TABLE 1. Distribution and relative frequencies of combinations of
G and P genotypes of the porcine group A rotavirus isolated
from diarrheic piglets in Chiang Mai, Thailand, from
June 2000 to July 2001

G genotype	No. (%) of specimens with P genotype				
	P[6]	P[7]	P[13][a]	P[19][a]	ND[b]
G2[a]				.	1 (2.6)
G3	3 (7.7)			13 (33.3)	1 (2.6)
G4[a]	17 (43.6)		1 (2.6)		
G5				2 (5.1)	
G9		1 (2.6)			

[a] G or P genotypes were identified by sequence analysis.
[b] ND, P genotype could not be identified.

maining one was with P[13]. The majority of G3 genotypes (13 of 17 isolates) were found in combination with P[19], the rare P genotype of porcine rotaviruses, whereas another three isolates were in combination with P[6]. In addition, the VP4 gene of one G3 isolate could not be amplified by the Con2 or Con3 primer. The G5 and G9 genotypes were found in combination with P[13] and P[7], respectively. It was interesting to note that the only isolate of G2 detected in this study was found in combination with the novel P genotype, since its VP4 sequence showed low nucleotide (45.7% to 67.4%) and amino acid (41.8% to 69.9%) sequence identities with all 26 currently recognized P genotypes. This G2 P[novel] isolate will be characterized further in more detail and reported separately in the future.

Analysis of VP4 nucleotide sequences of porcine G3P[19] strains. Despite the generation of 876-bp VP4 amplicons by PCR, the P genotypes of the 13 isolates of the G3 PoRV strains were initially designated P nontypeable strains, since they could not be typed by multiplex PCR using all of the P-type-specific primer sets described previously (8, 17, 20, 57). The P genotypes of these isolates were, therefore, identified based on their VP4 nucleotide sequence analysis, and all were found to be P[19] strains, as shown in Table 1. The VP4 sequence identities among the G3P[19] strains in the year 2000 (CMP029, CMP031, and CMP039) were 99.8% to 100%, while those in the year 2001 (CMP072, CMP087, CMP090, CMP092, CMP094, CMP095, CMP096, CMP098, CMP099, and CMP100) were 98.4% to 100%. However, the percent nucleotide sequence identities were slightly decreased (95.0% to 96.1%) when comparing the G3P[19] isolates from the year 2000 with those from the year 2001 (data not shown).

The VP4 nucleotide sequences of these G3P[19] strains were also compared with the P[19] reference strains of porcine (4F) and human (RMC321, Mc323, and Mc345) origins. Different P types of porcine strains, P[5], P[6], P[7], and P[13], as well as a bovine outlier reference strain, P[17], were also included. The results revealed that all of the porcine G3P[19] strains detected in this study were most closely related to human P[19] reference strains (RMC321, Mc323, and Mc345), with a nucleotide sequence identity of 95.4% to 97.4%, while the nucleotide sequence identity to the porcine P[19] reference strain (4F) was only 87.6% to 89.1%. Rotavirus strains representing other P types exhibited much lower nucleotide sequence identities (60.1% to 72.5% for different P types of porcine and 49.4% to

50.6% for an outlier strain of bovine) than our P[19] strains (data not shown).

Phylogenetic analysis of VP4 nucleotide sequences (Fig. 1) confirmed the finding that our PoRV P[19] strains, in both 2000 and 2001 isolates, which were clustered with human P[19] reference strains in a monophyletic branch, separated from the porcine P[19] prototype strain (4F). It was interesting to point out that the human P[19] strains Mc323 and Mc345 had been isolated in 1989 from children hospitalized with acute gastroenteritis in Chiang Mai city, which was the same geographical area where our PoRV P[19] strains were isolated.

Analysis of VP7 nucleotide sequences of porcine G3P[19] strains. The G genotypes of G3P[19] PoRV strains isolated in this study were identified by multiplex PCR using G-type-specific primers. To confirm the G genotype assignment for these strains and compare their relationships to other human and animal rotavirus strains, their VP7 genes were sequenced. Analysis of VP7 nucleotide sequences confirmed the that the G genotypes of these isolates were G3 and that they shared 99.8% to 100% nucleotide identities among the G3P[19] strains isolated in the same epidemic season and 93.2% to 93.4% identities of the strains in different epidemic seasons (data not shown).

Comparison of the VP7 nucleotide sequence of G3P[19] strains with those of G3 strains of human, porcine, and other animal origins, including bovine, caprine, simian, equine, lapine, and canine, revealed that they were most closely related (97.7% to 98.3% nucleotide identities) to PoRV G3 strains (CMP213 and CMP214) isolated in 2002 from the same geographical area (data not shown). However, it should be noted that the VP7 sequences of G3P[19] strains also shared a high level of nucleotide identity (92.2% to 93.7%) with bovine G3 reference strains (CP-1 and PP-1) (data not shown). On the contrary, nucleotide sequence identities with other porcine G3 strains (4F, 4S, A131, A411, and A138), human G3 strains (AU-1, YO, TK03, TK28, P, AU-17, MaCH09404, M, HCR3, B4106, and CMH222), and G3 strains of other animals were 85.0% to 88.6%, 81.9% to 88.0%, and 81.1% to 83.1%, respectively. The close genetic relationships of PoRV G3P[19] strains with other PoRV G3 strains isolated from the same geographical regions and with bovine rotavirus (BoRV) G3 strains (CP-1 and PP-1) were also reflected in the phylogenetic analysis (Fig. 2) in which our G3P[19] strains clustered tightly together with CMP213 and CMP214 PoRV strains.

Specificity of the newly designed primer for P[19]. As shown in Table 1, 33.3% of the strains with the P genotype of the porcine rotavirus identified in this study were P[19]. The data suggested that quite a high number of P[19] strains were circulating in this area. To our knowledge, there was no typing primer for P[19] available in the literature, and this explained why the P[19] rotavirus strains isolated in this study had been identified by VP4 nucleotide sequence analysis. For the sake of convenience and efficient identification of P[19] in a large number of clinical isolates, there was a need to develop a PCR-based genotyping method by designing a P[19]-specific primer used in conjunction with a Con2 primer.

The newly designed primer, designated VP4P19, was evaluated for its specificity by being tested with the 13 isolates of P[19] and 3 strains of P[13] isolated in this study, together with other P types, including P[3], P[4], and P[12] (data not shown).
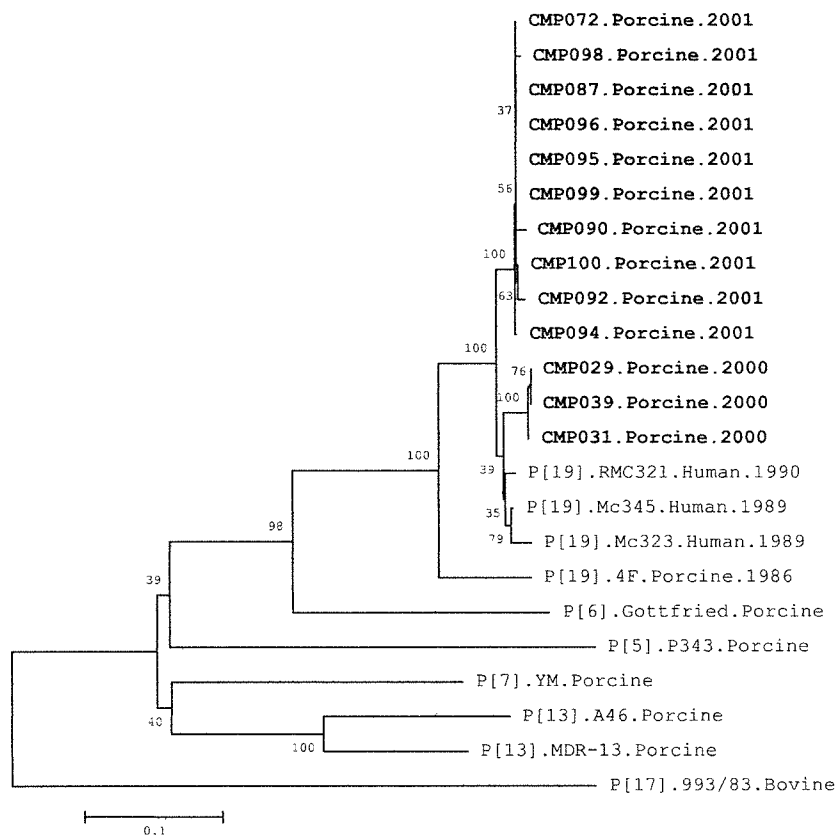
FIG. 1. Phylogenetic tree of VP4 nucleotide sequences of G3P[19] porcine rotavirus strains isolated from 2000 to 2001, demonstrating a close genetic relationship with human rotavirus reference strains (Mc323, Mc345, and RMC321) that were isolated from 1989 to 1990. Bootstrap values supporting some clusters are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

The results indicated that the VP4P19 primer specifically amplified the VP4 gene of all 13 isolates of P[19] with the expected PCR product size of 415 bp and that there was no cross-reaction with other P types tested thus far. Analysis of the 415-bp PCR products of the 3 representatives of the 13 P[19] isolates confirmed their P[19] specificities. In addition, the P[19] primer sequence was evaluated for its specificity by using the BLAST program and it was found that the P[19] primer sequence was identical only to human and porcine rotavirus P[19] strains. Furthermore, the P[19]-specific primer was used successfully for the detection of five G3P[19] isolates from diarrheic piglets in a subsequent surveillance from November 2001 to July 2003 (50). The data implied that the newly designed primer in this study might be useful for the PCR-based genotyping of P[19] strains circulating in this geographical area and perhaps for the P[19] strains in other areas as well.

