Rotavirus Antigenemia in Patients with Acute Gastroenteritis

Thea K. Fischer,[1,3] Deanna Ashley,[4] Tara Kerin,[1] Erica Reynolds-Hedmann,[4] Jon Gentsch,[1] Marc-Alain Widdowson,[1] Larry Westerman,[1] Nancy Puhr,[2] Reina M. Turcios,[1] and Roger I. Glass[1]

[1]Viral Gastroenteritis Team, Division of Viral and Rickettsial Diseases, and [2]Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, and [3]Epidemic Intelligence Service, Centers for Disease Control and Prevention, Atlanta, Georgia; [4]Health Promotion and Protection Division, Ministry of Health, Kingston, Jamaica

Although rotavirus infections are generally considered to be confined to the intestine, recent reports suggest that extraintestinal disease occurs. We studied whether rotavirus infection was associated with antigenemia during a major outbreak of gastroenteritis in the Kingston metropolitan area, during July–August 2003. Rotavirus antigen was identified in 30 of 70 acute-phase serum samples (including from 2 deceased individuals) but in only 1 of 53 control samples. Serum antigen levels were inversely associated with time since symptom onset and were directly associated with antigen levels in stool ($P = .02$). Serum antigen levels were significantly elevated during primary infections (acute-phase serum immunoglobulin G [IgG] titers, <25), compared with those in subsequent infections (acute-phase serum IgG titers, ≥25) ($P = .02$). Antigenemia was common in this outbreak and might provide a mechanism to help explain rare but well-documented reports of findings of extraintestinal rotavirus. In situations in which stool samples are not readily available (i.e., patients with severe dehydration or those recently recovered or deceased), serum testing by enzyme immunoassay offers a new and practical diagnostic tool.

Rotavirus is the major cause of acute dehydrating diarrhea among infants and young children worldwide. The clinical spectrum of rotavirus disease varies from asymptomatic infection to acute, severe, dehydrating diarrhea with vomiting that can be fatal. Rotavirus infection has always been considered to be confined to the intestine, but recent reports suggest that extraintestinal manifestations occur: rotavirus RNA has been detected in the heart and liver and, in patients with seizures, in cerebrospinal fluid [1–5]. Recently, rotavirus antigen was detected in 22 of 33 serum samples from children with confirmed rotavirus infection but in 0 of 35 samples from control individuals, and rotavirus RNA was detected by reverse-transcription (RT) polymerase chain reaction (PCR) in 3 of 6 rotavirus-positive serum samples. Viremia was demonstrated in animals with rotavirus infection, suggesting a possible mechanism for both extraintestinal and fatal disease [6]. These preliminary data prompted us to consider that rotavirus infection might be diagnosed by detection of rotavirus antigen or RNA in serum, and we questioned whether severe or fatal disease might be a direct consequence of this viremia.

In early June 2003, the Jamaican Ministry of Health (MoH) identified a sharp increase in the number of cases of acute gastroenteritis reported among children <5 years of age and children ≥5 years of age throughout the country that was accompanied by a parallel increase in hospitalizations and 10–15 deaths [7]. By July 2003, >3000 cases of gastroenteritis and 14 deaths were reported, and the outbreak was not subsiding; therefore, we began an investigation to determine the epidemiologic and etiologic cause of the outbreak, to prevent further cases [7]. When the outbreak was determined

to be due to rotavirus alone, we were able to establish the unexpected presence of rotavirus antigen in serum samples from patients with acute disease but not from convalescent-phase serum samples, and we found rotavirus in tissues and serum samples from 2 children who had died. Both findings were confirmed by independent molecular assays for double-stranded RNA (dsRNA) and have expanded our thinking about the pathogenesis and diagnosis of severe and fatal rotavirus disease.

PATIENTS AND METHODS

The outbreak was identified from weekly surveillance data on diarrhea cases reported from 55 sentinel sites (outpatient clinics and hospitals) throughout Jamaica to the National Sentinel Surveillance System, which is maintained by the MoH. Reports of diarrhea-related deaths occurring during the outbreak period were compared with the numbers reported during other peak seasons for diarrhea and were investigated to identify potentially preventable causes of death.

