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Outbreak of Sapovirus Infection Among Infants and Children With Acute Gastroenteritis in Osaka City, Japan During 2004–2005

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One hundred and twenty five fecal specimens were collected from sporadic cases of acute gastroenteritis in a pediatric clinic in Osaka City, Japan from July 2004 to June 2005 and tested for the presence of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus by RT-multiplex PCR. Among diarrheal viruses detected, norovirus was the most prevalent (19.2%, 24 of 125), followed by group A rotavirus (18.4%, 23 of 125), astrovirus (1.6%, 2 of 125), and adenovirus (0.8%, 1 of 125), respectively. Interestingly, sapovirus infection was identified with high incidence of 17.6% (22 of 125). Sapovirus was subjected to molecular genetic analysis by sequencing. It was found that sapovirus detected in this study was classified into four genotypes (GI/1, GI/4, GI/6, and GI/8), and sapovirus GI/6 was predominant, followed by GI/8 and accounted for 81.8% (18 of 22) and 9.1% (2 of 22), respectively. It was noteworthy that sapovirus GI/6 infection was apparently confined within the period of 5 months (August 2004 through December 2004). This pattern of infection implied the outbreak of sapovirus GI/6 in these patients, which was the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City. Another interesting feature of the study was the appearance of two novel, naturally occurring recombinant sapoviruses circulating in the Japanese population studied. This report confirmed the presence as well as the importance of sapovirus causing acute gastroenteritis among Japanese infants and children. ***J. Med. Virol. 78:839–846, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: sapovirus; Osaka; outbreak

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths [Murray and Lopez, 1997; Parashar et al., 2003a]. Among different types of diarrheal viruses, rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [Parashar et al., 2003b]. Sapovirus, however, is also considered to be a significant global enteropathogen in association with sporadic cases as well as outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing home for the elderly [Chiba et al., 1979; Matson et al., 1989; Chiba et al., 2000; Akihara et al., 2005; Zintz et al., 2005]. Etiologic studies of acute gastroenteritis in infants conducted in Japan with RT-PCR showed that sapovirus was one of the most common causes of outbreaks of viral gastroenteritis among infants in Sapporo [Chiba et al., 1979, 2000]. Immunological and seroepidemiological studies have indicated a worldwide distribution of sapovirus [Sakuma et al., 1981; Nakata et al., 1985; Lopman et al., 2002]. The age-related prevalence of antibody against this virus also has shown that infections commonly occur in children less than 5 years old. It was also found that serum antibody level to sapovirus was lowest in the first year of

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life, increasing after 2 years of age [Matsui and Greenberg, 2000].

Norovirus (formerly known as "Norwalk-like virus") and sapovirus (formerly known as "Sapporo-like virus") are distinct genera within the family *Caliciviridae*. These viruses contain a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2, and 3). The ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF 2 encodes the capsid proteis and ORF3 enodes a small capsid protein. In the two sapovirus ORFs (ORF 1 and 2), however, it is ORF 1 that encodes the non-structural as well as the capsid proteins while ORF2 encodes a small protein. The prototype strain of sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was discovered originally during an outbreak a home for infants in Sapporo, Japan in 1977 [Chiba et al., 1979]. Sapovirus has a typical "Star of David" configuration by electron microscopy (EM), and sapovirus strains are antigenically identical to each other by immune EM [Chiba et al., 2000]. Sapovirus is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans. Recently, the diversity of sapoviruses was described in which genogroup I and II sapoviruses could be classified into eight and five genotypes, respectively [Farkas et al., 2004; Akihara et al., 2005; Phan et al., 2005b].

The objectives of this study were: to determine the incidence of diarrheal virus infection in infants and young children with acute gastroenteritis in Osaka City, Japan; to characterize the detected sapovirus according to genogroup and genotype; and to analyze the genetic diversity among these viruses. Additionally, the age-related distribution and seasonal pattern of sapovirus infection were also studied.

MATERIALS AND METHODS

Fecal Specimens

One hundred twenty-five fecal specimens were collected from sporadic cases of acute gastroenteritis in a pediatric clinic in Osaka City, Japan during the period of July 2004 to June 2005. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000*g* for 10 min. The supernatants were collected and stored at −30°C until use for the detection of diarrheal viruses.

Extraction of Viral Genome

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

Reverse Transcription (RT)

For reverse transcription (RT), except for adenovirus, 4 μl of extracted viral genome was added with a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen), random primer (1μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total of reaction mixture is 8 μl. The RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C [Phan et al., 2005a].

Polymerase Chain Reaction (PCR)

The first group of viruses including astrovirus, norovirus (GI, GII), and sapovirus and the second group including group A, B, and C rotaviruses and adenovirus were detected by mutiplex PCR with specific primers as reported previously by Phan et al. [2005a]. These mutiplex PCR assays are the sensitive and specific methods that have been used with the success to accurately define the true burden of diarrheal disease due to viruses. The identification of the first group of viruses was performed with specific primers Beg9 and VP7-1', B5-2 and B3-3, G8NS1 and G8NA2, Ad1 and Ad2 for group A, B, and C rotaviruses and adenovirus with four different amplicon sizes of 395, 814, 352, and 482 bp, respectively in one PCR tube. For the detection of the second group of viruses, specific primers PreCAP1 and 82b; G1SKF and G1SKR; COG2F and G2SKR, SLV5317 and SLV5749 specifically generated four different sizes of amplicons of 719, 330, 387, and 434 bp for astrovirus, norovirus (GI, GII), and sapovirus, respectively (Table I). Sapovirus polymerase region was also amplified to identify recombinant sapovirus using primers P290 and P289 [Jiang et al., 1999]. To eliminate the possibility of co-infection of two different genotypes in case of sapovirus recombination, flanking polymerase and capsid regions with their junction was amplified with primers P290 and SLV5749 to generate 1,163-bp product [Jiang et al., 1999; Phan et al., 2005a]. 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), dNTPs (2.5 mM/μl), primers (33 μM/μl), 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 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C. In order to monitor the quality of extraction and RT-PCR, Hu/SVGII/Karachi/928/1994, and MilliQ water were used as positive and negative controls, respectively. In experiment, to avoid false positive results instructions (autoclaved solutions, reagents into aliquots, disposable gloves, avoidance of splashes, and positive displacement pipettes) to prevent cross contamination were followed strictly [Kwok and Higuchi, 1989].

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide

TABLE I. A List of Specific Primers Used in the Study to Detect Multiple Target Viruses Among Infants and Children With Diarrhea in Japan by RT-PCR

Virus	Target region	Primer name	Polarity[a]	Sequence (5′–3′)[b]	Amplicon size (bp)	Position (nt number)	Representative strain's accession number
Group A rotavirus	VP7	Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	395	1–395	D16343
		VP7-1′	−	ACTGATCCTGTTGGCCATCCTTT			
Group B rotavirus	VP7	B5-2	+	GGCAATAAAATGGCTTCATTGC	814	1–814	AY539856
		B3-3	−	GGGTTTTTACAGCTTCGGCT			
Group C rotavirus	VP7	NG8S1	+	ATTATGCTCAGACTATCGCCAC	352	353–704	AB086966
		NG8S2	−	GTTTCTGTACTAGCTGGTGAAC			
Adenovirus	Hexon	Ad1	+	TTCCCCATGGCICAYAACAC	482	1834–2296	M12411
		Ad2	−	CCCTGGTAKCCRATRTTGTA			
Astrovirus	Capsid	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	719	4235–4953	L23513
		82b	−	GTGAGCCACCAGCCATCCCT			
Norovirus GI	Capsid	G1-SKF	+	CTGCCCGAATTYGTAAATGA	330	5342–5671	M87661
		G1-SKR	−	CCAACCCARCCATTRTACA			
Norovirus GII	Capsid	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	387	5003–5389	X86557
		G2-SKR	−	CCRCCNGCATRHCCRTTRTACAT			
Sapovirus	Capsid	SLV5317	+	CTCGCCACCTACRAWGCBTGGTT	434	5083–5516	X86560
		SLV5749	−	CGGRCYTCAAAVSTACCBCCCCA			
Sapovirus	Polymerase	P290	+	GATTACTCCAAGTGGGACTCCAC	331	4354–4684	X86560
		P289	−	TGACAATGTAATCATCACCATA			

[a]+, Forward primer; −, Reverse primer.
[b]Within nucleotide sequence of primers, B = C, G or T; H = A, C or T; N: Any base; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

for 20 min, then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for sapovirus were determined directly with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples 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). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. The capsid and polymerase sequences of sapovirus strains 5786/Osaka/JP, 5800/Osaka/JP, 5836/Osaka/JP, 5862/Osaka/JP, 5862/Osaka/JP, and 5821/Osaka/JP, had been submitted to the DDBJ DNA/GenBank database and had been assigned accession numbers AB242322 and DQ307499, AB242323 and DQ 307500, AB242324 and DQ307501, DQ401095 and DQ 401096, AB242325, and AB242326, respectively. Reference sapovirus 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 (AB 181227), Karachi/876/1993 (AB181132), Houston/90 (U95644), Stockholm/97 (AF194182), 12/DCC/Tokyo/Japan/44 (AB236380), Karachi/872/1991 (AB181231), 4408/Maizuru/Japan (AB180209), and Mex14917/2000 (AF435813).

