CHAPTER 5

Results

5.1 Prevalence of human and porcine group A rotaviruses

A total of 401 fecal specimens collected during January 2013 to February 2014 from children hospitalized with diarrhea, 137 (34.2%) were positive for group A rotaviruses by RT-PCR screening method. In addition, 113 out of 491 (23.0%) stool specimens collected from diarrheic piglets during January 2011 to March 2014 were positive for group A rotaviruses.

5.2 Molecular characterization of group A rotaviruses

5.2.1 Identification of G and P genotypes of human and porcine rotaviruses by multiplex RT-PCR

The fecal specimens that were positive for human (137 samples) and porcine (113 samples) group A rotaviruses were further determined for their G and P genotypes by RT-multiplex PCR using specific primers for amplifications of VP7 and VP4 genes and using a pool of specific primers for each G and P genotypes. The genotypes of positive samples were assigned based on the sizes of PCR products by comparing with those of reference strains and compared with GeneRuler^{TM} 100 bp Plus DNA Ladder marker (Fermentas, Glen Burnie, MD, USA). The example of agarose gel electrophoresis of G and P genotyping of human rotaviruses is shown in Figure 5.1 while G and P genotyping of porcine rotaviruses is shown in Figure 5.2. For human rotavirus, five different G-genotypes were detected

in this study. The G3 was the most prevalent genotype (69 of 137; 50.3%), followed by G1 (30 of 137; 21.9%), G2 (22 of 137; 16.1%), G9 (4 of 137; 2.9%), G8 (3 of 137; 2.2%), and 6.6% (9 of 137) were nontypeable strains. For P genotypes, P[8] was detected as the most prevalent genotype (109 of 137; 79.6%), followed by P[4] (25 of 137; 18.2%), mix-infection of P[8] and P[4] (1 of 137; 0.7%) and 1.5% (2 of 137) were nontypeable strains. The G and P genotypes of human rotavirus determined by multiplex PCR are shown in Table 5.1. The rotavirus isolates of which their G genotype and/or P genotype could not be identified by multiplex PCR method, so called nontypeable strains, were subjected further to nucleotide sequencing. The prevalence of G and P genotypes of porcine rotaviruses in piglets with diarrhea were also determined. The G4 was the most predominant genotype (63 of 113; 31.9%), followed by G3 (17 of 113; 15.0%), G5 (12 of 113; 10.6%), and G9 (6 of 113; 5.3%). For P genotype, P[6] was the most predominant genotype (17 of 113; 15.0%), followed by P[19], and P[7] with the prevalent rate of 4.4% (5 of 113), and 3.5% (4 of 113), respectively. Of these, G nontypeable strains were 13.3% (15 of 113) and P nontypeable were 77.1% (87 of 113). The G and P genotypes of porcine rotavirus identified by multiplex PCR are shown in Table 5.2. The G and P nontypeable strains of porcine rotaviruses were also subjected further to nucleotide sequencing.

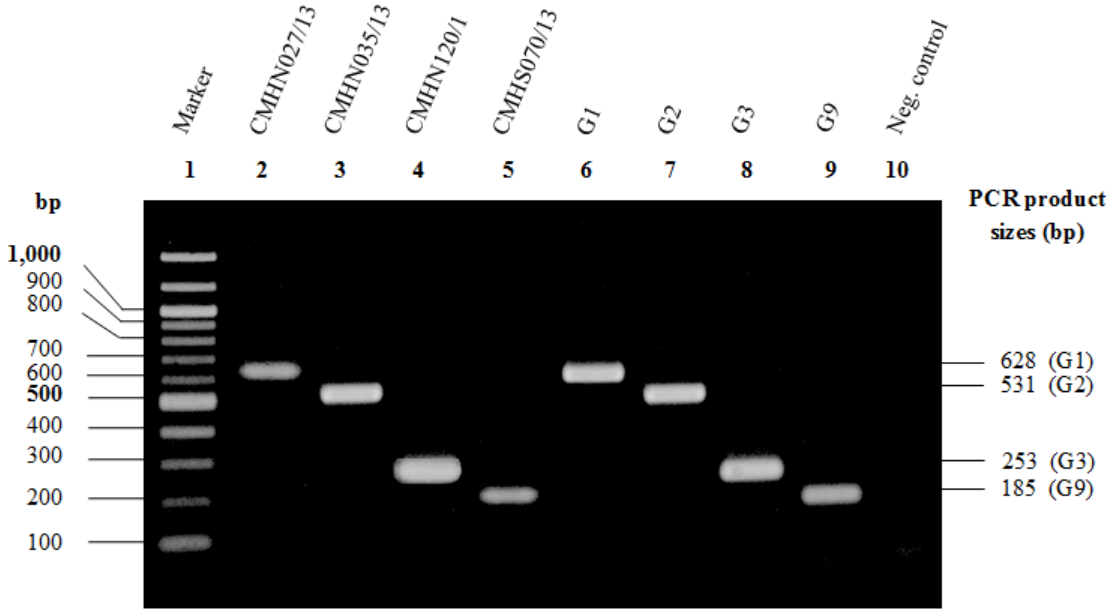

Figure 5.1 Agarose gel electrophoresis demonstrated the PCR product sizes of G genotypes (G1, G2, G3, and G9) of human rotaviruses in comparison with the reference strains. Lane 1 is 100 bp Plus DNA Ladder marker. Lanes 2-5 are test samples that were positive for G1, G2, G3, and G9 with the PCR product sizes of 628, 531, 253, and 185 bp, respectively. Lanes 6-9 are reference strains of G1, G2, G3, and G9. Lane 10 is a negative control reaction in which the DNA template was omitted. The molecular sizes of marker are shown on the left and the expected fragment length of each G genotype is shown on the right sides of the gel.

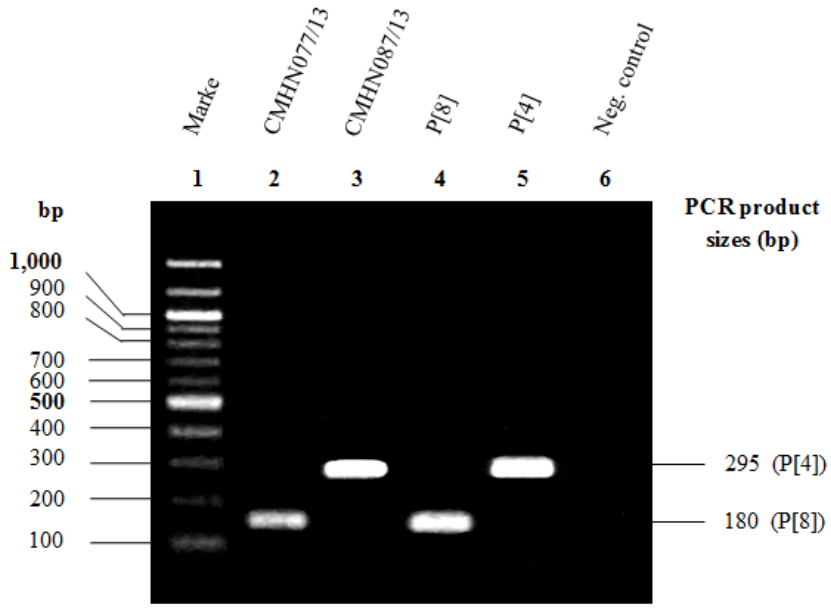

Figure 5.2 Agarose gel electrophoresis demonstrated the PCR product sizes of P genotypes (P[8] and P[4]) of human rotaviruses in comparison with the reference strains. Lane 1 is 100 bp Plus DNA Ladder marker. Lanes 2-3 are test samples that were positive for P[8] and P[4] with the PCR product sizes of 180 and 295 bp, respectively. Lanes 4-5 are reference strains of P[8] and P[4]. Lane 6 is a negative control reaction in which the DNA template was omitted. The molecular sizes of marker are shown on the left and the expected fragment length of each P genotype is shown on the right sides of the gel.

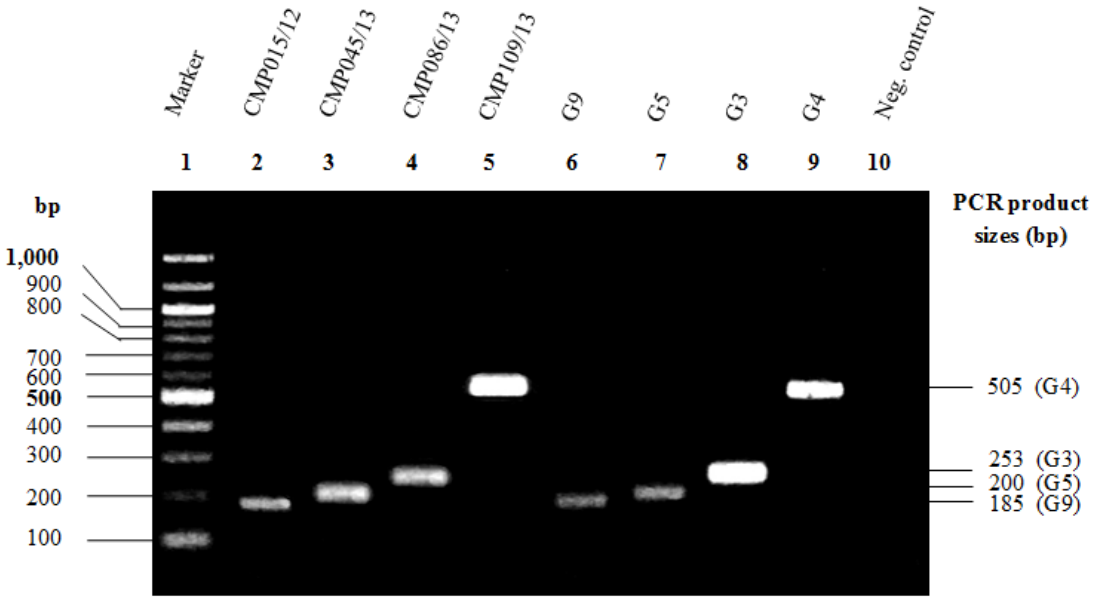

Figure 5.3 Agarose gel electrophoresis demonstrated the PCR product sizes of G genotypes (G9, G5, G3, and G4) of porcine rotaviruses in comparison with the reference strains. Lane 1 is 100 bp Plus DNA Ladder marker. Lanes 2-5 are test samples that were positive for G9, G5, G3, and G4 with the PCR product sizes of 185, 200, 253, and 505 bp, respectively. Lanes 6-9 are reference strains of G9, G5, G3, and G4. Lane 10 is a negative control reaction in which the DNA template was omitted. The molecular sizes of marker are shown on the left and the expected fragment length of each G genotype is shown on the right sides of the gel.