Surveillance. From 25 July to 12 August, we surveyed patients <12 years of age with diarrhea who attended 1 of 2 major pediatric hospital facilities—Bustamante Hospital for Children in Kingston and Spanish Town Hospital in St. Catherine. We developed a questionnaire to collect demographic data such as age, sex, symptoms, and time from onset of symptoms, and we collected stool samples or rectal swab samples to test for a range of enteric viruses, bacteria, and parasites. In addition, on the basis of hospital admission records, we identified children admitted with a diagnosis of gastroenteritis during the 2 weeks before July 25, visited the children at home to collect stool and/or serum samples to establish an etiologic diagnosis, and recorded the time interval from the onset of illness to the time of sample collection. We later collected convalescent-phase serum from 25 children, for testing of immune responses to other suspected etiologic agents, such as norovirus, in case no pathogen was identified in the stool. Since the samples and data were collected as part of an outbreak investigation and were obtained to guide the public health response, institutional review board regulations do not apply.

Laboratory analyses. All stool samples were stored in coolers, delivered to the Central Public Health Laboratory in Kingston, and tested for rotavirus by use of a commercial EIA kit (Rotaclone; Meridian Diagnostics) and for parasites by wet microscopy and Ziehl-Nielsen staining before storage at −20°C. Twenty-five fecal swabs in Cary-Blair transport medium were sent to the Centers for Disease Control and Prevention (CDC; Atlanta, GA) and were tested for the following bacterial pathogens, by use of standard bacteriologic procedures: *Salmonella* species, *Shigella* species, *Vibrio* species, *Escherichia coli* O157:H7, *Yersinia enterocolitica, Campylobacter jejuni,* and diarrheagenic *E. coli* (non-O157 Shiga toxin–producing, enterotoxigenic, enteropathogenic, and enteroinvasive). A subset of 32 stool samples

from children for whom serum samples were also available were analyzed for norovirus, sapovirus, astrovirus, and adenovirus by PCR, in accordance with procedures described elsewhere [8].

Once rotavirus was identified as the causative agent, we screened all serum samples by EIA for evidence of rotavirus antigenemia and by RT-PCR for viral RNA, a method recently reported by Blutt et al. [6]. Undiluted serum samples (50 µL) were tested for rotavirus antigen by use of RotaClone, in accordance with the manufacturer's instructions, and the optical density was read by spectrophotometry at a wavelength of 450 nm. This commercial EIA for rotavirus was intended to screen stool samples, and, when we applied this assay to test undiluted serum samples from control subjects (i.e., individuals without a rotavirus infection), 15% gave positive results with optical density values above the 0.1 level prescribed as a cutoff value for stool testing. To establish an appropriate cutoff value for discriminating between rotavirus-positive and rotavirus-negative undiluted serum samples, we tested serum samples from 53 American adults seen as outpatients in an emergency room who had diarrhea of unknown etiology, and we established the cutoff point to be 3 SDs above the mean optical density value. Subsequently, we compared the optical density values from a total of 4 groups of patients: 43 children with diarrhea who had rotavirus detected in their stool (group A), 27 children recovering from diarrhea who did not have rotavirus detected in their stool 1–2 weeks after the onset of their episode (group B), the 53 adult control individuals (group C), and children from whom 25 convalescent-phase serum samples were obtained 8–10 weeks after the acute-phase serum samples (group D). The frequency distributions of the optical density values were plotted for each of the 4 groups, to test for differences. The presence of rotavirus RNA in serum was assessed by RT-PCR, using the Qiagen 1-step RT-PCR kit with the cycling conditions described by Iturriza Gomara et al. [9], and by applying a degenerate primer pair for a VP6 gene fragment. These results were confirmed by Southern hybridization with chemiluminescent detection, as described elsewhere [10], using a degenerate 5′ digoxigenin–labeled oligonucleotide probe homologous to an internal region of the PCR product corresponding to nt 912–931 of the VP6 gene (T. Kerin, personal communication). We tried to cultivate rotavirus from serum samples by treating 100 µL of serum with trypsin before inoculation in MA-104 cells and cultivation for 3–6 days, in accordance with standard methods for cultivation of rotavirus-positive stool samples [11].

