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Molecular Epidemiology of Astrovirus Infection in Barcelona, Spain

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A 3-year study involving 2,347 gastroenteritis samples was conducted to determine the prevalence, time distribution, and medical significance of human astrovirus infection in Barcelona, Spain. The overall incidence of astrovirus was found to be 4.9%. Mixed infections with other enteric agents were detected in 17.2% of all astrovirus-positive samples. During the 3-year period, the highest astrovirus incidence was reported in the winter months, although infections also occurred in summer. The peak detection rate was observed in children between 2 and 4 years of age. Overall, HAstV-1 was the most prevalent type, followed by HAstV-4, HAstV-3, HAstV-8, and HAstV-2. HAstV-5, HAstV-6, and HAstV-7 were not detected during these 3 years. From our serotype data for each age group, we observed that HAstV-1, HAstV-2, and HAstV-3 affected mostly children younger than 3 years of age, while HAstV-4 and HAstV-8 had a greater impact in older children. Genetic variability was analyzed between astroviruses isolated in Barcelona and strains isolated in other parts of the world. A fourth lineage was described for HAstV-1, most likely due to the large number of assayed samples, which may also explain the high level of genetic variability observed in the astrovirus isolates.

Astroviruses are nonenveloped single-stranded RNA viruses that were first detected in 1975 by electron microscopy in stool specimens from children with acute gastroenteritis (14). The astrovirus genome contains three open reading frames (ORFs): ORF1a and ORF1b, which encode the viral protease and polymerase, respectively, and ORF2, which encodes the capsid precursor. A subgenomic RNA that contains ORF2 may be detected in the cytoplasm of astrovirus-infected cells.

Human astroviruses have been increasingly identified as important agents of diarrheal disease in children and the elderly (17). Outbreaks of diarrhea due to astrovirus have frequently been reported (1, 20, 24, 26, 32), and astroviruses have also been associated with nosocomial infections in hospitals (29, 33). They have also been detected in immunocompromised (24) and AIDS-infected patients (13). Astrovirus infections occur worldwide, and their incidence in children with gastroenteritis in both developing and developed countries ranges from 2 to 9% (2, 5, 6, 22, 31, 34), although some studies report prevalences up to 26% (15).

Although astrovirus epidemiological studies have been commonly based on electron microscopy and enzyme immunoassay techniques, during the past few years the number of surveys using molecular techniques, mainly reverse transcription-PCR (RT-PCR), has substantially increased. There is a widespread belief that astrovirus incidence may have been underestimated, since enzyme immunoassay is far less sensitive than RT-PCR (6, 20). Furthermore, seroprevalence studies indicate that most children acquire astrovirus antibodies during the first years of life (10, 11). Consequently, a new appreciation for the role of astrovirus in diarrheal disease has evolved, and in many cases, astroviruses are regarded as the second most common cause of viral gastroenteritis in children after rotavirus (7, 9).

Presently, astroviruses are classified into seven (HAstV-1 to HAstV-7) serotypes according to the reactivity of the capsid proteins with type-specific antibodies. These seven antigenic groups (serotypes) correlate perfectly with seven genotypes that can be determined according to the nucleotide sequence of a 348-bp region of ORF2 (25). The existence of an eighth type (HAstV-8) has been suggested based on three complete capsid protein gene sequences deposited in GenBank. Most studies in different countries around the world indicate that HAstV-1 is the most common serotype, while HAstV-6 and HAstV-7 have rarely been isolated (6, 12, 22, 24, 28, 33).

The aim of the present study was to determine the prevalence, time distribution, serotype frequencies, and medical significance of astrovirus infections from children with gastroenteritis in Barcelona, Spain, during a 3-year period. In addition, we analyzed the genetic diversity of astrovirus isolates in order to further investigate the molecular epidemiology of this gastroenteritis agent.

