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Persistent Gastroenteritis in Children Infected With Astrovirus: Association With Serotype-3 Strains

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The relationship between cases of persistent diarrhoea and the levels and type of human astrovirus was investigated. The potential correlation between human astrovirus excretion levels and the occurrence of protracted gastroenteritis was elucidated after quantifying astroviruses in faecal samples by a competitive RT-PCR. This assay was developed employing an internal RNA standard constructed for this purpose and showed a threshold of positivity of 3.4×10^4 genomes per gram of faeces. By this procedure, the levels of astrovirus, belonging to serotypes 1, 2, 3, 4, and 8, in faecal samples could be ascertained to range from 3.4×10^8 to 1×10^{13} per gram of faeces. The mean viral titre in the serotype 3-containing faeces was higher than in any of the other serotype-containing samples. In children with no background disease, persistent gastroenteritis cases were detected in 8.5% of the astrovirus infections, and 37.5% of those were associated with astrovirus type 3 infection. In addition, 42.9% of astrovirus 3 isolates were implicated with persistent cases, some of them lasting for 3 months. Other type 3 isolates, detected in the faeces in very large numbers, caused severe gastroenteritis. ***J. Med. Virol. 71:245–250, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: epidemiology; quantification; competitive RT-PCR; stool

INTRODUCTION

Human astroviruses were first described in 1975 in stool specimens of newborns with gastroenteritis using electron microscopy [Appleton and Higgins, 1975]. Astrovirus infections occur worldwide and are most frequent in young children and the elderly [Matsui and Greenberg, 2001]. These viruses constitute a family of 27–40-nm non-enveloped viruses, *Astroviridae*, with a single-stranded positive RNA genome of about 6.8 kb [van Regenmortel et al., 2000]. The astrovirus genome includes three open reading frames (ORFs), ORF1a with a protease motif, ORF1b with a polymerase motif, and ORF2 coding for the capsid proteins [Matsui and Greenberg, 2001].

Human astroviruses are classified into seven serotypes based on the reactivity of capsid proteins with type-specific antibodies [Kurtz and Lee, 1984]. These 7 antigenic groups (serotypes) perfectly correlate with 7 genotypes that may be determined according to the nucleotide sequence of a 348-bp region of the ORF2 [Noel et al., 1995]. The existence of an eighth type has been suggested based on three complete capsid protein gene sequences deposited in GenBank.

Astrovirus infections induce a mild watery diarrhoea that typically lasts for 2–3 days, associated with vomiting, fever, anorexia, abdominal pain, and various constitutional symptoms that last for less than 4 days [Kurtz and Lee, 1987; Kurtz and Cubitt, 1989; Guix et al., 2002]. Protracted diarrhoea and viral shedding in immunocompromised patients have been described in several studies [Kurtz and Lee, 1987; Wood et al., 1988; Björkholm et al., 1995; Coppo et al., 2000]. Additionally, astroviruses have been associated with persistent gastroenteritis in non-immunocompromised individuals in a study conducted in Bangladesh, although no data were given on any specific serotype or strain link [Unicomb et al., 1998].

In a previous study [Guix et al., 2002], we determined the astrovirus prevalence in the Barcelona area. The overall incidence was 4.9%, with type 1 as the most prevalent followed by types 4, 3, 8, and 2. This study allowed us to gather a collection of strains belonging to the above-mentioned serotypes.

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In the present study, the incidence of persistent diarrhoea among the astrovirus-associated gastroenteritis cases and the potential relationship with a specific serotype were evaluated. The potential correlation between the virus numbers in faeces and some pathogenic aspects were also analysed. For this purpose, an internal RNA standard has been constructed and included in the amplification method used [Guix et al., 2002], in order to develop a competitive RT-PCR system to quantify astroviruses in faecal samples. This internal standard control may be used as a tracer of the presence of inhibitors of the RT-PCR procedure.

