MAJOR ARTICLE

The Pathology of Rotavirus-Associated Deaths, Using New Molecular Diagnostics

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Rotavirus, the most common cause of severe, dehydrating gastroenteritis among children worldwide, annually causes ~500,000 deaths among children aged <5 years. The primary site of rotavirus infection is the small intestine. Pathologic investigations of patients who died of rotavirus infection are limited to data from a few reported autopsies, and dehydration with electrolyte imbalance is believed to be the major cause of death. Several recent reports suggest that children who died during a rotavirus illness were viremic before death, because rotavirus was detected at several extraintestinal sites. We report 3 rotavirus-associated deaths among children, 2 of whom had evidence of rotavirus genome in extraintestinal tissues detected by use of novel molecular diagnostic methods. The part played by rotavirus in fatal cases is unclear and requires additional investigation of diarrhea-associated deaths, because a better understanding might alter the approach to treatment and the need for antiviral therapy.

Rotavirus gastroenteritis remains the most common cause of severe, dehydrating gastroenteritis among children worldwide, resulting in 400,000–500,000 deaths each year among children aged <5 years [1]. In the United States, rotavirus infection leads to ~50,000 hospitalizations and ~500,000 clinic visits per year but to only 20–40 deaths [2]. The deaths occur mainly among children with poor access to medical care and who die, presumably, of dehydration and electrolyte imbalance [3]. A key question in considering rotavirus-related deaths is why they occur far more frequently among children in less-developed countries than in industrialized countries. It remains unclear whether these deaths are due to abnormal fluid and electrolyte levels treatable by rehydration or to viremia associated with extraintestinal manifestations requiring other interventions. Extraintestinal manifestations of rotavirus infections have been reported in immunodeficient children, in whom rotavirus has been detected in liver and kidney specimens [4], and in children with seizures who had rotavirus detected in CSF specimens [5, 6]. However, although rotavirus has been identified in extraintestinal sites, its causal role in fatal cases has not been clearly established [6].

Most of the pathologic features of rotavirus gastroenteritis were described soon after its discovery in 1973, when electron microscopic examination of biopsy specimens obtained from children with acute nonbacterial gastroenteritis demonstrated virus particles in the duodenal mucosa [7]. From these studies, rotavirus infection was found to result in structural abnormalities of the small intestine, including blunting of villi, increased crypt depth, flattening of epithelial cells, and an increase in inflammatory cells in the lamina propria [8–10]. As methods for diagnosing rotavirus gastroenteritis, endoscopy and biopsy were soon replaced by

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electron microscopy and rapid antigen detection methods with fecal specimens [11]. Since then, further description of the pathology of human rotavirus infections has been limited to data from a few reported autopsies [3, 12, 13].

We recently developed new diagnostic assays to detect evidence of rotavirus infection in fixed tissue. We applied these methods to the examination of postmortem tissue specimens obtained from 3 previously healthy children who died during a rotavirus illness. In each case, tissue samples were examined for rotavirus using immunohistochemistry (IHC), in situ hybridization (ISH), and RT-PCR [14].

CASE REPORTS

We describe 3 children who died during a rotavirus illness and report the findings of examinations of postmortem tissue specimens using newly developed rotavirus-specific methods (IHC, ISH, and RT-PCR).

Patient 1. A 20-month-old white boy with cerebral palsy was transferred to the hospital with a 3-day history of diarrhea, vomiting, decreased oral intake, and change in behavior. The child was administered intravenous rehydration, and a fecal specimen was positive for rotavirus antigen by EIA. He developed seizures on the day of hospitalization and was transferred to the second hospital in status epilepticus. At admission, his pupils were dilated and poorly reactive but became reactive after he received a loading dose of anticonvulsants and sedatives.

Shortly after hospital admission, the patient had a respiratory arrest, was hypotensive and hyponatremic, and underwent intubation for ventilation. A CT scan of his brain showed cerebral edema, and an electroencephalogram (EEG) indicated no evidence of electrocerebral activity. Ventilatory support was discontinued, the child was pronounced dead, and permission was given for an autopsy.

Histopathologic examination of the brain and spinal cord showed moderate congestion, with no evidence of inflammation. The small intestine showed sloughed mucosal epithelia, mildly edematous submucosa with increased inflammatory cell infiltrates, and poorly defined Peyer patches, with focal necrosis. Mild reactive changes were present in mesenteric lymph nodes.

