CHAPTER 4

Materials and methods

4.1 Specimen collection

A total of 401 fecal specimens were collected from children hospitalized with diarrhea from two hospitals, (Nakorn Ping Hospital and Maharaj Nakorn Chiang Mai Hospital [Chiang Mai University Hospital]) in Chiang Mai, Thailand. The age of the patients ranged from neonate up to 14 years old. The specimen collection period was from January 2013 through February 2014. In addition, 491 stool samples were also collected from diarrheic piglets from 9 farms in Chiang Mai and 35 farms in Lumphun provinces, Northern Thailand. The specimen collection period was from January 2011 to March 2014. All fecal sample materials were stored at -20^{o}C until used. The study was conducted with the approval of the ethical committee for human rights related to human experimentation, Faculty of Medicine, Chiang Mai University (No. 181/2554).

4.2 Stool sample preparation and screening for group A rotavirus by reverse transcription (RT) and multiplex polymerase chain reaction (multiplex PCR)

4.2.1 Sample preparation

Stool samples were prepared as 10% suspension in phosphate buffered saline (PBS) and then clarified by centrifugation at 5,000 rpm for 5 min at room temperature. Supernatant of each sample was collected for viral RNA genome extraction.

4.2.2 Viral RNA extraction

Viral RNA was extracted from the supernatant of stool sample of 4.2.1 by using Geneaid Viral Nucleic Acid Extraction Kit II (Geneaid, Taipei, Taiwan), according to the manufacturer's protocol. Firstly, transfered a 200 µl of the supernatant into a 1.5 ml microcentrifuge tube and added 400 µl of VB lysis buffer to the sample and mixed by vortexing, and incubated the mixture at room temperature for 10 min. Then, add 450 µl of AD buffer (ethanol added) to the mixture and shaked vigorously. Secondly, transfered 600 µl of the mixture to the VB spin column. The column was centrifuged for 1 min at 13,400 rpm and discarded the flow-through from collecting tube. After that, placed the VB column back into a 2 ml collecting tube. The remaining lysate mixture was transferred to the VB column and centrifuged for 1 min at 13,400 rpm and then discarded the flow-through from collecting tube. Thirdly, the column was washed with 400 µl of W1 buffer and centrifuged at 13,400 rpm for 1 min. Then, the column was washed again with 600 µl of wash buffer (ethanol added) and centrifuged at 13,400 rpm for 3 min, and placed into a 1.5 ml microcentrifuge tube. Finally, the viral RNA was eluted by adding 50 µl of RNase-free water to the center of the column and then centrifuged for 1 min at 13,400 rpm. The viral RNA was collected and stored at -70^{o}C until used.

4.2.3 Reverse transcription (RT) reaction

The viral RNA was reversed transcribed by reverse transcriptase enzyme using protocol according to manufacturer's instruction (Thermo Scientific, USA). Firstly, 10 µl of viral genome extract was mixed with 1 µl of 50% dimethyl sulfoxide (DMSO), and heated at 95°C for 5 min to denature the double-stranded RNA. Then, complementary DNA (cDNA) was synthesized by using random hexamer primers (Takara, Shiga, Japan) and RevertAid^{TM} M-MuLV reverse transcriptase enzyme (Thermo Scientific, USA). For a 20 µl reverse transcription reaction, 10 µl of DMSO-treated

viral RNA was mixed with 1 µl of random hexamer primer (0.25 µg/µl), heated at 65ºC for 5 min to denature the secondary structure of template and chilled on ice. After that, the mixture was mixed with 4 µl of 5X reaction buffer (Thermo Scientific, USA), 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs) mix (Roche, Indianapolis, USA). RNase-free water was added to give a total volume of 18.5 µl. Then, 0.5 µl of RNase inhibitor (Ribolock ™ RNase Inhibitor 40 unit/µl; Thermo Scientific, USA) and 1 µl of RevertAid ™ M-MuLV reverse transcriptase (200 units/µl; Thermo Scientific, USA) were added and incubated at 25ºC for 10 min. The reverse transcription reaction was performed at 42ºC for 1 hr, followed by heating at 72ºC for 10 min to inactivate the enzyme, and cooling at 4ºC immediately. The cDNA was used as a template in multiplex polymerase chain reaction (PCR) or stored at -20ºC for later used.

4.2.4 Detection of group A rotaviruses

The presence of group A rotavirus (RVA) in fecal specimens was detected by polymerase chain reaction (PCR) using a protocol described previously (Yan et al., 2004). For amplification of RVA genome, a forward primer Beg9 was used in combination with the reverse primer VP7-1', which specifically amplify the VP7 gene to generate a PCR product size of 395 bp.

The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of specific-primer pair (20 µM), 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition; 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 48ºC for 1 min, 72ºC for 1 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR

product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min and then visualized under UV light. Negative control was also concurrently included along with the test samples in order to monitor any possible contamination that might occur in the PCR process. The expected fragment lengths of RVAs was 395 bp.

