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Genetic Analysis of Group B Human Rotaviruses Detected in Bangladesh in 2000 and 2001

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Group B rotaviruses detected in Bangladesh in 2000 and 2001 were analyzed genetically to clarify relatedness to human group B rotaviruses reported previously in China and India, and to animal group B rotaviruses. *VP7* gene sequences of the Bangladeshi group B rotaviruses (Bang373, Bang544, Bang334, and Bang402) were almost identical to each other and also showed high sequence identity to the Indian strain CAL-1 (98%) and Chinese strain adult diarrhea rotavirus (ADRV) (92%), while identities to bovine and murine viruses were considerably low (60–63%). Other genes of Bang373 and Bang544 encoding VP2, VP4, VP6, and NSP1 ~ NSP5 also showed much higher sequence identities to those of CAL-1 (97.7–99.4%) than to those of ADRV (89.9–93.9%). Characterization of nucleotide substitutions among Bang373, CAL-1, and ADRV suggested that all the gene segments might have evolved neutrally at similar mutation rates, while some of the gene segments (e.g., *VP2* gene) were suggested to be more conserved than others. In conclusion, group B rotaviruses detected in Bangladesh represented by Bang373 and the Indian virus CAL-1 were considered as virtually identical viruses which are distinct genetically from ADRV, and it was suggested that Bang373 (CAL-1)-like group B rotavirus (Bengali strains) might be distributed primarily in an area around the Bay of Bengal. ***J. Med. Virol. 72:149–155, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: rotavirus; group B; human; Bangladesh; gene

INTRODUCTION

Group B rotavirus is genetically and antigenically quite distinct from group A rotavirus, which is a most common cause of infantile gastroenteritis, and is unique because it causes severe cholera-like diarrhea mostly in adults [Mackow, 1995]. This virus was identified first as adult diarrhea rotavirus (ADRV) in nationwide outbreaks in China in 1982 and 1983 [Hung et al., 1983, 1984; Wang et al., 1985; Su et al., 1986]. Thereafter, diarrheal cases due to group B rotavirus have been reported exclusively in China [Dai et al., 1987; Fang et al., 1989], and accordingly this virus was regarded as an endemic pathogen in China. However, seroepidemiological studies suggested that group B rotavirus might have existed in some other countries at a low prevalence [Hung, 1988].

In 1997 and 1998, group B rotaviruses were detected in sporadic adult cases of diarrheal disease in Calcutta in eastern India, which was the first detection of human group B rotavirus outside China [Krishnan et al., 1999]. Genetic analysis revealed that the Indian group B rotavirus strain CAL-1 is genetically closer to the Chinese strain ADRV than animal (bovine and murine) group B rotaviruses reported so far [Kobayashi et al., 2001]. However, some different characteristics in gene sequences between CAL-1 and ADRV suggested that CAL-1 may be a distinct virus from ADRV, although

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both viruses might have evolved from a common ancestral strain. The detection of CAL-1 suggested the distribution of a similar group B human rotavirus in and around eastern India.

During a period between 2000 and 2001, group B rotaviruses were detected in 14 sporadic cases (12 adults and 2 children) through our virological investigation of diarrheal pathogens in Mymensingh, Bangladesh [Sanekata et al., 2003]. Mymensingh is located in the north of Dhaka, and is approximately 400 km distant from Calcutta. Thus, it is significant to analyze genetically these group B rotaviruses in Bangladesh to know their origin, i.e., their genetic relatedness to the previously reported human group B rotaviruses (CAL-1 and ADRV) and animal group B rotaviruses. For this purpose, we determined and analyzed the gene sequences of the Bangladeshi group B rotaviruses, and characterized further nucleotide substitutions in individual viral RNA segments to understand the mode of generation of genomic divergence among group B rotaviruses.

MATERIALS AND METHODS

Four group B rotaviruses, Bang373, Bang544, Bang334, and Bang402, which were derived from adult patients (male, 30 years old; female, 35 years old; female, 28 years old; female, 27 years old, respectively) were examined. Fecal specimens containing group B rotaviruses were collected in the SK Hospital, Mymensingh, Bangladesh, in Dec. 2000 (Bang334 and Bang373), Feb. 2001 (Bang402), and June 2001 (Bang544). Viral RNA was extracted from a 10% suspension of a fecal specimen in PBS with a commercially available kit (RNAID kit, BIO101, Inc., La Jolla, CA) according to the manufacturer's instructions.

The viral genes encoding VP2, VP4, VP6, VP7, and NSP1 ~ NSP5 were amplified by RT-PCR as described previously [Taniguchi et al., 1992] with some modifications. Oligonucleotide primers which are complementary to ADRV or CAL-1 gene sequences published previously [Mackow, 1995; Kobayashi et al., 2001] were synthesized and used for RT-PCR (Table I). A mixture of extracted dsRNA (1 μl), dimethyl sulfoxide (3.5 μl), and distilled water (4 μl) was heated in a reaction tube at 97°C for 5 min and rapidly cooled in ice. To this tube, 1 μl each of primer pair (60 pmol/μl each), 0.5 μl (15 U) of reverse transcriptase (AMV) (Seikagaku Co., Tokyo, Japan), 0.5 μl (1.8 U) of thermostable DNA polymerase mix (Expand High Fidelity PCR System, Roche, Mannheim, Germany), and 41.5 μl of reaction buffer were added. The reaction buffer contained Expand HF buffer (containing $MgCl_2$, final 1.5 mM), dATP, dCTP, dGTP, and dTTP (final 200 μM each), dithiothreitol (final 4.5 mM), and RNase inhibitor (RNA guard, Porcine, Amersham Biosciences Corp., Piscataway, NJ).

The reaction mixture was covered with mineral oil and the tube was placed in a thermal cycler (PC-700; Astec Co., Fukuoka, Japan). Following incubation for reverse transcription at 42°C for 30 min, samples were subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C, and a final cycle of 1 min at 94°C, 1 min at 55°C, and 7 min at 72°C. PCR products (8 ~ 10 μl) were analyzed by electrophoresis in 1% agarose gel in Tris-acetate–EDTA buffer containing ethidium bromide (1 μg/ml).

Nucleotide sequences were determined directly from PCR products by the dideoxynucleotide chain termination method using the Sequenase PCR Product Sequencing Kit (United States Biochemical, Cleveland, OH). In addition to group B rotaviruses detected in Bangladesh, CAL-1 genes encoding VP2, NSP2, NSP4, and NSP5 were also determined in this study. Sequence data were analyzed by using the GeneWorks software package (IntelliGenetics, Inc., Mountain View, CA).

RESULTS AND DISCUSSION

VP7 gene sequences were determined for four group B rotaviruses detected in Bangladesh, and other viral gene sequences encoding VP4, VP6, NSP1 ~ NSP5 were determined for Bang373 and Bang544. The *VP2* gene was sequenced for Bang373. Viral structural proteins VP7 and VP4 constitute outer capsids of rotavirus particles, and are important immunologically because these proteins contain neutralization antigens. Similarly to ADRV and CAL-1, *VP7* gene sequences of Bangladeshi group B rotaviruses comprise 814 nucleotides and are considered to encode a VP7 protein consisting of 249 amino acids. Nucleotide sequences of *VP7* genes were almost identical (99.8 ~ 100%) among the four group B rotaviruses detected in Bangladesh. The *VP7* gene sequence of Bang373 was completely identical to that of Bang334, and differed from *VP7* genes of Bang402 and Bang544 by only one and two nucleotides, respectively. *VP7* (gene) sequence identities of Bang373 and Bang544 to other group B rotaviruses are shown in Table II. Bang373 and Bang544 showed the highest nucleotide sequence identities to CAL-1 (98.4% and 98.2%, respectively) followed by ADRV (92.0% and 92.3%, respectively). In contrast, identities to bovine and murine group B rotaviruses were considerably low (59.8 ~ 63.6%). As shown in Figure 1, *VP7* genes of group B rotaviruses were discriminated into three clusters, i.e., groups of human, bovine, and murine rotaviruses. Bangladeshi group B rotaviruses were classified into a cluster of human group B rotavirus, and are more closely related to CAL-1 than to ADRV.

Sequence identities of other genes of Bang373 and Bang544 to those of CAL-1, ADRV, and IDIR (murine rotavirus) are shown in Table III. Since sequence identities between Bang373 and Bang544 were 99.4 ~ 99.9% for all the genes analyzed, these viruses were considered to be virtually the same. As seen in the *VP7* gene, Bang373 and Bang544 showed higher sequence identities to CAL-1 and ADRV than to IDIR.

It is known that in group A rotavirus, the outer capsid protein VP4 is cleaved into VP8 and VP5 with the presence of protease, which facilitates infectivity of the rotavirus [Kapikian et al., 2001]. When the coding

TABLE I. Primers and Their Sequences Used for RT-PCR Amplification of Human Group B Rotavirus Genes