MATERIALS AND METHODS

Viruses and Cells

A cell-adapted strain (p23795) of human astrovirus serotype 4 (HAsV-4) was kindly provided by W.D. Cubitt (Great Ormond Street Hospital for Children, London), and propagated in CaCo-2 cells, as described previously [Pintó et al., 1994]. Viral stocks were obtained from the cell fraction at 72 hours post-infection by lysing the cells in TNE buffer (Tris-HCl 50 mM pH 7.4, NaCl 100 mM, EDTA 10 mM) supplemented with 1% of NP40.

Purification of HAsV-4 by Sucrose Gradient Centrifugation

Viral stocks were subjected to sonication (3×50 W for 30 s) and purified further in a sucrose gradient (0–45% wt/wt) in TNE buffer. The gradient was spun at 205,000*g* for 2 hr and 45 min at 4°C, and separated in fractions of 0.3 ml.

To detect the antigenicity of each fraction, a sandwich ELISA consisting of an antigen-capture through the MAb 8E7 and detection with a rabbit polyclonal anti-astrovirus antibody (kindly provided by Dorsey Bass from the Department of Pediatrics, Stanford University, Palo Alto, CA) was used. All the incubations were carried out in blocking buffer (PBS, 0.3% BSA, and 0.05% Tween-20) at 37°C, and the final detection performed with a peroxidase-labelled goat anti-rabbit antibody.

Nucleic acids from the antigenic fractions were extracted by Boom's method [Boom et al., 1990], blotted onto a nylon membrane and fixed under UV light. The presence of astrovirus genomes was assayed by hybridisation with a digoxigenin-labelled probe from a region of the ORF2 (nt 4,544–4,956 of the HAsV-1 Oxford reference strain Accession number L23513) at a final concentration of 25 ng/ml at 42°C. After washing the membrane under stringent conditions, detection was performed following the manufacturer's specifications with a chemiluminescent substrate (CSPD: Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate, Roche, Barcelona, Spain).

The number of infectious viruses in the antigenic fractions was determined by a method described previously [Abad et al., 1997] based on an integrated cell-culture RT-PCR (CC-RT-PCR). Briefly, tenfold dilutions of the antigenic fractions were inoculated on CaCo2 cell monolayers. At 72 hr post-infection, the presence of astrovirus RNA in the infected cell suspension was assayed by RT-PCR (see below), and expressed as CC-RT-PCRu/ml [Abad et al., 1997].

To confirm the presence of viral particles, the antigenic fractions were examined by electron microscopy (EM) of negatively stained suspensions. Fractions from each of the antigenic peaks were pooled separately and dialysed against TNE buffer. Ten microliters of the viral suspension were placed on carbon copper grids (400 mesh) for 15 min and stained with 2% phosphotungstic acid pH 6.5 (PTA) for 1 min. The grid was dried and observed in a Hitachi HT600 AB microscope. To quantify the number of physical particles, 10 μl of the viral suspension were mixed with 1 μl of a colloidal gold particle (42 nm) suspension (7.35×10^{10} colloids/ml). The number of viruses and colloidal gold particles present in several fields were counted and the number of viruses was estimated in relation to the known number of colloidal gold particles.

Construction of an Internal RNA Standard

An internal standard for the RT-PCR reaction described by Guix and co-workers [Guix et al., 2002] was generated. The starting material was the amplimer obtained after the RT-PCR of the cell-adapted HAsV-4 strain mentioned above. This RT-PCR procedure is based on the use of the previously described primers A1 (5′-CCTGCCCCGAGAACAACCAAGC-3′) and A2 (5′-GTAAGATTCCCAGATTGGTGC-3′) [Willcocks et al., 1994], which amplify a specific region, of variable size (192–237 bp) depending on the strain, of the ORF1a. Five microliters of a HAsV-4 suspension were heated at 99°C for 5 min and the cDNA was synthesised at 42°C for 60 min by adding 2 μM of primer A2, 0.2 mM of each dNTP, and 4 U of M-MLV reverse transcriptase (Promega, Inogenetics, Barcelona, Spain) in a 10-μl final volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT. Five microliters of the RT product were amplified by using 0.5 U of *Pwo* polymerase (Roche) in a final volume of 50 μl, containing 1 μM of each primer A1 and A2, 2 mM $MgSO_4$, and 0.2 mM of each dNTP. After a denaturation step of 3 min at 95°C, 40 cycles of amplification (94°C, 30 s; 55°C 30 s; 72°C, 30 s) followed by a final extension of 7 min at 72°C were performed. The use of the *Taq* polymerase was avoided in order to generate blunt-ended fragments.

