

Prevalence of Astroviruses in a Children's Hospital

SUNITA SHASTRI, ANNE MARTIN DOANE, JAIME GONZALES,
USHA UPADHYAYULA, AND DORSEY M. BASS*

Department of Pediatrics and Center for Digestive Disease,
Stanford University, Stanford, California 94305-5208

Received 26 November 1997/Returned for modification 30 January 1998/Accepted 19 June 1998

An enzyme immunoassay for astrovirus was used to screen 357 stool samples from 267 symptomatic inpatients at a tertiary-care children's hospital. Thirty stool samples from 26 patients contained astrovirus antigen, while rotavirus was found in 34 samples and *Clostridium difficile* toxin was found in 40. Half of the astrovirus infections were nosocomial. Additional pathogens were identified in six of the astrovirus antigen-positive stool samples. Most (80%) of the astroviruses recovered were of serotype 1. Astrovirus infections were significantly more common than rotavirus or *C. difficile* infections in very young infants and in those with surgical short-bowel syndrome.

Astroviruses are small, 28 to 34 nm, single-stranded RNA viruses which are part of a recently described family, *Astroviridae* (25). Astroviruses cause diarrhea in a variety of species and have been observed by electron microscopy in human infantile diarrheal stools since 1975 (1, 22). Recently, simple and sensitive assays such as enzyme-linked immunoassays (EIAs) and reverse transcriptase-PCR have replaced electron microscopy and have shown astroviruses to be a common cause of diarrhea (7, 14, 24).

Astroviruses have been increasingly identified as important agents of diarrheal disease in infants, immunocompromised patients (6, 12), and the elderly in nursing homes (11) and of nosocomial diarrhea in children's hospitals (9, 10). Here we describe a new, sensitive, monoclonal antibody (MAb)-based EIA which detects all seven serotypes of human astrovirus and its application in surveillance of astrovirus among symptomatic patients at a tertiary children's hospital in the United States.

MATERIALS AND METHODS

Stool samples. Stool samples were submitted for microbiologic analysis based on symptoms of vomiting, diarrhea, or feeding intolerance. A portion of each inpatient stool sample submitted to the Microbiology Laboratory at Lucile Salter Packard Children's Hospital, Stanford, Calif., between 1 July 1996 and 1 July 1997 was aliquoted for this study. Samples were held at -70°C until the time of assay. For the EIAs, stools were suspended at 10 or 0.2% (wt/vol) in phosphate-buffered saline containing 15 to 20% fetal bovine serum (FBS) and pelleted at $1,000 \times g$ for 5 min.

Antibodies. Polyclonal rabbit antiserum to cesium chloride-purified serotype 1 human astrovirus was prepared (3). Reference rabbit antisera against human astrovirus serotypes 1 to 7 were kindly provided by J. B. Kurtz and T. W. Lee (Oxford, England). MAb 8G4 is an immunoglobulin G1 (IgG1) mouse hybridoma which reacts with human astrovirus serotypes 1 to 7 by immunoperoxidase staining of infected cells and by EIA (3). It was purified by protein A-Sepharose chromatography and biotinylated (30). MAb 191 is an IgG1 mouse hybridoma which reacts with rotavirus (RV) nonstructural protein NSP3 (2). MAb 5B7 is an IgG3 antibody which specifically neutralizes serotype 1 astrovirus (3).

Viruses. Astrovirus serotypes 1 to 7 adapted to tissue culture by J. B. Kurtz and T. W. Lee were the kind gift of S. Matsui, Stanford University. Astroviruses were propagated in Caco 2 cells in RPMI medium without FBS and in the presence of 10 $\mu\text{g/ml}$ trypsin (type IX; Sigma), while rhesus RV (RRV) (strain G3,P5[3]) was grown in MA 104 cells (3).

EIA. Three EIAs were devised, two for detection of astrovirus group antigen in stool samples and one for detection of the presence of a serotype 1-specific epitope recognized by MAb 5B7 (3).

The initial astrovirus antigen EIA used rabbit polyclonal anti-serotype 1 human astrovirus serum as the capture antibody. A biotinylated MAb (8G4) directed against human astrovirus group antigen was used for detection. In preliminary experiments, this EIA was able to detect cell culture supernatants from all seven human astrovirus serotypes.