Viral gene	Primer name	Primer sequence(5′-3′)	Position[a]	Size of PCR product (bp)
VP2	GB2-1	(+) GGCAATTGTCGTGATGGATTC	1–21	702
	GB2-2	(–) GTACGATCTGCAATTATGTTG	702–682	
	GB2-3	(+) GGAAAAAGAAGTGAACCCTTTC	613–634	777
	GB2-4	(–) GTCGGCCGATCAACGGTCCA	1389–1370	
	GB2-5	(+) GACAATTTGTGTAATTTGGT	1331–1350	881
	GB2-6	(–) GCTCTGACAGCATCACGAAT	2211–2192	
	GB2-7	(+) GCTTAATAATGAGATAAGGC	2143–2162	687
	GB2-8	(–) GGGTTTTTAAAATATCATGT	2848–2829	
VP4	GB4-1	(+) GGCAATATATTTGCTATGTTGAC	1–23	580
	GB4-9	(–) TGGTGAGGAGATTTGTTTGTG	580–560	
	GB4-10	(+) CAATCTGATTTACACATTGA	217–236	1,113
	GB4-17	(–) ACAGCCTTGTCTGTAAAAGTC	1329–1309	
	GB4-24	(+) GTATATATGCGGGATGGCATG	811–831	912
	GB4-26	(–) CAAATTTGGCACGTCAACAG	1722–1703	
	GB4-16	(+) GCGGTTTTTACGGCTATAACC	1543–1563	588
	GB4-15	(–) GGTCGGTGTGTCAAATGAGT	2130–2111	
	GB4-20	(+) GCTACCAACTTTTCAGATCA	1867–1886	437
	GB4-4	(–) GGGTTTTTATATGTATTTGCAAC	2303–2281	
VP6	GB6-1	(+) GGTTTAAATAGCCCAACCGGTG	1–22	606
	GB6-3	(–) TACAACCCCTTTATTACCTG	606–587	
	GB6-4	(+) CATAGACCGAATATAGGTATG	529–549	741
	GB6-2	(–) GGGTTTTATTGCTTATTTTTTCG	1269–1247	
VP7	B5-2	(+) GGCAATAAAATGGCTTCATTGC	1–22	814
	B3-3	(–) GGGTTTTTACAGCTTCGGCT	814–795	
NSP1	BN1-1	(+) GGTATAATTAGATTGTCAGTATCC	1–24	655
	BN1-11	(–) TCCAGGTATCCATGTTGGA	655–637	
	BN1-10	(+) CAGGAGACTGAAAAGAACGA	237–256	1,019
	BN1-2	(–) GGGTTTTTATTTAGATATGTAG	1255–1276	
NSP2	GB8-1	(+) GGTAGAAATTAATCTATTCAGTG	1–23	1,006
	GB8-3	(–) GGGTTTTAAAAATAGCCGTTAAC	1006–984	
NSP3	BS7-1	(+) GGTATAATTACGTTTGATTCAG	1–22	637
	BS7-4	(–) GATTGGTTTTGTCACACTCGAG	637–616	
	BS7-3	(+) TTCCCATCAGAGGCAGTTGCT	436–456	743
	BS7-2	(–) GGGTTTTTATTACTTTGGTTGG	1178–1157	
NSP4	GB10-1	(+) GGCAATTAAAAGTCCAGTTATGG	1–23	751
	GB10-2	(–) GGGTCCTTATCAGTTTGATCA	(3′-End)	
NSP5	GB11-1	(+) GGTATATAAAAGTCAGTAGAC	1–21	631
	GB11-2	(–) GGGTTTTTTAAATATAACTC	631–612	

[a]Positions of primer sequences are expressed as nucleotide numbers in individual genes of adult diarrhea rotavirus (ADRV).

region of VP8 (636 bp) and VP5 (1,649 bp) of human group B rotaviruses are compared, the VP5-coding region showed slightly higher sequence identities than the VP8-coding region among Bang373, Bang544, CAL-1, ADRV, and IDIR. Similar findings have also been reported in group A rotavirus [Gorziglia et al., 1988; Sereno and Gorziglia, 1994], suggesting that the VP4 of group A and group B rotaviruses may be structurally and functionally similar. Deduced VP4 sequences of Bang373 and Bang544 consisted of 750 amino acids, as is reported for CAL-1 [Kobayashi et al., 2001], which is longer than ADRV VP4 by one amino acid [Mackow et al., 1993]. The additional amino acid in VP4 found in Bang373, Bang544, and CAL-1 resulted from additional nucleotides at nos. 401, 407, and 408, which are located in the VP8-coding region. Similarly, deduced VP2 sequences of Bang373 and CAL-1 consisted of 934 amino acids, which is one amino acid longer than ADRV VP2. The one extra amino acid was also due to additional nucleotides, at nos. 1971, 1974, and 1975, which were commonly found in Bang373 and CAL-1 (data not shown).

As observed previously in CAL-1, Bang373 and Bang544 possessed an *NSP3* gene which is one nucleo-

TABLE II. Identities (Percentage) of *VP7* Gene Sequences (Upper Right) and Deduced Amino Acid Sequences (Lower Left) Among Human (Bang373, Bang544, CAL-1, ADRV), Bovine (WD653, ATI, Mebus, Nemuro), and Murine (IDIR) Group B Rotaviruses

	Identity with strain[a]								
Strain	Bang373	Bang544	CAL-1	ADRV	WD653	ATI	Mebus	Nemuro	IDIR
Bang373		99.8	98.4	92.0	62.7	63.4	63.1	62.9	59.8
Bang544	99.6		98.2	92.3	63.1	63.6	63.3	63.1	60.3
CAL-1	98.4	98.0		92.1	63.6	63.8	63.2	63.2	60.1
ADRV	94.8	95.2	95.2		62.9	63.8	62.9	63.7	61.7
WD653	62.0	62.7	62.8	62.2		93.1	91.7	94.3	63.3
ATI	60.4	60.6	61.2	60.2	93.9		94.0	94.0	62.3
Mebus	59.2	59.4	60.0	59.0	92.3	95.1		92.8	61.8
Nemuro	62.0	62.2	62.8	61.8	96.4	97.6	96.1		63.7
IDIR	48.8	50.0	47.6	48.8	50.4	50.8	49.2	50.8	

[a]References of sequence (Genbank accession no.) used in this analysis: CAL-1, [Kobayashi et al., 2001] (AF184083); ADRV, [Chen et al., 1990] (M33872); WD653, [Chang et al., 1997] (U84141); ATI, [Chang et al., 1997] (U84472); Mebus, [Chang et al., 1997] (U84473); Nemuro, [Tsunemitsu et al., 1999] (AB016818); IDIR, [Sato et al., 1989] (D00911).

tide longer than *ADRV* due to the absence of one nucleotide (corresponding to no. 1104 of ADRV *NSP3* gene sequence) and two additional nucleotides (after nos. 999 and 1151 of ADRV *NSP3* gene sequence), which are located around the 3′-end portion [Kobayashi et al., 2001]. An additional nucleotide in the *NSP3* gene (at nucleotide no. 1000) of Bang373, Bang544, and CAL-1 was considered to cause different coding frames from ADRV *NSP3* gene, generating a 23 amino acids-longer product than the ADRV NSP3. Although the whole NSP3 amino acid sequence of ADRV showed relatively low identities (82.4–82.7%) to those of Bang373, Bang544, and CAL-1, higher identities (89.5–89.8%) were observed in a partial sequence (amino acid nos. 1∼315) corresponding to a region without the frameshift. Similarly, critical genetic divergence was also found near the 3′-end portion in *NSP2* genes. Compared with the ADRV *NSP2* gene, an additional nucleotide at no. 782 was commonly found in *NSP2* genes of Bang373, Bang544, and CAL-1. Due to a frameshift caused by the additional nucleotide, the NSP2 of these viruses is considered to be 22 amino acids longer than the ADRV NSP2. Identity of partial NSP2 sequences (amino acid nos. 1–242) corresponding to the nucleotide sequence without the frameshift was 95.9–100% among the group B human rotaviruses.

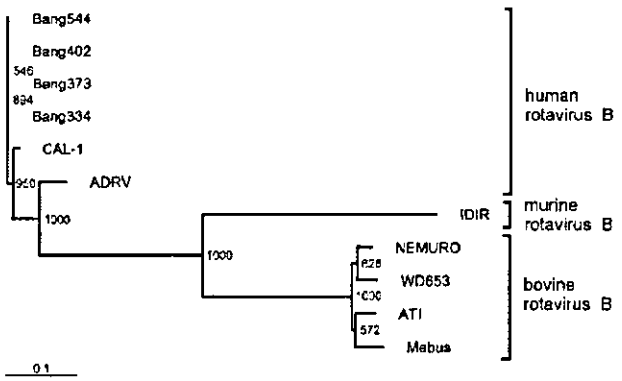

Fig. 1. Phylogenetic tree for the *VP7* genes of group B rotaviruses. The tree was constructed by the neighbor-joining method using ClustralW program. Scale bar represents a genetic distance that is equivalent to 0.1 substitutions per site. Bootstrap probabilities of 1,000 trials are indicated at diverging points of branches.

Nucleotide substitutions among Bang373, Bang544, and CAL-1 were characterized in terms of synonymous or missense substitutions, and transition (A:T ↔ G:C) or transversion (A:T ↔ C:G, A:C ↔ T:G) (Table IV). Throughout the genes analyzed, rates of synonymous substitutions and transitions ranged generally between 60∼90% and 70∼90%, respectively, and extremely low rates were not found in any RNA segments. Among the synonymous substitutions, transitions accounted for 83∼100%. These findings suggested that the three viruses might have evolved neutrally at a similar mutation rate to each other or been derived from a common ancestral strain, without an event of reassortment with a different virus strain.

In most gene segments, transition rates in synonymous substitutions were higher than in missense substitutions, although at the gene level mutations are considered to occur neutrally in a similar mode irrespective of the type of amino acid codon. This finding suggests that some missense substitutions due to transition might not be maintained because of functional defects of the protein product or exclusion of replicated viruses by immune response in the host. It was of note that between Bang373 and ADRV, higher synonymous substitution rates as well as synonymous/missense ratios were observed for the *VP2*, *VP6*, and *NSP2* genes than for other gene segments. Furthermore, in these genes, transition rates in missense substitutions were considerably lower than those in synonymous substitutions. Considering that VP2, VP6, and a portion of NSP2 (amino acid nos. 1–242) are relatively more conserved than other proteins, functions of these proteins are suggested to more stringently depend on their amino acid sequences than those of other viral proteins of group B rotaviruses.