The resulting 192-bp amplimer contained two *Dde I* restriction sites separated by 57 bp. After digestion of the amplimer with this restriction enzyme, a ligation of the resulting DNA fragments, of 71, 57, and 64 bp, was performed with the T4 ligase (Roche). Among all ligated products, a 135-bp DNA band, corresponding to the ligation of the 71- and 64-bp fragments, was purified from an agarose gel by using the High-Pure PCR Product (Roche). This DNA was cloned in the pCAP^{s} cloning vector using the Blunt-Ended PCR Cloning Kit (Roche) and transformed into competent *Escherichia coli* cells.

Clones containing the proper insert were identified by sequencing and used to produce RNA in an in vitro transcription reaction with the T7 RNA polymerase. A PCR using 0.5 U of the *Pwo* polymerase and 50 nM of the primers P1and P2, supplied with the Blunt-Ended PCR Cloning Kit, was carried out in a final volume of 50 µl, containing 0.2 mM of each dNTPs and 2 mM $MgSO_4$. Cycling conditions were performed following the manufacturer's instructions. The *Taq* polymerase was not used in order to avoid the generation of protruding ends, which could interfere in the in vitro transcription reaction [Triana-Alonso et al., 1995]. The resulting 511-bp blunt-ended amplicon was used in the in vitro transcription reaction with the T7 RNA polymerase. One microgram of the amplicon was in vitro transcribed in a 50-µl volume containing 50 U RNasin (Promega), 10 mM DTT, 0.5 mM rNTPs, and 20 U of the T7 RNA polymerase, for 2 hr at 37°C. The DNA was removed by digestion with RNase-free DNase I, and the RNA purified with phenol:chloroform:isoamylic alcohol (25:24:1), and precipitated with 7.5 M ammonium acetate: absolute ethanol (1:5). The RNA was suspended in 50 µl of RNase-free water and quantitated by spectrophotometry at a wave length of 260 nm. The amount of internal standard usually obtained after the in vitro transcription was around 100 ng RNA/µl which corresponded to 3.4×10^{11} RNA molecules/µl.

Competitive RT-PCR

Quantitation of human astroviruses was carried out by RT-PCR with primers A1/A2 in the presence of the internal RNA standard, thus establishing competitive conditions. The 135-bp internal standard amplimer is easily distinguishable from the viral amplimers whose length ranges from 192 to 237 bp, depending on the strain [Guix et al., 2002]. The sensitivity of detection of the RNA internal standard in this RT-PCR, in the absence of viral RNA, was 17 molecules per reaction. A fixed number of RNA molecules of the internal standard was added to each of the threefold serial dilutions of the viral RNA. Five microliters of the viral RNA dilution were heated to 99°C for 5 min and placed on ice, and 5 µl of an RNA suspension of the internal standard containing 1,700 molecules added to each virus RNA dilution. An RT-PCR assay was carried out with each of these virus/internal standard dilutions under the conditions described above, but replacing the *Pwo* pol by the Expand High Fidelity PCR System enzyme mix (Roche) in the PCR amplification. Twenty microliters of the PCR product were analysed on a 9% polyacrylamide gel and detected by ethidium bromide staining. The number of astrovirus contained in the sample was estimated from the dilution where the viral PCR product (192–237 bp length) showed similar fluorescence intensity to that of the internal RNA standard PCR product (135 bp length). In this way, it could be assumed that in this dilution there would be an approximately equal number of molecules of the RNA internal standard (1,700 molecules) and of the viral RNA. Having in mind the sample dilution and the processed volume, the number of viral genomes in a given unit of volume could be estimated.

