Detection of Astrovirus in Pediatric Stool Samples by Immunoassay and RNA Probe

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Two new astrovirus assays, a rapid biotin-avidin enzyme immunoassay (EIA) and RNA probe hybridization, were developed and compared with an established astrovirus assay, an indirect EIA, and immune electron microscopy. Sensitivity and specificity were evaluated by using a screening panel of 22 astrovirus-positive and 305 astrovirus-negative fecal specimens. The biotin-avidin assay was equivalent in performance to the reference indirect assay, and both could detect about 10 ng of viral protein. Although the probe was more sensitive than either EIA and could detect higher dilutions of virus in tissue culture and stool specimens, it did not detect more astrovirus-positive fecal specimens. Of the 22 astrovirus-positive specimens detected by the EIAs, 20 were confirmed by immune electron microscopy with hyperimmune rabbit antiserum. To determine the usefulness of EIAs for large epidemiologic studies, EIAs were used to screen 1,289 stool specimens from three studies of children with and without diarrhea. Astrovirus was detected in 3.5% of specimens from children with diarrhea and 1.9% of specimens from those without diarrhea. Our results indicate that the biotin-avidin EIA is an efficient, sensitive, and specific method for routinely screening large numbers of fecal samples and that its application in epidemiologic studies may yield higher rates of astrovirus infection than have been found previously by other methods.

Astroviruses were first described in 1975 by Appleton and Higgins (1), who examined stool samples from infants with gastroenteritis by electron microscopy. Because of the characteristic starlike appearance of these 28- to 30-nm particles, the name "astrovirus" was suggested by Madeley and Cosgrove (18). Astroviruses appear to occur worldwide and have been detected in the People's Republic of China (29), Japan (11), Thailand (7), Australia (5), South Africa (27), Malawi (24), Italy (2), Sweden (3), the United Kingdom (19), and the United States (15, 16, 23, 25). These reports have generally been associated with epidemic gastroenteritis in institutions with infants or the elderly and also with endemic illness in young children. The incubation period is typically 3 to 4 days, and illness is characterized by fever, headache, diarrhea, malaise, and occasional vomiting. Virus excretion can persist for up to 12 days (21).

Astrovirus can be transmitted by food and water (13), although person-to-person transmission is the more likely cause in institutional settings where outbreaks have occurred. Nosocomial astrovirus infections have been documented in Sweden (3), Great Britain (22), and Canada (4). Lew et al. (15) observed that more than 50% of all hospital-based detections of these agents in North America occurred in patients who had been hospitalized for 7 days or more. Electron microscopy surveillance studies suggest that astrovirus-associated diarrhea has a winter seasonality and that children under 1 year of age are most commonly affected (5, 15, 19). Data from the public health laboratory surveillance system in the United Kingdom show that astrovirus made up

3% of all electron microscopic detections reported from 1985 to 1987 (19).

Antibody prevalence surveys suggest widespread exposure in childhood, with antibodies present in only 7% of British infants (age, <1 year) but in more than 70% of children by the age of 5 years (13). The high antibody prevalence rate appears to be inconsistent with the relatively low frequency of astrovirus detection among children with diarrhea and suggests that astrovirus is difficult to detect or that infection may not often lead to disease. Until recently, astrovirus infection could be diagnosed only by electron microscopy or by primary culture of stool samples in human embryonic kidney cells, followed by immunofluorescence (14). The development of monoclonal antibodies to astrovirus and enzyme immunoassays (EIAs) (6) has allowed large-scale screening of stool sample collections for examination of the epidemiologic characteristics of astrovirus infection and disease. Recent work on the molecular biology of astrovirus has increased our understanding of the viral genome and has led to the development of astrovirus clones which can be used for diagnostic purposes (20).