Patient 2. A previously healthy 23-month-old white girl was seen in the emergency department at Memorial Hospital (Colorado Springs, CO) after a 2-day history of fever, vomiting, diarrhea, dehydration, and lethargy. Rotavirus antigen was detected in a fecal specimen by EIA. The child was sent home with oral rehydration therapy, only to be readmitted 1 day later for intravenous rehydration. At admission, she had a cardiorespiratory arrest and was transferred to a second hospital, where a CT scan of the brain showed diffuse edema. An electrocardiogram (ECG) indicated inferior and anteroseptal subendocardial injury. The child developed diabetes insipidus, became hypernatremic, and died 24 h later.

At autopsy, the child was found to have had marked cerebral edema, with cerebellar tonsillar and cerebral uncal herniation, but no inflammation in the CNS. The small bowel showed degenerative mucosal epithelia, focal moderately increased inflammatory infiltrates in the lamina propria, and hyperplasia of Peyer patches, with prominent immunoblastic cells. Pathologic findings consistent with a reaction to viral infection included follicular lymphoid hyperplasia, with increased immunoblasts in lymph nodes and the spleen, mild hemophagocytosis in the spleen, and lymphoid nodules in the bone marrow.

Patient 3. A 15-month-old white girl with recent onset of a seizure disorder was admitted to the emergency department at Alberta Children's Hospital (Calgary, Alberta, Canada) after experiencing 3 tonic-clonic type seizures without fever in the previous 3 months. The findings of investigations at that time, including EEG, ECG, and brain CT, were normal. The child had had chickenpox 1–2 weeks before admission and presented with nausea, vomiting, and fever. She was treated with lorazepam and phosphophenytoin, and she was given ampicillin and cefotaxime intravenously after blood and CSF samples were obtained. No neurologic abnormalities were found in the initial examination. She developed diarrhea, samples of which tested positive for rotavirus antigen by EIA. Plasma electrolyte levels were normal at the time of admission, and maintenance intravenous fluid therapy was began for the child.

Later on that day, her neurologic status deteriorated: she had no response to auditory stimuli and had small fixed pupils. Over the subsequent 2 h, the child's level of consciousness progressively decreased. Liver enzyme levels were slightly elevated, with an alanine aminotransferase (ALT) level of 240 IU/L (normal range, 1–35 IU/L), an aspartate aminotransferase (AST) level of 249 IU/L (normal range, 10–55 IU/L), and a lactate dehydrogenase (LDH) level of 866 IU/L (normal range, 125–320 IU/L). The glucose level was normal, and blood gases showed a compensatory metabolic acidosis. A brain CT scan showed bilateral thalamic hypodensities, with no edema, hemorrhage, elevated intracranial pressure, or sinus thrombosis. The child underwent elective ventilatory support. A lumbar puncture was done, and the level of CSF protein was elevated (0.81 g/L; normal range, 0.15–0.45 g/L); the CSF glucose level was 3.5 mmol/L (normal range, 2.2–3.9 mmol/L), with no cells. The blood glucose level was 4.7 mmol/L (normal range, 3.9–6.1 mmol/L). Her neurologic status deteriorated, despite the initiation of dexamethasone and acyclovir therapy. The girl's liver enzyme levels increased (ALT level, 884 IU/L; and AST level, 241 IU/L), and a liver biopsy performed on the day after admission showed microvesicular steatosis, a histopathologic feature suggestive of Reye syndrome. The child had no history of aspirin exposure.

Potentially hepatotoxic drugs (fosphenytoin and lorazepam) were discontinued; carnitine therapy was initiated, and the results of a plasma amino acid screen were normal. Liver function improved, but the girl's neurologic status continued to deteriorate. A second CT scan showed increased areas of hypodensity involving the thalami, perithalamic regions, and right caudate nucleus. The clinical picture was consistent with a diagnosis of acute necrotizing encephalopathy. In view of the continued deterioration, ventilatory support was discontinued 5 days after admission, the child was pronounced dead, and autopsy was performed.

