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Typing of Human Astroviruses from Clinical Isolates by Enzyme Immunoassay and Nucleotide Sequencing

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A typing enzyme immunoassay (TYPE-EIA) was used to determine the antigenic types of 64 astrovirus-positive specimens from nine collections from seven countries. Six of the seven known astrovirus types were detected in the collections, with HAstV-1 predominating in all collections except for one from the United Kingdom. Selected specimens were analyzed further by reverse transcriptase PCR and nucleotide sequencing of 348 bp within the capsid protein precursor region of the genome. The phylogenetic groupings (genotypes) determined from the sequences were entirely consistent with the antigenic groupings (serotypes) of isolates obtained by using the TYPE-EIA. The genetic variation within genotypes was small compared with the variation between genotypes, allowing unambiguous categorization of all specimens. Although some strains from widely separated geographic areas had identical sequences, in general, within a region most strains of the same type were identical. The TYPE-EIA may help further our understanding of the epidemiology of astrovirus and the possible role of serotype-specific immunity, while further knowledge of sequences could facilitate the development of simpler molecular methods of typing astrovirus strains.

Astrovirus, recently classified into a new family of RNA viruses, *Astroviridae* (31), was first identified in stool specimens from children with diarrhea (1, 7). Initially, detection was limited to electron microscopy (EM), and the star-like appearance of some viral particles led to the name of this agent (25). Astroviruses are currently classified into seven antigenic types, HAstV-1 to HAstV-7, on the basis of immune EM and immunofluorescence techniques (18–20) using polyclonal and monoclonal antibodies (10) to cell culture-adapted virus. Epidemiologic studies using EM (2, 16, 21, 22, 30, 40) and the more recently developed enzyme immunoassays (EIAs) (3, 9, 11, 12, 17, 23, 28, 29, 33) have demonstrated the worldwide distribution of astroviruses.

The complete nucleotide sequence and genomic organization have been determined for HAstV-1 (37) and HAstV-2 (14). The genome contains three open reading frames (ORFs), designated ORF1a, ORF1b, and ORF2. The first two ORFs contain amino acid motifs homologous to protease and polymerase proteins involved in the replication of other positive-strand RNA viruses and are expressed via a ribosomal frameshifting mechanism (14, 24, 26, 37). A subgenomic RNA, corresponding to the 3′ third of the astrovirus genome and containing ORF2, can be detected in astrovirus-infected cells (27, 31, 32). Recently, a viral capsid protein containing a neutralization epitope has been mapped to the subgenomic RNA region of the genome, confirming that ORF2 encodes the capsid precursor protein (24, 35, 38).

Epidemiologic studies of astrovirus using EIAs have yielded a large number of isolates. In this study, we first produced a typing EIA (TYPE-EIA) to type astrovirus-positive specimens without the cross-reactivities between type-specific sera that had been encountered previously (10, 13). We then went on to determine if the genetic grouping, based on a partial capsid region sequence, would correlate with the antigenic grouping based on the TYPE-EIA results. Our findings should help us in the development of simpler molecular methods for typing. Knowledge of the distribution of astrovirus types will enable better understanding of the epidemiology of astrovirus gastroenteritis.

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MATERIALS AND METHODS

Specimens. We examined specimens that had been found to contain astrovirus antigen during previous screening of nine collections from seven countries (8, 22, 29, 34). All specimens were retested by using an astrovirus antigen detection EIA (AG-EIA) to ensure that sufficient virus remained for cultivation and typing. A total of 64 specimens that tested positive were culture adapted in Caco-2 cells and typed with our TYPE-EIA. For the 34 fecal specimens with sufficient volume remaining, both the stool and the matched culture specimens were typed to assess whether adaptation to culture may have altered the type (36).

Ten-percent stool suspensions were prepared in 0.01 M phosphate-buffered saline (PBS) (pH 7.2)–3% bovine serum albumin (BSA) fraction V (Sigma, St. Louis, Mo.)–0.01% Tween 20 buffer (PBS/BSA/T), vortexed, and clarified at $1,600 \times g$ for 10 min for both EIAs. Culture supernatants were tested undiluted.