Rotavirus-specific IgG was detected by an immunoassay, as described elsewhere [12, 13], using the rotavirus strain RRV and mock-infected MA-104 cells as positive and negative antigens, respectively [12, 13]. In addition, since a key concern in this investigation was whether the outbreak strain represented a novel rotavirus strain or whether it was a common type, rotaviruses in a subset of stool samples ($n = 29$) and

autopsy specimens of small-intestinal tissues ($n = 2$) were characterized by RT-PCR, in accordance with previously described methods [11, 14–17].

   Statistical analyses.   Data were collected and tabulated in Excel (version 2000; Microsoft). Statistical analyses were performed using SAS (version 9.1; SAS Institute). Two or more groups were compared using Student's *t* test, the Wilcoxon rank sum test, or analysis of variance ("Proc ANOVA" in SAS), and the χ^2 test for trend was applied when the outcome was binary and several exposure groups were involved. Serum antigen optical density distributions were compared using the Kolmogorov-Smirnoff goodness-of-fit test [4].

RESULTS

We documented the presence of an outbreak of gastroenteritis by plotting the weekly number of visits at 55 sentinel sites reporting to Jamaica's National Sentinel Surveillance System (figure 1). Outbreak case reports were stratified into 2 groups—children <5 years of age and children ≥5 years of age; roughly equal numbers of children were in each group. The greatest increase in cases was noted among children <5 years of age in the southeastern parishes of Kingston and St. Andrews. The increase in the number of cases of gastroenteritis was accompanied by parallel increases in the number of hospital admissions and deaths from gastroenteritis reported to the MoH.

   From 29 July to 10 August, we surveyed 150 patients (100 through prospective surveillance and 50 through review of hospital records) at the 2 hospital sites, to determine their presenting symptoms and clinical course. Patients ranged in age from 1 month to 12 years (mean, 32.2 months); 8% were ≥5 years of age, and 43% (64/150) were female. All patients had diarrhea as their presenting complaint; 84% (126/150) had vomiting (with a mean of 5.0 episodes [SD, 5.8 episodes] during the 24-h period before the hospital visit), 56% (85/150) had fever, and 85% (107/125) were dehydrated. We also interviewed the primary caregivers of 14 children who died during the outbreak and confirmed that 10 of the deaths were attributable to diarrhea.

