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Detection and Sequencing of Rotavirus VP7 Gene from Human Materials (Stools, Sera, Cerebrospinal Fluids, and Throat Swabs) by Reverse Transcription and PCR

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Human rotavirus RNAs from stool samples, sera, cerebrospinal fluids, and throat swabs of 15 children with rotavirus gastroenteritis were detected and serotyped by reverse transcription and PCR. The reverse transcription-PCR method may allow us to consider rotavirus infections in other parts of the body in addition to the gastrointestinal tract. Moreover, sequence analysis of the VP7 gene was performed on seven samples (one stool, two serum, three cerebrospinal fluid, and 1 throat swab sample). There were no appreciable differences in viral sequences between samples from cerebrospinal fluids, sera, or stools.

Rotavirus is an important viral agent of acute gastroenteritis worldwide, especially in developing countries. The virus may also cause chronic gastroenteritis (12) and sometimes hepatitis (2) in immunocompromised children. Furthermore, rotavirus gastroenteritis associated with convulsions or encephalopathy (5, 11, 14) as well as with rash (9) has been observed in nonimmunocompromised patients. Convulsions may not be related to dehydration, because electrolyte levels in cerebrospinal fluid have been reported to be normal in patients in Japan (5, 11, 14). The incidence of convulsions associated with rotavirus gastroenteritis in Japan, Taiwan, and India were 2.9, 5.3, and 3.7%, respectively, in all patients with rotavirus gastroenteritis (1). However, convulsions are not commonly associated with rotavirus gastroenteritis in the United States (6). There have been no reliable reports of rotavirus isolation and cultivation from cerebrospinal fluids (CSFs), blood, or throat swabs (6) except by Wong et al. (18) and Kaidan et al. (5). Reverse transcription-PCR (RT-PCR) is a sensitive method (3, 16) and facilitates the detection of the virus genome in such samples.

In the study described in this report we used RT-PCR to detect rotaviruses not only from stool samples but also from sera, CSFs, and throat swabs from patients with rotavirus gastroenteritis. Moreover, we sequenced and compared the VP7 genes from these specimens to elucidate whether significant alterations exist between rotaviruses from CSFs and sera.

MATERIALS AND METHODS

Specimens. Stool samples from the patients in question were previously examined with commercially available latex agglutination or enzyme immunoassay kits (13).

Seven throat swabs and four stool samples from seven children from 1 month to 1 year of age in the acute stage of gastroenteritis (patients 1 to 7) were collected from the Department of Pediatrics, Teikyo University School of Medicine, Tokyo, Japan, in 1992 (Table 1). The throat swabs were collected from patients who had not vomited just after the first diarrhea. Stool samples from three patients were not available. However, three stool samples were previously determined to be rotavirus positive with commercial latex agglutination or enzyme immunoassay kits. As negative controls, 48 throat swabs were collected from adult patients without gastroenteritis.

Eight CSF samples, five stool samples, and seven serum samples from patients in the acute stage of disease as well as two CSF samples, two stool samples, and two serum samples from patients in the convalescent stage of disease were collected in the Tokyo area between January and April 1992 from eight children (10 months to 3 years of age) with convulsions associated with rotavirus gastroenteritis (patients 8 to 15). The samples were free of contamination. Patients 8 to 13 were at the Department of Pediatrics, Tokyo Women's Medical College, patient 14 was at the Department of Pediatrics, Juntendo University Urayasu Hospital, and patient 15 was at the Department of Pediatrics, Teikyo University School of Medicine. CSF samples from patients 8, 9, 10, 11, 12, 13, 14, and 15, who were in the acute stage of disease, were collected 2, 6, 2, 2, 3, 3, 2, and 3 days after the first diarrhea and 1, 2, 1, 1, 2, 2, 1, and 2 days after the first convulsion, respectively. Details of the symptoms and signs of patients 8, 9, 10, 11, 12, and 13 were reported previously (8, 10). Data for patients 14 and 15 were briefly reported at the meetings of Japanese pediatricians. Stool samples from three patients in the acute stage of disease, patients 8, 9, and 10, were not available. However, the patients were previously determined to be rotavirus positive with a commercial kit.

