were identified (data not shown). In some reports, NoV was prevalent in the cold season, whereas several studies did not find a seasonal correlation [20-24]. In strong agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, the main peak of NoV infection was between November, December, and January [25, 26]. In this study the highest incidence of cases was in the 1-year age group, and the incidence decreased with increasing age over 1 year. Quite possibly, 1-year old children might lack antibody protection to NoV, whereas by the time they have reached the age of 2 years they have begun to acquire viral immunity.

The results of the study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.4% and 98.6%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric populations. Extensive epidemiological studies of NoV infection worldwide, including Japan, which characterized NoV strains and identified the prevalent genotypes circulating in infants and children with acute gastroenteritis, have indicated that NoV GII/4 was the most prevalent genotype [5, 9, 23, 25]. However, the emergence of new NoV GII/3 was identified, and this strain became the leading genotype (43.9%) in Japan during 2003 to 2004 [27]. At the same time, the prevalence of NoV GII/4 rapidly dropped from 75.6% in 2002-2003 [28] to 35.1% in 2003-2004 [27]. In this study, the changing pattern of genotype distribution of NoV infection in children with acute gastroenteritis has been demonstrated. Of note, the NoV GII/4 re-emerged to be the most prevalent with a high frequency (77.7%) compared to the lower frequency of NoV GII/3 (15.8%) and NoV GII/6 (4.4%), which were the second and third prevailing genotypes, respectively. We hypothesized that the insufficient antibody protection from acquired viral immunity against NoV GII/4 in Japanese pediatric populations was due to the lack of immunization by the previous NoV GII/4 infection during 2003-2004. This hypothesis was in strong agreement with recent findings that the detection rate of NoV GII/4 infection was low during 2003-2004 [27]. Interestingly, NoV GII/4 strains detected in this study (2004-2005) made a distinct cluster, which was separate from NoV strains in 2002-2003, and 2003-2004 even all of them belonged to the same genotype. However, this might be due to the co-existence of multiple factors such as changes of climate, water, and others.

Another interesting finding of this study was the identification of the Picton/03/AU-like strain. The Picton/03/AU was isolated from an outbreak of vomiting and diarrhea at a care facility for the elderly in New South Wales, Australia in July 2003 and had been reported to be a rare recombinant with GII/1 capsid and GIIb polymerase [29]. Surprisingly, our strain, the 6322JP, was not recovered from an elderly patient but from a 1-year old male child with acute gastroenteritis in Maizuru City in 2005. The 6322JP shared a high identity with the Picton/03/AU and therefore it also was a NoV recom-

binant. This is the first report of the detection of the rare Picton/03/AU-like recombinant in Japan. More interestingly, the high detection rate of "new NoV variant with GIIb polymerase" 5424Saga-like strains was identified. "New NoV variant with GIIb polymerase" was recently found to cause outbreaks as well as sporadic cases of acute gastroenteritis throughout European countries [30-32]. In Japan, the strain 5424Saga, recognized as a "new NoV variant with GIIb polymerase", was first recovered from a male patient aged 2 years who developed symptoms of acute gastroenteritis in Saga City in 2003 and had been reported to be a recombinant with GII/3 capsid and GIIb polymerase based on the genetic analysis [27]. The sudden increase in the number of the variant strain from 4% in 2003-2004 to 45% in 2004-2005 indicated that this variant was still virulent in causing the illness in Japan. Further surveillance of diarrheal viruses should be conducted to determine whether this recombinant NoV variant will be dominant in Japan in the coming year.

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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: 41-48)

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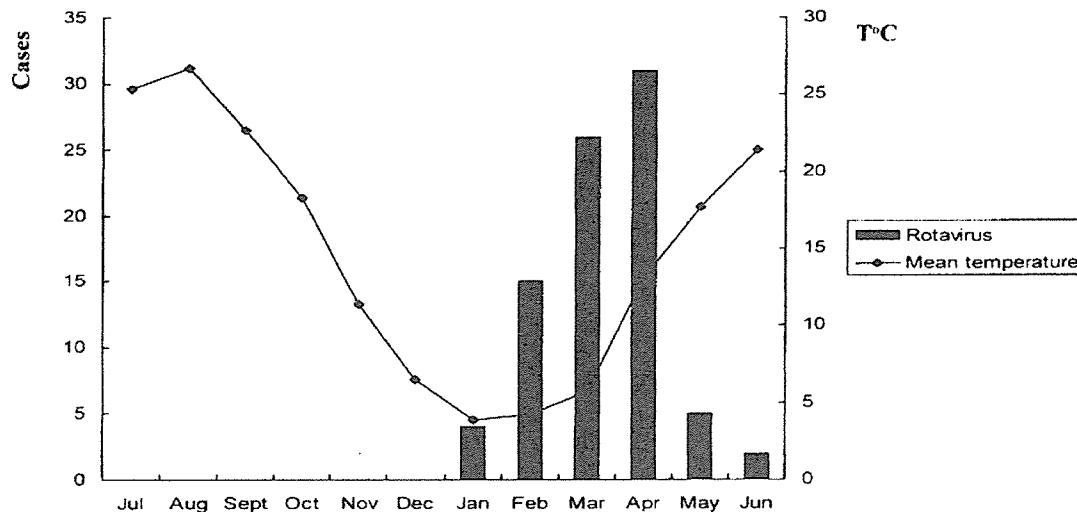
Figure 1: Seasonal variation of rotavirus infection among infants and children with acute gastroenteritis in five different regions (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2004. The mean temperature for the five regions obtained from the Japan Meteorological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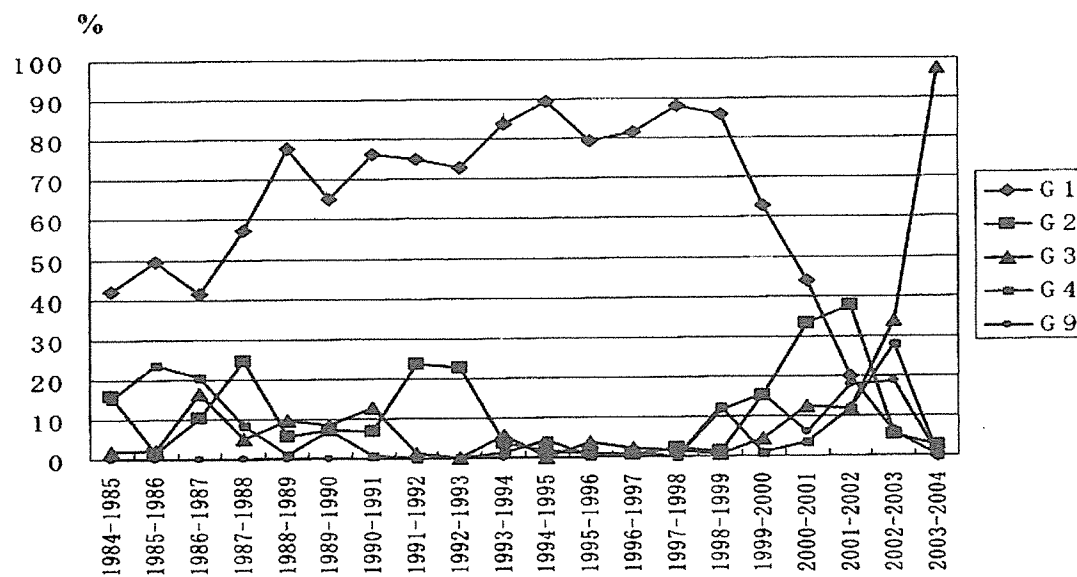