DISCUSSION

The segmented nature of a rotavirus genome can undergo genetic reassortment between strains during mixed infections and lead to the generation of progeny viruses with novel or atypical phenotypes (42, 48). The isolation of unusual strains possessing a gene segment(s) of human and/or of heterologous animal origin suggests interspecies transmission and reassort-

ment between the viruses of humans and animals, as well as animals and animals in nature (7, 26, 38, 42, 55, 56). In this study, we reported 13 isolates of the rare G3P[19] porcine rotaviruses. Among 13 isolates of G3P[19] strains described in this study, 3 were isolated in the year 2000 and 10 in 2001, and these accounted for 33.3% of the group A rotaviruses isolated. The data indicated that the G3P[19] strains are currently circulating in a relatively high proportion in the pig population of the Chiang Mai area. Although rotavirus strain surveillances in a pig population have been performed extensively in various parts of the world, so far only one isolate of G3P[19], designated 4F, has been reported, from a diarrheic pig in China (39). The VP4 and VP7 genes of 4F have been analyzed, and strain 4F has been identified as a G3P[19] strain (2). In addition, the pathogenicity of 4F has been evaluated, and it was found that in the first three passages the 4F strain showed no significant pathogenicity in pigs but that it became highly pathogenic at passages four and five (2). However, the significance of the epidemiological impact of 4F is unknown. Here, we report the isolation of G3P[19] porcine rotavirus strains that caused severe diarrhea in piglets at several farms in Chiang Mai province, Thailand. The G3P[19] strains in the year 2000 were isolated from diarrheic piglets in farms located in Mae Rim district, which is about 65 kilometers from the farms in Mae Wang district, where the G3P[19] strains were isolated in 2001. The data indicate that G3P[19] strains are
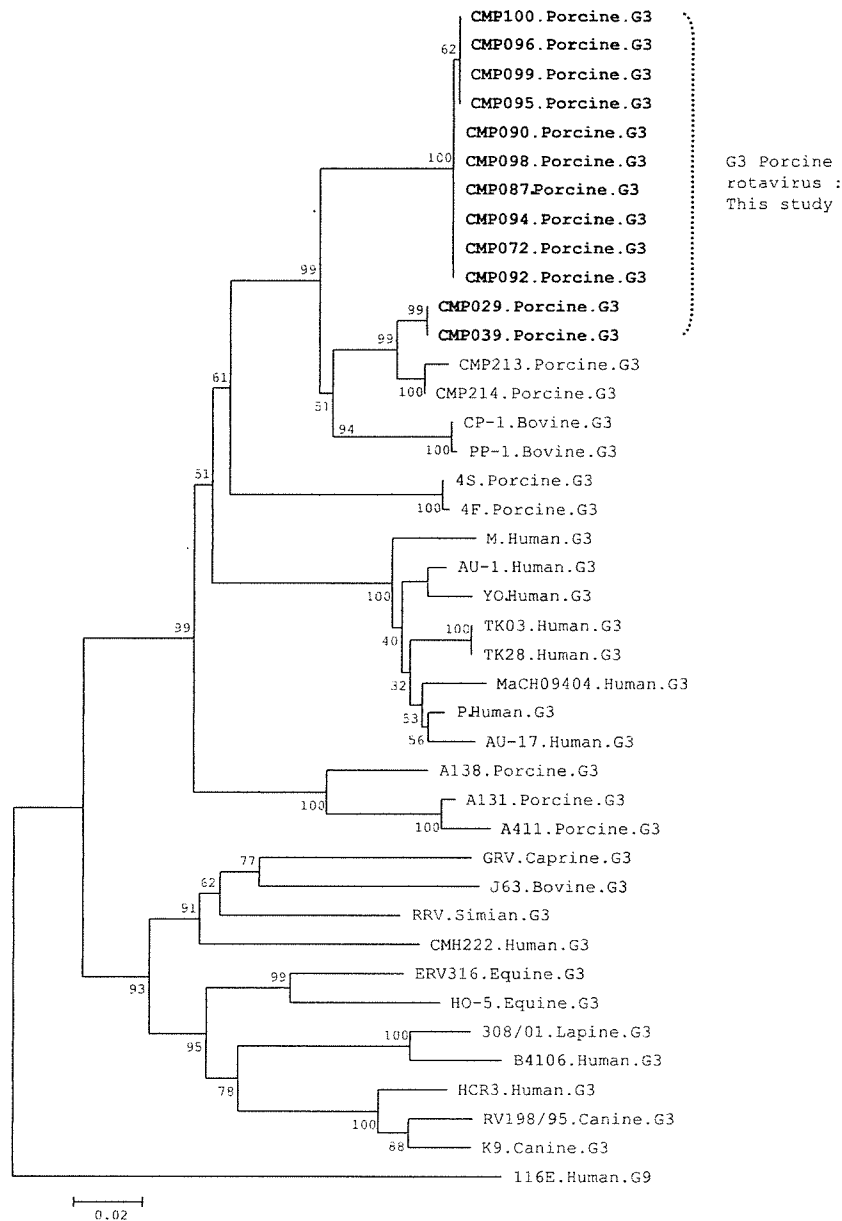
FIG. 2. Phylogenetic tree of VP7 nucleotide sequences of G3P[19] strains, demonstrating a close genetic relationship between G3P[19] strains isolated from 2000 to 2001 and PoRV G3 strains (CMP213 and CMP214) isolated in 2002 from the same geographical area. Bootstrap values supporting some clusters are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

currently circulating among the pig populations in several farms located at different distances around the area of Chiang Mai province.

Analysis of the VP4 genes revealed a high level of homology among viruses isolated in the same year, with nucleotide identities of 99.8% to 100% for strains isolated in the year 2000 and 98.4% to 100% for strains isolated in 2001. However, nucleotide sequence comparisons between the viruses isolated in the year 2000 and 2001 indicated that the nucleotide identities were slightly decreased (95.0% to 96.1%). The data imply that these strains belong to different sublineages of the same genotype and that the decrease of nucleotide identities among the isolates in the years 2000 and 2001 might be due to a genetic

evolutionary diversity of the viruses. Interestingly, the VP4 nucleotide sequence of these strains was most closely related with those of human P[19] strains (Mc323 and Mc345) which were isolated in 1989 from children admitted to a hospital in the same geographical area (Chiang Mai city), with nucleotide identities of 95.4% to 97.4%. Urasawa et al. (54) demonstrated, by RNA-RNA hybridization, that Mc323 and Mc345 were genetically more closely related to porcine than to human rotaviruses. Although sequence analyses of NSP1 (29) and VP4 (41) genes of Mc323 and Mc345 provided evidence suggestive of the porcine origin of these strains, direct evidence to support the origin of their VP4 genes remained undetermined. In this study, we could detect 13 isolates of G3P[19] porcine

rotavirus strains in the Chiang Mai area, where Mc323 and Mc345 were isolated. These porcine rotavirus strains accounted for 33.3% of all rotaviruses detected in this study and carried the VP4 gene of P[19] specificity that shared nucleotide sequence identities with Mc323 and Mc345 at a range of 95.4% to 97.6%. This finding provides peculiar evidence for verifying the origin of the VP4 genes of Mc323 and Mc345 rotavirus strains. It is possible that the VP4 genes, with a P[19] specificity of Mc323 and Mc345, might have derived through reassortment from PoRV G3P[19] or other P[19] PoRV strains that circulated in the region prior to the isolation of Mc323 and Mc345 from humans. However, the P[19] rotavirus strain was not detected in humans in the study carried out in the same epidemic season (28). In fact, Mc323 and Mc345 (G9P[19]) have been proposed as the porcine-human reassortant strains, since they possess many gene segments that are closely related to porcine rotaviruses (54), including NSP5 (29), while their VP7 genes are closely related to human G9 strains (41, 54). In addition, Mc323 and Mc345 exhibited a long RNA electropherotype with subgroup I specificity, a phenotype that is common to animal rotaviruses (54).