The second EIA used MAb 8G4 as the capture antibody and biotinylated 8G4 for detection. Microtiter wells were coated with alternating columns of MAbs 8G4 and 191. Sample application and detection were done as described for the first EIA, except that stool suspensions were at 0.2% (wt/vol).

The third EIA used MAb 5B7 as the capture antibody. The remainder of the EIA was performed as described above, with biotinylated MAb 8G4 as the detector.

In all three EIAs, avidin peroxidase (Sigma, St. Louis, Mo.) was used as the probe and TMB substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used as the substrate and the plates were read on a Bio-Rad EIA reader at 450 nm after the reaction had been stopped with 1 M phosphoric acid. Cell culture-grown astrovirus and RRV served as positive and negative controls. All results were considered positive when the mean A_{450} was greater than twice that of the negative controls.

Statistics. Minitab 8.0 for Macintosh (Addison-Wesley, Reading, Mass.) was used to perform chi-square and Mann-Whitney nonparametric tests.

Growth of virus in Caco 2 cells. Supernatants of stool suspensions were passed blindly one to three times as previously described (27), and infectious virus was detected and quantitated by titration with an immunoperoxidase assay on Caco 2 cells in 96-well plates (3).

Neutralization assay. Virus neutralization by serotype-specific polyclonal sera and MAbs was performed in a microneutralization test (3) with reference antisera used at a dilution of 1:10,000.

Microbiology. Bacterial cultures and examination for ova and parasites were conducted by standard microbiologic procedures. RV was detected by using the Abbott Testpack, while *Clostridium difficile* toxin was detected with the Becton-Dickinson Toxin-CD EIA. Adenovirus detection was performed as requested by the primary physician, by using a commercial EIA (MRL, Cypress, Calif.) which recognizes all adenovirus serotypes, including 40 and 41.

RESULTS

Assay development. Our initial astrovirus antigen EIA, similar to one previously described by Noel et al. (27), uses polyclonal rabbit serum as a capture antibody and provided a good signal-to-noise ratio both on tissue culture-grown virus and in mock-positive samples made by adding cell culture virus to stool samples. The sensitivity of the assay using purified serotype 1 astrovirus was approximately 10 to 20 ng/ml, and all seven human serotypes were detected equally well. RRV and reovirus serotype 1 consistently tested negative.

After assaying 334 clinical samples and finding 63 (18.8%) positive, we noted several problems with the assay. We found that many positive samples gave equally high absorbance read-

* Corresponding author. Mailing address: Department of Pediatrics and Center for Digestive Disease, Stanford University, Stanford, CA 94305-5208. Phone: (650) 723-5070. Fax: (650) 723-2137. E-mail: Dorsey.Bass@Forsythe.stanford.edu.

TABLE 1. Antigenic characteristics of astrovirus-positive stools^a

Sample no.	Result of EIA based on:			Cell culture result	Serotype
	Rabbit antiserum	MAB 8G4	MAB 5B7		
3	+	+	NT	NT	NT
43	±	+	-	+	2
50	+	+	±	NT	NT
72	-	+	-	NT	NT
78	+	+	+	NT	NT
82	-	+	±	+	1
96	-	+	±	-	NT
103	+	+	-	NT	NT
108	+	+	±	+	1
111	+	+	+	+	1
119	NT	+	-	+	2
132	+	+	NT	+	1
144	+	+	NT	+	1
182	+	+	+	NT	NT
201	+	+	+	+	1
203	+	+	+	-	NT
199	-	+	-	NT	NT
227	-	+	+	+	1
255	-	++	±	+	1
263	+	+	±	-	NT
264	-	+	±	-	NT
265	+	+	±	+	1
271	+	+	+	+	1
275	+	+	NT	-	NT
315	NT	+	+	+	1
338	NT	+	-	+	1
353	NT	+	-	+	3
359	NT	+	+	+	1
385	NT	+	-	+	1
386	NT	+	+	+	1

^a NT, not tested. Cell culture, adapted to cell culture on Caco 2 cells. Serotype, determined by microneutralization assay. ++, >200 OD units; +, >100 OD units; ±, equivocal; -, negative.

