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Detection and Genetic Characterization of Group A Rotavirus Strains Circulating among Children with Acute Gastroenteritis in Japan[▽]

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A total of 603 fecal specimens collected from July 2005 to June 2006 from children with acute gastroenteritis, encompassing five different localities in Japan, were screened for group A rotavirus by reverse transcription-PCR. It was found that 117 fecal specimens (19.4%) were positive for group A rotavirus. Rotavirus infection was detected continuously from November to June, with the highest prevalence in April. The G (VP7 genotypes) and P (VP4 genotypes) types were further investigated. The predominant genotype was G1P[8] (70.1%), followed by G3P[8] (17.9%), G9P[8] (6.8%), and G2P[4] (2.6%). A number of unusual G1P[4] combinations were also detected during this study period. A novel nomenclature for G1 is proposed, in which worldwide rotavirus G1 strains are classified into 11 lineages with 17 sublineages. A wide range of amino acid substitutions (up to 34) in VP7 that are specific for G1 lineages and sublineages were identified. Interestingly, only short amino acid motifs located at positions 29 to 75 and 211 to 213 of VP7 defined correctly the phylogenetic G1 lineages and sublineages. Examination of the deduced sequences of antigenic regions of VP7 also revealed multiple particular amino acid substitutions that correlated with the phylogenetic G1 lineages and sublineages. Of note, at least three distinct clusters of rotavirus G1 isolates were cocirculating in the Japanese pediatric population studied.

Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality of children in both developed and developing countries. It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age (17, 18, 22, 31). The rotaviruses, which comprise a genus in the family *Reoviridae*, are spherical in appearance and measure about 70 nm in diameter. Rotaviruses contain 11 segments of double-stranded RNA. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B, and C rotaviruses. Of these, group A rotavirus is the most important, being a significant cause of severe gastroenteritis in children worldwide (7, 8, 12, 13, 27, 29). The two outer capsid proteins, VP4 and VP7, allow classification of rotavirus into P and G genotypes, respectively. In rotavirus, at least 15 G genotypes have been recognized by neutralization assay and 27 P genotypes have been identified by hybridization or sequence analysis (13, 14, 16, 25, 28). Of these, four rotavirus G-P combinations, i.e., G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], are the most common globally and are therefore the targets for current vaccine development strategies (28). Since effective antirotavirus drugs have not been developed, a rotavirus vaccine would be very useful. The first rotavirus vaccine, Rotashield, was licensed for use in the United States in 1998. This vaccine was, however, withdrawn from use after reports of association with intestinal intussusception. Two current rotavirus vaccines, Rotarix and RotaTeq, have been licensed in many countries (2, 20, 23, 28, 32, 33).

Genetic and antigenic drift is one of the major driving forces of rotavirus evolution (10). Study of the molecular epidemiology of the rotaviruses provides knowledge on the diversity of the specific VP7 types found in humans. Phylogenetic analysis of the VP7 gene sequences of G1 rotavirus strains in Italy showed the emergence of novel antigenic variants which might be responsible for the continuous circulation of G1 rotaviruses with various lineages appearing and disappearing over a 19-year period (1). In Melbourne, Australia, it was found that two distinct genetic and antigenic G1 rotavirus lineages, I and II, were exclusively present in 1990 to 1992 and 1994 to 1995 (6). Due to genetic diversity in different parts of the world, knowledge of the molecular epidemiology of rotavirus in circulation is important, and appropriate rotavirus surveillance in a community before, during, and after the introduction of a vaccine campaign is essential to detect uncommon and novel types which might help to explain vaccine failure. For diarrheal disease control to be successful through vaccination, continuous monitoring of the rotavirus types is needed.

The objectives of the present study were to describe briefly the molecular epidemiology of rotavirus infections in children with acute gastroenteritis in Japan during 2005 to 2006 and to propose a novel classification scheme for standardization of G1 rotavirus nomenclature.

MATERIALS AND METHODS

Fecal specimens. A total of 603 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics encompassing five localities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) from July 2005 to June 2006. These localities are located in the northeast, the middle east, the north, the southwest, and the middle south of Japan, respectively. The fecal specimens were diluted with distilled water to 10% suspensions and clarified by centrifugation at

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TABLE 1. Distribution of different G types and P types of rotaviruses in Japan in 2005 to 2006

G type	No. (%) of isolates with P type:	
	P[4]	P[8]
G1	3 (2.6)	82 (70.1)
G3		21 (17.9)
G4		3 (2.6)
G9		8 (6.8)

10,000 × *g* for 10 min. The supernatants were collected and stored at −30°C until used for the detection of group A rotavirus.