Analysis of the VP7 gene of our G3P[19] strains confirmed the initial assignment of their G genotype as G3 strains by PCR-based genotyping. Comparison of the VP7 nucleotide sequences of these strains revealed two clusters of G3P[19], one being strains isolated in 2000 (CMP029 and CMP039) and the other being strains isolated in 2001 (CMP072, CMP087, CMP090, CMP092, CMP094, CMP095, CMP096, CMP098, CMP099, and CMP100). Strains in the same cluster shared nucleotide sequence identities ranging from 99.8% to 100%, while strains from different clusters shared 93.2% to 93.4% (data not shown). The data imply that at least two clusters of G3P[19] strains were circulating in the pig population in the Chiang Mai area from 2000 to 2001. The phylogenetic analysis of the VP7 nucleotide sequences of these G3P[19] strains supports the detection of two clusters of G3P[19] strains in this study (Fig. 2). In addition, a comparison between the VP7 nucleotide sequences of these G3P[19] strains and those of other G3 strains of different P genotypes (CMP213 and CMP214), which were isolated in 2002 from the same geographical area, revealed nucleotide identities of 97.7% to 98.3% (data not shown). Moreover, phylogenetic analysis also revealed that CMP213 and CMP214 were grouped with the G3P[19] strains and showed the closest relationship with CMP029 and CMP039 (Fig. 2). The results indicated that the VP7 genes of G3P[19] strains most likely derived from those of porcine rotaviruses that circulated in the region. However, it is worthwhile to note that even though the VP7 genes of G3P[19] strains were most closely related to each other (99.8% to 100%) and to other G3 porcine rotaviruses (97.7% to 98.3%) circulated in the area, they also shared 92.2% to 93.7% nucleotide sequence identities with BoRV G3 strains CP-1 and PP-1, which were isolated from an outbreak of calf diarrhea in the United Kingdom in 1973 (12, 13, 58). The similarity of the G3P[19] VP7 gene with bovine G3 strains CP-1 and PP-1 might be due to the genetic diversity of the G3 VP7 gene, which naturally occurs among porcine rotaviruses. Since G3 is not common in bovines, the acquisition of the PoRV G3 VP7 gene by BoRV via genetic reassortment and the evolution of bovine

and porcine rotavirus G3 from a common ancestor cannot be ruled out.

Taken together, this study describes the detection of G3P[19], a rare porcine rotavirus strain, from diarrheic piglets in Chiang Mai, Thailand, which is the same geographical area where the Mc323 and Mc345 human rotaviruses were isolated. The finding provides peculiar evidence that supports the porcine origin of VP4 genes of Mc323 and Mc345 human rotaviruses.

ACKNOWLEDGMENTS

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Genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan in 2004–2005

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Abstract

A total of 752 fecal specimens collected from July 2004 to June 2005 from children with acute gastroenteritis in four localities in Japan (Maizuru, Tokyo, Sapporo, and Osaka) were screened for group A rotavirus by RT-PCR. It was found that 82 (10.9%) specimens were positive for group A rotavirus. The G-(VP7 genotypes) and P-(VP4 genotypes) types were further investigated. The P-types of 18 rotavirus strains, which could not be typed by RT-PCR, were determined by sequencing analysis. Of these, 94% (17/18) were P[8] with multiple point mutations at the VP4 primer-binding site. Another sample turned out to be a rare genotype P[9], which was closely related to feline rotavirus. The predominant genotype was G1P[8] (46.4%), followed by G3P[8] (32.9%) and G2P[4] (12.2%). A number of unusual combinations including, G1P[4] (1.2%), G2P[8] (1.2%), G3P[9] (1.2%), G1G3P[8] (1.2%), and G2G3P[8] (3.7%), were also detected. A new nomenclature of P[8] was proposed, in which worldwide rotavirus P[8] strains were classified into four sub-lineages, namely IA, IB, IIA, and IIB. A wide range of amino acid substitutions (up to 22) specific for P[8] lineages and sub-lineages were also identified. Interestingly, only short amino acid motifs located at positions 32–35, 121–135, and 195–236 of VP4 correctly defined the phylogenetic P[8] lineages and sub-lineages. Of note, at least two distinct clusters of rotavirus P[8] were co-circulating in the Japanese pediatric population studied.
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Keywords: Rotavirus; Genotype; Gastroenteritis; Japan

1. Introduction

Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality among infants and young children in both developed and developing countries (Murray and Lopez, 1997; Thapar and Sanderson, 2004). Among different enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals (Parashar et al., 2003; Mulholland, 2004). Rotaviruses are classified into seven groups (A–G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with groups A–C rotaviruses. Of these, group A rotavirus is the most important, being a major cause of severe

gastroenteritis in infants and young children worldwide (Murray and Lopez, 1997; Mulholland, 2004). Group A rotavirus is known to have the highest prevalence and pathogenicity, causing an estimated nearly one million deaths every year, predominantly in developing countries. Recent studies suggest that as global deaths from childhood diarrhea have decreased during the past two decades, the proportion of diarrhea hospitalizations attributable to rotavirus may have increased (Parashar et al., 2006).

Rotavirus, which is a member of the family Reoviridae, possesses a genome of 11 dsRNA segments that are enclosed in a triple-layered capsid. According to the antigenic and genetic diversity of two of rotavirus's outer capsid proteins, VP7 and VP4, P genotypes and G genotypes have been defined, respectively. In group A rotavirus, at least 15 G genotypes have been recognized by neutralization assay and 25 P genotypes have been identified by hybridization or sequence analysis. The four predominant rotavirus genotypes G1P[8], G2P[4], G3P[8], and G4P[8], comprise nearly 83% of all the rotavirus infections in the world (Estes, 1996; Kapikian et al., 2001). Of these, G1 is

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reported to be most common cause of acute gastroenteritis in most countries, followed by G2, G3, and G4 in various countries of Europe, North and South America, Africa, and Asia (Maneekarn and Ushijima, 2000; Kapikian et al., 2001; Santos et al., 2003; Okitsu-Negishi et al., 2004). Therefore, these genotypes are the targets for current vaccine development strategies. Unlike antimicrobial therapies that are effective against some bacterial and parasitic agents, no specific treatment for rotavirus infection is available; therefore, a rotavirus vaccine is sorely needed. The first human rotavirus vaccine, which was known as a human–animal tetravalent vaccine with limited genotypic cover (G1–G4), was licensed in the United States in August 1998. This vaccine was, however, withdrawn from use after an alleged association with cases of bowel intussusceptions (Mulholland, 2004). Due to genetic diversity in different parts of the world, knowledge of molecular epidemiology of rotavirus in circulation is important in the effort to develop a suitable and efficacious vaccine.

Therefore, the objectives of the present study were to: (1) determine the detection rate of rotavirus infections in children with acute gastroenteritis in four different localities in Japan in 2004–2005; (2) characterize the detected rotaviruses according to G- and P-types and (3) describe the genetic diversity among them.

2. Materials and methods

2.1. Fecal specimens

A total of 752 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics in four localities (Maizuru, Tokyo, Sapporo, and Osaka) in Japan from July 2004 to June 2005. Of these, 231 specimens were from Osaka, 51 from Sapporo, 24 from Tokyo, and 446 from Maizuru. The case definition of diarrhea used in this study was as at least three passings of unformed (loose and watery) stool a day. Acute gastroenteritis was defined as the occurrence of diarrhea and other symptoms such as vomiting, fever, and abdominal pain. The ages of the subjects ranged from 2 months to 15 years, with a median of 27 months. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at $10,000 \times g$ for 10 min. The supernatants were collected and stored at $-30\,^{\circ}\text{C}$ until use for the detection of group A rotavirus.

2.2. Extraction of viral genome

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

2.3. Reverse transcription

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, USA), dNTPs (10 mM/μl) (Roche, Mannheim, Germany), DTT (Invitrogen),

superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total 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 held at 4 °C (Phan et al., 2005).

2.4. Polymerase chain reaction (PCR)

Using PCR with specific primers as previously reported resulted in the identification of rotavirus. Primers Beg9 (5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3') and VP7-1' (5'-ACTGATCCTGTTGGCCATCCTTT-3') was used to amplify VP7 of rotavirus and specifically generated a size of amplicon of 395 bp (Phan et al., 2005). PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega) 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, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.5. Group A rotavirus G-typing

G-typing of group A rotavirus was preformed using the protocol from the method previously presented by Das et al. (1994). The full-length of the VP7 gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length VP7 gene was 1062 bp in length. 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) for downstream priming and 9con1 for upstream priming in an amplification of G1–G4 and G9 VP7 genes, respectively. These primers specifically generated five different sizes of amplicons: 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively. The samples whose G-type could not be identified by the first set of primers, described by Das et al. (1994) were then identified using another alternative set of type-specific primers previously reported by Gouvea et al. (1990).