MATERIALS AND METHODS

Stool samples. Between May 1997 and April 2000, 2,291 fecal samples were collected from infants and children with gastroenteritis at four hospitals in the Barcelona area (Hospital de la Vall Hebron, Hospital de Terrassa, Hospital de Sant Joan de Deu, and Hospital de la Santa Creu i Sant Pau). Fifty-six adult samples were also analyzed. Routine diagnostic tests for common bacterial pathogens, rotavirus, adenovirus, and several parasites were carried out for a substantial number of samples following the responsible physician's advice (Table 1). For astrovirus detection, stools were suspended (10%, wt/vol) in phosphate-buffered saline containing 2 M $NaNO_3$, 1% bovine serum albumin; frac-

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TABLE 1. Incidence of enteropathogens detected in stool specimens

Enteropathogen[a]	No. (%) of specimens		
	Tested/total	Positive	Negative
Bacteria and yeasts	1,896/2,347	171 (9)	1,725 (91)
Parasites	888/2,347	10 (1)	878 (99)
Rotavirus	1,265/2,347	151 (12)	1,114 (88)
Adenovirus	404/2,347	8 (2)	396 (98)
Astrovirus	2,347/2,347	116 (5)	2,229 (95)

[a] Bacteria and yeasts were detected by standard culture techniques. Parasites were detected by optical microscopy and immunological procedures. Rotaviruses and adenoviruses were detected by latex agglutination tests or enzyme-linked immunosorbent assay procedures.

tion V), and 0.1% Triton X-100 (pH 7.2) and pelleted at 1,000 × *g* for 5 min, and the resulting supernatant was stored at −70°C for later analysis.

Astrovirus detection. Astrovirus was detected by RT-PCR after extraction of its RNA and subsequently confirmed by Southern blot hybridization with an internal probe. RNA was purified from 50 μl of fecal supernatant by guanidine thiocyanate extraction, as previously described (3). RT-PCR was carried out with primers A1(5′-CCTGCCCCGAGAACAACCAAGC-3′) and A2(5′-GTAAGA TTCCCAGATTGGTGC-3′), which amplify a fragment of ORF1a (35). Five microliters of the extracted RNA was heated to 99°C for 5 min and immediately placed on ice. First-strand cDNA was synthesized at 42°C for 60 min by adding 1 μM primer A2 and 3 U of reverse transcriptase (Expand; Roche) in 10 μl (final volume) containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM Tween 20, and 0.2 mM concentrations of each deoxynucleoside triphosphate. Five microliters of the RT product was amplified by using 0.5 U of the Expand high-fidelity PCR system enzyme mix (Roche) and 0.5 μM (each) primers A1 and A2 in a total volume of 50 μl containing 5 μl of Expand high-fidelity buffer (Roche), 2 mM $MgCl_2$, and each deoxynucleoside triphosphate at 0.2 mM. 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) were performed followed by a final extension of 7 min at 72°C. Ten microliters of the PCR product was analyzed on a 1.5% agarose gel and detected by ethidium bromide staining. PCR products were confirmed by Southern blot hybridization with an internal digoxigenin-labeled probe (5′-AAGAAAGAGAAACAACCAG-3′) under stringent conditions.

Astrovirus typing. All specimens positive for astrovirus were typed by sequencing the RT-PCR product as previously described by Noel et al. (25), after amplification of a 413-bp region of ORF2. Conditions for RT-PCR reactions with primers Mon244 and Mon245 were identical to those with primers A1 and A2, but primer and $MgCl_2$ concentrations in the PCR were 1 and 1.5 μM, respectively. The PCR program consisted of 3 min at 95°C followed by 40 cycles of amplification (94°C, 1 min; 55°C, 30 s; 72°C, 1 min) and a final extension of 7 min at 72°C. Forty microliters of the RT-PCR product was run on a 1% agarose gel, and the DNA was purified with a High Pure PCR product purification kit (Roche), according to the manufacturer's instructions. The nucleotide sequence of a 348-bp fragment of the PCR product was determined from 2 to 7 μl of the purified DNA by using the Thermo Sequenase II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech) and the primers Mon244 and Mon245. Each nucleotide sequence was compared to those of reference strains by using the BLAST program (National Center for Biotechnology Information) in order to assign a serotype.