Extraction of viral genomes. The viral genomes were extracted from 140 μl of 10% fecal suspensions by using a spin column technique according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

RT. For reverse transcription (RT), 4 μl of extracted viral genome was added to 4 μl of a reagent mixture consisting of 5× first-strand buffer (Invitrogen, Carlsbad, CA), deoxynucleoside triphosphates (10 mM/μl) (Roche, Mannheim, Germany), dithiothreitol (Invitrogen), Superscript reverse transcriptase III (Invitrogen, Carlsbad, CA), random primer (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total volume of the reaction mixture was 8 μl. The RT step was carried out at 50°C for 1 h, followed by 99°C for 5 min, and then the mixture was held at 4°C (34).

PCR. The use of PCR with previously reported specific primers resulted in the identification of rotavirus (34). Beg9 and VP7-1′ primers was used to amplify VP7 of rotavirus and specifically generated a size of amplicon of 395 bp. PCR was carried out with 1 μl of cDNA in 10 μl of reagent mixture containing 10× *Taq* DNA polymerase buffer (Promega, Madison, WI), deoxynucleoside triphosphates (2.5 mM/μl), primers (33 μM), *Taq* DNA polymerase (5 U/μl) (Promega, Madison, WI), and MilliQ water. PCR was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s and a final extension at 72°C for 7 min, and then the mixture was held at 4°C.

Group A rotavirus G typing. G typing of rotavirus was preformed using the protocol from the method previously presented by Das et al. (5). The full-length VP7 gene was reverse transcribed and then further amplified with Beg9 and End9 primers. The second amplification was performed using the first PCR product as the template with G type-specific mixed primers (9T1-1, 9T1-2, 9T-3P, 9T-4, and 9T-B) and 9con1 primer. These primers specifically generated amplicons of 158 bp, 224 bp, 466 bp, 403 bp, and 110 bp for G1, G2, G3, G4, and G9, respectively.

Group A rotavirus P typing. P typing was conducted by using a method modified from that of Gentsch et al. (9). The RT-PCR was performed by using Con2 and Con3 primers for the amplification of the partial VP4 gene. In the second amplification, a mixture of primers 1T-1, 2T-1, 3T-1, 4T-1, 5T-1, and Con3 was utilized for the identification of P[8], P[4], P[6], P[9], and P[10], with amplicons of 346 bp, 484 bp, 268 bp, 392 bp, and 584 bp, respectively.

Electrophoresis. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min, and then visualized under UV light, and the results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis. PCR products positive for rotavirus was sequenced. Genetic analysis was performed using CLUSTAL X. A phylogenetic tree with 100 bootstrap replicates of the nucleotide alignment data sets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP).

Nucleotide sequence accession numbers. The sequences of rotavirus isolates detected in this study have been submitted to GenBank and assigned accession numbers EF079064 to EF079070. Reference rotavirus strains and their accession numbers used in this study were as follows: porcine C60 (L24164), porcine C95 (L24165), bovine T449 (M92651), porcine SW2021 (AF426162), AU19 (AB018697), Kor-64 (U26378), Kor-54 (U26377), JP421 (D16326), 88H249 (AB081795), Ban-59 (U26366), Fin-804 (Z80303), Fin-431 (Z80314), Fin-111-1 (Z80278), Au81 (M64666), PA5/03 (DQ377596), PA2/04 (DQ377598), PA164 (DQ377588), PA430 (DQ377591), PA378 (DQ377589), Fin-429 (Z80312), AU007 (AB081799), Fin-101-1 (Z80271), Fin-425-1 (Z80309), G192B (AF043678), DC03 (AF183859), Oh-64 (U26387), Cos70 (U26370), Fin-110 (Z80277), Fin-220 (Z80294), Fin-308 (Z80297), PA5 (Q377573), PA3c (DQ377566), PA17c (DQ377602), PA32 (DQ377574), ISO-4(AY098670), Thai-1604 (DQ512981), Dhaka8 (AY631049), VN-281 (DQ508167), VN-355 (DQ512968), Thai-804 (DQ512979), Mvd9816 (AF480293), Mvd9810 (AF480288), 97′S6 (AF260945J), CH631 (AF183857), China-45 (U26371), JP471 (D16328), JPTE1 (D17721), Egypt-7 (U26373), Wa (K02033), D/JP (AB118022), HOU8697 (U88717), Brazil-5 (U26367), Brazil-6 (U26368), Brazil-4 (U26365), Israel-56 (U26376), KU (D16343), K2 (D16323), K8 (JP/D16344), Russia-1407 (S83903), Egypt-8 (U26374), PA8 (DQ377592), and PA19 (DQ377593).