Cell counts, protein and sugar contents, and electrolyte levels in the CSF samples were within normal limits during the period of observation. CSF samples were examined for occult blood and were found to be negative. Electrolyte levels in sera were also within the normal limits; less than 20 cells/mm^3, 10 to 60 mg of protein per dl, 125 to 150 meq of sodium per liter, 2.3 to 4.6 meq of potassium per liter, and 115 to 130 meq of chloride per liter are normal ranges in CSF, while 4,000 to 8,000 cells/mm^3, 6.0 to 8.0 g of protein per dl, 140 to 145 meq of sodium per liter, 4.0 to 5.0 meq of potassium per liter, and

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TABLE 1. Detection by RT-PCR of rotavirus from throat swabs and stool specimens in patients with rotavirus gastroenteritis and from CSF, serum, and stool samples from patients with rotavirus gastroenteritis accompanied by convulsions[a]

Patient group and no.	Age	Sex	Stage	Latex or EIA	RT-PCR											
					Throat swab			Stool			CSF			Serum		
					I	II	III	I	II	III	I	II	III	I	II	III
Gastroenteritis																
1	10 mo	F	A	+	−	+	4	+	+	4						
2	1 mo	M	A	+	−	+	3	+	+	3						
3	6	M	A	+	−	+	−	ND	ND	ND						
4	8 mo	M	A	+	−	+	−	ND	ND	ND						
5	1 yr	M	A	+	−	+	−	ND	ND	ND						
6	8 mo	M	A	+	−	+	1	+	+	1						
7	1 yr	M	A	+	−	+	1	−	+	1						
Gastroenteritis and convulsions																
8	1 yr	F	A	+				ND	ND	ND	−	+	1	−	+	1
			C	−				ND	ND	ND	−	−	−	−	−	−
9	3 yr	M	A	+				ND	ND	ND	−	+	1	−	+	1
			C	−				ND	ND	ND	−	−	−	−	−	−
10	1 yr	F	A	+				ND	ND	ND	−	−	−	ND	ND	ND
11	1 yr	F	A	+				+	+	1	−	+	1	−	−	−
12	1 yr	M	A	+				+	+	1	−	+	1	−	−	−
13	1 yr	M	A	+				−	+	1	−	+	1	−	−	−
14	10 mo	M	A	+				+	+	1	−	+	1	−	−	−
15	1 yr	M	A	+				−	+	1	−	+	1	−	−	−

[a] Abbreviations: F, female; M, male; A, acute stage; C, convalescent stage; EIA, enzyme immunoassay; ND, not done; I, RT-PCR with primer set A; II, RT-PCR with primer set B; III, RT-PCR with primer set C. 1, 3, and 4, serotypes 1, 3, and 4.

103 to 117 meq of chloride per liter are normal ranges in serum.

Twenty CSF samples from children without rotavirus gastroenteritis or convulsions were kindly donated by the Laboratory Center of Teikyo University School of Medicine in Tokyo.

Extraction of RNA. For RT-PCR, we extracted viral RNA from either the supernatants of stool suspensions, throat swabs, CSFs, or sera by the modification of the guanidine thiocyanate and glass powder method described by Gentsch et al. (1a). Briefly, (i) about 20% stool suspensions in phosphate-buffered saline (PBS) were centrifuged at 2,000 × *g* for 20 min. Freon was added to the stool suspensions at the same volume as the supernatant, and these were then mixed together for about 15 min. The mixture was again centrifuged at 2,000 × *g* for 20 min. (ii) The throat swab was dipped in about 1 ml of PBS, and the solution was squeezed out of the throat swabs. (iii) CSF and serum specimens were used after centrifugation.

Two hundred fifty microliters of 6 M guanidine thiocyanate and 10 μl of RNAID (Bio 101, Inc., La Jolla, Calif.) were added to 250 μl of the sample, and these were mixed together for 15 min. Then the sample was centrifuged at 50 × *g* for 2 min, and the supernatants were aspirated. The pellet was washed three times with the RNAID kit wash buffer and once with 100% ethanol; this was followed by centrifugation at 50 × *g*. After the last wash, however, the pellet was centrifuged at 8,000 × *g*. The sample was dried for 20 min in a vacuum centrifuge to remove all residual alcohol. The sample was resuspended in 30 μl of sterile, deionized water, and the solution was mixed vigorously until the RNAID was completely in suspension; finally, the mixture was incubated for 10 min at 65°C and was then centrifuged at 8,000 × *g* for 10 min. The supernatant was stored at −30°C until use.

