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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.

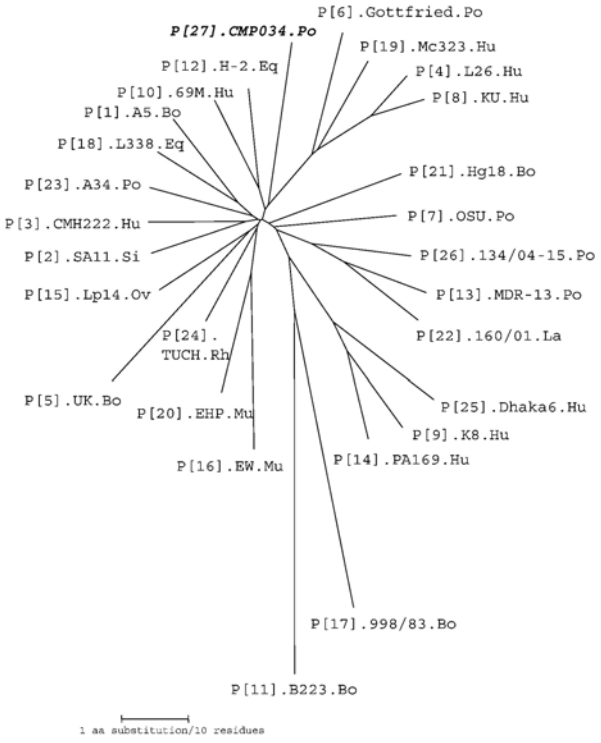

Fig. 1. Phylogenetic tree of the VP4 gene nucleotide sequence displaying the relationships between PoRV CMP034 and 26 known P genotypes. GenBank accession numbers of the VP4 sequences of all known 26 P genotypes are given in the legend to Table 1. The tree was generated on the basis of the neighbor-joining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

