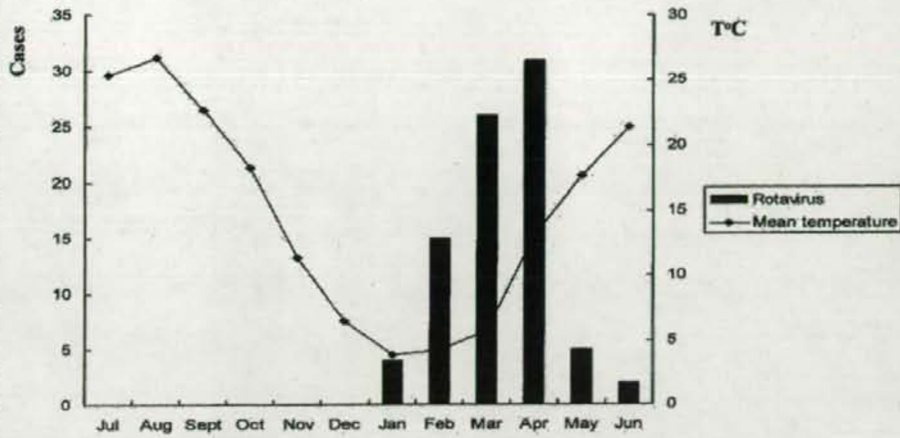

Figure 1: Seasonal variation of rotavirus infection among infants and children with acute gastroenteritis in five different regions (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2004. The mean temperature for the five regions obtained from the Japan Meteorological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

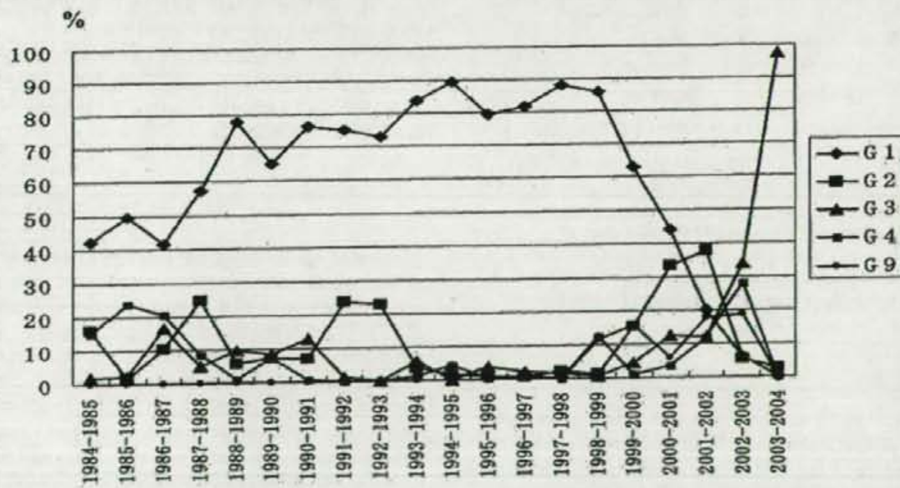

Figure 2: Prevalence pattern of rotavirus G-types with an emergence of rotavirus G3 together with a sudden disappearance of G1, G4, and G9 during the 21-year survey of rotavirus infection among infants and children in the same age group with acute gastroenteritis in five different regions (Maizuru, Tokyo,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

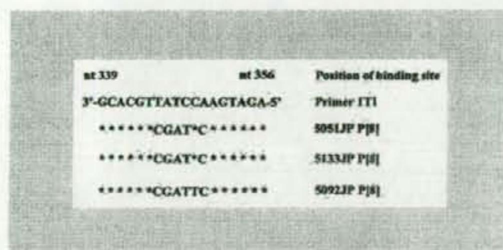

Figure 3: Alignment of fragment of the VP4 gene of Japanese rotavirus strains that were not typed by RT-PCR and the reverse complementary sequences of the original primer 1T-1. Residues that match primer 1T-1 are denoted by asterisks.

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

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

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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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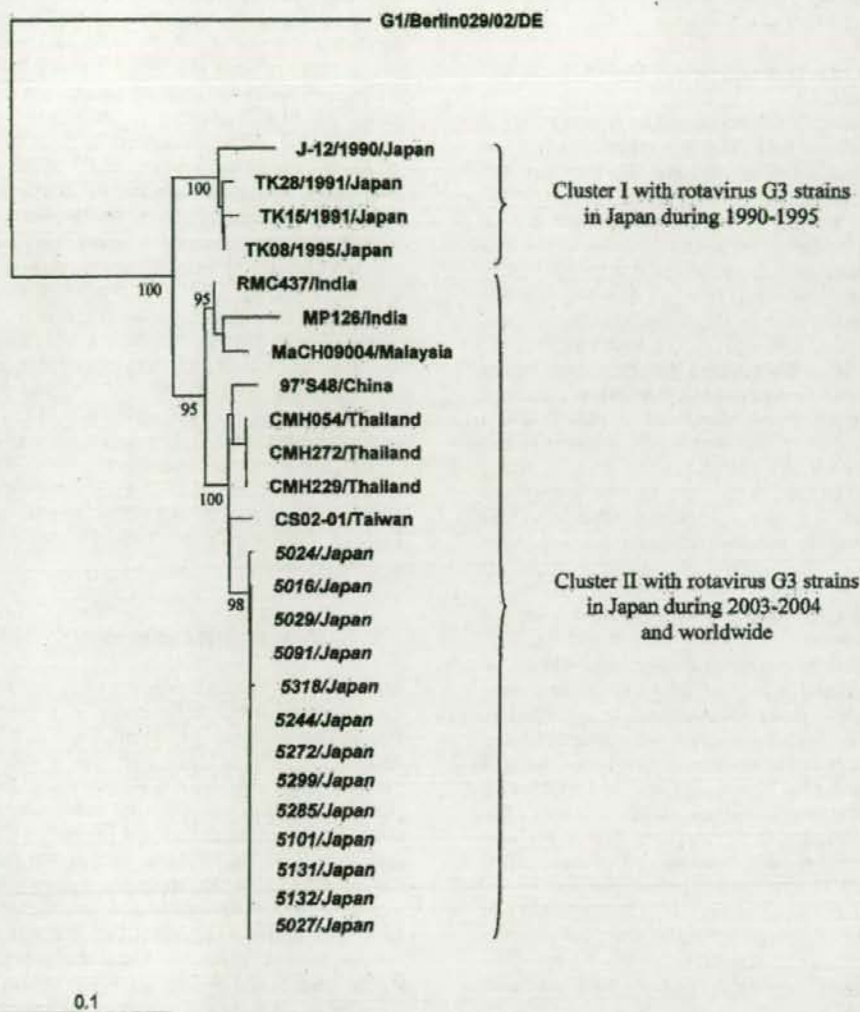

Figure 4: Phylogenetic tree of nucleotide sequences of rotavirus G3 strains detected in infants and children with acute gastroenteritis in five different places in Japan. The tree was constructed from nucleotide sequences of VP7 of rotavirus G3 strains. Reference rotavirus strains were selected from 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.