Culture in Caco-2 cells. Caco-2 cells (ATCC-HTB37) were propagated in minimal essential medium (MEM) with Earle's salts supplemented with nonessential amino acids (splMEM) and 10% fetal bovine serum (all from GIBCO, Grand Island, N.Y.). Astrovirus was cultured in Caco-2 cells by the method of Willcocks et al. (39), with the following modifications. Ten-percent stool suspensions were prepared in splMEM, clarified at $1,000 \times g$ for 10 min, filtered (0.2 μm), and then diluted 1:10 (vol/vol) in splMEM containing 10 μg of trypsin (porcine pancreatic trypsin; Sigma T-0134) per ml (splMEM/T). Flasks (25-cm^2) of Caco-2 cells that were >95% confluent were inoculated with 2 ml of the diluted stool suspension and incubated for 2 h at 37°C without rocking. A further 8 ml of splMEM/T was added, and the incubation was continued for 3 days. The cells were not monitored for cytopathic effect, as they became detached 12 to 24 h postinfection because of the presence of trypsin. Specimens were passaged three times in Caco-2 cells, and the harvests were stored at −70°C.

AG-EIA. All specimens were first screened for astrovirus antigen by using the AG-EIA, which was distinct from the assay used previously to screen the collections (29). Production of a new EIA was necessary since supplies of reagents used previously were exhausted. Protein A-purified [Immunopure(A) immunoglobulin G purification kit; Pierce, Rockford, Ill.] pre- and postimmune rabbit (CDC9303) sera to HAstV-2 were used as negative and positive capture sera,

respectively, at a concentration of 5 μg/ml. Microtiter plates (Immulon II; Dynatech, Chantilly, Va.) were coated with 100 μl of the purified sera diluted in carbonate-bicarbonate buffer (0.06 M, pH 9.6) per well, incubated for 1 h at 37°C, washed six times in 0.01 M PBS (pH 7.2)–0.01% Tween 20 (PBS/T), and blocked for 1 h at 37°C with 100 μl of PBS/BSA/T per well. Between all subsequent steps, plates were washed (with PBS/T) and incubated as described above, with a final wash in distilled water prior to addition of substrate. One hundred microliters of the clarified stool suspension or cell culture supernatant was incubated in duplicate wells with the pre- or postimmune rabbit serum. Bound antigen was detected by using a 1:60,000 dilution of monoclonal antibody 8E7 (kindly provided by J. Herrmann and N. Blacklow, University of Massachusetts, Worcester) that had been prepared by ammonium sulfate precipitation of the hybridoma culture supernatant containing fetal bovine serum followed by a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce). A solution of 5 mg of 3,3′,5,5′-tetramethyl benzidine (TMB) (Sigma) per ml was used as the substrate for color development, the reaction was stopped after 10 min by using 2 N H_2SO_4, and the A_{450} was read with a microplate spectrophotometer (MR5000; Dynatech).

Specimens were considered positive if the average absorbance of the positive capture (*P*) and negative capture (*N*) wells met the following criteria: $P/N > 2$ and $P - N \geq 0.07$.

TYPE-EIA. All culture-adapted strains and matched stool specimens of sufficient volume were typed by the TYPE-EIA. Specimens were initially screened with antisera to HAstV-1 through HAstV-5 because HAstV-6 and HAstV-7 have only recently been characterized (20). All specimens that gave indeterminate results in the initial typing assay were rescreened when antisera to HAstV-6 and HAstV-7 became available. Unpurified hyperimmune rabbit sera to astrovirus reference types HAstV-1 through HAstV-7, prepared in the Virology Department, John Radcliffe Hospital, Oxford, United Kingdom, were used as capture sera at dilutions of 1:10,000, 1:10,000, 1:2,500, 1:5,000, 1:5,000, 1:2,500, and 1:2,500, respectively. Normal rabbit serum (Pierce) was used as a negative capture serum at a dilution of 1:10,000.

Duplicate wells of microtiter plates (Immulon II) were incubated at 4°C overnight, with 100 μl of the appropriately diluted type-specific serum per well. Plates were washed and blocked as described for the AG-EIA. After the blocking, 100 μl of the clarified stool suspension or cell culture supernatant was incubated against all six or eight capture sera at 37°C for 3 h. Bound antigen was detected by using a 1:10,000 dilution of monoclonal antibody 8E7, with the dilutions of the conjugate and TMB as for the AG-EIA. The substrate reaction was stopped with 2 N H_2SO_4 after 15 min. Specimens were determined to be reactive or nonreactive against a particular serum by using the criteria for *P* and *N* as described above.