The present study indicated that group B rotaviruses detected in Bangladesh (Bang373 and Bang544) and

TABLE III. Sequence Identities of Rotavirus Gene Segments Among Group B Rotaviruses Bang373, Bang544, CAL-1, ADRV, and IDIR

	Nucleotide (amino acid) sequence identity (%) between group B rotavirus strains									
	Bang373 with strain				Bang544 with strain			CAL-1 with strain		
Gene segment	Bang544	CAL-1[b]	ADRV[c]	IDIR[d]	CAL-1	ADRV	IDIR	ADRV	IDIR	ADRV with IDIR
VP2 gene	—[a]	98.5	91.5	73.6	—	—	—	91.8	73.5	73.4
		(98.9)	(97.5)	(85.9)				(97.5)	(85.7)	(85.8)
VP4 gene	99.4	99.0	91.5	63.2	98.9	91.3	63.1	92.0	63.0	63.7
	(99.1)	(99.2)	(94.1)	(57.9)	(98.8)	(93.7)	(57.7)	(94.6)	(57.7)	(57.2)
VP8-coding region	98.9	98.6	89.5	53.1	97.8	90.0	52.9	89.9	53.9	52.6
	(98.1)	(98.1)	(92.2)	(36.4)	(96.1)	(90.3)	(37.2)	(92.2)	(35.5)	(34.6)
VP5-coding region	99.6	99.3	92.8	66.3	99.3	92.3	66.4	92.8	66.0	66.8
	(99.4)	(99.6)	(95.1)	(66.5)	(99.8)	(95.3)	(66.5)	(95.5)	(66.7)	(66.1)
VP6 gene	99.9	98.9	93.7	73.5	99.0	93.6	73.3	94.3	73.5	72.2
	(99.7)	(99.0)	(96.7)	(84.4)	(99.2)	(96.9)	(84.1)	(97.5)	(84.1)	(83.4)
VP7 gene	99.8	98.4	92.0	59.8	98.2	92.3	60.3	92.1	59.8	61.5
	(99.6)	(98.4)	(94.8)	(48.8)	(98.0)	(95.2)	(50.0)	(95.2)	(47.6)	(48.8)
NSP1 gene	99.4	98.8	92.6	65.7	98.8	92.6	65.3	92.8	65.8	66.8
Peptide 1	(98.1)	(98.1)	(97.2)	(63.5)	(98.1)	(97.1)	(61.7)	(99.1)	(63.5)	(62.6)
Peptide 2	(99.1)	(98.4)	(91.9)	(62.0)	(98.8)	(92.8)	(62.0)	(92.8)	(62.3)	(63.2)
NSP2 gene	98.9	99.1	93.9	79.3	99.4	93.5	79.4	93.6	79.4	78.0
	(99.7)	(99.6)	(79.4)	(88.0)	(99.7)	(79.7)	(88.4)	(79.7)	(88.0)	(72.4)
NSP3 gene	99.6	98.8	90.8	59.0	98.8	89.9	63.6	90.9	59.5	60.4
	(99.4)	(98.3)	(82.7)	(50.3)	(98.3)	(82.4)	(50.3)	(82.4)	(50.0)	(46.5)
NSP4 gene	99.9	98.3	—	67.0	98.4	—	67.5	—	67.4	—
	(99.5)	(98.2)		(63.0)	(98.6)		(63.4)		(62.6)	
NSP5 gene	99.8	97.9	91.4	72.1	97.7	91.3	72.0	91.6	72.1	71.4
	(100)	(97.6)	(92.9)	(67.8)	(97.6)	(92.9)	(67.8)	(90.6)	(68.4)	(67.8)

[a] *VP2* gene of Bang544 and of *NSP4* gene of ADRV were not sequenced or reported. Sequences of both terminal regions of ADRV *NSP4* gene have been published [Mackow, 1995].

[b–d] GenBank accession nos. of genes from CAL-1, ADRV, and IDIR which have been published previously and used in this analysis are as follows; CAL-1: AF184084 (*VP4* gene), AB037931 (*VP6* gene), AF184083 (*VP7* gene), AF230975 (*NSP1* gene), AF230974 (*NSP3* gene); ADRV: M91433 (*VP2* gene), M91434 (*VP4* gene), M55982 (*VP6* gene), M33872 (*VP7* gene), M91435 (*NSP1* gene), M91437 (*NSP2* gene), M91436 (*NSP3* gene), M34380 (*NSP5* gene); IDIR: U00673 (*VP2* gene), X16949 (*VP4* gene), M84456 (*VP6* gene), D00911 (*VP7* gene), U01164 (*NSP1* gene), U03558 (*NSP2* gene), L09722 (*NSP3* gene), U03557 (*NSP4* gene), D00912 (*NSP5* gene).

India (CAL-1) are genetically closely related, and may be regarded as virtually identical viruses. Considering that these viruses were detected in geographically proximal sites recently, it might be possible that group B rotaviruses like Bang373 or CAL-1 existed endemically in eastern India and Bangladesh, i.e., the area around the Bay of Bengal. Based on the genetic distances and remarkable characteristics in some gene segments as described above, it seems likely that these Bengali strains may be distinct from ADRV in China, although these human group B rotaviruses might have diverged from a common ancestral virus. Since genetic analysis of Chinese group B rotavirus has been done only for ADRV, which was detected in 1982, the mutation rate of this virus is still not clear. Accordingly, it will be of significance to analyze gene sequences of group B rotaviruses detected in China recently, to calculate mutation rates of this virus and also to estimate the time when divergence of ADRV and Bengali strains occurred. However, alternatively, mutation rates of group B rotavirus may be inferred by analogy to mutation frequencies reported in group A rotavirus.

Through in vitro experiments using group A porcine rotavirus, the mutation rate of RNA segment 11 (NSP5 gene) was calculated as having a maximum value of 5×10^{-5} per replicated base [Blackhall et al., 1996]. Furthermore, the substitution of eight nucleotides in the *VP4* gene (2,356 bases) was observed through 33 times-multiple passage of group A human rotavirus [Kitamoto et al., 1993], which may indicate the mutation rate of the *VP4* gene as 1×10^{-4} per replicated base. On the other hand, in an epidemiologic study of serotype G4 human rotaviruses, 0–2 amino acid changes together with 0–10 nucleotide changes were observed in the VP4 hypervariable region encoding VP8 (amino acid nos. 71–203 encoded by a sequence of 399 nucleotides in length) during a period of 16 years [Palombo et al., 1993]. Assuming that mutations in VP4 occurred at a rate of 5×10^{-5} or 1×10^{-4} per base and 10 nucleotide changes occurred in a 399 bases-portion of the hypervariable region for 16 years, it is suggested that rotavirus replication might have occurred up to about 32 or 16 times per year, respectively. Based on the assumption that nucleotide change occurs at either of the above rates, a 1% nucleotide substitution is suggested to occur every 6–7 years in a variable region of VP4 (VP8-coding region). Since the prevalence of group B rotavirus is considerably lower than group A rotavirus, replication frequency is also suggested to be lower. Thus, an 8.5% difference in the *VP4* gene (10.5% difference in VP8-coding region) between Bang373 and ADRV is suggested to have been generated during the last several decades at least.

Another matter of concern regarding Bengali group B rotaviruses is the width of their present distribution in

TABLE IV. Characterization of Nucleotide Substitutions Between Bang373 and ADRV or Bang373 and CAL-1

Gene segment	Sequence compared (length, bp)	Group B rotavirus compared with Bang373	Total no. of nucleotides substituted	No. (%) of synonymous substitution	Ratio of synonymous/ missense substitution	No. (%) of transition	Rate (%) of transition in	
							Missense substitutions	Synonymous substitutions
2 (*VP2* gene)	Whole sequence (2,847)	ADRV	244	220 (90.2)	9.6	210 (86.1)	66.7	88.2
		CAL-1	43	30 (69.8)	2.3	33 (76.7)	46.2	90
4 (*VP4* gene)	Whole sequence (2,306)	ADRV	192	140 (72.9)	2.7	163 (84.9)	59.6	94.3
		CAL-1	22	16 (72.7)	2.7	20 (90.9)	66.7	100
	VP8-coding region (636)	ADRV	62	45 (72.6)	2.6	53 (85.5)	70.6	91.1
		CAL-1	9	5 (55.6)	1.3	8 (88.9)	75	100
	VP5-coding region (1,649)	ADRV	127	93 (73.2)	2.7	108 (85.0)	55.9	95.7
		CAL-1	12	10 (83.3)	5	11 (91.7)	50	100
5 (*VP6* gene)	Whole sequence (1,269)	ADRV	79	63 (79.7)	3.9	66 (83.5)	56.3	90.5
		CAL-1	14	10 (71.4)	2.5	12 (85.7)	75	90
9 (*VP7* gene)	Whole sequence (814)	ADRV	65	48 (73.8)	2.8	58 (89.2)	82.4	91.7
		CAL-1	13	10 (76.9)	3.3	12 (92.3)	100	90
6 (*NSP1* gene)	5′-UTR + orf1 (363)	ADRV	23	19 (82.6)	4.8	20 (87)	50	94.7
		CAL-1	4	2 (50)	1.0	2 (50)	0	100
	3′-UTR + orf2 (1,020)	ADRV	76	43 (56.6)	1.3	63 (82.9)	72.7	90.7
		CAL-1	13	7 (53.8)	1.2	9 (69.2)	50	85.7
7 (*NSP3* gene)	Whole sequence (1,180)	ADRV	106	66 (62.3)	1.7	94 (88.7)	82.5	92.4
		CAL-1	13	7 (53.8)	1.2	11 (84.6)	66.7	100
8 (*NSP2* gene)	Whole sequence (1,007)[a]	ADRV	60	48 (80)	4.0	47 (78.3)	58.3	83.3
		CAL-1	9	7 (77.8)	3.5	7 (77.8)	50	85.7
10 (*NSP4* gene)	Whole sequence (751)	CAL-1	13	9 (69.2)	2.3	10 (76.9)	50	88.9
11 (*NSP5* gene)	Whole sequence (631)	ADRV	54	38 (70.4)	2.4	48 (88.9)	93.8	86.8
		CAL-1	13	9 (69.2)	2.3	9 (69.2)	50	77.8

[a]For characterization of synonymous or missense substitution, a portion of sequence (nucleotide number 1–781) was analyzed.

southern Asia. Group B rotavirus infection is important in view of public health policy because this virus causes severe diarrhea in adults but may have been underestimated as a cause of diarrhea because rotavirus is regarded as a pathogen in children. Further epidemiologic study of group B rotavirus in and around the Bengal region, as well as in China, will contribute to the control of group B rotavirus infection.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequences of Bang373 and CAL-1 genes analyzed in this study have been deposited in GenBank under the following accession nos. Bang373 *VP2* gene, AY238390; Bang373 *VP4* gene, AY238388; Bang373 *VP6* gene, AY238389; Bang373 *VP7* gene, AY238385; Bang373 *NSP1* gene, AY238391; Bang373 *NSP2* gene, AY238393; Bang373 *NSP3* gene, AY238392; Bang373 *NSP4* gene, AY238384; Bang373 *NSP5* gene, AY238394; *CAL-1 VP2* gene, AB037932; CAL-1 *NSP2* gene, AY238383; CAL-1 *NSP4* gene, AY238387; CAL-1 *NSP5* gene, AY238386.