Phylogenetic analysis. Both nucleotide and amino acid multiple-sequence alignments were carried out with the CLUSTALW program. Phylogenetic trees were constructed, on the basis of the 348-bp DNA sequences from ORF2 described above, by the neighbor-joining method. Nucleotide distance matrices were calculated by Kimura's two-parameter method (PHYLIP package version 3.5 c; distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle). Phylogenetic relationships were analyzed with and without a bootstrap of 100 replicates. The consensus tree was visualized by using TreeView software, and it was rerooted by using feline astrovirus sequence as an outgroup (AF056197). Sequence information for HAstV-1 to HAstV-7 was obtained from Oxford reference strains and also from astrovirus type 1 Newcastle (36). For HAstV-8, three sequences available at GenBank were used (Z66541, AF175261, and AF260508), as well as some sequences from astroviruses isolated elsewhere (18, 22). The *Pi* parameter was used as a measure of nucleotide diversity (nucleotide changes per site), and it was calculated with DnaSP 3.0 software (26).

TABLE 2. Mixed infections in astrovirus-positive cases

Enteropathogen	No. of specimens tested (n = 116)	No. of coinfections (%)
Rotavirus	60	7 (11.7)
Campylobacter spp.	87	5 (5.7)
Salmonella spp.	87	4 (6.7)
Aeromonas caviae	87	2 (3.3)
Candida albicans	87	1 (1.7)
Cryptosporidium	47	1 (2.1)
Adenovirus	17	0 (0)

Statistical analysis. The chi-square test was used to compare prevalence rates among 1-year periods of the study and also to evaluate the differences between astrovirus incidence among age groups. Association between astrovirus serotype and age group was studied by analysis of variance (ANOVA).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in GenBank and have been assigned accession numbers AF348753 to AF348801.

RESULTS

Epidemiology of astrovirus infections. Over a 3-year period, from May 1997 to April 2000, a total of 2,347 gastroenteritis fecal samples were analyzed for the presence of astrovirus. The overall incidence of astrovirus was found to be 4.9% (116 out of 2,347 samples).

A significant number of samples were also subjected to other microbiological analyses at the different hospitals (Table 1). A total of 325 samples were positive for some of the following agents: rotavirus, adenovirus, *Salmonella* spp., *Campylobacter* spp., *Aeromonas* spp., *Yersinia* spp., *Enterococcus* spp., *Staphylococcus* spp., *Candida* spp., *Giardia*, *Entamoeba*, and *Cryptosporidium*. Additionally, mixed infections were detected in 20 of the 116 astrovirus-positive samples, which represent 17.2% of all positive samples (Table 2).

Clinical features of astrovirus infections were documented for a group of 52 pediatric patients from the Hospital Vall d'Hebron whose samples were positive. Of these children, 51.9% did not require hospitalization, while 34.6% were hospitalized because of gastroenteritis (the mean number of days in the hospital was 10.7; range, 1 to 57), and 13.5% of the infections were considered nosocomial infections in nongastroenteritis patients (mainly suffering from oncologic diseases). Although the disease induced by astrovirus in our community was mild, the role of astrovirus as a cause of nosocomial infections should not be neglected. Clinical symptoms were diarrhea (86.3%), temperature above 37.8°C (54.9%), and vomiting (49.0%). Resolution was good in all patients, although one child suffered from two episodes of astroviral gastroenteritis within the same year (see below).

For the pediatric population (up to 15 years), the average age was 2.8 years (33.5 months), and the median age was 1.6 years (19 months). For astrovirus-positive samples, the average and median ages were 2.5 years (29.6 months) and 1.9 years (23 months). Although astrovirus infections occurred in most age groups (Fig. 1), the number of cases was higher for children between 2 and 3 years of age, and 80% of astrovirus infections occurred in children under 3 years of age (Fig. 1A). Three astrovirus cases were also detected in newborns. Detection rates for each age group are shown in Fig. 1B. The highest detection rate was observed among children of 13 to 14 years.