RNA extraction from Caco-2 cell lysates. RNA was extracted from the Caco-2 cell culture lysates by the method of Jiang et al. (15) for Norwalk virus, with the following modifications. One hundred and fifty microliters of cell culture supernatant was diluted in 150 μl of H_2O prior to extraction with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane. The final RNA pellet was resuspended in 25 μl of H_2O, and 5 μl per reaction mixture was used for the PCR.

Reverse transcriptase PCR (RT-PCR). First-strand cDNA was synthesized at 42°C for 60 min by using 5 μl of template RNA with 1 μM primer Mon245 (TTAGTGAGCCACCAGCCATC) and 10 U of avian myeloblastosis virus RT (Molecular Genetic Resources, Tampa, Fla.) in 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.01% gelatin (Fisher Scientific, Fair Lawn, N.J.), and 1.25 mM each deoxynucleoside triphosphate (Pharmacia LKB Biotechnology, Piscataway, N.J.). Amplification was performed by using 2.5 U of native *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) and 0.5 μM primer Mon244 (GGTGTCACAGGACCAAAACC) in a total volume of 100 μl (50 μl from the RT reaction mixture) containing final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.625 mM deoxynucleoside triphosphates, and 5% dimethyl sulfoxide. Forty cycles of amplification (94°C, 1 min; 50°C, 1 min; and 72°C, 1 min) were performed. The products were analyzed on 3% agarose gels and detected by ethidium bromide staining. Primer Mon245 corresponds to the complement of nucleotides 4934 to 4953 in the HAstV-2 genome, while primer Mon244 corresponds to nucleotides 4541 to 4560, resulting in a predicted product size of 413 bp. This part of ORF2 was chosen for genetic analysis since the priming sites were relatively conserved among the available sequences (HAstV-1, HAstV-2, and HAstV-5 [35b]), while the internal regions were only about 70% identical between types (data not shown). As HAstV-6 and HAstV-7 specimens gave positive, although weak, results with primer set Mon244/245, a second set of primers was designed by using a similarity plot of the sequence data obtained during the project. Mon270 (TCAGATGCATTGTCATTGGT), the complement of nucleotides 4955 to 4974, and Mon269 (CAACTCAGGAAACAGGGTGT), nucleotides 4526 to 4545, with a product with a predicted size of 449 bp, gave better results than the original primers for these specimens. Although not extensively tested yet, the primer set Mon270/269 may be more universal than primer set Mon245/244.

Sequencing of astrovirus. The RT-PCR products of a selection of 36 specimens representing different types as determined by the TYPE-EIA and the Oxford reference virus types (HAstV-1 to HAstV-7) were sequenced. All isolates representing types 2 to 7 were included in this selection together with three arbitrarily chosen type 1 specimens (when available) from each collection. Forty microliters of the RT-PCR product was run on a 1% agarose gel, and the product band was excised and purified by using a Qiaex gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cycle sequencing was performed on 3 to 6.5 μl of the Qiaex-purified product with a Prism Ready Reaction Dyedeoxy Terminator Cycle sequencing kit (Perkin-Elmer Cetus), using the same primers as for RT-PCR. Excess nucleotides were removed by using Centri-Sep columns (Princeton Separations, Inc., Adelphia, N.J.), and the purified DNA was sequenced with an ABI 373A automatic sequencer. Sequence data from both strands of the PCR products were aligned and compared by using the xbap program of the Staden sequence analysis package (4). A multiple-sequence alignment was generated from the consensus sequence of each of the isolates by using the Pileup program of the Genetics Computer Group sequence analysis package (6). The sequences were further analyzed by using the DNAML program of the PHYLIP 3.5 package (5) to produce a dendrogram of predicted phylogenetic relatedness.

Nucleotide sequence accession numbers. The nucleotide sequences of HAstV-3, -4, -6, and -7 have been submitted to GenBank under accession no. L38505, L38506, L38507, and L38508, respectively.