4.3 Molecular genetic characterizations of group A rotaviruses

The detected RVAs were characterized further for their G and P genotypes by multiplex PCR method using genotype-specific primers. The RVAs that their G or P genotype could not be identified were subjected further to nucleotide sequencing and phylogenetic analysis.

4.3.1 G genotyping

For human RVA, the G genotypes of human RVA were identified by using the multiplex PCR method described previously by Govea et al. (1990) with minor modifications. All the oligonucleotide primers used in the multiplex PCR for identification of G genotypes are listed in Table 4.1. The amplification of VP7 gene was performed using a pool of forward primers BT1, CT2, ET3, DT4, AT8, and FT9 in combination with a reverse primer (End9(s)) for amplification of the VP7 genes of G1-G4, G8, and G9, respectively. Each G genotype of human RVA was assigned based on the size of PCR product by comparing with reference strains of human RVA G1-G4, G8, and G9. The expected fragment lengths of G1, G2, G3, G4, G8, and G9 genotypes were 628, 531, 253, 462, 764, and 185 bp, respectively, as demonstrated in Figure 4.1. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 1.20 µl of a pool of specific mixed primers (20 µM

each), 0.20 µl of 20 µM End9(s) primer, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The reaction were performed under the following thermal cycling condition; 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product sizes were detected by electrophoresis (at 100 volts for 40 min) though 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed with tap water for 20 min and then visualized under UV transilluminator. Negative control was also concurrently included along with the test samples in order to monitor any possible contamination that might occur in the PCR process. However, the viruses of which their G genotypes could not be identified with this primer set were subjected further to multiplex PCR using alternative sets of primers previously reported by Gouvea et al. (1994a), Das et al. (1994) and Winiarczyk et al. (2002), as listed in Table 4.1.

For porcine RVA, the G genotypes were identified by using the method described previously by Winiarczyk et al. (2002) with minor modifications. All the oligonucleotide primers used in the multiplex PCR for identification of G genotypes are listed in Table 4.1. A forward primer (Beg9) was used in combination with a pool of reverse primers SG3, SG4, SG5, and SG10 for amplification of the VP7 genes of G3-G5, and G10, respectively. Each G genotype of porcine RVA was assigned based on the size of PCR product by comparing with reference strains of porcine RVA G3-G5, and G10. The expected fragment lengths of G3, G4, G5, and G10 genotypes were 714, 505, 200, and 692 bp, respectively, as demonstrated in Figure 4.2. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of 20 µM Beg9 primer, 0.80 µl of a pool of specific mixed primers (20 µM each), 0.05 µl of

GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The reaction was performed under the following thermal cycling condition; 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 42ºC for 1 min, 72ºC for 1 min, and the final extension step at 72ºC for 10 min in thermal cycler (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product sizes were detected by electrophoresis (at 100 volts for 40 min) through 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min and then visualized under UV transilluminator. Negative control was also concurrently included along with the test samples in order to monitor any possible contamination that might occur in the PCR process. However, the viruses of which their G genotypes could not be identified with this primer set were subjected further to multiplex PCR using alternative sets of primers previously reported by Gouvea et al. (1990, 1994a), and Das et al. (1994), as listed in Table 4.1.

Table 4.1 Oligonucleotide primers for G genotyping of group A rotaviruses

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	G-genotype
Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Consensus for VP7
End9	-	GGTCACATCATACAATTCTAATCTAAG	1062-1036	Consensus for VP7
End9(s)	-	GTATARAAHACTTGCCACCAT	941-921	Consensus for VP7
SG3	-	TTCAGCTGTTGCAACTTC	714-697	G3
SG4	-	ATATATCTAGCTCTTCTCCAG	505-485	G4
SG5	-	TGAGCTTTAATGAGCGGTGCA	200-180	G5
SG10	-	AACGTTCTAGTATTTGTGGTCT	692-671	G10
Winiarczyk et al., 2002				
End9(s)	-	GTATARAAHACTTGCCACCAT	941-921	Consensus for VP7
BT1	+	CAAGTACTCAAATCAATGATGG	314-335	G1
CT2	+	CAATGATATTAACACATTTTCTGTG	411-435	G2
ET3	+	CGTTTGAAGAAGTTGCAACAG	689-709	G3
DT4	+	CGTTTCTGGTGAGGAGTTG	480-498	G4

Table 4.1 (continued)