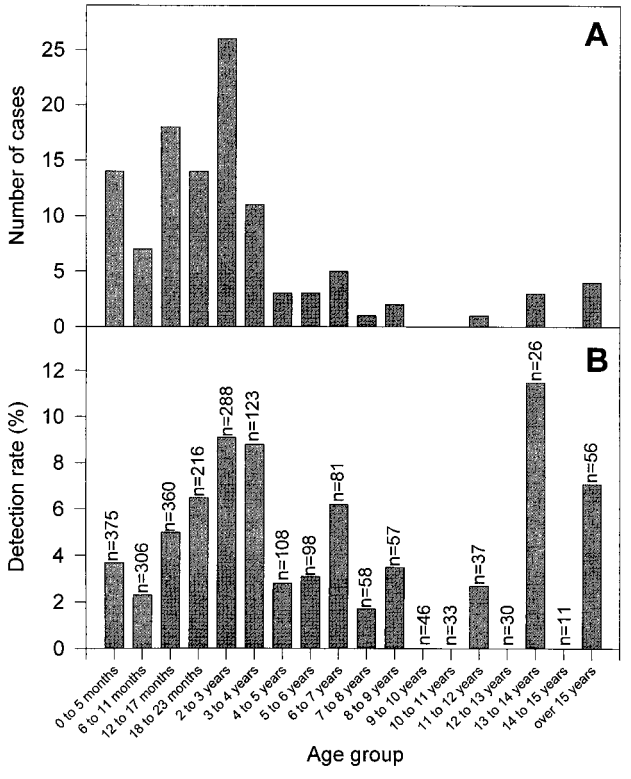

FIG. 1. Age distribution of patients with astrovirus gastroenteritis from April 1997 to May 2000. (A) Number of astrovirus-positive samples in every age group; (B) astrovirus detection rate in every age group. In, number of samples analyzed. Age information was available for 2,309 samples.

However, since the number of analyzed samples is remarkably higher in the age groups of less than 5 years and the number of cases is significantly higher in infants between 2 and 4 years of age than in those of 13 to 14 years, it can be concluded that the maximum detection rate occurs in patients between 2 and 4 years of age.

A group of adult patients with ages between 15 and 82 years was also analyzed. Astrovirus RNA was found in 4 of 56 samples (ages: 27, 28, 59, and 69 years), which represents 7% of the adult patients, who were immunocompromised patients (bone marrow transplantation or multiple myeloma) or suffered from severe syndromes (brain stroke or ulcerous colitis).

The monthly distribution of astrovirus incidence is shown in Fig. 2. During the 3-year period, detection rates were higher in the winter months, although astrovirus infections also occurred in summer. In our study, the incidence of astrovirus between May 1998 and April 1999 was higher than those observed in the 1-year periods 1997–1998 and 1999–2000, being 8, 2.6, and 2.5%, respectively. When analyzed by a χ^2 test, astrovirus incidence in 1998–1999 was significantly higher than incidences in 1997–1998 and 1999–2000 ($\chi^2 = 37.43$, $P < 0.01$).

The annual incidence of each serotype is shown in Fig. 3. In 1997–1998, HAstV-1 was the only serotype detected, while in 1998–1999, five different serotypes affected the studied population, with HAstV-3 being the most prevalent, followed by HAstV-4. In the following 1-year period, HAstV-1 reappeared as the most common type accounting for 67% of the cases, while HAstV-4 and HAstV-8 showed a decreasing incidence and HAstV-2 and HAstV-3 were not detected. The analysis of the genetic variability of isolates allowed us to describe the emergence of a different HastV-1 strain during 1999–2000 (see below). During the study period, one child suffered from an episode of HAstV-3 diarrhea in November 1998 and had a subsequent episode of HAstV-1 infection 9 months later, suggesting a lack of heterotypic immunity between the different antigenic types, which could be responsible for the changes in serotype distribution observed in consecutive years.

Overall, HAstV-1 was the most prevalent type (38%) followed by HAstV-4 (26%), HAstV-3 (19%), HAstV-8 (11%), and HAstV-2 (6%). HAstV-5, HAstV-6, and HAstV-7 were not detected during these 3 years.

