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## RESEARCH NOTE

### Molecular epidemiology of astrovirus infection in Italian children with gastroenteritis

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### ABSTRACT

A 1-year study involving 157 gastroenteritis samples was conducted to investigate the role of human astrovirus (HAstV) as a cause of gastroenteritis in Italian children aged <2 years. The overall incidence of HAstV was 3.1%. Most cases

occurred between March and May, and four of the five isolates were of the HAstV-1 type, the other being HAstV-3. Analysis of genetic variability showed that the three HAstV-1 isolates collected in 2000 clustered together, but separately from the 1999 isolate. The results indicated that HAstV should be considered as a potential diarrhoeal pathogen in Italian children.

**Keywords** Astrovirus, children, diarrhoea, gastroenteritis, typing

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Human astroviruses (HAstVs) have been identified increasingly worldwide as agents of infantile gastroenteritis [1]. Associated initially with focal outbreaks of diarrhoea, they have also been implicated occasionally in nosocomial infections and with persistent gastroenteritis in immunocompromised hosts [2,3]. Their incidence in children with gastroenteritis in both developed and developing countries is usually 2–9% [1]. The astrovirus virion is composed of a non-enveloped capsid and a single-stranded (positive) RNA genome, which includes three open reading frames (ORFs). A 348-bp nucleotide sequence of ORF2, which codes for the capsid proteins, has been used to classify HAstVs into eight genotypes [4]. Previous studies in various countries have shown that HAstV-1 is the most common type causing disease, whereas types HAstV-6, -7 and -8 have been detected only rarely [4]. The aims of the present study were: (1) to define the epidemiological role of HAstV as a cause of gastroenteritis in Italian children; (2) to compare HAstV-related illnesses with those caused by other enteric viruses; and (3) to analyse the potential genetic correlation between any HAstV isolates obtained and prototype strains from other geographical areas.

The study was performed with 439 children aged <2 years who were admitted to the G. Di Cristina Children's Hospital (Palermo, Italy) with acute diarrhoea (at least three watery stools in 24 h, with a sudden onset) between August 1999 and July 2000. Medical staff interviewed the accompanying adults, examined the children, and recorded demographic data (sex and age)

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and clinical symptoms (duration of diarrhoea and number of bowel movements/day, occurrence and duration of vomiting and fever, >5% dehydration). A 14-point scoring system, described previously by Cascio *et al.* [5], was used to summarise the clinical severity of the cases. A stool specimen from each patient was collected within 12 h of admission in order to exclude cases of nosocomial infection. Specimens were divided into aliquots and stored at  $-70^{\circ}\text{C}$  until assayed.

Routine diagnostic tests for rotavirus and bacterial pathogens were carried out on all specimens. In total, 157 specimens negative for rotavirus and bacteria were available in adequate amounts and were tested for the presence of HAstV, adenovirus and norovirus with commercially available tests. Adenovirus group antigen was detected by a latex test (Orion Diagnostica, Helsinki, Finland). Latex-positive specimens were tested for enteric sub-genus F serotypes 40 and 41 adenoviruses with the Premier Adenoclone-type 40/41 enzyme immunoassay (EIA) (Cambridge Bioscience, Worcester, MA, USA). Norovirus and HAstV were also detected with EIAs (Dako Diagnostics, Cambridge, UK). All HAstV-positive specimens were confirmed by reverse transcription (RT)-PCR amplification, carried out before and after cultivation on the PLC/PRF/5 human hepatoma cell line [6]. Virus RNA was obtained by binding to chromatographic cellulose fibre powder as described previously [7], followed by amplification with HAstV-specific primers Mon269 and Mon270, as described by Noel *et al.* [8], following the protocol of Guix *et al.* [4]. The 348-bp HAstV RT-PCR product was sequenced (MWG-Biotech, Ebersberg, Germany) and compared to the sequences of reference

strains with the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) in order to assign each isolate to a known serotype.

Data were analysed with Statistica (StatSoft, Tulsa, OK, USA) and EpiInfo (CDC, Atlanta, GA, USA) software, as described previously [5]. The Mann-Whitney *U*-test, Student's *t*-test and the Pearson chi-square test were used as appropriate, with  $p < 0.05$  considered to be significant.