Here we report the development of two diagnostic assays for astrovirus, a biotin-avidin EIA and dot blot hybridization with an astrovirus RNA probe, and compare these methods with two reference methods, an indirect EIA developed by Herrmann et al. (6) and immune electron microscopy (IEM). Using the indirect EIA, we prepared a reference collection of 22 astrovirus-positive and 305 astrovirus-negative stool samples from specimens collected in a study of children in day-care centers in Phoenix, Ariz. (16). These samples were then tested with the biotin-avidin assay, and all EIA-positive and a selection of EIA-negative samples were also examined by IEM and RNA probe hybridization for confirmation by a

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different method. We then compared the EIAs with each other and with dot blot hybridization in terms of their sensitivities, specificities, and limits of detection. Finally, we used the EIAs to screen two additional collections of stool samples from children with and without gastroenteritis. Our results demonstrate the value of EIAs for examining the epidemiologic features of astrovirus infection and disease and indicate that the use of these assays in the routine screening of fecal specimens from different populations may show higher rates of astrovirus infection than were previously observed.

MATERIALS AND METHODS

Virus. Cell culture-adapted astrovirus type T2 from John Kurtz (Oxford, England) was grown in LLCMK2 cells in the presence of trypsin (10 µg/ml) and was harvested after 48 h. The virus was purified by banding in a cesium chloride gradient, and virus-containing fractions (density, 1.39 g/ml) were dialyzed against Vogt's Tris-buffered saline. Rabbits were inoculated with purified astrovirus, first in Freund's incomplete adjuvant and subsequently with MDP adjuvant (Syntex Corp., Palo Alto, Calif.). Viral protein was determined by using a dye-binding assay (Bio-Rad, Richmond, Calif.) with a bovine gamma globulin protein standard.

Reference strains of human astrovirus serotypes T1, T4, and T5 were obtained from John Kurtz (12) and were grown at the Centers for Disease Control (CDC) in a similar fashion. These were used to examine the ability of the EIAs and RNA probe hybridization to detect each of the different serotypes.

Antibodies. Hyperimmune rabbit serum (CDC909) was purified by ammonium sulfate precipitation and protein G chromatography (Gammabind-G; Genex, Gaithersburg, Md.). The purity of immunoglobulin G (IgG) in the eluted fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (28). IgG-containing fractions were pooled and concentrated by using centrifugal ultrafiltration devices (Amicon, Danvers, Mass.) or by ammonium sulfate precipitation. The protein concentration of the purified IgG was adjusted to 1 mg/ml with sodium bicarbonate and biotinylated with *N*-biotinyl- ω -aminocaproic acid-*N*-hydroxysuccinimide ester according to the protocol of the manufacturer (Enzo Biochem, Inc., New York, N.Y.). An antiastrovirus monoclonal antibody, 8E7 (8), was used as unpurified ascitic fluid.

Stool samples. The reference collection of stool samples screened by the indirect EIA was a subset of samples obtained from children younger than 36 months of age participating in a study on diarrheal disease in day-care centers in Phoenix and surrounding Maricopa County, Ariz. (16). To determine astrovirus prevalence in different populations, we examined two additional collections of samples chosen because they included specimens from children with and without diarrhea. One collection was from children younger than 2 years of age in a pediatric hospital diarrhea ward and outpatient controls in Seoul, Republic of Korea (10). The other was from children younger than 3 years being followed in a longitudinal study in a poor periurban community near Lima, Peru. Specimens from the Phoenix collection that contained rotavirus, adenovirus, calicivirus, or Norwalk virus (as identified by EIA or electron microscopy) were used to check the specificity of both astrovirus EIAs. Stool suspensions (10 to 20%) were prepared in phosphate-buffered saline (PBS) and were clarified by low-speed centrifugation.

Direct electron microscopy and IEM. Examination of astrovirus by solid-phase IEM was performed by using a modification of a technique described previously (9). Stool suspensions were diluted 1:2.5, and hyperimmune rabbit serum (CDC909) was diluted 1:50. Glow discharge-exposed carbon-Formvar grids (Lanum Company, Homewood, Ill.) pretreated with protein A were exposed to 20 µl of the antigen-antibody mixture, incubated at room temperature for 1 to 2 h, and stained with 2% phosphotungstic acid at pH 6.6. Selected samples were also examined by direct electron microscopy.