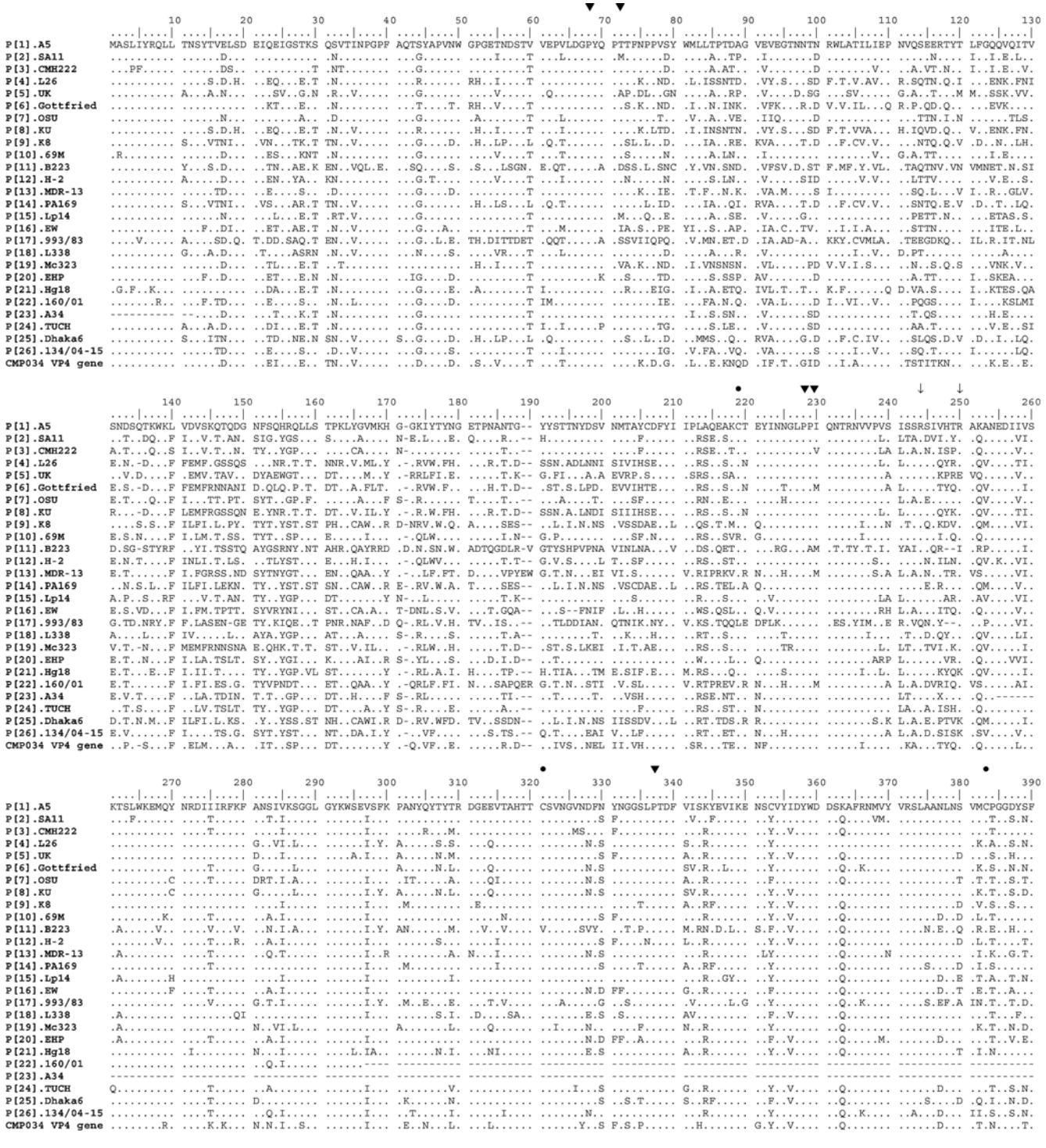

Fig. 2. Comparison of the deduced amino acid sequence of protein VP4 of the porcine rotavirus strain CMP034 with those of 26 known P genotypes. The potential trypsin cleavage site (↓) and highly conserved cysteine (●) and proline (▼) residues are indicated.