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Isolation and Characterisation of Poliovirus Mutants Resistant to Heating at 50°C for 30 min

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Poliovirus is heat-labile; on heating at 50°C for 30 min its infectivity decreases drastically and its antigenicity reverts from N to H. However, mutants resistant to heating at 50°C for 30 min from the Sabin 1 and 2 viruses were isolated by repeating the process of incubation of the virus stock at 50°C for 30 min and multiplication of the remaining virus in a cell culture. The isolated mutants were stable genetically, and maintained the rct and d markers of the parent virus. On electron microscopical examination, the mutants were observed to retain the intact morphology after being heated at 50°C for 30 min, while the parent virus was converted to empty particles devoid of RNA under the same conditions. On determination of the nucleotide sequence of the P1 region, a single nucleotide sequence substitution was detected at nucleotide no. 2741, resulting in an amino acid change from valine to alanine at the 87th position of VP1. This amino acid might be associated with the heat-resistance of the mutants. Furthermore, it was found that the thermostable mutants obtained in this study, which are resistant to "high" temperature (50°C) for a short time (30 min), were not stable against heating at the ambient temperature (37°C) for a long time (5 or 7 days). This suggests that the inactivation at high temperature for a short time and that at ambient temperature for a long time involve different mechanisms. ***J. Med. Virol. 74:484–491, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: poliovirus; mutant; vaccine; thermostability; Sabin strain

INTRODUCTION

Poliovirus, the causative agent of poliomyelitis, is the prototype of genus *Enterovirus* of family *Picornaviridae*. It is a small, nonenveloped, icosahedral virus. Each particle consists of 60 copies each of capsid proteins VP1, VP2, VP3, and VP4. Three proteins, VP1 to VP3, form the outer surface, while VP4 is a capsid-internal protein. The genome of poliovirus comprises a single-stranded RNA (ssRNA) of plus-strand polarity encoding a single polypeptide, the polyprotein of which is processed into structural and functional viral proteins by virus-encoded proteinases [Rueckert, 1996]. Poliovirus has three serotypes (PV1, PV2, and PV3). The serotype specificity is defined by the N antigen associated with infectious complete particles. The N antigen is converted to the H antigen on exposure to heat (50°C for 30 min), UV radiation, or high pH [Mayer et al., 1957; Breindl, 1971].

After the successful eradication of smallpox in the late 1970s, poliomyelitis caused by poliovirus is the next target disease for eradication, and extensive vaccination against poliomyelitis has been carried out worldwide, especially in developing countries [Melnick, 1992]. For this eradication programme, the use of potent and safe vaccines is of utmost importance. Live oral poliovirus vaccine (OPV) comprising the three serotypes of attenuated Sabin strains has been employed successfully throughout the developed world, but less so in some tropical developing countries. Since poliovirus is one of the most thermolabile viruses, the maintenance of a cold chain is very important for the delivery of potent OPV in the field. The implementation of a cold chain from the manufacturer to the final vaccination sites is still defective in several developing countries, and the vaccine is exposed frequently to ambient temperature. With this

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background, the efficacy of regular OPV should be improved by increasing the stability of the vaccine.

In this study, poliovirus mutants resistant to heating at a high temperature (50°C) for a short time (30 min) were obtained and characterised, and the substitution of amino acid residue on VP1, one of the viral proteins, which might be associated with the thermal resistance, was detected first. The mutants were, however, not stable against heating at ambient temperature (37°C) for a long time (5 or 7 days). Thermal inactivation on short-term heating at high temperature, and long-term heating at low temperature appear to occur through different mechanisms.

MATERIALS AND METHODS

Viruses and Cells

The Sabin strains of poliovirus types 1, 2, and 3 (Sabin 1, Sabin 2, and Sabin 3) were provided by the Japan Polio Institute. Primary Green monkey kidney (primary GMK) cells were used for selection of heat-resistant virus clones and their multiplication. Primary Cynomolgous monkey kidney (primary CMK) cells were employed for the multiplication test at high temperature (39.5°C). For measurement of virus infectivity, an established GMK2 cell line was used. These three kinds of cells, all of which were provided by the Japan Poliomyelitis Research Institute, were cultivated in Eagle's minimum essential medium (Eagle's MEM) supplemented with 10% bovine foetal calf serum (Flow Lab, North Ryde, Australia; mycoplasma-free and bovine origin agent-free). The Sabin strains were routinely grown at 35.5°C.

Isolation of Poliovirus Clones Resistant to Heating at 50°C for 30 min

Sabin 1, Sabin 2, and Sabin 3 virus suspensions were heated at 50°C for 30 min by completely immersing test tubes containing the viruses in a water bath. The surviving viruses were grown at 35.5°C in primary GMK cells. The obtained virus preparations were designated as $S1t_1$ for Sabin 1, $S2t_1$ for Sabin 2, and $S3t_1$ for Sabin 3, respectively. After successive heating and multiplication treatment had been repeated three times, each virus preparation was subjected to the fourth heating, and virus clones were obtained from the treated viruses ($S1t_4$, $S2t_4$, and $S3t_4$) by means of plaque purification using primary GMK cells.

Titration of Virus Infectivity

Virus infectivity was determined as described previously [Urasawa et al., 1976] by plaque assay using 6-well plates with GMK2 monolayer cells. Virus titers were expressed as plaque forming units (PFU)/0.1 ml.

Multiplicity Test at 39.5°C (rct Marker Tests)

Virus multiplication at 39.5°C was examined using primary GMK cells. Capped glass tubes each containing a virus-infected cell monolayer were immersed completely in a water bath maintained at 39.5 ± 0.1°C for 7 days, the cytopathic effect (CPE) of the infected cells being observed on days 5 and 7. The virus infectivity on day 7 was expressed as 50% tissue culture infective dose ($TCID_{50}$)/0.1 ml. The rct marker was defined as the difference between the $\log_{10}$ virus titers after incubation for 7 days at 37 and 39.5°C.

Lyophilisation Test

Virus preparations before and after heat treatment were suspended in prelyophilisation buffer (2.5% sorbitol, 0.06% glutamine, 0.06% Mg_2SO_4, and 0.01% bovine serum albumin), and then lyophilised under the conditions of −45°C for 3 days, first lyophilisation at −5°C for 2 days, second lyophilisation, as described previously [Shiomi et al., 2003]. Each lyophilised virus was solubilised in DDW or 10% bactocasitone just before virus infectivity determination.

Electron Microscopy

Purified viruses were prepared by differential centrifugation, fluorocarbon treatment, and banding by CsCl density gradient centrifugation. Negative staining was carried out in a routine manner as described previously [Taniguchi et al., 1983]. A drop of a virus preparation was placed on a 400-mesh carbon-collodion-coated grid, excess fluid being withdrawn with the edge of a filter paper disk. The grid was placed in a Hitachi H500 electron microscope and then examined. The stability of the particles was assessed by counting the intact, staining solution-penetrated, and empty particles in micrographs taken at an instrumental magnification of 30,000.

Nucleotide Sequence Determination

Poliovirus RNA was extracted from the purified virions with phenol–chloroform and then precipitated with ethanol. The entire nucleotide sequences of the P1 regions of the parent Sabin 2 and its heat-resistant mutants were determined by the dideoxynucleotide method using the purified virus RNA. For Sabin 1 mutants, the region around the sequences encompassing the point mutation found in Sabin 2 mutants was amplified by reverse transcription-polymerase chain reaction (RT-PCR), and then the nucleotide sequences were determined.

RESULTS

Preparation of Sabin Poliovirus Clones Resistant to Heating at 50°C

When Sabin viruses were incubated at 50°C for 30 min, the infectivity of the Sabin 1, 2, and 3 viruses decreased to almost $1/10^5$, $1/10^3$, and $1/10^6$, respectively (Table I). In contrast, surviving viruses were obtained after the heating; 8.0×10 PFU/0.1 ml, 4.6×10^3 PFU/0.1 ml, and 2.0×10 PFU/0.1 ml for Sabin 1, 2, and 3, respectively. After three cycles of heating-replication and the following fourth heating, the Sabin 1, 2, and 3

TABLE I. Virus Infectivity of Sabin 1, 2, and 3 Virus Preparations After Heating at 50°C for 30 min and Subsequent Multiplication in Primary GMK Cells

Virus type	Heating and multiplication (1st)			Heating and multiplication (2nd)			Heating and multiplication (3rd)			Heating (4th)	
	Heating			Heating			Heating			Heating	
	Before	After	Multiplication	Before	After	Multiplication	Before	After	Multiplication	Before	After
Sabin 1	6.0×10^6 (S1)	8.0×10 ($S1t_1$)	2.8×10^7 ($S1t_1C$)	2.8×10^7 ($S1t_1C$)	8.1×10^6 ($S1t_2$)	2.0×10^7 ($S1t_2C$)	2.0×10^7 ($S1t_2C$)	5.5×10^6 ($S1t_3$)	1.9×10^7 ($S1t_3C$)	1.9×10^7 ($S1t_3C$)	4.3×10^7 ($S1t_4$)
Sabin 2	5.0×10^8 (S2)	4.6×10^3 ($S2t_1$)	5.0×10^7 ($S2t_1C$)	5.0×10^7 ($S2t_1C$)	4.8×10^5 ($S2t_2$)	2.3×10^7 ($S2t_2C$)	2.3×10^7 ($S2t_2C$)	5.0×10^8 ($S2t_3$)	2.4×10^7 ($S2t_3C$)	2.4×10^7 ($S2t_3C$)	1.6×10^7 ($S2t_4$)
Sabin 3	1.5×10^7 (S3)	2.0×10 ($S3t_1$)	2.4×10^6 ($S3t_1C$)	2.4×10^6 ($S3t_1C$)	1.5×10^6 ($S3t_2$)	1.9×10^7 ($S3t_2C$)	1.9×10^7 ($S3t_2C$)	1.5×10^8 ($S3t_3$)	2.3×10^7 ($S3t_3C$)	2.3×10^7 ($S3t_3C$)	1.9×10^7 ($S3t_4$)

Virus infectivity is expressed as PFU/0.1 ml.

virus preparations (S1t4, S2t4, and S3 t_4) showed little decreases in infectivity (Table I).