Immunoassays. Our reference assay was the indirect double-antibody EIA developed by Herrmann et al. (6), which uses the monoclonal antibody (8E7) as capture antibody and hyperimmune rabbit serum (CDC909) as detector antibody. This assay was modified in our laboratory, in that the plates were coated with the monoclonal antibody diluted in carbonate-bicarbonate buffer (pH 9.6) for 2 h at 37°C before they were washed with PBS-Tween 20 (0.05%) and were then blocked overnight at 4°C with 1% bovine serum albumin in 0.1 M PBS (pH 7.0).

A fundamental difference between how this assay was performed in our laboratory and how it was performed previously (6) was our use of a control capture antibody for each sample rather than the use of stool diluent as the negative control. A 100-µl sample of each stool suspension was added to four wells (two test wells coated with antiastrovirus monoclonal antibody and two control wells coated with anti-parainfluenza virus type 1 monoclonal antibody) and was incubated for 2 h at 37°C. The plates were again washed with PBS-Tween 20, and 50 µl of rabbit antiserum to astrovirus, diluted 1:1,000 in 50% fetal calf serum and 50% 0.1 M Tris hydrochloride buffer (pH 7.2) with 0.15% Tween 20 and 1% goat antirotavirus serum, was added to each well. After incubation for 1.5 h at 37°C, the plates were washed and 50 µl of peroxidase-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), which was diluted to 1 µg/ml in the diluent described above, was added to each well. The plates were again incubated for 1.5 h at 37°C and washed with PBS-Tween 20.

The biotin-avidin immunoassay used an identical configuration, except that the rabbit antiserum detector was purified and biotinylated as described above. The plates were coated with the monoclonal antibodies (8E7 and anti-parainfluenza virus type 1) for 2 h at 37°C and washed with PBS-Tween 20 before adding the stool suspensions. The detector (diluted in PBS with 0.05% gelatin and 0.15% Tween 20 [PBS-GT]) was titrated to determine its optimal dilution and was used at a concentration of about 2 µg of protein per ml. The biotinylated detector (50 µl) was added to each well and was allowed to incubate for 1.5 h at 37°C. After washing with PBS-Tween 20, 50 µl of a 1:3,000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham Corp., Arlington Heights, Ill.) in PBS-GT was added to each well and was incubated for 20 min at 37°C.

Both immunoassays used 3,3',5,5'-tetramethylbenzidine hydrochloride substrate (Sigma, St. Louis, Mo.) for color development. After 10 min, the reaction was stopped with 2 M H₂SO₄, and the A₄₅₀ of the solution was measured in a plate reader spectrophotometer (Dynatech MR5000).

RNA probe hybridization. cDNA was prepared from a poly(A)-containing fraction of RNA isolated from astrovirus-infected cells. Following the addition of *Eco*RI adapters, the cDNA was cloned into an *Eco*RI-linearized plasmid vector, pBluescript II (Stratagene, La Jolla, Calif.). The specificities of the clones were determined by blot hybridization of in

vitro RNA transcripts to RNA isolated from astrovirus-infected and uninfected cells. One clone containing a 1,200-bp insert that hybridized to astrovirus RNA was used to generate ^{32}P -labeled probes by in vitro transcription.

Serial dilutions (fivefold) of one strong EIA-positive sample, an astrovirus T2-infected cell lysate, and undiluted stool samples were treated with proteinase K (100 $\mu\text{g}/\text{ml}$) in the presence of yeast tRNA (25 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. The samples were then mixed with an equal volume of a 3:2 (vol/vol) mixture of 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–37% formaldehyde and heated at 60°C for 15 min. Each sample (200 μl) was applied to a nylon membrane under suction through a 96-well dot blot manifold. After baking for 2 h at 80°C, the filters were prehybridized for 30 min and hybridized in sealed plastic bags with radiolabeled probes for 16 h at 50°C in 50% formamide–5 $\times$ Denhardt's solution–5 $\times$ SSC–1% sodium dodecyl sulfate–50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–0.1% sodium pyrophosphate–200 μg of yeast tRNA per ml. The filters were washed twice at 65°C for 15 min in 1 $\times$ SSC and 0.1 $\times$ SSC, both containing 0.1% sodium dodecyl sulfate, and were exposed to X-ray film without intensifying screens at room temperature for 24 to 48 h.