Figure 2: Prevalence pattern of rotavirus G-types with an emergence of rotavirus G3 together with a sudden disappearance of G1, G4, and G9 during the 21-year survey of rotavirus infection among infants and children in the same age group with acute gastroenteritis in five different regions (Maizuru, Tokyo,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

nt 339	nt 356	Position of binding site
3'-GCACGTTATCCAAGTAGA-5'		Primer 1T1
* * * * * *CGAT*C * * * * * *		5051JP P[8]
* * * * * *CGAT*C * * * * * *		5133JP P[8]
* * * * * *CGATTC * * * * * *		5092JP P[8]

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 fol-lows: 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 rota-virus. 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 identi-fied 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 Decem-ber. The highest prevalence of rotavirus infection was
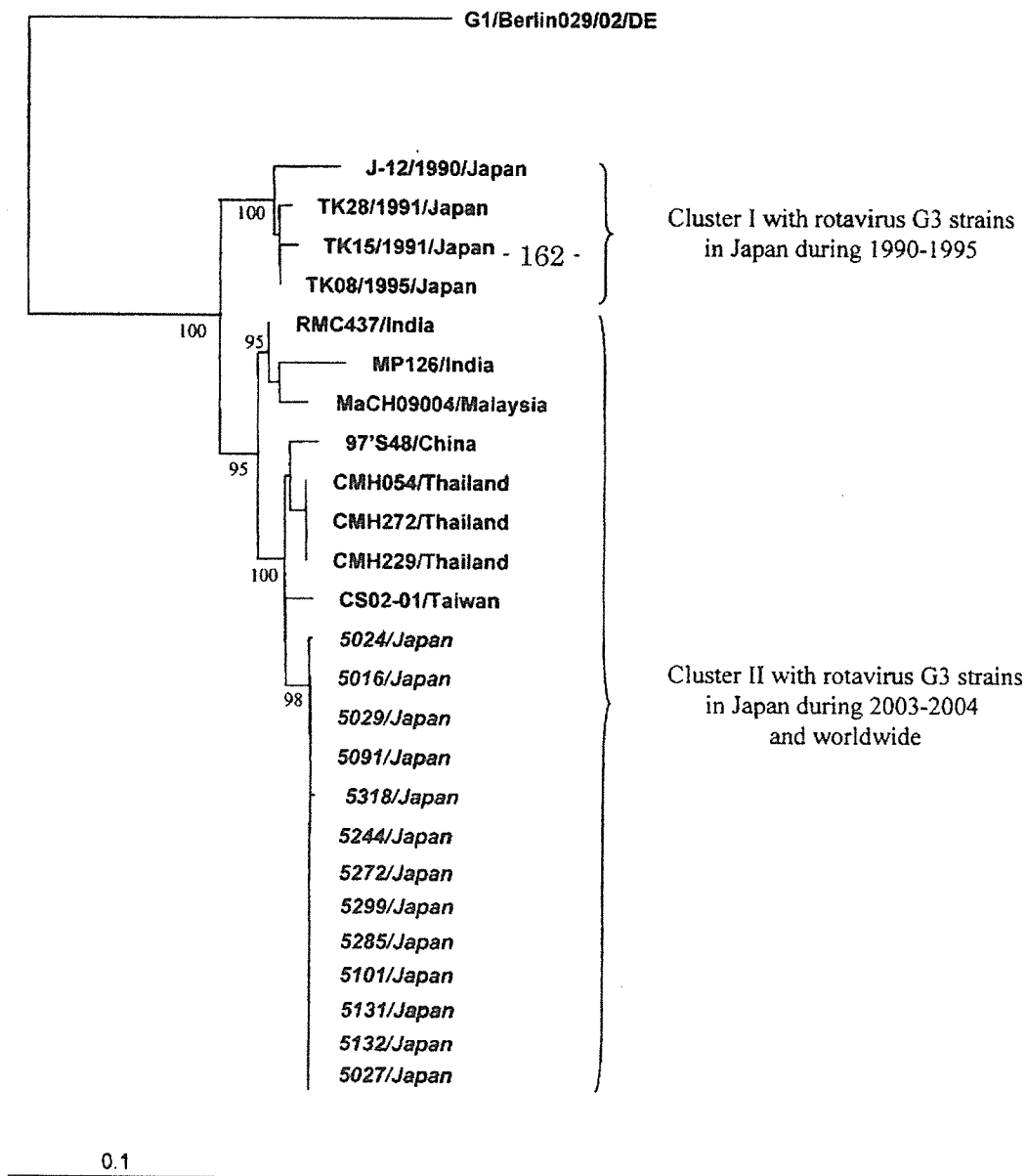
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                                    ——————— G1/Berlin029/02/DE

                                   ┌——— J-12/1990/Japan          ⎫
                              100 ┌┤  TK28/1991/Japan             ⎪
                                  │├  TK15/1991/Japan - 162 -     ⎬  Cluster I with rotavirus G3 strains
                                  │└  TK08/1995/Japan             ⎭      in Japan during 1990-1995
                100              95 ┌ RMC437/India                ⎫
                              95 ┌┤  ┌ MP126/India                ⎪
                                │└  └ MaCH09004/Malaysia          ⎪
                                │┌ 97'S48/China                   ⎪
                                ││ CMH054/Thailand                ⎪
                                ││ CMH272/Thailand                ⎪
                             100 │ CMH229/Thailand                ⎪
                                 ┌ CS02-01/Taiwan                 ⎪
                                 │ 5024/Japan                     ⎪
                                 │ 5016/Japan                     ⎬  Cluster II with rotavirus G3 strains
                              98 │ 5029/Japan                     ⎪      in Japan during 2003-2004
                                 │ 5091/Japan                     ⎪          and worldwide
                                 ├ 5318/Japan                     ⎪
                                 │ 5244/Japan                     ⎪
                                 │ 5272/Japan                     ⎪
                                 │ 5299/Japan                     ⎪
                                 │ 5285/Japan                     ⎪
                                 │ 5101/Japan                     ⎪
                                 │ 5131/Japan                     ⎪
                                 │ 5132/Japan                     ⎪
                                 │ 5027/Japan                     ⎭

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           0.1
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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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Novel Recombinant Norovirus in China

To the Editor: Norovirus (NoV), the distinct genus within the family *Caliciviridae*, is a major cause of sporadic cases and outbreaks of acute gastroenteritis in humans (*1*). NoV possesses a positive-sense, single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains 3 open reading frames (ORFs). ORF1 encodes non-structural proteins, ORF 2 encodes capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). NoV is still uncultivable by standard culture with different cell lines. However, expression of either VP1 or both VP1 and VP2 with recombinant baculoviruses formed viruslike particles that are morphologically and antigenically similar to the native virion (*2*).