At autopsy, the main findings were in the brain and were consistent with the diagnosis of acute necrotizing encephalopathy of childhood [15, 16], including massive systemic necrosis and hemorrhage of cerebral white matter, thalami, tegmentum, and pons. Histologic examination revealed acute necrosis of grey and white matter, with a symmetrical appearance; this was seen especially in the tegmentum of the brainstem. Within the necrotic grey matter, the small calibre vessels were characterized by various degrees of thrombosis and fibrinoid necrosis, with rings of hemorrhage around the vessels but no associated inflammatory reaction. An examination of the liver showed mild diffuse microdroplet steatosis but was otherwise unremarkable.

METHODS

Some of the tissue samples were submitted to the Centers for Disease Control and Prevention (CDC; Atlanta) for rotavirus testing by IHC, ISH, and RT-PCR, as described elsewhere [14]. For IHC, a polyclonal rabbit antibody directed against human rotavirus Wa strain was used with formalin-fixed, paraffin-embedded cell controls and experimentally infected piglet small intestine tissues to determine its specificity and cross-reactivity. The IHC assay used the preimmune serum for the negative antibody control and the polyclonal rabbit antibody as the positive control, and Wa-infected cells served as positive tissue controls [14]. For ISH, the RNA probes were generated from PCR products amplified from the rotaviral genes VP4, NSP4, and NSP1 and tailored with the T7 promoter [17]. Human rotavirus Wa–infected cells were used as positive controls, and rhesus rotavirus (RRV)–infected cells served as negative controls [14]. For RT-PCR, RNA was isolated from formalin-fixed tissue using a commercial RNaid Plus kit (Bio101), as described elsewhere [14]. Negative controls included extracts from rotavirus-negative tissues. Positive controls were RNAs extracted from Wa (P1A[8] G1) or DS-1(P1B[4] G2) rotavirus–infected cell lysates. A 1-step RT-PCR kit [14] and a multiplex RT-PCR method specific for the major human rotavirus G and P types were used to genotype rotaviruses present in tissue specimens [18, 19]. VP4 gene detection primers that were broadly reactive for major human rotavirus P types were also included (J.R.G., unpublished data).

RESULTS

Patient 1. Sections of the small intestine, lymph node, and spleen were found by RT-PCR to be positive for rotavirus that was characterized as genotype P[8] G1 (table 1 and figure 1). Sections of heart, lung, kidney, testes, and bladder were found

Table 1. Results of immunohistochemistry (IHC), in situ hybridization (ISH), and RT-PCR rotavirus testing of various postmortem tissue samples obtained from 3 patients with fatal diarrhea-associated illnesses.

Patient, tissue sample source	Diagnostic assay result		
	IHC	ISH	RT-PCR
1			
Intestinal sites[a]	Negative	Negative	Positive[b]
Extraintestinal sites			
Spleen	Negative	Negative	Positive[b,c]
Heart	Negative	Negative	Positive[c]
Lung	Negative	Negative	Positive[c]
Kidney	Negative	Negative	Positive[c]
Liver	Negative	Negative	Negative
Testes	Negative	Negative	Positive[c]
Bladder	Negative	Negative	Positive[c]
CNS	Negative	Negative	Negative
2			
Intestinal sites[a]	Positive[d]	Positive[e]	Positive[b]
CNS	Negative	Negative	Negative
3			
Intestinal sites[a]	Negative	Negative	Positive[f]
Extraintestinal sites			
Spleen	Negative	Negative	Positive[c]
Adrenal gland	Negative	Negative	Positive[c]
Liver	Negative	Negative	Negative
Lymph nodes	Negative	Negative	Negative
Heart	Negative	Negative	Negative
Lung	Negative	Negative	Negative
Pancreas	Negative	Negative	Positive[c]
Kidney	Negative	Negative	Positive[c]
Thymus	…	…	Negative
CNS	Negative	Negative	Negative

[a] Samples obtained from intestinal sites for all 3 patients were mainly from the small intestine. For patient 3, they also contained a sample from the cecum.
[b] Genotype P[8] G1, using both VP4 detection primers and multiplex P and G genotype–specific primers, with confirmation by probe hybridization.
[c] Determined using a probe of RT-PCR product.
[d] Determined using polyclonal rabbit antibody.
[e] Determined using human rotavirus Wa probe.
[f] Determined using VP4 detection primers with probe hybridization. Genotype P [NT], G2 determined by multiplex RT-PCR with ethidium bromide staining.