ings if we used preimmune serum as the capture antibody. We also noted that when we attempted to confirm selected positive samples by tissue culture adaptation in Caco 2 cells, only 3 of 10 samples were confirmed. We then developed the second EIA, which uses MAB 8G4 for both capture and detection, increased the amount of FBS in the sample buffer to 20%, and retested samples with high background levels as 0.2% rather than 10% stool suspensions. With these modifications, our high-background problems diminished substantially. Eighteen of 23 available stool samples positive by this assay have been successfully adapted to cell culture (Table 1). Comparing results from samples tested by both assays, 39 samples which were initially positive or uninterpretable by assay 1 due to a high background level were negative by assay 2. Eight samples which were initially negative by assay 1 later tested positive by assay 2. Of the four available samples which became positive with the second, all-MAB assay, three have been successfully tissue culture adapted. All further results in this report are based on our second assay.

Pathogens identified. During the 11 months of the study, 357 stool samples from 267 symptomatic children throughout the hospital were submitted to microbiological analysis. A total of 121 pathogens were found in the samples. Ninety-one percent of the identified pathogens were accounted for by three agents. The three common pathogens were *C. difficile* toxin (40 samples, 34 patients), RV (34 samples, 31 patients), and astrovirus (30 samples, 26 patients). The remaining 18 pathogens were

TABLE 2. Ages of patients infected with common pathogens

Organism	Age (yr)			
	Mean	Median	SE	Range
Astrovirus	2.5 ^a	0.7	0.75	0.1-16
RV	4.5	3.0	0.76	0.3-19
<i>C. difficile</i>	5.3	2.5	0.86	0.2-16

^a $P < 0.0036$ by Mann-Whitney test.

bacteria (nine samples), protozoans (five samples), or adenovirus (four samples).

Thus, of the 267 symptomatic patients, 9.7% were positive for astrovirus, 11.6% were positive for RV, and 12.7% were positive for *C. difficile*. Other pathogens were identified in six (23%) of the astrovirus antigen-positive patients, including two RV and four *C. difficile* isolates. Thirteen astrovirus infections were considered to be nosocomial, as evidenced by a previous negative EIA during admission (8 patients) and/or onset of symptoms more than 72 h after admission (11 patients). The remaining 13 astrovirus infections were considered to be community acquired.

Assessment of the duration of astrovirus shedding by four patients with astrovirus infections was possible. Two patients had shedding for less than 14 days, one had shedding for at least 10 days, and shedding by one was documented on three occasions over a span of 45 days.

Patient age at infection. When the ages of patients shedding astrovirus, RV, and *C. difficile* toxin were compared, it was evident that astrovirus infected a significantly younger population (median age, 0.7 year) than RV (median age, 3.0 years) or *C. difficile* (median age, 2.5 years) (Table 2). Patients shedding astrovirus were also significantly younger than the entire cohort of patients.

Diagnosis at admission. We examined the diagnoses at admission and the underlying diagnoses of patients identified with the three major pathogens. The most common diagnoses are presented in Table 3. The most striking finding is the association of astrovirus infection with short-bowel syndrome. Six of the 26 astrovirus-positive patients (including one who shed for at least 45 days) carried this diagnosis. It is unclear whether their underlying medical condition, their physical proximity on a surgical ward, or their panel of care providers explains this clustering. Very few (two) of the hematology/oncology patients shed astrovirus, perhaps as a function of their greater age and prior exposure. All three microorganisms appeared to be capable of causing diarrhea severe enough to warrant hospitalization in normal infants and children.

Serotyping of isolates. The 18 tissue culture-adapted viruses were tested by neutralization assay against serotype-specific polyclonal rabbit sera (Table 1). Fifteen (83%) of 18 were of

TABLE 3. Diagnoses at admission and underlying diagnoses

Pathogen	No. of patients			
	Kidney/liver Tx ^a	BM ^b Tx/cancer	Short bowel syndrome	Diarrhea
Astrovirus	3	2	6 ^c	3
RV	3	6	1	5
<i>C. difficile</i>	3	9	0	4

^a Tx, transplant.

^b BM, bone marrow.

^c $P < 0.05$ by chi-square test.

serotype 1, 2 were of serotype 2, and 1 was of serotype 3 by neutralization assay.