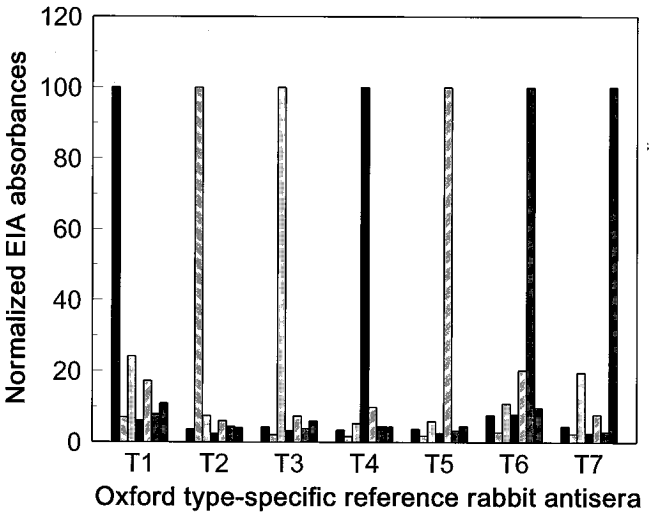

FIG. 1. Graph of the normalized EIA absorbances obtained by using the Oxford type-specific reference sera to HAstV-1 to -7 (left to right, respectively, for each group of bars) against the homotypic reference viruses in the TYPE-EIA. For each type, the absorbance against the homotypic virus is five or more times greater than that against the heterotypic viruses.

RESULTS

The TYPE-EIA was able to distinguish clearly the reference strains of astrovirus (Fig. 1). For each type, the absorbance of the homotypic virus was five or more times greater than that of heterotypic virus, with the largest degree of cross-reactivity (22%) occurring between HAstV-3 virus and antiserum to HAstV-1. Samples were defined as representing coinfections when there was a less than threefold difference between in absorbance values any two antisera.

Sixty-four AG-EIA-positive specimens were cultured in Caco-2 cells and typed with the TYPE-EIA (Table 1). HAstV-1 was the predominant type in all collections except number 7, from the United Kingdom, in which HAstV-3 predominated. Three collections contained specimens with mixed infections, only one case of HAstV-5 infection was detected, and one outbreak was attributed to HAstV-6. No cases of infection with HAstV-7 were identified. Thirty-four specimens had sufficient volumes to allow typing directly from the stool. The results were identical to those for the matched Caco-2 culture-adapted specimens.

The selected two HAstV-1 specimens from collection 1 and three HAstV-1 specimens each from collections 4, 5, 7, 8, and 9 ($n = 17$) and the majority of specimens classified as other types by our TYPE-EIA ($n = 19$), together with the Oxford

TABLE 1. Results of typing astrovirus-positive specimens from children

Collection no.	Country (reference[s])	Source	No. of specimens								
			Total	With antigenic type:							With mixed infections
				1	2	3	4	5	6	7	
1	Australia (8)	Hospital	6	5	1						
2	Israel (3a)	Field study	4	1	1	1	1				
3	Japan (34)	School outbreak	1[a]						1		
4	Korea (15a)	Hospital	10	6			2				2
5	Peru (35a)	Field study	8	4	2		1	1			
6	Peru (6a)	Field study	3		1		1				1
7	United Kingdom (23a)	Public health laboratory	17	6	1	9	1				
8	United States (Colorado) (22, 29)	Hospital	4	3							1
9	United States (New York) (22, 29)	Hospital	8	7	1						
Total (%)			61	32 (52)	7 (11)	10 (16)	6 (10)	1 (2)	1 (2)	0 (0)	4 (7)

[a] The outbreak included a total of four positive specimens. Only one is included in the table to avoid bias in the prevalence of HAstV-6.

