between genotypes I and VI, within serotype 4 there was a statistically significant difference between genotypes V and VI, indicating that besides serotype, HVR genotype may influence viral excretion levels. Interestingly, no differences were found in viral loads between serotypes 2 and 8, which belonged to the same HVR genotype II, nor within genotype VI, which contained samples belonging to serotypes 1 and 4. Overall, our data indicate that samples belonging to genotypes IV and V were excreted at significantly higher titers than other genotypes.

DISCUSSION

In this study, we examined the sequence heterogeneity of the HVR of the HAstV C-terminal nsP1a gene, using a large collection of isolates from different long-term studies and from disperse geographical locations. Since C-terminal nsP1a protein has been shown to be related to the RNA replication process, and its variability may affect its functional properties [Guix et al., 2005], a genetic analysis of this HVR has allowed us to develop a typing method to trace these viral differences.

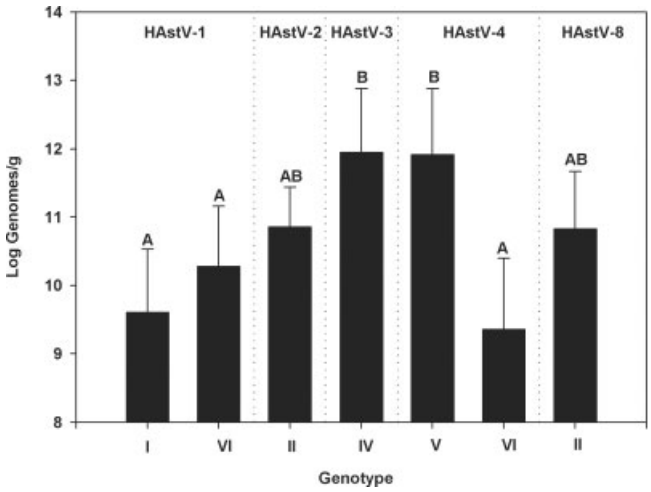

Fig. 5. Analysis of HAstV viral load according HVR genotype for samples belonging to serotypes HAstV-1, HAstV-2, HAstV-3, HAstV-4, and HAstV-8. Data plotted represent the mean of the $\log_{10}$ genome copies/g of stool. Capital letters indicate statistical differences ($P < 0.05$).

Despite information of viral load present in stool was not available for all genotypes, we could associate certain genotypes with increased excretion. Isolates belonging to genotypes IV and V were excreted in stools at significantly higher levels than other genotypes whilst the level of viral excretion of genotypes I and VI, which included samples from two different serotypes, 1 and 4, was significantly lower. Although not significantly different, the level of excretion of genotype II, which includes samples from serotypes 2 and 8 was also lower. While belonging to different serotypes, the fact that these samples shared the same HVR genotype could probably explain their similar level of viral excretion. Consistently, in a previous study using recombinant viral mutants it was observed that HVR genotypes IV and V replicated with significantly higher levels of subgenomic RNAs in CaCo-2 cells, resulting in higher levels of infectious progeny, while genotype VI produced lower levels of subgenomic RNA and infectious progeny [Guix et al., 2005]. Recently, a quantitation study of HAstV by real-time RT-PCR reported that children coinfected with rotavirus had lower viral RNA titer in stool samples than those without coinfection, as well as a correlation between higher viral load and longer duration of diarrhea for the episode [Zhang et al., 2006]. In the present study, all samples from children that were coinfected with other pathogens were excluded from the analysis. Although Zhang et al. [2006] did not show a trend of change of RNA titer with change of serotype of infection, the association between HVR-derived genotype and viral load for those was not analyzed. While in a previous study we found that protracted diarrhea was associated with higher titers of serotype 3 [Caballero et al., 2003], we know nowadays that the correlation is actually with genotype, and particularly in that case with genotype IV. In addition, the enterotoxin-like properties of the astrovirus capsid have been very recently reported, being dose-dependent but serotype-independent [Moser et al., 2007]. Since the role of the C-terminal nsP1a protein in regulating the levels of subgenomic RNA has also been described [Guix et al., 2005] and subgenomic RNA directs the level of capsid synthesis, an association of this protein with the toxic phenotype is foreseen. To confirm this hypothesis the disruption of the actin cytoskeleton as well as the relocalization of the tight junction protein occludin induced by different genotypes should however be tested.

