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Molecular Analysis of Outer Capsid Glycoprotein (VP7) Genes from Two Isolates of Human Group C Rotavirus with Different Genome Electropherotypes

MITSUTAKA KUZUYA,[1]* RITSUSHI FUJII,[1] MASAKO HAMANO,[1] JUN NAKAMURA,[2] MASAO YAMADA,[2] SHIRO NII,[2] AND TADASHIGE MORI[1]

Department of Microbiology, Okayama Prefectural Institute for Environmental Science and Public Health, Okayama 701-02,[1] and Department of Virology, Okayama University Medical School, Okayama 700,[2] Japan

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Nucleotide sequences of the VP7 gene of human group C rotavirus were determined for two strains isolated in Okayama, Japan, during a 1988-to-1990 epidemic. These isolates, OK118 and OK450, were selected as prototypes of two different electropherotypes, patterns I and II, respectively. The genes were identical in size (1,063 bp), and both contained single open reading frames encoding 332 amino acids. The alignment of two sequences revealed 46 nucleotide substitutions, 11 of which were predicted to give amino acid changes. The deduced amino acid sequence of VP7 from OK118 was similar to published sequences of a Japanese isolate and three foreign isolates (more than 98.4% identity), whereas the VP7 sequence of OK450 revealed around 96% identity with these isolates and had nine unique amino acid substitutions. The VP7 genes of nine Okayama isolates were then analyzed by dot blot hybridization with the VP7 probes of OK118 and OK450. Under highly stringent conditions, the OK118 probe produced strong hybridization signals with the genes of five pattern I strains and one pattern II strain, while the OK450 probe strongly reacted only with those of three pattern II strains. Our results concluded that relative sequence diversity in the VP7 gene was observed between two different electropherotypes prevalent in a limited area.

Rotaviruses are members of the *Reoviridae* family and are the major viral agents of diarrheal illness of infants and young children. On the basis of antigenic cross-reactivity and the relative electrophoretic mobilities of their double-stranded RNA (dsRNA) genome segments, rotaviruses are classified into seven groups (A to G) (20). Only three of these groups (A, B, and C) are known to infect humans (20).

Group C rotaviruses were first recognized as a causative agent of gastroenteritis in piglets (1, 21). Bridger et al. (3) definitely characterized them as human pathogens in 1986. Since then, many workers have reported human group C rotavirus (CHRV) infections in several continents (2, 6, 18). Recently, outbreaks of CHRV were also reported in some areas in Japan (15, 17) and the United Kingdom (5). Thus, CHRV is globally distributed and is thought to be one of the emerging pathogens of medical importance.

In group A rotaviruses, 14 serotypes are recognized on the basis of cross-neutralization tests with hyperimmune sera (4, 7, 22). The major serotype-specific epitopes of group A rotaviruses were demonstrated to be located in the major outer capsid glycoprotein VP7, which was encoded by genome segment 7, 8, or 9 (22). Although serological diversity within group C rotaviruses was suggested in a preliminary cross-neutralization study (24), no serotyping has so far been done. Nevertheless, an outer capsid glycoprotein corresponding to the VP7 of group A rotaviruses was identified in group C rotaviruses (13) and was shown to be encoded by the eighth genome segment (19). Sequence variation of the VP7 gene between animal group C rotaviruses and CHRVs was demonstrated by cross-hybridization studies (12). In contrast, surprisingly high levels of sequence conservation of the VP7 gene among three CHRV strains were recently demonstrated by Grice et al. (11).

We previously demonstrated that genome electropherotypes of CHRVs isolated in Okayama, Japan, were classified into two patterns (patterns I and II) (8). In the present study, we performed a comparative nucleotide and amino acid sequence analysis of the VP7 genes from two strains with different electropherotypes in order to investigate the genetic and antigenic divergence of CHRVs.

MATERIALS AND METHODS

Viruses. Epidemiological data from 11 CHRV strains used in this study are shown in Table 1. Most of the strains were isolated in or around Okayama City. The strains were identified as CHRV by specific monoclonal antibodies (8, 14). Two samples (OK118 and OK450) were used as templates of cDNA cloning.