Antigenic diversity of astrovirus isolates. To gain some insight into the diversity of astroviruses found in our studies, we devised another EIA based on MAb 5B7, which is serotype 1 specific in neutralization assays using the prototype astrovirus strains (3). Initial testing of this EIA showed that it reacted only with the prototype serotype 1 and 7 strains (data not shown). When we applied it to our clinical samples which were previously positive by the 8G4-based EIA, we found that 10 of the 26 were unequivocally positive. An additional eight were equivocal, and eight (including the three non-serotype 1 isolates) were clearly negative (Table 1). Thus, MAb 5B7 seems to recognize an epitope present on many, but not all, serotype 1 astroviruses. The strength of the signal in the 5B7-based EIA bore no relation to the signal strength in the 8G4 assay, suggesting that the quantity of astrovirus antigen in the stool specimen was not critical for 5B7 reactivity. Furthermore, the reactivity of stool samples with the 5B7 EIA had no correlation with the virus titer found after one passage on Caco 2 cells.

DISCUSSION

There is no widely accepted "gold standard" for detection of astroviruses in stool samples. Electron microscopy and cell culture isolation are expensive and cumbersome and may miss positive samples due to lack of sensitivity. On the other hand, highly sensitive assays based on EIA and PCR may be prone to false-positive results. In this report, we describe the development of an EIA based entirely on a group-specific MAb which reacts with human astrovirus serotypes 1 to 7. The advantages of a MAb-based assay include unlimited supplies of consistent reagents and avoidance of possible detection bias due to the use of polyclonal serum raised to a single serotype of astrovirus. Our EIA appears to be reasonably specific, with 78% of positives confirmed by tissue culture isolation.

In our survey, a possible etiologic agent was identified in 45% of stool samples from symptomatic pediatric inpatients, similar to the 47% reported in a prior study of nosocomial diarrhea in a Swedish pediatric hospital (4). Previous studies of inpatient pediatric diarrhea from around the world have consistently shown that viral infections, particularly those due to RV, account for the majority of cases (4, 8, 10, 13, 18, 31). Among bacterial pathogens, *C. difficile* is the most common cause of inpatient/nosocomial pediatric diarrhea (5, 23, 29). Astrovirus, RV, and *C. difficile* accounted for over 90% of the pathogens identified in our study, which included both community-acquired and nosocomial infections. We found that astroviruses accounted for approximately 10% of the diarrhea cases (21% of the identifiable cases) in this setting, which is comparable to previous reports (4, 10). We also observed an increased incidence of RV and astrovirus infections during winter months (data not shown), which is also consistent with previous reports (19, 21).

As seen in prior long-term surveys (20, 27), the majority (80%) of our clinical isolates were serotype 1 astroviruses. Serotype 1 strains were also predominant in two studies from children's hospitals in England and Australia (26, 28). The variable reactivity of our serotype 1 isolates with MAb 5B7 and the isolation of three different serotypes demonstrate that multiple astrovirus strains were circulating in this children's hospital during a single season.

Astrovirus infections occurred in a significantly younger patient population than either RV or *C. difficile* infections in our studies. The decreased susceptibility of older patients to astrovirus infection suggests that astrovirus-infected infants may

develop protective immunity to resist subsequent exposures. Very little is known about such immunity, although it is known that young children develop serum antibodies to astroviruses early in life (15, 16, 17).

A novel observation in our study was the high rate of astrovirus infection in patients with short-bowel syndrome. Although this may have been due to ongoing environmental contamination or an unidentified carrier, other children on the same ward did not have as high an attack rate. These patients had undergone significant surgical intestinal resections and had lost not only absorptive surface area but large amounts of gut-associated lymphoid tissue, both of which losses may have rendered them particularly susceptible to symptomatic mucosal infection. Indeed, a 2 year old in our study with a very short gut excreted high titers of astrovirus for at least 45 days. Others have noted that patients with "underlying gastrointestinal disorders" were at increased risk for astrovirus-associated nosocomial diarrhea (9).

All three of the major pathogens identified in this study may be found in stools of asymptomatic pediatric patients. Examination of 20 control stool samples from a ward with the highest number of symptomatic astrovirus-shedding patients showed that 6 were positive, suggesting that asymptomatic astrovirus infection can be quite common (data not shown). Prior studies of day care center outbreaks have also shown that asymptomatic astrovirus shedding is common among older attendees (24). Further prospective case-control studies are warranted to fully assess the role of each of these pathogens in diarrheal disease of hospitalized pediatric patients.