As shown in Table 1, rotavirus was the most common pathogen isolated from the patients studied, being detected in 179 (40.7%) cases (full details have been published previously [9,10]). HAstVs were isolated from five (3.1%) cases, adenovirus types 40/41 from eight (5%) cases, non-enteric adenoviruses from five (3.1%) cases, and norovirus from nine (5.7%) cases. All HAstV-positive samples were confirmed by RT-PCR before and after cultivation. Concurrent infection with HAstV and norovirus was detected in only one patient, but a sample from a second patient was co-infected with adenovirus and norovirus. All HAstV-infected children were aged <14 months, and HAstV infections were concentrated between March and May. In contrast, rotavirus, norovirus and adenovirus type 40/41 infections occurred from December to April, while non-enteric adenovirus infections occurred in June and July.

The disease caused by HAstV was mild, with an overall mean score of enteritis that was 1.5 points lower than that caused by rotavirus ( $p 0.15$ ) and comparable to those caused by norovirus and adenovirus types 40/41. Surprisingly, the overall mean score of enteritis caused by non-enteric adenoviruses was 3 points higher than that caused by adenovirus types 40/41

Characteristic	Virus detected				
	Rotavirus	Astrovirus	Adenovirus 40/41	Non-enteric adenovirus	Norovirus
No. tested	439	157	157	157	157
No. (%) of children infected	179 (40.7)	5 (3.1)	8 (5)	5 (3.1)	9 (5.7)
No. (%) male	99 (55.3)	4 (80)	3 (37.5)	2 (40)	4 (44.4)
Age in months	14.7 (7.9–23.2)	13.4 (3–13.6)	6.7 (2.8–18.2)	12.2 (2–17.3)	12.2 (7.5–22.9)
Days of diarrhoea	5 (3–6.5)	4 (4)	4 (3–5.5)	6 (3.5–7.5)	4.5 (4–6.5)
Maximum number of stools/day	6 (4–10)	6 (3–8)	5.5 (3–6)	8.5 (6–11.5)	7 (5.5–9)
Vomiting (%)	153 (85)	3 (60)	6 (75)	3 (60)	3 (33.3)
Days of vomiting	1.5 (1–2)	1 (0–1)	1.5 (0.5–2.5)	2.5 (1–3.5)	1 (1.5–1.5)
Fever (%)	135 (75.4)	2 (40)	3 (37.5)	3 (60)	6 (66.6)
Maximum fever ( $^{\circ}\text{C}$ )	38.5 (38–39)	38.5 (38–39)	38.2 (37–39.2)	39 (38.5–39)	39.1 (37.7–39.3)
No. (%) of dehydrated children	71 (40)	2 (40)	1 (12.5)	2 (40)	1 (11.1)
Days of hospitalisation	4 (4–6)	5 (4.5–5.5)	4 (3.5–4.5)	5.5 (4.5–6.5)	3.5 (3–5.5)
Mean severity score (SD)	9 (8–11)	8 (3–9)	7 (6–9)	11	8 (6–9)

If not otherwise specified, data shown are median values (lower and upper quartiles).

**Table 1.** Demographic and clinical findings associated with single viral infections in children hospitalised with acute gastroenteritis in Palermo, Italy, 1999–2000