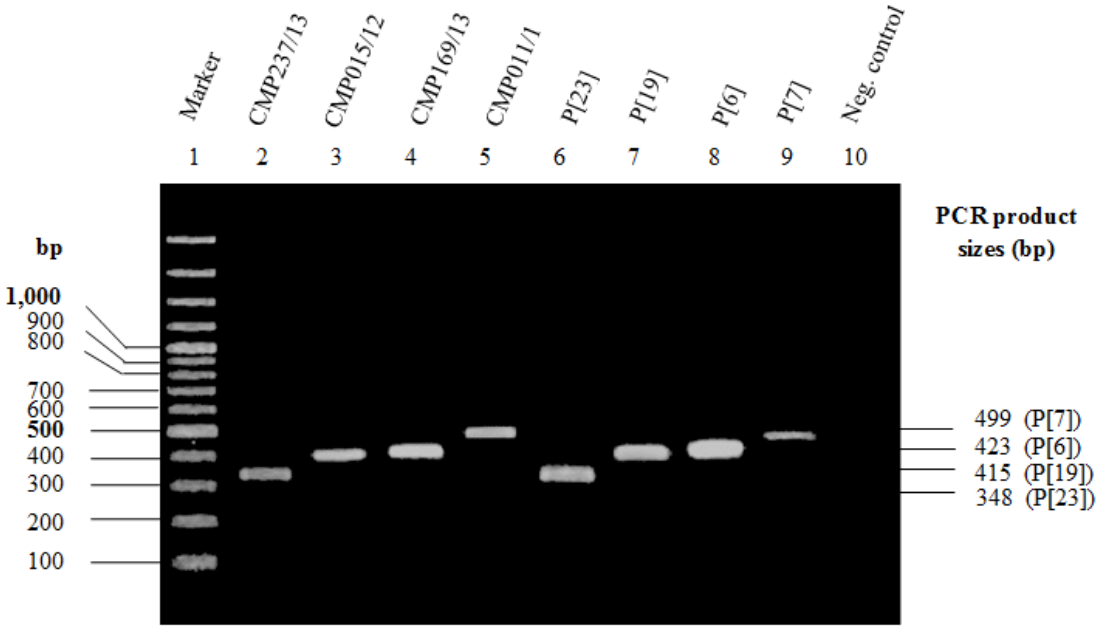

Figure 5.4 Agarose gel electrophoresis demonstrated the PCR product sizes of P genotypes (P[23], P[19], P[6], and P[7]) of porcine rotaviruses in comparison with the reference strains. Lane 1 is 100 bp Plus DNA Ladder marker. Lanes 2-5 are test samples that were positive for P[23], P[19], P[6], and P[7] with the PCR product sizes of 348, 415, 423, and 499 bp, respectively. Lanes 6-9 are reference strains of P[23], P[19], P[6], and P[7]. Lane 10 is a negative control reaction in which the DNA template was omitted. The molecular sizes of marker are shown on the left and the expected fragment length of each P genotype is shown on the right sides of the gel.

Table 5.1 The G and P genotypes of human group A rotaviruses as determined by RT-multiplex PCR

Number of human RV positive/ total specimens tested	G-genotypes (%)						P-genotypes (%)			
	G1	G2	G3	G8	G9	NT[a]	P[4]	P[8]	P[4]+P[8]	NT[b]
137/401	30 (21.9)	22 (16.1)	69 (50.3)	3 (2.2)	4 (2.9)	9 (6.6)	25 (18.2)	109 (79.6)	1 (0.7)	2 (1.5)

Table 5.2 The G and P genotypes of porcine group A rotaviruses as determined by RT-multiplex PCR

Number of porcine RV positive/ total specimens tested	G-genotypes (%)					P-genotypes (%)			
	G3	G4	G5	G9	NT[a]	P[6]	P[7]	P[19]	NT[b]
113/491	17 (15.0)	63 (55.8)	12 (10.6)	6 (5.3)	15 (13.3)	17 (15.0)	4 (3.5)	5 (4.4)	87 (77.1)

NT[a], G genotype could not be identified by RT-multiplex PCR

NT[b], P genotype could not be identified by RT-multiplex PCR

5.2.2 Identification of P genotypes of porcine rotaviruses by P[13] specific primer

In the present study, there were 87 out of 113 samples of porcine rotaviruses in which P genotype remained unidentified by using RT-multiplex PCR of group A rotaviruses in piglets with diarrhea. Therefore, 10 out of 87 representative strains of P nontypeable were selected and subjected further to nucleotide sequencing. It was interesting to note that 6 and 4 out of 10 isolates were identified as P[13] and P[23] genotypes, respectively. The P genotypes of porcine rotavirus identified by nucleotide sequence analysis are shown in Table 5.3.

Table 5.3 The representative of P genotypes of nontypeable porcine rotavirus strains identified by nucleotide sequence analysis

Porcine rotavirus strains (n=10)	P genotypes
CMP-007-11	P[13]
CMP-008-11	P[13]
CMP-013-11	P[13]
CMP-029-11	P[23]
CMP-031-11	P[23]
CMP-032-11	P[23]
CMP-033-11	P[23]
CMP-048-11	P[13]
CMP-050-11	P[13]
CMP-001-12	P[13]

In the previous study, P[13] genotype was a common genotype in pigs and the epidemiological surveillance in Thailand between 2002 to 2003 demonstrated that P[13] genotype was also detected as a major genotype (Chan-it et al., 2008). In this study, there were 87 isolates in which their P-genotypes were P nontypeable by RT-multiplex PCR while the random sampling of 10 representative strains of P nontypeable were identified as P[13] and P[23] genotype by nucleotide sequencing. Therefore, the specific primer for P[13] genotype was designed in this study while specific primer for P[23] genotype has already been designed in the previous study (Saikruang et al., 2013). The specific primer for P[13] and P[23] were used for P genotyping of the remaining P nontypeable strains. The newly designed primer for P[13] is shown in Figure 5.5 and the examples of PCR products of P[13] genotype in agarose gel electrophoresis are shown in Figure 5.6. By using specific primers for P[13] and P[23] genotype demonstrated that 40 out of 77 isolates of P nontypeable strains turned out to be P[13] genotype and 35 out of 77 isolates as P[23] genotype. The P genotypes of nontypeable porcine rotavirus strains identified by specific primer for P[13] and P[23] genotypes are summarized in Table 5.4.

Table 5.4 The P genotypes of nontypeable porcine rotavirus strains identified by specific primer for P[13] and P[23] genotypes

Number of nontypeable porcine RV/ total specimens tested	**G-genotypes (%)**		
	P[13]	**P[23]**	**NT[a]**
77/113	40 (51.9%)	35 (45.5%)	2 (2.6%)

NT[a], P genotype could not be identified by RT-multiplex PCR

Molecular characterization of P nontypeable by multiplex PCR using type-specific primers of two isolates remained could not be identified. Therefore, these two strains were further characterized by nucleotide sequence analysis.