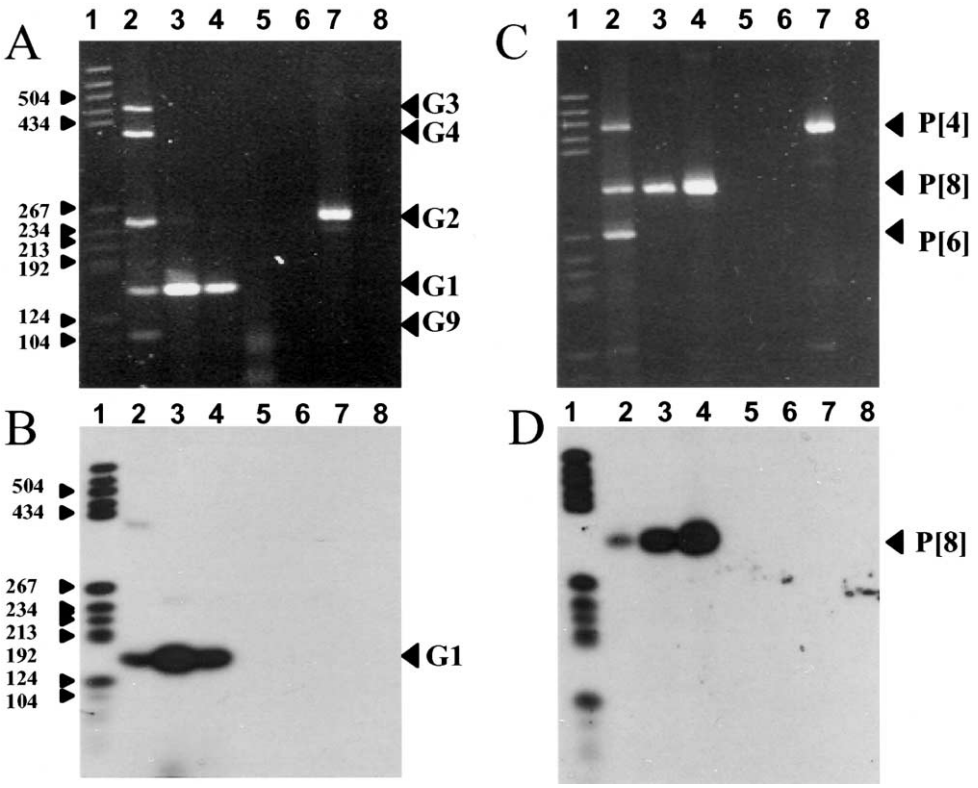

Figure 1. P and G genotyping of human rotavirus in tissue specimens by RT-PCR and probe hybridization. Shown are ethidium bromide–stained agarose gels of products amplified from RNA extracts of tissue sections by use of a 1-round RT-PCR with multiplex G *(A)* or P *(C)* genotyping primers. *Lanes 1A* and *1C,* digoxigenin-labeled molecular weight markers (size given in base pairs for selected fragments on the left); *lanes 2A* and *2C,* mixtures of G and P genotype–specific PCR amplicons, respectively (specificities as indicated on right); *lanes 3A, 3C, 4A,* and *4C,* 2 different bowel sections from patient 1; *lanes 5A, 5C, 6A,* and *6C,* 2 different negative bowel sections; *lanes 7A* and *7C,* strain DS-1; and *lanes 8A* and *8C,* negative (water) control. Results of Southern hybridization and chemiluminescent detection of the PCR products with human rotavirus VP4–specific digoxigenin-labeled probe AVP4-c1 are shown in panels *B* and *D* (corresponding to panels *A* and *C,* respectively).

to be positive for rotavirus by probe analysis of RT-PCR products. Rotavirus was not detected in CNS sections or in CSF specimens by RT-PCR. Results of IHC and ISH tests of gastrointestinal tissue specimens were negative (table 1).

Patient 2. Sections of the small intestine showed increased lymphoplasmacytic infiltrates in the lamina propria and focal areas of superficial ulceration (figure 2*A* and 2*B*). Sections of small intestine were positive for rotavirus by IHC (figure 2*C*–2*E*), with rotavirus antigens present in the sloughing epithelial cells, lamina propria, and lymphoid tissue (figure 2*F*). ISH showed rare inconspicuous staining in the mucosa. RT-PCR testing identified the genotype of the rotavirus strain in intestinal sections as P[8] G1. Rotavirus was not detected in CNS sections.