A fecal specimen was collected from an infant hospitalized with acute gastroenteritis in Kunming, China, in November 2004 and was tested for diarrheal viruses in a cooperative laboratory in Japan. The viral genome was extracted by using a Qiagen kit (Qiagen, Hilden, Germany). Polymerase chain reaction with specific primers resulted in the identification of astrovirus, rotavirus, sapovirus, adenovirus, and NoV genogroup I (GI) and GII (*3*). NoV polymerase was also amplified to identify recombinant NoV with primers Yuri22F and Yuri22R (*4*). Products were sequenced directly, and sequence analysis was performed by using ClustalX and SimPlot.

The fecal specimen was positive for NoV GII. The Figure shows that the 146/Kunming/04/China sequence clustered into the distinct GII genotype 7 (Leeds/90/UK cluster). 146/Kunming/04/China was classified into the Saitama U4 cluster (GI/6) when polymerase-based grouping was performed. Altogether, 146/Kunming/04/China was expected to be the

recombinant NoV with GII/7 capsid and GII/6 polymerase.

To eliminate the possibility of co-infection with 2 different NoV genotypes, to localize the potential recombination site, and to clarify a possible recombination mechanism, the ORF1/ORF2 overlap and flanking polymerase and capsid regions of 146/Kunming/04/China was amplified with primers Yuri22F and GIISKR to produce a 1,158-bp amplicon (*3,4*). When the sequence of 146/Kunming/04/China was compared with that of Saitama U4 by using SimPlot, a recombination site was found at the ORF1/ORF2 overlap. Before this junction, 146/Kunming/04/China and Saitama U4

were homologous. After the ORF1/ORF2 overlap, however, the homology was notably different. SimPlot showed a sudden drop in the nucleotide identity for 146/Kunming/04/China. ClustalX showed that 146/Kunming/04/China shared a high identity (93%) in the polymerase region and a low identity (78%) in the capsid region with Saitama U4. In contrast, high identity (95%) in the capsid region was found between 146/Kunming/04/China and Leeds/90/UK. Since Leeds/90/UK polymerase was not available in GenBank, the polymerase homology between 146/Kunming/04/China and Leeds/90/UK was unknown. Polymerase of 146/Kunming/04/China was almost
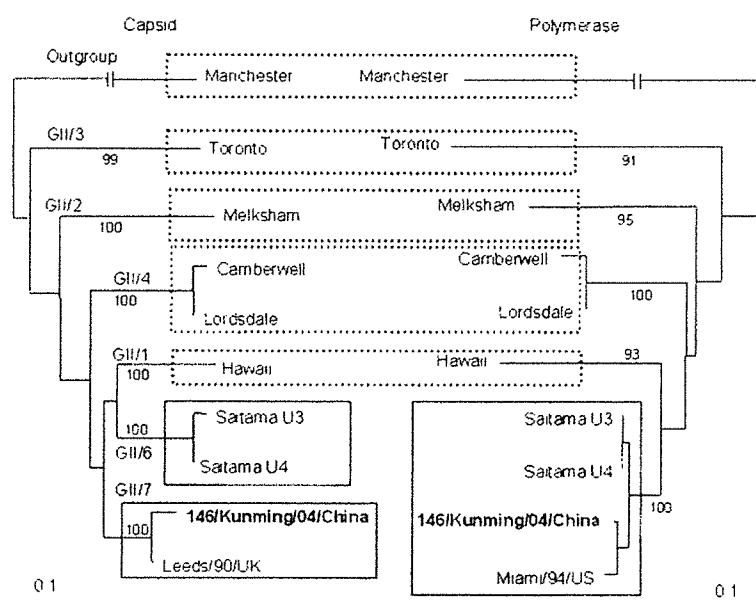

Figure. Changes in norovirus (NoV) genotypes on the basis of phylogentic trees of nucleotide sequences of 146/Kunming/04/China. Trees were constructed from partial nucleotide sequences of capsid and polymerase regions of 146/Kunming/04/China. 146/Kunming/04/China is **boldface**. Dashed boxes indicate the maintenance of genotypes of reference NoV strains, and solid boxes indicate the involvement of NoV genotypes with recombinant NoV 146/Kunming/04/China. A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using the Kimura 2-parameter method (PHYLIP). The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Manchester strain was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence of NoV strain 146/Kunming/04/China had been submitted to GenBank and has been assigned accession no. DQ304651. Reference NoV strains and accession nos. used in this study are as follows: Manchester (X86560), Toronto (U02030), Melksham (X81879), Camberwell (AF145896), Leeds/90/UK (AJ277608), Lordsdale (X86557), Hawaii (U07611), Saitama U3 (AB039776), Saitama U4 (AB039777), and Miami/94/US (AF414410).

identical with that of Saitama U4, but the capsids of 146/Kunming/04/China and Leeds/90/UK were distinctly different from that of Saitama U4. This genetic pattern of 146/Kunming/04/China implied a novel, naturally occurring recombinant NoV with GII/7 capsid and GII/6 polymerase.

RNA recombination is a mechanism for virus evolution (5). Literature documenting recombination in NoV is fairly rich, but none is from China (6). Therefore, 146/ Kunming/04/China was not only the first but also the first recombinant NoV from China. This isolate shared the closest sequences of polymerase and capsid with Saitama U4 and Leeds/90/UK, respectively. Strain Saitama U4 was detected in 1997 in Japan (7), whereas strain Leeds/90/ UK was detected in 1990 in the United Kingdom (8). Quite possibly, Saitama U4 and Leeds/90/UK were parental strains of 146/Kunming/04/China. However, the distant geographic relationship of these strains obscured evidence of where and when the recombination event occurred. This phenomenon also suggested that these parent strains or this progeny strain might be more prevalent than is often assumed.

Recombination depends on various immunologic and intracellular constraints. Recombinant viruses are all alike in that they successfully pass through 5 stages: 1) successful co-infection of a single host, 2) successful co-infection of a single cell, 3) efficient replication of both parental strains, 4) template switching, and 5) purifying selection (9). In this study, 146/Kunming/04/China was recovered from a patient with diarrhea, fever, and vomiting. This observation indicated that this strain theoretically fulfilled all prerequisites for recombination.

The NoV capsid is predicted to be well suited for genotype classification (10). In this study, 146/Kunming/04/China belonged to 2 distinct genotypes, 7 and 6, by capsid- and poly-

merase-based groupings, respectively. Moreover, the recent demonstration of recombination in an increasing number of NoVs suggests that it is a more widespread event than was previously realized. Consequently, the phylogenetic classification of NoV on the basis of on capsid sequence is questionable. We suggest that classification of NoV strains should rely on not only capsid sequence but also polymerase sequence.

In conclusion, our results described the genetic characterization of novel, naturally occurring recombinant NoV and increased evidence for the worldwide distribution of recombinant NoV. This report is the first to describe acute gastroenteritis caused by recombinant NoV in China and warns of the threat it poses.

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Instructions for Emerging Infectious Diseases Authors

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

closely related to *E. coli,* as was previously observed in Tanzania (*9*). This finding is also the first report of CTX-M-3 in sub-Saharan Africa.

Multidrug resistance profiles involving non–β-lactam antimicrobial drugs coselected these ESBL-producing isolates. We suggest that the misuse of antimicrobial drugs in the Central African Republic and the migratory flux of regional populations could result in emergence and selection of these ESBL phenotypes in the community. We could not establish a relationship between the different strains isolated in hospitalized and ambulatory patients. Because of the implications for treating such infections, particularly in developing countries, the spread of ESBL-producing *Enterobacteriaceae* merits close surveillance in the Central African Republic.