2.6. Group A rotavirus P-typing

P-typing was conducted using the method modified from Gentsch et al. (1992). The RT-PCR was performed 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, ND2, and Con3 primers were utilized for the identification of P[8], P[4], P[6], P[9], P[10], and P[11] with six different sizes of amplicons of 346, 484, 268, 392, 584, and 123 bp, respectively.

2.7. Electrophoresis

PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr) for 20 min

then visualized under ultraviolet light, and the results were recorded by photography.

2.8. Nucleotide sequencing and phylogenetic analysis

The group A rotavirus isolate whose P-type could not be determined by RT-PCR method was then subjected to nucleotide sequence analysis. The nucleotide sequences of PCR products (DNA) positive for the rotavirus VP4 gene were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP4 nucleotide sequences were compared to each other as well as to those of reference rotavirus strains available in GenBank by BLAST. Sequence analysis was performed using CLUSTAL X software (Version 1.6). The phylogenetic tree with 100 bootstrap replicates of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). The sequences of group A rotavirus strains 6672/JP, 6226/Japan, and 6299/Japan, had been submitted to GenBank and had been assigned accession numbers DQ479964, DQ479965, and DQ479966, respectively. Reference group A rotavirus strains and their accession numbers used in this study were as follows: BP785/00/Hungary (AJ605315), VA70/USA (AJ540229), WA/USA (L34161), HOCHI/Japan (AB039943), ODELIA/Japan (AB039942), MW670/Malawi (AJ302146), OP530/Malawi (AJ302152), AI-75/Japan (AB008285), MW258/Malawi (AJ302143), OP511/Malawi (AJ302151), CH927B/China (AB008273), MO/Japan (AB008278), Kagawa/90-513/Japan (AB039944), OP601/Malawi (AJ302153), CU132P8/Thailand (DQ235955), DK V98-893/Denmark (AY509908), DK V00-2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), TF101/Taiwan (AF183870), Hun9/Hungary (AJ605320), WH-1194/China (AY856445), and WH-624/China (AY856444).

3. Results

3.1. Molecular epidemiology of group A rotavirus infection

Rotavirus was detected in 82 out of 752 (10.9%) specimens tested. The rotavirus detection rates were different between the four localities: 7.4% in Maizuru, 3.9% in Sapporo, 4.2% in Tokyo, and 19.9% in Osaka. Rotavirus was identified throughout the 6-month period from December 2004 to June 2005. However, none of rotaviruses was detected from July to November 2004. The rotavirus incidence was found to be highest in April (30.5%), followed by March (26.8%) and May (22%). The lowest rotavirus detection rate occurred in December (1.2%).

3.2. Distribution of G- and P-types with re-emergence of G1

The distributions of G- and P-types of group A rotavirus during the study period from July 2004 to June 2005 are shown in Table 1. Three different G-types, G1–G3, were detected. Of

Table 1
Distribution of different G-type and P-type of rotaviruses in Japan in 2004–2005

G-type/P-type	P[4]	P[8]	P[9]
G1	1 (1.2%)	38 (46.4%)	–
G2	10 (12.2%)	1 (1.2%)	–
G3	–	27 (32.9%)	1 (1.2%)
Mix G1G3	–	1 (1.2%)	–
Mix G2G3	–	3 (3.7%)	–

these, G1 was the most prevalent genotype (37.6%), followed by G3 (34.1%) and G2 (13.4%). When examined for their P-types, P[4] (13.4%) and P[8] (64.6%) were identified. However, there were 18 rotavirus isolates (22%) whose P-types could not be determined by RT-PCR using specific primers previously reported in the literature.

3.3. Nucleotide sequence analysis of P-nontypeable group A rotavirus isolates

Eighteen group A rotavirus isolates whose P-types could not initially be determined using the RT-PCR method, even though their VP4 genes were successfully amplified by RT-PCR. Therefore, their P-types were assigned based on nucleotide sequence analysis by direct sequencing of VP4 genes using the consensus Con3 as a sequencing primer. After sequence analysis, 94% (17/18) were P[8]. Fig. 1 revealed that these rotavirus P[8] isolates contained four point mutations at the VP4 primer-binding site. As many as 16 of 17 (94.1%) P[8] isolates approved by sequence analysis were highly homologous at the nucleotide level, ranging from 99% to 100%, and were highly homologous (98%) with the Malaysian P[8] strain WH-1194. On the other hand, another rotavirus P[8] isolate, the 6690/Japan strain, had a low homology (only 86%) at the nucleotide level with other rotavirus P[8] isolates detected in the study. The 6690/Japan strain shared the highest identity (99%) with the BP785/00, rotavirus P[8] strain from Hungary. Of note, another P-nontypeable isolate, the 6299/Japan, turned out to be P[9] by sequencing, which was a rare human P-type. Its VP4 nucleotide sequence shared greater homology with the feline rotavirus (100%) P[9] strain FRV-1 (accession number D14618) than with human P[9] reference strains, 95% for strain K8 (accession number D90260) and 94% for strain PA151 (accession number D14623).

nt 339	nt 356	Position of binding site
3'-GCACGTTATCCAAGTAGA-5'		Primer 1T1
******CGAT*C******		6672JP P[8]
******CGAT*C******		6226JP P[8]
T**C*C**C***		6690JP P[8]

Fig. 1. Alignment of the VP4 gene fragment of Japanese group A rotavirus isolates that were not typed by RT-PCR and the reverse complementary sequences of the original primer 1T-1. Residues that match primer 1T-1 are denoted by asterisks.

The frequencies of various combinations of the G- and P-types of rotavirus detected in this study were also investigated. G1P[8] was the most predominant combination (46.4%), followed by G3P[8] (32.9%), and G2P[4] (12.2%). A number of unusual combinations such as G1P[4], and G2P[8] were also detected.

3.4. A new nomenclature for group A rotavirus P[8] genotypes

In an attempt to understand the molecular basis of rotavirus genetic diversity within the P[8] genotype, partial sequences of the VP4 gene (VP8*) of P[8] isolates detected in this study and worldwide rotavirus reference P[8] strains were used for genetic analysis. Phylogenetic analysis identified two distinct lineages, I and II, which were further divided into sub-lineages

IA and IB, IIA and IIB (Fig. 2). It should be noted that sub-lineage IA consisted of both human rotavirus P[8] strains and the porcine rotavirus S8 strain. The high nucleotide homology of rotavirus strains within each sub-lineage ranged from 95% to 100%, indicating less than 5% of genetic difference among them. The nucleotide sequence divergence between sub-lineage IA and sub-lineage IB (within lineage I) was 11–14%. In contrast, the sequence variation among strains between sub-lineage IIA and sub-lineage IIB (within lineage II) was considerably lower, ranging from 7% to 9%.