Statistical analyses. To determine which stool samples were positive for astrovirus by both the indirect and biotin-avidin immunoassays, we examined the relationship between the absorbance of the test wells (P) and the absorbance of the control wells (N) for each sample. P values were plotted against N values on a logarithmic scale. A regression curve was fit to the data, and the calculated cutoff curve was set at three standard errors of prediction above the regression curve. For practical application, we determined that simplified criteria of $P - N \geq 0.07$ and $P/N \geq 2$ provided a good approximation of the cutoff curve in the mid to upper range of negative well readings.

RESULTS

Variability in indirect and biotin-avidin EIAs. A total of 327 specimens were tested by both the indirect and the biotin-avidin EIAs. Analysis of the relationship between P and N indicated that the 22 samples that were positive by the indirect assay by using the simplified cutoff criteria (Fig. 1A) were also positive by the biotin-avidin assay (Fig. 1B). Absorbance values for positive samples in the indirect assay ranged from 0.15 to 0.80, with a mean of 0.39 ± 0.18 ; for the biotin-avidin assay, absorbance values for positive samples ranged from 0.13 to 1.05, with a mean of 0.33 ± 0.23 .

Sensitivity and specificity. The biotin-avidin and indirect assays gave identical results for 22 positive and 305 negative samples. Five samples initially identified as positive by the indirect assay and negative by the biotin-avidin assay were retested by both assays on the same day and gave concordant results. Examination of the 22 EIA-positive samples by IEM confirmed the presence of astrovirus particles in 20 samples. The two samples that were IEM negative had absorbance values in the biotin-avidin assay of 0.14 and 0.33 but may not have had sufficient quantities of intact viral particles to be detected by IEM.

The specificities of both assays were also examined by testing specimens that contained rotavirus ($n = 25$), adenovirus ($n = 43$), calicivirus ($n = 2$), or Norwalk virus ($n = 5$) to check for cross-reactivity. Of these, only one sample previously identified as positive for calicivirus gave a positive reaction in both the indirect and biotin-avidin immunoassays. This sample may have contained both astrovirus

FIG. 1. Average P plotted against average N for 327 stool samples assayed by the indirect EIA (A) and the biotin-avidin EIA (B). The $P - N$ and P/N cutoff criteria for determining positive samples are shown with a solid line ($P - N = 0.07$) and a dashed line ($P/N = 2$), respectively. Samples meeting both of these criteria were considered positive. ■, negative samples; *, positive samples.

and calicivirus since another calicivirus-positive sample did not react in the astrovirus EIA.

Both immunoassays were able to detect the four serotypes of astrovirus (T1, T2, T4, and T5) grown in tissue culture.

Threshold of detection. The detection limit of the indirect assay was compared with that of the biotin-avidin assay by titrating cesium chloride-purified astrovirus type 2 (Fig. 2). Both tests had similar levels of detection and could detect 5 to 15 ng of viral protein. Both assays exhibited similar dose-response curves for titrations of astrovirus type 2 cell harvest and astrovirus-containing stool samples (data not shown).

Comparison of immunoassay with RNA probe. The RNA probe was evaluated as a diagnostic test against the EIAs for sensitivity, specificity, limit of detection, and application to clinical specimens. A dot blot membrane was prepared with RNA from fivefold dilution series of each of the astrovirus serotypes grown in tissue culture (Fig. 3). Before RNA extraction, the quantities of viral antigen for each of the four serotypes were normalized on the basis of their absorbance values in an EIA. This permitted us to compare the limit of detection of the probe with the EIAs, even though equivalent amounts of antigen may not represent equivalent amounts of viral RNA on the membrane.