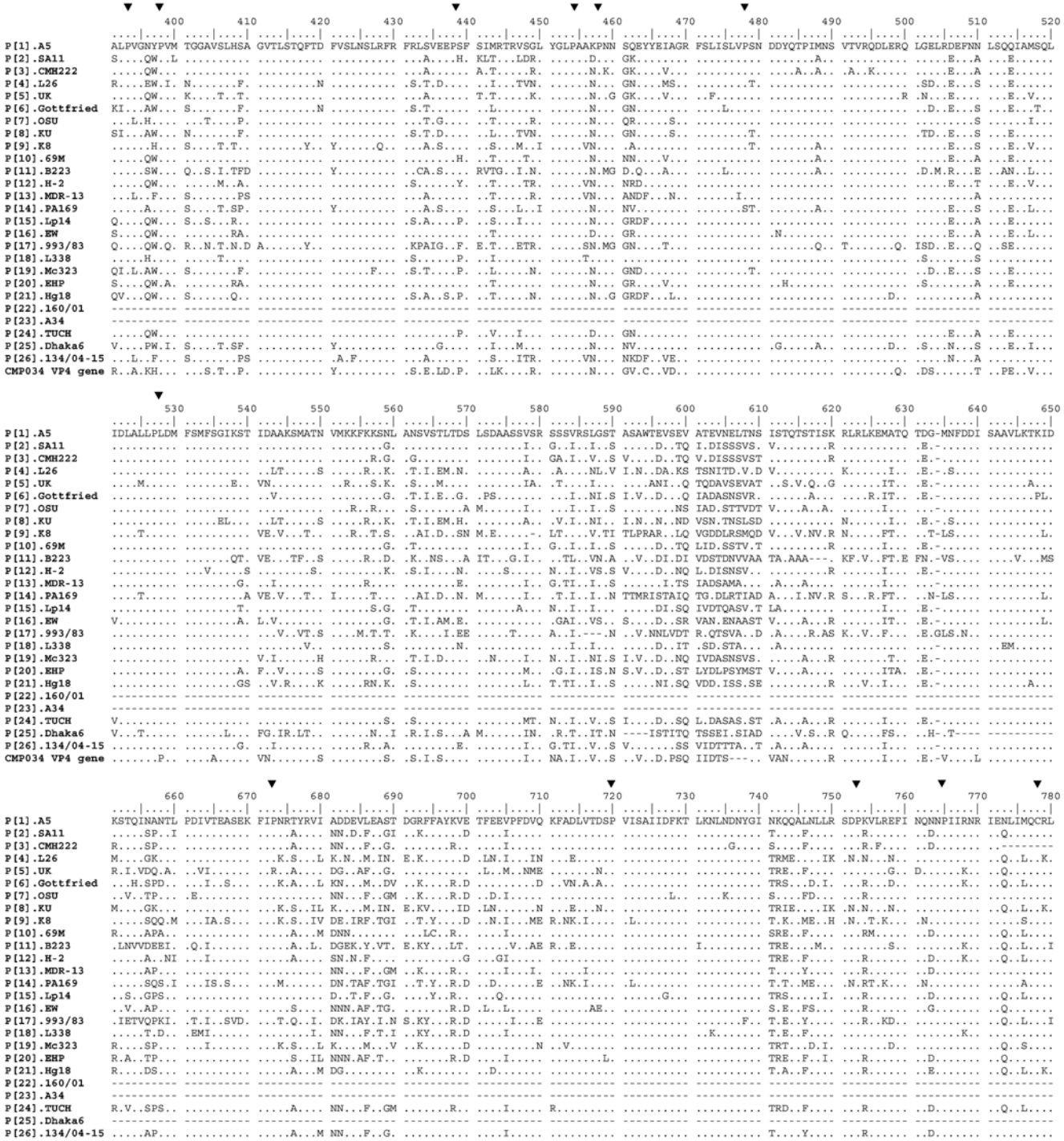

Fig. 2 (*continued*).

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

Table 2
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP7 of strain CMP034 with those of 15 known G rotavirus genotypes

Strain	Species	G genotype	Identity (%)	
			Nucleotide	Amino acid
KU	Human	G1	72.4	75.1
DS-1	Human	G2	81.3	88.0
S2	Human	G2	82.6	87.7
TA20	Human	G2	82.9	88.0
KUN	Human	G2	82.0	88.0
906SB/98	Human	G2	83.0	88.0
92C	Human	G2	83.5	88.0
34461-4	Porcine	G2	90.9	94.7
CMH222	Human	G3	73.6	76.6
Hochi	Human	G4	72.5	72.3
OSU	Porcine	G5	74.1	76.0
NCDV	Bovine	G6	72.3	75.7
Ch2	Avian	G7	61.0	56.9
B37	Human	G8	73.5	57.1
116E	Human	G9	77.0	77.3
61A	Bovine	G10	71.7	74.2
YM	Porcine	G11	72.9	75.1
L26	Human	G12	74.8	75.1
L338	Equine	G13	72.0	72.6
CH3	Equine	G14	73.5	73.9
Hg18	Bovine	G15	72.1	72.3

The GenBank accession numbers of the following strains are given in parentheses: KU (D16343), DS-1 (AB118023), S2 (M11164), TA20 (AF106281), KUN (D50124), 906SB/98 (AY261347), 92C (U73949), 34461-4 (AY766085), CMH222 (AY707792), Hochi (AB012078), OSU (X04613), NCDV (M12394), Ch2 (X56784), B37 (J04334), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666).

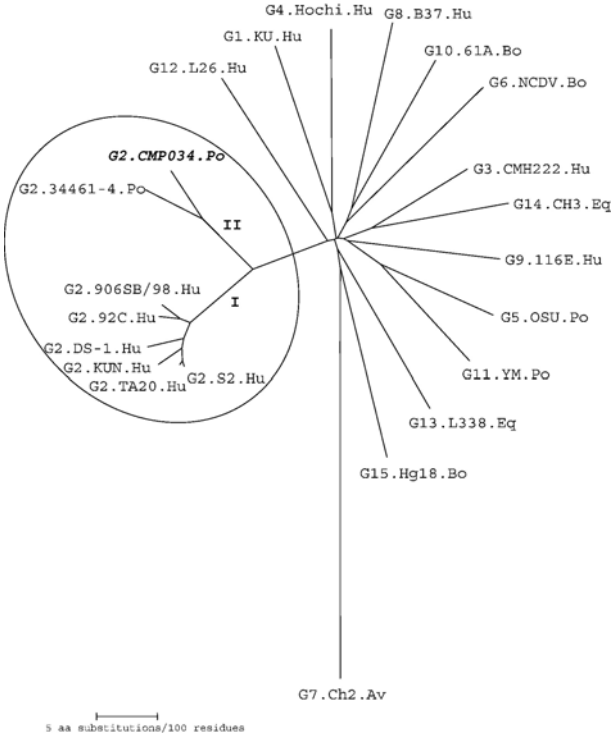

Fig. 4. Phylogenetic tree based on the VP7 nucleotide sequences. The tree displays the relationships among strain CMP034 and of the 15 known G genotypes. GenBank accession numbers of the VP7 sequences of all known 15 G genotypes are given in the legend to Table 2. The tree was generated on the basis of the neighbor-joining method using program MEGA 3.1.