RT-PCR. RT-PCR was performed as described previously (3, 16). Briefly, RT of the RNA was done with the avian myeloblastosis virus reverse transcriptase XL (Life Science Inc., St. Petersburg, Fla.), the Beg 9 and End 9 group A-specific primers (primer set A) of the VP7 gene, and the deoxynucleoside triphosphates. The first PCR was conducted with the same primers and *Tth* DNA polymerase (Toyobo Co., Osaka, Japan). For the second (nested) PCR we used a mixture of type-specific primers (aBT1, aCT2, aET3, aDT4, aAT8, and aFT9) for upstream priming and the same End 9 downstream primer (primer set C). Another primer common to the group A rotavirus, VP7-1′, was also used together with Beg 9 (primer set B) in order to confirm the presence of group A rotaviruses (16). After electrophoresis on a 1.5% agarose gel with ethidium bromide at 100 V for 1 h, the gel was photographed under UV light. The cDNA was isolated from the gel for the purpose of cloning and sequencing.

Cloning. The cloning of PCR products into the M13 vector and dideoxynucleotide sequencing were performed as described previously (19). The nucleotide sequences and the deduced amino acid sequences were compared with those of a standard strain of serotype 1 (Wa) and the previously analyzed sequences from a different set of patients (19).

Miscellaneous procedures. To exclude contamination extraction of RNAs and RT-PCR were conducted carefully by two examiners who were blinded as to the samples and in different laboratories. Stools were treated at separate times from CSFs, sera, and throat swabs.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers against human rotavirus RNA for VP7: D17717 (C1 strain), D17718 (C2 strain), D17719 (C3 strain), D17720 (C4 strain), D17721 (TE1 strain), D17722 (TE2 strain), and D17723 (TE3 strain).

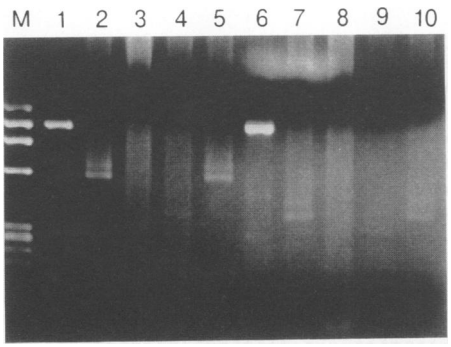

FIG. 1. Electrophoretic patterns of rotavirus RT-PCR products from stool and throat swab samples of patients 1 and 2, *Hae*III-digested ϕX174 DNA size markers (lane M), RT-PCR products of rotavirus gene 9 RNA from a stool specimen from patient 1 (primer set A, lane 1; primer set C, lane 2) and from a stool specimen from patient 2 (primer set A, lane 6; primer set C, lane 7), and RT-PCR products of rotavirus gene 9 DNA from a throat swab from patient 1 (primer set A, lane 3; primer set B, lane 4; primer set C, lane 5) and from a throat swab from patient 2 (primer set A, lane 8; primer set B, lane 9; primer set C, lane 10) are shown. Lanes 1, 2, 4, 5, 6, 7, 9, and 10 tested positive and lanes 3 and 8 tested negative. Patient 1 was infected with serotype 4 rotavirus, and patient 2 was infected with serotype 3 rotavirus. See text for details regarding the primer sets.

RESULTS

Detection of rotavirus in throat swabs and stool samples. All seven throat swabs were positive for rotavirus by RT-PCR. Three of them were positive only with the group-specific primers Beg 9 and VP7-1′, while the remaining four were also positive with type-specific primers (Table 1). Rotaviruses in two of the throat swabs in the latter group were found to be serotype 1; the rotaviruses in the other two were types 3 and 4. The serotypes of the rotaviruses found in the throat swabs were identical to those observed for the rotaviruses in the stool samples from the respective patients. All 48 throat swabs from patients without rotavirus gastroenteritis were negative in the first and second PCRs. The electrophoretic patterns of rotavirus RT-PCR products from stool and throat samples of patients 1 and 2 are presented in Fig. 1 (those from the other patients are not shown).