Virus samples ($S1t_4$, $S2t_4$, and $S3t_4$) were subjected to plaque purification. Sixty, 60, and 142 plaques (designated as $S1t_4Cl$ 1–60, $S2t_4Cl$ 1–60, and $S3t_4Cl$ 1–142) from the $S1t_4$, $S2t_4$, and $S3t_4$ viruses were picked up, respectively, and then grown in primary GMK cells. The thermostability of the clones against heating at 50°C for 30 min was examined. The heat stability of each clone was placed in one of four classes (rated as A–D) according to the difference in infectivity ratio before and after heating at 50°C for 30 min (Table II): class A (ratio of infectivity after heating to that before heating, >2/3), class B (2/3 to 1/3), class C (1/3 to 1/10), and class D (<1/10).

Most of the 60 clones ($S1t_4Cls$) from Sabin 1 were of class B or C. Twenty-four and 21 of the 60 clones of $S2t_4Cls$ were grouped into classes A and B, respectively. In contrast, 7, 6, and 21 clones from $S3t_4Cls$ were of classes A, B, and C, respectively, the remaining 108 clones exhibiting low heat stability (class D).

Multiplicity at 39.5°C of Heat-Resistant Virus Clones

The clones which showed adequate stability (class A or B) on heating at 50°C for 30 min were subjected to the reproductive capacity (rct marker) test which examines the multiplicity at 39.5°C in primary CMK. Most of the clones (17 of 25 $S1t_4Cls$, 43 of 45 $S2t_4Cls$, and 13 of 14 $S3t_4Cls$) exhibited almost the same multiplicity to that of the original Sabin 1, 2, and 3 strains (Table III). Thus, the heat-resistant virus clones obtained were found to have a similar rct marker to that of the original Sabin strains. Furthermore, the efficiency of plating at low bicarbonate concentrations (d marker) of the heat-resistant mutants was examined. The resistant mutants showed the same d marker as the heat-sensitive parent virus from which they were selected (Table IV).

Genetic Stability of Heat-Resistant Virus Clones

In order to determine the genetic stability of the heat-resistant clones, ten subclones from each of the selected five $S1t_4Cl$, two $S2t_4Cl$, and one $S3t_4Cl$ clones were obtained by plaque formation, and their heat stability against heating at 50°C for 30 min was examined. As shown in Table V, the $S2t_4Cl$ subclones showed the same heat stability (class A) as the $S2t_4Cl$ clones. In contrast, most $S1t_4Cl$ subclones exhibited lower heat stability (class C or D) than the $S1t_4Cl$ clones, and all the $S3t_4Cl$ subclones almost completely lost the heat stability (class D). Thus, the heat-resistant mutants from Sabin 2 were most stable genetically, and thus they were again cloned and used for further experiments.

Preparation of more genetically stable S1 heat-resistant mutants were tried by repeated subcloning. Three $S1t_4Cl$ clones (21C, 26C, and 33C) exhibiting the best heat-resistance (rated as class B) were subjected to subcloning in primary GMK cells. Of the 25 subclones

TABLE II. Thermostability Against Heating at 50°C for 30 min of Heat-Resistant Sabin Virus Clones Obtained After Heating and Multiplication

Virus clones	Number of virus clones showing the following ratio of virus titer after heating to that before heating					
	A: >2/3	B: 2/3–1/3	C: 1/3–1/10	D: <1/10	ND	Total
$S1t_4Cl$-1C–60C	1	27	30	2	0	60
$S2t_4Cl$-1C–60C	24	21	7	5	3	60
$S3t_4Cl$-1C–142C	7	6	21	104	4	142

ND, not determined.

after the 2nd cloning, 5 and 16 were placed in classes A and B, respectively (data not shown). However, even when the cloning was repeated further, the heat stability of the clones could not be increased further.

Electron Microscopical Examination of Heat-Resistant Mutants

Purified particles from heat-resistant mutant $S2t_4Cl$-17 and the parental Sabin 2 were suspended in 10 mM phosphate buffer (pH7.5), and then heated at 50°C for 30 min. The heated samples were examined for morphological changes by electron microscopy. As shown in Figure 1, the morphology of the heat-resistant mutant did not change apparently in comparison to that of the parental Sabin 2 virus particles, most of which were partially penetrated by phosphotungstic acid or were empty, being devoid of RNA from the virion. The frequency of the change in the appearance of the particles after heating was examined by counting the empty particles or penetrated particles for the mutant and original virus preparations. While 98.3% of the particles were empty or partially penetrated by the staining solution for the parent Sabin 2 virus, only 13.4% of the particles of heat-resistant $S2t_4Cl$-17 were found to be empty or partially penetrated, as judged from the penetration of the staining solution into the particles (Table VI).

Identification of the Amino Acid Responsible for the Heat Stability

The genetic factor responsible for the thermal stability at 50°C for 30 min is conceivable to be limited to a gene coding for structural protein, because growth in cells is not involved in this phenotype. In order to identify the genetic locus for the thermal stability, the complete nucleotide sequence of the P1 region was determined by direct sequencing using purified virus genome RNA. The nucleotide sequence of the entire P1 coding region of heat-resistant mutant $S2t_4Cl$-17 was compared with that of the parent Sabin 2. Only one point mutation (from U to C) was detected at nucleotide no. 2741, resulting in an amino acid change from valine to alanine, which corresponds to the 87th amino acid of VP1. Also, for the other four clones, $S2t_4Cl$-1, $S2t_4Cl$-2, $S2t_4Cl$-7, and $S2t_4Cl$-41, the same nucleotide substitution at the same position was detected on direct sequencing of the PCR products emcompassing the VP1 encoding sequence.

In Sabin 1 mutants $S1t_4Cl$-21-5-6 and $S1t_4Cl$-26-8-4, the nucleotide sequence between positions 2541 and 2931 was determined, and the same nucleotide substitution corresponding to the same position, nucleotide no. 2739, from U to C was detected in $S1t_4Cl$-21-5-6, but no nucleotide change was detected in $S1t_4Cl$-26-8-4.

Heat Stability at Low Temperature for a Long Time of the Mutants Resistant to Heating at High Temperature for a Short Time

By using heat-resistant clones $S2t_4Cl$-1, $S2t_4Cl$-2, $S2t_4Cl$-7, and $S2t_4Cl$-41, the stability at low temperature (37°C) for a long time (5 days) was examined. As shown in Table VII, the infectivities of the clones resistant to heating at 50°C decreased by 1.08 to 2.10 $\log_{10}$, which is comparable to the decrease found for the parent

TABLE III. Reproductive Capacity Test (rct Marker) of Virus Clones Resistant to Heating at 50°C Prepared From Sabin Type 2 Virus

Virus subclone	Infectivity titer ($\log_{10}$ PFU/ml) in the cultivation at the indicated temperature (°C)			
	36.0	40.0	39.5	39.0
$S2t_4Cl$-1C	7.92	0.39	1.20	2.78
$S2t_4Cl$-2C	7.72	0.39	0.39	0.39
$S2t_4Cl$-7C	7.68	0.39	0.39	1.70
$S2t_4Cl$-17C	7.54	0.39	0.39	1.40
$S2t_4Cl$-41C	7.65	0.39	0.39	1.40
Sabin type 2	7.98	0.39	0.09	1.70
MEF-1	8.94	7.24	NT	NT

NT, not tested.

TABLE IV. d-Marker Test of Virus Clones Resistant to Heating at 50°C Prepared From Sabin Type 2

Subclone virus	Infectivity titer ($\log_{10}$) at different bicarbonate concentration		Difference of infectivity
	0.255%	0.05%	
$S2t_4Cl$-1C	7.92	0.39	7.53
$S2t_4Cl$-2C	7.72	3.80	3.92
$S2t_4Cl$-7C	7.68	2.40	5.28
$S2t_4Cl$-27C	7.54	3.70	4.05
$S2t_4Cl$-41C	7.65	4.63	3.02
Sabin type 2	7.98	3.30	4.62
MEF-1	8.94	7.92	1.02

TABLE V. Stability of Heat-Resistant Mutants

Virus clone	Rating class of stability	Subcloned virus	Number of the subcloned virus with the indicated rating class of stability				
			A[a]	B	C	D	ND
S1t$_4$Cl-21C	B	S1t$_4$Cl-21C-1–10	0	0	2	7	1
S1t$_4$Cl-26C	B	S1t$_4$Cl-26C-1–10	0	0	3	7	0
S1t$_4$Cl-33C	B	S1t$_4$Cl-33C-1–10	1	0	4	5	0
S1t$_4$Cl-47C	B	S1t$_4$Cl-47C-1–10	0	1	4	4	0
S1t$_4$Cl-59C	B	S1t$_4$Cl-59C-1–10	0	0	3	7	0
S2t$_4$Cl-1C	A	S2t$_4$Cl-1C-1, 5, 7	3	0	0	0	0
S2t$_4$Cl-2C	A	S2t$_4$Cl-2C-9	1	0	0	0	0
S3t$_4$Cl-59C	A	S3t$_4$Cl-59C-1–4	0	0	0	4	0

ND, not determined.
[a]Ratios of the infectivity after heating to that before heating in A–D are >2/3, 2/3 to 1/3, 1/3 to 1/10, and <1/10, respectively.

Sabin 2 (1.77 $\log_{10}$). Similar results were obtained in the stability experiment for 7 days (data not shown). These results showed that the heat stabilities at high temperature and low temperature are quite different.

Lyophilisation Efficiency of the Heat-Resistant Mutants

The relationship between heat resistance (50°C for 30 min) and lyophilisation efficiency (heat stability of a lyophilised sample) was also examined. After S2t4Cl-17C had been lyophilised, a sample was incubated at 37°C for 7, 14, and 21 days or at 45°C for 7 days. The virus infectivity of the treated samples of the mutants was decreased remarkably, similar to that of the original Sabin 2 (data not shown).