Preparation of the Stool Samples

Faecal samples from children with astroviral gastroenteritis (HAsV-1, HAsV-2, HAsV-3, HAsV-4, and HAsV-8) were quantified by the competitive RT-PCR. RNA from 50 µl of 10% faecal suspensions was extracted by Boom's method [Boom et al., 1990] and resuspended in 50 µl of TE buffer (Tris-HCl pH 8 and EDTA 1 mM). Five microliters of the extracted RNA were used in the competitive RT-PCR described above.

The time elapsed between the onset of symptoms and sample collection was 1–3 days in the acute cases, and around two weeks in the protracted diarrhoeas, with the sole exception of one sample taken three months after the initial discharge.

RESULTS AND DISCUSSION

Quantitation of a Sucrose Purified HAsV-4 Suspension

Sucrose gradient purified viral suspensions were used to evaluate the potential usefulness of the competitive RT-PCR procedure hereby described for the quantitation of astroviruses.

Sucrose gradient centrifugation of HAsV-4 suspensions revealed the presence of three different antigenic peaks (Fig. 1), with densities of 1.05, 1.12, and 1.14 g/ml. Only the 1.05 and 1.14 g/ml antigenic peaks were positive by the molecular hybridisation assay (Fig. 1). These results suggested that the 1.14 g/ml peak corresponded to the nucleic acid-containing virus particle fraction (infectious viruses), the 1.12 g/ml peak corresponded to the nucleic acid-free virus particle fraction (non-infectious empty viruses), and the 1.05 g/ml corresponded to non-assembled viral compounds (proteins and nucleic acids). To confirm the nature of these fractions, the associated infectivity was assayed by CC-RT-PCR. The 1.14 g/ml peak contained 1×10^{10} CC-RT-PCRu/ml, which represents 98.5% of the total infectious viruses, the 1.12 g/ml peak contained 1×10^{8} CC-RT-PCRu/ml, and the 1.05 g/ml peak 5×10^{7} CC-RT-PCRu/ml. Finally, the EM observation of the three antigenic peaks showed no structured material in the 1.05 g/ml fraction and the presence of viral particles in both the 1.14 g/ml and 1.12 g/ml peaks, with no clear morphological differences between them (Fig. 1). Additionally, the average concentration of physical particles was 3×10^{10} particles/ml in the 1.14 g/ml and 2×10^{10} particles/ml in the 1.12 g/ml peak. These results confirmed the existence of two viral capsid structures corresponding to low sedimentation empty particles void of RNA, and high sedimentation RNA-containing particles, as demonstrated previously by separation of astroviruses structures in CsCl gradients [Willcocks et al., 1990; Matsui et al., 1993].

The number of HAsV-4 genomes, determined by competitive RT-PCR, in the 1.14 g/ml peak was estimated as