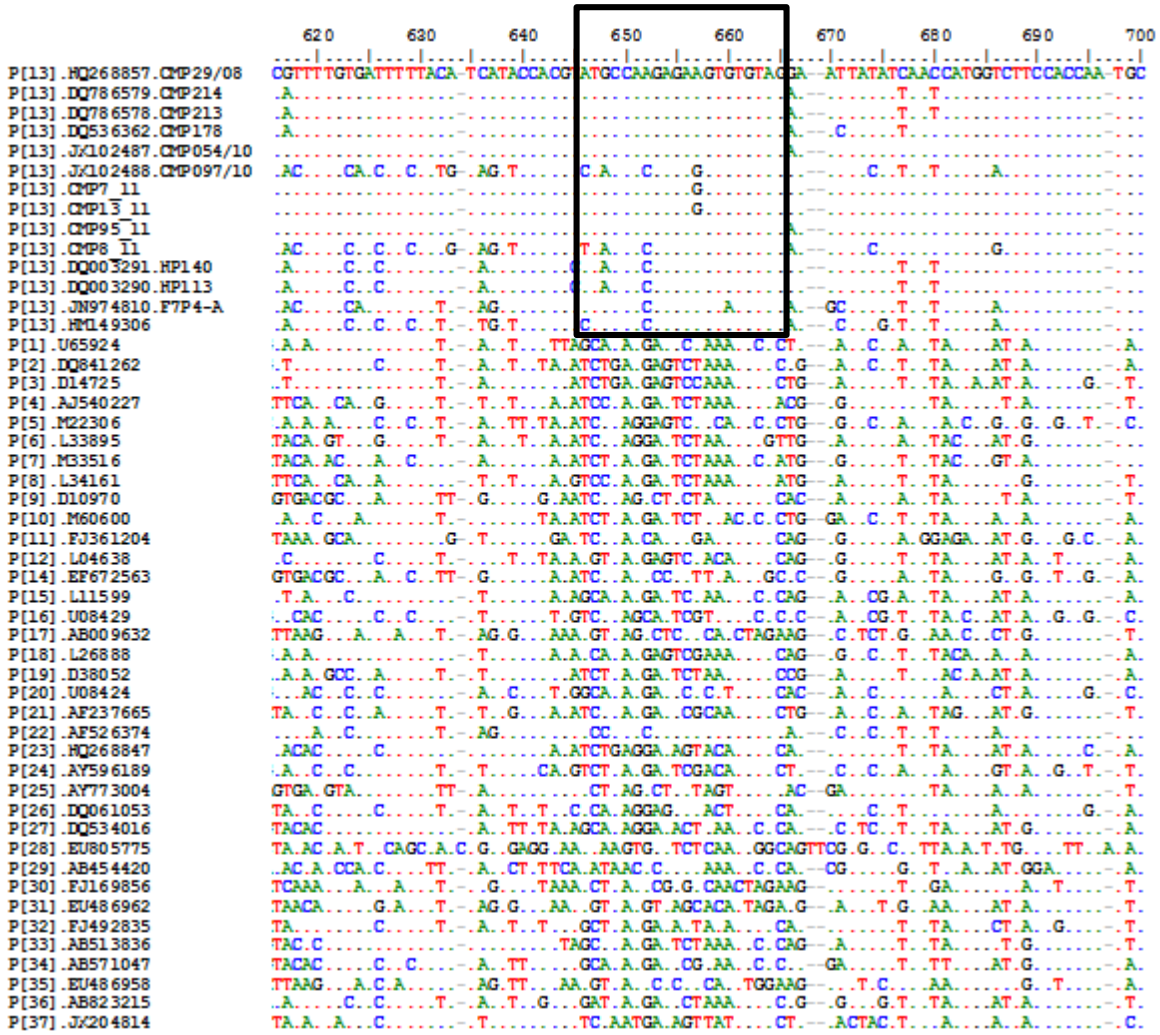

Figure 5.5 The newly designed primer for P[13], namely P[13]F. The VP4 nucleotide sequences of P[13] porcine rotaviruses detected in this study and sequences of other P[13] reference strains were aligned using the ClustalX and BioEdit program. The region which was highly conserved among P[13] strains but divergent from other P-genotypes was selected as a target primer sequence, nucleotides (nt) 646 to 665 of the VP4 gene. The oligonucleotide sequence of the primer is as follows: 5'- HTRCCAMGAGARGTRTGTAG -3'.

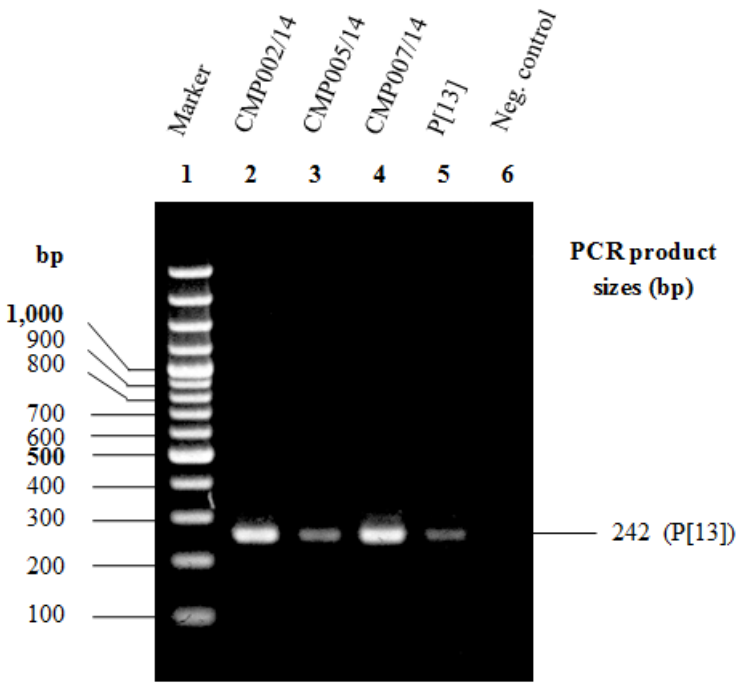

Figure 5.6 Agarose gel electrophoresis demonstrated the PCR product sizes of P[13] genotype in comparison with the P[13] reference strain of porcine rotavirus. Lane 1 is 100 bp Plus DNA Ladder marker. Lanes 2-4 are test samples that were positive for P[13] which showed the PCR product sizes of 242 bp. Lanes 5 is the reference strain of P[13]. Lane 6 is a negative control reaction in which the DNA template was omitted. The molecular sizes of marker are shown on the left and the expected fragment length of P[13] is shown on the right sides of the gel.

5.2.3 Identification of G and P genotypes of nontypeable strains by nucleotide sequence analysis

For human rotavirus, G and P nontypeable strains were 9 and 2 out of 137 isolates, respectively. For porcine rotavirus, G and P nontypeable strains were 15 and 2 of 113 isolates, respectively. For identification of G and P nontypeable strains of both human and porcine rotaviruses, the VP7 and VP4 genes were amplified by using consensus primers or alternative primers specific for each gene. The partial VP7 gene of human and porcine rotaviruses of G nontypeable strains were amplified by consensus Beg9 (forward primer) in combination with the reverse primer VP7-1' or End9(s) to generate the PCR product size of 395 or 941 bp, respectively. The partial VP4 gene of human and porcine rotaviruses of P nontypeable strains were amplified by consensus Con3 (forward primer) in combination with the reverse primer Con2 to generate the PCR product size of 877 bp. The PCR product of partial VP7 and VP4 genes were purified and subjected further to nucleotide sequencing and nucleotide sequence analysis. The obtained sequences were searched for a close genetic relationship with reference sequences available in GenBank database by using NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The G and P genotypes of nontypeable human rotavirus strains identified by nucleotide sequence analysis are listed in Table 5.5 and 5.6, respectively. The G genotypes of nontypeable porcine rotavirus strains identified by nucleotide sequence analysis are listed in Table 5.7. However, there were 2 strains of porcine rotavirus of which their P genotypes remained unidentified because they could not be amplified by those primers.

Table 5.5 The G genotypes of nontypeable human rotavirus strains as identified by nucleotide sequence analysis

Human rotavirus strains (n=9)	G genotypes
CMH-N023-13	G1
CMH-N066-13	G1
CMH-N078-13	G1
CMH-N082-13	G1
CMH-N085-13	G1
CMH-N086-13	G1
CMH-N091-13	G1
CMH-S106-13	G8
CMH-N067-14	G1

Table 5.6 The P genotypes of nontypeable human rotavirus strains as identified by nucleotide sequence analysis

Human rotavirus strains (n=2)	P genotypes
CMH-N078-13	P[8]
CMH-S070-13	P[19]

Table 5.7 The G genotypes of nontypeable porcine rotavirus strains as identified by nucleotide sequence analysis

Porcine rotavirus strains (n=15)	G genotypes
CMP-008-11	G5
CMP-034-11	G5
CMP-036-11	G5
CMP-048-11	G5
CMP-050-11	G5
CMP-001-12	G5
CMP-002-12	G5
CMP-018-12	G3
CMP-002-13	G11
CMP-015-13	G11
CMP-217-13	G5
CMP-016-14	G5
CMP-048-14	G5
CMP-135-14	G4
CMP-138-14	G5

5.2.4 Distribution of G and P genotype combinations of human and porcine rotaviruses

A total of 137 and 113 of positive human and porcine group A rotaviruses were characterized for their G and P genotypes by RT-multiplex PCR and by nucleotide sequencing, respectively. Among these, five different G genotypes including G1, G2, G3, G8, and G9 were identified in this study. For P genotypes, P[4], P[8], and P[19] were detected. Moreover, one sample was identified as mixed-infection of P[8] and P[4] genotypes. For G and P genotypes of porcine rotaviruses, five different G genotypes, including G3, G4, G5, G9, and G11 and five different P genotypes, including P[6], P[7], P[13], P[19], and P[23], were detected. However, there were 2 strains of which their P genotypes remained unidentified.