Trial to Obtain Heat-Resistant Mutants at 38.5°C for a Long Time

By repeating the incubation at 38.5°C for 7 days and selection of resistant mutants in a cell culture, it was tried to obtain mutants resistant to heating at ambient temperature (37°C) for a long time (7 days). Sabin 1 showed, to some extent, a tendency for increased heat resistance on early treatment. However, further resis-

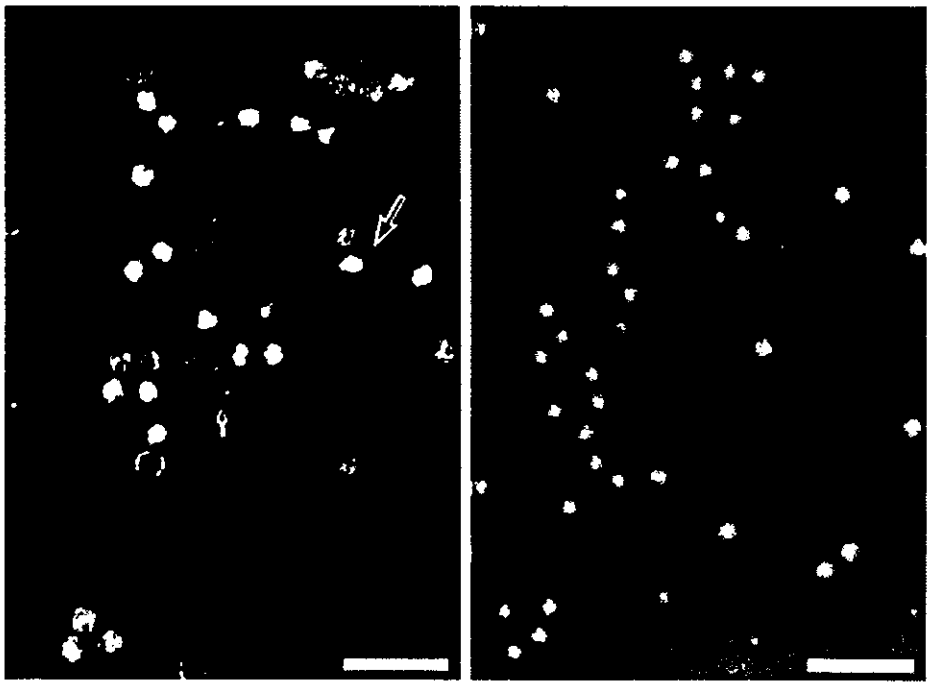

Fig. 1. Electron micrographs of the heat-resistant Sabin 2 mutant (**right plate**) and the parental Sabin 2 virus (**left plate**) after heating at 50°C for 30 min. Small and large arrows in the left plate indicate an empty particle and a penetrated particle, respectively. Scale bar, 100 nm.

TABLE VI. Morphologic Appearance of the Particles After Heating at 50°C for 30 min as Examined by Electron Microscopy

Virus strain	Number of particles (%)			
	Intact	Partially penetrated	Empty	Total
$S2t_4Cl$-17	482 (86.5)	65 (11.7)	10 (1.8)	557
Sabin 2	9 (1.6)	151 (27.8)	384 (70.6)	544

tance was not obtained (data not shown). This was also the case for Sabin 2 and 3 viruses. Thus, no mutants resistant to heating at 38.5°C for 7 days could be obtained with the same methods as those used for the preparation of heat-resistant mutants at 50°C for 30 min.

DISCUSSION

The strategies for the eradication of poliomyelitis are based on extensive immunisation with OPV and surveillance for every possible case of poliomyelitis [Hull et al., 1997]. They have been successful in many countries, and have resulted in the recent elimination of endogenously transmitted poliovirus in the Western hemisphere. In mass poliovirus immunisation programmes, particularly in developing tropical countries where it is difficult to maintain or to transport a vaccine under frozen conditions, a stabilised vaccine offers numerous advantages. For the accomplishment of the programme to eradicate poliovirus, the preparation of a thermostable poliovirus vaccine would be very useful. In 1993, the Expanded Programme on Immunisation of the World Health Organisation set an enhanced-thermostability target for OPV, i.e., a vaccine that can withstand 37°C for 7 days, compared to 2 days with the current vaccine [Milstien et al., 1997].

There are several ways to improve the thermal stability of the OPV. The first one is the addition of a capsid-stabilising compound to the vaccine preparation. At present, the Sabin vaccine is stabilised with 1 M $MgCl_2$, so it can be kept for over 1 year at 4°C and for a month at room temperature without losing any potency

TABLE VII. Thermostability at 37°C of Virus Clones Resistant to Heating at 50°C Isolated From Sabin type 2

Virus subclone	Incubation for 5 days		Rate of inactivation
	At −20°C	At 37°C	
$S2t_4Cl$-1C	7.67[a]	5.95	1.72
$S2t_4Cl$-2C	7.44	6.36	1.08
$S2t_4Cl$-7C	7.72	6.35	1.37
$S2t_4Cl$-27C	7.90	6.14	1.76
$S2t_4Cl$-41C	7.90	5.80	2.10
Sabin type 2	8.09	6.32	1.77

[a]Infectivity titer (log_{10} PFU/ml).

[Melnick and Wallis, 1963]. Its mechanism of action, although not well defined, might involve the nonspecific binding of Mg^{2+} to charged residues in the viral proteins. Other compounds have the ability to bind to the hydrophobic pocket in the canyon of a particle. They include pyridines, isoxazoles (such as WIN51711), pyridazine derivatives, flavans, chalcones, and so on [Bauer et al., 1981; Rombaut et al., 1991; Andries et al., 1994]. They are uncoating inhibitors. Secondly, the improvement of lyophilisation is conceivable, since the lyophilisation of poliovirus has been found to cause drastic decrease of infectivity. In a previous study [Shiomi et al., 2003], the loss of infectivity after lyophilisation was much reduced by modifying the lyophilisation conditions and the formula of the dissolving solution. The third method is the preparation of thermostable poliovirus by isolating heat-resistant mutants from the poliovirus population or by artificially manipulating the poliovirus genome. In this study, the last method was selected, and thermostable clones were isolated from the Sabin vaccine population.

Several changes have been reported to occur after incubation of poliovirus at 50°C for 30 min: loss of infectivity, antigenicity conversion, a morphological change, a change of pI of the particles, and so on. RNA viruses such as poliovirus consist of heterogeneous mixtures of related genomes, referred to as quasispecies. Based on this, heat-resistant mutants could be isolated from a Sabin virus preparation by repeating the process of incubation of the virus stock at 50°C for 30 min and multiplication of the remaining virus in a cell culture. The heat-resistant mutants thus obtained were found to exhibit genetic stability, since the thermal stability of the mutants remained unchanged in four successive passages, showing that the characteristic was hereditary. Furthermore, it was found that the mutants remained the original rct marker and d marker (the plating efficiency at low bicarbonate concentrations), which are used as markers for attenuation. This implies that the resistance to inactivation at 50°C varies independently of the d and rct markers. A similar observation was reported by Papaevangelou and Youngner [1961], but in contrast Wallis and Melnick [1963] stated that heat-resistant mutants represent phenotypic rather than genetic variation. Since the antibody reactivity of the mutants was not examined in this study, it cannot be stated if antigenic property of the mutants unchanged.

The amino acid substitution at the 87th amino acid residue of VP1 was detected in the mutants, in the comparison of the nucleotide sequences between parent virus and its heat-resistant mutants. The mutation was found in the five different clones of Sabin 2 and one clone of Sabin 2, although the Sabin 2 clones were obtained from the same heat-selected preparation. Although the 87th amino acid residue is near the B–C loop of VP1 (amino acids 94–102), it is in a very hydrophobic region [Toyoda et al., 1984], and should affect directly or allosterically the interaction of RNA with viral proteins. The change in this amino acid of VP1 might destabilise

the viral RNA–protein interaction, which may be responsible for the thermal stabilisation. It cannot be denied that other or additional mutation(s) is present in the P2, P3, or noncoding region as suggested by the different, though not much, property of $S2t_4Cl$-1C was observed (Table III) and by the absence of mutation in one clone of Sabin 1 mutant $S1t_4Cl$-26-8-4. In order to conclude the association of the nucleotide change with the thermostability, it is necessary to examine the properties of the virus by manipulating the Sabin 2 or Sabin 1 virus by cDNA mutagenesis.

The natures of the particles subjected to treatment with heat, UV, or alkaline pH, and the particles detached from the cells after cell attachment appear to be the same. The interaction between capsid proteins and RNA may be associated with this stability. Furthermore, there might be a relationship between viral uncoating and RNA release from virions in vivo and in vitro as found for heated empty particles. Mouse adaptation determinants enhance viral uncoating by facilitating receptor-mediated conformational changes and the release of RNA from the inside of the particles [Couderc et al., 1996]. In addition, the neurovirulence phenotype is related to the stability of the virions. The determinants associated with neurovirulence in mice affect the themostability of viral particles: mouse-adapted mutants are more sensitive to thermal inactivation (more themolabile) than the parental virus (PV1 Mahoney) without neurovirulence in mice. The mutations in the mouse-adapted mutants are mapped to the 22nd position of VP1 and 31st position of VP2 [Couderc et al., 1996]. Other amino acids in viral capsid proteins have also been identified as ones involved in uncoating and stability [Kirkegaard, 1990; Moscufo and Chow, 1992; Mosser et al., 1994]. Relationship between these amino acid residues and the 87th residue identified in this study is unknown. However, it is of interest to examine the properties in uncoating and cell attachment of heat-resistant mutants.

The mutants obtained in this study did not exhibit any resistance to ambient temperature (37°C) for a long time (5 or 7 days), as Youngner [1957] reported. In addition, mutants resistant to heating at 37°C for 7 days could not be obtained by the same procedure as that used to obtain the mutants resistant to heating at 50°C for 30 min. There might be different mechanism(s) for the stability at high and low temperature [Dimmock, 1967]. The difficulty in isolating mutants resistant to heating at ambient temperature for a long time could mean that multiple genetic changes are associated with the thermostability at ambient temperature. Stanley et al. [1956] also isolated heat-resistant (50°C for 30 min) mutants of poliovirus. They also found that the heat-resistant mutants were not stable on incubation at 37°C for a long time. Rombaut et al. [1994] reported that on heating at 37°C and over 47°C, RNA degradation and inactivation of viral proteins occur, respectively, and with moderate heating (at 42–45°C), the effects on protein and RNA are related. It has been shown that deuterium oxide inhibits the degradation of viral RNA, and pirodavir, a capsid-binding compound, inhibits the thermodenaturation of viral capsid proteins [Crainic et al., 1996; Verheyden et al., 2001]. The mechanism underlying the thermostability of the heat-resistant mutants obtained in this study should be examined further.