season to verify whether P[19] was really circulating in the pig population in Chiang Mai city. Therefore, we conducted a rotavirus surveillance in both humans and pigs simultaneously in the same epidemic season in Chiang Mai city during the year 2000–2001, and we found P[19] strains circulating in pig populations in Chiang Mai city (Maneekarn et al., 2006). These PoRV strains were G3P[19], and shared a level of VP4 P[19] sequence identity with those of P[19] Mc323 and Mc345 as high as 95.4–97.6% nucleotide sequence identity. This finding indicates that gene VP4 of Mc323 and Mc345 might have been derived through reassortment from VP4 P[19] of PoRV strains that circulated in this region. However, P[19] rotavirus was not detected in humans in the study that was carried out in the same epidemic season (Khamrin et al.,

	A		B		C		F	
	87	101	142	152	208	221	235	242
G2.CMP034	IYYPTEAKNEISDTE		NVVLMRYDTTS		GIGCKTTEVNTFET		NGVNHKIS	
G2.34461-4	.....		.....K.....		.....		.....	
G2.92C	L.....D.		.....N..		.....D.S...I		.....N	
G2.906SB/98	L...A.....D.		.....GN..		.....D.S....		.....N	
G2.S2	L...A.....D.		.....N..		.....D.D...I		.....N	
G2.TA20	L...A.....D.		.....N..		.....D.D...I		.....N	
G2.KUN	L.....D.		.....N..		.....D.....I		.....N	
G2.DS-1	L...A.....D.		.....N..		.....D.....I		.....N	
G1.KU	L.....STQ.N.GD		.L...K..QSL		....Q..N.DS..M		D.I....N	
G3.CMH222	L.....AT..N.NS		.....K..A.L		....L..DTT...E		D.....LD	
G4.Hochi	L...S..PTQ.....		....I.FASGE		....Q..NTA....		DS....LD	
G5.OSU	L...N..AT..A..K		.I...K..GNL		....S..DI.S...		D.....LD	
G6.NCDV	L...V..S..MA...		.L...K..S.Q		....LI.NPD....		D.....LN	
G7.Ch2	....KA.DT..A.P.		.I..AH.TNDV		....Q..NTD...I		D.....VD	
G8.B37	L...V..ET..A.SS		.I...K.NAN.		R...L..DTT...E		D...Y..N	
G9.116E	L...I..STQ.G...		....VK.NS.L		....T..NTA...E		D.....LD	
G10.61A	L.....RT..N.N.		.I.....NSSL		....Q..NTR...E		D.....LD	
G11.YM	L...H..ATQ.A.DK		.I...K..GN.		....L..DPT...E		D.....LD	
G12.L26	L...NSVTT..T.PD		.I..VQ.QNSL		....T..D.A...E		D.....N	
G13.L338	L...N.VVS.LN.DS		.I.VVK.S.EL		....L..DTE...E		D.....N	
G14.CH3	L.S....ATQ.D.SS		.....K..EAL		....L..N.E...E		D..D...N	
G15.Hg18	.....I.S.DLA.PD		.I...K.ESDL		....L..DTSS...		D..S..LD	

Fig. 3. Comparison of the amino acid sequences of antigenic regions A, B, C, and F of porcine CMP034 with those of 15 known G genotypes. Dots indicate amino acid residues identical with those in the sequence of strain CMP034.