RESULTS

Detection of Diarrheal Viruses

A total of 125 fecal specimens were collected from infants and children with acute gastroenteritis in Osaka City, Japan, during the period of July 2004 to June 2005. For the pediatric population, the lowest age was 5 months, the highest was 8 years, and the average age was 1.2 years (14 months). Among all children with acute gastroenteritis, 91.2% were aged less than 36 months. Males accounted for 57%. All fecal specimens were tested for the presence of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus by RT-multiplex PCR. Diarrheal viruses were detected in 72 out of 125 (57.6%) specimens tested. Among diarrheal viruses detected, norovirus was the most prevalent (19.2%), followed by 18.4% of group A rotavirus, 1.6% of astrovirus, and 0.8% of adenovirus, respectively. It was interesting that sapovirus infection was identified with the high incidence and accounted for 17.6%. No group B and C rotaviruses were found in these patients.

Nucleotide Sequencing and Phylogenetic Analysis of Sapovirus Genogroup and Genotype

The PCR products of sapovirus were sequenced in order to characterize further the genetic relationship among the sapovirus isolates detected in infants and children with acute gastroenteritis in Osaka City, Japan. Their partial nucleotide sequences were compared to each other as well as to those of reference sapovirus strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5′ ends of the sapovirus capsid gene was determined by direct sequencing with the amplified fragments. This region has been shown to be suitable for genotyping [Farkas et al., 2004; Phan et al., 2005b]. A total of 22 sapovirus sequences were analyzed by phylogenetic analysis and grouped using the recent sapovirus capsid region classification scheme [Farkas et al., 2004; Akihara et al., 2005; Phan et al., 2005b]. In the present study, all of the sapovirus sequences were classified into only one distinct genogroup I (Fig. 1). The results indicated that sapovirus group I was a dominant genogroup. The sapovirus GI sequences clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8) and these presented 4.5% (1 of 22), 4.5% (1 of 22), 81.8% (18 of 22), and 9.1% (2 of 22), respectively. Using CLUSTAL X, it was also noted that these sapoviruses had a high identity on the amino acid level and the nucleotide level with other reference sapovirus strains previously registered in the DDBJ DNA/GenBank database in the same genotype ranged from 90% to 100%.

Nucleotide Sequencing and Genetic Analysis of Sapovirus Polymerase

The polymerase regions of all sapovirus strains were amplified and sequenced to examine genetically the virus found during this study. In 20 sapoviruses belonging to GI/1, GI/4, and GI/6, the genotypes were maintained in both polymerase and capsid regions. They also shared a high identity at the nucleotide level and the amino acid level of the polymerase with other reference sapovirus strains registered previously in the DDBJ DNA/GenBank database in the same genotype ranged from 95% to 100%. It was found that two sapoviruses with GI/8 capsid, the 5862/Osaka/JP and the 5821/Osaka/JP, were classified into the Sapporo/82 cluster (known as GI/1), not into the 12/DCC/Tokyo/Japan/44 cluster (known as GI/8) when polymerase-based grouping was performed (Fig. 2).

Novel Recombinant Sapoviruses

It was found that the polymerase and capsid regions of the 5862/Osaka/JP and the 5821/Osaka/JP shared 96% and 85% of the amino acid similarity with the Sapporo/82, respectively. However, they had a high homology (99%) at the amino acid level of the capsid region with the 12/DCC/Tokyo/Japan/44. Therefore, these two strains were all recombinant viruses with a GI/8 capsid and GI/1 polymerase. When the sequences of the 5862/Osaka/JP and the 5821/Osaka/JP were compared with that of the Sapporo/82 using the SimPlot, an apparent region of genetic recombination was found at the polymerase- and capsid-junction. Before this junction, the 5862/Osaka/JP, the 5821/Osaka/JP, and the Sapporo/82 were homologous. After the junction, however, the homology was notably different. The SimPlot analysis showed a sudden drop in the identity for the 5862/Osaka/JP and the 5821/Osaka/JP. The 100% homology at the nucleotide as well as the amino acid levels of polymerase and capsid among the 5862/Osaka/JP and the 5821/Osaka/JP was also noted.

Outbreak of Sapovirus

The results shown in Figure 3 indicated that although the fecal specimens were collected over the period of 12 months (July 2004 to June 2005), the sapovirus GI/6 infection was apparently confined within a period of 5 months (August 2004 through December 2004). This pattern of infection indicated an outbreak of sapovirus GI/6 in these subjects and this would be the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City. It was found that the nucleotide as well as the amino acid sequences of the capsid and polymerase regions among 18 sapovirus isolates were identical (100%). A phylogenetic tree of the amino acid sequences of these isolates and the reference strains was constructed and all of 18 sapovirus GI/6 isolates formed a distinct cluster. These results indicated that sapovirus GI/6 isolates detected in infants and children in Osaka City were closely homologous to each other, suggesting that they were probably derived from the same source of infection. In addition, majority (94.4%, 17 of 18) of sapovirus GI/6 infected cases were confined to infants and young children with an age of less than 3 years. This observation demonstrated that sapovirus infection in this outbreak occurred mainly in infants and young children.

DISCUSSION

Viral gastroenteritis is still a health burden and one of the most frequently encountered problems in developed and developing countries [Parashar et al., 2003a; Mulholland, 2004]. In this study, diarrheal viruses were detected in 57.6% of fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Osaka City about 57.6% might be due to the diarrheal viruses and 42.4% caused by other etiologic agents. Among the diarrheal viruses detected, norovirus was found to be the most prevalent and became a leading cause of viral gastroenteritis in infants and children in Osaka City, followed by group A rotavirus, sapovirus, astrovirus, and adenovirus. These viruses were also considered to be significant global enteropathogens and were associated with sporadic cases as well as outbreaks of gastroenteritis [Noel and Cubitt, 1994; Carter and Willcocks, 1996; Bon et al., 1999; Deneen et al., 2000; Marks et al., 2000].