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	G-genotype
AT8	+	GTCACACCATTTGTAAATTCG	178-198	G8
FT9	+	CTAGATGTAACTACAACTAC	757-776	G9
Gouvea et al., 1990				
Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Consensus for VP7
FT5	-	CATGTACTCGTTGTTACGTC	779-760	G5
DT6	-	CTAGTTCCTGTGTAGAATC	499-481	G6
HT8	-	CGGTTCCGGATTAGACAC	273-256	G8
ET10	-	TTCAGCCGTTGCGACTTC	714-697	G10
BT11	-	GTCATCAGCAATCTGAGTTGC	336-316	G11
Gouvea et al., 1994a				
9Con1	+	TAGCTCCTTTTAATGTATGG	37-56	Consensus for VP7
9Con2	-	GTATAAAATACTTGCCACCA	941-922	Consensus for VP7

Table 4.1 (continued)

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	G-genotype
9T-1	-	TCTTGTCAAAGCAAATAATG	195-176	G1
9T-2	-	GTTAGAAATGATTCTCCACT	281-292	G2
9T-3P	-	GTCCAGTTGCAGTGTAGC	501-484	G3
9T-4	-	GGGTCGATGGAAAATTCT	440-423	G4
106	-	TCTTCAAAAGTCGTAGTG	697-681	G8
9T-9B	-	TATAAAGTCCATTGCAC	147-131	G9
Das et al., 1994				

*IUB (International Union of Biochemistry) code: H = A, C or T, R = A or G

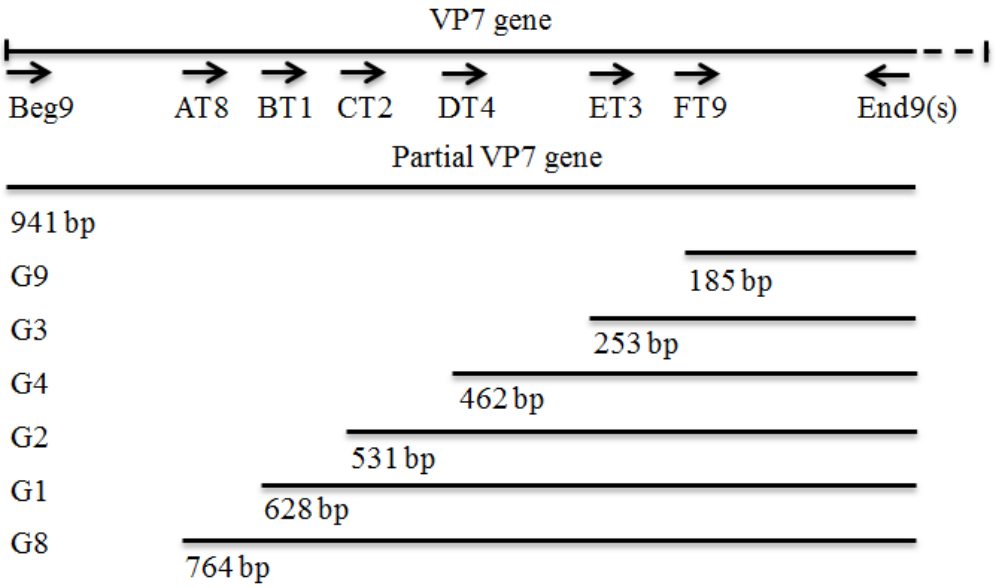

Figure 4.1 Diagram illustrating the amplification of VP7 gene and G genotyping of human group A rotaviruses by multiplex PCR.

The diagram indicates the positions and directions of amplification relative to the plus sense strand of genomic RNA for binding of consensus primer Beg9 (forward) and End9(s) (reverse) and mixed primers FT9, ET3, DT4, CT2, BT1, and AT8, for amplification the VP7 genes of G9, G3, G4, G2, G1, and G8, respectively. The PCR amplification was carried out using a pool of forward primers in combination with a reverse primer End9(s). The expected PCR product sizes of G9, G3, G4, G2, G1, and G8 were 185 bp, 253 bp, 462 bp, 531 bp, 628 bp, and 764 bp, respectively.