ACKNOWLEDGMENTS

This work was supported by grants R29 DK45036 and DK38707 from the NIH.

We thank Suzanne Matsui for reagents and helpful discussions and David Kiang for assistance with ascites production. We also gratefully acknowledge the assistance of the nursing staff and the Department of Clinical Microbiology at the Lucile Packard Children's Hospital.

REFERENCES

1. Appleton, H., and P. G. Higgins. 1975. Viruses and gastroenteritis in infants. *Lancet* **i**:1297.
2. Bass, D. M., E. R. Mackow, and H. B. Greenberg. 1990. NS35 and not vp7 is the soluble rotavirus protein which binds to target cells. *J. Virol.* **64**:322-330.
3. Bass, D. M., and U. Upadhyayula. 1997. Characterization of human serotype 1 astrovirus-neutralizing epitopes. *J. Virol.* **71**:8666-8671.
4. Bennet, R., K. O. Hedlund, A. Ehrnst, and M. Eriksson. 1995. Nosocomial gastroenteritis in two infant wards over 26 months. *Acta Paediatr.* **84**:667-671.
5. Collignon, A., C. Chaumard, I. Vallet-Collomb, and N. Delepine. 1988. *Clostridium difficile* in children and adolescents undergoing anticancer and antimicrobial chemotherapy. The possibility of nosocomial acquisition. *Pathol. Biol.* **36**:754-758.
6. Cox, G. J., S. M. Matsui, R. S. Lo, M. Hinds, R. A. Bowden, R. C. Hackman, W. G. Meyer, M. Mori, P. I. Tarr, and L. S. Oshiro. 1994. Etiology and outcome of diarrhea after marrow transplantation: a prospective study. *Gastroenterology* **107**:1398-1407.
7. Cruz, J. R., A. V. Bartlett, J. E. Herrmann, P. Caceres, N. R. Blacklow, and F. Cano. 1992. Astrovirus-associated diarrhea among Guatemalan ambulatory rural children. *J. Clin. Microbiol.* **30**:1140-1144.
8. Dutta, P., S. K. Bhattacharya, M. R. Saha, D. Dutta, M. K. Bhattacharya, and A. K. Mitra. 1992. Nosocomial rotavirus diarrhea in two medical wards of a pediatric hospital in Calcutta. *Indian Pediatr.* **29**:701-706.
9. Esahli, H., K. Breback, R. Bennet, A. Ehrnst, M. Eriksson, and K. O. Hedlund. 1991. Astroviruses as a cause of nosocomial outbreaks of infant diarrhea. *Pediatr. Infect. Dis. J.* **10**:511-515.
10. Ford-Jones, E. L., C. M. Mindorff, R. Gold, and M. Petric. 1990. The incidence of viral-associated diarrhea after admission to a pediatric hospital. *Am. J. Epidemiol.* **131**:711-718.
11. Gray, J. J., T. G. Wreghitt, W. D. Cubitt, and P. R. Elliot. 1987. An outbreak of gastroenteritis in a home for the elderly associated with astrovirus type 1 and human calicivirus. *J. Med. Virol.* **23**:377-381.
12. Grohmann, G. S., R. I. Glass, H. G. Pereira, S. S. Monroe, A. W. Hightower, R. Weber, and R. T. Bryan. 1993. Enteric viruses and diarrhea in HIV-