   Sample testing: stool.   Subsets of stool samples from the outbreak were tested for a range of viral, bacterial, and parasitic agents; rotavirus was the main etiologic agent identified. Rotavirus was identified by EIA in 55% of samples (29/53) obtained within the first 7 days after the onset of diarrhea; of these, 78% (28/35) were found to be positive by RT-PCR confirmed by probe hybridization. Detection rates assessed by EIA declined gradually during the 2 weeks after onset of diarrhea, from 22% (4/18) at week 2 to 0% (0/8) at week 3 by EIA
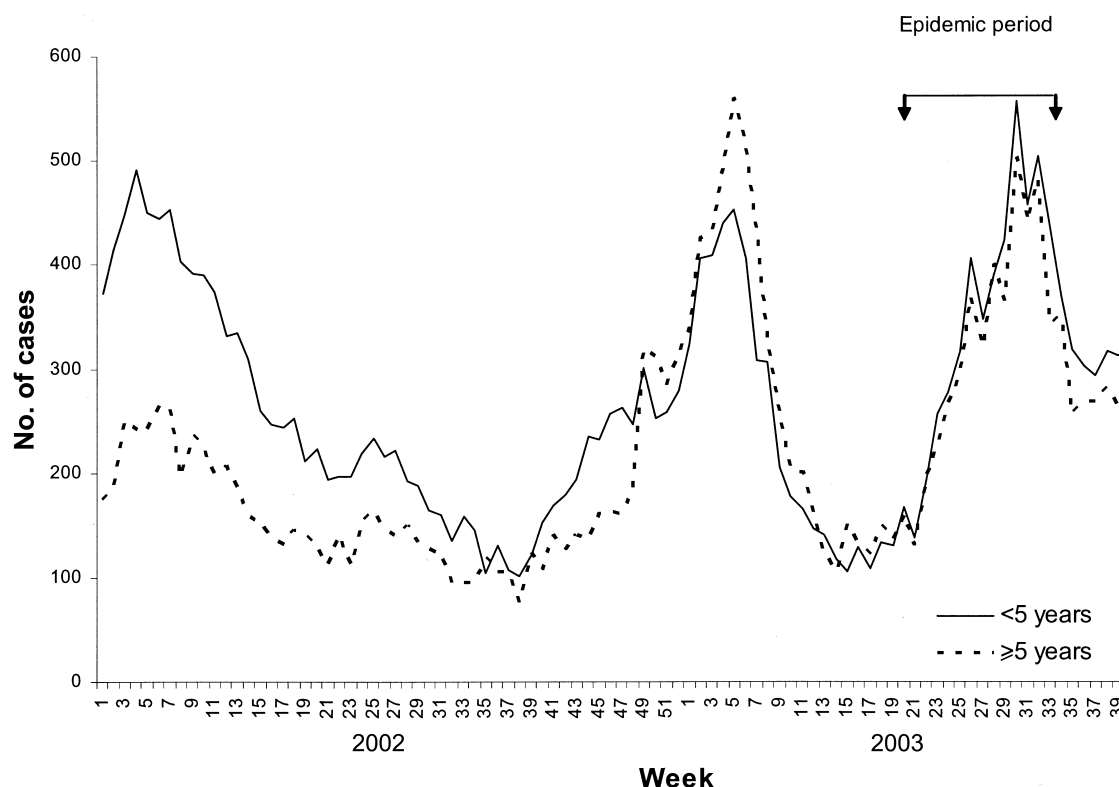

Figure 1.   No. of acute gastroenteritis cases in children <5 years of age and children ≥5 years of age, by month—National Sentinel Surveillance System, Jamaica, January 2002–October 2003. The epidemic period of the summer outbreak is indicated by arrows.
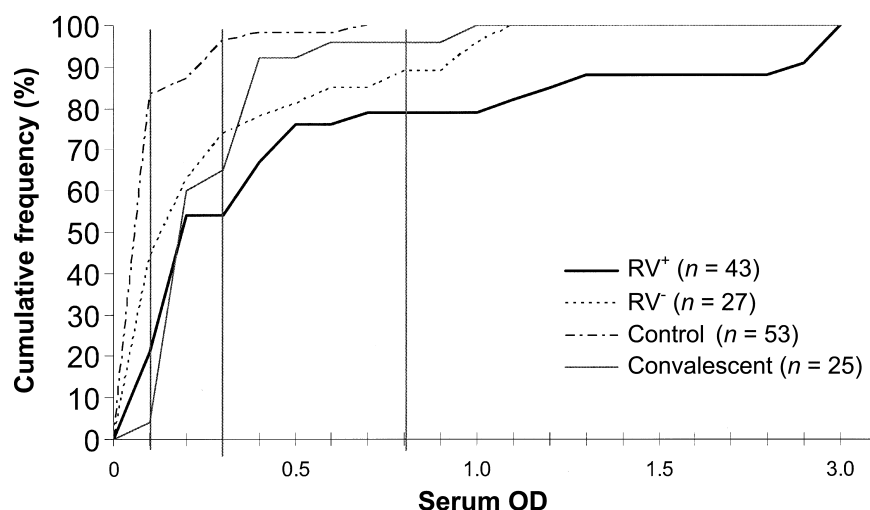
Figure 2. Cumulative frequency of optical density results from a rotavirus (RV) antigen–detection assay applied to serum from 3 groups of chidren with either acute, recent, or convalescent history of diarrhea and from a control group of adult patients with diarrhea.