For human group A rotavirus, G3 was the most prevalent genotype (69 of 137; 50.3%) followed by G1 (38 of 137; 27.8%), G2 (22 of 137; 16.1%), G9 and G8 each of 2.9% (4 each of 137), respectively. For P genotypes, P[8] was the most prevalent genotype (110 of 137; 80.3%), followed by P[4] (25 of 137; 18.3%), mix-infection of P[8] and P[4] (1 of 137; 0.7%), and P[19] (1 of 137; 0.7%). For G-P genotype combinations, nine different G-P combinations were detected in the present study. The G3P[8] was the most predominant genotypes with the prevalence of 49.6% (68 of 137), followed by G1P[8] (32 of 137; 23.4%), G2P[4] (19 of 137; 13.9%), G1P[4] (6 of 137; 4.4%), G8P[8] (4 of 137; 2.9%), G2P[8] and G9P[8] each of 2.2% (3 of 137), and mixed-infection of G3 in combination with P[8] and P[4] (1 each of 137; 0.7%). Interestingly, one uncommon strain of human rotavirus G9P[19] (1 of 137; 0.7%) was detected in this study. Distribution of G and P genotype combinations of human rotaviruses are summarized in Table 5.8.

For porcine group A rotavirus, G4 was the most predominant genotype with the prevalence of 56.6% (64 of 113) while G3, G5, G9, and G11 were

detected with lower frequency of 15.9% (18 of 113), 20.3% (23 of 113), 5.4% (6 of 113), and 1.8% (2 of 113), respectively. For P genotypes, P[13] was identified as the most predominant genotype (46 of 113; 40.6%), followed by P[23] (39 of 113; 34.4%), P[6] (17 of 113; 15.1%), P[19] (5 of 113; 4.5%), and P[7] (4 of 113; 3.6%), whereas two samples were P-nontypeable. For the combinations of G and P genotypes in porcine rotaviruses, 16 different combinations were detected in this study. The G4P[13] was the most predominant strain with the prevalence of 29.2% (33 of 113), followed by G4P[23] (16 of 113; 14.1%), G5P[23] (13 of 113; 11.5%), G4P[6] (11 of 113; 9.7%), G3P[23] (8 of 113; 7.0%), G5P[13] (7 of 113; 6.1%), G3P[13] (5 of 113; 4.4%), G3P[6] and G5P[6] each of 2.7% (3 of 113). Moreover, the other G-P combinations detected at the prevalence lower than 2.0% per individual combinations were divergent, including G3P[19], G4P[7], G9P[19], G9P[23], G9P[7], G4P[19], and G11P[13]. In addition, two porcine rotavirus strains, which the P genotype could not be identified, one was found in combination with G4 while the other one in combination with G11 genotype. Distribution of G and P genotype combinations of porcine rotaviruses are summarized in Table 5.9.

Table 5.8 Distribution of G and P genotype combinations of human rotaviruses

G genotypes	P genotypes (%)				Total (%)
	P[4]	P[8]	P[4]+P[8]	P[19]	
G1	6 (4.4)	32 (23.4)	-	-	38 (27.8)
G2	19 (13.9)	3 (2.2)	-	-	22 (16.1)
G3	-	68 (49.6)	1 (0.7)	-	69 (50.3)
G8	-	4 (2.9)	-	-	4 (2.9)
G9	-	3 (2.2)	-	1 (0.7)	4 (2.9)
Total (%)	25 (18.3)	110 (80.3)	1 (0.7)	1 (0.7)	137 (100)

Table 5.9 Distribution of G and P genotype combinations of porcine rotaviruses

G genotypes	P genotypes (%)						Total (%)
	P[6]	P[7]	P[13]	P[19]	P[23]	ND[a]	
G3	3 (2.7)	-	5 (4.4)	2 (1.8)	8 (7.0)	-	18 (15.9)
G4	11 (9.7)	2 (1.8)	33 (29.2)	1 (0.9)	16 (14.1)	1 (0.9)	64 (56.6)
G5	3 (2.7)	-	7 (6.1)	-	13 (11.5)		23 (20.3)
G9	-	2 (1.8)	-	2 (1.8)	2 (1.8)	-	6 (5.4)
G11	-	-	1 (0.9)	-	-	1 (0.9)	2 (1.8)
Total (%)	17 (15.1)	4 (3.6)	46 (40.6)	5 (4.5)	39 (34.4)	2 (1.8)	113 (100)

ND[a]; P genotype could not be identified.

5.2.5 Phylogenetic analysis of VP7 and VP4 gene sequences of human and porcine rotaviruses

1) Phylogenetic analysis of VP7 gene sequences

The phylogenetic analyses were performed using the partial nucleotide sequences of VP7 gene from 39 representative strains of group A rotaviruses detected in this study. The representative strains divided into 17 strains of human rotaviruses and 22 strains of porcine rotaviruses. The VP7 sequences of these strains were determined and analysed by comparing with those of rotavirus reference strains of all 27 G genotypes obtained from the Genbank database using BLAST server. Nucleotide sequence identity at 80% was used as the cut-off point for rotavirus of the same G genotype (Matthijnssens et al., 2008). Analysis of VP7 nucleotide sequences confirmed the results of G genotyping obtained by multiplex RT-PCR of those strains detected in the present study.

The phylogenetic analysis of 39 representative strains described in this study revealed eight different G genotypes including G1, G2, G3, G4, G5, G8, G9, and G11 (Figure 5.7 A and Figure 5.7 B). The VP7 genes of G1 genotype (Figure 5.7 A) of human rotavirus representative strains were classified into 3 clusters. One of the representative strains of G1 (CMH-N067-14) shared high level of nucleotide sequence identity (97.3%) with AU007 (AB081799) strain which was isolated previously from Japan. Second cluster, two strains of G1, CMH-N023-13 and CMH-N066-13, were closely related to OH3625/2012 (AB796448) strain which was isolated from Japan in 2012 at 84.7-98.8%. In addition, five strains of G1, CMH-N078-13, CMH-N082-13, CMH-N085-13, CMH-N086-13, and CMH-N091-13, were most

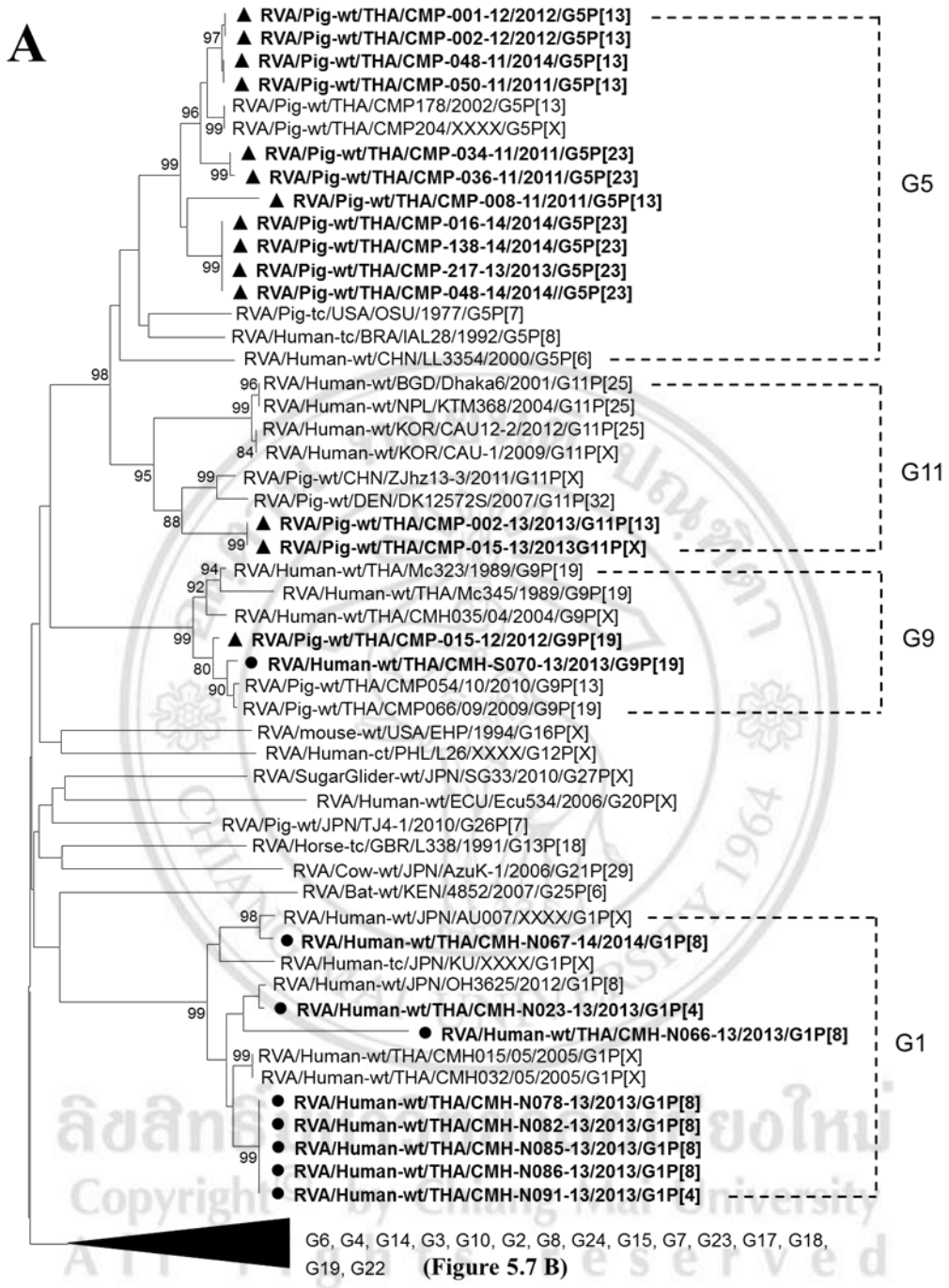

Figure 5.7 A The phylogenetic analysis of partial nucleotide sequences of VP7 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

Figure 5.7 B The phylogenetic analysis of partial nucleotide sequences of VP7 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

closely related to CMH015/05 (GU288622) and CMH032/05 (GU288623), which were previously isolated from Chiang Mai in 2005 at 95.9% nucleotide sequence identity.