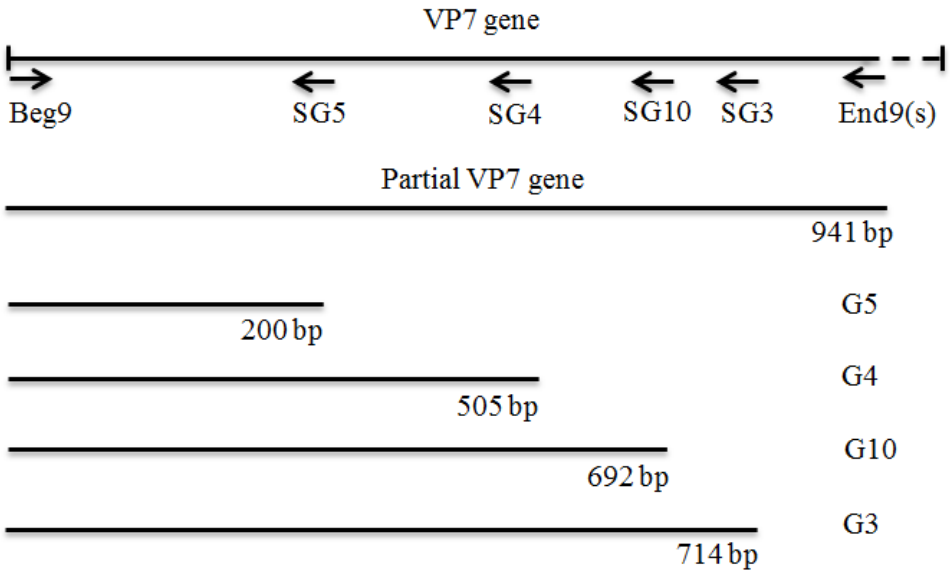

Figure 4.2 Diagram illustrating the amplification of VP7 gene and G genotyping of porcine group A rotaviruses by multiplex PCR.

The diagram indicates the positions and directions of amplification relative to the plus sense strand of genomic RNA for binding of consensus primer Beg9 (forward) and End9(s) (reverse) and mixed primers SG5, SG4, SG10, and SG3, for amplification the VP7 genes of G5, G4, G10, and G3, respectively. The PCR amplification was performed using a forward primer (Beg9) in combination with a pool of reverse primers (SG5, SG4, SG10, and SG3). The expected PCR product sizes of G5, G4, G10, and G3 were 200, 505, 692, and 714 bp, respectively.

4.3.2 P genotyping

The P genotypes of RVA were identified by using the method described previously by Gentsch et al. (1992) with slight modifications. All oligonucleotide primers used in the P genotyping are listed in Table 4.2.

The amplification of VP4 gene was performed using a forward primer (Con3) in combination with a pool of reverse primers (1T-1, 2T-1, 3T-1, 4T-1, 5T-1, and ND2) for identification of human rotaviruses P[8], P[4], P[6], P[9], P[10] and P[11] genotypes, respectively (Gentsch et al., 1992) as depicted in Figure 4.3, while the amplification of porcine rotaviruses were amplified by using a reverse primer Con2 in combination with a pool of forward primers pNCDV, pUK, pOSU, pGott, and pB223 for amplification the VP4 genes of P[1], P[5], P[7], P[6], and P[11], respectively (Gouvea et al., 1994c) as depicted in Figure 4.4. The expected fragment lengths of human rotaviruses P[8], P[4], P[6], P[9], P[10] and P[11] genotypes were 346, 484, 268, 392, 584, and 123 bp, respectively and the expected fragment lengths of porcine rotavirus P[1], P[5], P[7], P[6], and P[11] genotypes are 619, 552, 499, 423, and 314 bp, respectively. The amplification reaction was performed under the following thermal cycling condition; 94^{o}C for 3 min, 35 cycles of 94^{o}C for 1 min, 45^{o}C for 1 min, 72^{o}C for 1 min, and the final extension step at 72^{o}C for 10 min in thermal cycler (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product sizes were detected by electrophoresis (at 100 volts for 40 min) through 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min and then visualized under UV transilluminator. Negative control was also concurrently included along with the test samples in order to monitor any possible contamination that might occur in the PCR process. However, the samples in which the P genotypes could not be identified by this primer set were subjected further to multiplex PCR using alternative sets of primers previously reported by

Gentsch et al. (1992), Gouvea et al. (1994b), Maneekarn et al. (2006), and Winiarczyk et al. (2002), as listed in Table 4.2.

The rotavirus isolates of which their G and P genotypes could not be identified by using these primer sets were analyzed further for identification of their G and P genotypes by nucleotide sequencing and phylogenetic analysis.

Table 4.2 Oligonucleotide primers for P genotyping of group A rotaviruses

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	P-genotype
Con3	+	TGGCTTCGCTCATTTATAGACA	11-32	Consensus for
1T-1	-	TCTACTTGGATAACGTGC	356-339	P[8]
2T-1	-	CTATTGTTAGAGGTTAGAGTC	494-474	P[4]
3T-1	-	TGTTGATTAGTTGGATTCAA	278-259	P[6]
4T-1	-	TGAGACATGCAATTGGACC	402-385	P[9]
5T-1	-	ATCATAGTTAGTAGTCGG	594-575	P[10]
ND2	-	AGCGAACTCACCAATCTG	133-116	P[11]
Gentsch et al., 1992				
Con3	+	TGGCTTCGCTCATTTATAGACA	11-32	Consensus for
SP6	-	ATTTCGGACCATTTATAACC	519-499	P[6]
SP7	-	TCTACTTGGATAACGTGC	279-259	P[7]
Winiarczyk et al., 2002				

Table 4.2 (continued)