Patient 3. Sections from the small intestine showed focal architectural distortion of the villi, with desquamation of surface epithelium and increased inflammatory cell infiltrates in the lamina propria. The liver showed mild fatty metamorphosis. Results of IHC tests of multiple organ specimens were negative (table 1), as were results of ISH tests of intestine and brain tissue specimens. Sections of duodenum, ileum, cecum, and lymph node were positive for rotavirus by RT-PCR. Sections of spleen, adrenal gland, and kidney were positive for rotavirus by probe analysis of RT-PCR products. Rotavirus was not detected in CNS sections by RT-PCR. PCR analysis of samples of the esophagus and gastroesophageal junction was negative for varicella-zoster virus DNA.

DISCUSSION

In this study, we describe the clinical courses and pathologic findings for 3 children who died after a course of severe diarrhea and vomiting in the hospital and in whom rotavirus antigen was detected in stool specimens by EIA. In all 3 patients, rotavirus was detected during autopsy in small intestine tissues by ⩾1 method (i.e., IHC, ISH, and/or RT-PCR). In 2 patients, rotavirus genome was detected in specimens of other organs by RT-PCR: spleen, lymph node, heart, lung, testes, kidney, and bladder. In none of these patients was it possible to establish the exact cause of death on the basis of histopathologic findings.