The probe detected all four astrovirus serotypes and gave

FIG. 2. Titration of cesium chloride-purified astrovirus T2 grown in tissue culture by both the indirect and biotin-avidin EIAs. The dashed line indicates the $P - N$ cutoff criterion for determining positive samples ($P - N = 0.07$). ■, biotin-avidin EIA; *, indirect EIA.

the strongest reaction with serotype 2. Probe hybridization could detect a 1:625 dilution of the normalized T2 sample which had been diluted an additional 25-fold from the original cell harvest. This detection level was 25-fold more sensitive than the EIAs, which were able to detect a 1:625 dilution of the original T2 cell harvest. There was less than a fivefold difference between the reactivity of the probe to serotypes 1 and 2. The probe was fivefold less reactive with serotype 5 and about 25-fold less reactive with serotype 4. These differences may have been due to differences in the concentration of viral RNA.

A fivefold dilution series of a strong EIA-positive stool sample indicated that the probe could detect a 1:625 dilution of the 10 to 20% stool suspension (Fig. 4, wells C7 [1:5] through C12 [1:15,625]) compared with the EIAs, which could detect between a 1:27 and a 1:81 dilution of the same suspension. Wells C1 through C4 contained samples of adenovirus, rotavirus A, poliovirus, and feline calicivirus, respectively, none of which reacted with the astrovirus RNA probe.

For clinical application, we compared the EIA with RNA probe hybridization by probing 22 EIA-positive specimens (Fig. 4, wells A1 through B11, excluding well A6) and 55 EIA-negative specimens (Fig. 4, wells A6 and D1 through D12; 42 additional negative wells not shown). For 71 specimens (92%), the biotin-avidin immunoassay and the probe gave the same results (Table 1). Four specimens that were positive by both the indirect and biotin-avidin EIAs were negative by probe hybridization, suggesting that the single-stranded viral RNA may have degraded but that sufficient viral protein was present to react with the antibodies in the EIA. Six samples were not consistently probe positive, indicating that probe hybridization may be sensitive to specimen handling and storage. Two EIA-negative samples were probe positive. Both of these samples were originally weakly positive by the indirect assay and negative by the biotin-avidin assay, but they were negative by both EIAs when they were retested.

Application of astrovirus immunoassays to routine diagnosis. The immunoassays were used to screen three collections

FIG. 3. RNA probe hybridization dot blot with fivefold serial dilutions of tissue culture harvests of four astrovirus serotypes (T1, T2, T4, and T5).

FIG. 4. RNA probe hybridization dot blot with (i) 22 EIA-positive stool specimens in positions A1 through B11, excluding A6; (ii) adenovirus, rotavirus A, poliovirus, and feline calicivirus in positions C1 through C4, respectively; (iii) a fivefold dilution series of a strong EIA-positive stool sample in positions C7 through C12; and (iv) 12 EIA-negative stool specimens in positions D1 through D12.

TABLE 1. Comparison of the biotin-avidin astrovirus EIA with RNA probe hybridization

EIA results	Probe hybridization results (no. of samples)		
	Positive	Negative	Total
Positive	18	4	22
Negative	2	53	55
Total	20	57	77

of stool specimens from studies of diarrheal disease in the United States, the Republic of Korea, and Peru. A total of 1,289 specimens were tested for astrovirus, which was detected in 2 to 4% of 867 specimens from children with diarrhea and in 1 to 2% of 422 specimens from children without diarrhea (Table 2). A significant difference was observed between the astrovirus detection rates from diarrhea cases and controls in the Phoenix collection (P [probability] = 0.04 by one-tailed Fisher's exact test). The results from the Korean and Peruvian collections suggest that asymptomatic infection may be common in these populations.

DISCUSSION

We began this research by evaluating astrovirus immunoassay formats. Several combinations of polyclonal-polyclonal, polyclonal-monoclonal, and monoclonal-monoclonal antibody sandwich-type immunoassays were tested in indirect, biotin-avidin, and direct formats. The best configuration was a biotin-avidin EIA with monoclonal capture antibody and a polyclonal detector antibody. We then compared this format with our reference method, the indirect EIA developed by Herrmann et al. (6), which uses the same monoclonal capture and hyperimmune rabbit detector, and confirmed our results by IEM. In addition, we developed an RNA probe for astrovirus and compared dot blot hybridization with both EIAs.