Table 3
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP6 of strain CMP034 with those of known subgroups

Strain	Species	Subgroup	Identity (%)	
			Nucleotide	Amino acid
BRV033	Bovine	I	76.8	90.1
NCDV	Bovine	I	79.1	90.9
UK	Bovine	I	80.0	91.4
SA11	Simian	I	78.7	91.1
I321	Human	I	80.7	90.9
US1205	Human	I	78.6	90.1
1076	Human	I	79.7	91.6
RMC321	Human	I	90.8	98.9
RMC/G60	Human	I	90.7	98.4
RMC/G7	Human	I	90.9	98.4
4F	Porcine	I	91.0	98.9
4S	Porcine	I	90.9	98.9
JL94	Porcine	I	92.6	98.4
A253	Porcine	I	86.0	99.2
A131	Porcine	I	87.5	99.2
OSU	Porcine	I	86.9	98.9
H-1	Equine	I	87.7	97.9
Wa	Human	II	82.1	93.7
116E	Human	II	83.4	93.9
TK159	Human	II	82.9	91.9
Gottfried	Porcine	II	83.6	93.7
FI-14	Equine	I+II	78.9	91.1
L338	Equine	I+II	80.5	92.1
H-2	Equine	Noni/nonII	78.9	90.2
FI-23	Equine	Noni/nonII	78.5	90.2

The GenBank accession numbers of the following strains are given in parentheses: BRV033 (AF317126), NCDV (AF317127), UK (X53667), SA11 (AY187029), I321 (X94618), US1205 (AF079357), 1076 (D00325), RMC321 (AF531913), RMC/G60 (AY601552), RMC/G7 (AY601551), 4F (L29184), 4S (L29186), JL94 (AY538664), A253 (AF317122), A131 (AF317124), OSU (AF317123), H-1 (AF242394), Wa (K02086), 116E (U85998), TK159 (AY661888), Gottfried (D00326), FI-14 (D00323), L338 (D82974), H-2 (D00324), FI-23 (D82971).

2006a). In this epidemiological study of the distribution of PoRV G and P genotypes in Chiang Mai province, we detected an unusual rotavirus strain, which appears to carry a novel P[27] genotype. As revealed by the analysis of the amino acid sequence, gene VP4 of PoRV strain CMP034 shares less than 77% amino acid sequence identity with the 26 known P genotypes.

It is well established that the VP4 sequence of animal rotaviruses contains 776 amino acid residues, while most human rotavirus strains contain 775 residues, with the loss of one residue between positions 134 and 136 of the VP8* fragment of gene VP4 (Gorziglia et al., 1988; Kantharidis et al., 1987). Analysis of the amino acid sequence of VP4 revealed that CMP034 has lost one amino acid residue at position 135, similar to human rotaviruses. Nevertheless, the deletion of three other amino acid residues in the hypervariable region of the VP5* portion of gene VP4, like those found in bovine rotavirus strain B223, implies an animal origin of CMP034. This unusual feature of amino acid deletion in the VP4 gene of CMP034, therefore, makes the origin of gene VP4 unclear. Nevertheless, it is interesting to note that sequence analyses of VP6 and NSP5/6 genes of CMP034 reveal a porcine-like specificity, whereas the NSP4 gene has some characteristics consistent with a human origin.

The distribution of rotavirus G and P genotypes in pigs has been reported from throughout the world (Estes, 2001; Liprandi et al., 1991; Pongsuwanna et al., 1996; Winiarczyk et al., 2002). On the basis of the accumulated evidence of transmission of rotaviruses between pigs and other animal species, including human, pigs are regarded as the potential reservoir for the emergence of unusual or novel strains of rotaviruses (Palombo, 2002; Martella et al., 2005, 2006b). Several novel strains of P genotypes have been identified recently in pigs, such as P[23] (Liprandi et al., 2003) and, most recently, P[26] (Martella et al., 2006a). In the present study, a novel P genotype has been isolated from a diarrheic piglet in Thailand.

Although G2 strains of rotavirus are commonly found in humans, it has not been identified from other animal sources (Estes, 2001), except for one recent report from Spain of a

Table 4
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein NSP4 of strain CMP034 with those of known NSP4 genetic groups