RNA extraction. Two hundred microliters of 20% fecal suspensions containing CHRV were extracted with an equal volume of trichlorotrifluoroethane, and the supernatants were adjusted to contain 10 mM EDTA, 0.6% sodium dodecyl sulfate (SDS), and 300 μg of proteinase K per ml. The suspensions were incubated for 1.5 h at 40°C and then extracted with phenol-chloroform. Viral dsRNA was further purified with the RNAID kit (BIO 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions. Finally, the RNAs were resuspended in 20 μl of diethyl pyrocarbonate-treated water and stored at −30°C until use.

Oligonucleotide primers. Oligonucleotide primers corresponding to the 5′ end (G8S, 5′-GGCATTTAAAAAAGAAGAAGCTGT-3′) and the 3′ end (G8A, 5′-AGCCACATGATCTTGTTTACGC-3′) of the published VP7 gene sequence (19) were synthesized and used for reverse transcription (RT)-PCR amplification of the VP7 gene. Two primers (G8SH, 5′-CCCAAGCTTGGCATTTAAAAAA GAAGAAGCTGT-3′; and G8AB, 5′-CCCGGATCCAGCCACATGATCTTG TTTACGC-3′) were also prepared for cDNA cloning of the full-length VP7 sequence. The underlined parts of these sequences were restriction sites introduced for cloning.

RT-PCR of the VP7 genes. Genomic dsRNA from the clinical isolates was dissolved in 15% dimethyl sulfoxide solution containing the primers (G8S and G8A) and heated at 97°C for 5 min. After rapid cooling, the denatured RNA was converted to cDNA with RNase H⁻ reverse transcriptase (Superscript; Life Technologies, Inc., Gaithersburg, Md.) at 42°C for 60 min. The remaining RNA was hydrolyzed by addition of NaOH, followed by neutralization with HCl and Tris-HCl (pH 8.0). The resultant cDNA was purified with a Suprec-02 column (Takara Shuzo Co., Ltd., Kyoto, Japan) and amplified with *Taq* DNA polymerase

* Corresponding author. Phone: 001-81-86-298-2681. Fax: 001-81-86-298-2088.

TABLE 1. Sources, genome patterns, and dot blot results for 11 clinical isolates

Sample	Date of isolation (mo/day/yr)	Genome pattern	Dot blot result with probe:	
			OK118	OK450
OK70	1/11/88	I	+	−
OK118	2/6/88	I	+	−
OK222	4/7/88	I	+	−
OK231	4/25/88	I	+	−
OK239	4/28/88	I	+	−
OK450	2/8/89	II	−	+
OK459	2/13/89	I	+	−
OK462	2/13/89	II	−	+
OK595	8/12/89	II	+	−
I54	6/7/90	II	−	+
I57	6/13/90	II	−	+

for an initial 3-min incubation at 72°C, followed by 30 cycles of PCR (94°C for 1 min, 48°C for 2 min, and 72°C for 2 min) and a final 7-min incubation at 72°C.

Cloning and sequencing of the VP7 genes. The VP7 genes of two clinical isolates (OK118 and OK450) were amplified by PCR with the G8SH and G8AB primers. The PCR products were digested with restriction endonucleases (*Bam*HI and *Hin*dIII) and then cloned into plasmid pUC18. Five individual recombinants were isolated, and both strands of the cloned DNA were sequenced by the dideoxynucleotide chain-termination method (Applied Biosystems, Foster City, Calif.).

Dot blot hybridization. The VP7 genes amplified from clinical isolates by RT-PCR were extracted with phenol-chloroform and precipitated with ethanol. The genes were dissolved in distilled water and purified with Suprec-02 columns. Equivalent amounts (200 ng) of the genes were denatured by boiling and were blotted onto nylon membranes. Probes were generated by labeling the VP7 genes from OK118 and OK450 with digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) by the PCR method. Hybridization was performed at 52°C for 16 h in hybridization buffer consisting of 50% formamide, 5× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0]), 7% SDS, 2% blocking reagent (Boehringer), 50 mM sodium-phosphate (pH 7.0), 0.1% *N*-lauroylsarcosine, 50 μg of denatured salmon sperm DNA per ml, and 10 ng of the digoxigenin-labeled probe per ml. After hybridization, the membranes were washed twice in 0.1× SSC containing 0.1% SDS at 68°C for 15 min. Colorimetric detection of bound probe was carried out as recommended by the manufacturer.