The biotin-avidin EIA for astrovirus appears to have the same sensitivity and specificity as the reference indirect EIA (91 and 98%, respectively, compared with IEM) (6), which was not unexpected, since the same basic reagents were used. It should be noted, however, that our modification of the indirect test used the absorbance of each sample in wells coated with a parainfluenza virus monoclonal antibody as the negative control (rather than stool diluent), and for determining positive samples, we used criteria different from those used in the assay described previously (6). Both assays displayed a lack of cross-reactivity with stool samples containing other gastrointestinal viruses and detected comparable levels of purified virus and virus in cell harvest supernatant and stool suspensions. In terms of routine practice, the biotin-avidin test is somewhat simpler to perform than the indirect EIA, because it does not include an overnight blocking step, has a shorter conjugate incubation period (20 min versus 1.5 h), and uses a simpler diluent (PBS-GT). In addition, we combined the biotin-avidin test for astrovirus with biotin-avidin tests for adenovirus and rotavirus that use the same general protocol but that use different capture and detector antibodies. This method allows us to test each stool specimen for astrovirus, adenovirus, and rotavirus simultaneously on the same plate, thus enabling us to screen samples for multiple agents.

Comparison of the EIAs with RNA probe hybridization suggests that while the probe had greater absolute sensitivity

TABLE 2. Astrovirus detection by EIA in pediatric stool samples

Collection location	Source	Age (yr)	No. positive/no. tested (%) in children:	
			With diarrhea	Without diarrhea
Phoenix	Day-care study	<3	21/524 (4)	1/138 (1)
Republic of Korea	Hospital cases, out-patient controls	<2	2/105 (2)	1/45 (2)
Peru	Longitudinal study	<3	7/238 (3)	6/239 (2.5)

and could detect higher dilutions of virus in tissue culture supernatant and stool samples, its clinical sensitivity in terms of identifying positive stool specimens was not markedly different from that of the EIAs. Furthermore, probe hybridization appears to be more sensitive to handling and storage conditions that may degrade the viral genome but leave enough intact viral protein to be detected by EIA. For routine testing, EIAs are simpler and more efficient; however, RNA probe hybridization is a useful confirmatory test for samples with small amounts of astrovirus that give equivocal results by EIA.

Our preliminary screening for astrovirus by EIA in several collections of stool specimens extends our understanding of the role of astrovirus in pediatric diarrhea. The rates of detection by immunoassay screening reported here (2 to 4%) are consistent with the rates of astrovirus detection by electron microscopy in young children. In a 6-year study of nonbacterial gastroenteritis, Grohmann (5) observed astrovirus particles in 4.3% of samples from Australian children younger than 5 years. Donelli et al. (2) reported a 3.7% detection rate of astrovirus by electron microscopy in a 3-year study of children aged 3 years and younger hospitalized for acute diarrhea in Rome, Italy. In a 2-month survey of children younger than 5 years who were treated for diarrhea at an outpatient clinic in Malawi, astrovirus particles were observed in 2 (1.2%) of 168 children (24).

The detection of astrovirus in children without diarrhea observed here suggests that asymptomatic infection may be common in some populations or age groups. Asymptomatic astrovirus infections have been noted by others (17, 26) and may explain in part the discrepancy observed between the relatively low astrovirus detection rates in fecal samples and the high prevalence of astrovirus antibodies in several populations.

A recent report of astrovirus detection by EIA in 8.6% of Thai children younger than age 5 with gastroenteritis and in 2% of age-matched children without gastroenteritis (7) illustrates the need for these assays to further clarify the epidemiologic features of this virus. Our results indicate that EIAs are sensitive, specific, and practical for testing large numbers of fecal specimens on a routine basis. Continued application of astrovirus screening by EIA to a variety of populations may reveal higher rates of astrovirus infection than previously detected by other methods and may identify those groups at greatest risk of astrovirus infection and disease.

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