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	P-genotype
Con2	-	ATTTCGGACCATTTATAACC	887-868	Consensus for VP4
pNCDV	+	CGAACGCGGGGGTGGTAGTTG	269-289	P[1]
pUK	+	GCCAGGTGTCGCATCAGAG	336-354	P[5]
pOSU	+	CTTTATCGGTGGAGAATACGTCAC	389-412	P[7]
pGott	+	GCTTCAACGTCCTTTAACATCAG	465-487	P[6]
pB223	+	GGAACGTATTCTAATCCGGTG	574-594	P[11]
Gouvea et al., 1994b				
Con3	+	TGGCTTCGCTCATTTATAGACA	11-32	Consensus for VP4
VP4P[19]	-	AACTTCCAYTTAYTTGAGGTATTAAC	425-400	P[19]
Maneekarn et al., 2006				
Con2	-	ATTTCGGACCATTTATAACC	887-868	Consensus for VP4
VP4P23	+	TAATGGTGAGACACCGAATGC	540-560	P[23]
Saikruang et al., 2013				

Table 4.2 (continued)

Primer	Polarity	Nucleotide sequences (5'-3')	Positions	P-genotype
Con2	-	ATTTCGGACCATTTATAACC	887-868	Consensus for VP4
P[13]F	+	MTRCCAMGARAAGTRTGTAG	646-665	P[13]
This study				

*IUB (International Union of Biochemistry) code: H = A, C or T, R = A or G

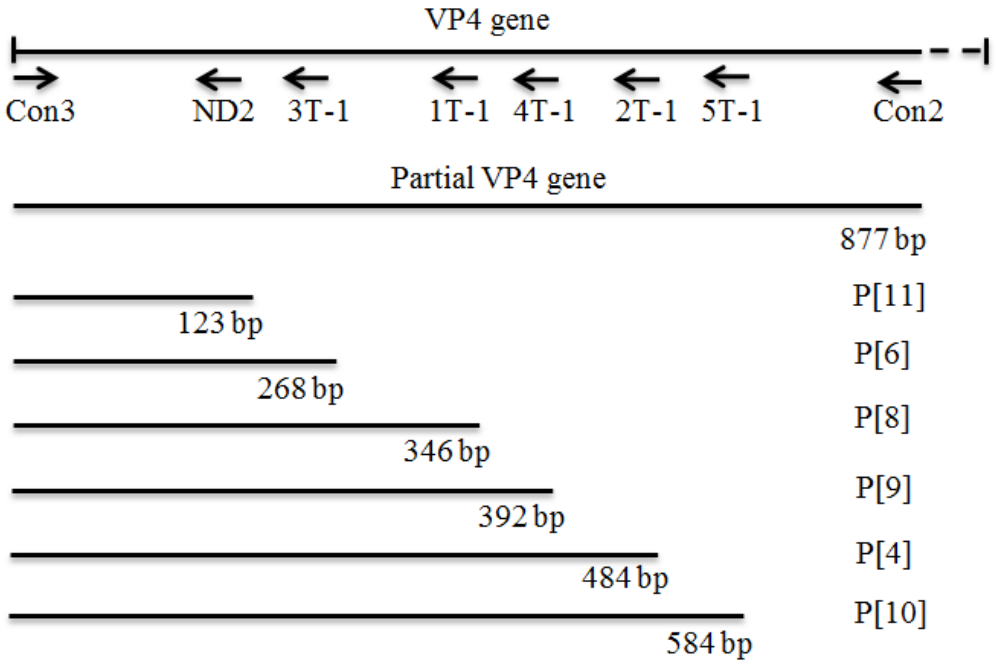

Figure 4.3 Diagram illustrating the amplification of VP4 gene and P genotyping of human group A rotaviruses by multiplex PCR.

The diagram indicates the positions and directions of amplification relative to the plus sense strand of genomic RNA for binding of consensus primer Con3 (forward) and Con2 (reverse) and mixed primers ND, 3T-1, 1T-1, 4T-1, 2T-1, and 5T-1, for amplification the VP4 genes of P[11], P[6], P[8], P[9], P[4], and P[10], respectively. The expected PCR product sizes of P[11], P[6], P[8], P[9], P[4], and P[10] were 123, 268, 346, 392, 484, and 584 bp, respectively.