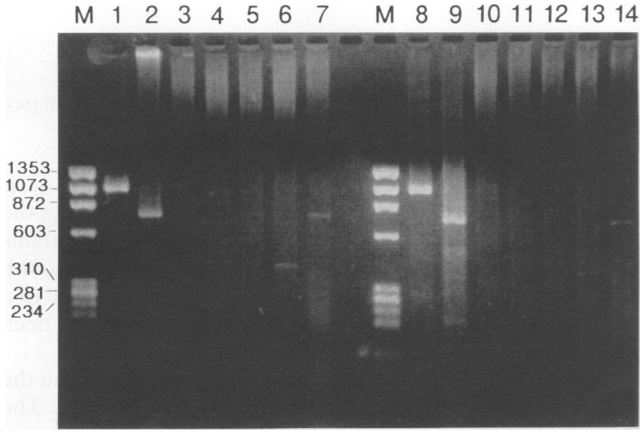

FIG. 2. Electrophoretic patterns of rotavirus RT-PCR products from stool, serum, and CSF samples from patients 11 and 12. *Hae*III-digested ϕX174 DNA size markers (lane M), RT-PCR products of rotavirus gene 9 RNA from a stool specimen from patient 11 (primer set A, lane 1; primer set C, lane 2) and from a stool specimen from patient 12 (primer set A, lane 8; primer set C, lane 9), RT-PCR products of rotavirus gene 9 DNA from a serum sample from patient 11 (primer set A, lane 3; primer set B, lane 4) and from a serum sample from patient 12 (primer set A, lane 10; primer set B, lane 11), RT-PCT products of rotavirus gene 9 DNA from the CSF of patient 11 (primer set A, lane 5; primer set B, lane 6; primer set C, lane 7), and RT-PCR products of rotavirus gene 9 DNA from the CSF of patient 12 (primer set A, lane 12; primer set B, lane 13; primer set C, lane 14) are shown. Lanes 1, 2, 6, 7, 8, 9, 13, and 14 tested positive, and lanes 3, 4, 5, 10, 11, and 12 tested negative. Patients 11 and 12 were infected with serotype 1 rotavirus.

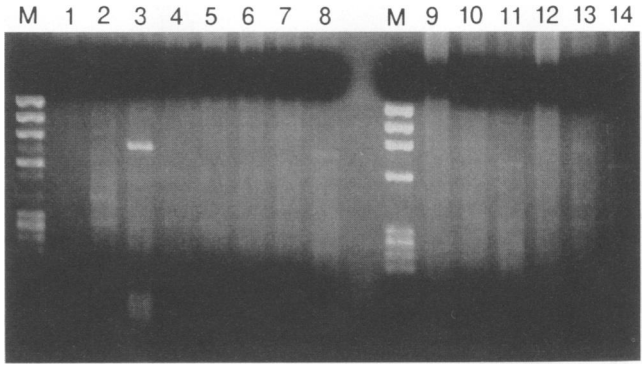

FIG. 3. Electrophoretic patterns of rotavirus RT-PCR products from stool, serum, and CSF samples from patients 15 and 8. *Hae*III-digested ϕX174 DNA size markers (lane M), RT-PCR products of rotavirus gene 9 RNA from a stool specimen from patient 15 (primer set A, lane 1; primer set B, lane 2, primer set C, lane 3), RT-PCR products of rotavirus gene 9 RNA from a serum specimen from patient 15 (primer set A, lane 4; primer set B, lane 5) and from a serum specimen from patient 8 (primer set A, lane 9; primer set B, lane 10; primer set C, lane 11), and RT-PCR products from the CSF of patient 15 (primer set A, lane 6; primer set B, lane 7; primer set C, lane 8) and from the CSF of patient 8 (primer set A, lane 12; primer set B, lane 13; primer set C, lane 14) are shown. Lanes 2, 3, 7, 8, 10, 11, 13, and 14 tested positive, and lanes 1, 4, 5, 6, 9, and 12 tested negative. Both patients 11 and 12 were infected with serotype 1 rotavirus.

Detection of rotavirus from CSF, sera, and stool samples. Rotaviruses in seven of the eight CSF samples, five of the five stool samples, and two of the seven serum samples from patients in the acute stage of disease were determined to be serotype 1. Two CSF samples, two stool samples, and two serum samples from patients in the convalescent stage of

TABLE 2. Percent homology of nucleotide sequences and amino acid sequences among the seven strains sequenced in the present study and the Wa strain

Strain	% Homology[a]							
	Wa	C1	C2	C3	C4	TE1	TE2	TE3
Wa		93	93	92	93	93	92	93
C1	97		99	99	99	96	98	98
C2	97	98		99	99	97	99	98
C3	97	98	99		99	97	99	99
C4	97	98	99	99		96	99	98
TE1	97	98	98	98	98		96	96
TE2	96	98	98	99	99	98		99
TE3	97	98	98	98	98	98	98	

[a] The percent homologies of nucleotide sequences are given above the blank space, and the percent homologies of amino acid sequences are given below the blank space. C1 and C2 are serum and CSF from patient 8, respectively; C3 and C4 are serum and CSF from patient 9, respectively; and TE1, TE2, and TE3 are stool, CSF, and throat swab, respectively, from patient 15.