The VP7 gene of three human rotavirus G2 representative strains (Figure 5.7 B) isolated in the present study were divided into 2 groups. One strain of G2, CMH-S084-13, was more closely related with human G2 reference strains previously isolated in Thailand CMH134/05 (GU288625) and in Bangladesh MMC6 (EU839923) in 2005 at 95.9-96.2% nucleotide sequence identity. However, the other two G2 strains (CMH-S055-13 and CMH-N106-13) were less closely related to CMH-S084-13 strain at 91.0% nucleotide sequence identity. In addition, when comparing the nucleotide sequences of three strains of G2 detected in this study (CMH-S055-13, CMH-S084-13, and CMH-N106-13) with those of human rotavirus G2 reference strains CMH008/05 (GU288621) and J-4777 (DQ904510), revealed the nucleotide sequence identities ranging from 86.9-92.1%.

Phylogenetic analysis of the VP7 gene of G3 representative strains (Figure 5.7 B) were classified into 2 lineages. The first lineage, comprised CMH-S016-13, CMH-N169-13, and CMH-N010-14 isolated from human with diarrhea in the present study, were similar to other human rotavirus strains previously identified in Thailand CMH055/07 (JQ043273), in Japan AU-1 (D86271), and in China L621 (EU708588) with the nucleotide sequence identities ranging from 88.4-100.0%. The second lineage consisted of porcine rotavirus strains CMP012-12, CMP-018-12, CMP-088-13, and CMP-161-13 were closely clustered together with other two porcine rotaviruses previously isolated in Chiang Mai in 2000 [CMP039 (AY707788) and CMP213 (DQ786576)] with nucleotide sequence identity of 90.7-98.5%. However, when comparing G3 representative strains of human with porcine rotaviruses detected in the present study, the nucleotide

sequence identities were ranging from 81.4-87.3%. These results implied that G3 strains of human were distantly related to G3 strains of piglets with diarrhea detected in the same study.

The VP7 gene of G4 porcine representative strains, which were in combination with different P genotypes, CMP-007-11 and CMP-107-14 shared the greatest homology with porcine rotavirus CMP77 (DQ683521), which was previously isolated in the same geological area, at 94.7-96.2% nucleotide sequence identity (Figure 5.7 B). In addition, CMP-071-11 and CMP-196-13 strains also clustered closely together with human G4 reference strains previously isolated in China E931 (EU708602) and in Vietnam HV157 (FR822302) in 2008 and 2011, respectively, at 95.9-97.3% nucleotide sequence identity. The results demonstrated that G4 rotavirus was circulating in both humans and piglets with diarrhea.

Phylogenetic analysis of 11 representative strains of G5 porcine rotaviruses (CMP-008-11, CMP-034-11, CMP-036-11, CMP-048-11, CMP-050-11, CMP-001-12, CMP-002-12, CMP-217-13, CMP-016-14, CMP-048-14, and CMP-138-14) detected in the present study revealed that they were closely related to CMP178 (DQ515961) and CMP204 (DQ683523) reference strains previously isolated in Chiang Mai, Thailand with nucleotide sequence identities ranging from 89.2-97.0% (Figure 5.7 A). These data suggested that G5 are the common genotypes of porcine rotaviruses circulating in Chiang Mai area.

In the phylogenetic tree of VP7 gene which contained G8 human rotavirus representative strains found in this study (CMH-S106-13 and CMH-N069-13) showed more than 93.3% nucleotide sequence identity to cow rotavirus reference strains, A5 (D01054), which was isolated in Thailand, 86 (GU984762) and 68 (GU984760) which were isolated from India in 2007, and UP (JX442786) which was isolated in

India in 2010 (Figure 5.7 B). It was interesting to note that these two rotavirus strains showed the nucleotide sequence identities (95.9-96.6%) with Taiwan human strain 04-97s379 (JX156636) as well as the cow-like human rotavirus which was previously identified in 2008.

The VP7 gene of G11 strains CMP-002-13 and CMP-015-13 showed the highest nucleotide sequence identity at 88.4-89.9% with porcine rotavirus strains ZJhz13-3 (JX498966) and DK12572S (JN410645), which were previously isolated in China (2011) and Denmark (2007), respectively (Figure 5.7 A). These G11 strains were lesser related with other human rotavirus strains [AY773003 (Dhaka6), KTM368 (GU199497), CAU12-2 (KC140587), and CAU-1 (HQ198807)], ranging from 82.8-83.2% nucleotide sequence identity.

The phylogenetic tree of G9 human rotavirus representative strain CMH-S070-13 identified in the present study was more closely related with porcine rotavirus strain CMP-015-12 with highest nucleotide sequence identity at 97.7% (Figure 5.7 A). Both of them showed 96.2-98.5% nucleotide sequence identity to porcine rotavirus strains CMP054/10 (JX102482) and CMP066/09 (JX102481) previously detected in Chiang Mai, Thailand. These findings imply that interspecies transmission among human and porcine rotaviruses might have been occurred in nature. The G9 human and porcine rotavirus strains detected in this study were characterized further of their full-length VP7, VP4, VP6, NSP4, and NSP5 genes, and the results were shown in section 5.2.6.

2) Phylogenetic analysis of VP4 gene sequences

The phylogenetic analyses were performed using the partial nucleotide sequences of VP4 gene from 17 representative strains of group A rotaviruses detected in the present study. The representative strains

divided into human rotavirus 9 strains and porcine rotavirus 8 strains. The VP4 nucleotide sequences of these strains were determined and analysed by comparing with those of rotavirus reference strains of all 37 P genotypes obtained from the Genbank database using BLAST server. Nucleotide sequence identity at 80% was used as the cut-off point for rotavirus of the same P genotype (Matthijnssens et al., 2008). Analysis of VP4 nucleotide sequences confirmed the results of P genotypes obtained by multiplex RT-PCR.

The phylogenetic analysis demonstrated that 17 representative strains described in this study were classified into 4 different P genotypes including P[4], P[8], P[13], and P[19] as shown in Figure 5.8 A and Figure 5.8 B. In the phylogenetic tree of VP4 gene which contained P[4] human rotavirus representative strains detected in the present study (CMH-S055-13 and CMH-N108-13) showed the highest nucleotide sequence identity at 96.1-98.4% with human rotavirus strains LB2744 (HM467941), which was previously isolated in USA in 2005 (Figure 5.8 B). In addition, both strains were similar to other human rotavirus strains previously identified in China TB-Chen (AY787644), in Japan KUN (AB733131), in Philippines L26 (EF672591), and in USA DS-1 (AJ540227) with the nucleotide sequence identities ranging from 89.4-96.3%.

Phylogenetic analysis of P[8] human representative strains (CMH-S084-13, CMH-N069-13, CMH-N078-13, CMH-N046-14, CMH-N047-14, CMH-N048-14), which were in combination with different G genotypes, revealed that they shared high level of nucleotide sequence identity with reference strains, RMC437 (AY603158), RMC100 (AF531911), CK00011 (JF490189), DRC88 (DQ005111), CK00088 (JX027876) ranging from 96.3-99.7% (Figure 5.8 B). The results implied that P[8] was the most common P genotype circulating in humans in Chiang Mai.