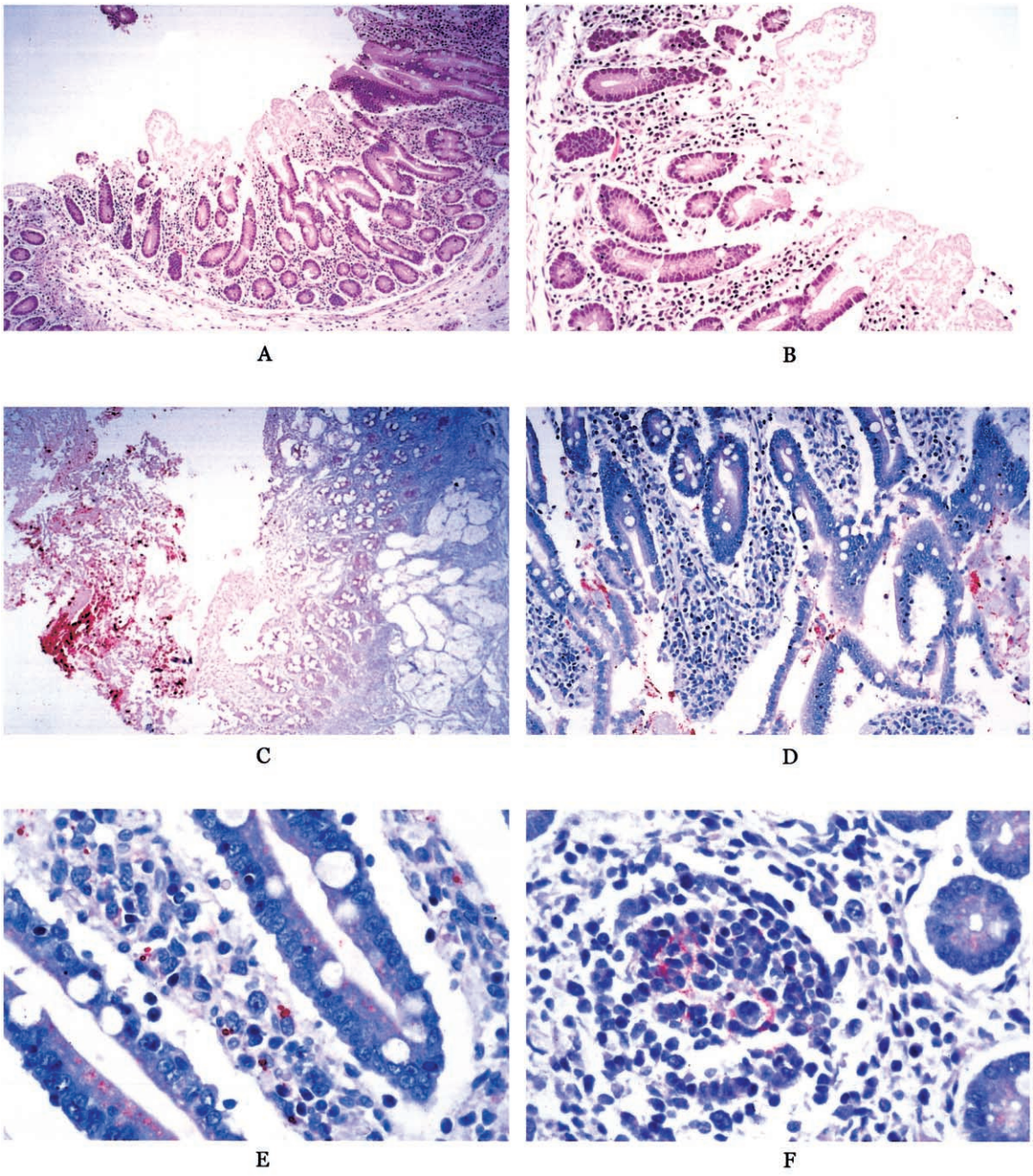

Figure 2. Histopathologic and viral immunolocalization findings for rotavirus infection. Sections of intestine show focal areas of superficial ulceration, epithelial necrosis, and increased lymphoplasmacytic infiltrates in the lamina propria (*A,* hematoxylin-eosin stain [original magnification, ×25]; *B,* hematoxylin-eosin stain [original magnification, ×50]). Immunoalkaline phosphatase with napthol fast red substrate and hemetoxylin counter staining shows that viral antigens are present in superficial epithelial cells of small intestine and cellular debris (*C* and *D;* original magnification, ×50), lamina propria (*E;* original magnification, ×158), and lymphoid tissue (*F;* original magnification, ×158).

However, the first 2 children were admitted to hospital for intravenous rehydration after a short prodromal illness of diarrhea and vomiting, and both children had electrolyte abnormalities shortly before death. The first child developed seizures after admission to the hospital that may have resulted in inappropriate antidiuretic hormone secretion and hyponatremia. The second child had neurologic findings suggestive of encephalopathy and was hypernatremic before death. The third child had clinical and autopsy features of acute necrotizing encephalopathy of childhood, a rare disorder described in Japan in 1995. The etiology of this disorder is unknown, but it is usually preceded by a viral infection [20]. However, it seems

doubtful that rotavirus caused the encephalopathy, because rotavirus was not detected in CNS specimens.

Tissue specimens obtained from all 3 patients were tested by IHC, ISH, and RT-PCR. The most sensitive method developed in our previous study is RT-PCR, the most specific is the ISH, and the one with the broadest range of activity is the IHC assay [14]. Taken together, these techniques have the sensitivity and specificity to detect human rotaviruses in naturally occurring infections [14]. The ability to detect rotavirus genome in various extraintestinal tissue extracts from 2 patients may reflect the increased sensitivity of the RT-PCR methods used, compared with ISH and IHC. Combined with Southern hybridization, the human rotavirus–specific RT-PCR methods used here can detect as few as 10–100 copies of the rotavirus genome (unpublished data). This is consistent with a study in which it was estimated that rotavirus PCR is 5000 times more sensitive than a typical dot-blot hybridization assay [21, 22]. In addition, a recent study from our laboratory demonstrated that RT-PCR/hybridization methods could detect rotavirus genome in intestinal tissue specimens from an experimentally infected monkey that tested negative by both rotavirus-specific ISH and IHC techniques [14].

Pathologic studies on formalin-fixed, paraffin-embedded tissue specimens obtained from all 3 children localized infection to the small intestine, the primary site of rotavirus infection [10]. However, the pathogenesis of rotavirus diarrhea is complex, and several mechanisms have been postulated to explain the diarrhea. One postulation is that there was malabsorbtion secondary to destruction of enterocytes—cells that normally carry digestive and absorptive functions [23]. Destruction of enterocytes results from increased intracellular calcium levels [24], and, when the rate of destruction exceeds that of production of new immature enterocytes in the crypts of the small intestine, which are secretory in nature, diarrhea results [10, 25]. A second hypothesis suggests that a nonstructural rotavirus protein, NSP4, functions as an enterotoxin [26, 27]. When injected into mice, NSP4 produces a secretory diarrhea by increased intracellular calcium concentrations. The increased intracellular calcium level causes an efflux of chlorine and a subsequent efflux of sodium and water resulting in secretory diarrhea. A third explanation is that the enteric nervous system may be involved in rotavirus diarrhea [28]. Researchers found that, in mice, rotavirus activates the nerves that control intestinal motility and fluid absorption and secretion. The activated nerves stimulate intestinal lining cells to boost their water secretion, resulting in diarrhea.