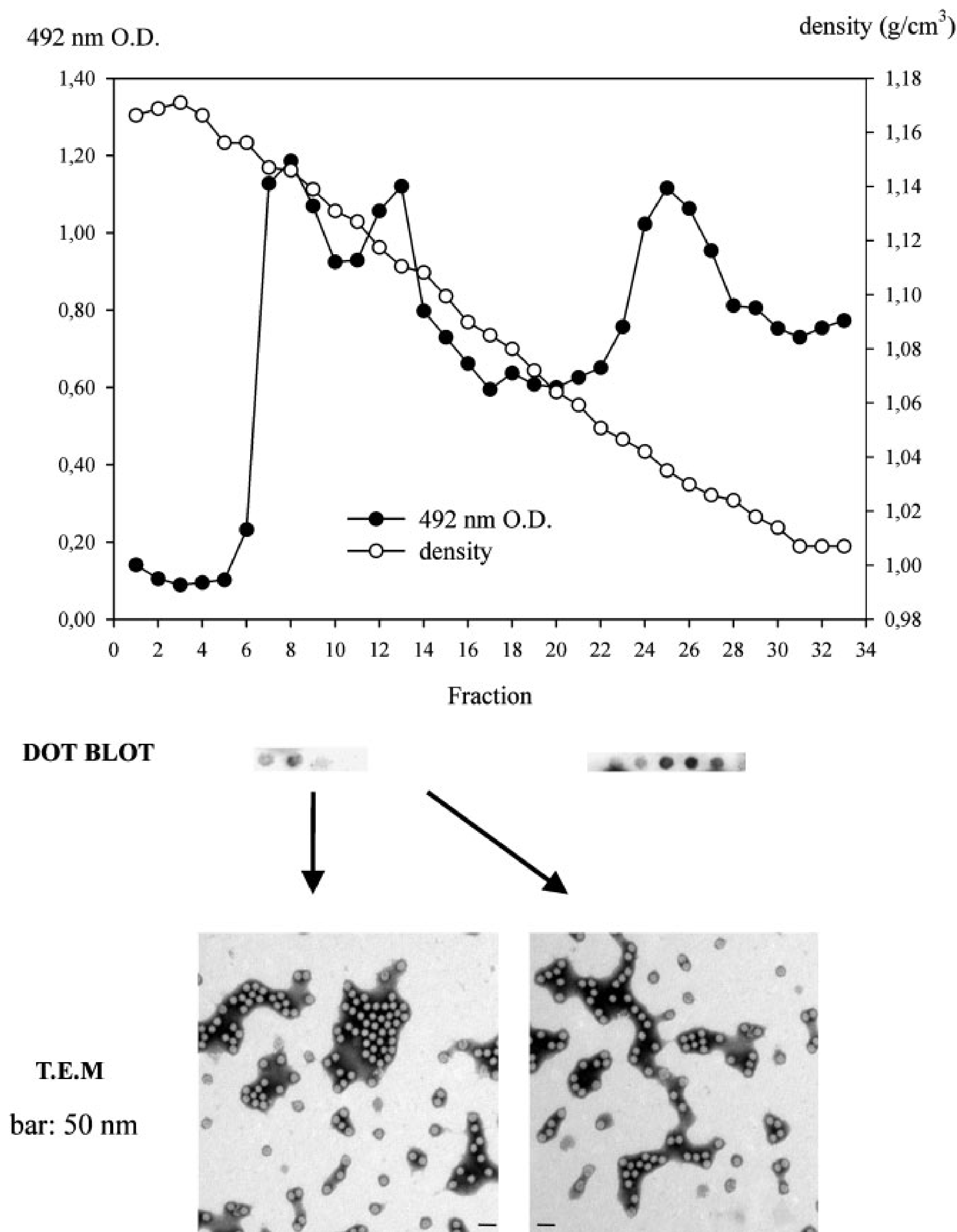

Fig. 1. Sucrose gradient purification of the cell-adapted astrovirus serotype 4. Antigenicity was measured by ELISA (absorbance at 492 nm). The presence of astrovirus RNA was assayed by dot-blot hybridisation and physical particles were visualised by transmission electron microscopy. Scale bar = 50 nm.

9×10^{10}genomes/ml. Although there is not an exact agreement between both physical measures, i.e., E.M. and competitive RT-PCR counting, it should be borne in mind that the microscopic technique is highly dependent on the colloidal gold suspension status (monodispersed or aggregated colloids) and thus less accurate.