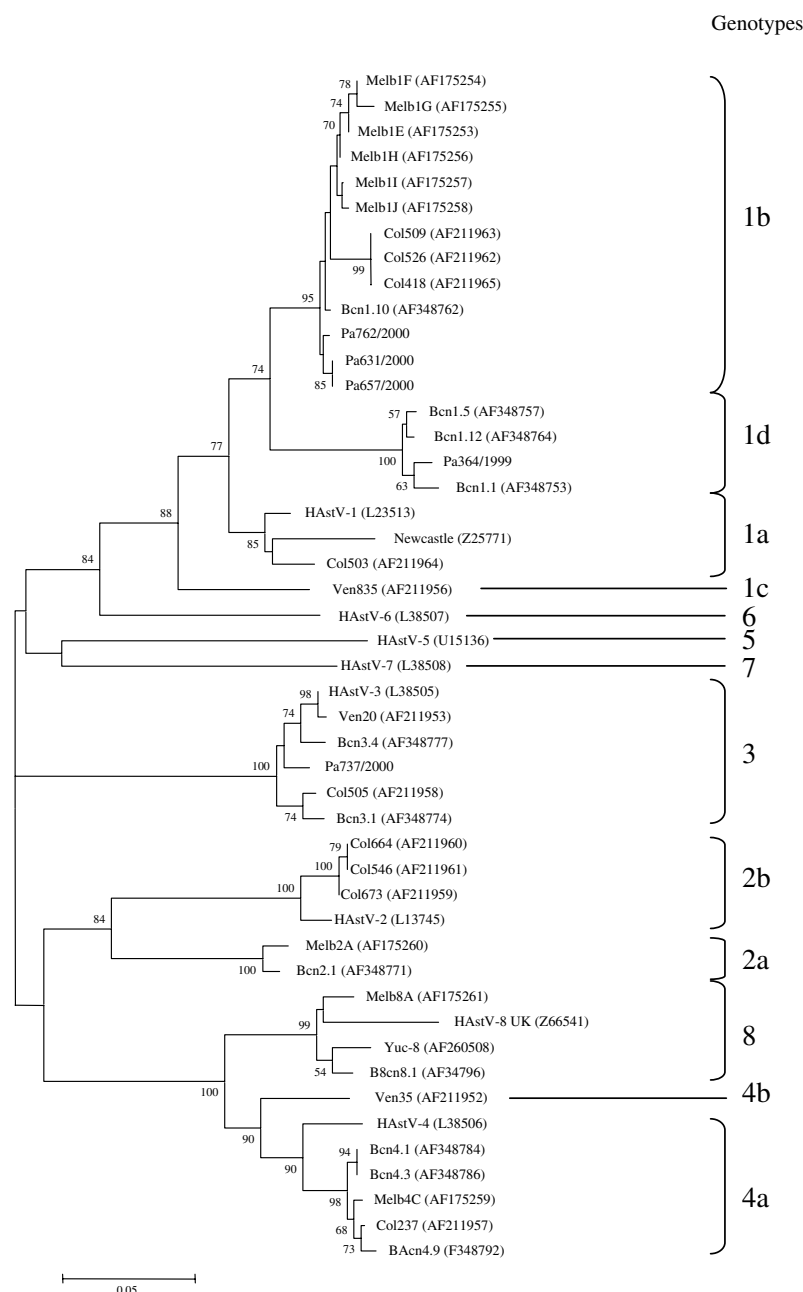
( $p$  0.043). Adenoviruses belonging to a sub-genus other than F have been involved occasionally in the aetiology of acute diarrhoea [11,12].

BLAST analysis of the ORF-2 sequences of the Italian isolates indicated that the single 1999 isolate and three of the four 2000 isolates were of serotype 1, while the fourth isolate from 2000 corresponded to serotype 3. The phylogenetic tree comparing the astroviruses isolated in this study with other published sequences (Fig. 1) clustered

the three type 1 isolates from 2000 into the major 'b' branch described by Guix *et al.* [4], while the type 1 isolate from 1999 clustered with a high confidence level in the major 'd' lineage, corresponding to the Barcelona 1.1 prototype sequence [4].

The five Italian isolates were analysed at the nucleotide and amino-acid levels based on the 348-bp fragment (116 amino-acids) of the capsid region determining the serotype. Genetic variab-

**Fig. 1.** Phylogenetic tree constructed from a 348-bp fragment of the ORF2 of five astrovirus isolates collected in Palermo (Pa), the seven Oxford reference strains (HAstV-1 to HAstV-7), the serotype 8 reference strain from the UK (HAstV-8 UK), 11 strains from Spain (Bcn), nine strains each from Australia (Melb) and Colombia (Col), three strains from Venezuela (Ven), and one strain each from the UK (Newcastle) and Mexico (Yuc8). Phylogenetic relationships were analysed with CLUSTALW and MEGA software and the neighbour-joining method. Nucleotide distance matrices were calculated by Kimura's two-parameter method. Significant bootstrap values are given at the branch points. The scale bar is proportional to evolutionary distance. Sequence data for the previously published HAstV capsid gene sequences were obtained from GenBank under the accession numbers shown in the tree.



ility was compared with that of HAstVs isolated in Palermo and other HAstVs isolated in Spain, the UK, Australia, Colombia, Venezuela and Mexico [4,13–16]. Nucleotide homologies were: 99.1–99.7% among HAstV-1b Italian isolates (corresponding to 1–3 nucleotide differences) and >97% compared with other HAstV-1b sequences; >97% between the Italian and the Barcelona HAstV-1d sequences; and >89% for the HAstV-3 isolate compared to the sequences of corresponding prototypic strains. At the amino-acid level, conservation was high (99–100%) within the serotype 1b isolates. These isolates were also >98% identical to 1b reference strains. Amino-acid similarity was lower (89–94%) between the type 3 isolate and reference strains. A conserved amino-acid change, not reported previously, of Ser → Pro at amino-acid 64 of the capsid protein was detected in the serotype 3 isolate.