From our serotype data for each age group, we observed that HAstV-1, HAstV-2, and HAstV-3 affected mostly children younger than 3 years of age, while HAstV-4 and HAstV-8 had a greater impact in older children (Fig. 4). Average ages for each serotype were 18.8 months for HAstV-1, 17.3 months for

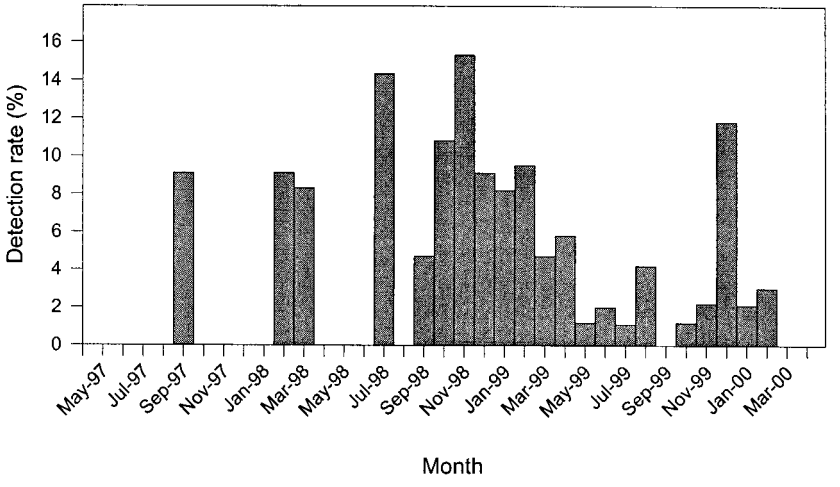

FIG. 2. Monthly distribution of astrovirus detected in stool specimens from May 1997 to April 2000.

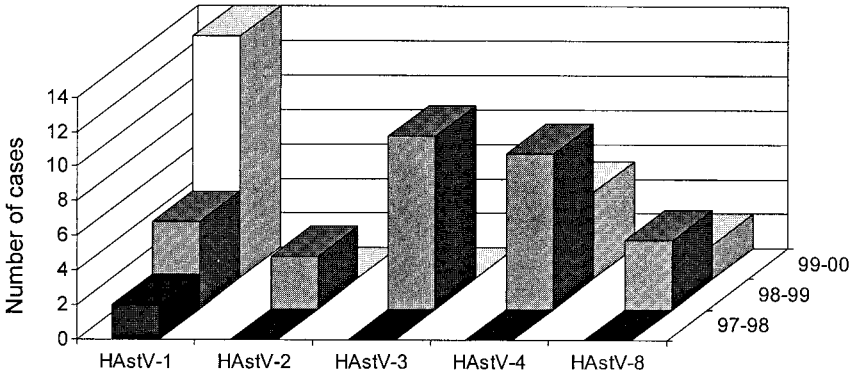

FIG. 3. Serotype distribution of astrovirus identified in every 1-year period of the study.

HAstV-2, 19.1 months for HAstV-3, 43.1 months for HAstV-4, and 57 months for HAstV-8. When we subjected these data to analysis of variance, we determined that the average ages for HAstV-8 and HAstV-4 were significantly different from those for HAstV-1 and HAstV-3 ($P < 0.05$). The lack of significance with HAstV-2 average age is due to the low number of positive samples for this serotype.

Genetic variation. Forty-nine Spanish samples were analyzed at the nucleotide and amino acid levels based on the 348-bp fragment (116 amino acids) of the capsid region determining the serotype (25). Genetic variability was analyzed between astroviruses isolated in Barcelona and other astroviruses isolated in Australia, Colombia, Venezuela, Mexico, and the United Kingdom (18, 19, 22, 36). The degree of genetic variability, measured as the number of nucleotide changes by nucleotide site (*Pi*), was calculated in all antigenic groups. The group with higher nucleotide diversity was shown to be serotype 2 (*Pi* = 0.08118), followed by serotype 1 (*Pi* = 0.05622), serotype 8 (*Pi* = 0.02083), serotype 4 (*Pi* = 0.01547), and finally serotype 3 (*Pi* = 0.01228).

Nucleotide variation was not detected among serotype 2 and serotype 8 Spanish isolates. Nucleotide homologies were 90 to 100% among HAstV-1 isolates, 97 to 100% among HAstV-3 isolates, and 95 to 100% among HAstV-4 isolates. Comparing Spanish isolates with prototypic strains, nucleotide identities were high in all cases (90 to 97%) except for HAstV-2, for which it was only 85%.