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Novel Recombinant Sapovirus, Japan

To the Editor: *Sapovirus* is the distinct genus within the family *Caliciviridae*; these viruses cause sporadic cases and outbreaks of gastroenteritis in humans worldwide (*1*). The sapovirus genome contains 2 open reading frames (ORFs). ORF1 encodes nonstructural and capsid proteins while ORF2 encodes a small protein (*2*). Sapovirus has a typical "Star of David" configuration by electron microscopic examination. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/ JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (*3*). Sapovirus is divided into 5 genogroups, among which only genogroups I, II, IV, and V are known to infect humans (*4*).

A fecal specimen was collected from a 1-year-old boy with acute gastroenteritis in Osaka, Japan, in March 2005. The viral genome was extracted by using a QIAamp kit (Quigen, Hilden, Germany). By using multiplex reverse transcription–polymerase chain reaction (RT-PCR), 2 groups of diarrheal viruses were identified. The first group included astrovirus, norovirus, and sapovirus; the second group included rotavirus and adenovirus (*5*). Sapovirus polymerase region was also amplified to identify recombinant sapovirus by using primers P290 and P289 (*6*). To eliminate the possibility of co-infection of 2 different sapovirus genotypes, to localize the potential recombination site, and to understand a possible recombination mechanism of recombinant sapovirus, flanking polymerase and capsid regions, with their junction of HU/5862/Osaka/JP, were amplified with primers P290 and SLV5749 to produce a 1,162-bp product (*5,6*). Products were directly sequenced, and capsid- and polymerase-based phylogenetic trees showed recombinant sapovirus.

The fecal specimen was positive for sapovirus. HU/5862/Osaka/JP clustered into the genogroup I genotype 8 (GI/8 the 8/DCC/Tokyo/JP/44 cluster) (Figure) by using the recent sapovirus capsid region classification (7). HU/5862/Osaka/JP with GI/8 capsid was classified into GI/1 (the Sapporo/82 cluster) when polymerase-based grouping was performed. When the sequence of HU/5862/Osaka/JP was compared with that of Sapporo/82 by using SimPlot Version 1.3 (available from http://sray.med.som.jhmi.edu/SCRoftware/simplot), the recombination site was identified at the polymerase-capsid junction. Before this junction, sequences of HU/5862/Osaka/JP and Sapporo/82 were highly homologous. However, homology between them was notably different after the junction, with a sudden drop in the identity for HU/5862/Osaka/JP. By using

ClustalX, HU/5862/Osaka/JP shared a 96% identity in polymerase sequence and an 85% identity in capsid sequence with Sapporo/82. In contrast, homology was 99% in the capsid region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44. Since a polymerase sequence of 8/DCC/Tokyo/JP/44 was not available in GenBank because of the unsuccessful amplification, homology in the polymerase region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44 was unknown.

Altogether, the findings underscored that HU/5862/Osaka/JP represented a novel, naturally occurring, recombinant sapovirus with GI/8 capsid and GI/1 polymerase. To determine whether the child was infected with this novel recombinant sapovirus or whether the novel recombinant sapovirus resulted from co-infection with 2 different viruses, Svppo

(Sapporo/82-specific primer), Svdcc (8/DCC/Tokyo/JP/44-specific primer), and SLV5749 were used to amplify the capsid region (5). However, no amplicon was found. These negative results indicate no co-infection in this child.

Even though many molecular epidemiologic studies on sapovirus infection have been performed worldwide, reports documenting recombination in sapovirus are still limited. The first recombinant sapovirus identified was the Thai isolate Mc10 or the Japanese isolate C12 (8); the Japanese isolate Ehime1107 and the SW278 isolate from Sweden were identified later (9). Recombination occurred only in sapovirus genogroup II, which is more capable of recombination than other genogroups (8,9). In this study, we identified HU/5862/Osaka/JP with a novel recombination between 2 distinct genotypes within genogroup I. This is the first report of acute gastroenteritis caused by recombinant sapovirus genogroup I. The findings underscore that natural recombination occurs not only in sapovirus genogroup II but also in genogroup I.

In recent studies of sapovirus recombination, evidence for the location of the recombination event is lacking because of the distant geographic relationship of parent and progeny strains. HU/5862/Osaka/JP shared the closest sequences of polymerase and capsid with Sapporo/82 and 8/DCC/Tokyo/JP/44, respectively. Sapporo/82 was first isolated in 1982, and 8/DCC/Tokyo/JP/44 was isolated in 2000, both in Japan. Possibly, Sapporo/82 and 8/DCC/Tokyo/JP/44 were parental strains of HU/5862/Osaka/JP, and the event leading to the novel recombination might have occurred in Japan.

The capsid region was used for genotype classification of sapovirus (7). When capsid-based grouping was performed, HU/5862/Osaka/JP distinctly belonged to genotype 8. When polymerase-based grouping was
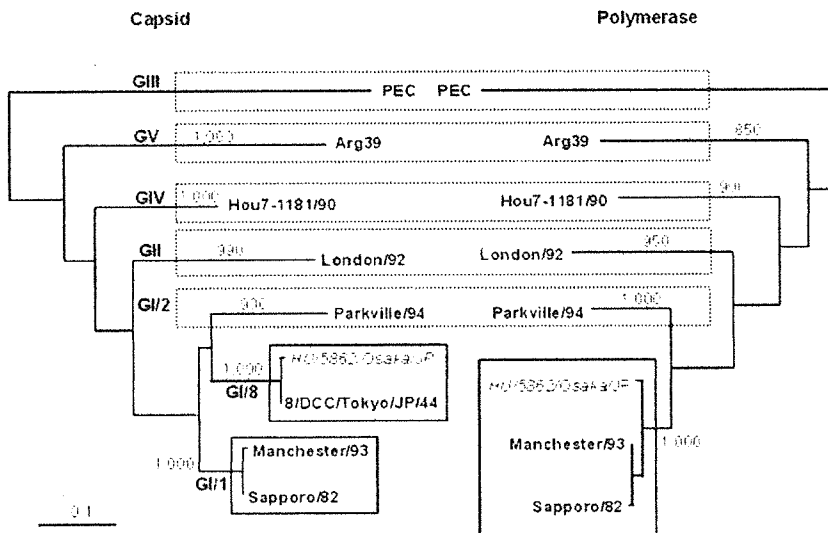

Figure. Changing genotypes of sapovirus on the basis of phylogenetic trees. Trees were constructed from partial amino acid sequences of capsid and polymerase of HU/5862/Osaka/JP highlighted in *italics*. Phylogenetic tree with 1,000 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using Kimura 2-parameter method (PHYLIP). The scale indicates amino acid substitutions per position. The numbers in branches indicate bootstrap values. Porcine enteric calicivirus was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence data of sapovirus strain HU/5862/Osaka/JP has been submitted to GenBank and has been assigned accession no. DQ318530. Reference sapovirus strains and accession nos. used in this study were as follows: PEC (AF182760), London/92 (U95645), Arg39 (AY289803), Parkville/94 (U73124), Manchester/93 (X86560), Sapporo/82 (U65427), Hou7-1181/90 (AF435814), and 8/DCC/Tokyo/Japan/44 (AB236377).

performed, HU/5862/Osaka/JP distinctly belonged to genotype 1. Therefore, sapovirus classification based on capsid sequence is questionable. We suggest that sapovirus classification should rely not only on capsid sequence but also on polymerase sequence.