Acknowledgements
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References

1. Estes MK. Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM, eds. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven Press, 1996; 1625-55
2. Kapikian AZ, Hoshino Y, Chanock RM. Rotaviruses. In: Knipe DM, Howley PM, eds. Fields virology. 4th ed. Philadelphia: Lippincott-Raven Press, 2001; 1787-833
3. Mulholland EK. Global control of rotavirus disease. Adv Exp Med Biol 2004; 549: 161-8
4. Parashar UD, Hummelman EG, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003; 9: 565-72
5. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol 2005; 15: 29-56
6. Parashar UD, Glass RI. Public health. Progress toward rotavirus vaccines. Science 2006; 12: 851-2
7. Bernstein DI, Ward RL. Rotarix: development of a live attenuated monovalent human rotavirus vaccine. Pediatr Ann 2006; 35: 38-43
8. Offit PA, Clark HF. RotaTeq: a pentavalent bovine-human reassortant rotavirus vaccine. Pediatr Ann 2006; 35: 29-34
9. Yan H, Tuan AN, Phan TG, Okitsu S, Yan L, Ushijima H. Development of RT-multiplex PCR assay for detection of adenovirus, group A and C rotaviruses in diarrheal fecal specimens from children in China. Kansenshogaku Zasshi 2004; 78: 699-709
10. Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, Kumar R, Bhan MK, Glass RI. Characterization of rotavirus strains from newborns in New Delhi, India. J Clin Microbiol 1994; 32: 1820-2
11. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, Das BK, Bhan MK. Identification of group A rotavirus gene 4 type by polymerase chain reaction. J Clin Microbiol 1992; 30: 1365-73
12. Wen L, Nakayama M, Yamanishi Y, Nishio O, Fang ZY, Nakagomi O, Araki K, Nishimura S, Hasegawa A, Muller WE, Ushijima H. Genetic variation in the VP7 gene of human rotavirus serotype 3 (G3 type) isolated in China and Japan. Arch Virol 1997; 142: 1481-9
13. Thapar N, Sanderson IR. Diarrhoea in children: an interface between developing and developed countries. Lancet 2004; 363: 641-53
14. Nishio O, Matsui K, Oka T, Ushijima H, Mubina A, Dure-Samin A, Isomura S. Rotavirus infection among infants with diarrhea in Pakistan. Pediatr Int 2000; 42: 425-7
15. Maneekarn N, Ushijima H. Epidemiology of rotavirus infection in Thailand. Pediatr Int 2000; 42: 415-21
16. Okitsu-Negishi S, Nguyen TA, Phan TG, Ushijima H. Molecular epidemiology of viral gastroenteritis in Asia. Pediatr Int 2004; 46: 245-52
17. Zhou Y, Li L, Okitsu S, Maneekarn N, Ushijima H. Distribution of human rotaviruses, especially G9 strains, in Japan from 1996 to 2000. Microbiol Immunol 2003; 47: 591-9

18. Yoshinaga M, Phan TG, Nguyen AT, Yan H, Yagyu H, Okitsu S, Muller WEG, Ushijima H. Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan. Arch Virol 2006; 151: 183-92

19. Kirkwood C, Masendycz PJ, Coulson BS. Characteristics and location of cross-reactive and serotype-specific neutralization sites on VP7 of human G type 9 rotaviruses. Virology 1993; 196: 79-88

20. Kirkwood C, Bogdanovic-Sakran N, Palombo E, Masendycz P, Bugg H, Barnes G, Bishop R. Genetic and antigenic characterization of rotavirus serotype G9 strains isolated in Australia between 1997 and 2001. J Clin Microbiol 2003; 41: 3649-54

21. Laird AR, Gentsch JR, Nakagomi T, Nakagomi O, Glass RI. 2003. Characterization of serotype G9 rotavirus strains isolated in the United States and India from 1993 to 2001. J Clin Microbiol 2003; 41: 3100-11

22. Santos N, Volotao EM, Soares CC, Albuquerque MC, da Silva FM, Chizhikov V, Hoshino Y. 2003. VP7 gene polymorphism of serotype G9 rotavirus strains and its impact on G genotype determination by PCR. Virus Res 2003; 93: 127-38

23. Steele AD, Ivanoff B. Rotavirus strains circulating in Africa during 1996-1999: emergence of G9 strains and P[6] strains. Vaccine 2003; 17: 361-7

24. Lazdins I, Coulson BS, Kirkwood C, Dyall-Smith M, Masendycz PJ, Sonza S, Holmes IH. Rotavirus antigenicity is affected by the genetic context and glycosylation of VP7. Virology 1995; 209: 80-9

25. Huang JA, Wang L, Firth S, Phelps A, Reeves P, Holmes I. Rotavirus VP7 epitope mapping using fragments of VP7 displayed on phages. Vaccine 2000; 18: 2257-65

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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-

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

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

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

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

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

The VP4 nucleotide sequences of these G3P[19] strains were also compared with the P[19] reference strains of porcine (4F) and human (RMC321, Mc323, and Mc345) origins. Different P types of porcine strains, P[5], P[6], P[7], and P[13], as well as a bovine outlier reference strain, P[17], were also included. The results revealed that all of the porcine G3P[19] strains detected in this study were most closely related to human P[19] reference strains (RMC321, Mc323, and Mc345), with a nucleotide sequence identity of 95.4% to 97.4%, while the nucleotide sequence identity to the porcine P[19] reference strain (4F) was only 87.6% to 89.1%. Rotavirus strains representing other P types exhibited much lower nucleotide sequence identities (60.1% to 72.5% for different P types of porcine and 49.4% to 50.6% for an outlier strain of bovine) than our P[19] strains (data not shown).

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

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

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

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

The newly designed primer, designated VP4P19, was evaluated for its specificity by being tested with the 13 isolates of P[19] and 3 strains of P[13] isolated in this study, together with other P types, including P[3], P[4], and P[12] (data not shown).

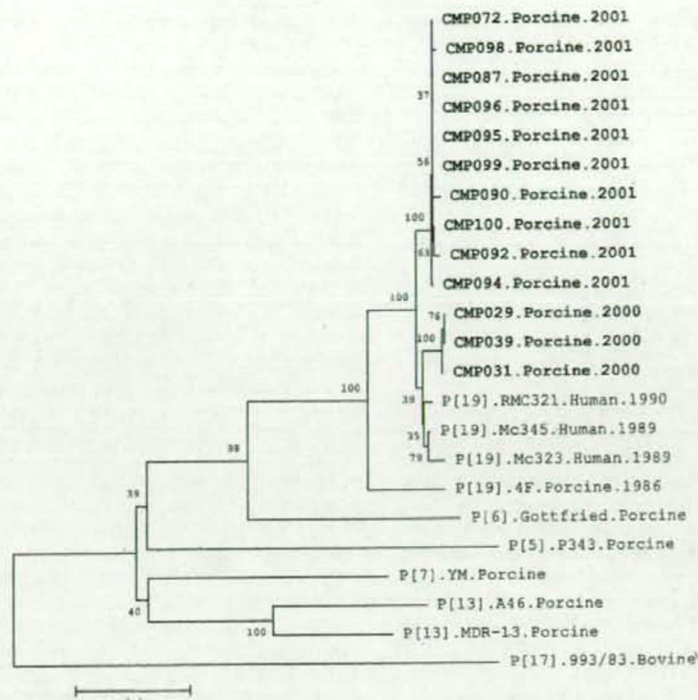

FIG. 1. Phylogenetic tree of VP4 nucleotide sequences of G3P[19] porcine rotavirus strains isolated from 2000 to 2001, demonstrating a close genetic relationship with human rotavirus reference strains (Mc323, Mc345, and RMC321) that were isolated from 1989 to 1990. Bootstrap values supporting some clusters are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