reference virus types, were analyzed by RT-PCR and nucleotide sequencing (Fig. 2). A dendrogram of the predicted phylogenetic relatedness was constructed by using a 348-nucleotide region within the 413-bp PCR product. For each of the 36 specimens examined, the phylogenetic grouping (genotype) predicted by sequence was identical to the antigenic grouping (serotype) obtained by using the TYPE-EIA. The pairwise sequence identity was greater within a genotype (86 to 100%) than between types (76 to 81%). In many cases, multiple specimens from the same collection had identical sequences, most clearly seen in the genotype 1 specimens: the three specimens examined from collections 1, 8, and 9 were identical. Specimens from Korea (collection 4) showed regional diversity, with specimen 76 collected in a different region from the other two. The same was not true for collections of specimens from the United Kingdom (collection 7) and Peru (collection 5), although the specimens from the United Kingdom were from a regional public health laboratory which has a larger catchment area than any of the collections mentioned above. In most cases, collections contained only a single representative spec-

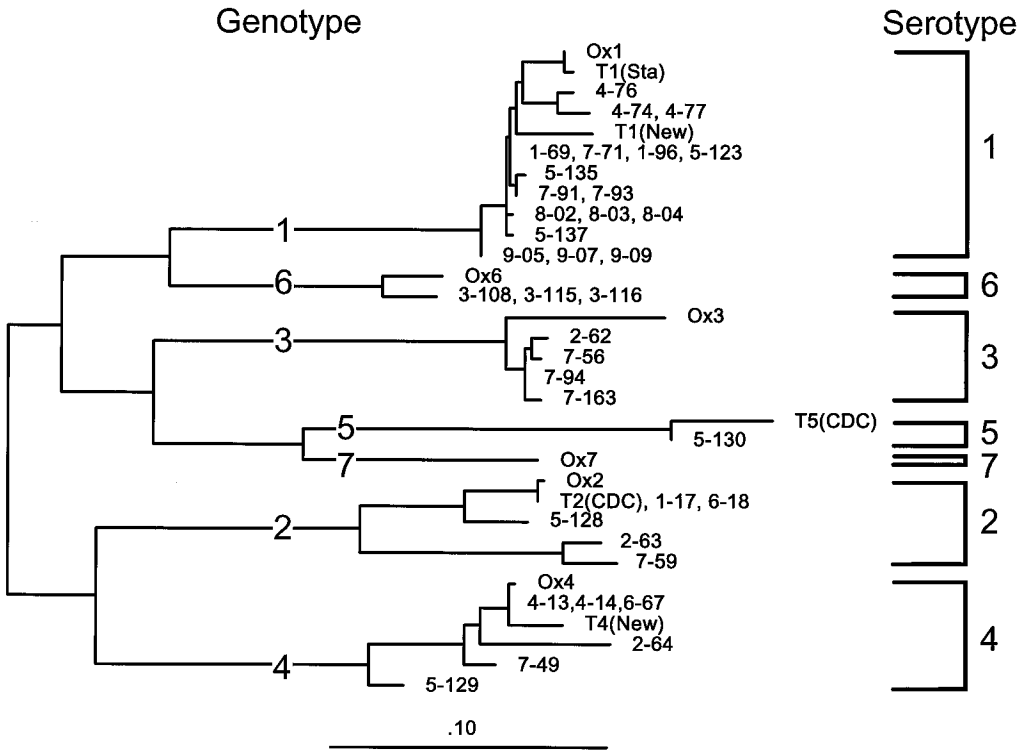

FIG. 2. Dendrogram demonstrating the genetic relatedness of astrovirus strains of seven known types. Each reference type (HAstV-1 to -7) was located on a separate and distinct branch of the dendrogram whose genotype was numbered according to the serotype. All specimens of a particular type clustered with the Oxford reference strain of the same type. Each strain is identified by the collection number from Table 1 and an arbitrary specimen number. Ox1, -2, -3, -4, -6, and -7 represent sequences of the Oxford reference strains determined in this study. T1(New), T1(Sta), T2(CDC), and T4(New) are taken from GenBank (accession no. Z25771, L23513, L13745, and Z33883, respectively). T5(CDC), GenBank accession no. U15136, is from a cDNA clone of the Oxford type 5 reference virus (31a). Sequence information for all specimens can be obtained by contacting the corresponding author.

imen for types 2 to 6. The sequences of all specimens from a school outbreak in Japan (collection 3) were identical.