($P = .004$, χ^2 test for trend) and from 57% (4/7) at week 2 to 0% (0/3) at week 3 by RT-PCR ($P = .02$, χ^2 test for trend). None of 32 randomly selected samples was positive for norovirus, sapovirus, or astrovirus. Four samples contained adenovirus, and rotavirus was present in 3 of these. Only 2 of 25 rectal swabs tested at the CDC contained bacterial pathogens—1 had *Salmonella* serotype Saint Paul and *C. jejuni,* and the other was positive for enterotoxigenic *E. coli* (serotype O6:H16, heat-labile and heat-stable toxins).

To investigate whether the outbreak was associated with the appearance of an unusual or novel strain, we characterized 31 randomly selected rotavirus specimens by RT-PCR and found 2 rather common serotypes, P[8],G1 (61%) and P[8],G9 (29%), as well as mixed infections with P[8],G1G9 (7%), but no novel strains. Furthermore, RT-PCR of dsRNA extracted from autopsy tissue from the small intestine yielded a P-nontypeable, G2 strain in a 17-month-old girl and a P[8],G1 strain in a 9-month-old girl. In the latter patient, the strain P[8],G1 was documented by genotypic characterization of dsRNA extracted from both serum and stool.

Sample testing: serum. The cumulative frequency of optical density results from the antigen-detection assay applied to serum was plotted for the 4 groups, to identify an appropriate cutoff value (figure 2). Patients with acute rotavirus diarrhea (group A) had significantly higher optical density values in serum than did all other groups ($P = .02$). Furthermore, patients with a history of diarrhea during the preceding 2 weeks but with rotavirus-negative stool samples (group B) were significantly more likely to have positive optical density results than were the control individuals (groups C and D) ($P = .01$). The mean OD of the 53 control serum samples was 0.05, and 3 SDs above the mean was 0.31. On the basis of data from the 2 control groups (C and D), an OD cutoff value of 0.3 seemed appropriate, since it included all but 1 of 53 serum samples from adult control individuals (specificity, 98%) and all but 2 of 25 serum samples from convalescent patients (sensitivity, 92%).

To determine whether serum samples with OD values >0.3 were true positive or false positive for rotavirus, we compared the levels of antigen detected by EIA in patient serum with results obtained by a confirmatory RT-PCR (table 1). Overall,

Table 1. Detection of rotavirus RNA by reverse-transcription polymerase chain reaction (RT-PCR) in serum samples screened for rotavirus antigen by EIA in patients with acute and recent rotavirus infection and adult control individuals.

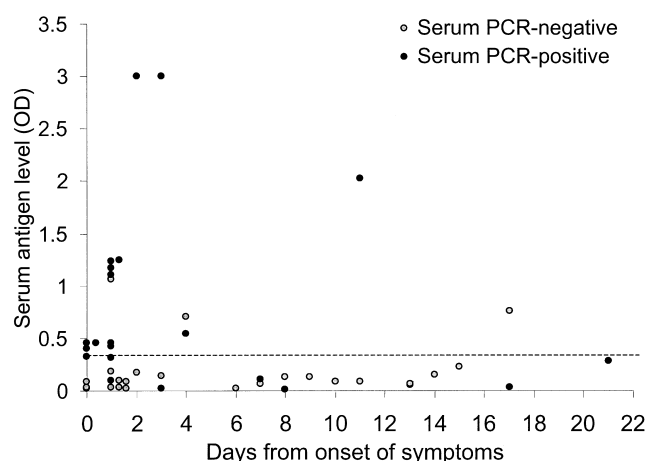| Group	Patient category	Stool EIA	Percentage positive by RT-PCR (no. positive/no. tested) OD <0.3	OD ≥0.3
A	Outbreak	Positive	43 (3/7)	82 (9/11)
B	Outbreak	Negative	31 (5/16)	60 (3/5)
C	Nonoutbreak (adult control individuals)	Unknown	0 (0/52)	0 (0/1)

Figure 3. Levels of serum rotavirus antigen, as assayed by EIA, by time from onset of diarrhea to collection of serum samples. (The broken line represents the cutoff value, at an OD of 0.3.) PCR, polymerase chain reaction.

the RT-PCR results corresponded well with the optical density measurements: the control individuals were uniformly negative by RT-PCR, even when optical density values were elevated, and the majority of patients with acute or recent infection with positive optical density results had positive RT-PCR results. However, some patients with diarrhea had confirmatory RT-PCR results even when the optical density was below the cutoff value, suggesting that, in some cases, RT-PCR is a more sensitive indicator of rotavirus antigen in serum.