RESULTS

Molecular epidemiology of group A rotavirus infection. Rotavirus was detected in 117 out of 603 (19.4%) specimens tested. The highest incidence of rotavirus was in the 12- to 23-month-old group (47%, 55 of 117), and the lowest was in the under-6-month-old group (4.3%, 5 of 117). The rate of rotavirus infection in children aged 3 to 15 years was 23% (27 of 117). Rotavirus was detected continuously in an 8-month period lasting from November to June. The highest prevalence

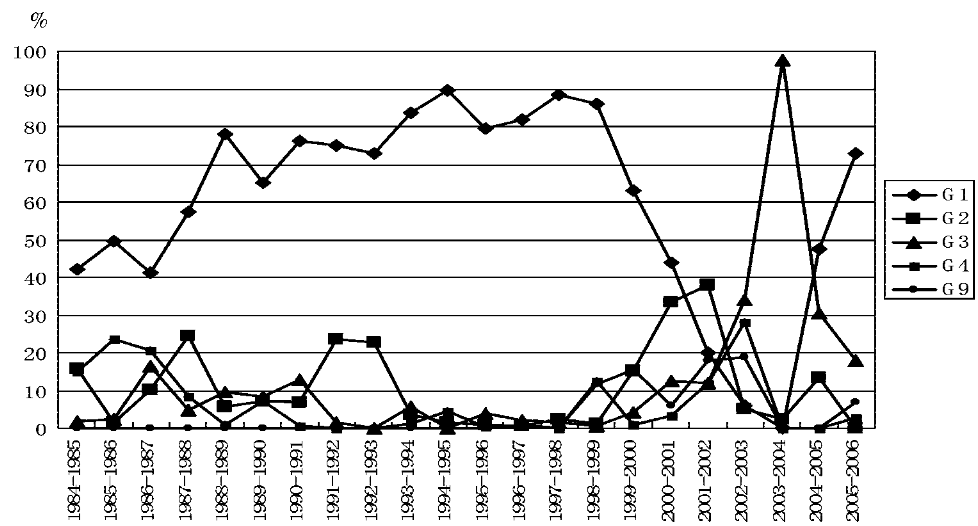

FIG. 1. Fluctuation of the prevalence of group A rotavirus G types, with the reemergence of G1 and the decrease of G3 among children with acute gastroenteritis during 2005 and 2006 in Japan.