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Postmortem Confirmation of Human Rabies Source

To the Editor: Rabies is a fatal encephalitis caused by a neurotropic RNA virus of the family *Rhabdoviridae*, genus *Lyssavirus*. The predominant rabies virus reservoir hosts are bats and carnivores. Among these, rabid dogs represent a substantial public health problem, particularly in developing countries (*1*).

Laboratory diagnosis of rabies is essential to guide control programs, epidemiologic surveys, and prophylactic measures (*2*). Among the laboratory tests recommended by the World Health Organization (WHO), the fluorescent antibody test (FAT) is the accepted standard for rabies diagnosis (*1*). Although rabies virus antigens can be detected in decomposed samples, FAT is less effective when such samples are tested. In those cases, polymerase chain reaction (PCR) can provide better results (*3*). Since the degree of decomposition at which FAT starts to become ineffective is unknown (*4*), when smears from decomposed samples are made for FAT, a suspension of the same brain tissues should be made in the appropriate diluents for the mouse

inoculation test (MIT), cell culture, or reverse transcription–polymerase chain reaction (RT-PCR) (*2*). However, if all test results are negative, rabies cannot be ruled out because of the condition of the sample.

On February 28, in the city of Carbonita, Minas Gerais State, in southeastern Brazil, a 62-year-old man was bitten by a bat on the right ankle. Approximately 50 days later, his leg began to feel numb, and he experienced a continuous headache, pain at the site of the bite, convulsions, frequent urge to clear his throat, hiccups, nausea, difficulty in swallowing, dry lips, slightly elevated body temperature (37°C–37.5°C), paralysis of superior and inferior left limbs, shaking, and hallucinations. On May 4, 16 days after clinical manifestations began, the patient died; the cause of death was registered as a cerebral vascular accident. One month later, the body was exhumed to obtain a sample from the central nervous system (CNS), which was sent to Instituto Pasteur, São Paulo, registered as sample 5341 M/04 and tested by FAT, MIT, and RT-PCR.

In total, 8 smears were prepared from the sample to be analyzed by FAT according to the method of Dean et al. (*5*) with fluorescein isothiocyanate–labeled polyclonal antinucleocapsid antibodies. MIT was carried out as described by Koprowski (*6*) with 7 mice. For RT-PCR, RNA was extracted from the CNS sample with TRIzol, according to the manufacturer's instructions (Invitrogen, Rockville, MD, USA). RT-PCR was carried out with modifications as described by Orciari et al. (*7*), with primers 504 (sense) and 304 (antisense), aiming at the amplification of a 249-bp fragment of rabies virus nucleoprotein (N) gene, by using Superscript II (Invitrogen) and Taq DNA-polymerase (Invitrogen).

Fluorescent inclusions were observed in 6 of the 8 slides prepared for the FAT. The RT-PCR of the RNA

Detection of Norovirus Antigens from Recombinant Virus-Like Particles and Stool Samples by a Commercial Norovirus Enzyme-Linked Immunosorbent Assay Kit

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The commercial norovirus enzyme-linked immunosorbent assay kit was evaluated for its reactivity to recombinant virus-like particles and the detection of natural viruses from stool samples of Japanese infants and children with sporadic acute gastroenteritis compared to reverse transcription-PCR. The kit had a sensitivity of 76.3% and a specificity of 94.9%. Our results clearly indicated that the kit allows the detection of the most prevalent genotype, GII/4. In order to increase the sensitivity of the kit, the reactivity with norovirus of GII/3 and GII/6 genotypes needs to be improved.

Norovirus (NoV) is one of the leading etiologic agents of nonbacterial sporadic acute gastroenteritis (AGE) in infants and children, and outbreaks of this infection may be due to contaminated water or food. At present, the reverse transcription-PCR (RT-PCR) assay is widely used to detect NoV in diarrheal stool samples. The development of immunological methods to detect NoV has been delayed due to the lack of viruses in cell culture and to diverse genotypes with distinct antigenicities. NoVs are currently divided into five genogroups, and most human NoV strains belong to two genogroups: genogroup I (GI) and genogroup II (GII). Furthermore, each genogroup contains at least 15 and 18 genotypes, respectively (13). RT-PCR is an expensive and complicated technique, and its use requires special equipment and skills. Thus, a faster and simpler method is needed. At present, three commercial enzyme-linked immunosorbent assay (ELISA) kits are available, the IDEIA NLV kit from Dako Cytomation, Ltd. (Ely, United Kingdom), the SRSV(II)-AD kit from Denka Seiken Co., Ltd. (Tokyo, Japan), and the RIDASCREEN norovirus (R-Biopharm AG, Darmstadt, Germany). According to previous evaluations of the ELISA kits, the first two kits cannot effectively replace RT-PCR for NoV detection due to their low sensitivities and/or specificities (1, 3, 19). To date, the RIDASCREEN norovirus assay has only been evaluated by one Australian group using outbreak specimens (2). No research has hitherto been conducted using recombinant virus-like particles (rVLPs) and sporadic stool samples.

Therefore, using the RIDASCREEN norovirus ELISA kit, we set out to measure the reactivity of 16 kinds of rVLPs, to detect the presence of NoV in fecal samples from infants and

children with sporadic AGE in Japan, and to compare the sensitivity and specificity data with those obtained with the RT-PCR.

We previously expressed one rVLP (strain 1207, GII/4) (14). The other 15 rVLPs were prepared from NoV isolated from stool samples among infants and children with diarrhea between 1995 and 2003 in Japan. The genotypic classification of these NoV was performed based on the method described by Kageyama et al. (6). These are genotypes 1 (strain 4656), 3 (strain 3634), 4 (strain 2876), 8 (strain 3006), and 11 (strain 2258) in genogroup I and genotypes 1 (strain 3101), 2 (strain 2840), 3 (strain 3229), 5 (strain 3611), 6 (strain 3612), 7 (strain 419), 12 (strain 2087), 13 (strain 3385), 14 (strain 2468), and 15 (strain 3625) in genogroup II. The production of recombinant bacmids was performed using the baculovirus expression system with Gateway Technology (Invitrogen Japan, Tokyo) and the transfection of bacmids into insect cells, as well as the purification of rVLPs, was performed according to the method of Hansman et al. (5). We used two sense primers: attB1NVGI (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAT GAT GGC GTC TAA GG) for GI strains and attB1NVGII (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAA GAT GGC GTC GAA TGA) for GII strains. Purified rVLPs from the cultured supernatants of the inset cells were examined for particle formation by electron microscopy. Protein concentration of each rVLP was measured by BCA Coomassie protein assay (Pierce Biotechnology, Inc., Rockford, IL), and 150 μg/ml was prepared as stock solutions. The assays were started from 10 μg/ml as the highest concentration.