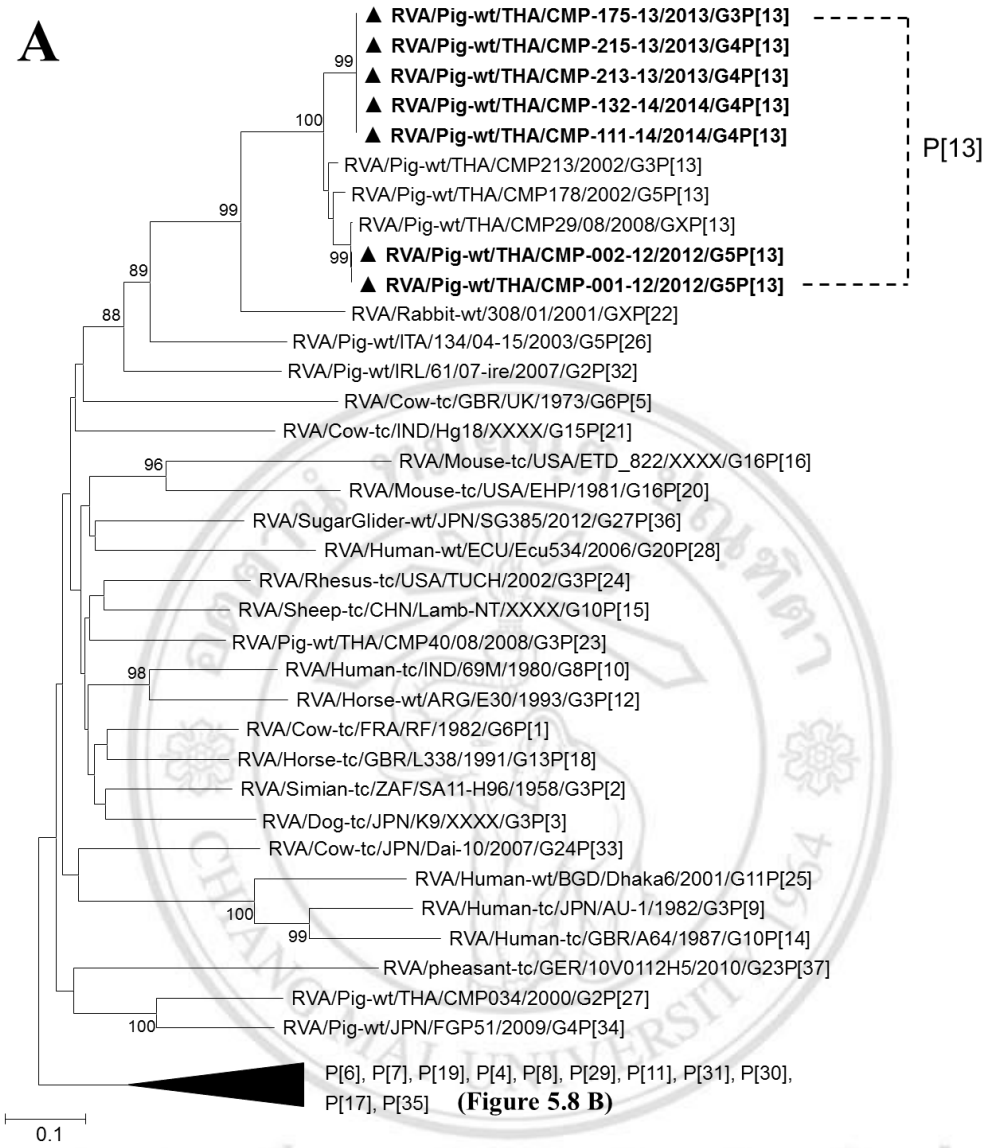

Figure 5.8 A The phylogenetic analysis of partial nucleotide sequences of VP4 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

Figure 5.8 B The phylogenetic analysis of partial nucleotide sequences of VP4 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

The VP4 gene of 7 porcine rotavirus P[13] representative strains isolated in the present study (CMP-001-12, CMP-002-12, CMP-175-13, CMP-213-13, CMP-215-13, CMP-111-14, and CMP-132-14) were divided into 2 groups. Two strains of P[13], CMP-001-12 and CMP-002-12 , formed exclusive cluster with P[13] porcine rotaviruses CMP213 (DQ786578), CMP178 (DQ536362), and CMP29/08 (HQ268857) previously identified in Chiang Mai in 2002 and 2008 at 96.4-99.7% nucleotide sequence identity (Figure 5.8 A). In addition, the other 5 strains of P[13] (CMP-175-13, CMP-213-13, CMP-215-13, CMP-111-14, and CMP-132-14) clustered in a monophyletic branch separated from P[13] reference strains. These 5 strains of P[13] were less closely related with CMP-001-12 and CMP-002-12 strains at 93.0% nucleotide sequence identity. The data suggested that P[13] were the common P genotype circulating in pigs in Chiang Mai area during the study period.

For VP4 gene of P[19] representative strains detected in the present study, human rotavirus strain CMH-S070-13 was most closely related to porcine rotavirus strain CMP-015-12 at 99.3% nucleotide sequence identity. In addition, when comparing the nucleotide sequence identity of both strains with those of porcine rotavirus reference strains, CMP138/10 (JX102490) and CMP066/09 (JX102481) previously detected in Chiang Mai, Thailand, showed nucleotide sequence identity at 97.6-98.4%. These results indicated that interspecies transmission among human and porcine rotaviruses might have been occurred in nature. The P[19] human and porcine rotavirus strains detected in the present study were characterized further for their full-length VP7, VP4, VP6, NSP4, and NSP5 genes, and the results were shown in section 5.2.6.

5.2.6 Genetic characterization and phylogenetic analysis of G9P[19] unusual human and porcine rotavirus strains

Human and porcine rotavirus unusual strains G9P[19] genotype were selected for molecular and phylogenetic analysis of full-length VP7, VP4, VP6, NSP4, and NSP5 gene segments, by nucleotide sequencing. Human rotavirus G9P[19] (CMH-S070-13) was isolated from fecal specimen of a child (3-year-old girl) admitted to the hospital with acute gastroenteritis in Chiang Mai in 2013, while porcine rotavirus G9P[19] (CMP-015-12) was isolated from a piglet with diarrhea in Lamphun province in 2012.

1) Genetic characterization and phylogenetic analysis of VP7 gene

The phylogenetic analysis of VP7 was performed using full-length nucleotide sequences from G9 strains of human and porcine rotaviruses detected in this study. The nucleotide sequences of these strains were determined and analyzed by comparing with those of rotavirus reference strains of all 27 G genotypes obtained from the Genbank database using BLAST server.

The VP7 gene of human rotavirus strain CMH-S070-13 shared high level of nucleotide sequence identity of 98.8% with those of porcine rotavirus strain CMP-015-12. Both strains showed most closely related to porcine rotavirus strains, CMP066/09 (JX102481) and CMP054/10 (JX102482), which were previously detected in the Chiang Mai in 2009 and 2010 (Saikruang et al., 2013) at 97.9-98.7% nucleotide sequence identity. The VP7 gene of these G9 strains in comparison with reference strains including porcine-like human rotaviruses G9 strains RMC321 (AF501578) isolated from India, Mc345 (D38055), and Mc323 (D38053), which were isolated from Chiang Mai, and also human G9 rotaviruses previously isolated in the same area (Chiang Mai) [CMH022/03 (EF199738) and CMH035/04

(EF199728)] showed closely related nucleotide sequence identity ranging from 92.8-94.4%. In addition, the sequences of these G9 strains were also closely related to those of G9 rotaviruses isolated from piglets with diarrhea in Japan and Korea, JP35-7 (AB176683), Hokkaido-14 (AB176677), and PRG9121 (JF796739), with a lesser degree of nucleotide sequence identity ranging from 90.0-90.3%. The data imply that interspecies transmission between human and porcine rotaviruses might have been occurred in nature. The phylogenetic tree of full-length nucleotide sequences of the VP7 gene is shown in Figure 5.9.

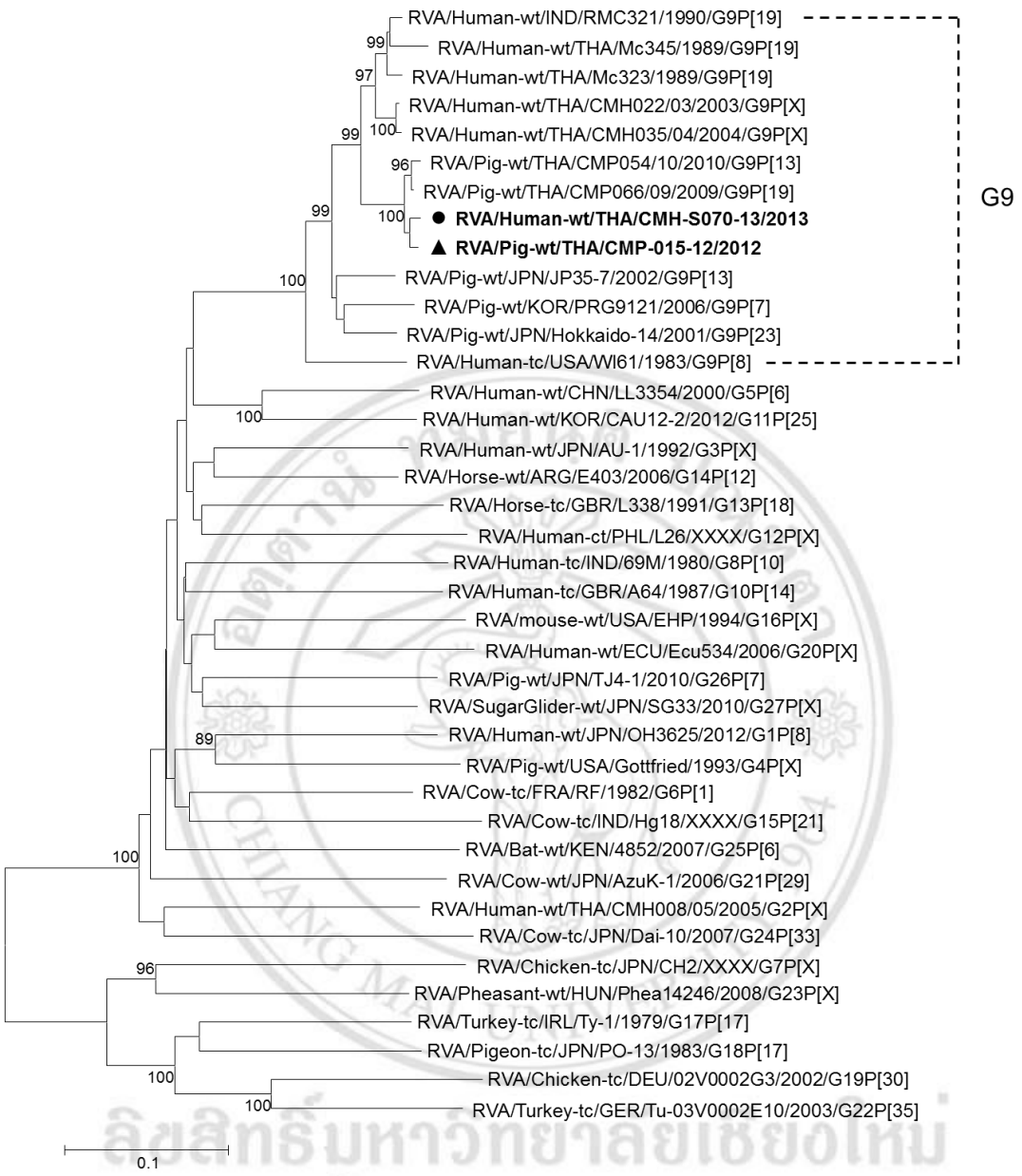

Figure 5.9 The phylogenetic analysis of full-length nucleotide sequences of VP7 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

2) Genetic characterization and phylogenetic analysis of VP4 gene

The phylogenetic analysis of VP4 was performed using full-length nucleotide sequences from P[19] strains of human and porcine rotaviruses detected in this study. The nucleotide sequences of these strains were determined and analyzed by comparing with those of rotavirus reference strains of all 37 P genotypes obtained from the Genbank database using BLAST server.