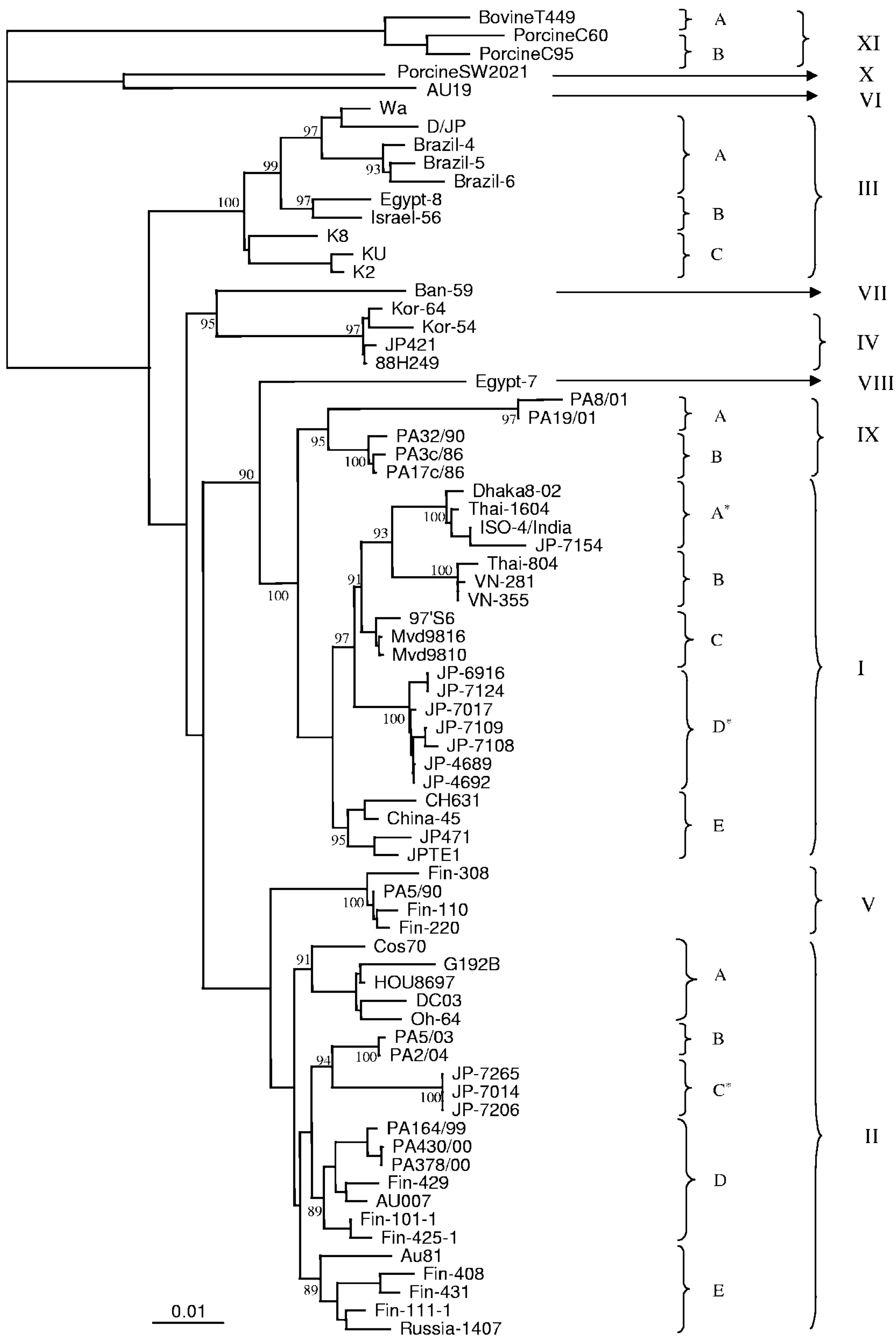

FIG. 2. Phylogenetic tree obtained from nucleotide sequences of group A rotavirus G1 VP7 genes. Reference group A rotavirus strains were selected from GenBank (accession numbers are indicated in the text). The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. *, sublineage containing rotavirus detected in this study.

TABLE 2. Amino acid substitutions in the VP7 protein that are specific for lineages and sublineages

Lineage	Sublineage	Strain	Substitution at position (amino acid)[a]:																													
			11 (I)	16 (I)	19 (L)	28 (R)	29 (I)	34–35 (IY)	37 (F)	42 (V)	43 (A)	50 (A)	55 (L)	57 (L)	65 (T)	68 (A)	72 (Q)	74 (G)	75 (I)	76 (F)	90 (S)	91 (T)	116 (V)	147 (N)	193 (V)	211–213 (NVD)	217 (M)	218 (V)	231 (D)	233 (I)	266 (S)	268 (I)	278 (N)
I	A	ISO-4	*	*	*	*	*	*	*	*	*	*	*	I	*	S	**R**	**E**	V	*	*	*	*	*	*	*	T	*	*	*	**A**	*	*
		Dhaka8	*	*	*	*	*	*	*	*	*	*	*	I	*	S	**R**	**E**	V	*	*	*	*	*	*	*	T	*	*	*	**A**	*	*
	B	Thai-804	*	**Q**	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	**A**	*	*
		VN-281	*	**Q**	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	**A**	*	*
	C	Mvd9816	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	*	V	*
		97′S6	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	*	V	*
	D	JP-6916	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	N	*	*	*	*	T	*	*	*	*	*	*
		JP-4689	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	N	*	*	*	*	T	*	*	*	*	*	*
	E	CH631	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	*	*	*
		JPTE1	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	*	*	*	*	*	*	*	T	*	*	*	*	*	*
II	A	Oh-64	*	*	*	*	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		Cos70	*	*	*	*	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
	B	PA5	*	*	*	*	M	*	S	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		PA2	*	*	*	*	M	*	S	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
	C	JP-7014	*	*	**F**	*	M	*	S	*	*	**T**	I	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		JP-7206	*	*	**F**	*	M	*	S	*	*	**T**	I	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
	D	AU0007	*	*	*	*	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		PA378	*	*	*	*	*	*	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
	E	Rus-1407	*	*	*	*	*	*	S	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		AU81	*	*	*	*	*	*	S	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
III	A	Wa	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	E	V	*	*	*	*	S	*	*	*	I	*	*	*	V	*
		Brazil-5	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	E	V	*	*	*	*	S	*	*	*	I	*	*	*	V	*
	B	Egypt-8	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	E	V	*	*	*	*	S	*	*	*	*	*	*	*	V	*
		Israel-56	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	E	V	*	*	*	*	S	*	*	*	*	*	*	*	V	*
	C	KU	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	E	V	*	*	*	*	S	*	*	*	*	*	*	*	V	*
		K8	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	E	V	*	*	*	*	S	*	*	*	*	*	*	*	V	*
IV		Kor-64	*	V	*	*	*	*	S	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	I	*	*	*	*	D
		88H249	*	V	*	*	*	*	S	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	I	*	*	I	*	*	*	*	D
V		PA10	*	*	*	*	*	*	S	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
		PA78	*	*	*	*	*	*	S	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	V	*
VI		AU19	*	V	F	*	M	*	*	M	T	*	*	*	*	*	*	E	T	*	*	N	*	*	I	*	I	*	*	*	*	*	*
VII		Ban-59	*	*	*	*	*	*	*	*	*	*	*	I	*	S	*	*	V	S	V	*	*	*	*	*	*	I	*	*	*	*	*
VIII		Egypt-7	*	*	*	*	*	MC	*	*	*	V	*	I	*	S	*	*	*	*	V	*	*	*	*	*	T	*	*	*	*	*	*