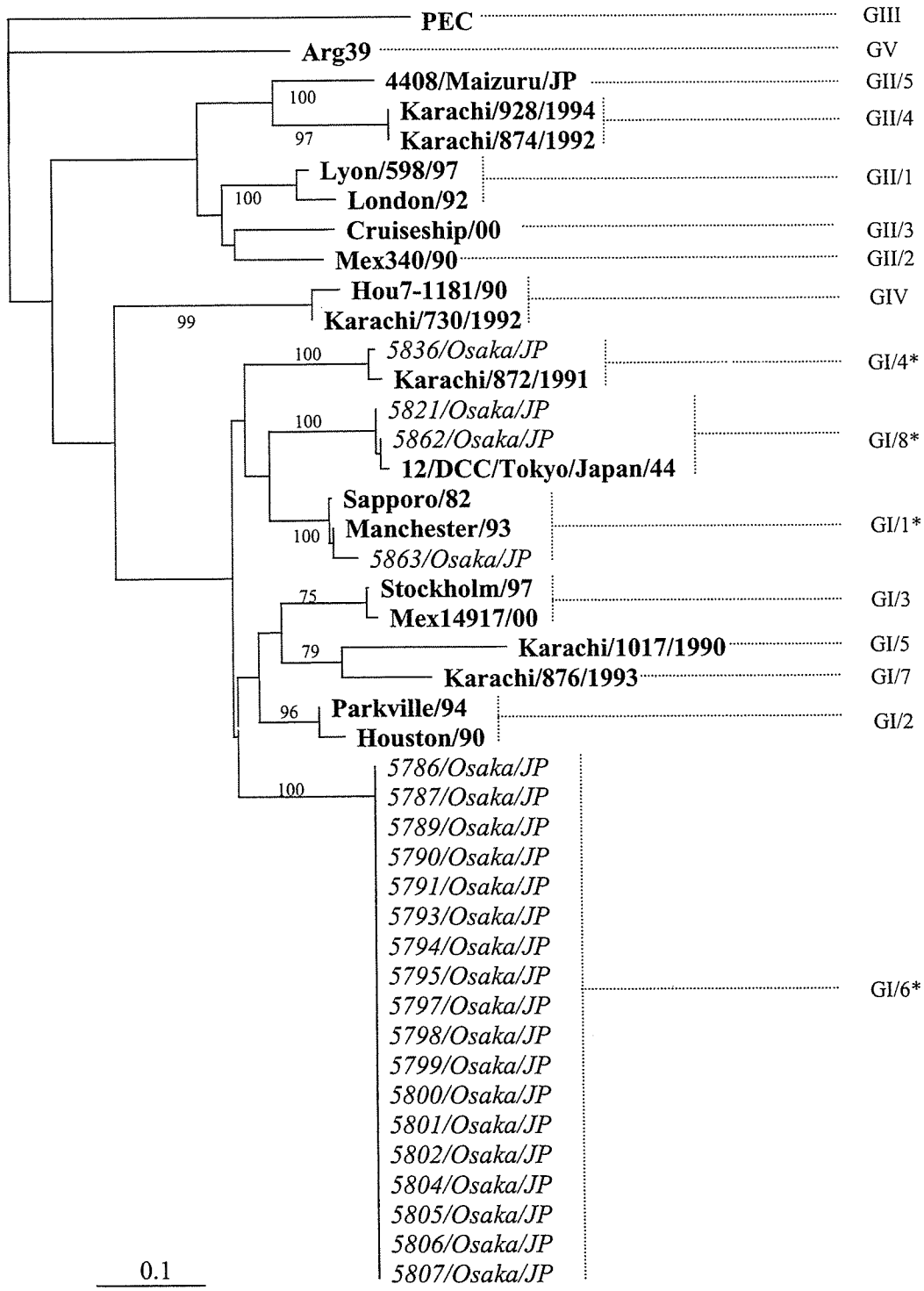

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese sapovirus. All of the sapovirus sequences were classified into only one distinct genogroup I and the sapovirus genogroup I sequences clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8). The tree was constructed from partial amino acid sequences of capsid region of sapovirus isolates detected in Osaka City, Japan. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Japanese sapovirus is highlighted in italic. PEC 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. *, Genotype contains Japanese sapovirus detected in the study.

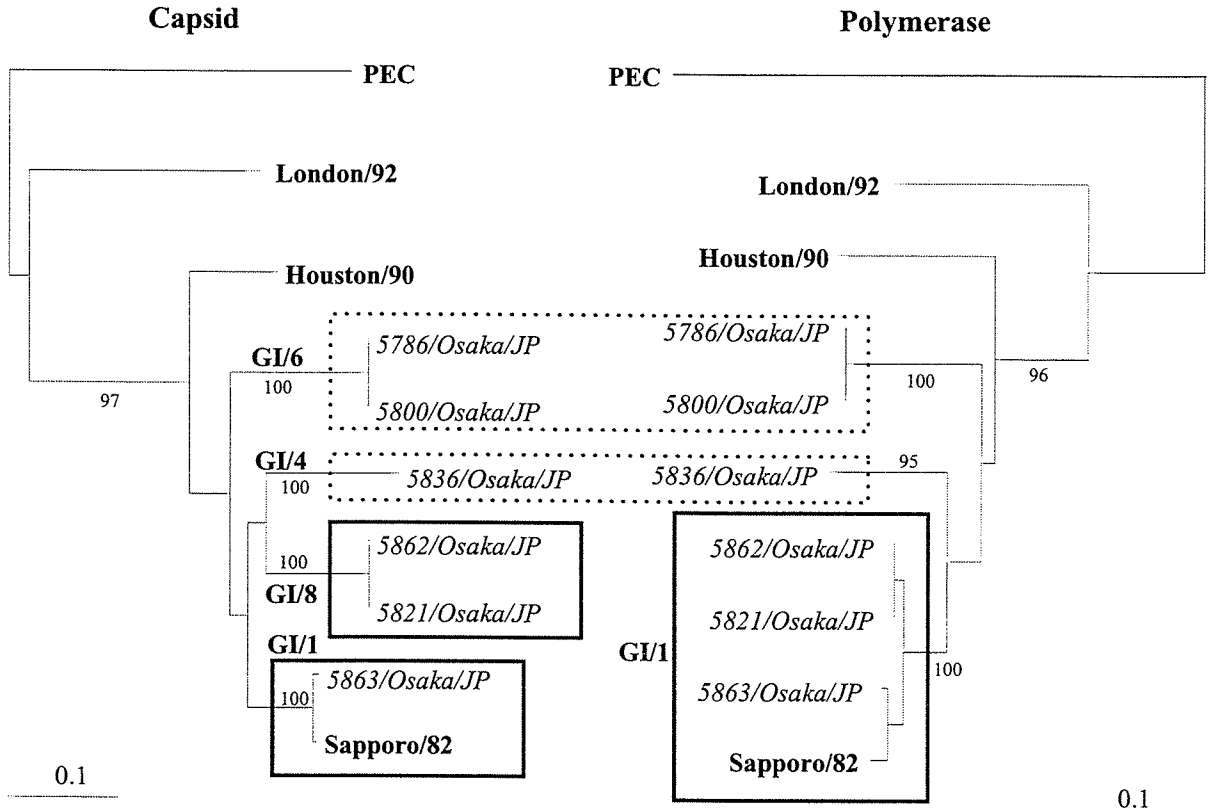

Fig. 2. Observation of changes of sapovirus genotypes on the basis of phylogentic trees of amino acid sequences. The novel recombinant sapoviruses, the 5862/Osaka/JP and the 5821/Osaka/JP, were classified into two distinct genotypes GI/1 and GI/8 when the polymerase- and capsid-based groupings were performed. In other sapoviruses detected, the genotypes were maintained in both polymerase and capsid regions. The trees were constructed from partial amino acid sequences of capsid and polymerase regions of Japanese sapovirus isolates. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Japanese sapovirus was highlighted in italic. PEC strain was used as an outgroup strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

Sapovirus is recognized as one of the causes of the diarrheal illness in humans worldwide and its prevalence was shown to range from 0.3% to 9.3% and usually much lower than norovirus [Oh et al., 2003; Yan et al., 2003; Phan et al., 2004]. It was interesting that sapovirus infection was identified with high prevalence, accounting for 17.6%. The results in this study showed that all Japanese sapovirus sequences belonged to only one sapovirus genogroup I with four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). Of note, the sapovirus GI/6 isolates detected were predominant (81.8%) and closely homologous to each other, suggesting that they came from the same source of infection. It was noted that the sapovirus GI/6 infection was confined only within a short period of 5 months (August 2004 through December 2004). Moreover, only two sapovirus GI/6 strains were found in 2002–2003 during a 7-year (1996–2004) survey of sapovirus infection in diarrheal fecal specimens of infants and children collected from Osaka City, Japan and no sapovirus GI/6 was detected in 2003–2004 [Phan et al., 2005b]. Taken together, this is the first report of an outbreak attributed to the sapovirus GI/6 among infants and children in Japan and this would be the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City.

In this outbreak, the majority of infants and children with sapovirus infection (94.4%) were aged less than 36 months. This result was in line with published reports on sapovirus epidemiology worldwide in which sapovirus infection causes acute gastroenteritis in all age group, through it occurs predominantly in infants and young children [Chiba et al., 2000; Okada et al., 2002; Akihara et al., 2005]. Our findings also confirmed sapovirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to most studies, sapovirus infection has been found mainly during the cold season [Chiba et al., 1979; Phan et al., 2004, Yan et al., 2005]. In contrast, the present study has demonstrated that the outbreak associated with sapovirus lasted continuously from August to December as the middle of summer to the end of autumn in Japan. This observation clearly indicated that sapovirus infection could occur not only in the cold season but also in the hot season.