Strain	Species	NSP4 genetic group	Identity (%)	
			Nucleotide	Amino acid
KUN	Human	A	78.6	78.8
UK	Bovine	A	79.0	80.0
NCDV	Bovine	A	78.1	80.0
SA-11	Simian	A	78.9	78.2
Wa	Human	B	85.1	84.0
RV4	Human	B	84.5	88.2
ST3	Human	B	83.1	81.1
M37	Human	B	83.9	81.7
116E	Human	B	85.9	83.4
97'SZ37	Human	B	85.4	83.4
GR856/86	Human	B	86.8	86.2
GR1106/86	Human	B	86.6	85.1
A131	Porcine	B	79.8	85.4
A253	Porcine	B	81.8	85.7
A411	Porcine	B	81.2	82.8
H-1	Equine	B	81.9	83.4
OSU	Porcine	B	85.7	85.1
A34	Porcine	B	86.0	85.1
FRV-1	Feline	C	77.8	77.1
AU1	Human	C	77.9	76.5
GRV	Caprine	C	61.0	78.2
CMH222	Human	C	78.0	77.1
CU-1	Canine	C	73.3	77.7
EW	Murine	D	66.2	60.0
EHP	Murine	D	66.1	59.4
EC	Murine	D	66.0	58.2
Ty-1	Avian	E	49.8	30.2
Ty-3	Avian	E	46.8	29.7
Ch-1	Avian	E	51.3	32.5

The GenBank accession numbers of the following strains are given in parentheses: KUN (D88829), UK (K03384), NCDV (X06806), SA11 (K01138), Wa (AF093199), RV4 (U59108), ST3 (U59110), M37 (U59109), 116E (U78558), 97'SZ37 (AF284778), GR856/86 (AF170832), GR1106/86 (AF170833), A131 (AF144798), A253 (AF144797), A411 (AF144799), H-1 (AF144800), OSU (D88831), A34 (AF165219), FRV-1 (D89874), AU-1 (D89873), GRV (AB055968), CMH222 (DQ288660), CU-1 (AF144806), EW (U96335), EHP (U96336), EC (U96337), Ty-1 (AB065285), Ty-3 (AB065286), Ch-1 (AB065287).

PoRV strain bearing a G2-like genotype (strain 34461-4) in a piglet (Martella et al., 2005). The VP7 sequence analysis of our CMP034 strain revealed a genetically close relationship to the G2-like PoRV strain 34461-4. A close genetic relationship between VP7 from strains CMP034 and 34461-4 was demonstrated by analysis of the VP7 antigenic regions A, B, C, and F. The only difference observed between the two strains was a Lys to Arg change at position 147 in the antigenic region B. The relatedness between the sequence of VP7 in strains CMP034 and 34461-4 was confirmed repeatedly by the phylogenetic analysis of the VP7 sequence, showing that CMP034 and 34416-4 are clustered closely together in a branch separate from that of other human G2 reference strains. These findings suggest that VP7 of PoRV strain CMP034 may have originated from the same ancestor as those of strain 34461-4. The isolation of two strains of rotaviruses with a close genetic relatedness of gene VP7 from two countries, Thailand and Spain, which are geographically far apart, may indicate that the gene VP7 of G2 specificity may have already been introduced into the porcine rotaviruses worldwide. To verify this notion, the epidemiological rotavirus surveillance in pigs may need to be performed extensively in several other regions of the world.

Materials and methods

Rotavirus antigen detection

Porcine rotavirus strain CMP034 was isolated during the surveillance of porcine rotavirus infection in Chiang Mai province, Thailand from June 2000 to July 2001. A total of 175 fecal specimens were collected from diarrheic piglets from six different farms and the age of the piglets ranged from 7 days to 49 days old. Group A rotavirus antigen was detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody against group A rotavirus as described previously (Hasegawa et al., 1987). Out of 175, 39 (22.3%) were positive for group A rotaviruses (Maneekarn et al., 2006). PoRV strain CMP034 was identified as group A rotavirus in stool sample from a diarrheic piglet of 49 days old at a farm in Mae Rim district, Chiang Mai province. Despite numerous attempts, using RNA PAGE of phenol/chloroform or acid phenol/guanidinium thiocyanate/chloroform RNA extraction methods, we were unable to visualize the dsRNA electrophoretic pattern.

RNA extraction, RT-PCR, and multiplex-PCR for G and P genotyping

The G and P genotypes of CMP034 were determined by RT-PCR and multiplex-PCR. Viral dsRNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). Viral dsRNA was denatured in 50% (v/v) dimethyl sulfoxide at 95 °C for 5 min. The RT-PCR was carried out with a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). For PCR amplification of the VP7 gene, a 1062 bp fragment was generated using Beg9 (forward) and End9 (reverse) primers (Gouvea et al., 1990). For PCR amplification of the VP4 gene, an 876 bp fragment was generated using Con3 as a forward primer and Con2 as a reverse primer (Gentsch et al., 1992). The G genotyping was performed using different pools of primers specific for G genotypes of human and animal rotaviruses (G1–G6 and G8–G11) as described previously (Das et al., 1994; Gouvea et al., 1990, 1994a; Winiarczyk et al., 2002). The VP4 characterization was performed using different pools of P genotype-specific primers for P[1], P[4]-P[11], and P[14] (Gentsch et al., 1992; Gouvea et al., 1994b; Mphahlele et al., 1999; Winiarczyk et al., 2002).