The literature describing rotavirus-associated deaths is limited. In an analysis of 21 fatal cases in the 1970s, death occurred within 3 days after onset of symptoms [3], and dehydration with electrolyte imbalance leading to cardiac arrest was the major cause of death in 16 of the 21 patients. Additional contributing factors included aspiration of vomitus in 3 cases and seizures in 2 others. These fatal cases were reported when the rate of infant mortality associated with diarrhea in the United States averaged 12.8 deaths per 100,000 live births [29]. In a study from 1968–1991 [29], deaths due to diarrhea were shown to have decreased 75% from 1968 (when there were ~1200 deaths per year) to 1985, but they have stabilized since then at ~300 deaths per year. Peaks in diarrhea-related deaths in winter previously associated with rotavirus infection were prominent in the early years of the study among infants aged 4–23 months, but these peaks virtually disappeared after 1985, leaving an estimated 20–40 rotavirus infection–associated deaths per year [3]. The decrease in diarrhea-associated deaths from 1968 to 1985 is likely the result of improvements in the care of children with severe dehydrating diarrhea through intravenous rehydration therapy and improved access to care [30]. Since 1985, the 300 diarrhea-related deaths that have occurred annually have represented the more complicated cases [29]. Among them are those involving patients from minority groups, for whom access to care remains a problem [31], and premature infants [32].

Several recent reports suggest that children who have died during a rotavirus illness may have had some extraintestinal manifestations of the disease [4–6, 12, 13]. In 4 patients with immunodeficiency, rotavirus was detected in tissue specimens of both liver and kidney obtained at autopsy [4]. Rotavirus has been detected in the CSF samples of patients with gastroenteritis who have seizures and encephalopathy, and 2 of 19 reported cases had fatal outcomes [5, 6]. In 1 of these patients, who had gastroenteritis and intractable seizures for 5 months [6], rotavirus RNA was detected in the CSF on 2 occasions 3 weeks apart. In the current report, all 3 patients had CNS symptoms, but none had evidence of CNS infection, as determined by testing brain tissue specimens by IHC, ISH, and RT-PCR methods. Because rotavirus RNA was not detected in the CSF of the first patient, it is likely that the CNS symptoms were related to electrolyte imbalance. In contrast to these findings, a recent report of 2 fatal cases presented controversial evidence of rotavirus in extraintestinal sites, including cardiac and CNS tissues [12]. However, the clinical course and autopsy findings indicated that both children were severely dehydrated, a factor that could explain their cardiac and CNS symptoms. In our study, rotavirus RNA was detected by RT-PCR in 2 cases at extraintestinal sites. However, the presence of viral genome does not necessarily indicate infection. For example, Brown et al. [33] found evidence of rotavirus proteins in macrophages in the circulation after oral inoculation of mice with murine rotavirus. Although findings in mice are not necessarily predictive of events that occur in humans, that study raises the possibility that rotavirus is taken up by macrophages (or other antigen-presenting cells) in gut-associated lymphoid tissue and enters the bloodstream. Therefore, rotavirus genome may be detected

in any organ that has a blood supply using a sensitive assay, such as RT-PCR.

In conclusion, we have reported 3 rotavirus-associated deaths among children in whom rotavirus was detected by pathologic and molecular diagnostic studies. Pathologic studies identified rotavirus infection in the small intestine of all 3 children and at extraintestinal sites in 2 of them. Although all children had CNS symptoms, there was no pathologic evidence to suggest a direct CNS infection. It is likely that the first 2 children died of common complications of severe diarrhea and that the third child died of acute necrotizing encephalopathy of childhood. The availability of novel molecular diagnostic methods has allowed the detection of rotavirus nucleic acids in extraintestinal sites in fatal cases, but its clinical significance needs additional investigation. Increased reporting of cases of fatal diarrhea and examination of specimens by the methods described here could elucidate the relative importance of dehydration versus viremia in rotavirus-associated deaths.

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