IX	A	PA8	*	*	*	*	*	*	*	*	*	*	*	*I*	*A*	*S*	*	***E***	*	*	*	*	*	*	*	*	T	*	*	**V**	*	*	*
		PA19	*	*	*	*	*	*	*	*	*	*	*	*I*	*A*	*S*	*	***E***	*	*	*	*	*	*	*	*	T	*	*	**V**	*	*	*
	B	PA3c	*	*	**I**	*	*	*	*	*	*	*	*	*I*	*	*S*	*	*	*	*	*	*	**I**	*	*	*	*T*	*	**N**	*	*	*	*
		PA17c	*	*	**I**	*	*	*	*	*	*	*	*	*I*	*	*S*	*	*	*	*	*	*	**I**	*	*	*	*T*	*	N	*	*	*	*
X		SW2021	*	*	*F*	*	***M***	*	*	*	*T*	*	*	*	*	*V*	*	***E***	*	*	*	*N*	**I**	*	*	*	*V*	*	*	*	*	*	*
XI	A	T449	*T*	*	*	*Q*	***M***	*	*	*	*T*	*	***I***	*	*	*	K	***E***	*T*	*	*	*	*	*	*	*QCG*	*I*	*	*	*	*	*	*
	B	C95	*T*	*	*	*Q*	***M***	*	*	*	*T*	*	***I***	*	*	*	*K*	***E***	*T*	*	*	*	*	*	*	*	*I*	*	*	*	*	*	*
		C60	*T*	*	*	*Q*	***M***	*	*	*	*T*	*	***I***	*	*	*	*K*	***E***	*T*	*	*	*	*	*	*	*	*I*	*	*	*	*	*	*

[a] The amino acid substitutions in italic are specific for lineages. The amino acid substitutions in boldface are specific for sublineages. The amino acid substitutions in both italic and boldface are specific for both lineages and sublineages. Asterisks indicate the lack of a substitution. Variable regions are located at amino acids 11 to 29, 37 to 50, 65 to 91, 147, and 211 to 218 in rotavirus VP7. The phylogenetic lineage- and sublineage-defining sequences are amino acids 29 to 75 and 211 to 213.

of rotavirus was found in April (41%, 48 of 117), and the lowest was in November (0.9%, 1 of 117).

Distribution of G and P types with reemergence of G1. Four different G types, G1, G3, G4, and G9, were detected during this study period (Table 1). Interestingly, G1 was the most prevalent genotype (72.7%), followed by G3 (17.9%), and accounted for the majority of rotavirus isolates detected (Fig. 1). When the isolates were examined for their P types, two common genotypes, P[4] and P[8], were identified. The majority of rotavirus isolates were P[8] (97.4%), and 2.6% were P[4]. The G1P[8] combination was the most predominant genotype and represented 70.1%, followed by G3P[8] (17.9%), G9P[8] (6.8%), and G2P[4] (2.6%). A number of unusual G1P[4] combinations (2.6%) were also detected.

Nucleotide sequence analysis of group A rotavirus G1 isolates. In an attempt to understand the molecular basis of rotavirus genetic diversity within G1, 14 rotavirus G1 isolates were randomly chosen and their VP7 genes were sequenced. Ten of the 14 G1 isolates (94.1%) had high identities to each other at the nucleotide level, ranging from 99% to 100%. The findings demonstrated that they came from the same source of infection and very likely represented the same strain, JP-6916. Another rotavirus G1 isolate, JP-7154, had an identity of only 97% at the nucleotide level with the JP-6916. However, JP-7154 shared the highest identity (99%) with ISO-4, a rotavirus G1 strain from India. On the other hand, 3 of the 14 rotavirus G1 isolates (21.4%), which had high identity (100%) each other and were represented by JP-7014, had a low identity (only 93%) at the nucleotide level with JP-6916 and JP-7154. By BLAST, JP-7014 had the closest match (97%) with HOU8697, a rotavirus G1 strain from the United States.