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

DISCUSSION

The segmented nature of a rotavirus genome can undergo genetic reassortment between strains during mixed infections and lead to the generation of progeny viruses with novel or atypical phenotypes (42, 48). The isolation of unusual strains possessing a gene segment(s) of human and/or of heterologous animal origin suggests interspecies transmission and reassortment between the viruses of humans and animals, as well as animals and animals in nature (7, 26, 38, 42, 55, 56). In this study, we reported 13 isolates of the rare G3P[19] porcine rotaviruses. Among 13 isolates of G3P[19] strains described in this study, 3 were isolated in the year 2000 and 10 in 2001, and these accounted for 33.3% of the group A rotaviruses isolated. The data indicated that the G3P[19] strains are currently circulating in a relatively high proportion in the pig population of the Chiang Mai area. Although rotavirus strain surveillances in a pig population have been performed extensively in various parts of the world, so far only one isolate of G3P[19], designated 4F, has been reported, from a diarrheic pig in China (39). The VP4 and VP7 genes of 4F have been analyzed, and strain 4F has been identified as a G3P[19] strain (2). In addition, the pathogenicity of 4F has been evaluated, and it was found that in the first three passages the 4F strain showed no significant pathogenicity in pigs but that it became highly pathogenic at passages four and five (2). However, the significance of the epidemiological impact of 4F is unknown. Here, we report the isolation of G3P[19] porcine rotavirus strains that caused severe diarrhea in piglets at several farms in Chiang Mai province, Thailand. The G3P[19] strains in the year 2000 were isolated from diarrheic piglets in farms located in Mae Rim district, which is about 65 kilometers from the farms in Mae Wang district, where the G3P[19] strains were isolated in 2001. The data indicate that G3P[19] strains are

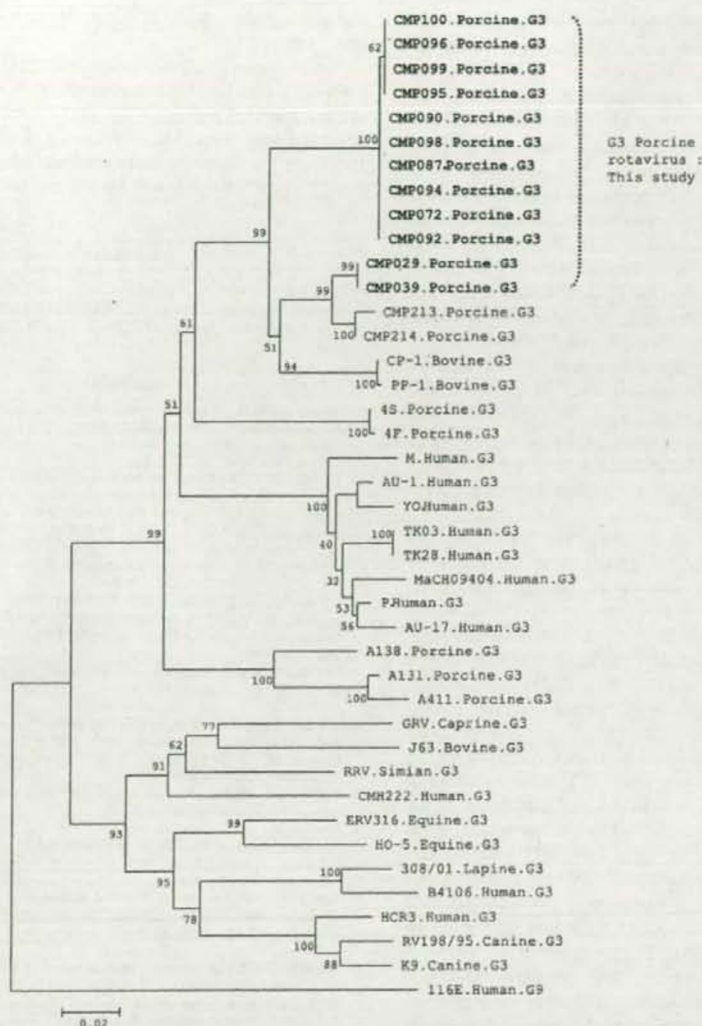

FIG. 2. Phylogenetic tree of VP7 nucleotide sequences of G3P[19] strains, demonstrating a close genetic relationship between G3P[19] strains isolated from 2000 to 2001 and PoRV G3 strains (CMP213 and CMP214) isolated in 2002 from the same geographical area. Bootstrap values supporting some clusters are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

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

Analysis of the VP4 genes revealed a high level of homology among viruses isolated in the same year, with nucleotide identities of 99.8% to 100% for strains isolated in the year 2000 and 98.4% to 100% for strains isolated in 2001. However, nucleotide sequence comparisons between the viruses isolated in the year 2000 and 2001 indicated that the nucleotide identities were slightly decreased (95.0% to 96.1%). The data imply that these strains belong to different sublineages of the same genotype and that the decrease of nucleotide identities among the isolates in the years 2000 and 2001 might be due to a genetic evolutionary diversity of the viruses. Interestingly, the VP4 nucleotide sequence of these strains was most closely related with those of human P[19] strains (Mc323 and Mc345) which were isolated in 1989 from children admitted to a hospital in the same geographical area (Chiang Mai city), with nucleotide identities of 95.4% to 97.4%. Urasawa et al. (54) demonstrated, by RNA-RNA hybridization, that Mc323 and Mc345 were genetically more closely related to porcine than to human rotaviruses. Although sequence analyses of NSP1 (29) and VP4 (41) genes of Mc323 and Mc345 provided evidence suggestive of the porcine origin of these strains, direct evidence to support the origin of their VP4 genes remained undetermined. In this study, we could detect 13 isolates of G3P[19] porcine