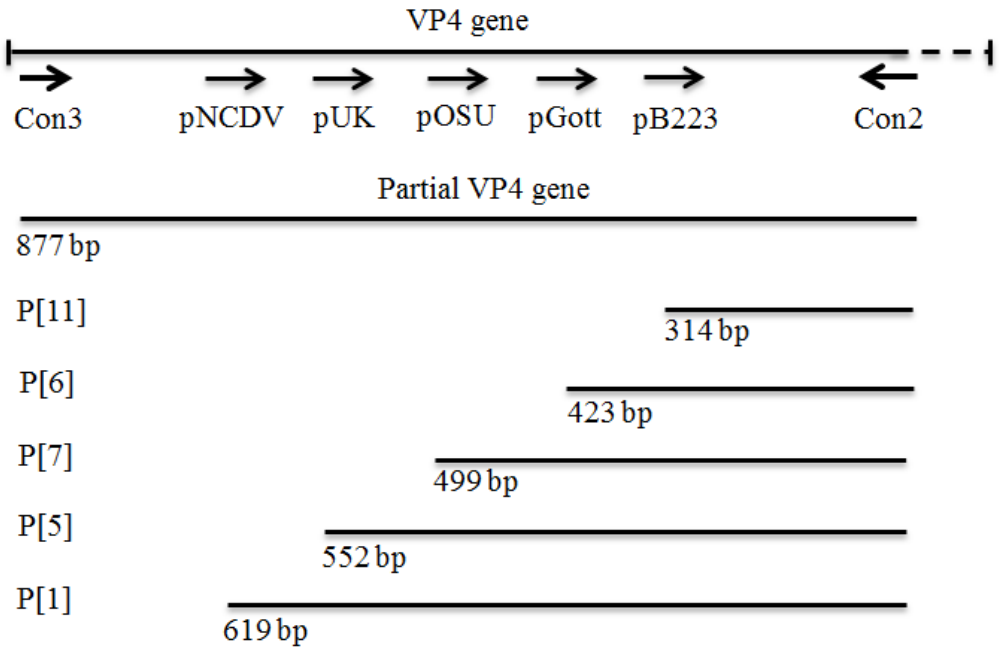

Figure 4.4 Diagram illustrating the amplification of VP4 gene and P genotyping of porcine group A rotaviruses by multiplex PCR.

The diagram indicates the positions and directions of amplification relative to the plus sense strand of genomic RNA for binding of consensus primer Con3 (forward) and Con2 (reverse) and mixed primers pNCDV, pUK, pOSU, pGott, and pB223, for amplification the VP4 genes of P[11], P[6], P[7], P[5], and P[1], respectively. The expected PCR product sizes of P[11], P[6], P[7], P[5], and P[1] were 314, 423, 499, 552, and 619 bp, respectively.

4.3.3 Newly designed P[13] primer

The P[13] genotype is a common genotype in pigs and has not been identified from other animal sources, including humans (Estes, 2001; Ghosh et al., 2007a; Teodoroff et al., 2005; Huang et al., 1993). In Northern Thailand, P[13] genotype has been known as a common genotype as P[6], P[7], P[23], and P[19]. The recent report of an epidemiological surveillance from Chiang Mai Province, Thailand between 2002 to 2003 revealed that apart from the P[6] and P[7] genotypes, P[13] genotype was also detected as a major genotype (Chan-it et al., 2008). However, the identification of P[13] porcine rotavirus strains had been done by VP4 nucleotide sequence analysis, because there was no specific primer for P[13] genotype available. Thus, P[13] specific primer was designed in the present study and used to identify rotavirus P[13] genotype in porcine stool samples.

The VP4 nucleotide sequences of P[13] porcine rotaviruses detected in the present study and sequences of other P[13] strains, such as DQ003290, DQ536362, HQ268857, L07886, and AB573876, as well as other P types of reference strains (P[1] to P[37]) obtained from the Genbank database, were aligned using the ClustalX program. The region which was highly conserved among P[13] strains and divergent in other P types were selected as a target primer sequence. The newly designed primer for P[13], namely, P[13]F, was targeted to nucleotide (nt) positions 646 to 665 of the VP4 gene. The oligonucleotide sequence of the newly designed primer was as follows: 5'-HTRCCAMGAGARGTRTGTAG-3'. For the PCR amplification, P[13]F forward primer was used in combination with the Con2 reverse primer (nt positions 868 to 887), which generated a PCR product of 242 bp.

The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of

20 µM of P[13]F, 0.20 µl of 20 µM of Con2, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition; 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 48ºC for 1 min, 72ºC for 1 min, and the final extension step at 72ºC for 10 min in thermal cycler (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min and then visualized under UV light. Negative control was also concurrently included along with the test samples in order to monitor any possible contamination that might occur in the PCR process. The P[13] genotype was identified by comparing the PCR product size with GeneRuler^{TM} 100 bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The DNA band of PCR product was 242 bp in length. To evaluate the specificity of newly designed primer, the obtained PCR amplicons of P[13] genotype were randomly selected an subjected to nucleotide sequencing and sequence analysis for the confirmation of P[13] genotype.

4.3.4 Genetic characterization of unusual rotavirus strains

All of non-typeable rotavirus strains detected in the present study were characterized further for full-length analysis of VP4, VP6, VP7, NSP4, and NSP5 gene segments by nucleotide sequencing, and the sets of primers used for amplification and sequencing of each gene are listed in Table 4.3.