Recombination is among the major driving forces of viral evolution. Recombination of viral RNA is known to

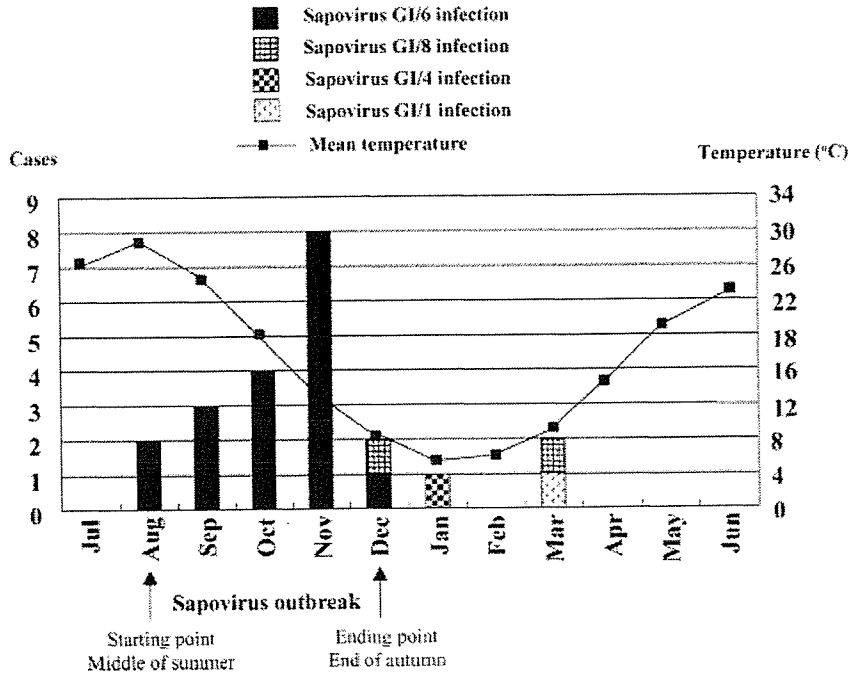

Fig. 3. Monthly distribution of sapovirus infection in infants and children with acute gastroenteritis in Osaka City, Japan, during the period July 2004 to June 2005. The sapovirus GI/6 infection was apparently confined within a period of 5 months (August 2004 through December 2004). The duration of outbreak of sapovirus GI/6 infection is shown.

depend upon various immunological and intracellular constraints that may allow the emergence of viable recombinant [Worobey and Holmes, 1999]. Recombination in viruses can greatly affect phylogenetic groupings, confuse molecular epidemiologic studies, limit the virus control programs, and have major implications in viral vaccine design [Matson, 2003; Bull et al., 2005]. Although several recombinant noroviruses causing sporadic cases and outbreaks of acute gastroenteritis have been reported, the data of recombinant sapovirus are limited. Another interesting feature of this study was the presence of two novel, naturally occurring recombinant sapoviruses circulating in the Japanese population studied. These isolates were obtained from a male patient and a female patient both aged 1 year and with clinical manifestations of acute gastroenteritis in Osaka City, Japan. These isolates had total homology (100%) at the nucleotide as well as amino acid levels of the capsid and polymerase. The findings demonstrated that they likely represented the same strain. Based on genetic analysis, these isolates shared a high level of sequence identity (96%) in the polymerase region and a low level of amino acid identity in the capsid region (85%) with the Sapporo/82 cluster (known as GI/1). However, they had a high level of amino acid identity (99%) in capsid region with 12/DCC/Tokyo/Japan/44 cluster (known as GI/8). Altogether, these sapovirus strains demonstrated a novel recombination with GI/1 polymerase and GI/8 capsid.

In conclusion, this is the first report of outbreak associated with the sapovirus GI/6 infection among infants and children with acute gastroenteritis in Osaka City, Japan and warns of the threat they pose. This is also the first study, to our best knowledge, demonstrating the existence of the novel recombinant sapovirus causing diarrheal illness in Osaka City, Japan.

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Clin. Lab. 2006;52:363-366

SHORT COMMUNICATION

Novel Intragenotype Recombination in Sapovirus

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SUMMARY

Based on the genetic analysis, a novel, naturally occurring recombination between two distinct sapovirus subtypes (subtype a and subtype b) within genogroup I genotype 1 was identified. Breakpoint analysis of recombinant sapovirus showed that the recombination site was at the polymerase-capsid junction. This is the first report of the existence of acute gastroenteritis caused by intragenotype recombinant sapovirus. The results also provided evidence that the natural recombination occurs not only in sapovirus genogroup II but also in sapovirus genogroup I. (Clin. Lab. 2006;52:363-366)

KEY WORDS

Sapovirus, recombination, intragenotype, Japan

The study

Acute gastroenteritis is a common disease with a high morbidity reported worldwide. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (1, 2). Sapovirus is recognized as a global enteropathogen, being a significant cause of acute gastroenteritis in such settings as kindergartens, schools, and nursing homes (3, 4). Immunological and seroepidemiologic studies have indicated a worldwide distribution of sapovirus (3, 5). Sapovirus (formerly known as "Sapporo-like virus") is the distinct genus within the family *Caliciviridae*. The sapovirus genome contains two ORFs. The ORF1 encodes non-structural and capsid proteins while ORF2 encodes a small protein. Sapovirus is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans (6).

A fecal specimen was collected from a 10-month old male child with acute gastroenteritis in Maizuru City, Japan in November 2005. The fecal specimen was diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 x g for 10 min. The supernatant was collected and the viral genomes were extracted by using a QIAamp Viral RNA kit (QIAGEN®, Hilden, Germany). Using PCR with specific primers as previously reported resulted in the identification of the first group of diarrheal viruses including astrovirus, norovirus, and sapovirus and the second group including rotavirus and adenovirus (7). Polymerase was also amplified to detect recombinant sapovirus using primers SR80 and JV33 (8). Products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X (Version 1.6). A phylogenetic tree with 1000 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). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. The nucleotide sequence data of sapovirus strain 6728/05/Maizuru/JP had been submitted to GenBank and had been assigned accession number

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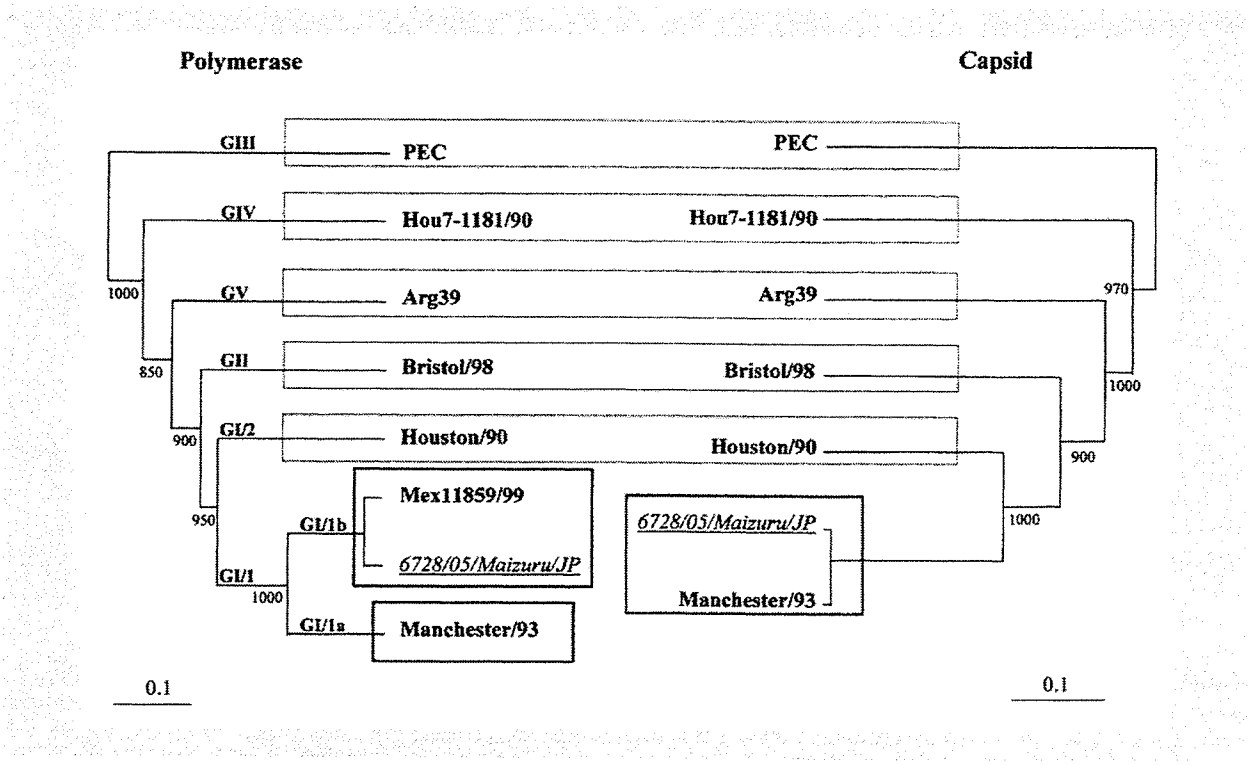

Figure 1: Observation of changes of sapovirus subtypes on the basis of phylogenetic trees of nucleotide sequences of the 6728/05/Maizuru/JP. The trees were constructed from partial nucleotide sequences of the capsid and polymerase regions of the 6728/05/Maizuru/JP. The 6728/05/Maizuru/JP is underlined and highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. PEC strain was used as an out-group strain for phylogenetic analysis.