3.5. Alignment of the partial amino acid sequences of VP4

Direct inspection of the partial sequence alignment of VP4 revealed that there are two kinds of amino acid
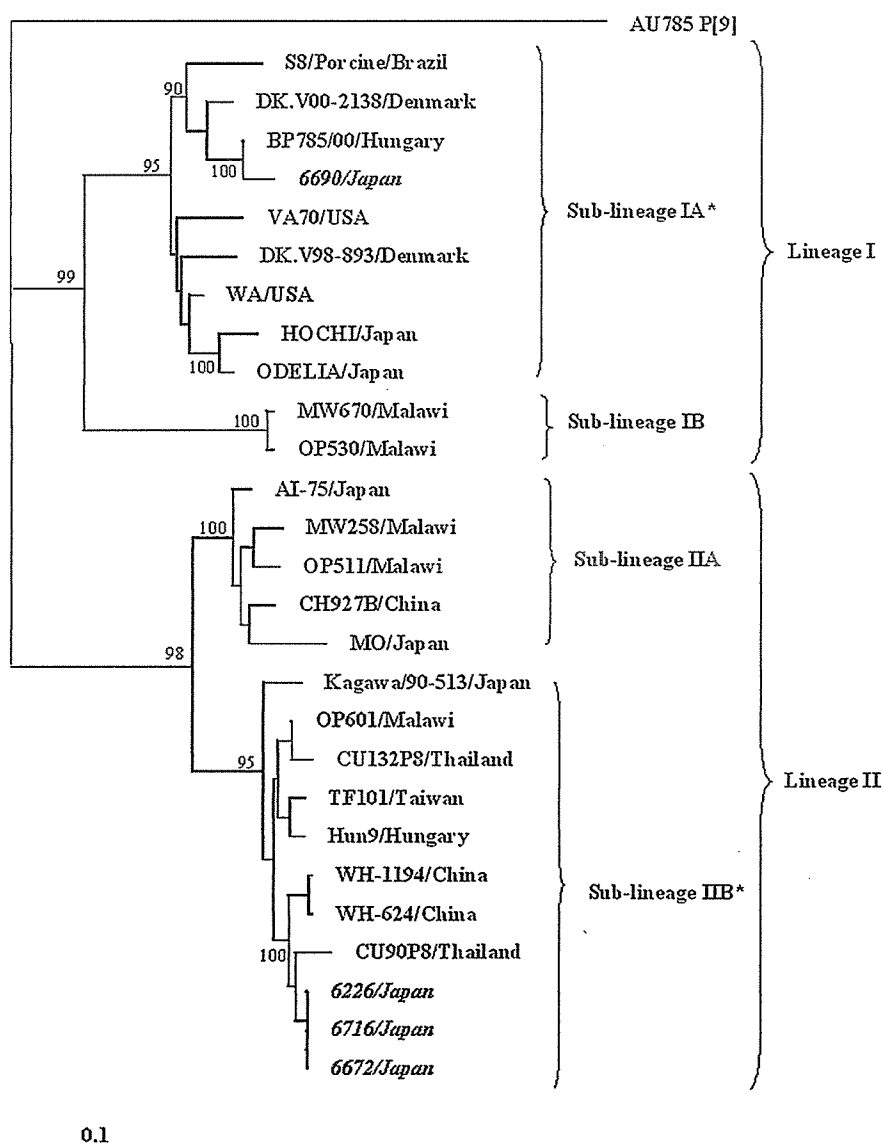
Fig. 2. Phylogenetic tree obtained from nucleotide sequences of the group A rotavirus P[8] VP8* gene. Reference group A rotavirus strains were selected from GenBank under the accession number indicated in the text. Japanese A rotavirus isolates detected are highlighted in italics. The AU785 P[9] strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. (*) Indicates the sub-lineage containing the rotavirus detected in this study.

Table 2
The amino acid substitutions from sequence data of the P[8] VP8* gene specific for lineages and sub-lineages. The amino acid substitutions highlighted in italics and in bold are specific for differentiation of lineages and sub-lineages, respectively. Shaded boxes indicate variable regions located at rotavirus VP8*. The phylogenetic lineage- and sub-lineage-defining sequences are indicated by a horizontal bar

Lineage	Sub-lineage	Position	32	35	55	78	108	113	120	121	125	135	150	178	182	184	187	191	192	194	195	216	221	236
		Amino acid	N	V	I	I	V	D	I	V	N	D	I	G	R	T	S	A	N	N	G	N	N	S
Lineage I	Sub-lineage IA	BP785/00	*	I	*	N	I	T	M	I	S	V	*	*	*	*	*	*	*	*	N	*	*	P
		S8 Porcine	*	I	*	N	I	N	M	I	S	A	*	*	*	*	*	*	*	*	N	*	*	P
		HOCHI	*	I	*	N	I	N	*	I	S	V	*	*	*	*	*	*	*	*	N	*	*	P
		ODELIA	*	I	*	N	I	N	*	I	S	V	*	*	*	*	*	*	*	*	N	*	*	P
		WA	*	I	*	N	I	N	*	I	S	V	*	*	*	*	*	*	*	*	N	*	*	P
		VA70	*	I	*	N	I	N	*	I	S	A	*	*	*	*	*	*	*	*	N	*	*	P
	Sub-lineage IB	MW670	D	*	V	K	*	*	*	I	S	A	*	K	G	V	G	S	D	T	S	I	V	*
		OP530	D	*	V	K	*	*	*	I	S	A	*	K	G	V	G	S	D	T	S	I	V	*
Lineage II	Sub-lineage IIA	MW258	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		OP511	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		CH927B	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		MO	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
	Sub-lineage IIB	CU90P8	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		WH-1194	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		WH-624	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		TF101	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		Hun9	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		OP601	*	*	*	*	*	N	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		CU132P8	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		Kagawa315	*	*	*	*	*	N	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6226/Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6716 Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6672 Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*

substitutions specific for lineages and sub-lineages (Table 2). For differentiation within lineages, three amino acid substitutions at positions 121, 125, and 135 of variable regions of VP8* were identified. Amino acid I, S, and N were specific for lineage I and amino acid V, N, and D were specific for lineage II. For differentiation within sub-lineages, up to 19 amino acid substitutions located at variable regions and conserved regions of VP8* were found. Of these, substitutions at 32 (D), 55 (V), 178 (K), 182 (G), 184 (V), 187 (G), 191 (S), 192 (D), 194 (T), 216 (I), and 221 (Y) were specific only for sub-lineage IB; substitutions at 35 (I), 108 (I), 113 (T or N), and 236 (P) for sub-lineage IA; substitution at 113 (S) for sub-lineage IIA. At positions 78

and 195, amino acid substitutions differed according to each sub-lineage, e.g., N for sub-lineage IA; K and S for sub-lineage IB.

Interestingly, only short signature sequences of VP4, which correctly defined the phylogenetic P[8] lineages and sub-lineages, were found. Three amino acids at positions 32, 35, and 55 formed an identification code of NII, DVV, and NVI for sub-lineages IA, IB and lineage II, respectively. Three amino acids at positions 121, 125, and 135 formed a code of ISN and VND for lineages I and II, respectively. Other amino acids at positions 195, 216, 221, and 236 also formed a code for sub-lineages, e.g., sub-lineage IA had a code of NNNP, and sub-lineage IB had a code of DNNS.

4. Discussion

In this study, the prevalence of rotavirus infection was 10.9% of children with acute gastroenteritis. This result was different from our previous report on rotavirus epidemiology in Japan from 2000 to 2003, with the detection rate ranging from 23% to 27% (Yoshinaga et al., 2006). This may result from the co-existence of multiple factors such as anti-rotavirus immunity in children, climate, water, and others, but direct evidence is lacking. In some reports, rotavirus was prevalent during the cold season, although several studies did not find a seasonal correlation (Nishio et al., 2000; Kang et al., 2002; Okitsu-Negishi et al., 2004). The findings in this study are in good agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of rotavirus infection was in the period of March and April (Zhou et al., 2003; Suzuki et al., 2005).

Extensive epidemiological studies of rotavirus infection worldwide including in Japan, which characterized rotavirus strains, indicated that G1 was the most prevalent genotype (Maneekarn and Ushijima, 2000; Kapikian et al., 2001; Santos et al., 2003; Zhou et al., 2003; Okitsu-Negishi et al., 2004). However, the emergence of new variant G2 and G3 was identified, and these strains became the leading genotypes in Japan in 2001–2004 (Yoshinaga et al., 2006; Phan et al., in press). At the same time, the prevalence of G1 rapidly dropped from 86% in 1998–1999 to 6% in 2002–2003 (Yoshinaga et al., 2006) and no G1 was found in 2003–2004 (Phan et al., in press). In this study, the changing pattern of the G-type distribution of rotavirus infection in children with acute gastroenteritis has been demonstrated. Of note, the G1 genotype re-emerged as the most prevalent with a high frequency (47.6%) compared to the lower frequency of G3 (34.1%) and G2 (13.4%), which were the second and third prevailing genotypes, respectively. The insufficient antibody protection from acquired viral immunity against G1 in a Japanese pediatric population due to the lack of a trigger of the previous G1 rotavirus infection during 2001–2004 was hypothesized. This hypothesis was in strong agreement with recent findings that the detection rate of G1 infection was very low during 2001–2004 (Yoshinaga et al., 2006; Phan et al., in press). Moreover, the common genotype G4 and the emerging genotype G9 were not detected in this epidemic season. Unlike the G-typing success of all rotaviruses, there were 18 rotavirus isolates whose P-types could not be determined by RT-PCR with specific primers. Therefore, their P-types were further approved by sequence analysis. After sequence analysis, 94% (17/18) had the closest relatives among rotavirus P[8] strains, demonstrating that our isolates belonged to P[8] genotype. These rotavirus P[8] isolates were found to contain four mismatches at the VP4 primer-binding site. Quite possibly, the initial failure in identifying a considerable fraction of the rotavirus P isolates in our study was due to those point mutations. Moreover, one P-nontypeable isolate turned out to be P[9], which is known to be a rare human P-type. Remarkably, this isolate shared the greatest homology (100%) of the VP4 gene nucleotide sequence with feline rotavirus strain FRV-1. This finding is additional evidence to support the notion that interspecies transmission of rotaviruses might be taking place in nature.