1) VP4 gene

P genotypes of the rotavirus nontypeable strains were determined by nucleotide sequencing, using the consensus primer for VP4 gene as listed in Table 4.3. The VP4 gene of human and porcine rotaviruses

were amplified by using Con3 forward primer in combination with VP4end reverse primer. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of 20 µM of Con3, 0.20 µl of 20 µM of VP4end, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 42ºC for 1 min, 72ºC for 1.30 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min, and then visualized under UV light. The VP4 gene was identified by comparing the PCR product size with GeneRuler™ 100 bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The expected fragment length of PCR product was 2,383 bp and the PCR product was analyzed further by nucleotide sequencing and phylogenetic analysis.

2) VP6 gene

VP6 genogroup of the rotavirus non-typeable strains were determined by nucleotide sequencing, using the consensus primers for VP6 gene as reported previously by Shen et al. (1994), as listed in Table 4.3. The VP6 gene of human and porcine rotaviruses were amplified by using VP6-F forward primer in combination with VP6-R reverse primer. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis,

USA), 0.20 µl of 20 µM of VP6-F, 0.20 µl of 20 µM of VP6-R, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 45ºC for 1 min, 72ºC for 1.30 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min, and then visualized under UV light. The VP6 gene was identified by comparing the PCR product size with GeneRuler^{TM} 100 bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The expected fragment length of PCR product was 1,356 bp and PCR product was analyzed further by nucleotide sequencing and phylogenetic analysis.

3) VP7 gene

G genotypes of the rotavirus non-typeable strains were determined by nucleotide sequencing, using the consensus primers for VP7 gene as listed in Table 4.3. The VP7 gene of human and porcine rotaviruses were amplified by using Beg9 forward primer in combination with End9 reverse primer. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of 20 µM of Beg9, 0.20 µl of 20 µM of End9, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 48ºC

for 1 min, 72ºC for 1.30 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min, and then visualized under UV light. The VP7 gene was identified by comparing the PCR product size with GeneRuler^{TM} 100 bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The expected fragment length of PCR product was 1,062 bp and PCR product was analyzed further by nucleotide sequencing and phylogenetic analysis.

4) NSP4 gene

NSP4 genogroups of the rotavirus non-typeable strains were determined by nucleotide sequencing, using the consensus primer for NSP4 gene as reported previously by Kudo et al. (2001), as listed in Table 4.3. The NSP4 gene of human and porcine rotaviruses were amplified by using NSP4-1a forward primer in combination with NSP4-2b reverse primer. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of 20 µM of NSP4-1a, 0.20 µl of 20 µM of NSP4-2b, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 45ºC for 1 min, 72ºC for 1 min, and the final extension step at 72ºC for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel

was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min, and then visualized under UV light. The VP6 gene was identified by comparing the PCR product size with GeneRuler^{TM} 100 bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The expected fragment length of PCR product was 750 bp and PCR product was analyzed further by nucleotide sequencing and phylogenetic analysis.

5) NSP5 gene

NSP5 genogroups of the rotavirus non-typeable strains were determined by nucleotide sequencing, using the consensus primers for NSP5 gene as reported previously by Matthijnssens et al. (2006), as listed in Table 4.3. The NSP5 gene of human and porcine rotaviruses were amplified by using GEN_NSP5F forward primer in combination with GEN_NSP5R reverse primer. The amplification reaction components consisted of 2.5 µl of 5X Green GoTaq® Flexi Buffer (Promega, Medison, WI, USA), 1.25 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix (Roche, Indianapolis, USA), 0.20 µl of 20 µM of GEN_NSP5F, 0.20 µl of 20 µM of GEN_NSP5R, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA), 1.50 µl of cDNA template, and adjusted to a final volume of 12.5 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94^{o}C for 3 min, 35 cycles of 94^{o}C for 1 min, 45^{o}C for 1 min, 72^{o}C for 1 min, and the final extension step at 72^{o}C for 10 min in thermal cycler machine (G-STORM GS1, Gene Technologies Ltd Braintree, Essex, UK). The PCR product was detected by electrophoresis (at 100 volts for 40 min) on 1.5% agarose gel. The gel was stained with 0.5 µg/ml ethidium bromide for 5 min. After that, the gel was washed twice with tap water for 20 min, and then visualized under UV light. The VP6 gene was identified by comparing the PCR product size with GeneRuler^{TM} 100

bp Ladder marker (Fermentas, Glen Burnie, MD, USA). The expected fragment length of PCR product was 667 bp and PCR product was analyzed further by nucleotide sequencing and phylogenetic analysis.