The VP4 sequence of human rotavirus strain CMH-S070-13 shared high level of nucleotide sequence identity of 99.5% with porcine rotavirus strain CMP-015-12 detected in this study. In addition, both strains were most closely related to CMP138/10 (JX102490) and CMP066/09 (JX102481) strains previously detected in the same geological area with the nucleotide sequence identity of 97.8-98.1% while the percent nucleotide sequence identities were slightly decreased (93.6-96.6%) when comparing with the P[19] isolates from human [Mc345(D38054) and Mc323 (D38052)] and piglets [CMP029 (AY689219) and CMP099 (AY689208)] in Thailand and from human [RMC321 (AF523677), NIV929893 (DQ887060), and mani-97/06 (GQ240618)] from India. In fact, all of human P[19] rotaviruses reference strains have been proposed as the porcine-human reassortant strains because they possess many gene segments that are closely related to porcine rotaviruses (Urasawa et al., 1992). The data imply that VP4 gene of human P[19] strain CMH-S070-13 may derive from rotavirus of porcine origin. The phylogenetic tree of full-length nucleotide sequences of the VP7 gene is shown in Figure 5.10.

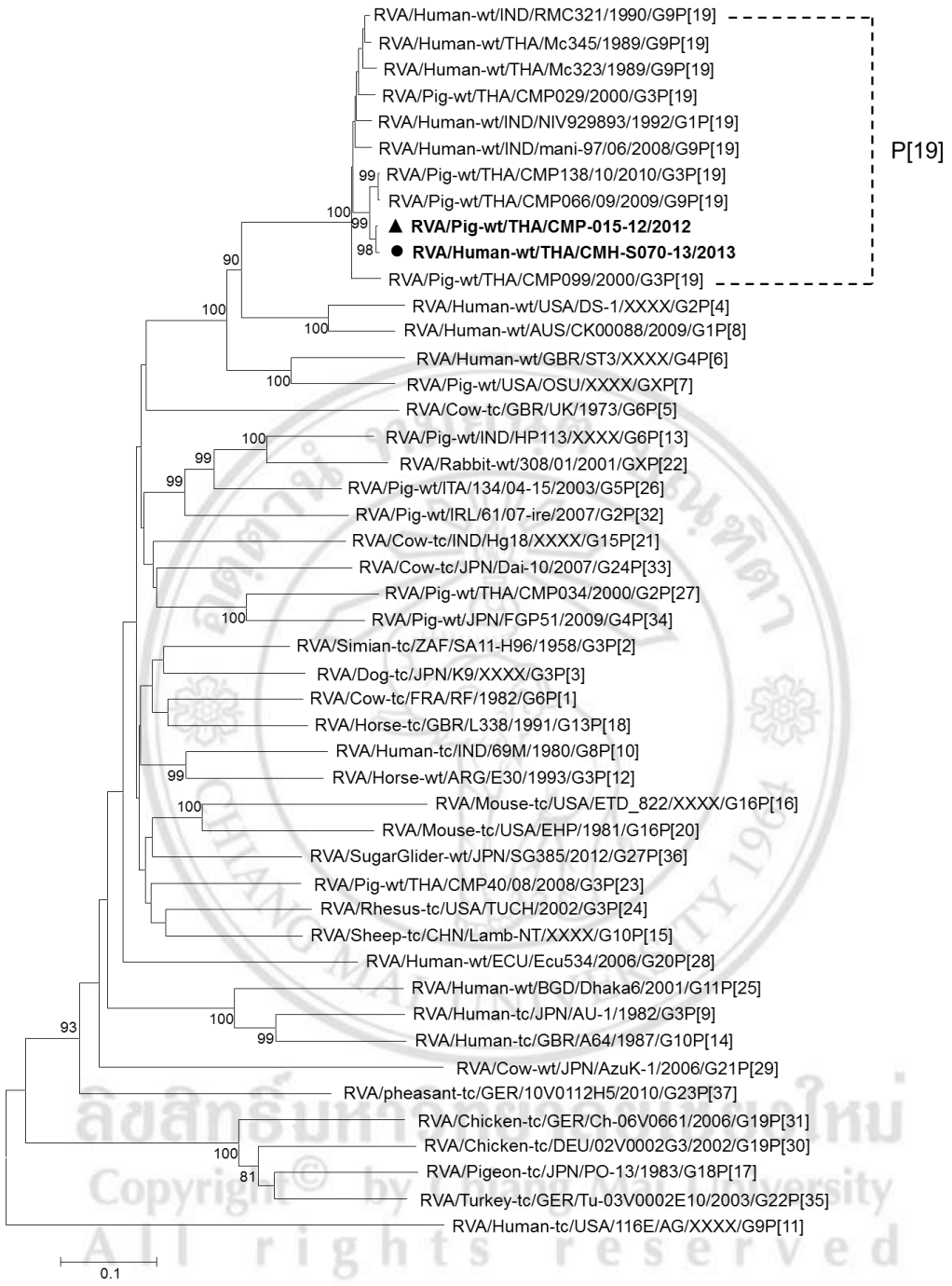

Figure 5.10 The phylogenetic analysis of full-length nucleotide sequences of VP4 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

3) Genetic characterization and phylogenetic analysis of VP6 gene

Based on a classification system of rotaviruses was recommended by the Rotavirus Classification Working Group in 2008. The VP6 gene of rotavirus detected in this study were assigned as I genotype by the cut-off value of VP6 nucleotide sequence identity of the same I genotype is at least 85% (Matthijnssens et al., 2008). The full-length nucleotide sequence of VP6 gene of G9P[19] human rotavirus (CMH-S070-13) detected in this study was compared with those of porcine rotavirus (CMP-015-12) detected in the same study. In addition, these strains were compared with those of the reference strains of all known 18 I genotypes available in NCBI Genbank database.

The phylogenetic tree of human CMH-S070-13 and porcine CMP-015-12 rotavirus strains belonged to the I5 genotype with the highest nucleotide sequence identity at 93.1% and showed a close genetic relationship to other porcine rotavirus I5 strains, CMP12/03 (EU372798), CMP31/01 (EU372786), CMP52/01 (EU372758), and CMP45/08 (HQ268858) which were previously isolated in Chiang Mai, Thailand at 92.1-97.9% nucleotide sequence identity. However, the nucleotide sequence of human rotavirus I5 (CMH-S070-13) was quite different from human I5 strains, LL3354 (EU330646) and RMC321 (AF531913). The results indicated that the VP6 gene of human rotavirus detected in the present study was more closely related to the porcine rotavirus than to human rotaviruses. The phylogenetic tree of full-length nucleotide sequences of VP6 gene is shown in Figure 5.11.