Amplification and sequence analysis of VP4, VP7, VP6, NSP4, and NSP5/6 genes

We could not initially identify the P genotype of PoRV CMP034 strain by multiplex PCR using several sets of genotype-specific primers, so the reverse primer 170 (Martella et al., 2006a), was used in combination with Con3 (forward primer) for amplification of gene VP4 (2341 bp). The full-length VP7 gene (1062 bp) was reverse transcribed and amplified using the primer pair of Beg9 and End9 (Gouvea et al., 1990). The G genotype of CMP034 strain was not

Table 5
Oligonucleotide primers used for the amplification and sequencing of genes VP4, VP6, VP7, NSP4, and NSP5/6

Primer	Gene	Sequence 5′ to 3′	Sense	Position	Reference
Con3	VP4	TGGCTTCGCTCATTTATAGACA	+	11–32	Gentsch et al. (1992)
170	VP4	GGTCACAWCCTCTAGMMRYTRCTTA	−	2362–2383	Martella et al. (2006a)
Con2	VP4	ATTTCGGACCATTTATAACC	−	868–887	Gentsch et al. (1992)
Con2R [a]	VP4	GGTTATAAATGGTCCGAAAT	+	868–887	Gentsch et al. (1992)
VP4-3R	VP4	CAATTCTRTTHCGAATTATTGGRTT	−	2287–2311	Khamrin et al. (2006a)
P34F665	VP4	GATTGCCACCAATACAGAAC	+	665–684	This study
P34F2089	VP4	GAGTAGACACGTTTGAGGAGG	+	2069–2089	This study
Beg9	VP7	GGCTTTAAAAGAGAGAATTTCCGTCTGG	+	1–28	Gouvea et al. (1990)
End9	VP7	GGTCACATCATACAATTCTAATCTAAG	−	1036–1062	Gouvea et al. (1990)
VP6-5F	VP6	GGCTTTTAAACGAAGTCTTC	+	1–20	Shen et al. (1994)
VP6-3R	VP6	GGTCACATCCTCTCACTA	−	1339–1356	Shen et al. (1994)
NSP4 1a	NSP4	GGCTTTTAAAAGTTCTGTTCCG	+	1–22	Kudo et al. (2001)
NSP4 2b	NSP4	GGTCACATTAAGACCGTTCC	−	731–750	Kudo et al. (2001)
GEN-NSP5F	NSP5/6	GGCTTTTAAAGCGCTACAG	+	1–24	Matthijnssens et al. (2006)
GEN-NSP5R	NSP5/6	GGTCACAAAACGGGAGTG	−	650–667	Matthijnssens et al. (2006)

[a] Con2R primer was modified from the original Con2 primer described by Gentsch et al. (1992).

determined, even by using several sets of genotype-specific primers. In order to determine the G genotype specificity, we analyzed the sequence of gene VP7. The full length of gene VP6 was amplified by primer pairs VP6-5F and VP6-3R, which were modified slightly from the original VP6-specific primers described by Shen et al. (1994). The NSP4 full-length gene was amplified by the NSP4-1a and NSP4-2b primer pair (Kudo et al., 2001). The full-length NSP5/6 gene was amplified with the pair of primers, GEN-NSP5F and GEN-NSP5R, described by Matthijnssens et al. (2006). The sequences of primers used for amplification and sequencing are shown in Table 5.

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences of genes VP4, VP7, VP6, NSP4, and NSP5/6 were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (Kumar et al., 2004).

Nucleotide sequence accession number

The nucleotide sequences of genes VP4, VP6, VP7, NSP4, and NSP5/6 of strain CMP034 have been deposited in GenBank with the accession numbers DQ534016, DQ534018, DQ534015, DQ534017, and DQ916134, respectively.

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