A novel nomenclature for the group A rotavirus G1 genotype. To investigate the heterogeneity and evolution of rotaviruses within G1 genotype, the VP7 genes of G1 isolates detected in this study and worldwide rotavirus reference G1 strains were used for genetic analysis. Phylogenetic analysis identified 11 distinct lineages with 17 sublineages (Fig. 2). Of note, lineages I to IX were found only in humans, the porcine rotavirus SW2021 strain was in lineage X, and lineage XI consisted of both the bovine rotavirus T449 strain and porcine rotavirus C60 and C95 strains. It was found that all 14 rotavirus G1 sequences in the study were classified into two distinct lineages, lineage I with 78.6% (11 of 14) and lineage II with 21.4% (3 of 14). Within lineage I, the rotavirus G1 isolate JP-6916 belonged to sublineage IA (known as the ISO-4 virus cluster). Ten other rotavirus G1 isolates, represented by JP-6916, did not group in any genetic cluster and therefore made a novel sublineage, ID. Within lineage II, three rotavirus G1 isolates, represented by JP-7014, also did not belong to any cluster and therefore made a novel sublineage, IIC. The nucleotide identities of rotavirus strains within each sublineage ranged from 98% to 100%, indicating less than 2% genetic difference among them. The nucleotide sequence divergence between sublineages within the same lineage was from 3% to 4%. In contrast, sequence variation among strains between lineages was considerably higher, ranging from 5% to 16%.

Alignment of the amino acid sequences of VP7. Direct inspection of the sequence alignment revealed that there are three kinds of amino acid substitutions specific for lineages and sublineages (Table 2). For differentiation within lineages, up to

TABLE 3. Deduced amino acid sequences of antigenic regions of the VP7 proteins of rotavirus G1 worldwide

Lineage	Sublineage	Strain	Sequence of antigenic region[a]:					
			A (87–101)	B (142–152)	C (208–221)	D (291K)	E (189–190)	F (233–242)
Wa[b]			TEASTQINDGDWKDS	MKYDQSLELDM	QTTNVDSFEMIAEN	K	SM	INHKINLTTT
I	A	ISO-4	.............S.....E.........	...........N.........	.................TV......	R	....	....
		Dhaka8	.............S.....E.........	...........N.........	.................TV......	R	....	....
	B	Thai-804	.............S.....E.........	...........N.........	.................TV......	R	F..	....
		VN-281	.............S.....E.........	...........N.........	.................TV......	R	....	....
	C	Mvd9816	.............S.....E.........	...........N.........	.................TV......	R	....	....
		97′S6	.............S.....E.........	...........N.........	.................TV......	R	....	....
	D	JP-6916	.........N..S.....E.........	...........N.........	.................TV......	R	....	....
		JP-4577	.........N..S.....E.........	R.........N.........	.................TV......	R	....	....
	E	CH631	.................E.........	...........N.........	......G.........TV......	R	....	....
		China-45	....	...........N.........	.................TV......	R	....	....
		JPTE1	.............S.....E.........	...........N.........	.................TV......	R	....	....
II	A	G192B	..................E.........	...........N.........	...............D...V......	..	....	....
		Cos70	....	...........N.........	...................V......	..	....	....
		DC03	.................RE........	...........N.........	...................V......	..	....	....
	B	PA5	...................E.........	...........N.........	....	..	....	....
		PA2	...................E.........	...........N.........	...................V......	..	....	....
	C	JP-7014	...................E.........	...........N.........	...................V......	..	....	....
		JP-7206	...................E.........	...........N.........	...................V......	..	....	....
	D	AU0007	...................E.........	...........N.........	...................V......	..	....	......Y.............
		PA378	...................E.........	...........N.........	...................V......	..	....	......Y.............
	E	Rus-1407	...................E.........	...........N.........	...................V......	..	....	....
		AU81	...................E.........	...........N.........	...................V......	..	....	....
III	A	Wa	....	....	....	..	....	....
		Brazil5	....	....	....	R	....	....
		Brazil6	....	....	......Y..................	R	....	....
	B	Egypt-8	....	....	...................V......	..	....	....
		Israel-56	....	....	...................V......	..	....	....
	C	Ku	....	....	...................V......	..	....	....
		K8	N...........................	....	...................V......	..	....	....
IV		Kor-64	.............S............T	...........N.........	....	R	....	......Y.............
		88H249	.............S....E.......T	...........N.........	....	R	....	......Y.............
V		PA10	.................E........	...........N.........	...................V......	..	....	....
		PA78	..................E.........	...........N.........	...................V......	..	....	....

VI		AU19	I.........N.........E	...........N.........	...................IV.....D	..	....	....
VII		Ban-59	.............S............	...........N.........	....	R	....	....
VIII		Egypt-7	....	...........N.........	.................TV......	R	....	....
IX	A	PA8	A......V...........E........	...........N.........	.................TV......	R	....	....
		PA19	A......V..........E........	...........N.........	.................TV......	R	....	....
	B	PA3c	..................E........	...........N.........	.................TV......	R	....	....
		PA17c	..................E........	...........N.........	.................TV......	R	....	....
X		SW2021	..........N..V.....E.......T	...........N.........	.................VV......	K	....	....
XI	A	T449	V........N.........E.......T	...........N.........	..................IV......	E	....	....
	B	C95	V........N.........E.......T	...........N.........	..................IV......	..	....	....
		C60	V........N.........E.......T	...........N.........	..................IV......	..	....	....