To gain further insights into the genetic variability within the P[8] genotype, VP8* of rotavirus P[8] strains were used for genetic analysis. The alignment of a partial amino acid sequence we compiled from a number of rotavirus P[8] strains detected in different parts of the world demonstrated two distinct lineages, I and II, which were further divided into sub-lineages IA and IB, IIA and IIB. Another interesting finding of this study was the discovery of a wide range (up to 22) of amino acid substitutions, which were specific for lineages and sub-lineages. These changes were exclusively present in only one or two lineages and/or sub-lineages, but they are absent in the other lineages and/or sub-lineages. Of these, as many as 14 amino acid positions were located at variable regions of VP8*. Interestingly, eight more positions were identified at the conserved sites of VP8*. At two positions, 78 and 195, amino acid substitutions consistently changed according to each sub-lineage. Consistent with a previous report (Maunula and von Bonsdorff, 1998), a short motif located in amino acids 121–135 of the VP4 variable region was found to differentiate rotavirus P[8] strains into phylogenetic lineages. The identification of two new amino acid motifs at positions 32–35 and 195–236 of VP4 conserved regions should be noted. These new signature motifs also correctly divided rotavirus P[8] strains not only into phylogenetic lineages but also into phylogenetic sub-lineages.

Based on the novel nomenclature of the P[8] genotype, at least two distinct rotavirus P[8] clusters (sub-lineages IA and IB) were co-circulating in the Japanese pediatric population studied. Sub-lineage IA contained both human rotavirus P[8] strains and the porcine rotavirus S8 strain from Brazil. This porcine rotavirus strain shared the high identities, ranging from 95% to 96%, at the nucleotide and the amino acid levels with other human rotavirus P[8] strains, including the 6690/Japan in this study. Taken together, in view of rotavirus evolution, a genomic relation might exist between human and porcine, and between human and feline strains.

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ARTICLE

Emergence of rare sapovirus genotype among infants and children with acute gastroenteritis in Japan

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Abstract A total of 1,154 fecal specimens from infants and children with acute gastroenteritis in five cities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka), collected from July 2003 to June 2005, were tested for the presence of diarrheal viruses by reverse transcriptase multiplex PCR. Overall, 469 of 1,154 (40.6%) were positive for diarrheal viruses, of which 49 (10.4%) were positive for sapovirus. The peak of sapovirus infection shifted from April–June in 2003–2004 to October–December in 2004–2005. The observations show that maximum sapovirus prevalence can occur during warmer seasons. Sapovirus was subjected to molecular genetic analysis by sequencing. The results indicated that sapovirus genogroup I was a dominant group (100%). Sapovirus strains detected in this study were further classified into four genotypes (GI/1, GI/4, GI/6, and GI/8). Of these, sapovirus GI/1 was the most predominant, followed by sapovirus GI/6; these accounted for 93% (13 of 14) and 7% (1 of 14), respectively, in 2003–2004. However, it was noteworthy that sapovirus GI/6 suddenly emerged to become the leading genotype, accounting for 77% (27 of 35) of isolates in 2004–2005. This is believed to be the first report of the changing distribution of sapovirus genotypes and of the emergence of the rare sapovirus GI/6.

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Introduction

Acute gastroenteritis is a common illness in humans worldwide and has a great impact on public health [1]. It is recognized as one of the leading causes of death by infectious disease [1, 2]. The mortality of infants and children due to acute gastroenteritis is greater in developing than in developed countries [3]. While rotavirus is recognized as the most important cause of severe gastroenteritis in infants and young children worldwide, sapovirus (SaV) is also considered a significant global enteropathogen of acute gastroenteritis [4–7].

SaV, previously referred to as Sapporo-like virus, is a distinct genus within the family *Caliciviridae*. The prototype strain of SaV is the Sapporo virus (Hu/SaV/Sapporo virus/ 1977/JP), which was derived from an outbreak in a home for infants in Sapporo, Japan, in 1977 [8]. SaV has a positive-sense single-strand RNA genome surrounded by an icosahedral capsid. The SaV genome is 7.3–7.5 kb long and contains two main open reading frames. The open reading frame 1 encodes a polyprotein that undergoes protease processing to produce several nonstructural proteins, including an RNA-dependent RNA polymerase and a capsid protein. The open reading frame 2 encodes a small basic protein with unknown function [8]. On the basis of the sequence analysis of the capsid gene, SaV is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans [9–12]. Recently, the diversity of SaV was described by Akihara et al. [13], who found that SaV genogroup I and genogroup II could be classified into eight and five genotypes, respectively. Immunological and seroepidemiologic studies have indicated a worldwide distribution of SaV. The age-related prevalence of antibody against this virus also has shown that infections commonly occur in children under 5 years of age. Furthermore, it was

found that serum antibody level to SaV was lowest in the first year of life but increased after 2 years of age [14–16].

The objectives of this study were to determine the incidence of diarrheal virus infections in infants and children with acute gastroenteritis in five different cities in Japan during 2003–2005; to characterize the SaV detected according to genogroup and genotype; and to describe the genetic diversity among them. Additionally, the age-related distribution and the seasonal pattern of SaV infection were determined.

Materials and methods

Fecal specimens

A total of 1,154 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics from five different cities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2005. The ages of the subjects ranged from 2 months to 15 years, the median age being 28 months. This age distribution in the periods 2003–2004 and 2004–2005 was similar. The fecal specimens were suspended in distilled water to prepare 10% suspensions, which were clarified by centrifugation at 10,000×g for 10 min; the supernatants were stored at −30°C.

Extraction of viral genome

The genomes of both RNA and DNA viruses were extracted from 140 µl of 10% fecal suspensions using a single QIAamp spin-column kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Reverse transcription

In reverse transcription (RT), 4 µl of extracted viral genome was added to a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/µl) (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (1 µg/µl) (Takara, Shiga, Japan), RNase inhibitor (33 U/µl) (Toyobo, Osaka, Japan), and MilliQ water. The total volume of reaction mixture was 8 µl. The RT step was carried out at 50°C for 1 h, then 99°C for 5 min, after which it was stored at 4°C [17].

Viral detection and polymerase chain reaction

Two multiplex polymerase chain reaction (PCR) procedures using mixtures of primers previously reported were used to identify two groups of diarrheal viruses. The first group includes astrovirus, norovirus (GI, GII), and SaV, and the second group includes group A, B, and C rotaviruses and adenovirus [17]. In the first multiplex PCR, the primer set included equimolar mixes of PreCAP1 and 82 b for astrovirus, G1SKF and G1SKR for norovirus genogroup I, COG2F and G2SKR for norovirus genogroup II, and SLV5317 and SLV5749 for SaV. The respective products of these reactions consisted of amplicons of 719, 330, 387, and 434 bp for astrovirus, norovirus (GI, GII), and SaV, respectively. In the second multiplex PCR, the primer set included equimolar mixes of Beg9 and VP7-1, B5-2 and B3-3, and G8NS1 and G8NA2 for group A, B, and C rotaviruses, respectively, and Ad1 and Ad2 for adenovirus. The respective products of these reactions consisted of amplicons of 395, 814, 352, and 482 bp for group A, B, and C rotaviruses and adenovirus, respectively. The PCR was performed at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min, after which the products were stored at 4°C.

Genotype of group A rotavirus

Genotyping of group A rotavirus was conducted using the G-type specific primers from the method described previously by Das et al. in 1994 [18]. The full-length of *VP7* gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length *VP7* gene was 1,062 bp in length. 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) for downstream priming and 9con1 for upstream priming in an amplification of *VP7* genes of G1–G4 and G9, respectively. These primers specifically generated five different sizes of amplicons of 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, after which they were stained with ethidium bromide for 20 min and then examined under ultraviolet light. The results were photographed.

Latex agglutination test

The Diarlex test (Orion Diagnostica, Espo, Finland), a commercial latex agglutination test, was used for the detection of group A rotavirus infection as a confirmatory test in the fecal specimens that were found by RT-PCR to harbor coinfection with SaV and group A rotavirus. A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and the reaction was observed after 2 min. Development

of distinct agglutination in the Diarlex reagent was considered a positive result. If agglutination was seen in the negative control latex, the result was considered uninterpretable.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of RT-PCR products (DNA) positive for SaV and norovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Clustal X software, version 1.6. A phylogenetic tree with 1,000 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with Clustal X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). The sequences of SaV strains detected in the present study—5786/Osaka/JP, 5800/Osaka/JP, 5836/Osaka/JP, 5862/Osaka/JP, 5862/Osaka/JP, and 5821/Osaka/JP—had been submitted to GenBank and had been assigned accession numbers AB242322, AB242323, AB242324, DQ401095, AB242325, and AB242326, respectively. Reference SaV strains and accession numbers used in this study were as follows: PEC (AF182760), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruiseship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Parkville/94 (U73124), Sapporo/82 (U65427), Manchester/93 (X86560), Karachi/730/1992 (AB126249), Karachi/874/1992 (AB181129), Karachi/928/1994 (AB181128), Karachi/1017/1990 (AB181227), Karachi/876/1993 (AB181132), Houston/90 (U95644), Stockholm/97 (AF194182), 12/DCC/Tokyo/Japan/44 (AB236380), Karachi/872/1991 (AB181231), 4408/Maizuru/JP (AB180209), 4724/Osaka/JP (AB180212), and Mex14917/2000 (AF435813).