Of note, genotype 2 contained the largest within-group differences in nucleotide identity (14%). Reexamination of the TYPE-EIA results for these strains indicated that these genetic differences were reflected in the absorbances against the type-specific rabbit serum. The specimens that clustered with the reference HAstV-2 virus all gave high ratios of the *P* value for the TYPE-EIA to that for the AG-EIA, whereas the corresponding ratios for the remaining specimens were low (data not shown).

DISCUSSION

New appreciation for the role of astrovirus as a cause of diarrhea in children has evolved with the development of sensitive EIAs for detecting astrovirus in fecal specimens. The types of astrovirus strains collected in field studies can now be rapidly and efficiently determined by using the TYPE-EIA rather than the immunofluorescence, immunosorbent EM, and immune EM methods originally used to establish the reference types. Such methods do not lend themselves so favorably to field studies. Moreover, our sequence results suggest that the development of molecular methods for typing (e.g., probe hybridization and PCR) may be possible, thus eliminating the need for currently limited EIA reference reagents.

Our TYPE-EIA was able to type all 64 culture-adapted, astrovirus-positive strains and 78% of the matched fecal specimens of sufficient volume. Using our selection of 36 specimens, we sought to obtain sufficient sequence information for each specimen to be able to distinguish genetic differences between the antigenic types. We analyzed approximately 15% (348 bp) of the 2,388 bp of ORF2, using RT-PCR with primers reactive to all known types of astrovirus that span a region of considerable sequence diversity. Although we are unsure if this region of the genome encodes any epitopes detected by the TYPE-EIA, there was 100% correlation between the antigenic groupings (serotypes) and the genotypic groupings (genotypes) we obtained. Overall, the sequence diversity within a genotype was small in comparison with the variation between genotypes. Interestingly, in the cases of genotypes 1, 2, and 4, the sequences of some specimens within each group were identical despite collection of the specimens from countries as widely separated as Australia and the United Kingdom in the case of genotype 1, Australia and Peru in the case of genotype 2, and Korea and Peru in the case of genotype 4. Although some global distribution of sequence types was evident, there appears to be a clustering of community strains that circulate in the same region and have identical sequences. Such community strains were apparent in collections of genotype 1 specimens from Australia, Colorado, and New York. Clustering of identical sequence types was also observed for two specimens from the Gang Nam province in Korea, while the sequence of a third specimen from another region was different. Identical sequences were also found in genotype 4 specimens collected in one region in Korea and genotype 6 specimens from a multischool outbreak in Japan, where it appears that the cause of the food-borne contamination was from a single source.

The specimens representing mixed infections by TYPE-EIA are being investigated further to determine whether they show true coinfection of two different types or infection with a single type giving a mixed reactivity. Preliminary results indicate the possibility of dual infections.

The concordance of results obtained by genotyping and serotyping supports the reliability of data that could be obtained with the TYPE-EIA, and this reliability will allow the convenient collection of epidemiologic data on astrovirus. In general, fresh, appropriately collected stool specimens contain sufficient viral antigen to be serotyped directly by TYPE-EIA, with culture in Caco-2 cells required only for specimens giving indeterminate results. The TYPE-EIA can be more easily used in the field than the traditional immune EM methods. However, its widespread use would be restricted to laboratories that have access to the limited reference reagents available. Genotype determination of the 36 specimens examined in this study provided us with the sequence data to begin the development of simple molecular methods such as probe hybridization, which may serve as a good proxy for serotyping assays. The effectiveness of digoxigenin-labeled probes as a method of typing astrovirus specimens is being evaluated. Such assays, although more time-consuming than EIAs, contain none of the previously mentioned limitations of the TYPE-EIA, require no specialized equipment or reagents, and, if successful, would provide a suitable alternative to the EIA.

Further epidemiologic studies are needed to determine the disease burden of astrovirus-associated diarrhea in children. Our findings are consistent with those of Lee and Kurtz (20), who showed that for a 17-year period HAstV-1 to -7 have been detected in the Oxford region of the United Kingdom, with HAstV-1 the predominant type for all but 1 year. The incidence of infection with HAstV-2 to -4 was low, and that with HAstV-5 to -7 was rare. The AG-EIA, TYPE-EIA, or probe hybridization could be used to study further the epidemiology of astrovirus infection, assess the likelihood of repeat infection, and examine whether immunity to astrovirus may be type specific.

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