[a] Residues that match sequences of antigenic regions are denoted by dots.
[b] Wa has been included as the G1 standard.

23 amino acid substitutions located in variable regions and conserved regions of VP7 were found, e.g., T at position 11 for lineage XI, V at position 16 for both lineages IV and VI, T at position 68 for lineage III, and S at position 147 for lineage III. For differentiation within sublineages, nine amino acid substitutions located in variable regions and conserved regions of VP7 were identified, e.g., Q at position 16 for sublineage IB, R at position 72 for sublineage IA, and V at position 233 for sublineage IXA. For specificity of lineages and sublineages, six amino acid substitutions located in variable regions and only one amino acid substitution located in a conserved region were found, e.g., I at position 55 for sublineages IIC and IIE and lineage IX and N at position 91 for sublineage ID and lineages VI and X.

Of interest was the identification of only short signature sequences of VP7 which correctly defined the phylogenetic G1 lineages and sublineages. Fourteen amino acids, at positions 29, 34, 35, 37, 42, 43, 50, 55, 57, 65, 68, 72, 74, and 75, formed identification codes for lineages; e.g., lineage I had a code of IIYFVAALITSQ(R)G(E)V, lineage III had a code of IIYFV AALLATQEV, and lineage XI had a code of MIYFVTAILT AKET. This motif also made an identification code for some sublineages. Within lineage II, sublineages B, C, and D had identification codes of MIYSVAALLAAQGI, MIYSVATILA AQGI, and MIYSVATILAAQGI, respectively, which were different from that of sublineages A and E. Within lineage IX, sublineage A had an identification code of IIYFVAALIA SQEI and sublineage B had an identification code of IIYFV AALIASQGI. A similar pattern was also noted for sublineages of lineages I and III. Other amino acids at positions 211, 212, and 213 formed a code QCG only for sublineage XIA.

Examination of the deduced sequences of antigenic regions of VP7. Table 3 shows the diversity of the amino acid substitutions in VP7 antigenic regions A to F of rotavirus G1 strains worldwide. All rotavirus G1 lineages except for lineage III (known as the Wa virus cluster) showed an amino acid substitution N at position 147 of antigentic region B. A similar finding with an amino acid substitution D at position 97 was also found for the majority of G1 lineages, except for lineages III, VII, and VIII. Moreover, multiple amino acid variations that were specific for lineages and sublineages were also identified, e.g., V at position 87 for lineage XI; A and V at positions 87 and 90, respectively, for sublineage IXA; S at position 94 for lineage IV and some others; and R at position 291 for lineage I and some others. Within lineage I, the novel genetic cluster D formed by the Japanese rotavirus isolates in the study demonstrated the distinct amino acid substitution N at position 91 of antigenic region A. In contrast, the novel sublineage IIC also formed by other Japanese rotavirus isolates did not have any specific amino acid substitution in comparison with other sublineages within lineage II.

DISCUSSION

Extensive epidemiological studies of rotavirus infection worldwide indicated that G1 was the most prevalent genotype (5, 13, 15, 19, 21, 28, 36). However, the new variants G2 and G3 emerged to become the leading genotypes in Japan from 2001 to 2004 (24, 35). At the same time, the prevalence of G1 rapidly dropped from 86% in 1998 to 1999 to 6% in 2002 to

2003 (35), and no G1 was found in 2003 to 2004 (24). In this study, the changing pattern of the G type distribution of rotavirus was demonstrated. Of note, G1 reemerged to be the most prevalent, with a high frequency (72.7%) compared to G3 (17.9%) and G9 (6.8%), which were the second and third prevailing genotypes, respectively. An insufficient antibody protection from acquired viral immunity against G1 in the Japanese pediatric population due to the lack of a trigger of previous G1 rotavirus infection during 2001 to 2004 was hypothesized. This hypothesis was in strong agreement with recent findings that the rate of detection of G1 infection was very low during 2001 to 2004 (24, 35). Moreover, the common genotype G4 was not detected in this epidemic season.