Results

Molecular epidemiology of viral infections

RT-PCR analysis revealed the presence of viruses in 469 fecal specimens (Table 1). The ages of the infants and children with viral gastroenteritis ranged from 2 months to 11 years. The highest rate of viral infection was in the age group 12–23 months (42%) and the lowest rate was in the age group <6 months (2%). There was no difference in age distribution between the periods 2003–2004 and 2004–2005. In 2003–2004, group A rotavirus was the most prevalent (43.3%, 78 of 180), followed by norovirus (GI and GII) (29.5%, 53 of 180). Interestingly, norovirus (GI and GII) dominated over group A rotavirus and became the leading cause of acute gastroenteritis in 2004–2005, accounting for 45.3% (131 of 289) of cases. Moreover, a

Table 1 Distribution of viral infection in infants and children with acute gastroenteritis in five cities in Japan

Year	No. of specimens tested	No. (%[a]) positive for viruses	Monoinfection (%[b]) RV A	B	C	NoV I	II	SaV	Ade	AsV	Mixed infection (%[b]) Ade+ AsV	SaV+ RA	Ade+ NoV GII	RA+ NoV GII	Ade+ RA	Ade+ SaV+ NoV GII	SaV+ AsV+ RA
2003–2004	402	180 (44.8)	78 (43.3)	0 (0)	1 (0.6)	1 (0.6)	52 (28.9)	12 (6.7)	22 (12)	5 (2.8)	1 (0.6)	2 (1.1)	3 (1.7)	2 (1.1)	1 (0.6)	0 (0)	0 (0)
2004–2005	752	289 (38.4)	77 (26.6)	0 (0)	0 (0)	2 (0.7)	129 (44.6)	30 (10.4)	32 (11.2)	8 (2.8)	0 (0)	0 (0)	1 (0.3)	2 (0.7)	1 (0.3)	5 (1.7)	2 (0.7)
Total	1,154	469 (40.6)	155 (33)	0 (0)	1 (0.2)	3 (0.6)	181 (38.6)	42 (9)	54 (11.5)	13 (2.8)	1 (0.2)	2 (0.4)	4 (0.9)	4 (0.9)	2 (0.4)	5 (1.1)	2 (0.4)

RV rotavirus, NoV norovirus, SaV sapovirus, Ade adenovirus, AsV astrovirus, NoV GI norovirus genogroup I, NoV GII norovirus genogroup II, RA group A rotavirus

[a] Refers to total number of specimens tested

[b] Refers to number of viral-positive specimens tested

Fig. 1 Monthly distribution of SaV infection in infants and children with acute gastroenteritis in five different cities of Japan, July 2003 to June 2005. The mean temperature in Japan during the molecular epidemiology study of SaV infection is shown
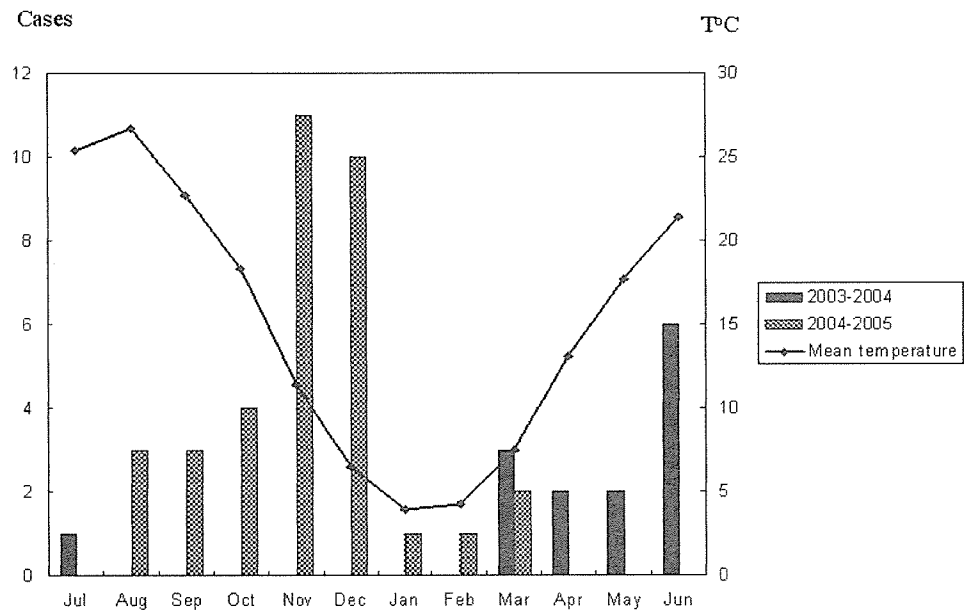
rather high rate (4.3%, 20 of 469) of viral coinfections was shown in this study during 2003–2005. It was found that the number of SaV infections increased sharply from 14 cases in 2003–2004 to 35 cases in 2004–2005. The incidence of SaV was highest in the 12–23-month-old group (45%, 22 of 49) and lowest in infants aged <6 months (2%, 1 of 49). Of note, most of the SaV infections (82%, 40 of 49) occurred in infants and children <3 years of age.

Seasonal variation of sapovirus infection

A comparison of rate of detection of SaV between the periods 2003–2004 and 2004–2005 is shown in Fig. 1. The monthly mean temperature in the five cities in Japan is also shown. The summer lasts from June to September, and the hottest temperature was recorded in August. The coldest month is January, when the temperature might dip as low as

4°C. The incidence of SaV was highest in April–June (ten cases) and was second highest in January–March (three cases). The lowest rate of detection was recorded in October–December (zero cases) in 2003–2004 ($p<0.001$). In contrast, SaV infection was identified continuously from August to February in 2004–2005, and the highest number of cases was recorded in October–December (25 cases), followed by July–September (six cases) ($p<0.001$). The peak of SaV infection shifted from April–June in 2003–2004 to October–December in 2004–2005.

Coinfection with sapovirus and other viral enteropathogens

In total, 49 fecal specimens were determined to be positive for SaV by RT-PCR. Seven (14%) of these specimens harbored coinfection with other viral enteropathogens: norovirus in five cases and group A rotavirus in two. The

Table 2 Characteristics of seven cases of mixed infection with SaV and other enteropathogens among the Japanese pediatric population

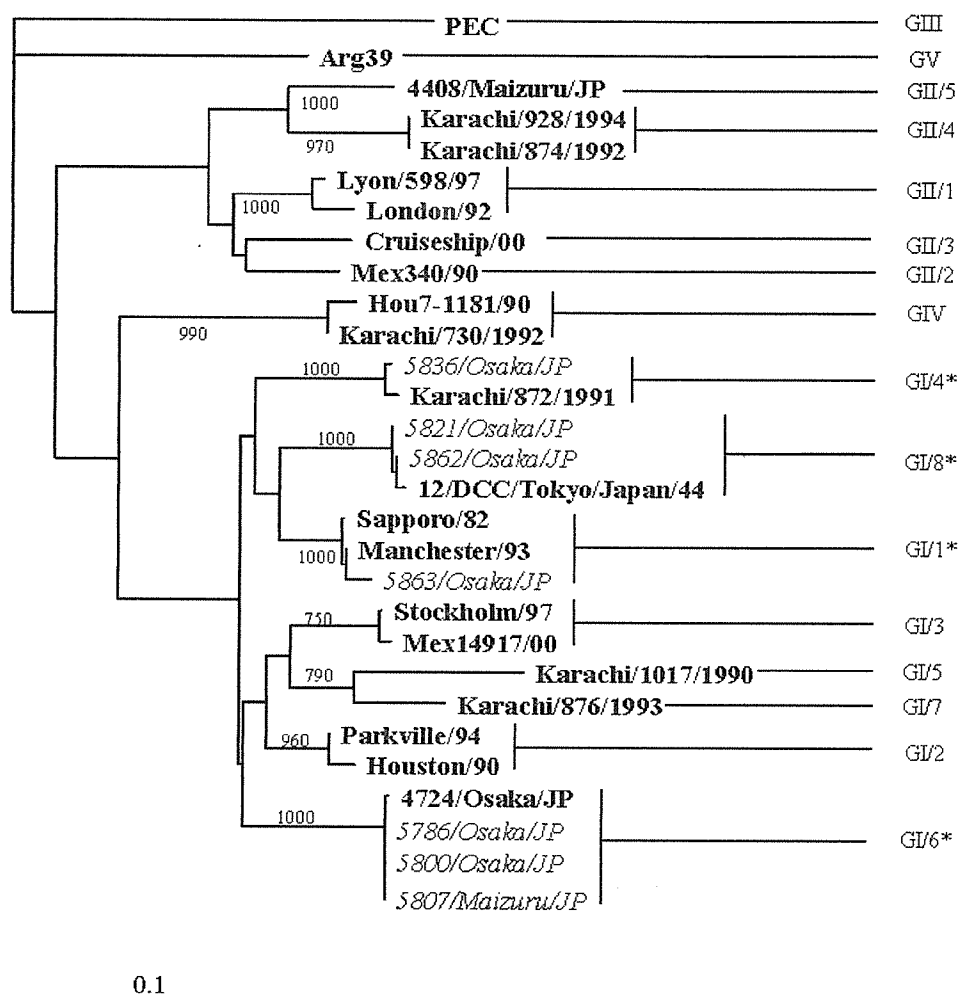| Case no.	Patient no.	Sex	Age (months)	City	Month	Year	Sapovirus		Other diarrheal viruses detected			
							Genogroup	Genotype	RT-PCR	Diarlex[a]	Sequence[b]	Genotype[c]
1	5,060	F	14	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
2	5,299	F	17	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
3	5,720	F	46	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
4	5,721	F	31	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
5	5,735	M	49	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
6	5,797	F	10	Osaka	Oct	2004	I	6	Norovirus	ND	GII/3	ND
7	5,806	M	11	Osaka	Nov	2004	I	6	Norovirus	ND	GII/4	ND