Sequence data analysis. Nucleotide sequence data were analyzed by the GENETYX-MAC version 6.0 program. The program MAlign, version 1.0, was also used for sequence alignments.

RESULTS

Observation of two different genome electropherotypes in an epidemic in Okayama Prefecture. Electropherotyping of 11 clinical isolates revealed two distinct genome profiles, patterns I and II (Table 1). The OK118 strain was selected as a representative of six isolates with pattern I, while the OK450 strain was selected as a representative of five isolates with pattern II. RNA profiles of OK118 and OK450 are shown in Fig. 1. Although both strains exhibited the typical 4-3-2-2 profile of group C rotaviruses, the 5th, 7th, and 10th genome segments of OK450 migrated slowly compared with those of OK118; other minor differences were observed in the 3rd, 4th, and 8th segments.

Nucleotide and deduced amino acid sequences of the VP7 genes from OK118 and OK450. A sequence comparison of the VP7 genes from OK118 and OK450 is shown in Fig. 2. Both sequences were 1,063 nucleotides in length and contained a single open reading frame beginning at the 49th nucleotide from the 5′ end and terminating 16 bases upstream from the 3′ end. The open reading frame consisted of 999 nucleotides and encoded 332 amino acids. There were 46 nucleotide substitutions between the two sequences, and these substitutions were scattered throughout the gene. The overall nucleotide identity value was 95.7%.

The VP7 genes of OK118 and OK450 were then compared with the published sequences of four CHRV strains. As shown in Table 2, a surprising level of sequence conservation was observed between OK118 and a Japanese isolate (88-220). The gene sequence of OK118 was also similar to those of foreign isolates (Bristol, Preston, and Belém), with overall identities of 98.0 to 98.2%, whereas the overall nucleotide identities between OK450 and the other strains were relatively low (from 95.3 to 95.7%).

Figure 3 shows the alignment of the deduced amino acid sequences of VP7 from OK118 and OK450 with the published sequences from four CHRV strains. The amino acid identity values among six strains are summarized in Table 2. The overall identity between the OK118 and OK450 sequences was 96.7%. Although there were 11 amino acid substitutions between the two sequences, no clustered amino acid-divergent region was observed. There were three conserved sites that were potential N-glycosylation sites (Asn-X-Ser/Thr) in both VP7 polypeptides. Among five of the six strains (all except OK450), high degrees of sequence identity (more than 98.4%) were observed, while there were around 96% identities between OK450 and the other strains. In addition, unique amino acid substitutions occurred at nine positions in the OK450 sequence.

Dot blot hybridization analysis of the VP7 genes from the clinical isolates with different electropherotypes according to OK118 and OK450 cDNA probes. Dot blot hybridization analysis was performed to evaluate the existence of genome divergence represented by OK118 and OK450 in nine clinical iso-

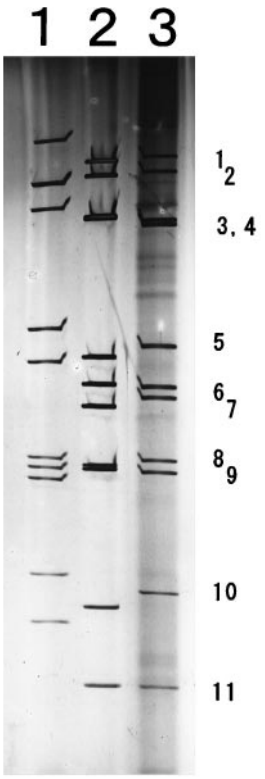

FIG. 1. RNA electropherotypes of human group A and group C rotavirus strains. The viral dsRNAs were dissociated in a 10% polyacrylamide slab gel. The RNA bands were visualized by silver nitrate. Lanes: 1, Wa strain of human group A rotavirus; 2 and 3, OK118 and OK450 strains of CHRV, respectively. The approximate positions of RNA segments of OK450 are indicated on the right.