Quantitation of Human Astroviruses (HAsVs) in Faecal Samples by Competitive RT-PCR

Thirty-two faecal samples, positive for astrovirus by RT-PCR, were quantified by competitive RT-PCR. These astrovirus strains belonged to serotype 1 (n = 6; 18.75%), 2 (n = 4; 12.50%), 3 (n = 7; 21.87%), 4 (n = 9; 28.12%) and

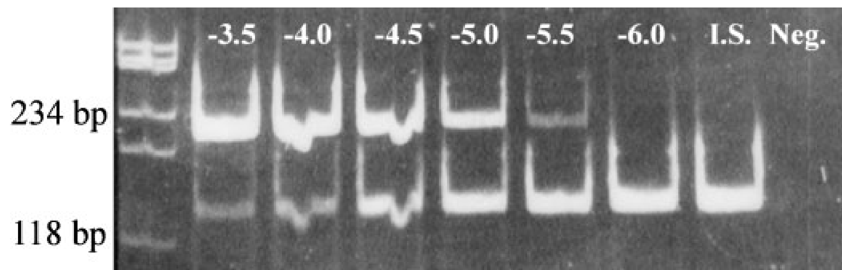

Fig. 2. Quantification of a faecal human astrovirus type 8 by competitive RT-PCR. The intensities of fluorescence of the viral (210 bp) and the internal standard amplimers (135 bp) were equivalent at the 10^{-5} dilution. Since 1,700 molecules of the internal standard were competing, an estimation of 1.7×10^8 RNA molecules/reaction, or 3.4×10^{10} genomes/ml of faecal suspension, or 3.4×10^{11} genomes/g could be made (see text). M: molecular weight marker; I.S.: sample containing only the RNA internal standard; Neg.: RT-PCR negative control.

8 (n = 6; 18.75%). Figure 2 shows the image of an stained polyacrylamide gel in which the quantitation of a faecal specimen containing HAsV-8 was performed. The intensities of fluorescence of the viral and the internal standard amplimers were equivalent at dilution 10^{-5}. Since 1,700 molecules of the internal standard were competing in the RT-PCR assay, it could be assumed that the number of viral RNA molecules contained in this sample was 1.7×10^8 molecules/reaction. Having in mind that the volume of viral RNA processed in each competitive RT-PCR was 5 μl, a concentration of 3.4×10^{10} genomes/ml of faecal suspension, or 3.4×10^{11} genomes/g of faeces could be estimated to be present in the sample.

A minimum number or a cut-off value of 3.4×10^4 genomes/g of faeces is required to be able to be quantified by the present competitive RT-PCR method.

The titres of viral RNA genomes in the samples included in the present study ranged from 3.4×10^8 to 1×10^{13} per gram of faeces, as shown in Table I. An analysis of variance (ANOVA) of the number of genomes per gram of faeces in samples belonging to different serotypes, revealed significant ($P < 0.05$) differences between serotypes 1 (10.28 ± 0.88) and 3 (11.95 ± 0.93), and between serotypes 3 and 4 (10.74 ± 1.64). The mean viral titre in the serotype 3-containing faeces was higher than in any of the other serotype-containing samples. However, the lack of significance in astrovirus levels between serotype 3, and serotypes 2 and 8 could probably arise from the low number of samples. The time of collection, which could be relevant in terms of the viral load, should not influence the differences regarding virus titres observed between the serotypes in acute gastroenteritis, since all samples, independently of serotype, were collected between days 1–3. Regarding the protracted diarrhoea cases, sample collection was usually around two weeks of the onset of symptoms. However, in a sample taken after 3 months of gastroenterits, the titre was still very high (3.4×10^{11} molecules per gram of faeces).

To confirm that differences in the viral titre between serotypes were not due to different primer annealing efficiencies in viral and internal standard RNA molecules, depending on the viral serotype, the sequences corresponding to the annealing regions were determined by means of a third external primer (data not shown). These sequences were determined for those viral strains showing maximum differences in titre. The number of mismatches detected, for all serotypes, between the viral and primer sequences was 1 or 2, either in the A1 or A2 region.