DQ395300. Reference sapovirus strains and accession numbers used in this study were as follows: PEC (AF182760), Arg39 (AY289803), Manchester/93 (X86560), Hou7-1181/90 (AF435814), Mex11859/99 (AY157857), Sapporo (U65427), Plymouth (X86559), Houston/86 (U95643), Dresden (AY694184), Houston/90 (U95644), and Bristol/98 (AJ249939).

The fecal specimen was positive for sapovirus. Figure 1 shows that the sequence of the 6728/05/Maizuru/JP was classified into genogroup I genotype 1a (GI/1a) known as the Manchester/93 cluster. Interestingly, the 6728/05/Maizuru/JP with GI/1a capsid belonged to the Mex11859/99 cluster (known as GI/1b) when polymerase-based grouping was performed. Taken together, the 6728/05/Maizuru/JP was expected to be a recombinant sapovirus with GI/1a capsid and GI/1b polymerase.

To eliminate the possibility of co-infection with two different sapovirus subtypes, to localize the potential recombination site as well as to understand a possible recombination mechanism of the novel recombinant sapovirus, flanking polymerase and capsid regions with their junction of the 6728/05/Maizuru/JP were amplified with primers SR80 and SLV5749 to produce 1,151-bp amplicon (7, 8). When the nucleotide sequence of the 6728/05/Maizuru/JP was compared with that of the Manchester/93 using SimPlot, an apparent region of genetic recombination was found at the polymerase-capsid junction. Before this junction, the homology of the 6728/05/Maizuru/JP and the Manchester/93 was different. After this junction, however, they were highly homologous. Using CLUSTAL X, the 6728/05/Maizuru/JP shared a low level of nucleotide identity (89%) in the polymerase region and a high level of the nucleotide identity (99%) in the capsid region with the Manchester/93. In contrast, there was a high level of identity (97%) in the polymerase region between the 6728/05/Maizuru/JP and the Mex11880/99. Since the capsid sequence of the Mex11859/99 was not available in GenBank, the homology in the capsid region between the 6728/05/Maizuru/JP and the Mex11859/99 was unknown. Altogether, the capsid sequences of the 6728/05/Maizuru/JP and the Manchester/93 were almost identical, but the polymerase sequences of the 6728/05/Maizuru/JP and the Mex11859/99 were different from that of the Manchester/93. This pattern of the genetic characterization of the 6728/05/Maizuru/JP implied a novel, naturally occurring recombinant sapovirus with GI/1a capsid and GI/1b polymerase.

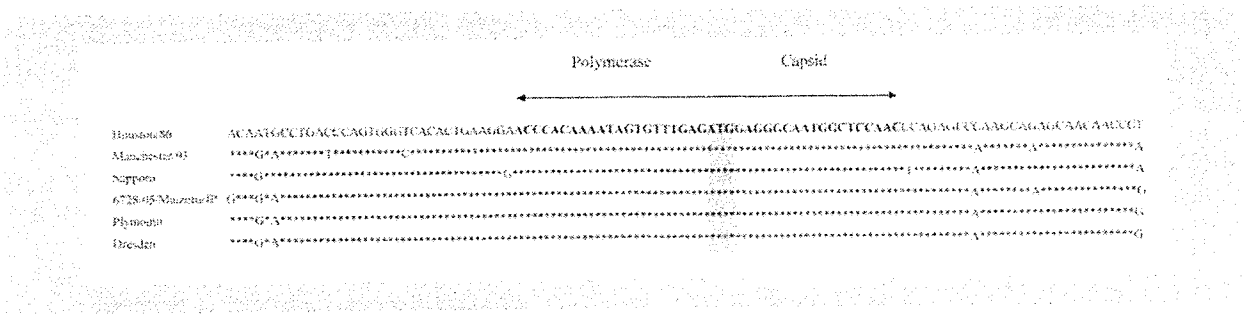

Figure 2: Nucleotide alignment of the 6728/05/Maizuru/JP, the Manchester/93, and reference sapovirus strains of GI/1 in GenBank, showing the highly conserved polymerase and capsid junction which is highlighted in bold type. The asterisks represent conserved nucleotides. The shaded nucleotides represent the putative capsid start codons.

RNA recombination is a mechanism for virus evolution (9). Even though there are now many molecular epidemiological studies on sapovirus infection worldwide, reports documenting recombination in sapovirus are still limited. To date, only 2 kinds of recombinant sapoviruses have been reported as intergenotype recombination and intergenogroup recombination (10, 11). Based on polymerase-based groupings, these recombinations had occurred only in sapovirus genogroup II, which was more capable of recombination than other genogroups (10, 11). However, we identified the 6728/05/Maizuru/JP with a novel recombination between two distinct subtypes (subtype a and subtype b) within genogroup I genotype 1. This is the first report of the existence of acute gastroenteritis caused by intragenotype recombinant sapovirus. The results also provided evidence that the natural recombination occurs not only in sapovirus genogroup II but also in sapovirus genogroup I.

In this study, the 6728/05/Maizuru/JP shared the closest sequences of polymerase and capsid with the Mex11859/99 and the Manchester/93, respectively. Strain Mex11859/99 was detected in 1999-2000 in Mexico (6), whereas strain Manchester/93 was detected in 1993 in the United Kingdom (12). Quite possibly, the Mex11859/99 and the Manchester/93 were parental strains of the 6728/05/Maizuru/JP. However, the very distant geography of these strains did not provide evidence for where and when a recombination event might occur in nature. Even that recombination event in this sapovirus is not completely understood, but it can be potentially dangerous for host species, and it likely limits the virus control programs and has in addition major implications in viral vaccine design.

The capsid gene is predicted to be well suited for the genotype classification of circulating sapovirus strains (4, 6). In this study, the HU/5862/Osaka/JP was recognized to belong to two distinct sapovirus subtypes by capsid- and polymerase-based groupings. Therefore, in order to monitor the evolution of sapovirus in nature, we suggest that classification of sapovirus strains should rely not only on the capsid sequence but also on the polymerase sequence. Altogether, this novel recombinant sapovirus could greatly affect phylogenetic groupings and confuse molecular epidemiological studies.

The primary mechanism involved in recombination in RNA viruses is the copy-choice model in which recombination is known to depend on various immunological and intracellular constraints, (i) successful co-infection of the host and in a single cell by two parental strains, (ii) efficient replication of parental viral genomes with template switching, (iii) adaptation to different environments to be transmitted (9). Furthermore, the observation of circulating 6728/05/Maizuru/JP in the community underscored that this strain theoretically fulfilled all prerequisites for its recombination. However, it was unclear whether the child was infected with this recombinant sapovirus, or whether the recombinant sapovirus resulted from co-infection with 2 different viruses in this child. Using CLUSTAL X it was interesting that the 6728/05/Maizuru/JP, the Manchester/93, and reference sapovirus strains of GI/1 in GenBank shared the 42 nucleotide-conserved region (100% identity) including the last 21 nucleotides of polymerase and the first 21 nucleotides of capsid (Figure 2). This highly conserved region might represent the break and rejoin site for recombination during viral replication. In fact, RNA recombination often takes a major part leading to the emergence of novel viral agents (9). Therefore, this novel recombinant sapovirus in our study will have an epidemiological importance in causing diarrheal diseases that warns of the threat it poses in future. Further epidemiological studies should be conducted to determine whether this recombinant strain would become more predominant in Japan in the coming year.

In conclusion, RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity. Our results have described the genetic characterization of novel, naturally occurring recombinant sapovirus as well as increased the evidence for the worldwide distribution of recombinant sapovirus. This report is also the first indication of intragenotype recombination in sapovirus.