To assess whether levels of serum antigen as assayed by EIA were correlated with the time from the onset of diarrhea to the time of sample collection, we plotted serum optical density values against this time interval (figure 3). Most of the elevated optical density values occurred within the first 5 days of diarrhea onset, but, in 2 patients, the optical density values remained elevated for 7–14 days; in these cases, antigenemia, as indicated by elevated optical density values, was confirmed by RT-PCR. Of note, there were a number of interesting discrepant results in which serum samples were rotavirus-positive by EIA and rotavirus-negative by RT-PCR, and vice-versa.

Last, we compared the optical density values in the acute- and convalescent-phase serum samples from 25 patients with acute diarrhea (figure 4). Overall, we observed a decline in serum antigen levels over time ($P = .06$). Six patients (24%) had elevated optical density values in their acute-phase serum samples, and, with 1 exception, these values all returned to normal (OD values <0.3) in the convalescent-phase serum samples. In the 1 patient who was the exception, the OD increased to 0.8, and we did not have adequate clinical history or a recent stool sample to determine whether the patient might have been reinfected after the acute-phase serum was collected. To investigate whether this child experienced a rotavirus reinfection at the time

the convalescent-phase serum was collected, we compared the paired IgM and IgG titers and found a decrease from 1600 to <25 and from 800 to 400, respectively. Thus, reinfection was not the likely explanation for the increase in optical density values.

We questioned whether children with low acute-phase IgG titers (<25) who had no prior exposure to rotavirus were more likely to develop antigenemia upon first infection than were children with higher acute-phase titers (i.e., >50), which would suggest past infections or, for young infants, the presence of maternal antibodies. The 13 children with low IgG titers had significantly higher optical density values of rotavirus antigen in their serum (median OD value, 0.22; range, 3) than did the 12 children with initially higher IgG titers (median OD value, 0.07; range, 0.22) ($P < .02$).

Although there was a tendency for the severity of illness to be associated with higher serum optical density values, this was not significant. We attempted cultivation of rotavirus in serum samples but could not detect any virus replication at any stage of cultivation.

DISCUSSION

A novel and unanticipated finding of this outbreak investigation came from our application of a simple commercial EIA to investigate rotavirus antigenemia in a group of patients with well-documented rotavirus diarrhea and in a second group of patients who had recently recovered from acute diarrhea presumed to be due to rotavirus and whose stools were now negative for the virus. Our findings of antigenemia, confirmed by RT-PCR, demonstrated that rotavirus antigen escapes the small intestine and is present in the serum of children with acute gastroenteritis on the first day of disease, that antigen levels peak 1–3 days after the onset of symptoms, and that serum of some children remains positive beyond 1 week. We observed
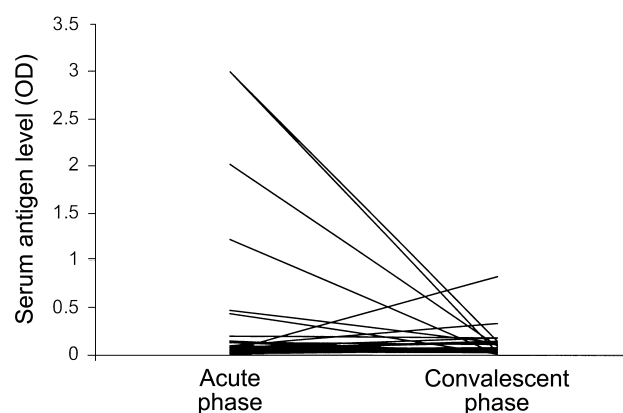