The rVLPs stock solutions were serially threefold diluted with the sample dilution buffer in the the RIDASCREEN norovirus ELISA kit and used to determine the minimal concentration of each rVLP for detection by ELISA according to the manufacturer's manual. All of the assays except that for

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TABLE 1. Minimal detected concentrations of rVLPs by ELISA

VLP	Mean minimal rVLP concn (μg/ml) ± SD[a]	
	Theoretical[b]	Detected[c]
GI/1	0.00106 ± 0.00042 (3)	
GI/3	0.0368 ± 0.0084 (3)	
GI/4		8.33 ± 2.89 (3)
GI/8	0.118 ± 0.084 (3)	.
GI/11	0.0752 ± 0.0273 (3)	
GII/1	0.456 ± 0.191 (4)	
GII/2	0.116 ± 0.012 (3)	
GII/3		6.00 ± 3.65 (5)
GII/4	0.00298 ± 0.00100 (3)	
GII/5	4.22 ± 3.26 (4)	
GII/6		10 (1)
GII/7	10 < (3)*	
GII/12	0.468 ± 0.393 (3)	
GII/13	10 < (3)*	
GII/14		3.33 (2)
GII/15		6.67 ± 4.71 (2)

[a] The values show three significant figures. Each assay was done in triplicate, and the assay for single rVLP was repeated three to six times. The number of samples is given in parentheses.

[b] The theoretical values were calculated from the absorbance given by the serial dilution of rVLPs. The calculations were performed by cubic logit-log analysis ($R^2 > 0.949$). *, these rVLPs (GII/7 and GII/13) could not be determined for values of <10 μg/ml in the assay.

[c] The rVLPs given in this column could not be used to calculate the theoretical values because a theoretical value of <10 μg/ml (used maximum concentration) could not be calculated in even one assay. In these cases, the minimal detected concentrations are given.

GI/3 were done with kits of the same lot number. In the manual, the cutoff value is calculated as an absorbance value of negative control plus 0.15. Values that are 10% above or below the cutoff value are considered to be in the gray zone and therefore need to be examined again. In view of this, the theoretical minimal detectable concentration of each rVLP was determined as a calculated value which gave an absorbance value that was 10% above the cutoff value in each assay. Each assay was conducted in triplicate, and the experiment for each rVLP was repeated three to six times.

Five hundred and three stool samples were collected from infants and children with AGE who visited six pediatric clinics in Sapporo, Tokyo, Maizuru, and Osaka, Japan, from July 2004 to March 2005. All of the stool samples were stored at −30°C until testing. Watery stool samples were diluted 1:2 with phosphate-buffered saline (PBS), and hard stool samples were suspended to 1:5 with PBS. The suspensions were clarified by centrifugation at 10,000 × g for 15 min. The supernatants were diluted to 1:3 with the sample dilution buffer of the kit and used for the assay. The positives or negatives of the samples were determined as mentioned above.

Ten percent stool suspensions of 503 samples were prepared with PBS from the same aliquots for ELISA, and viral RNA was extracted by the QIA amp viral RNA mini kit (QIAGEN, Tokyo, Japan). The detection of NoV (GI and GII), astrovirus, sapovirus, rotavirus, and adenovirus was performed by two sets of multiplex PCR (21, 22). NoV-negative samples were examined by using two sets of monoplex PCRs, for NoV GI and GII. Twenty samples were further assayed by seminested PCR using a primer set, which were G2SKF and G2SKR for NoV GII (9). The genotypes of NoV were determined according to the method of Phan et al. (16).

TABLE 2. Sensitivity, specificity, and agreement of ELISA and RT-PCR[a]

ELISA	Detection (no. of samples) by RT-PCR		
	+	−	Total
+	87	20[b]	107
−	27	369	396
Total	114	389	503

[a] Sensitivity = 76.3% (87/114); specificity = 94.9% (369/389); agreement = 90.7% (456/503).

[b] These samples were positive as determined by seminested PCR.

The minimal detectable sensitivity is indicated in Table 1. The kit could detect GI/1 and GII/4 rVLPs at concentrations of <0.01 μg/ml. rVLPs of GI/3, GI/8, GI/11, GII/1, GII/2, and GII/12 were detectable within a range between 0.04 and 1 μg/ml. On the other hand, rVLPs of GI/4, GII/3, GII/5, GII/6, GII/14, and GII/15 were detected at more than 1 μg/ml. In cases where the assays for GI/4, GII/3, GII/6, GII/14, and GII/15 could not be detected at concentrations of <10 μg/ml and theoretical detectable concentrations could not be calculated, minimal concentrations given by the assay have been indicated (Table 1). GII/6 rVLP could be detected once at the highest concentration, 10 μg/ml. Two rVLPs of GII/7 and GII/13 could not be detected at a concentration of <10 μg/ml.

NoV in stool samples collected from sporadic cases in Japan was examined using both the ELISA kit and the RT-PCR, and the kit was evaluated based on the RT-PCR (Table 2). The calculated percent sensitivity, specificity, and agreement were 76.3, 94.9, and 90.7%, respectively. Twenty samples were determined to be positive by the kit but negative by the RT-PCR. These samples became positive when tested by the seminested PCR using NoV GII-specific primer pair. A total of 27 samples were positive with the RT-PCR but negative with the kit. The genotypes of 134 positive stool samples recorded by the RT-PCR were identified by using the clustering determined by Kageyama et al. (6) (Table 3). The genotypes of kit-positive, PCR-positive samples were 1 GI/1, 3 GII/3, 82 GII/4, and 1 GII/6, and the sensitivities of GI/1, GII/3, GII/4, and GII/6 were 50, 23.1, 85.4, and 33.3%, respectively. The low sensitivities of GII/3 and GII/6 were comparable to the results for the rVLPs. RT-multiplex PCR detected four other species of viruses in 503 stool samples. These were 7 group A rotavirus, 27 adenovirus, 30 sapovirus, and 1 astrovirus samples, and the stool samples containing these viruses were determined to be negative by ELISA. Furthermore, multiplex-PCR indicated that 8 of 112 NoV GII-positive samples were mixed infected

TABLE 3. Genotypes of norovirus in positive stool samples as determined by RT-PCR

Genogroup/ genotype	No. of samples	Frequency (%)	No. of samples positive by ELISA (%)
GI/1	2	1.8	1 (50.0)
GII/3	13	11.4	3 (23.1)
GII/4	96	84.2	82 (85.4)
GII/6	3	2.6	1 (33.3)
Total	114	100.0	87

with other viruses (5 sapovirus, 2 group A rotavirus, and 1 adenovirus).

Some studies showed that the strains belonging to GII/4 cluster were most predominant not only in stool samples from sporadic cases involving infants and children but also from the outbreaks (8, 10, 12, 15, 16, 19). On the other hand, it was found that various genotypes of NoV strains were detected in the outbreak cases, and there were no predominant genotypes in outbreak strains (20). Furthermore, a change in the distribution of NoV genotypes in the sporadic cases and the emergence of recombinant viruses has been reported (7, 11, 17, 18).

The ELISA kit could detect two kinds of rVLPs (GI/1 and GII/4) with a high sensitivity. Meanwhile, the GII/3 and GII/6 rVLPs formed a group that was responsive at higher concentrations. A total of 23.1% of the stool samples containing GII/3 NoV, and 33.3% of the samples with the GII/6 genotype could be effectively examined by the kit. NoV genotypes with low reactivity levels in the stool samples could be detected by the kit in cases with a sufficient viral load. On the other hand, the genotypes of 20 samples, which were ELISA positive and seminested PCR positive, were 7 GII/3 and 13 GII/4. It would appear that these samples have a smaller viral load than monoplex PCR-positive stools. This suggests that there are other factors, such as inhibitors, that may cause the lower sensitivity of ELISA.