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

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

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

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REFERENCES

1. **Bellinzoni, R. B., N. M. Mattion, D. O. Matson, J. Blackhall, J. L. La Torre, E. A. Scodeller, S. Urasawa, K. Taniguchi, and M. K. Estes.** 1990. Porcine rotaviruses antigenically related to human rotavirus serotypes 1 and 2. J. Clin. Microbiol. **28:**633–636.
2. **Bridger, J. C., B. Burke, G. M. Beards, and U. Desselberger.** 1992. The pathogenicity of two porcine rotaviruses differing in their in vitro growth characteristics and gene 4. J. Gen. Virol. **73:**3011–3015.
3. **Burke, B., M. A. McCrae, and U. Desselberger.** 1994. Sequence analysis of two porcine rotaviruses differing in growth in vitro and in pathogenicity: distinct VP4 sequences and conservation of NS53, VP6 and VP7 genes. J. Gen. Virol. **75:**2205–2212.
4. **Ciarlet, M., M. K. Estes, and M. E. Conner.** 1997. Comparative amino acid sequence analysis of the outer capsid protein VP4 from four lapine rotavirus strains reveal identity with genotype P[14] human rotavirus. Arch. Virol. **142:**1059–1069.
5. **Ciarlet, M., and F. Liprandi.** 1994. Serological and genomic characterization of two porcine rotaviruses with serotype G1 specificity. J. Clin. Microbiol. **32:**269–272.
6. **Ciarlet, M., J. E. Ludert, and F. Liprandi.** 1995. Comparative amino acid sequence analysis of the major outer capsid protein (VP7) of porcine rotaviruses with G3 and G5 serotype specificities isolated in Venezuela and Argentina. Arch. Virol. **140:**437–451.
7. **Cook, N., J. Bridger, K. Kendall, M. Iturriza-Gomara, L. El-Attar, and J. Gray.** 2004. The zoonotic potential of rotavirus. J. Infect. **48:**289–302.
8. **Das, B. K., J. R. Gentsch, H. G. Cicirello, P. A. Woods, A. Gupta, M. Ramachandran, R. Kumar, M. K. Bham, and R. I. Glass.** 1994. Characterization of rotavirus strains from newborns in New Delhi, India. J. Clin. Microbiol. **32:**1820–1822.
9. **Das, B. K., J. R. Gentsch, Y. Hoshino, S. Ishida, O. Nakagomi, M. K. Bhan, R. Kumar, and R. I. Glass.** 1993. Characterization of the G serotype and genogroup of New Delhi newborn rotavirus strain 116E. Virology **197:**99–107.
10. **Desselberger, U., M. Iturriza-Gomara, and J. J. Gray.** 2001. Rotavirus epidemiology and surveillance, p. 82–100. *In* D. Chadwick and J. A. Goode (ed.), Gastroenteritis viruses. Wiley, New York, N.Y.
11. **Dunn, S. J., H. B. Greenberg, R. L. Ward, O. Nakagomi, J. W. Burns, P. T. Vo, K. A. Pax, M. Das, K. Gowda, and C. D. Rao.** 1993. Serotypic and genotypic characterization of human serotype 10 rotaviruses from asymptomatic neonates. J. Clin. Microbiol. **31:**165–169.
12. **El-Attar, L., W. Dhaliwal, C. R. Howard, and J. C. Bridger.** 2001. Rotavirus cross-species pathogenicity: molecular characterization of a bovine rotavirus pathogenic for pigs. Virology **291:**172–182.
13. **El-Attar, L., W. Dhaliwal, M. Iturriza-Gomara, and J. C. Bridger.** 2002. Identification and molecular characterization of a bovine G3 rotavirus which causes age-independent diarrhea in cattle. J. Clin. Microbiol. **40:**937–942.
14. **Estes, M. K.** 2001. Rotaviruses and their replication, p. 1747–1785. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, Pa.
15. **Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bhan.** 1992. Identification of group A rotavirus gene 4 type by polymerase chain reaction. J. Clin. Microbiol. **30:**1365–1373.
16. **Gorziglia, M., G. Larralde, A. Z. Kapikian, and R. M. Chanock.** 1990. Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. Proc. Natl. Acad. Sci. USA **87:**7155–7159.
17. **Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z. Y. Fang.** 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. **28:**276–282.
18. **Gouvea, V., and M. Brantly.** 1995. Is rotavirus a population of reassortants? Trends Microbiol. **3:**159–162.