An early study of the divergence of VP7 genes of G1 rotaviruses from infants vaccinated with reassortant rhesus rotaviruses in the United States identified four lineages of G1 rotaviruses (11). A recent study to investigate the heterogeneity and dynamics of the evolution of G1 rotaviruses during 19 consecutive years from 1986 to 2004 in Italy assigned these strains to seven lineages (1). Based on a phylogenetic analysis, each lineage makes a distinct cluster, and the degree of interlineage nucleotide divergence is 5% or higher (1, 11). With limited sequence variation, however, some other G1 rotavirus strains cannot be classified into any clusters in the abovementioned classification schemes. In order to gain further insights into the genetic variability within the G1 genotype, the VP7 genes of rotavirus G1 strains were used for genetic analysis. The alignment of VP7 sequences that we compiled from a number of rotavirus G1 strains detected in different parts of the world that were available at the time of the study demonstrated 11 distinct lineages with nucleotide divergence ranging from 5% to 16%, which were further divided into 17 sublineages. This novel nomenclature should aid molecular characterization and description of sporadic cases as well as outbreak G1 rotavirus strains and also should provide genetic and antigenic insights for future studies with this virus. Even current vaccines have been proven to be highly efficacious against G1 rotaviruses, with some studies showing that symptomatic reinfection of vaccinated children with G1 rotaviruses is possible (26, 32, 33); these G1 rotavirus strains were different from the vaccine virus (26, 32). Thus, the novel nomenclature in the study should help to classify accurately G1 rotaviruses into their proper clusters and to identify introduction and emergence of uncommon or new strains not only in Japan but also in other countries, especially those which currently have a rotavirus vaccine program. Therefore, it could be a useful tool to demonstrate the genetic diversity of G1 rotaviruses circulating in a community and also to explain the situation of vaccine failure.

Another interesting finding of this study was the discovery of a wide range of amino acid substitutions (up to 34) which were specific for lineages and sublineages. These changes are exclusively present in only one or more lineages and/or sublineages, but they are absent in the other lineages and/or sublineages. Of these, as many as 23 amino acid positions were located in variable regions of VP7. Interestingly, 11 other positions were identified at the conserved sites of VP7. The results clearly indicated that the amino acid differences between lineages and/or sublineages were found not only in variable regions but also in conserved regions of the VP7 gene. To date, information on localization of amino acids involved in conformational changes of rotavirus VP7 structure is not available. Therefore, it is still unclear whether the amino acid substitutions found in the study could induce conformational changes of G1 rotavirus VP7 protein. Remarkably, a short motif located in amino acids 29 to 75 in both variable and conserved regions of VP7 was found to differentiate rotavirus G1 strains into phylogenetic lineages. This signature motif consistently changed according to each lineage. Moreover, this motif also correctly divided rotavirus G1 strains into the majority of phylogenetic sublineages. The identification of another amino acid motif at positions 211 to 213 of the VP7 variable region, which existed exclusively in bovine rotavirus, should be noted. Examination of the deduced sequences of antigenic regions of VP7 also revealed multiple particular amino acid substitutions that correlated with the phylogenetic G1 lineages and sublineages. Of note, the diversity of the amino acid substitutions covering all six VP7 antigenic regions A to F of rotavirus G1 strains worldwide was identified. Importantly, some of the substitutions at positions 94, 97, 147, and 291 have been recognized as critical components of neutralization epitopes, and they are essential in further division of G1 rotaviruses into monotypes by a panel of monoclonal antibodies (4, 30). Taking the data together, it is possible that the different G1 rotavirus lineages might have different antigenic properties. This notion is supported by previous findings that the VP7 protein of the RRV-S1 vaccine strain, belonging to lineage III, was antigenically distinct from U.S strains and most circulating G1 strains worldwide, belonging to other lineages, as determined by using serum neutralizing antibodies from postvaccinated children and that the amino acid substitutions in antigenic regions might play role in vaccine failure due to antigenic changes (11). The high intragenotypic and antigenic heterogeneity is a possible reason for the consistent predominance of G1 rotavirus throughout the world over the years (1).

As determined from the genetic diversity, at least three distinct clusters (sublineage IA, sublineage ID, and sublineage IIC) of rotavirus G1 have been cocirculating in the Japanese pediatric population studied. Of these, sublineage ID and sublineage IIC were recognized as novel clusters and were detected in Japan for the first time. These findings are important to provide baseline data prior to a possible widespread use of rotavirus vaccines in Japan in the near future to subsequently assess the impact of the vaccines on circulating strains. Using the novel nomenclature of the G1 genotype, it was found that porcine rotaviruses were divided into two separate lineages, X and XI. Interestingly, the porcine rotavirus strain SW2021 of lineage X had higher identities, ranging from 86% to 90%, at the nucleotide level, with other human rotavirus G1 lineages than other porcine rotavirus strains C60 and C95 of lineage XI, with 84% and 85%, respectively. More interestingly, three rotavirus strains, T449, C60, and C95, from two different species, bovine and porcine, clustered in only one lineage, lineage XI. Bovine rotavirus T449 had high homologies of 96% to 98% at the nucleotide and the amino acid levels with porcine rotavirus strains C60 and C95. However, no further characterization has been performed on the bovine G1 rotavirus strain in terms of other structural or nonstructural proteins (3). Therefore, further research should be conducted to investigate any evidence on interspecies transmission of rotaviruses taking place in nature between the bovine and porcine species.

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