The sensitivity, specificity, and agreement of the kit were superior to those of the Denka and Dako kits (1). Dimitriadis and Marshall showed in 2005 that the RIDASCREEN ELISA kit could not be recommended for the study of stool samples in Australian outbreaks (2). In that report, the sensitivity and specificity of the kit were 71 and 47%, respectively, with the same cutoff calculations as our own. The difference between their sensitivity value and our own, which was 76.3%, was not large. On the other hand, the specificity was very different. In the present study, the specificity of the kit based on RT-PCR assay was 94.9%. There were the false-positive samples in their results. The reason for the difference in the specificities is unclear. We have been unable to obtain either the Denka kit or the Dako kit and have not been able to compare the RIDAS-CREEN kit with these kits using the same stool samples.

In conclusion, our results indicated that the kit could be a useful tool for sporadic diarrheal samples. However, it is quite possible to contain many kinds of genotypes in diarrheal samples derived from food-borne sources, and the particular kinds of genotypes found in such cases are not always the same as the genotypes found in sporadic cases. All in all, the reactivity for GII/3 and GII/6 needs to be improved in order to facilitate the detection of etiological agents in outbreaks.

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Detection of Rare G3P[19] Porcine Rotavirus Strains in Chiang Mai, Thailand, Provides Evidence for Origin of the VP4 Genes of Mc323 and Mc345 Human Rotaviruses[▽]

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Among 175 fecal specimens collected from diarrheic piglets during a surveillance of porcine rotavirus (PoRV) strains in Chiang Mai, Thailand, 39 (22.3%) were positive for group A rotaviruses. Of these, 33.3% (13 of 39) belonged to G3P[19], which was a rare P genotype seldom reported. Interestingly, their VP4 nucleotide sequences were most closely related to human P[19] strains (Mc323 and Mc345) isolated in 1989 from the same geographical area where these PoRV strains were isolated. These P[19] PoRV strains were also closely related to another human P[19] strain (RMC321), isolated from India in 1990. The VP4 sequence identities with human P[19] were 95.4% to 97.4%, while those to a porcine P[19] strain (4F) were only 87.6 to 89.1%. Phylogenetic analysis of the VP4 gene revealed that PoRV P[19] strains clustered with human P[19] strains in a monophyletic branch separated from strain 4F. Analysis of the VP7 gene confirmed that these strains belonged to the G3 genotype and shared 97.7% to 98.3% nucleotide identities with other G3 PoRV strains circulating in the regions. This close genetic relationship was also reflected in the phylogenetic analysis of their VP7 genes. Altogether, the findings provided peculiar evidence that supported the porcine origin of VP4 genes of Mc323 and Mc345 human rotaviruses.

Rotavirus is the major cause of acute gastroenteritis in infants and young children and in young animals of a large variety of species (27). It contains two outer capsid proteins, VP7 and VP4, which independently elicit neutralizing antibodies and specify the G and P genotypes of the virus, respectively (14, 16, 24, 27, 40). To date, 15 distinct G genotypes and 26 P genotypes have been identified (14, 27, 30, 31, 32, 33, 35, 46, 48). Epidemiological studies of porcine rotaviruses (PoRV) in several countries have identified at least four main G types, G3, G4, G5, and G11, which are the most common (14). However, other porcine rotaviruses, such as G1, G2, G6, G8, G9, and G10, have also been reported occasionally (1, 5, 6, 19, 25, 32, 34, 43, 45, 52, 57). For the P type, P[6] and P[7] were found to be the most common genotypes in pigs, while other P types, such as P[13], P[14], P[19], P[23], and P[26], were seldom reported (3, 14, 20, 25, 27, 30, 31, 59). In Thailand, the epidemiological study of the group A rotavirus in pigs has been limited, and G3 had been the only G type detected in the last decade (44), until G10 was recently reported (43). Rotaviruses belonging to the same G serotype usually share at least 90% amino acid sequence identity (21), whereas viruses of the same P genotype normally share more than 89% amino acid sequence identity (4, 14, 16). The increased detection of rotavirus strains bearing an unusual combination of phenotypes of human and animal rotaviruses has been well documented (10,

12, 42, 47). This observation supports the hypothesis that interspecies transmission of rotaviruses from one animal species to others, including humans, might take place in nature (7, 18, 37, 42). The interspecies transmission could be the result of infection with an animal rotavirus virion (38) or could occur via genetic reassortment between humans and animal rotavirus strains during coinfection of the same cell (9, 11, 54, 55).

Two strains of human G9 rotaviruses (Mc323 and Mc345) isolated in 1989 in Chiang Mai, Thailand, had been shown by RNA-RNA hybridization to be more closely related to the porcine G9 rotavirus than to human rotaviruses (54). Recently, analyses of VP7 and VP4 nucleotide and deduced amino acid sequences of Mc323 and Mc345 revealed that both strains belonged to G9P[19] genotype (41), with the VP7 sequences closely related to the G9 human rotaviruses WI61 and F45, while the VP4 sequence revealed a close genetic relatedness to that of the P[19] porcine rotavirus 4F reported previously (2). Most recently, a human rotavirus G9P[19] isolate (RMC321) with porcine rotavirus characteristics was also reported following an outbreak of infantile gastroenteritis in India (55). Currently, G9 is a common genotype of humans and pigs (32), while P[19] is a rare one in both of them (31). It is possible that human G9P[19] strains Mc323 and Mc345 might have arisen by natural reassortment among rotavirus strains of human and porcine origins that circulated in the Chiang Mai area. Unfortunately, in that study, the rotavirus strain surveillance in pigs was not carried out simultaneously. It is, therefore, tempting to verify whether the P[19] rotavirus, a rare genotype, is really circulating in the porcine population of the Chiang Mai area.

In this study, 13 strains of G3P[19] PoRV were isolated from diarrheic piglets raised in several pig farms located in Chiang

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Mai, Thailand. The VP4 genes of these strains were most closely related to those of Mc323 and Mc345, the human rotaviruses that were also isolated from Chiang Mai. These findings provided evidence that supported the porcine origin of VP4 genes of Mc323 and Mc345 strains.

MATERIALS AND METHODS

Specimen collection. A total of 175 fecal specimens were collected from diarrheic piglets from six different farms located in Chiang Mai province, Thailand. The ages of the piglets ranged from 7 to 49 days old. The specimens were collected from June 2000 to July 2001 and stored at −20°C until use.

Screening and subgrouping of the group A rotavirus by ELISA. The presence of the group A rotavirus in fecal specimens was detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody against it, as described previously (22). The subgroup specificities of the virus were determined by ELISA using monoclonal antibodies (MAbs) specific to subgroup I and subgroup II rotaviruses (Serotec, Japan), as described previously (53).

RNA extraction and polyacrylamide gel electropherotyping. Viral genomic RNA was extracted from fecal specimens by use of a phenol-chloroform (22) or an acid phenol-guanidinium thiocyanate-chloroform (51) extraction method. The extract was subjected to polyacrylamide gel electrophoresis for the detection of viral genomic RNA and characterization of RNA electrophoretic pattern, as described previously (49). The localization of RNA genome fragments migrated in the gel was detected by silver staining, as described previously (23).