19. **Gouvea, V., N. Santos, and C. M. Timenetsky.** 1994. Identification of bovine and porcine rotavirus G types by PCR. J. Clin. Microbiol. **32:**1338–1340.
20. **Gouvea, V., N. Santos, and C. M. Timenetsky.** 1994. VP4 typing of bovine and porcine group A rotaviruses by PCR. J. Clin. Microbiol. **32:**1333–1337.
21. **Green, K. Y., J. F. Sears, K. Taniguchi, K. Midthun, Y. Hoshino, M. Gorziglia, K. Nishigawa, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and J. Flores.** 1988. Prediction of human rotavirus serotype by nucleotide sequence analysis of the VP7 protein gene. J. Virol. **62:**1819–1823.
22. **Hasegawa, A., A. Mukoyama, H. Suzuki, S. Inouye, S. Chearskul, and P. Thongkrajai.** 1987. Rotavirus infection of Thai infants: antigen detection, RNA electrophoresis and virus cultivation. J. Diarrhoeal Dis. Res. **5:**165–170.
23. **Herring, A. J., N. F. Inglis, C. K. Ojeh, D. R. Snodgrass, and J. D. Menzies.** 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J. Clin. Microbiol. **16:**473–477.
24. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82:**8701–8704.
25. **Huang, J., H. S. Nagesha, M. L. Dyall-Smith, and I. H. Holmes.** 1989. Comparative sequence analysis of VP7 genes from five Australian porcine rotaviruses. Arch. Virol. **109:**173–183.
26. **Iturriza-Gómara, M., B. Isherwood, U. Desselberger, and J. Gray.** 2001. Reassortment in vivo: driving force for diversity of human rotavirus strains isolated in the United Kingdom between 1995 and 1999. J. Virol. **75:**3696–3705.
27. **Kapikian, A. Z., Y. Hoshino, and R. M. Chanock.** 2001. Rotaviruses, p. 1787–1833. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, Pa.
28. **Khamrin, P., S. Peerakome, L. Wongsawadi, S. Tonusin, P. Sornchai, V. Maneerat, C. Khamwan, F. Yagyu, S. Okitsu, H. Ushijima, and N. Maneekarn.** 2006. Emergence of human G9 rotavirus with an exceptionally high frequency in children admitted to hospital with diarrhea in Chiang Mai, Thailand. J. Med. Virol. **78:**273–280.
29. **Kojima, K., K. Taniguchi, and T. Urasawa.** 1996. Sequence analysis of normal and rearranged NSP5 genes from human rotavirus strains isolated in nature: implications for the occurrence of the rearrangement at the step of plus strand synthesis. Virology **224:**446–452.
30. **Liprandi, F., M. Gerder, Z. Bastidas, J. A. Lopez, F. E. Pujol, J. E. Ludert, D. B. Joelsson, and M. A. Ciarlet.** 2003. A novel type of VP4 carried by a porcine rotavirus strain. Virology **315:**373–380.
31. **Martella, V., M. Ciarlet, K. Banyai, E. Lorusso, A. Cavalli, M. Corrente, G. Elia, S. Arista, M. Camero, C. Desario, N. Decaro, A. Lavazza, and C. Buonavoglia.** 2006. Identification of a novel VP4 genotype carried by a serotype G5 porcine rotavirus strain. Virology **346:**301–311.
32. **Martella, V., M. Ciarlet, R. Baselga, S. Arista, G. Elia, E. Lorusso, K. Banyai, V. Terio, A. Madio, F. M. Ruggeri, E. Falcone, M. Camero, N. Decaro, and C. Buonavoglia.** 2005. Sequence analysis of the VP7 and VP4 genes identifies a novel VP7 gene allele of porcine rotaviruses. Virology **337:**111–123.
33. **Martella, V., M. Ciarlet, A. Camarda, A. Pratelli, M. Tempesta, G. Greco, A. Cavalli, G. Elia, N. Decaro, V. Terio, G. Bozzo, M. Camero, and C. Buonavoglia.** 2003. Molecular characterization of the VP4, VP6, VP7, and NSP4 genes of lapine rotaviruses identified in Italy: emergence of a novel VP4 genotype. Virology **314:**358–370.
34. **Martella, V., A. Pratelli, G. Greco, M. Tempesta, M. Ferrari, M. N. Losio, and C. Buonavoglia.** 2001. Genomic characterization of porcine rotaviruses in Italy. Clin. Diagn. Lab. Immunol. **8:**129–132.
35. **McNeal, M. M., K. Sestak, A. H. Choi, M. Basu, M. J. Cole, P. P. Aye, R. P. Bohm, and R. L. Ward.** 2005. Development of a rotavirus-shedding model in rhesus macaques, using a homologous wild-type rotavirus of a new P genotype. J. Virol. **79:**944–954.
36. **Mphahlele, M. J., I. Peenze, and A. D. Steele.** 1999. Rotavirus strains bearing the VP4P[14] genotype recovered from South African children with diarrhea. Arch. Virol. **144:**1027–1034.
37. **Nakagomi, O., and T. Nakagomi.** 1993. Interspecies transmission of rotaviruses studied from the perspective of genogroup. Microbiol. Immunol. **37:** 337–348.
38. **Nakagomi, T., and O. Nakagomi.** 2000. Human rotavirus HCR3 possesses a genomic RNA constellation indistinguishable from that of feline and canine rotaviruses. Arch. Virol. **145:**2403–2409.
39. **Ni, Y. W.** 1986. Isolation and identification of porcine and bovine rotaviruses. Chin. J. Virol. **2:**36–40.
40. **Offit, P. A., and G. Blavat.** 1986. Identification of the two rotavirus genes determining neutralization specificities. J. Virol. **57:**376–378.
41. **Okada, J., T. Urasawa, N. Kobayashi, K. Taniguchi, A. Hasegawa, K. Mise, and S. Urasawa.** 2000. New P serotype of group A human rotavirus closely related to that of a porcine rotavirus. J. Med. Virol. **60:**63–69.
42. **Palombo, E. A.** 2002. Genetic analysis of group A rotaviruses: evidence for interspecies transmission of rotavirus genes. Virus Genes **24:**11–20.
43. **Pongsuwanna, Y., K. Taniguchi, M. Chiwakul, T. Urasawa, F. Wakasugi, C. Jayavasu, and S. Urasawa.** 1996. Serological and genomic characterization of porcine rotaviruses in Thailand: detection of a G10 porcine rotavirus. J. Clin. Microbiol. **34:**1050–1057.
44. **Pongsuwanne, Y., K. Taniguchi, M. Choonthanom, M. Chiwakul, T. Susansook, S. Saguanwongse, C. Jayavasu, and S. Urasawa.** 1989. Subgroup and serotype distributions of human, bovine, and porcine rotavirus in Thailand. J. Clin. Microbiol. **27:**1956–1960.
45. **Rácz, M. L., S. S. Kroeff, V. Munford, T. A. R. Caruzo, E. L. Durigon, Y. Hayashi, V. Gouvea, and E. A. Palombo.** 2000. Molecular characterization of porcine rotaviruses from the southern region of Brazil: characterization of an atypical genotype G9 strain. J. Clin. Microbiol. **38:**2443–2446.
46. **Rahman, M., J. Matthijnssens, S. Nahar, G. Podder, D. A. Sack, T. Azim, and M. van Ranst.** 2005. Characterization of a novel P[25], G11 group A rotavirus. J. Clin. Microbiol. **43:**3208–3212.
47. **Ramig, R. F.** 1997. Genetics of rotaviruses. Annu. Rev. Microbiol. **51:**225–255.
48. **Rao, C. D., K. Gowda, and B. S. Y. Reddy.** 2000. Sequence analysis of VP4 and VP7 genes of nontypeable strains identifies a new pair of outer capsid proteins representing novel P and G genotypes in bovine rotaviruses. Virology **276:**104–113.
49. **Rodger, S. M., and I. H. Holmes.** 1979. Comparison of the genomes of simian, bovine, and human rotaviruses by gel electrophoresis and detection of genomic variation among bovine isolates. J. Virol. **30:**839–846.
50. **Saekhow, P.** 2005. Prevalence of G- and P-genotypes of group A rotavirus in bovines and porcines in Chiang Mai. Master's thesis in microbiology. Chiang Mai University, Chiang Mai, Thailand.
51. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning, 3rd ed., vol. 1, p. 7.4–7.8. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. **Santos, N., R. C. C. Lima, C. M. Nozawa, R. E. Linhares, and V. Gouvea.** 1999. Detection of porcine rotavirus type 9 and of a mixture of types G1 and G5 associated with Wa-like VP4 specificity: evidence for natural human-porcine genetic reassortment. J. Clin. Microbiol. **37:**2734–2736.
53. **Taniguchi, K., T. Urasawa, S. Urasawa, and T. Yasuhara.** 1984. Production of subgroup-specific monoclonal antibodies against human rotaviruses and their application to an enzyme-linked immunosorbent assay for subgroup determination. J. Med. Virol. **14:**115–125.
54. **Urasawa, S., A. Hasegawa, T. Urasawa, K. Taniguchi, F. Wakasugi, H. Suzuki, S. Inouye, B. Pongprot, J. Supawadee, S. Suprasert, P. Rangsiyanond, S. Tonusin, and Y. Yamazi.** 1992. Antigenic and genetic analyses of human rotaviruses in Chiang Mai, Thailand: evidence for a close relationship between human and animal rotaviruses. J. Infect. Dis. **166:**227–234.
55. **Varghese, V., S. Das, N. B. Singh, K. Kojima, S. K. Bhattacharya, T. Krishnan, N. Kobayashi, and T. N. Naik.** 2004. Molecular characterization of a human rotavirus reveals porcine characteristics in most of the genes including VP6 and NSP4. Arch. Virol. **149:**155–172.
56. **Watanabe, M., T. Nakagomi, Y. Koshimura, and O. Nakagomi.** 2001. Direct evidence for genome segment reassortment between concurrently-circulating human rotavirus strains. Arch. Virol. **146:**557–570.
57. **Winiarczyk, S., P. S. Paul, S. Mummidi, R. Panek, and Z. Gradzki.** 2002. Survey of porcine rotavirus G and P genotype in Poland and in the United States using RT-PCR. J. Vet. Med. B **49:**373–378.
58. **Woode, G. N., J. C. Bridger, G. A. Hall, and M. J. Dennis.** 1974. The isolation of a reovirus-like agent associated with diarrhoea in colostrums-deprived calves in Great Britain. Res. Vet. Sci. **16:**102–105.
59. **Zaberezhny, A. D., Y. S. Lyoo, and P. S. Paul.** 1994. Prevalence of P types among porcine rotaviruses using subgenomic VP4 gene probes. Vet. Microbiol. **39:**97–110.

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Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain

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Abstract

A novel and unusual strain of porcine rotavirus (PoRV) CMP034 was isolated from a 7-week-old piglet during the epidemiological survey of porcine rotavirus infection in Chiang Mai province, Thailand from June 2000 to July 2001. Molecular characterization of gene VP4 by sequence analysis showed a low level of amino acid sequence identity, ranging from 56.7% to 76.6%, while comparison of VP8* portion showed 41.8% to 69.9% identity, with the 26 P genotypes recognized to date. Phylogenetic analysis of the VP4 sequence revealed that CMP034 was only distantly related to the other 26 P genotypes and was located in a separate branch. Sequence analysis of gene VP7 showed the highest level of amino acid identity (94.7%) with the PoRV G2-like reference strain 34461-4 but a lower level of identity with those of human G2 rotaviruses, ranging from 87.7% to 88.0%. Phylogenetic analysis of gene VP7 revealed two major lineages among G2 rotavirus strains based on the host origin. PoRV strain CMP034 clustered exclusively with G2-like PoRV strain 34461-4 in a novel lineage that is distinct from the major G2 human lineage. Moreover, strain CMP034 displayed a porcine-like VP6 and NSP5/6 with subgroup I specificity, while bearing an NSP4 with some genetic group B human-like characteristics. These findings provide evidence that CMP034 should be considered as a novel VP4 genotype P[27].

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Keywords: Rotavirus; Porcine; P genotype; G genotype; Sequence analysis; Thailand

Introduction

Infection with group A rotavirus is the main cause of acute gastroenteritis in infants and young children worldwide, and in the young of many animal species, including neonatal piglets. Rotavirus belongs to the *Reoviridae* family and contains 11 segments of double-stranded RNA. The two outer-layer proteins VP7 and VP4 form the basis of the current dual classification system of group A rotavirus into G and P genotypes (Estes, 2001). To date, at least 15 G and 26 P genotypes have been identified globally, with various combinations of G and P genotypes (Estes, 2001; Martella et al., 2006a; McNeal et al., 2005; Rahman et al., 2005; Rao et al., 2000). Epidemiological studies have demonstrated that rotavirus genotypes G1, G2, G3, G4, and G9 combined with the P genotypes P[8] and P[4] are the types of VP7 and VP4 most frequently associated with human rotavirus infection globally (Gentsch et al., 1996, 2005).