In general, the results were in agreement with those of previous studies showing detection rates of HAstV infection in children with gastroenteritis in developed countries, such as France, Spain, Australia and the USA [2,4,14,17–19]. It was possible to confirm the high specificity of EIA for detection of HAstV in stool specimens and the usefulness of the human hepatoma cell line PLC/PRF/5 for isolation of HAstV. Type-specific RT-PCR primers and sequence analysis allowed genotyping information to be obtained from the isolates. With a few exceptions, HAstV-1 has been considered to be the most frequent and dominant type in most parts of the world [1]. The observation that HAstV-1d was circulating in 1999, coupled with the finding of three HAstV-1b viruses in 2000, supports the suggestion of Guix *et al.* [4] that 1b strains had replaced 1d strains over time. In Spain, Caballero *et al.* [3] found that HAstV-3 isolates were associated with more severe and protracted diarrhoea, but this was not observed in the HAstV-3 infected child in the present study.

In conclusion, this is the first report describing the molecular characterisation of HAstV isolates in Italy. The results provide additional information about HAstV strains causing enteritis in children, but further studies that span at least two consecutive years are warranted to assess fully the role of HAstV in diarrhoeal disease of hospitalised paediatric patients, and to enable the formulation of adequate prevention strategies.

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## RESEARCH NOTE

### High seroprevalence of *Pneumocystis* infection in Spanish children

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### ABSTRACT

*Pneumocystis* infection occurs worldwide, and most individuals test seropositive for *Pneumocystis* early in childhood. Little is known about the epidemiology of this infection in western Europe. The seroprevalence of *Pneumocystis* infection in 233 Spanish children was determined in a community study by immunoblot analysis of sera. The overall seroprevalence was 73%, with an age-related increase from 52% at 6 years to 66% at 10 years and 80% at 13 years. The data indicated a high seroprevalence of *Pneumocystis* infection in healthy Spanish children, thereby demonstrating that this pathogen is widespread in southern Spain.

**Keywords** Immunoblotting, *Pneumocystis*, pneumocystosis, seroepidemiological studies, serology

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Despite recent advances in our knowledge of *Pneumocystis* disease in immunocompromised individuals, the natural history and epidemiology of *Pneumocystis* infection in normal subjects are still poorly understood. Serological studies have shown that healthy adults frequently have serum antibodies to this pathogen [1–3], and that the primary contact usually occurs during childhood, with age-related differences in the prevalence rate being reported [2,4–8]. However, little is known about the acquisition of *Pneumocystis jirovecii* infection in western Europe [4,6,7], and there is no published information on spread among children in the Mediterranean area. This information would be of particular interest, since *P. jirovecii* colonisation rates of 10–40% have been reported in southern European patients with chronic bronchial disease [9,10], which suggests wide dissemination of the organism among the general population in this area. Therefore, the objective of the present study was to investigate the seroprevalence of *Pneumocystis* infection in Spanish children.

A cross-sectional study was performed with children aged 6, 10 and 13 years from three rural villages (Coronil, Pruna and Palmar) located in Andalucía, Spain. Community doctors obtained demographic information and serum samples at the children's school after informed consent. An immunoblot assay [11,12] was used to detect *Pneumocystis* antibodies in serum diluted 1:50. *Pneumocystis carinii* antigen was obtained from the lungs of Wistar female rats with dexamethasone-induced pneumocystosis as described previously [13,14]. Anti-*Pneumocystis* antibodies were detected with alkaline phosphatase-conjugated anti-human IgG, IgA and IgM antibodies (Dako, Glostrup, Denmark). An anti-mouse *P. carinii* monoclonal antibody (MAb) (Dako) was used as a positive control. All immunoblots were examined independently by two investigators. The presence of an immunoreactive band of 120 kDa (the molecular size of the MAb) was interpreted as a positive result regardless of the staining intensity of the band.

Overall, the presence of serum antibodies against *Pneumocystis* was detected in 169 (73%) of 233 children. A significant increase in the