Acknowledgements

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ORIGINAL ARTICLE

Emergence of New Variant Rotavirus G3 among Infants and Children with Acute Gastroenteritis in Japan during 2003-2004

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SUMMARY

A total of 402 fecal specimens from infants and children with acute gastroenteritis in five places (Tokyo, Maizuru, Saga, Sapporo, and Osaka) in Japan from July 2003 to June 2004 were collected and then tested for the presence of rotavirus by RT-PCR. Of these, 83 were positive for rotavirus and this accounted for 20.6%. Rotavirus was further characterized to G-types (VP7 genotypes) and P-types (VP4 genotypes). Interestingly, an emergence of rotavirus G3 was identified with an exceptionally high prevalence (97.5%; 81 of 83), followed by rotavirus G2 (2.5%; 2 of 83). The P-types of 19 rotavirus strains, which could not be typed by RT-PCR, were determined as P[8] with multiple point mutations at the VP4 primer-binding site by sequencing analysis. The predominant genotype was G3P[8] (95.2%, 79 of 83), followed by a number of unusual combinations G3P[4] (2.4%, 2 of 83), and G2P[8] (2.4%, 2 of 83). Another interesting feature of the study was the demonstration of a great genetic diversity in new variant rotavirus G3 strains circulating in Japan. In comparison with rotavirus G3 strains circulating in 1990-1995 in Japan, a wide range of amino acid substitutions (up to 16) of new variant rotavirus G3 VP7 genes was identified. Of note, the changes at positions 96, 99, and 100 were revealed to be located in the antigenic region A, and 213 in the antigenic region C. To the best of our knowledge, this is the first reporting of an emergence of new variant rotavirus G3 together with a sudden disappearance of G1, G4, and G9 in infants and children with rotavirus infection-associated gastroenteritis in Japan. (Clin. Lab. 2007;53:XXX-XXX)

KEY WORD

rotavirus, G3, emergence, gastroenteritis, Japan

INTRODUCTION

Rotavirus is considered to be a significant global enteropathogen, being a major cause of acute gastroenteritis in infants and children [1, 2]. It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age [3, 4]. 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. The two outer capsid proteins, VP4 and VP7, allow the rotavirus classification into P and G genotypes, respectively [1, 2]. In rotavirus, at least 15 G genotypes have been recognized by neutralization assay and 26 P genotypes have been identified by hybridization or sequence analysis. Of these, four rotavirus G-P combinations G1P[8], G2P[4], G3P[8], and G4P[8] are the most common globally and are therefore the targets for current vaccine development strategies [5].

Since effective anti-rotavirus drugs have not been developed, a rotavirus vaccine would be very useful. The first rotavirus vaccine (Rotashield; Wyeth Lederle Vaccines, Philadelphia, PA, USA) licensed for use was a live-attenuated tetravalent rhesus-human reassortant vaccine incorporating G1 to G4 specificity. This vaccine was, however, withdrawn from use after reports of asso-

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ciation with intestinal intussusception. Recent developments with rotavirus vaccines provide great promise for the prevention of severe dehydrating diarrhea. Two rotaviruses vaccines have recently been released onto the market. Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) has been licensed in more than 30 countries, and RotaTeq (Merck Vaccines, Whitehouse Station, NJ, USA) is now only available in the United States [6-8]. Moreover, study of the molecular epidemiology of the rotaviruses provides knowledge on the diversity of the specific VP7 types found in humans. 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 determine the occurrence of rotavirus infections in infants and children with acute gastroenteritis in five different places in Japan during 2003 and 2004; to characterize the detected rotaviruses according to G-and P-types; and to demonstrate the genetic diversity among them.

MATERIALS AND METHODS

Fecal specimens

A total of 402 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics in five different places (Tokyo, Maizuru, Saga, Sapporo, and Osaka) in Japan between July 2003 through June 2004. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 x g for 10 min. The supernatants were collected and stored at –30 °C until use for the detection of rotavirus.

Extraction of viral RNA

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

Reverse transcription (RT)

For reverse transcription (RT), 7.5 µl of extracted viral RNA was added to a reagent mixture consisting of 2.05 µl of 5x first strand buffer (Invitrogen, Carlsbad, CA, USA), 0.75 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 µl of 10 mM DTT (Invitrogen), 0.75 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, USA), 0.375 µl (1µg/µl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl (33 U/µl) of RNase inhibitor (Toyobo, Osaka, Japan), and 2.325 µl MilliQ water. The total volume of the reaction mixture was 15 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 [9].

Polymerase chain reaction (PCR)

PCR with specific primers was used for rotavirus identification as previously reported [9]. Primers Beg9 (5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3') and VP7-1' (5'-ACTGATCCTGTTGGCCATCCTTT-3') were used to amplify VP7 of rotavirus and specifically generated an amplicon of 395 bp. PCR was carried out with 2.5 µl of cDNA in 22.5 µl of the reagent mixture containing 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/µl), primers (33 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI, USA) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles at 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.

Rotavirus G-typing

G-typing of rotavirus was performed using the protocol of the method previously presented by Das et al. [10]. The full-length of the VP7 gene was reverse transcribed and then further amplified with primers Beg9 and End9. 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 primer 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 bp, 244 bp, 466 bp, 403 bp, and 110 bp for G1, G2, G3, G4, and G9, respectively.

Rotavirus P-typing

P-typing was conducted by using a modification of the Gentsch et al. method [11]. The RT-PCR was performed by using primers Con2 and Con3 for 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 six different sizes of amplicons of 346 bp, 484 bp, 268 bp, 392 bp, and 584 bp, respectively. The samples whose P-types could not be identified by RT-PCR were then determined by nucleotide sequence analysis.

Electrophoresis

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

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of PCR products (DNA) positive for rotavirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) in order to further characterize the genetic relationship among the G3 strains detected among infants and children with acute gastroenteritis in Japan. Their VP7 nucleotide sequences were compared to each

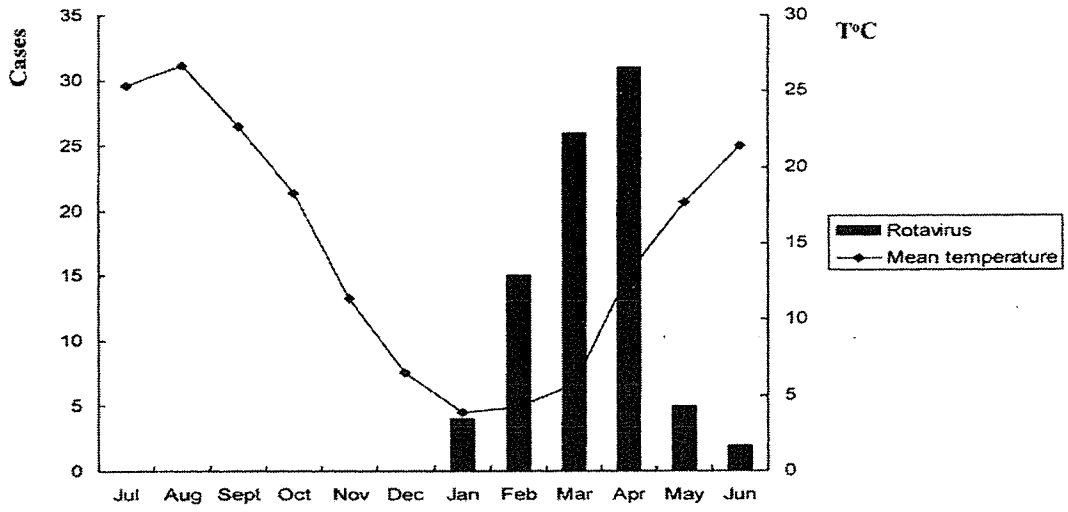

Figure 1: Seasonal variation of rotavirus infection among infants and children with acute gastroenteritis in five different regions (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2004. The mean temperature for the five regions obtained from the Japan Meteorological Agency is also shown.