In recent years, several epidemiological studies designed to monitor the appearance of novel or atypical rotavirus antigenic types have provided evidence for the increasing antigenic diversity of group A rotaviruses (Banyai et al., 2005; Gentsch et al., 2005; Yoshinaga et al., 2006). Usually, rotavirus strains sharing >89% of VP4 amino acid sequence identity are considered to belong to the same P genotype, while those sharing VP4 amino acid sequence identities <89% belong to different genotypes (Estes, 2001; Ciarlet et al., 1997; Gorziglia et al., 1990). At least 26 rotavirus P genotypes have been recognized, and the latest, P[26], was isolated from an Italian diarrheic piglet in an epidemiological study carried out in 2003–2004 (Martella et al., 2006a). Animal rotaviruses are regarded as a potential reservoir for genetic/antigenic diversity of human rotaviruses, and the potential of such transmission has been

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reported in several studies (De Leener et al., 2004; Khamrin et al., 2006b; Nakagomi et al., 1990; Palombo, 2002). The study of animal rotaviruses is a key step to acquiring an in-depth understanding of the ecology and evolution of rotaviruses.

Group A rotaviruses are recognized as a common cause of enteric disease and gastroenteritis in neonatal piglets (Estes, 2001; Woode et al., 1976). Among porcine rotaviruses, two main P genotypes, P[6] and P[7], have been described that are commonly associated with G3, G4, G5, and G11 genotypes (Estes, 2001; Liprandi et al., 1991; Pongsuwanna et al., 1996; Winiarczyk et al., 2002). Moreover, several additional combinations of G and P genotypes have been isolated occasionally from or detected in pigs; i.e. G1, G2-like, G6, G8, G9, G10, and P[13], P[19], P[23] genotypes (Burke et al., 1994; Ciarlet and Liprandi, 1994; Gouvea et al., 1994a,1994b; Huang et al., 1993; Liprandi et al., 2003; Martella et al., 2001, 2005; Pongsuwanna et al., 1996; Racz et al., 2000).

Although G2 rotavirus strains are commonly found in humans, they were not identified from animal sources (Estes, 2001) until 2005, when PoRV strain 34461-4 bearing the G2-like genotype was reported in a piglet in Spain (Martella et al., 2005). Accordingly, a comprehensive genotypic characterization of circulating rotavirus strains among domestic animal populations is important to define the extent of rotavirus diversity.

Here, we report the isolation of a PoRV strain with a novel VP4 genotype, tentatively proposed as P[27]. Characterization of gene VP7 reveals the G2-like rotavirus genotype.

Results

Failure of both G and P genotyping of porcine rotavirus strain CMP034

During an epidemiological survey of porcine rotavirus from June 2000 to July 2001, a total of 175 fecal specimens were collected from diarrheic piglets from 6 different farms located in Chiang Mai province, Thailand. Of these, 39 (22.3%) specimens were shown by ELISA to be positive for group A rotavirus. Initial G and P genotyping was done by reverse transcription-polymerase chain reaction (RT-PCR) and multiplex-PCR assays using different pools of primers specific for human and animal VP7 and VP4 genes. Interestingly, the G and P genotypes of one rotavirus-positive specimen, CMP034, could not be identified by multiplex-PCR typing, despite that the VP7 and VP4 genes were readily amplified by RT-PCR. Therefore, the CMP034 strain was further characterized by nucleotide sequencing of genes VP4, VP6, VP7, NSP4, and NSP5/6.

VP4 and VP8 sequence analyses and determination of P genotype*

Initially, P genotype characterization of PoRV strain CMP034 by multiplex PCR genotyping was unsuccessful. In order to determine the P genotype, the forward primer Con3 was used in combination with reverse primer 170 (Martella et al., 2006a) for amplification of gene VP4 (2341 nucleotides, coding for 772 amino acids). The VP4 and VP8* nucleotide and deduced amino acid sequences of gene VP4 were compared with those of the established reference strains P[1] to P[26] available in the GenBank database (Table 1). It was observed that the nucleotide and deduced amino acid sequences of PoRV strain CMP034 shared very low levels of sequence identity with those of other P genotypes (57.4–71.9% nucleotide identity and 56.7–76.6% amino acid identity for VP4 gene, and 45.7–67.4% nucleotide identity and 41.8–69.9% amino acid identity for VP8*). Rotavirus strains that exhibit a VP4 amino acid identity of approximately >89% are considered to belong to the same P genotype, while those sharing <89% identity belong to different genotypes (Estes, 2001; Ciarlet et al., 1997; Gorziglia et al., 1990). Our results indicated that PoRV strain CMP034 was likely a novel P genotype and, therefore, tentatively proposed as a P[27] VP4 genotype.

The phylogenetic tree was constructed on the basis of the VP4 nucleotide sequence of CMP034 and those of 26 P genotypes (Fig. 1). Phylogenetic analysis clearly confirmed that

Table 1
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP4 and VP8* of porcine strain CMP034 with those of 26 known P rotavirus genotypes

Strain	Species	P genotype	VP4 identity (%)		VP8* identity (%)	
			Nucleotide	Amino acid	Nucleotide	Amino acid
A5	Bovine	P[1]	69.7	74.7	64.4	68.6
SA11	Simian	P[2]	70.5	74.7	67.3	66.5
CMH222	Human	P[3]	70.3	73.9	64.2	64.5
L26	Human	P[4]	67.2	68.5	62.1	60.2
UK	Bovine	P[5]	66.3	70.5	60.5	62.7
Gottfried	Porcine	P[6]	67.9	70.6	63.2	62.6
OSU	Porcine	P[7]	68.9	72.7	64.1	63.8
KU	Human	P[8]	69.1	68.4	63.3	60.6
K8	Human	P[9]	64.5	63.8	57.7	56.9
69M	Human	P[10]	70.7	76.6	67.4	69.9
B223	Bovine	P[11]	57.4	56.7	45.7	41.8
H-2	Equine	P[12]	70.6	74.8	66.7	66.5
MDR-13	Porcine	P[13]	69.1	70.4	61.9	60.0
PA169	Human	P[14]	63.3	64.7	55.9	56.6
Lp14	Ovine	P[15]	70.3	74.3	66.2	66.8
EW	Murine	P[16]	65.4	71.3	60.8	62.7
993/83	Bovine	P[17]	61.0	59.3	51.3	45.3
L338	Equine	P[18]	70.8	72.2	65.2	63.8
Mc323	Human	P[19]	68.5	71.1	61.7	59.5
EHP	Murine	P[20]	66.2	72.8	61.3	63.4
Hg18	Bovine	P[21]	69.6	71.1	65.8	61.4
160/01	Lapine	P[22]	60.3[a]	58.9[a]	60.3	58.9
A34	Porcine	P[23]	62.5[a]	53.5[a]	62.5	53.5
TUCH	Rhesus	P[24]	71.9	76.1	67.4	68.6
Dhaka6	Human	P[25]	63.0	63.1	55.5	55.6
134/04-15	Porcine	P[26]	69.6	72.6	63.7	66.2

The GenBank accession numbers of the following strains are given in parentheses: A5 (D13395), SA11 (M23188), CMH222 (DQ288661), L26 (M58292), UK (M22306), Gottfried (M33516), OSU (X13190), KU (M21014), K8 (D90260), 69M (M60600), B223 (D13394), H-2 (L04638), MDR-13 (L07886), PA169 (D14724), Lp14 (L11599), EW (U08429), 993/83 (D16352), L338 (D13399), Mc323 (D38052), EHP (U08424), Hg18 (AF237665), 160/01 (AF526374), A34 (AY174094), TUCH (AY596189), Dhaka6 (AY773004), 134/04-15 (DQ061035).