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Characterization of Human Rotavirus Strains With G12 and P[9] Detected in Japan

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Two G12 human rotavirus strains, CP727 and CP1030, were isolated from the respective diarrheic stools of an infant and an adult in Japan. *VP7* gene sequences of strains CP727 and CP1030 showed high identity with that of the G12 prototype strain L26, and with those of G12 strains reported recently from Thailand, the United States, and India. *VP4* gene sequences of strains CP727 and CP1030 showed the highest identity with those of P[9] rotaviruses. In Northern blot hybridization, strains CP727 and CP1030 were found to be closely related to strain AU-1 (G3P[9]); nine RNA segments hybridized to each other. Moreover, all segments each of the two Japanese G12 strains hybridized to those of the Thai G12 strain T152. These results suggest that Japanese G12 strains detected in this study are reassortants between a L26-like strain and a strain in the AU-1 genogroup. A similar reassortant was found in the Thai G12 strain T152. ***J. Med. Virol. 73:612–616, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: human rotavirus; Japan; VP4; VP7; reassortant

INTRODUCTION

Rotavirus is the most common agent of acute gastroenteritis in infants and young children worldwide. In developing countries, rotavirus infection is associated with high mortality; it has an annual death rate of 500,000–600,000 persons [Miller and McCann, 2000]. In developed countries, rotavirus infection causes high morbidity and high frequency of hospitalization. Vaccination is thought to be the most effective way to protect against rotavirus disease.

Rotavirus has two outer capsid proteins, VP4 and VP7, which are implicated independently in neutralization, and are defined as P and G types, respectively. Thus far, 15 G serotypes and 22 P types have been reported [Rao et al., 2000; Martella et al., 2003]. Global surveillances have demonstrated that the most prevalent strains in humans are G1P[8], G2P[4], G3P[8], and G4P[8] [Gentsch et al., 1996; Griffin et al., 2000]. Recently, uncommon G or P types and rare combinations of G and P types have been described more frequently [Gentsch et al., 1996; Unicomb et al., 1999; Griffin et al., 2000].

G12 strains were first detected from diarrheic children in the Philippines in 1990 [Taniguchi et al., 1990; Urasawa et al., 1990]. After more than 10 years, human G12 strains were found in Thailand, the United States, and India [Griffin et al., 2002; Pongsuwanna et al., 2002; Das et al., 2003]. In this study, we report the first isolation and characterization of human rotaviruses with G12 and P[9] in Japan.

MATERIALS AND METHODS

Stool Specimens

A total of 740 stool specimens were collected from children and adults with diarrhea at hospitals in the Chiba Prefecture, Japan, between 1999 and 2002. The specimens included 687 from children and 53 from adults. Each specimen was prepared as a 10% stool suspension in PBS. Samples were screened by an enzyme-linked immunosorbent assay (ELISA; Rotaclone, Meridian Diagnostics, OH) for the presence of rotavirus. Virus isolation was attempted using MA104 cells in roller tube culture [Urasawa et al., 1981]. Stool specimens were pretreated with 30 μg of trypsin (TypeIX, from porcine pancreas and crystallized; Sigma, MO) per ml, inoculated onto MA104 cells in the presence of trypsin (3 μg/ml), and harvested 5–7 days after infection. This cultivation process was repeated three times.

ELISA

G serotyping of rotavirus was carried out by ELISA using G serotype-specific monoclonal antibodies to G1, G2, G3, and G4 (Serotech, Hokkaido, Japan).

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Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Identification of G and P types of rotavirus in stools was carried out by semi-nested RT-PCR. The primers employed for G and P typing were the same ones described by Gouvea et al. [1990] and by Gentsch et al. [1992], respectively.

Sequence Determination

Direct sequencing of RT-PCR products was carried out using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, CA) with an automated sequencer, the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Nucleotide sequences were compared with published strains using a phylogenetic tree constructed by the Neighbor-Joining Method.

Polyacrylamide Gel Electrophoresis (PAGE)

Rotavirus dsRNA was extracted from cultured fluid with a disruption comprised of 1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, and 50 mM EDTA, followed by phenol-chloroform. The RNA was electrophoresed on 10% acrylamide gels (2 mm thick) for 16 hr at 20 mA at room temperature. RNA segments were visualized by silver staining. For Northern blot analysis, RNA was extracted from purified virus preparation and RNA segments were stained with ethidium bromide.

Northern Blot Hybridization

Northern blot hybridization was carried out as described previously [Pongsuwanna et al., 1996]. In brief, after PAGE analysis, dsRNA was denatured by soaking the gel in 0.1 N NaOH and 0.25 M NaCl for 20 min, and then neutralized in 4× Tris-acetate-EDTA (TAE) for 20 min twice and in 1× TAE for 20 min. Electrotransfer of rotavirus RNA to Hybond N+ (Amersham, NJ) was conducted at 0.2 mA overnight at 4°C. Hybridization was performed with an enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham) according to the instructions of the manufacturer. Stringency was regulated by changing the concentration of the SSC solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for primary and secondary wash buffers.

RESULTS

Serotype Distribution

A total of 740 stool specimens were screened for rotavirus by ELISA, and 240 specimens were found to be positive. Of these positive specimens, 231 were obtained from infants and children, 9 from adults. For G typing, the specimens were examined by ELISA using G serotype-specific monoclonal antibodies and/or by RT-PCR. G serotype or G type of the 214 specimens could be determined. G1, G2, G3, and G9 types were detected in 89, 67, 46, and 7 specimens, respectively. The predominant G type was G1 from 1999 to 2000, G2 in 2001, and G3 in 2002. G9 type was found only in 2002 (data not shown).

Detection of G12 Strains

The G type could not be determined in 26 specimens. Of the 26 specimens, two specimens produced cDNA products in the first PCR. These two specimens were derived from an infant 1 year of age and an adult 45 years of age in April and July of 2001, respectively. The first PCR products from the two specimens were subjected to the sequence determination for the *VP7* gene to determine their G types. The partial nucleotide and deduced amino acid sequences of the *VP7* genes of these strains (CP727 and CP1030) were obtained; they were 1,007 bp and 326 deduced amino acids. The VP7 sequences of the two strains were highly homologous to each other (99.3% nt, 99.4% aa). When compared with prototype strains of G1–G15, these two strains were the most similar to strain L26 of G12, with 91.0% identity at the nucleotide level and 94.2–94.5% identity at the amino acid level (Table I). Further, these two strains showed very high identity with several recently reported G12 strains: T152 in Thailand, Se585 in the USA, and ISO1 in India. The highest identity was found with T152 (99.3–99.4% nucleotides, 99.7% amino acids).

To determine the P types of strains CP727 and CP1030, part of the *VP4* gene was sequenced and it was found that they were very similar to each other

TABLE I. VP7 Nucleotide and Amino Acid Sequence Identity of Japanese Strains to G12 Strains Detected Recently and Standard Strains G1–G15

		Identity (%)			
		CP727		CP1030	
G serotype	Strain	nt	aa	nt	aa
G1	Wa	74.7	79.1	74.6	78.8
G2	S2	73.1	76.7	72.9	76.7
G3	AU-1	75.3	81.9	75.2	82.2
G4	ST3	73.1	75.2	73.2	75.5
G5	OSU	75.3	80.7	75.4	81.0
G6	PA151	76.0	81.9	75.7	81.6
G7	Ty-1	64.7	60.7	64.7	61.0
G8	69M	72.9	78.8	72.6	79.1
G9	W161	76.9	81.6	76.5	81.3
G10	Mc35	73.4	79.4	73.3	79.8
G11	YM	74.2	81.3	74.6	81.8
G12	L26	91.0	94.2	91.0	94.5
G12	T152	99.4	99.7	99.3	99.7
G12	Se585	97.8	98.1	97.7	98.1
G12	ISO1	97.6	98.2	97.5	98.2
G13	L338	75.1	78.8	75.1	78.8
G14	F123	74.9	79.8	74.9	80.1
G15	Hg18	71.1	68.5	71.0	68.8

The VP7 sequences were obtained from published reports and the GenBank database by accession number; Wa (K02033), S2 (M11164), AU-1 (D86271), ST3 (X13603), OSU (X04613), PA151 (L20881), Ty-1 (L01098), 69M [Green et al., 1989], W161 [Green et al., 1989], Mc35 (D14033), YM (M23194), L26 (M58290), T152 (AB071404), Se585 (AJ311741), ISO1 (AY206861), Se585 (AJ311741), ISO1 (AY206861), L338 (M58290), F123 (M61876), Hg18 (AF237666).

TABLE II. VP4 Nucleotide and Amino Acid Sequence Identity of Japanese Strains to Other Rotavirus Strains

		Identity (%)			
		CP727		CP1030	
VP4 genotype	Strain	nt	aa	nt	aa
P[4]	DS-1	62.4	53.8	62.3	53.8
P[6]	ST3	61.1	53.0	61.6	53.0
P[8]	Wa	61.4	53.0	61.0	53.0
P[9]	AU-1	90.1	94.3	90.2	94.3
P[9]	Cat2	89.4	92.8	89.6	92.8
P[9]	FRV-1	90.1	94.3	90.2	94.3
P[9]	K8	90.1	95.3	90.0	95.3
P[9]	T152	99.8	100.0	99.6	100.0
P[10]	69M	64.3	63.4	64.2	63.4
P[14]	Mc35	78.5	86.4	78.2	86.4

The VP4 sequences were obtained from the GenBank databaseusing accession numbers; DS-1 (P11196), ST3 (L33895), Wa (M96825), AU-1 (D10971), Cat2 (D13403), FRV-1 (D10971), K8 (D90260), T152 (AB077766), 69M (M60600), Mc35 (D14032).