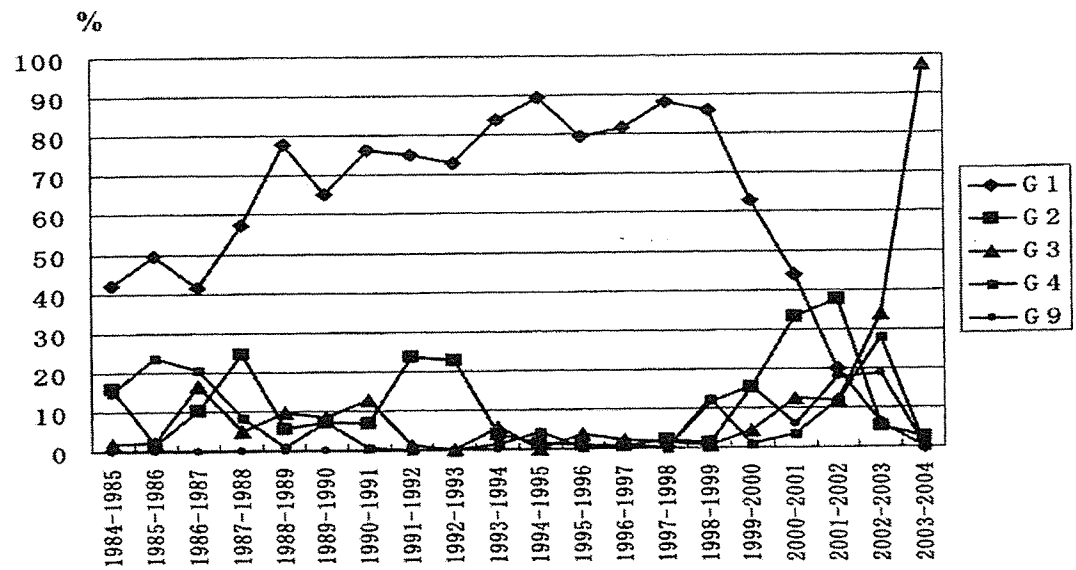

Figure 2: Prevalence pattern of rotavirus G-types with an emergence of rotavirus G3 together with a sudden disappearance of G1, G4, and G9 during the 21-year survey of rotavirus infection among infants and children in the same age group with acute gastroenteritis in five different regions (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan.

Table 1: Comparison of amino acid sequence of VP7 genes among Japanese and global rotavirus G3 strains

Strain	Place	Time	Amino acid identity																		
										A (87-101)			C (208-221)#								
			16	40	41	66	96	99	100	108	116	121	213	256	266	278	303	320	323	324	325
5244	Japan	2003-2004	V	I	I	P	N	K	D	I	Y	Y	N	N	P	M	V	F	Y	Y	R
5091	Japan	2003-2004	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5272	Japan	2003-2004	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5299	Japan	2003-2004	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
J-12	Japan	1990	I	V	*	L	D	P	G	T	*	N	K	*	S	T	A	*	T	T	G
TK15	Japan	1991	I	V	*	L	D	*	*	T	*	*	*	H	S	T	*	*	*	S	G
TK28	Japan	1991	I	V	*	L	D	*	*	T	*	*	*	*	S	T	*	*	*	*	*
TK08	Japan	1995	I	V	*	L	D	*	*	T	*	*	*	*	S	T	*	*	*	*	*
CS02-01	Taiwan	2001-2002	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CMH054	Thailand	2000-2001	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CMH229	Thailand	2000-2001	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CMH272	Thailand	2000-2001	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
MaCH09404	Malaysia	2004	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
MaCH09004	Malaysia	2004	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
RMC437	India	2004	*	*	*	*	*	*	*	*	V	*	*	*	*	*	*	*	*	*	*
97'S48	China	1997	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*

Note: #, A and C antigenic regions of rotavirus VP7 gene; *, Amino acids identical with the G3 strain 5244/Japan in 2003-2004

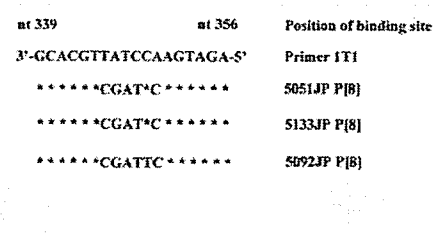

Figure 3: Alignment of fragment of the VP4 gene of Japanese rotavirus strains that were not typed by RT-PCR and the reverse complementary sequences of the original primer 1T-1. Residues that match primer 1T-1 are denoted by asterisks.

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). A 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 rotavirus strains detected in the study had been submitted to GenBank and had been assigned accession numbers DQ779048-DQ779054. Reference rotavirus strains and their accession numbers used in this study were as follows: J-12/1990/Japan (D86279), TK08/1995/Japan (D86281), TK28/1991/Japan (D86283), TK15/1991/Japan (D86282), CMH054/Thailand (AY707794), CMH229/Thailand (AY707791), CMH272/Thailand (AY707790), RMC437/India (AY603153), CS02-01/Taiwan (AY165009), MP126/India (AF386915), MaCH09004 (AY900173), MaCH09404 (AY870661), and 97'S48 (AF260957).

RESULTS

Molecular epidemiology of rotavirus infection

A total of 402 fecal specimens collected from infants and children with acute gastroenteritis in Japan from 2003 to 2004 were examined for the presence of rotavirus. For the pediatric population, the lowest age was 2 months, the highest was 11 years, and the average age was 2.5 years (29 months). Out of 402 diarrheal fecal specimens, 83 were found to be positive for rotavirus, and this represented 20.6%. The highest incidence of rotavirus was in the 12-23 months old group (43.4%, 36 of 83). Only one case of rotavirus infection was identified among infants aged less than 6 months. It was also found that infants and children aged less than 3 years had a high rate of rotavirus infection (81.9%, 68 of 83). In the study, rotavirus was detected continuously in 6-month period lasting from January to June (Figure 1). No rotavirus was found between July through December. The highest prevalence of rotavirus infection was

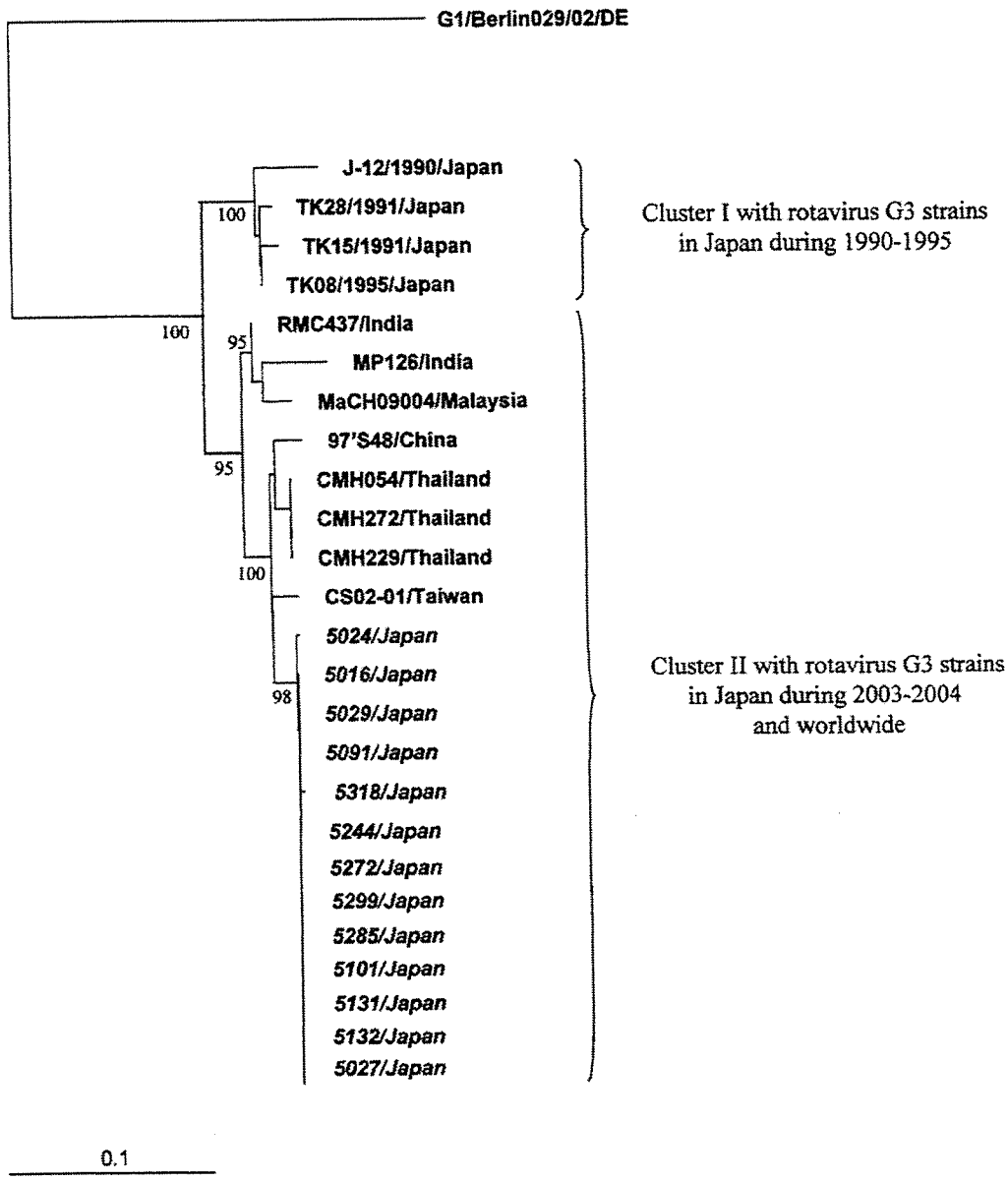

Figure 4: Phylogenetic tree of nucleotide sequences of rotavirus G3 strains detected in infants and children with acute gastroenteritis in five different places in Japan. The tree was constructed from nucleotide sequences of VP7 of rotavirus G3 strains. Reference rotavirus strains were selected from GenBank under the accession number indicated in the text. Japanese rotavirus G3 strains in 2003-2004 are highlighted in italics. The G1/Berlin029/02/DE 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.