The clinical significance of the higher viral titres in the faeces of individuals infected with HAsV-3 is not clear. However, these results suggest higher virus production levels of these strains. Interestingly, although persistent gastroenteritis was only detected in 8.5% of the astrovirus infections diagnosed during the period 1997–2000 (unpublished results), 42.9% of the HAsV-3 isolates (3 out of 7 total samples) were associated with persistent gastroenteritis. On the contrary, only 16.7% (1 out of 6 total samples) and 5.5% (1 out of 18 total samples) of the HAsV-8 and HAsV-1 isolates, respectively, were associated with persistent diarrhoeas, and

TABLE I. Analysis of Faeces From Individuals Infected With Different HAsV Serotypes*

Serotype	Genomes/g	Mean ± SD of log genomes/g
HAsV-1	3.4×10^9 (3)* 3.4×10^{10} (1) 1.0×10^{11} (1) 3.4×10^{11} (1)	10.28 ± 0.88
HAsV-2	1.0×10^{10} (1) 3.4×10^{10} (1) 1.0×10^{11} (1) 3.4×10^{11} (1)	10.77 ± 0.65
HAsV-3	3.4×10^{10} (1) 1.0×10^{11} (1) 3.4×10^{11} (1) 3.4×10^{12} (3) 1.0×10^{13} (1)	11.95 ± 0.93
HAsV-4	$3. 4 \times 10^8$ (1) 1.0×10^9 (2) 3.4×10^{10} (2) 3.4×10^{11} (1) 1.0×10^{12} (1) 3.4×10^{12} (1) 1.0×10^{13} (1)	10.74 ± 1.64
HAsV-8	$3. 4 \times 10^9$ (1) $3. 4 \times 10^{10}$ (1) 1.0×10^{11} (2) 3.4×10^{11} (2)	10.86 ± 0.75

*The number of samples is shown in parenthesis.

no serotype 2 or serotype 4 associated cases were reported. Overall, 3 out of 8 persistent gastroenteritis samples (37.5%) contained HAsV-3, one contained HAsV-1 (12.5%), one contained HAsV-8 (12.5%), and the other three could not be typed (37.5%), probably because the peak of viral excretion was over at the sampling time. The mean $\pm$ SD of the logarithm of the genomes/gram of faeces of these HAsV-3-associated persistent gastroenteritis samples was 12.20 ± 0.58 and the gastroenteritis could last as long as over 3 months. No other pathogens or basal diseases were reported in these children. Further studies are required, with an increased number of samples, to verify whether the association of these HAsV-3 isolates with persistent diarrhoeas is a frequent phenomenon of this serotype or whether it is just a specific characteristic of these particular strains. However, since serotype 3 represents less than 20% of the total astrovirus isolates in our area [Guix et al., 2002], it will be difficult to conduct these studies. Two out of the other four HAsV-3 isolates were associated with severe gastroenteritis, which required admission to hospital. The mean $\pm$ SD of the log of genomes/gram of faeces in these samples was 12.76 ± 0.33.

It is not clear whether this higher viral productivity is linked to a capsid-related phenomenon such as the interaction with the cellular receptor/s or to other viral protein functions, such as a protein coded in the ORF1a which includes a nuclear localization signal and that has been related to the adaptability of astrovirus to cell culture [Willcocks et al., 1994]. The higher stool titres of the HAsV-3 isolates could be the consequence not only of a higher replicative potential but also of some enhanced immune resistance of these strains. In this sense, a relationship between long-lasting astrovirus infection and a chemotherapic $CD4^+$ T-cell depletion has been suggested [Coppo et al., 2000]. It may then be speculated that a central role of anti-astrovirus specific immunoglobulins is required to discontinue the viral infection. Consequently, any mechanism decreasing the immunoglobulin concentration, including a high viral load, may result in protracted or severe diarrhoea. However, the function of other T-cell populations such as $CD16^+$ and $CD8^+$ in the control of the infection should not be neglected [Cubitt et al., 1999].

Although human astrovirus is associated usually with cases of mild and self-limiting gastroenteritis, its relation with severe and/or protracted diarrhoea, in patients with no other basal disease, deserves further attention.

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