FIG. 2. Nucleotide sequences of cDNA corresponding to the VP7 genes of OK118 and OK450. Identical nucleotides are represented by a dot. Initiation and termination codons are underlined. The nucleotide sequence data have been submitted to the DDBJ DNA database and have been assigned the accession numbers D87543 (OK118) and D87544 (OK450).

lates. First, various hybridization conditions were examined to differentiate the VP7 gene of OK118 from that of OK450. As a result, under highly stringent conditions (50% formamide, 5× SSC, and 52°C), each probe could strongly hybridize with the gene of homologous strains and weakly reacted with that of heterologous strains.

Amplified VP7 cDNAs from 11 isolates, including the prototypes, were spotted on nylon membranes and hybridized with the labeled probe under the same high-stringency conditions. The OK118 probe produced strong hybridization signals with the corresponding genes of all pattern I strains and one pattern II strain (OK595), and weak signals were observed with those of other strains. In contrast, the OK450 probe strongly reacted only with the genes of four pattern II strains (Table 1).

DISCUSSION

Genome electropherotyping is widely applied to epidemiological studies of human group A rotavirus infections, and various genome electropherotypes have been recognized to date (22). Although some investigators reported that RNA profiles of CHRV isolates were different from those of porcine group C rotavirus isolates (3, 18), little is known about the variation of genome electropherotypes among the CHRVs.

TABLE 2. Nucleotide and deduced amino acid sequence homologies of VP7 genes from six CHRV strains

Strain (country of origin)	% of homology to strain[a]:						Reference
	OK450	OK118	88-220	Bristol	Preston	Belém	
OK450 (Japan)		**95.7**	**95.6**	**95.6**	**95.4**	**95.3**	
OK118 (Japan)	96.7		**99.3**	**98.2**	**98.0**	**98.1**	
88-220 (Japan)	96.7	99.4		**98.3**	**98.1**	**98.0**	19
Bristol (England)	96.7	98.8	99.4		**99.8**	**97.8**	11
Preston (England)	96.7	98.8	99.4	100		**97.8**	11
Belém (Brazil)	96.4	98.5	99.1	99.1	99.1		11

[a] The percent homologies of nucleotide sequences are given in boldface. Other data represent the percent homologies of deduced amino acid sequences.

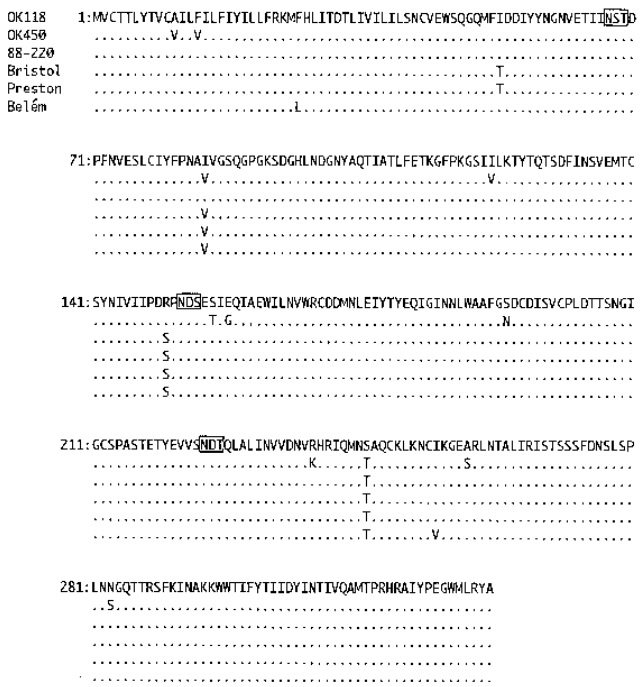

FIG. 3. Alignment of the deduced amino acid sequences of VP7 from OK118 and OK450 with the published sequences from four CHRV strains. Identical amino acids are represented by a dot. Potential N-linked glycosylation sites in the OK118 sequence are boxed.