The comparison of genetic parameters between the HVR and other regions of the viral genome revealed that unlike most nonstructural viral proteins, C-terminal nsP1a protein can easily tolerate the occurrence of

amino acid substitutions, as well as *indels*. In general, genomic regions where nucleotide structures play key biological roles can display a genetic heterogeneity that is higher at the amino acid level than at the nucleotide level. The high degree of amino acid variability would be the result of the need to maintain certain secondary structures in the viral RNA genome. However, since all *indels* maintain the reading frame, and the C-terminal nsP1a protein can be detected using an antibody designed against the HVR [Guix et al., 2004], we believe that the biological function of the translated protein is also essential for the virus. Supporting this idea, some years ago, an epitope reacting against antiserum to purified viral particles was identified in the HVR of the genomic RNA [Matsui et al., 1993]. A recent analysis of rates of synonymous and nonsynonymous substitutions in astrovirus genes also showed that ORF1a HVR is prone to positive selection, which is something expected at domains in charge of communication and interaction with the environment, such as host range and immune responses [van Hemert et al., 2007]. Hypervariability in this region provides strong evidence for selective pressure in favor of changes in the C-terminal nsP1a coding sequence that confers an advantage for the virus. Accordingly, a 15 amino acid deletion described some years ago in the HVR region in several HAstV isolates after human embryo kidney cell adaptation seemed to be necessary to enable the virus to grow in LLC-MK2 cells [Willcocks et al., 1994].

Based on genetic variability of the HVR, 12 genotypes have been established, and a RFLP typing method was developed to consistently distinguish between genotypes. Our results support the validity of the RT-PCR/RFLP-based method for the typing of HAstV in clinical and epidemiological settings. In addition, the RFLP assay provides a reliable and sensitive system that can easily be applied to all kinds of samples when rapid identification of astrovirus is needed. Unlike other methods based on sequencing reactions, the RFLP enables the coidentification and typing of different viruses present within a sample, which is particularly important for environmental virology.

ACKNOWLEDGMENTS

S. Guix was recipient of a FI fellowship from the Generalitat de Catalunya. We gratefully acknowledge D. W. Cubitt (Great Ormond Street Hospital for Children, UK), M. Divizia (Universitá di Roma Tor Vergata, Italy), E. Kohli (Centre Hospitalier Universitaire, France), and M. Koopmans (Rijksinstituut voor Volksgezondheld en Milieu RIVM, The Netherlands) for providing specimens. We also acknowledge the technical expertise of Núnà Pujol and the Serveis Científic-Tècnics of the University of Barcelona.

REFERENCES

Belliot G, Laveran H, Monroe SS. 1997. Detection and genetic differentiation of human astroviruses: Phylogenetic grouping varies by coding region. Arch Virol 142:1323–1334.

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28:495–503.

Caballero S, Guix S, El-Senousy WM, Calico I, Pintó RM, Bosch A. 2003. Persistent gastroenteritis in children infected with astrovirus: Association with serotype-3 strains. J Med Virol 71:245–250.

Geigenmüller U, Chew T, Ginzton N, Matsui SM. 2002. Processing of nonstructural protein 1a of human astrovirus. J Virol 76:2003–2008.

Gibson CA, Chen J, Monroe SA, Denison MR. 1998. Expression and processing of nonstructural proteins of the human astroviruses. Adv Exp Med Biol 440:387–391.

Glass RI, Noel J, Mitchell D, Herrmann JE, Blacklow NR, Pickering LK, Dennehy P, Ruiz-Palacios G, de Guerrero ML, Monroe SS. 1996. The changing epidemiology of astrovirus-associated gastroenteritis: A review. Arch Virol 12:287–300.

Guix S, Caballero S, Villena C, Bartolomé R, Latorre C, Rabella N, Simó M, Bosch A, Pintó RM. 2002. Molecular epidemiology of astrovirus infection in Barcelona, Spain. J Clin Microbiol 40:133–139.