G genotyping. The G genotype was determined by using a protocol modified from a method previously described (17). Briefly, the RNA genome of the rotavirus was first extracted from 10% fecal supernatant by use of a QIAamp viral RNA mini kit (QIAGEN). Reverse transcription-PCR (RT-PCR) was performed using a OneStep RT-PCR kit (QIAGEN). The full length of the VP7 gene was reverse transcribed and simultaneously amplified by using Beg9 and End9 primers in a single tube reaction. 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 together with G-genotype-specific mixed primers BT1, CT2, ET3, DT4, FT8, and FT9 for upstream priming and the End9 primer for downstream priming of VP7 genes for identifications of genotypes G1 to G4, G8, and G9. The samples for which the G genotype could not be identified by the first set of primers described by Gouvea et al. (17) were later identified by using alternative sets of type-specific primers reported by Das et al. (8), Gouvea et al. (19), and Winiarczyk et al. (57). These primer sets covered a wide range of rotavirus genotypes, i.e., G1 to G4, G5, G6, and G8 to G11.

P genotyping. The P genotype was identified by using a method modified from that described by Gentsch et al. (15). Briefly, the partial sequence of the VP4 gene was reverse transcribed and simultaneously amplified by using Con2 and Con3 primers. In the second amplification, a mixture of primers 1T-1, 2T-1, 3T-1, 4T-1, 5T-1, and ND2 with Con3 primers was utilized for the identification of P[8], P[4], P[6], P[9], P[10], and P[11], respectively. The samples for which the P genotype could not be identified by the first set of type-specific primers were then genotyped by using alternative sets of type-specific primers, as previously reported (20, 36, 57). These primer sets were specific for P[1], P[4] to P[11], and P[14] genotypes.

Nucleotide sequence analysis. The rotavirus isolates of which the G or P genotypes could not be determined by type-specific primers were then subjected to nucleotide sequencing. The PCR products of VP7 or VP4 genes obtained from the first amplification of each nontypeable isolate were purified by a QIAquick PCR purification kit (QIAGEN) and then subjected to direct nucleotide sequencing according to the manufacturer's instructions by using a BigDye Terminator cycle sequencing kit (PE Biosystems). The nucleotide sequences were analyzed by comparison with those of the reference strains available in the GenBank database.

Design of the new typing primer for P[19] and PCR optimization. The VP4 nucleotide sequences of 13 isolates of the P[19] porcine rotavirus detected in this study, together with the sequences of other P[19] strains, such as Mc323, Mc345, and 4F, as well as other P types (P[1] to P[26]) selected from the GenBank database, were aligned using the ClustalX program. The region highly conserved among P[19] strains and that divergent in other P types were selected as a primer sequence. The newly designed primer for P[19], namely, VP4P19, was targeted to nucleotides (nt) 400 to 425 of the VP4 gene. The nucleotide positions and sequence of the primer (5′ to 3′) were as follows: AAC TTC CAY TTA YTT GAG GTA TTA AC (nt 400 to 425; Y = C or T). The VP4P19 primer (forward)

was used in combination with the Con2 primer (reverse) (nt 868 to 887) in the second-round PCR to generate a 415-bp product.

The specificity of the VP4P19 primer was evaluated by testing the primer with all 13 isolates of P[19], three isolates of P[13], and one each of P[3], P[4], and P[12]. Briefly, 3 μl of RNA genome was added to 0.3 μl of 50% dimethyl sulfoxide before being heated at 97°C for 5 min and then rapidly cooled on ice. The denatured RNA was then reverse transcribed for 1 h at 37°C in 25 μl of reaction mixture containing 15.8 μl of RNase-free water and 2.5 μl of 10× PCR buffer containing 12.5 mM MgCl$_2$, 2.0 μl of 10 mM of each deoxynucleoside triphosphate mix (10 mM of each deoxynucleoside triphosphate), 0.4 μl of each (33-pmol/μl) primer pair of Con3 (forward) and Con2 (reverse), 0.5 μl of avian myeloblastosis virus-RT enzyme (200 U/μl), and 0.2 μl of RNase inhibitor. The cDNA was then amplified further with 0.5 μl of *Taq* DNA polymerase (5 U/μl) for 35 cycles, with a thermocycling condition at 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min and a final extension at 72°C for 7 min. The first PCR product was diluted at 1:100 and subjected to the second-round PCR, in which VP4P19 and Con2 were used as typing primers. The thermal cycling profile was 35 cycles of 94°C for 1 min, 45°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 7 min. The second PCR product was detected by electrophoresis on 1.5% agarose gel in Tris-borate-EDTA buffer and stained with ethidium bromide. The P[19] genotype was identified based on the presence of the DNA band of a PCR product of 415 bp in length and confirmed by nucleotide sequence analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of G3P[19] porcine rotavirus strains described in this study were deposited in the GenBank database. The accession numbers for the VP4 sequences of the 13 strains of porcine P[19] described in this study were as follows: for strain CMP029, accession no. AY689219; for strain CMP031, AY689218; for strain CMP039, AY689217; for strain CMP072, AY689216; for strain CMP087, AY689215; for strain CMP090, AY689214; for strain CMP092, AY689213; for strain CMP094, AY689212; for strain CMP095, AY689211; for strain CMP096, AY689210; for strain CMP098, AY689209; for strain CMP099, AY68208; and for strain CMP100, AY689207. The accession numbers for the VP7 sequences of the five representative strains of G3 described in this study were as follows: for strain CMP039, accession no. AY707788; for strain CMP096, DQ256502; for strain CMP099, DQ256503; for strain CMP213, DQ786576; and for strain CMP214, DQ786577.

RESULTS

Prevalence and characteristics of the porcine group A rotavirus. The group A rotavirus was detected in 39 out of 175 (22.3%) fecal specimens collected from diarrheic piglets in Chiang Mai, Thailand, from June 2000 to July 2001. The characteristics of the viruses are as follows. Among 39 isolates of the group A rotavirus, 8 isolates (20.5%) belonged to subgroup I (SG I), 2 isolates (5.1%) belonged to SG II, and 5 isolates (12.8%) showed a dual subgroup specificity, i.e., they were reactive to both MAbs specific for SG I and SG II. The majority of the virus isolates (24 out of 39 [61.6%]) were not reactive to MAbs specific for either SG I or SG II. The electrophoretic pattern of genomic viral RNA could be demonstrated in only 17 out of 39 (43.6%) fecal samples, and all of these isolates displayed a long electropherotype, while another 22 isolates (56.4%) were in a smear pattern. Therefore, their electropherotype could not be assigned by polyacrylamide gel electrophoresis. However, the RNA genome of these isolates could be amplified by RT-PCR using the consensus primers Con2 and Con3 for VP4 and Beg9 and End9 for VP7.

Distributions of G and P genotypes of the porcine rotavirus. Among 39 isolates of porcine group A rotaviruses, five different G genotypes, G2, G3, G4, G5, and G9, were detected in this study (Table 1). The G3 and G4 genotypes were copredominant genotypes, with a prevalence of 43.6% and 46.2%, respectively, followed by much less prevalent G5 (5.1%) and G2 (2.6%) genotypes. Most of the G4 genotypes (17 of 18 isolates) were found in combination with P[6], while the re-