At the amino acid level, conservation was high within all antigenic types, even serotype 2. All samples were 100% identical to reference strains, except one HAstV-3, all HastV-4, and all HastV-8 isolates. One conserved amino acid change, not previously reported, was detected in one serotype 3 isolate (Bcn3.10). It contained an Arg→His change at amino acid 84 of the capsid protein. This position is quite variable, being Arg in HAstV-1, HAstV-2, HAstV-3, HAstV-5, and HAstV-7, Lys in HAstV-6, and Thr in HAstV-4 and HAstV-8.

Regarding HAstV-4, all serotype 4 Spanish isolates had a Pro→Ala change with regard to the prototype Oxford strain at the amino acid 87. Ala is also present in all HAstV-4 sequences available at the databases from other studies, and it happens also to be a highly conserved amino acid in all serotypes. In addition, a Val→Ile substitution was observed at residue 192 of the capsid region in the Bcn4.9 isolate. This position appears to also be variable in HAstV-8 isolates. Four Spanish HAstV-8 isolates had a Val and two isolates displayed an lle residue. This substitution is also present in the Mexico isolate Yuc-8, in the prototype sequence of HAstV-6, and in the South American serotype 1 isolates (Col418, Col509, and Col526). Furthermore, it should be noted that at the amino acid level, serotype 8 isolates were 100% similar to most serotype 4 isolates, showing a close relationship in this capsid region.

A phylogenetic tree was constructed to study the genetic relationship between the astroviruses isolated in this study and other published sequences (Fig. 5). In the present study, type 1 isolates clustered into 4 major branches with a high confidence level. Within serotype 2 isolates, only two major branches were observed. Serotype 4 isolates could also be distributed into 2 major lineages, showing at least 7% sequence diversity. Regarding serotypes 3 and 8, no major lineages could be discriminated.

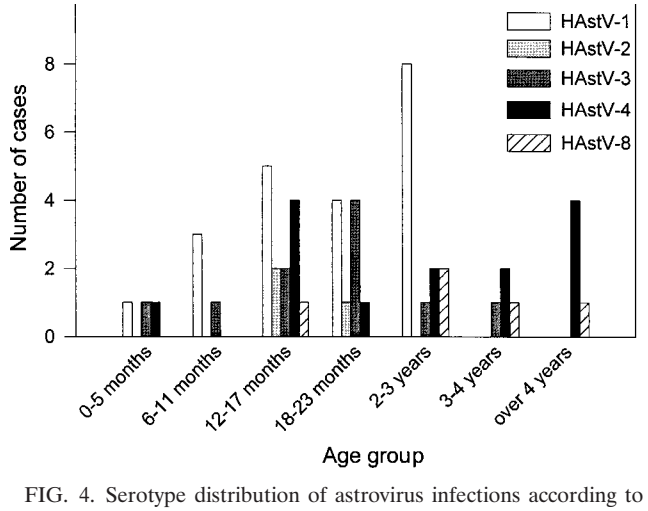

FIG. 4. Serotype distribution of astrovirus infections according to age group.

DISCUSSION

This is the first long-term study of astrovirus infection in Spain, and it reveals an overall incidence of 4.9%. This result is consistent with other studies, which report rates of 2 to 9%