ND not done
[a] Used only to detect group A rotavirus
[b] Used only to determine genotype of norovirus
[c] Used only to genotype group A rotavirus

Fig. 2 Phylogenetic tree of nucleotide sequences of SaV strains detected in infants and children with acute gastroenteritis in five different cities in Japan. All of the SaV sequences were classified into only one distinct genogroup (I), and the SaV genogroup I sequences were clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8). The tree was constructed from partial amino acid sequences of the capsid region of SaV strains. Reference SaV strains were selected from GenBank under the accession numbers indicated in the text. Japanese SaV is highlighted in *italic*. The PEC strain was used as an outgroup strain for phylogenetic analysis. The scale indicates amino acid substitutions per position. The numbers in the branches indicate the bootstrap values.
*Genotype contains Japanese SaV detected in the study

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                            ┌──────────────── PEC ────────────────────────  GIII
                            │──────── Arg39 ───────────────────────────────  GV
                            │        ┌──── 4408/Maizuru/JP ───────────────  GII/5
                            │   1000 │   ┌ Karachi/928/1994 ┐
                            │        │970 └ Karachi/874/1992 ┘──────────────  GII/4
                            │   ┌──── Lyon/598/97 ┐
                            │1000└──── London/92   ┘─────────────────────────  GII/1
                            │        ┌──── Cruiseship/00 ────────────────────  GII/3
                            │        └──── Mex340/90 ────────────────────────  GII/2
                   990      │   ┌── Hou7-1181/90 ┐
                            │   └ Karachi/730/1992 ┘─────────────────────────  GIV
                            │ 1000  ┌ 5836/Osaka/JP ┐
                            │       └ Karachi/872/1991 ┘──────────────────────  GI/4*
                            │ 1000  ┌ 5821/Osaka/JP
                            │       │ 5862/Osaka/JP
                            │       └ 12/DCC/Tokyo/Japan/44 ──────────────────  GI/8*
                            │ 1000 ┌ Sapporo/82
                            │      │ Manchester/93 ──────────────────────────  GI/1*
                            │      └ 5863/Osaka/JP
                            │ 750 ┌ Stockholm/97
                            │     └ Mex14917/00 ────────────────────────────  GI/3
                            │ 790 ┌──── Karachi/1017/1990 ─────────────────  GI/5
                            │     └──── Karachi/876/1993 ───────────────────  GI/7
                            │ 960 ┌ Parkville/94 ┐
                            │     └ Houston/90   ┘────────────────────────────  GI/2
                            │ 1000 ┌ 4724/Osaka/JP
                            │      │ 5786/Osaka/JP
                            │      │ 5800/Osaka/JP ─────────────────────────  GI/6*
                            │      └ 5807/Maizuru/JP
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0.1

five coinfections with norovirus were detected in Osaka and the two with group A rotavirus in Maizuru. All diarrheal viruses detected as coinfections with SaV (norovirus and group A rotovirus) were confirmed and further characterized by G typing, latex agglutination test, or sequencing analysis. The five noroviruses belonged to genogroup I, genotype 4 in four cases and genogroup I, genotype 3 in one case, while both group A rotaviruses were identified as G3 genotype 3 (Table 2).

Nucleotide sequencing and phylogenetic analysis of sapovirus

The PCR products of SaV were sequenced in order to further characterize the genetic relationship among the SaV strains detected in infants and children with acute gastroenteritis in Japan. The sequence of the 5' end of the *SaV* capsid gene was targeted for genotyping [10, 13]. Their partial amino acid sequences were compared to each other as well as to those of reference SaV strains available in GenBank by BLAST. A total of 49 SaV sequences were

analyzed by phylogenetic analysis and were grouped using the recent SaV capsid region classification scheme of Akihara et al. [13]. All of SaV sequences clustered into a single genogroup, genogroup I (GI). Most of the SaV GI sequences (93%, 13 of 14) in 2003–2004 belonged to genotype 1 (SaV GI/1) (typified by the Manchester virus cluster). Only one SaV GI sequence (7%, 1 of 14) clustered

Table 3 Distribution of SaV genotypes based on the sequencing genetic analysis among infants and children with acute gastroenteritis in five different cities in Japan

Date of fecal specimen collection	No. of specimens positive for sapovirus	No. (%) identified as sapovirus genogroup I			
		Genotype 1	Genotype 4	Genotype 6	Genotype 8
July 2003– June 2004	14	13 (93)	0 (0)	1 (7)	0 (0)
July 2004– June 2005	35	4 (11)	2 (6)	27 (77)	2 (6)

into the 4724/Osaka/JP virus cluster (known as SaV GI/6) (Fig. 2). In 2004–2005, SaV strains were classified into four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). Interestingly, SaV GI/6 emerged as the predominant genotype in 2004–2005, representing 77% (27 of 35) of the strains, followed by SaV GI/1 (11%, 4 of 35) (Table 3).

Discussion

SaV infection causes acute gastroenteritis in all age groups, through it occurs predominantly in infants and young children [5, 6, 19, 20]. Overall, 49 of 1,154 fecal specimens tested were positive for SaV, and positive specimens were found in all age groups of the subjects included in the study. However, most (82%) of the SaV infections occurred in infants and children <3 years of age. These results were in line with previously published reports on SaV epidemiology worldwide, in which SaV prevalence was shown to range from 0.3 to 9.3%, far below the prevalence of either rotavirus or norovirus [19–22]. Our findings also confirmed SaV as one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to surveillance on pediatric cases of viral gastroenteritis in Japan, the main peak of SaV infection was in winter [6, 9]. Interestingly, the highest number of SaV infections in our study was identified in the April–June period in 2003–3004 and in the October–December period in 2004–2005. The observations show that maximum SaV prevalence can occur during warmer seasons.

To date, coinfections with various enteric viruses have been widely reported [12, 20]. Interestingly, we found a rather high rate (14%, 7 of 49) of coinfections with SaV and other viral pathogens. Coinfection of SaV with rotavirus and with norovirus was confirmed by the Diarlex test and G typing. These results underscore that coinfection with SaV and other enteropathogens is not rare.

All of the Japanese SaV sequences belonged to only one SaV genogroup I with four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). The findings clearly indicated that SaV GI was the dominant group to cause acute gastroenteritis among the pediatric population in Japan. In strong agreement with previous reports [7, 10, 13], SaV strains most frequently detected in sporadic gastroenteritis in infants and children in 2003–2004 belonged to the Manchester cluster (GI/1). Of note, SaV GI/6 strains were the most predominant (77%) in 2004–2005 and closely homologous to each other, suggesting that they came from the same source of infection. Moreover, only two SaV GI/6 strains were found in 2002–2003 during a 7-year (1996–2004) survey of SaV infection in diarrheal fecal specimens from Japanese infants and children [20]. In the present study, only one SaV GI/6 was detected in 2003–2004. Taken together, there was an

emergence of rare SaV GI/6, and this was the first evidence of the changing distribution of the SaV genotype in association with acute gastroenteritis in Japan. This sudden emergence of SaV GI/6 indicates that the pediatric population in Japan might lack antibody protection to these strains and that these rare strains could be more virulent than those usually associated with pediatric gastroenteritis. Continuous surveillance of SaV infection in Japan is recommended in order to determine whether these rare strains remain dominant in the coming years.

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