Guix S, Caballero S, Bosch A, Pintó RM. 2004. C-terminal nsP1a protein of human astrovirus colocalizes with the endoplasmic reticulum and viral RNA. J Virol 78:13627–13636.

Guix S, Caballero S, Bosch A, Pintó RM. 2005. Human astrovirus C-terminal nsP1a protein is involved in RNA replication. Virology 333:124–131.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Kang YH, Park YK, Ahn JB, Yeun JD, Jee YM. 2002. Identification of human astrovirus infections from stool samples with diarrhea in Korea. Arch Virol 147:1821–1827.

Kiang D, Matsui SM. 2002. Proteolytic processing of a human astrovirus nonstructural protein. J Gen Virol 83:25–34.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Kumar S, Tamura K, Nei M. 1993. MEGA: Molecular evolutionary genetics analysis. University Park, PA: Pennsylvania State University.

Lukashov VV, Goudsmit J. 2002. Evolutionary relationships among Astroviridae. J Gen Virol 83:1397–1405.

Matsui SM, Greenberg HB. 2001. Astroviruses. In: Fields BN, Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. Fields virology. Lippincott Williams & Wilkins. Philadelphia PA: pp 875–893.

Matsui SM, Kim JP, Greenberg HB, Young LM, Smith LS, Lewis TL, Herrmann JE, Blacklow NR, Kupuis K, Reyes GR. 1993. Cloning and characterization of human astrovirus immunoreactive epitopes. J Virol 67:1712–1715.

Méndez E, Salas-Ocampo MP, Munguia ME, Arias CF. 2003. Protein products of the open reading frames encoding nonstructural proteins of human astrovirus serotype 8. J Virol 77:11378–11384.

Méndez-Toss M, Romero-Guido P, Munguia ME, Méndez E, Arias CF. 2000. Molecular analysis of a serotype 8 human astrovirus genome. J Gen Virol 81:2891–2897.

Moser LA, Carter M, Schultz-Cherry S. 2007. Astrovirus increases epithelial barrier permeability independently of viral replication. J Virol 81:11937–11945.

Nei M, Gojobori T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molec Biol Evol 3:418–426.

Noel JS, Lee TW, Kurtz JB, Glass RI, Monroe SS. 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. J Clin Microbiol 33:797–801.

Oh D, Schreier E. 2001. Molecular characterization of human astroviruses in Germany. Arch Virol 146:443–455.

Silva PA, Cardoso DD, Schreier E. 2006. Molecular characterization of human astroviruses isolated in Brazil, including the complete sequences of astrovirus genotypes 4 and 5. Arch Virol 151:1405–1417.

Taylor MB, Walter J, Berke T, Cubitt WD, Mitchell DK, Matson DO. 2001. Characterisation of a South African human astrovirus as type 8 by antigenic and genetic analyses. J Med Virol 64:256–261.

van Hemert FJ, Lukashov VV, Berkhout B. 2007. Different rates of (non-)synonymous mutations in astrovirus genes; correlation with gene function. Virol J 4:25.

Walter JE, Mitchell DK. 2003. Astrovirus infection in children. Curr Opin Infect Dis 16:247–253.

Walter JE, Mitchell DK, Guerrero ML, Berke T, Matson DO, Monroe SS, Pickering LK, Ruiz-Palacios G. 2001. Molecular epidemiology of human astrovirus diarrhea among children from a periurban community of Mexico City. J Infec Dis 183:681–686.

Willcocks MM, Ashton N, Kurtz JB, Cubitt WD, Carter MJ. 1994. Cell culture adaptation of astrovirus involves a deletion. J Virol 68: 6057–6058.

Willcocks MM, Boxall AS, Carter MJ. 1999. Processing and intracellular location of human astrovirus non-structural proteins. J Gen Virol 80:2607–2611.

Zhang Z, Mitchell DK, Afflerbach C, Jakab F, Walter J, Zhang YJ, Staat MA, Azimi P, Matson DO. 2006. Quantitation of human astrovirus by real-time reverse-transcription-polymerase chain reaction to examine correlation with clinical illness. J Virol Methods 134:190–196.