- infected patients. Enteric Opportunistic Infections Working Group. *N. Engl. J. Med.* **329**:14–20.
13. **Gusmao, R. H., J. D. Mascarenhas, Y. B. Gabbay, Z. Lins-Lainson, F. L. Ramos, T. A. Monteiro, S. A. Valente, and A. C. Linhares.** 1995. Rotaviruses as a cause of nosocomial, infantile diarrhoea in northern Brazil: pilot study. *Mem. Inst. Oswaldo Cruz* **90**:743–749.
 14. **Herrmann, J. E., D. N. Taylor, P. Echeverria, and N. R. Blacklow.** 1991. Astroviruses as a cause of gastroenteritis in children. *N. Engl. J. Med.* **324**:1757–1760.
 15. **Kotloff, K. L., J. E. Herrmann, N. R. Blacklow, R. W. Hudson, S. S. Wasserman, J. Morris, Jr., and M. M. Levine.** 1992. The frequency of astrovirus as a cause of diarrhea in Baltimore children. *Pediatr. Infect. Dis. J.* **11**:587–589.
 16. **Kriston, S., M. M. Willcocks, M. J. Carter, and W. D. Cubitt.** 1996. Seroprevalence of astrovirus type 1 and 6 in London, determined using recombinant virus antigen. *Epidemiol. Infect.* **117**:159–164.
 17. **Kurtz, J., and T. Lee.** 1978. Astrovirus gastroenteritis: age distribution of antibody. *Med. Microbiol. Immunol.* **166**:227–230.
 18. **Lam, B. C., J. Tam, M. H. Ng, and C. Y. Yeung.** 1989. Nosocomial gastroenteritis in paediatric patients. *J. Hosp. Infect.* **14**:351–355.
 19. **LeBaron, C. W., J. Lew, R. I. Glass, J. M. Weber, and P. G. Ruiz.** 1990. Annual rotavirus epidemic patterns in North America. Results of a 5-year retrospective survey of 88 centers in Canada, Mexico, and the United States. Rotavirus Study Group. *JAMA* **264**:983–988.
 20. **Lee, T. W., and J. B. Kurtz.** 1994. Prevalence of human astrovirus serotypes in the Oxford region 1976–92, with evidence for two new serotypes. *Epidemiol. Infect.* **112**:187–193.
 21. **Lew, J. F., R. I. Glass, M. Petric, C. W. LeBaron, G. W. Hammond, S. E. Miller, C. Robinson, J. Boutilier, M. Riepenhoff-Talty, and C. M. Payne.** 1990. Six-year retrospective surveillance of gastroenteritis viruses identified at ten electron microscopy centers in the United States and Canada. *Pediatr. Infect. Dis. J.* **9**:709–714.
 22. **Madeley, C. R., and B. P. Cosgrove.** 1975. Viruses in infantile gastroenteritis. *Lancet* **ii**:124.
 23. **Meropol, S. B., A. A. Luberti, and A. R. De Jong.** 1997. Yield from stool testing of pediatric inpatients. *Arch. Pediatr. Adolesc. Med.* **151**:142–145.
 24. **Mitchell, D. K., R. Van, A. L. Morrow, S. S. Monroe, R. I. Glass, and L. K. Pickering.** 1993. Outbreaks of astrovirus gastroenteritis in day care centers. *J. Pediatr.* **123**:725–732.
 25. **Monroe, S. S., B. Jiang, S. E. Stine, M. Koopmans, and R. I. Glass.** 1993. Subgenomic RNA sequence of human astrovirus supports classification of *Astroviridae* as a new family of RNA viruses. *J. Virol.* **67**:3611–3614.
 26. **Noel, J., and D. Cubitt.** 1994. Identification of astrovirus serotypes from children treated at the Hospitals for Sick Children, London 1981–93. *Epidemiol. Infect.* **113**:153–159.
 27. **Noel, J. S., T. W. Lee, J. B. Kurtz, R. I. Glass, and S. S. Monroe.** 1995. Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing. *J. Clin. Microbiol.* **33**:797–801.
 28. **Palombo, E. A., and R. F. Bishop.** 1996. Annual incidence serotype distribution, and genetic diversity of human astrovirus isolates from hospitalized children in Melbourne, Australia. *J. Clin. Microbiol.* **34**:1750–1753.
 29. **Rohner, P., D. Pittet, B. Pepey, T. Nije-Kinge, and R. Auckenthaler.** 1997. Etiological agents of infectious diarrhea: implications for requests for microbial culture. *J. Gen. Virol.* **78**:1427–1432.
 30. **Shaw, R. D., P. T. Vo, P. A. Offit, B. S. Coulson, and H. B. Greenberg.** 1986. Antigenic mapping of the surface proteins of rhesus rotavirus. *Virology* **155**:434–451.
 31. **Struve, J., R. Bennet, A. Ehrnst, M. Eriksson, K. O. Hedlund, P. Herin, and A. Samuelsson.** 1994. Nosocomial calicivirus gastroenteritis in a pediatric hospital. *Pediatr. Infect. Dis. J.* **13**:882–885.