We showed that genome electropherotypes of CHRV strains isolated in an epidemic in Okayama Prefecture during 1988 to 1990 were classified into patterns I and II and that dominant patterns changed from year to year.

In this study, we demonstrated that electropherotypes of CHRVs were related to variations in the outer capsid glycoprotein VP7 gene. Two clinical isolates (OK118 and OK450) were selected as prototypes of patterns I and II, respectively, and a comparative nucleotide and amino acid sequence analysis of the VP7 gene of these strains was performed. Although the sequences were identical in size and both contained single open reading frames with the same size, there were 46 nucleotide substitutions between them. The comparison of the predicted VP7 sequence also showed that amino acid substitutions occurred at 11 positions. These data indicate that the OK118 and OK450 strains are genetically and perhaps antigenically distinct.

Our dot blot analysis revealed almost exclusive cross-hybridization of the OK118 and OK450 cDNA probes to the clinical isolates with patterns I and II, respectively, suggesting that relative sequence diversity in the VP7 gene exists between the two electropherotypes. However, an exception was the OK595 strain, which exhibited the pattern II electropherotype but possessed the OK118-type VP7 gene. Nakagomi et al. (16) and Ward et al. (25) have recently described naturally occurring reassortants between human group A rotaviruses belonging to different genogroups. It is therefore of interest to know whether the OK595 strain is a reassortant between patterns I and II.

We compared the VP7 gene sequences of OK118 and OK450 with published sequences of VP7 genes from four CHRV strains. The VP7 gene of OK118 exhibited very high levels of homology with the genes of all four strains. In contrast, the overall nucleotide identities between OK450 and the other strains were from 95.3 to 95.7%, and 35 nucleotide substitutions, 9 of which were predicted to give amino acid changes, were uniquely observed in the OK450 sequence, indicating that OK450 had a distinct VP7 gene. It has been proposed that CHRV was originally derived from pigs through interspecies transmission (18). The VP7 genes of OK450 and OK118 were then aligned with the corresponding gene of a porcine group C rotavirus (Cowden strain). The nucleotide identity value between OK450 and the Cowden strain (83.7%) was similar to the value between OK118 and the Cowden strain (84.1%). These data imply that OK450 is not an intermediate strain between porcine and human viruses. Very recently, Tsunemitsu et al. (23) have described the sequence comparison of the VP7 gene among human, porcine, and bovine group C rotaviruses. Although the Cowden strain was most closely related to a human strain compared with the other strains, there was a limited (81.9%) homology between the Cowden strain and the human strain. Additional sequence analyses of the VP7 genes from group C rotavirus strains isolated from humans, pigs, and other species are needed to yield insights into the evolutionary origin of CHRV.

In group A rotaviruses, isolates belonging to the same serotype are highly homologous (more than 90%) in the VP7 sequence (10), while isolates in different serotypes show significant VP7 divergence, and several discrete regions of variable amino acid sequence are defined (9). It is thought that these variable regions are involved in the determination of serotype specificity. Among six CHRV strains, sequence heterogeneity of the VP7 protein was relatively small (less than 4%) in comparison with that of the group A rotaviruses, and no clustered amino acid variable region was observed. It seems likely that these strains belong to the same group C rotavirus serotype. However, further studies with serologic assays are required to define whether serotypes exist within CHRVs, because the minor amino acid changes may affect the antigenic properties of the VP7 protein.

In conclusion, we have demonstrated that CHRV strains with two different electropherotypes were prevalent in Okayama Prefecture and also have shown that the VP7 gene of CHRV varied in accordance with the changes in its genome electropherotypes. An attempt to generate monoclonal antibodies for differentiation of the OK118 and OK450 strains is under way to clarify the antigenic relationship between the two different electropherotypes.

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