[a] Amino acid identity was calculated based on VP8* region of VP4 gene.

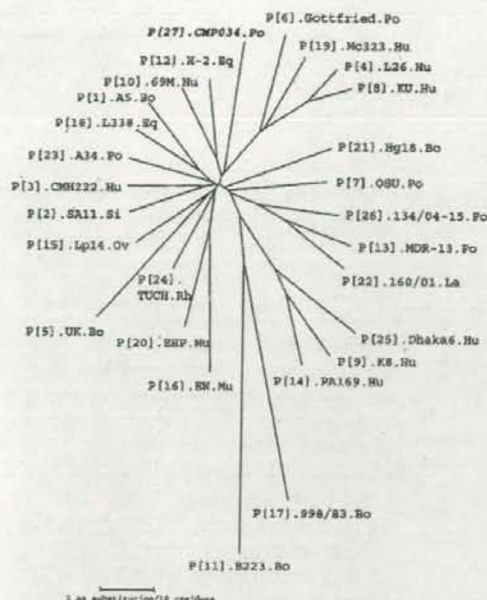

Fig. 1. Phylogenetic tree of the VP4 gene nucleotide sequence displaying the relationships between PoRV CMP034 and 26 known P genotypes. GenBank accession numbers of the VP4 sequences of all known 26 P genotypes are given in the legend to Table 1. The tree was generated on the basis of the neighbor-joining method using program MEGA 3.1.

PoRV strain CMP034 was located in a separate branch, which was only distantly related to the other P genotypes. The VP4 deduced amino acid sequence alignment of CMP034 with the representative rotavirus strains of all other P genotypes is shown in Fig. 2. The overall picture from this alignment showed the distinction between the CMP034 amino acid sequence and those of other representative strains of P[1] to P[26] genotypes. It was interesting to note that the complete deduced amino acid sequence of PoRV strain CMP034 had lost one amino acid residue at position 135, which was a unique feature of human, but not animal, rotavirus strains (Gorziglia et al., 1988; Kantharidis et al., 1987). However, the deletion of 3 amino acids observed in the hypervariable region of the VP5* portion of VP4 could also be found in bovine rotavirus strain B223 (Fig. 2). Accordingly, the origin of the CMP034 VP4 gene remained inconclusive. Furthermore, the potential trypsin cleavage sites at arginine 240 and 246 were conserved. The proline residues 68, 71, 224, 225, 333, 389, 394, 434, 450, 454, 474, 523, 665, 712, 745, and 757, and the cysteine residues at positions 215, 317, 379, which were highly conserved among the VP4 gene of rotaviruses, were maintained in the PoRV strain CMP034.

VP7 sequence analysis and determination of G genotype

Similar to the P genotype, the G genotype of PoRV strain CMP034 could not be determined by multiplex-PCR using the pools of G typing primer sets reported previously (Das et al., 1994; Gouvea et al., 1990). The full length of the nucleotide and deduced amino acid sequences of gene VP7, generated from RT-PCR, was determined and compared with VP7 sequences of the existing G1 to G15 strains. The highest sequence identity was found in G2 rotavirus strains (81.3–90.9% at the nucleotide level and 87.7–94.7% at the amino acid level), and showed the highest level of identity with PoRV G2-like reference strain 34461-4, 90.9% at the nucleotide level and 94.7% at the amino acid level (Table 2). Comparison between the VP7 sequences of PoRV CMP034 and human genotype G2 strains (DS-1, S2, TA20, KUN, 906SB/98, 92C) revealed nucleotide and amino acid identities ranging from 81.3 to 83.5% and from 87.7 to 88.0%, respectively.

In order to determine whether the failure of G genotyping of CMP034 was due to nucleotide mismatches at the primer binding region, the VP7 sequence of CMP034 was aligned with the sequences of G2 type-specific primers used in this study, aCT2 and 9T1-2 (Das et al., 1994; Gouvea et al., 1990). Several nucleotide mismatches were detected at the primer binding sites on VP7 gene of CMP034. The nucleotide mismatches were detected at 11 of 25 nucleotides for the aCT2 primer and at 5 of 20 nucleotides for the 9T1-2 primer, respectively (data not shown).

The amino acid sequences of VP7 antigenic regions A, B, C, and F of 15 established rotavirus G genotypes, as well as several G2 strains of human and porcine rotaviruses, were aligned with the respective sequence of the PoRV strain CMP034 (Fig. 3). Within the VP7 antigenic regions A, B, C, and F, PoRV strain CMP034 was identical with the G2 PoRV strain 34461-4, with the exception of one amino acid change from Lys to Arg in antigenic region B at position 147. In contrast, when comparing with human G2 reference strains (92C, 906SB/98, S2, TA20, KUN, and DS-1), a higher number of amino acid substitutions were found: 2 or 3 in antigenic regions A and C, 1 or 2 in antigenic region B, and 1 in antigenic region F. Thus, analysis of VP7 hypervariable regions A, B, C, and F confirmed the closest relationship of the PoRV strain CMP034 with G2-like PoRV strain 34461-4. A phylogenetic tree that included the VP7 sequences of all rotavirus G genotypes recognized to date from both human and non-human origins was constructed (Fig. 4). The result of phylogenetic analysis confirmed that PoRV CMP034 strain clustered with G2 rotavirus reference strains. Two major lineages were found among G2 rotavirus strains; one included most of the human G2 rotaviruses, including 92C, 906SB/98, S2, TA20, KUN, and DS-1, while another was formed exclusively by G2-like porcine rotavirus strains 34461-4 and CMP034.

VP6, NSP4, and NSP5/6 sequence analysis

Comparative analysis of the nucleotide and deduced amino acid sequences of full-length VP6 with those of four representative established subgroups allowed the identification of PoRV strain CMP034 as having subgroup I specificity. In addition, VP6 of strain CMP034 showed the highest level of nucleotide sequence identity (92.6%) with PoRV strain JL94, whereas the amino acid sequence is related most closely (99.2%) to that of PoRV strains A253 and A131 (Table 3). Strains JL94, A253, and A131 all have subgroup I specificity.

Analysis of the NSP4 sequences revealed that CMP034 was related most closely to human rotavirus NSP4 genetic group B strain GR856/86, with 86.8% nucleotide sequence identity, and to strain RV4, with 88.2% amino acid sequence identity (Table 4). These results indicate that CMP034 belongs to NSP4 genetic group B.

The complete nucleotide sequence (667 nucleotides) of NSP5/6 gene of CMP034 was analyzed. By comparison to sequences in the GenBank database, the highest level of sequence identity (98.0% nucleotide identity) was with PoRV strain YM (data not shown).