found in April (37.4%, 31 of 83), followed by March and February with 31.3% (26 of 83) and 18.1% (15 of 83), respectively. The lowest rotavirus detection rate was in June (2.4%; 2 of 83).

Distribution of rotavirus G- and P-types with an emergence of G3

The distribution of rotavirus G- and P-types of rotavirus from July 2003 to June 2004 was reported. Only two different rotavirus G-types, G2 and G3, were detected during the study period. Interestingly, rotavirus G3 emerged and became the most prevalent genotype. An exceptionally high prevalence of rotavirus G3 was found, accounting for the majority of rotavirus strains detected in our study (97.5%; 81 of 83). The rotavirus G2 was identified only in 2 specimens and represented 2.5%. In contrast, no G1, G4 or G9, which before 2004 were the common or emerging genotypes (see Figure 2), was detected in the present study. When examined for their P-types, two common genotypes, P[4] and P[8], were identified. The majority of rotavirus strains were P[8] with 97.5% (81 of 83) and 2.5% (2 of 83) were P[4]. However, there were 19 rotavirus strains (22.9%) whose P-types could not be determined by RT-PCR using specific primers previously reported in the literature.

Determination of rotavirus P-types by nucleotide sequence analysis

Nineteen rotavirus strains whose P-types could not be determined initially by 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, all of them were P[8]. Figure 3 shows that these rotavirus P[8] strains contained 5-6 point mutations at the VP4 primer-binding site. As many as nineteen P[8] strains proven by sequence analysis had great homology at the nucleotide level of each other, ranging from 99% to 100%. By using BLAST these strains were highly homologous (98%-100%) with the Thai P[8] strain CU90 (accession number DQ235978).

The frequencies of various combinations of the G- and P-types of rotavirus detected in this study were also investigated. The G3P[8] combination was the most predominant genotype and represented 95.2% (79 of 83). A number of unusual combinations, G3P[4] (2.4%, 2 of 83) and G2P[8] (2.4%, 2 of 83) [5], were also detected during this study period.

Nucleotide sequencing and phylogenetic analysis of new variant rotavirus G3 strains

To establish the changing epidemiology of rotavirus genotypes, the VP7 genes of rotavirus G3 strains were sequenced. Rotavirus G3 sequences were analyzed by phylogenetic analysis and grouped using the rotavirus G3 VP7 region classification scheme [12]. It was found that the Japanese rotavirus G3 strains in 2003-2004 had high homologies at the nucleotide as well as the amino acid level, ranging from 99% to 100% of each other. However, it was also shown that these G3 strains had the closest matches of only 94%-96% at the nucleotide level to those in Japan in 1990-1995 mentioned in Table 1. Moreover, there were high identities (97%-100%) between rotavirus G3 strains in 2003-2004 and recent worldwide G3 strains mentioned in Table 1. Figure 4 shows that the Japanese rotavirus G3 strains in 1990-1995 and in 2003-2004 made two distinct clusters, I and II, respectively. Compared with the Japanese G3 strains in 1990-1995, the Japanese G3 strains in 2003-2004 further demonstrated 16 substitutions at amino acids 16, I to V; 40, V to I; 66, L to P; 96, D to N; 99, P to K; 100, G to D; 108, T to I; 121, N to Y; 213, K to N; 256, H to N; 266, S to P; 278, T to M; 303, A to V; 323, T to Y; 324, T or S to Y; and 325, G to R. Of these, point mutations at amino acids 96, 99, and 100 were located in the antigenic region A, and amino acid 213 in the antigenic region C (Table 1). In contrast, no point mutation or only few point mutations at amino acids 41, T to I; 116, V to Y; and 320, A to F, were found between the Japanese G3 strains in 2003-2004 and G3 strains in Thailand, Malaysia, Taiwan, India, China previously registered in GenBank.

DISCUSSION

Rotavirus G1 is reported to be the most common causative agent of diarrhea in the majority of various countries of Europe, North and South America, Africa, and Asia [1, 4, 13-15]. In Japan, rotavirus G1 was a leading genotype since 1984 [16-18]. However, the prevalence pattern of rotavirus genotypes has been changing with a rapid decrease of G1 and a slight increase of G2 and G3 since 2000 [18]. Of particular interest was the finding that rotavirus G3 in this study had a dramatic increase with an exceptionally high prevalence (97.5%) in 2003-2004. More interestingly, other very common rotavirus G-types, such as G1 and G4, could not be detected during the same period of time. Rotavirus G9 has been recognized as the most widespread of the emerging genotypes since 1996 and to be the frequent cause of severe acute gastroenteritis in many countries, covering all continents of the world [19-23]. In Japan, only one rotavirus G9 was firstly detected in Tokyo in 1996-1997 [17]. And then, rotavirus G9 was determined to be the prevailing genotype in 1998-2003 with 15.3% [17, 18]. However, rotavirus G9 appeared to vanish in 2003-2004. Thus, taking into account the experience of 21 years of rotavirus surveillance in Japan, the period 2003-2004 was unusual in that a previously rare genotype (G3) rose to dominance whereas genotypes prominent in earlier years became rare or disappeared altogether. It is possible that the Japanese pediatric population might have enough antibody protection from acquired viral immunity against G1, G4, and G9 which

had been triggered by the previous rotavirus infection. This hypothesis is in line with the recently published findings in which rotavirus strains were genetically and antigenically similar, even though they had circulated in Japan during a long period of time (1999 to 2003) [18]. Unlike the successful G-typing of all rotaviruses, there were 19 rotavirus strains whose P-types could not be determined by standard RT-PCR with specific primers. Therefore, their P-types were further proven by sequence analysis. After sequence analysis, they had the closest relatives among rotavirus P[8] strains, demonstrating that these rotavirus strains belonged to the P[8] genotype. Interestingly, these rotavirus P[8] strains were found to contain 5-6 mismatches at the VP4 primer-binding site. Quite possibly, the initial failure to identify a considerable fraction of the rotavirus P strains in our study was due to those point mutations.

To investigate the changing distribution of rotavirus genotypes and to understand the genetic evolution of rotavirus G3 in Japan, the VP7 gene was subjected to sequencing analysis. The Japanese rotavirus G3 strains in 2003-2004 were found to be highly identical to each other and to recent worldwide rotavirus G3 strains. In contrast, these rotavirus G3 strains demonstrated a low identity in comparison with those circulating in Japan in 1990-1995. Of interest, a wide range of amino acid substitutions (up to 16) of rotavirus G3 VP7 genes was identified among them. According to the study of the neutralization sites on VP7 of rotavirus G3, mutations at amino acid position 96 in the region A was selected by antibodies and recognized as neutralization-escape mutants [24, 25]. Furthermore, the motif located at positions 211-213 in the antigenic region C was the critical region of neutralizing antibody [24]. Altogether, the amino acid mutations of VP7, especially in the antigenic regions A and C, played crucial roles in altering antigenicity that might lead to the emergence of new variant rotavirus G3 strains in Japan. This emergence of new variant rotavirus G3 indicated that the pediatric population might lack antibody protection against these strains, and that these strains might be more virulent. Surveillance of rotavirus infection should be continuously done to determine whether these strains continue to be dominant in Japan in the coming years.

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