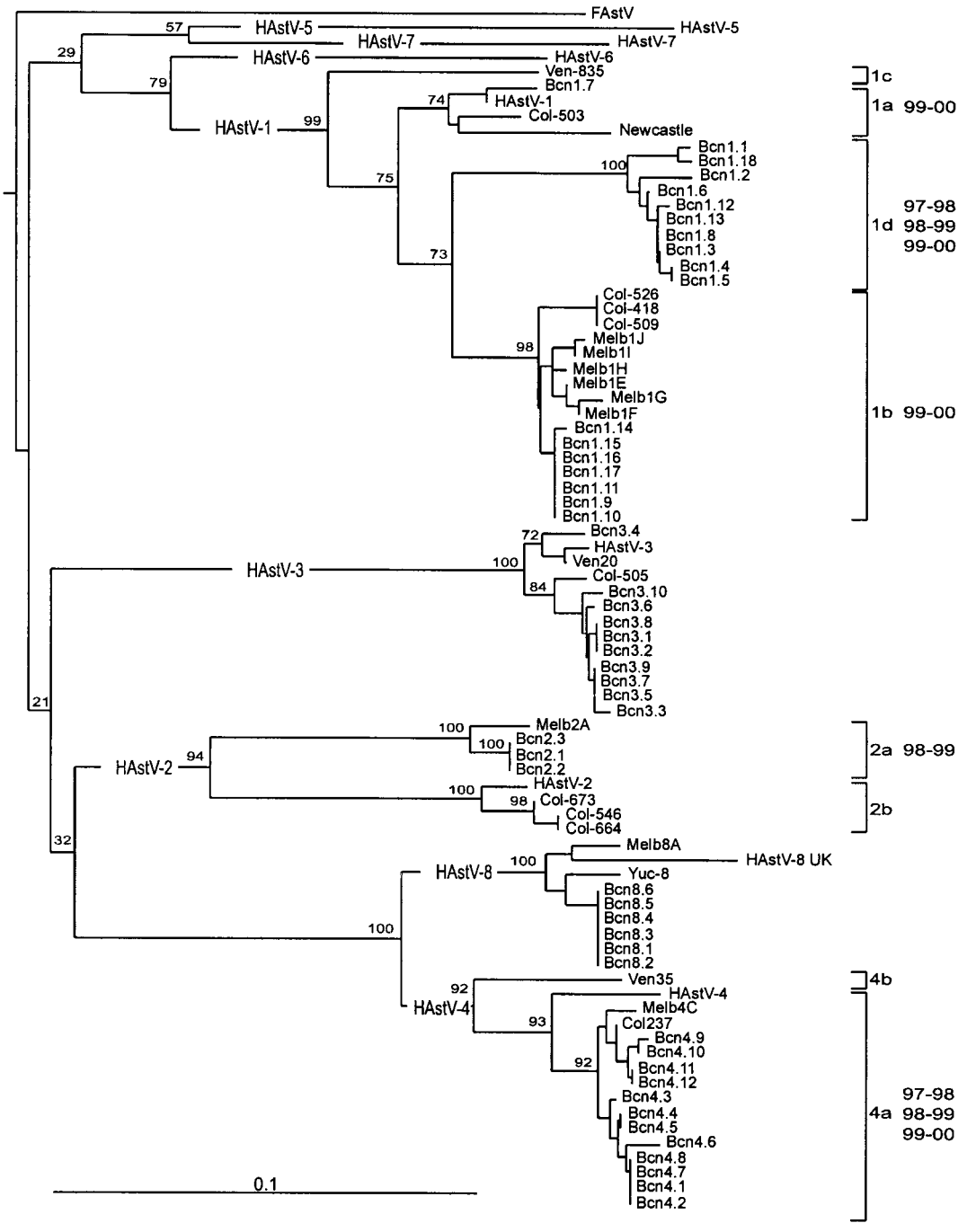

FIG. 5. Phylogenetic tree of the 348-bp region of ORF2 of 49 astrovirus isolates from Barcelona (Bcn), the 7 Oxford reference strains (HAstV-1 to HAstV-7), 2 serotype 8 strains from Mexico (Yuc-8) and the United Kingdom (HAstV-8 UK), 9 strains from Australia (Melb), 9 strains from Colombia (Col), 3 strains from the United Kingdom (Newcastle), and 1 strain from Venezuela (Ven). Bootstrap values are given at the branch points. The scale bar indicates an evolutionary distance of 0.10 nucleotide per position in the sequence. Sequences were obtained from the GenBank database with the following accession numbers: Oxford strains; L23513, L13745, L38505, L38506, U15136, L38507, and L38508; serotype 1 Newcastle strain, Z25771; serotype 8 strains, Z66541, AF260508, and AF175261; other Australian strains, AF175253, AF175254, AF175255, AF175256, AF175257, AF175258, AF175259, and AF175260; Colombian strains, AF211957, AF211958, AF211959, AF211960, AF211961, AF211962, AF211963, AF211964, and AF211965; Venezuelan strain, AF211952, AF211953, and AF211956.

in developed countries (2, 6, 22). Coinfections with other pathogens were detected in 17.2% of the astrovirus-positive samples, although this percentage ranges from 33 to 65% in the literature (4, 8, 33). Most of these studies were carried out in developing countries where poor hygienic conditions may contribute to multiple enteric infections.