Discussion

Several review articles have described the overview concerning the genetic and antigenic diversities of rotavirus

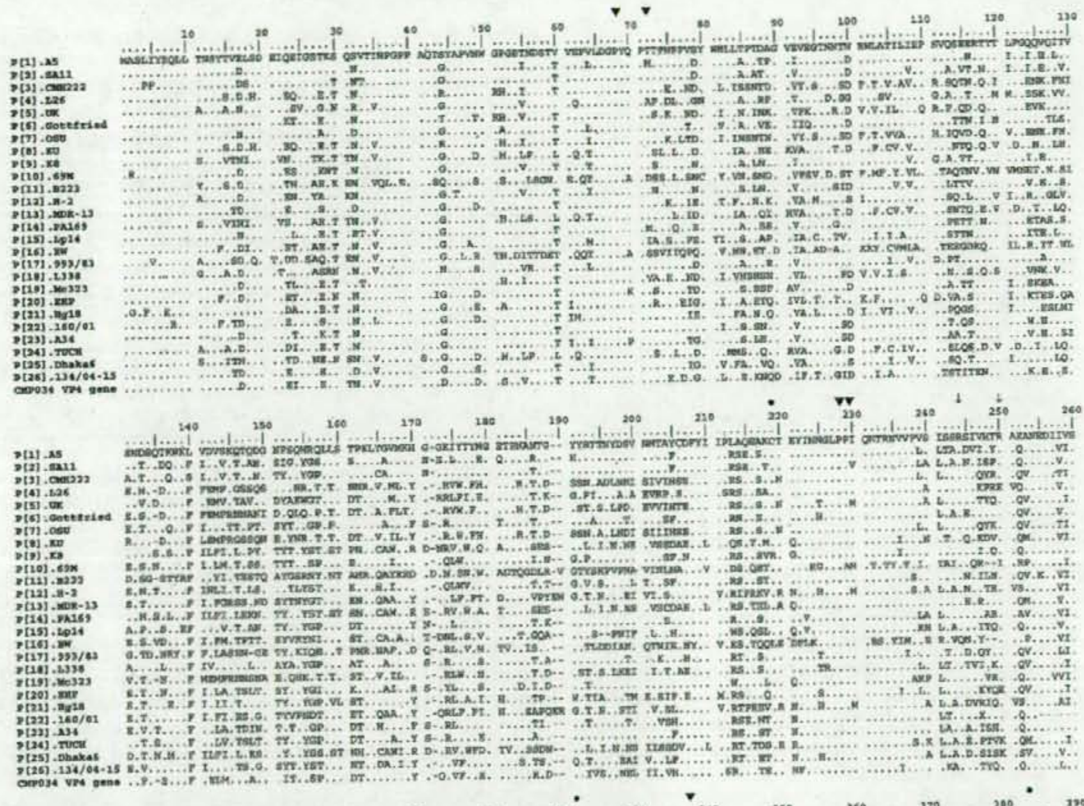

Fig. 2. Comparison of the deduced amino acid sequence of protein VP4 of the porcine rotavirus strain CMP034 with those of 26 known P genotypes. The potential trypsin cleavage site (↓) and highly conserved cysteine (●) and proline (▼) residues are indicated.

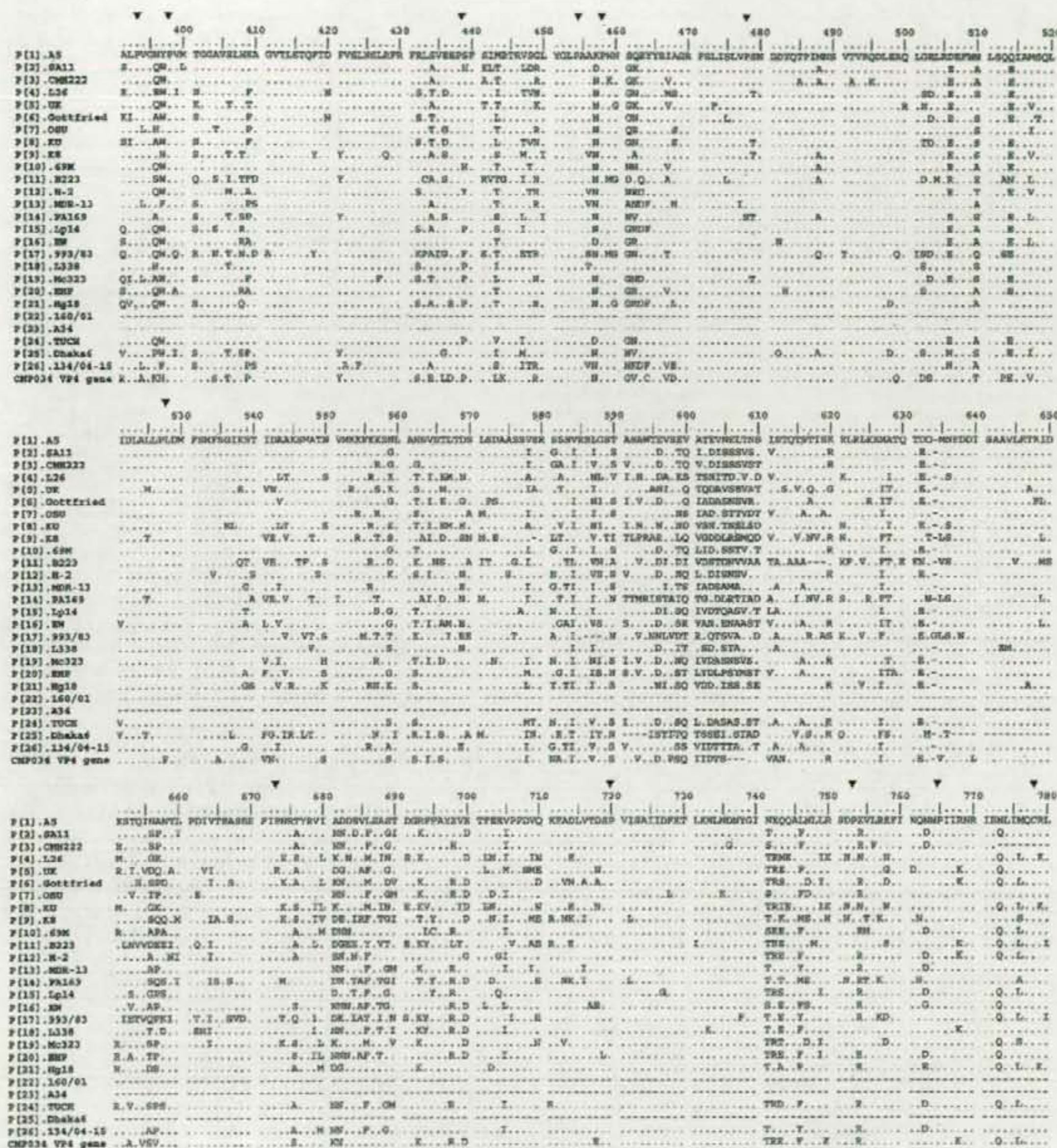

Fig. 2 (*continued*).

strains, and showed the potential evidence of close genetic relationships among rotavirus strains isolated from domestic animals and human host as a consequence of interspecies transmission (De Leener et al., 2004; Gentsch et al., 2005; Nakagomi et al., 1990; Palombo, 2002;). Therefore, simultaneous surveillance of animal and human rotavirus infections is a key step to understanding the ecology and evolution of rotaviruses.

Detection of human rotavirus strains bearing P[19] specificity (Mc323 and Mc345) was first reported from Chiang Mai city, Thailand in 1987–1989 (Urasawa et al., 1992). Analysis of the VP4 and VP7 sequences of these two strains revealed a G9P[19] specificity and close genetic relatedness to P[19] porcine rotavirus strain 4F (Okada et al., 2000). In that study, unfortunately, the epidemiological survey of porcine rotavirus was not done simultaneously in the same epidemic