(99.6% nt, 100.0% aa). These strains were compared with reference strains of various P types (Table II), and were found to be the most similar to P[9] rotaviruses, with 89.4–99.8% identity at the nucleotide level and 92.8–100.0% identity at the amino acid level. The highest identity was found with T152 (99.6–99.8% nucleotides, 100.0% amino acids). A phylogenetic tree was constructed for the VP8* region, which has been shown to be P type-specific, of the *VP4* gene of human and feline rotaviruses with P[9] specificity (Fig. 1). Strains CP727 and CP1030 were closely related to strain T152, and were phylogenetically distinct from other P[9] rotaviruses.

Northern Blot Hybridization Analysis

Strains CP727 and CP1030 were adapted to growth in MA-104 cell culture. RNA samples extracted from the culture fluid of strains CP727 and CP1030 were analyzed by Northern blot hybridization. The RNA-PAGE profiles of these strains were very similar to that of strain T152 (Fig. 2). The probe of T152 hybridized to all the RNA segments of strains CP727 and CP1030. A reciprocal assay with CP727 and CP1030 probes showed the same results. The probes of strains CP727 and CP1030 reacted with nine segments of strain AU-1 and with only segments 7 and 11 of strain L26. In contrast, the CP727 and CP1030 probes gave no reaction with the RNA from strains KU and S2, which are the representative strains of the Wa and DS1 genogroups [Nakagomi et al., 1989], respectively.

DISCUSSION

G12 human rotaviruses were first detected in the Philippines in 1990 [Taniguchi et al., 1990; Urasawa et al., 1990]. Since then, there have been no reports on the detection of G12 strains for more than 10 years. Recently, however, human G12 strains have been found in Thailand, the United States, and India [Griffin et al., 2002; Pongsuwanna et al., 2002; Das et al., 2003]. In this study, we isolated two G12 strains, CP727 and CP1030, respectively, from the diarrheic stools of an infant and an adult in Chiba, Japan.

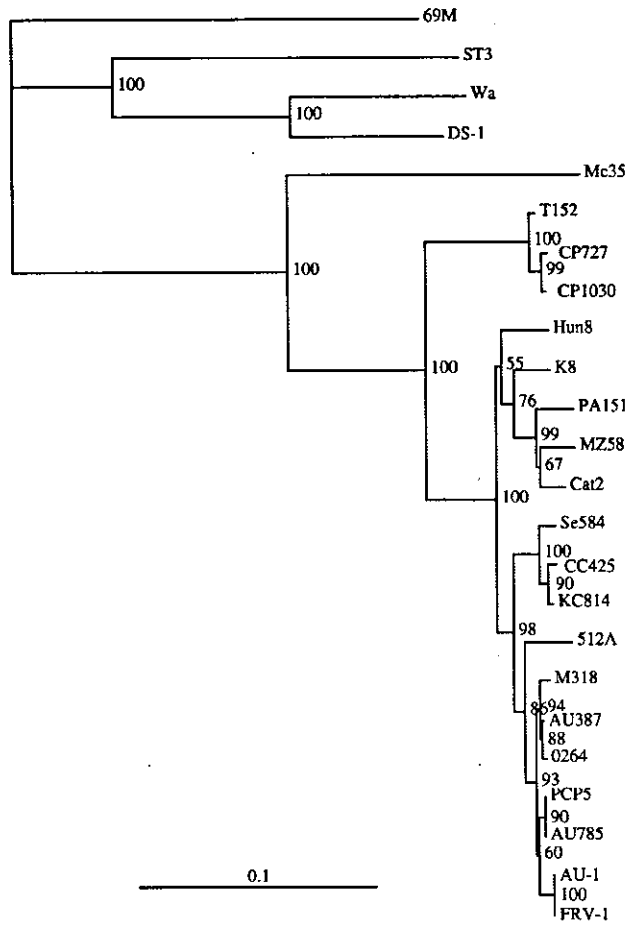

Fig. 1. Phylogenetic tree for the nucleotide sequence of the VP8* region of the *VP4* gene of strains CP727 and CP1030, other P[9] rotaviruses, and representative rotaviruses. The tree was constructed using the Neighbor-Joining Method provided by the Clustal X multiple alignment program. The bar indicates the variation scale. The following VP4 sequences were taken from the GenBank database (accession numbers are included): DS-1 (P11196), ST3 (L33895), Wa (M96825), AU-1 (D10971), Cat2 (D13403), FRV-1 (D10971), K8 (D90260), T152 (AB077766), 69M (M60600), Mc35 (D14032), Hun8 (AJ488142), PA151(D14623), MZ58 (D14622), Se584 (AJ311736), CC425 (AJ311734), KC814 (AJ311735), 512A (AB008668), M318 (AB008667), AU387 (D14617), 0264 (AB008665), PCP5 (D14624), AU785 (D14619).

The *VP4* gene of strains CP727 and CP1030 showed the highest identity with P[9] rotaviruses (89.4–99.8% nt, 92.8–100.0% aa). Gorziglia et al. [1990] showed that strains belonging to the same P genotype are required to have more than 89% homology at the amino acid level in the *VP4* gene. Thus, it was confirmed that the P genotypes of strains CP727 and CP1030 were P[9]. P[9] rotaviruses were classified into two clusters in the phylogenic analysis of the VP8* region of the *VP4* gene, as described by Nakagomi et al. [1993]. However, strains CP727 and CP1030 were distinct from these clusters,

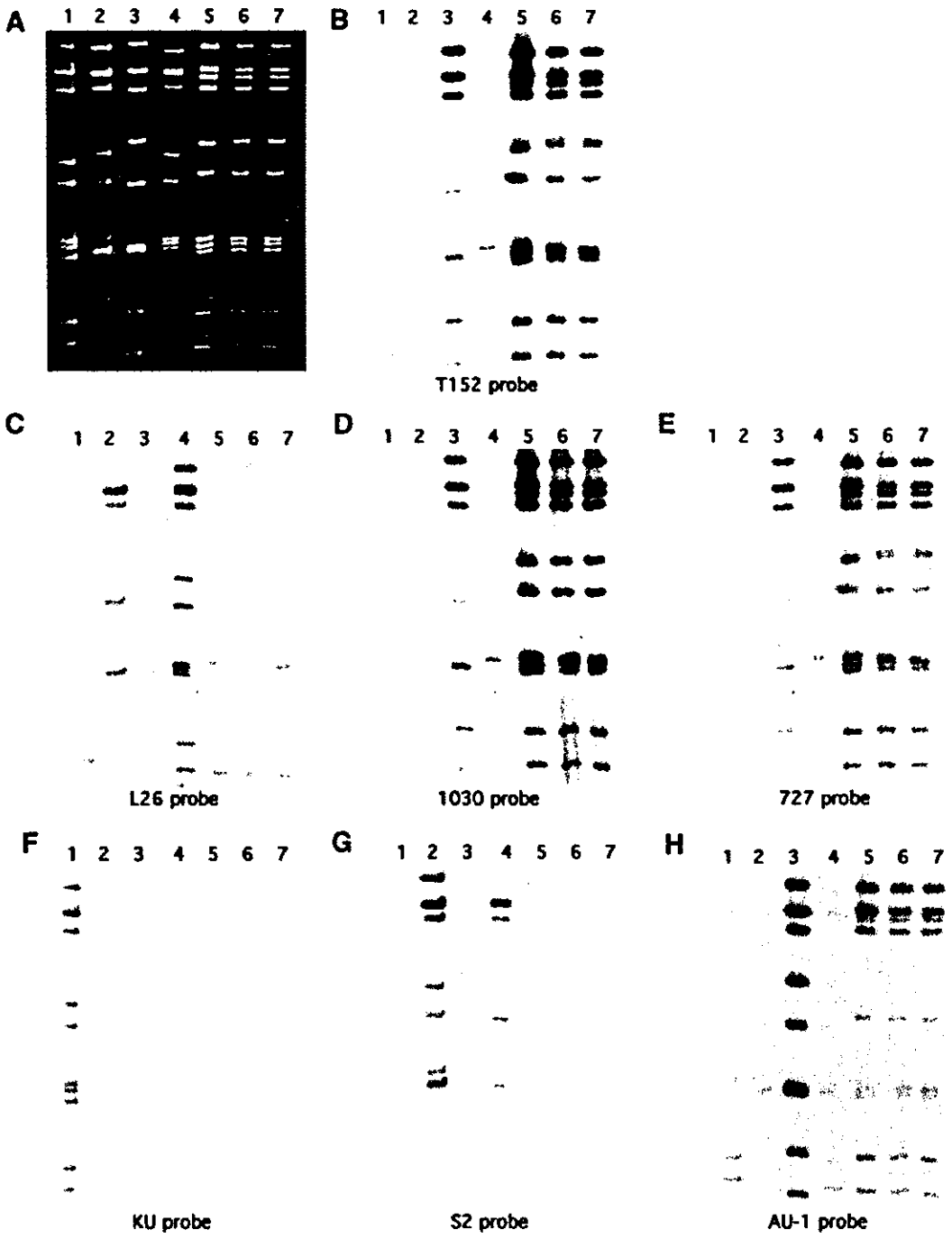

Fig. 2. Northern blot analysis of strains CP727 and CP1030. **A**: RNA profiles on PAGE. **Lanes**: 1, KU; 2, S2; 3, AU-1; 4, L26; 5, T152; 6, CP727; and 7, CP1030. **B–H**: Northern blot analysis using T152 (B), L26 (C), CP1030 (D), CP727 (E), KU (F), S2 (G), and AU-1 (H) probes. Northern blot analysis in panels from B to H was performed using the same blot, which was transferred from the polyacrylamide gel shown in panel A.

and made a distinct cluster with strain T152. These three strains, CP727, CP1030, and T152, diverge from the P[9] strains reported previously. These strains may be a subtype of P genotype 9.

On Northern blot hybridization, strains CP727 and CP1030 were closely related to strain AU-1; nine segments hybridized to each other. The *VP7* gene of these strains, which may be RNA segment 7, was related to L26. All of the respective segments of these two strains and those of T152 all hybridized to each other. These results suggest that Japanese G12 strains are reassortants between a L26-like strain and a strain belonging to the AU-1 genogroup. Similar reassortant was found in the strain T152 in Thailand [Wakuda et al., 2003]. In