The maximum detection rate was observed in children between 2 and 4 years of age. Reports from other countries like

Mexico, Thailand, Guatemala, France, Australia, Colombia, and Venezuela have shown higher incidences in younger populations (2, 4, 8, 9, 18, 22). However, in Guatemala and France (2, 4), the detection rate at the age of 2 was also high.

Detection rates were higher in winter, although astrovirus infections also occurred in summer months. This seasonal pattern is consistent with other epidemiological studies from temperate regions (17). However, reports exist which describe high astrovirus incidences during spring and summer (9, 24). Nevertheless, some other long-term studies (22) describe a slightly different pattern without a distinct winter peak in each year. Our study, although limited to 3 years, seems to support studies in the United Kingdom, South Africa, and Australia (12, 22, 30) that suggest a greater impact on the pediatric population in alternate years. In the United Kingdom, higher frequency of serotype 1 in alternate years was observed during a 5-year period. This observation was also reported by Mustafa et al. (22) in Australia, together with the lack of an evident winter peak in the years with a lower incidence of serotype 1. However, in our study, serotype 1 showed an increasing incidence every year (see below) and a winter peak was also detected in each 1-year period. Consequently, different reasons should account for this apparent biennial pattern.

Fifty-four of 116 astrovirus-positive samples could be serotyped using the method described by Noel et al. (25). This apparent lack of typing sensitivity has been reported previously (23). Overall, the most prevalent type was HAstV-1, followed by HAstV-4, HAstV-3, HAstV-8, and HAstV-2, while serotypes 5, 6, and 7 were not detected during these 3 years. Serotype 1 has been considered the most frequent and dominant type in most parts of the world, with a few exceptions (8). The second most common serotype differs depending on the country. In the present study, it appears to be HAstV-4, as in Australia and Bangladesh (22, 33).

More than 50% of serotype 1 and 3 samples were from children under 2 years of age, while the majority of serotype 4 and 8 samples were from children older than 3 years. This statistically significant different age pattern suggests that the immune protection against the most prevalent serotypes acquired earlier in life fails to confer any protection against new serotypes or variants appearing thereafter.

The genetic variability of the 348-bp fragment determining the serotype (25), measured as the number of mutations per nucleotide site, was highest in the serotype 2 sequences, followed by serotypes 1, 8, 4, and 3. In fact, a high degree of variation among serotype 2 isolates has been previously described (22). Spanish HAstV-2 isolates were much closer to Australian strains than to South American isolates, which clustered with prototype Oxford strains.

The genetic relationship among the present astrovirus isolates and the previously published isolates allowed us to distinguish several lineages or clusters into each serotype. In the case of serotype 1, together with the three previously described lineages (18) a new cluster (lineage 1d) is reported, including at the moment only sequences from Spain. Other Spanish isolates clustered in lineages 1a and 1b, meaning that Spanish type 1 isolates may represent variants of three different astrovirus strains. Additionally, strains belonging to lineage 1d were mostly isolated in 1997–1998 and 1998–1999 and occasionally in 1999–2000, while strains 1a and 1b were found only in 1999–2000. This suggests that strains 1a and 1b may have replaced strain 1d over time. This observation could also explain the remarkable increase in the prevalence of serotype 1 in 1999–2000, assuming that infection with 1d strains does not confer complete protection against strains 1a and 1b. However, this hypothesis could be accepted only provided that the epitope structure includes amino acids not only from the present fragment of the capsid region but also from other parts of ORF2, or even residues from other ORFs, such as ORF1a, that seem to be involved in the antigenic structure (16), since the region studied here is 100% identical among the different serotype 1 lineages at the amino acid level.

Within serotype 2 and within serotype 4, two lineages are reported. Although all Spanish serotype 4 isolates clustered in lineage 4a, two subgroups of samples (with 4% sequence diversity) belonging to different 1-year periods could be distinguished. This may also indicate that these two strains would not have cocirculated in the community.

Finally, no lineages could be described for serotypes 3 and 8.

We emphasize the description of the fourth lineage for serotype 1, as it may be a consequence of the large number of samples analyzed but may on the other hand also explain the high level of genetic variability observed in the astrovirus isolates.

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