FIG. 4. Comparison of the deduced VP7 amino acid sequences among the seven strains examined in the present study and the Wa strain (see Table 2 for details). The amino acids that were identical to those of the Wa strain are indicated with an asterisk (*).

disease were negative for rotavirus (Table 1). All 20 CSF samples from patients without rotavirus gastroenteritis were negative in the first and second PCRs. The electrophoretic patterns of rotavirus RT-PCR products from CSF, serum, and stool samples from patients 11 and 12 are presented in Fig. 2, and those of patients 15 and 8 are presented in Fig. 3.

Nucleotide and amino acid sequences. Rotaviruses in seven samples were sequenced, i.e., virus from the serum and CSF of two patients and from the stool, CSF, and throat swab of a third patient (Table 2 and Fig. 4).

Homologies of more than 96% were noted among the seven PCR products on both nucleotide and amino acid sequences; however, the homologies of the nucleotide sequences between strain Wa and the seven isolates were only 92 or 93% (Table 2). There were no consistent differences in rotavirus amino acid sequences between samples from the three types of material tested (Table 2). The nucleotide sequence data are not reported here because they will appear in the DDBJ, EMBL, and GenBank databases (see above).

DISCUSSION

Rotavirus has been assumed to be transmitted by the fecal-oral route. Oral administration of rotavirus-positive stool material induced diarrheal illness in volunteers (6). It is possible, however, that these viruses are also transmitted by the respiratory route. The source of infection for young infants who are normally not in contact with other children with gastroenteritis is not well documented. With the exception of a few studies, neither rotavirus nor rotavirus antigen has been detected in respiratory secretions (6).

In the present study we demonstrated for the first time the presence of rotavirus RNAs in throat swabs by RT-PCR. The patients had no signs of vomiting, and the swabs were collected carefully from the early part of the acute stage of disease. We are collecting more throat swab samples for RT-PCR to confirm our results. The presence of rotavirus genomes was also demonstrated in sera and CSFs by RT-PCR. Our results are supported by the recent detection by RT-PCR of the rotavirus genome in the CSF of a patient with rotavirus gastroenteritis accompanied by encephalitis by RT-PCR (20). Rotaviruses were not always detected in the CSFs and sera from patients with rotavirus gastroenteritis and convulsions. The negative results may be due to either of the following two reasons: (i) Rotaviruses are usually present for only a short time in the CSF or (ii) the concentration of virus is below the detection limit.

We have previously reported that the rotavirus RNA electrophoretic patterns and the rotavirus serotypes found in stool samples from patients with gastroenteritis and convulsions are not different from those found in patients only with gastroenteritis (15). This suggests that a specific strain or serotype with neurovirulence does not exist. We were not able to isolate rotaviruses or obtain RNA electrophoretic patterns from CSF samples (14). However, rotaviruses can infect neuronal cell lines, although only for a few passages (14). Further examination of the maturation and transportation of rotaviruses in neurons has been performed (17). The existence of the rotavirus genome in CSF by RT-PCR in the present study may be due to (i) the fact that the genomes permeate through the brain-blood barrier during convulsions, (ii) occasional rotavirus infection in neuronal cells and the release of the virus from the cells to CSF, or (iii) rotavirus encephalitis or encephalopathy like human herpesvirus 6 meningoencephalitis-associated convulsions with exanthema subitum in infants (4).

Moreover, the effect of rotavirus components, cytokines, and neurotoxins on neurons should be investigated. The relationship between convulsions and the existence of the rotavirus genome is not clear at this time and requires further study.

Small variations in nucleotide and amino acid sequences were detected among the seven samples sequenced. These data as well as sequence data from a previous report (19) do not indicate the existence of neurotropic strains.

A human rotavirus strain (Wa) that was passaged several times in a liver cell line (HepG2) exhibited alterations in the sequence of its VP4 gene (7). It may therefore be interesting to examine the VP4 sequences of our strains.

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