Figure 4. Rotavirus antigen levels as assayed by EIA in paired acute-phase and convalescent-phase serum samples from 25 children, Jamaica 2003.

a decline in serum antigen values to baseline over a 3–4-week interval ($P = .06$) by testing paired serum samples from 25 children. We also found that children with primary rotavirus infection, indicated by low titers of rotavirus antibodies in acute-phase serum samples, had more antigen detected in their serum than did children with higher acute-phase titers, which suggested either past infection or the presence of maternal antibodies. This finding indicates that antigenemia and, perhaps, viremia may occur with more-severe clinical symptoms, as is seen in patients with primary rotavirus infections versus secondary or tertiary infections [18].

The role of this antigenemia and viremia in rotavirus disease is puzzling, since rotavirus infections have not traditionally been linked to illness outside the gut. Only a few studies have identified extraintestinal manifestations of rotavirus infection, and the credibility of these findings have been questioned, because of limited observations and the possibility that the extraintestinal findings represented contamination with viral particles from feces. The demonstration by Morrison et al. [19] of rotavirus RNA in the heart and central nervous system of 2 deceased children was supported by 2 Japanese groups [20, 21] who documented rotavirus RNA in serum by RT-PCR. Blutt et al. [6] recently demonstrated rotavirus antigenemia and viremia by RT-PCR in 3 of 6 immunocompetent children with documented rotavirus diarrhea. We were able to document the presence of rotavirus antigen in serum by RT-PCR in 12 of 18 children with acute rotavirus infection, suggesting that antigenemia is more common than first anticipated. Our study confirms the demonstration of rotavirus in blood in a large number of patients and in intestinal tissue of 2 deceased individuals. The fact that several rotavirus antigen–positive serum and tissue samples were obtained from children from whom no stool samples were collected eliminates previous concerns of contamination as an explanation for these novel findings. Further characterization of rotavirus RNA in serum by G genotyping RT-PCR proved that full-length VP7 gene segments of rotavirus RNA were present in serum and that the rotavirus strains circulating in blood are similar to those identified in the stools of the same patient. Rotavirus strain G1 has been identified in cerebrospinal fluid [21], and, in a recent case report, the common rotavirus strain P[8],G1 was demonstrated in both the cerebrospinal fluid and stool of a child with central nervous system disease. We have further documented the presence of the P[8],G1 strain in serum as well as in stool and intestinal tissue of a deceased child. The presence of full genomes might help to explain why some serum samples had optical density values below the cutoff level but the virus was still detectable by RT-PCR: the virus might either be present in low doses or not be intact in these serum samples. This would cause the EIA to fail to detect the VP6 antigen, which is either missing or incomplete, although the genomes would be detectable by RT-PCR. We were not able to detect live virus in the serum by virus cultivation, but whether this was because of the absence of live virus or the use of inappropriate methodology is not known. Additional studies may be needed to determine the optimal trypsin concentration to overcome the trypsin inhibitors present in serum and to activate virus infectivity.

The novel use of EIA to test serum enabled us to confirm rotavirus infection in several epidemiologically important groups of patients for whom stool testing was difficult, such as dehydrated patients with decreased purging, patients with recent diarrhea that was resolving, and patients who died from whom serum was collected, even if it was collected for other purposes. Our comparison of serum antigen values in groups of patients with and without rotavirus diarrhea encouraged us to use an OD cutoff value of 0.3, which is substantially higher than the value recommended for testing stool samples. At this level, 98% of control serum samples were rotavirus negative. Although serum from adults might not be the ideal control sample, since antigenemia in adults has not yet been well studied, it proved valuable for this purpose, since 98% of adult serum samples had rotavirus antigen OD values <0.1. Our findings challenge our understanding of the pathogenesis of this common childhood disease, previously thought to be localized to the gut, by adding the prospect that primary as well as severe and/or fatal rotavirus infections are associated with antigenemia and/or viremia.

Acknowledgments

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