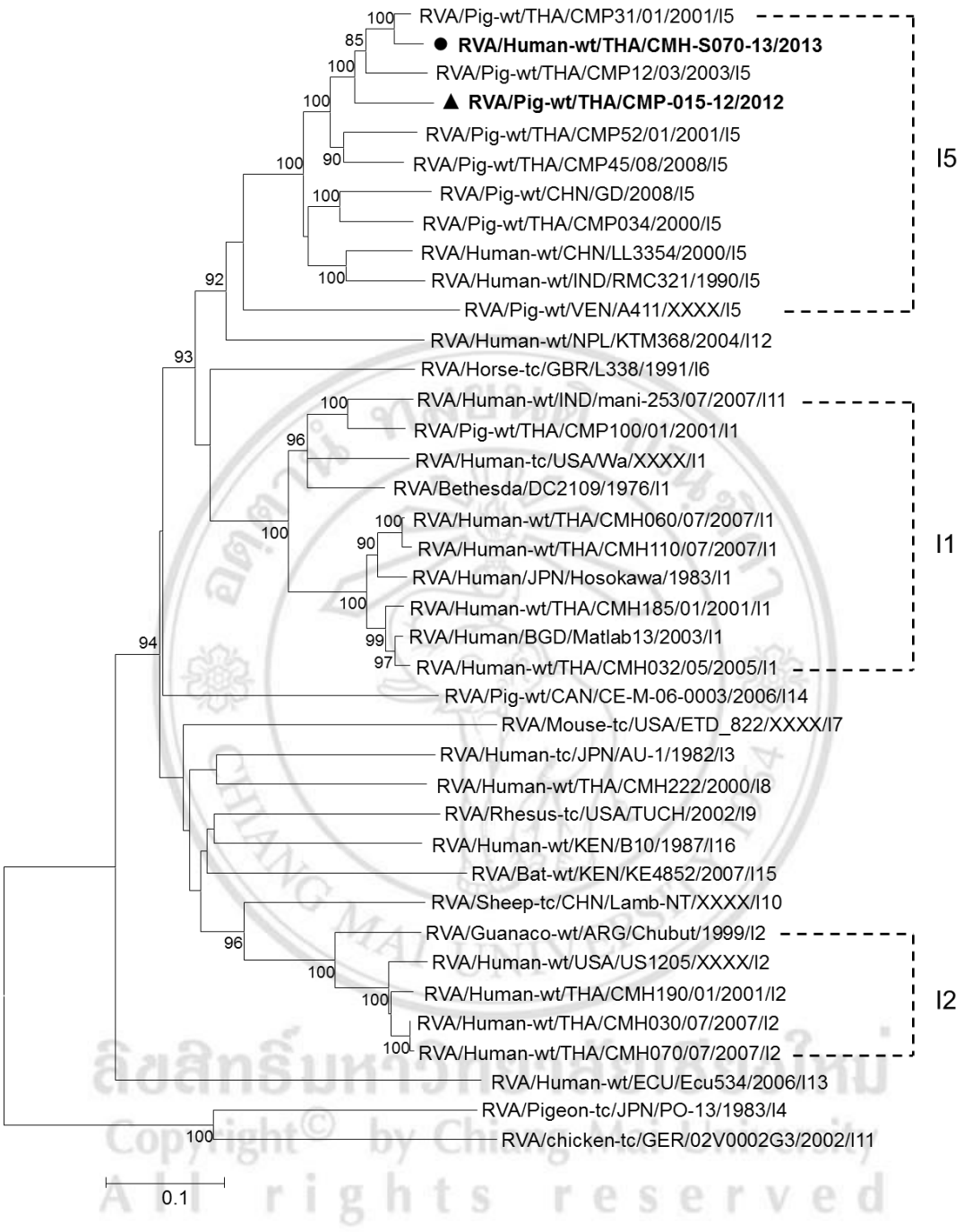

Figure 5.11 The phylogenetic analysis of full-length nucleotide sequences of VP6 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

4) Genetic characterization and phylogenetic analysis of NSP4 gene

Based on a classification system of rotaviruses NSP4 gene segment is assigned as E genotype by the cut-off value of NSP4 nucleotide sequence identity of the same E genotype is at least 85% (Matthijnssens et al., 2008). The full-length nucleotide sequence of NSP4 gene of G9P[19] human rotavirus (CMH-S070-13) was also compared with those of porcine rotavirus (CMP-015-12) detected in this study. In addition, these strains were compared with those of the reference strains of all known 15E genotypes available in NCBI Genbank database.

The phylogenetic tree of full-length NSP4 gene shown in Figure 5.12 clearly demonstrated that both human CMH-S070-13 and porcine CMP-015-12 rotavirus strains belonged to the E1 genotype and shared high level of nucleotide sequence identity at 93.8%. These strains clustered closely together with other Thai porcine E1 rotavirus strains, CMP40/08 (HQ268838) and CMP48/08 (HQ268841) which were previously isolated in the same geological area in 2008 at 93.6-97.8% nucleotide sequence identity. However, the E1 human strains, CMH146/05 (GU288650) and CMH014/07 (JQ043300), which were previously isolated in Chiang Mai, Thailand in 2005 and 2007, clustered in a separate branch distant from human rotavirus strain CMH-S070-13 detected in the present study with nucleotide sequence identity less than 86.9%.

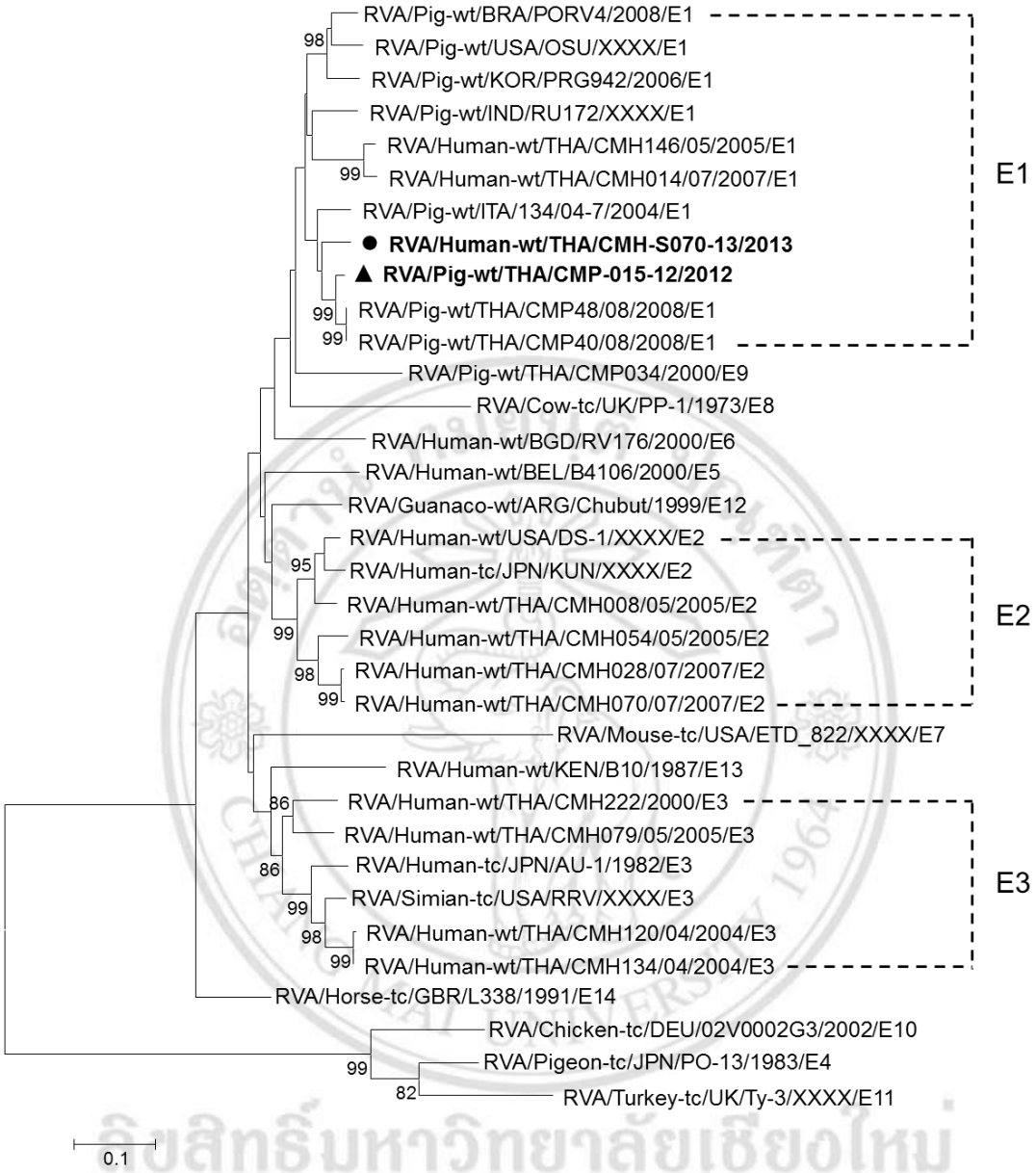

Figure 5.12 The phylogenetic analysis of full-length nucleotide sequences of NSP4 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.

5) Genetic characterization and phylogenetic analysis of NSP5 gene

Based on a classification system of rotaviruses, NSP5 gene segment is assigned as H genotype by the cut-off value of NSP5 nucleotide sequence identity of the same H genotype is at least 91% (Matthijnssens et al., 2008). The full-length nucleotide sequence of NSP5 gene of G9P[19] human rotavirus was compared with porcine rotavirus detected in this study. In addition, these strains were compared with those of the reference strains of all known 11H genotypes available in NCBI Genbank database.

The NSP5 nucleotide sequence analysis of human CMH-S070-13 and porcine CMP-015-12 rotavirus strains revealed that they belonged to the H1 genotype. The NSP5 gene of CMH-S070-13 strain showed the highest sequence identity with CMP-015-12 strain, with nucleotide sequence identity of 98.3%. Moreover, these strains of human rotaviruses were found to be more related to porcine H1 genotype strains [SB1A (EU169874), LS00008 (KJ659441), and ES51 (DQ189249)] with nucleotide sequence identity ranging from 98.3-99.4% than to human H1 genotype strains [Ryukyu-1120 (AB741659), BP271 (KF835969), KTM368 (GU199502), LB2719 (HM467916), and Wa (AB091726)] with nucleotide sequence identity ranging from 93.6-98.6%. The phylogenetic tree of full-length nucleotide sequences of the NSP5 gene is shown in Figure 5.13.

From the results of molecular and phylogenetic analysis of VP4, VP6, VP7, NSP4, and NSP5 gene segments of unusual rotavirus strains of G9P[19] human and porcine rotavirus indicated that they belong to G9-P[19]-I5-E1-H1 genotype constellation for the VP7-VP4-VP6-NSP4-NSP5 genes.

Figure 5.13 The phylogenetic analysis of full-length nucleotide sequences of NSP5 gene of selected human and porcine rotavirus strains. The tree was generated by using the neighbor-joining method in MEGA v5.05. The human and porcine rotavirus strains detected in the present study are presented in boldface with black dot and black triangle symbols, respectively. The scale bar indicates the branch length for 10% nucleotide difference.