Table 4.3 Oligonucleotide primers for full-length genotyping of VP4, VP6, VP7, NSP4, and NSP5 genes of group A rotaviruses

Gene	Primer	Polarity	Nucleotide sequences (5'-3')	Positions	Reference
VP4	VP4_1-17F	+	GGCTATAAAATGGCTTCGC	001-020	Modified from Fujii et al., 2012
	Con3	+	TGGCTTCGCTCATTTATAGACA	11-32	Gentsch et al., 1992
	Con2ed(F)	+	GGDTAYAARTGGTCNGARRT	868-887	unpublished
	VP4RVF	+	TWGAYATGTTYTCWATGTTYKCDGG	1593-1617	unpublished
	Con2	-	ATTTCGGACCATTTATAACC	887-868	Gentsch. et al., 1992
	VP4RVR	-	CCHGMRAACATWGARAACATRTCWA	1617-1593	unpublished
	GEN_VP4_P6_2359R	-	GGTCACATCCTCTATAGAGCTCTC	2359-2336	Matthijnssens et al., 2008
	VP4-3R	-	CAATTCTRTTHCGAATTATTGGRTT	2287–2311	Khamrin et al. (2006a)
	VP4-End	-	GGTCACAWCCTCTAGMMRYTRCTTA	2362–2383	Martella et al. (2006a)
VP6	VP6F	+	GGCTTTTAAACGAAGTCTTC	001-020	Shen et al., 1994
	VP6R	-	GGTCACATCCTCTCACTA	1356-1339	Shen et al., 1994
VP7	Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Gouvea et al.,1990
	End9	-	GGTCACATCATACAATTCTAATCTAAG	1062-1032	Gouvea et al.,1990

Table 4.3 (continued)

Gene	Primer	Polarity	Nucleotide sequences (5'-3')	Positions	Reference
NSP4	NSP4-1a	+	GGCTTTTAAAAGTTCTGTTCCG	1-22	Kudo et al., 2001
	NSP4-2b	-	GGTCACATTAAGACCGTTCC	750-731	Kudo et al., 2001
NSP5	GEN_NSP5 F	+	GGCTTTTAAAGCGCTACAG	001-024	Matthijnssens et al., 2006a
	GEN_NSP5 R	-	GGTCACAAAACGGGAGTG	667-650	Matthijnssens et al., 2006a

*IUB (International Union of Biochemistry) code: H = A, C or T, R = A or G

4.4 Nucleotide sequence analysis of rotavirus nontypeable strains

4.4.1 Purification of PCR product

The PCR products of VP7 and VP4 genes of rotavirus strains that G genotype and/or P genotype could not be identified by multiplex PCR method, so called nontypeable strains, and full length of VP4, VP6, VP7, NSP4, and NSP5 genes of nontypeable strains were subjected further to nucleotide sequencing. Initially, the PCR products of VP4, VP6, VP7, NSP4, and NSP5 genes were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) according to the manufacturer's protocol. The first PCR products were electrophoresed (at 100 volts for 40 min) in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide, followed by excision of the agarose gel containing the specific DNA fragment and put the excised gel into 1.5 ml microcentrifuge tube. After that 500 µl of Gel/PCR buffer were added to the sample and mix by vortexing and incubated at 60^{o}C for 10 min until the gel piece had completely dissolved. Then, the mixture was applied onto a DF column in a 2 ml collecting tube and centrifuged at 13,400 rpm for 30 sec. The flow-through filtrate was discarded and the column was placed back in the same collecting tube. To wash the DNA that bound to the column, 600 µl of W1 buffer was added and let stranded for 1 min and then centrifuged at 13,400 rpm for 30 sec. The flow-through filtrate was discarded. Then, 600 µl of washed buffer (ethanol added) were added into the column and let stranded for 1 min and then centrifuged for 30 sec. The flow-through filtrate was discarded again and the column was centrifuged for an additional 3 min to remove residual ethanol. The DF column was placed into a clean 1.5 ml microcentrifuge tube and then 50 µl of elution buffer (10 Mm Tria-Cl, pH 8.5) was added into the center of column matrix. The column was standed for at least 2 min and then centrifuged at 13,400 rpm for 2 min to elute the purified DNA. The quantity and quality of the purified DNA was assessed by

spectrophotometric analysis at 260 nm as well as by electrophoresis in 1.5% agarose gel.

4.4.2 Nucleotide sequencing

The purified PCR products were sent to First Base laboratories (Selanger Daru Ehsan, Malaysia) for sequencing. Briefly, the purified PCR products were sequenced directly by fluorescence based cycle sequencing method using BigDye® Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems, Foster city, CA, USA). Each sequencing reaction consisted of 8.0 µl of premix, 1.0 µl of sequencing primer, 100 ng of purified PCR product, and H_2O to yield a total volume of 20 µl. Cycle sequencing was performed in a thermocycler machine (Eppendorf, Hauppauge, NY, USA) using the following profiles: 96ºC for 1 min, 25 cycles of 96ºC for 10 sec, 50 for 5 sec. and 60ºC for 4 min. The sequencing primers for VP4, VP6, VP7, NSP4, and NSP5 genes, are listed in Table 4.3. Then, the sequencing DNA product was precipitated in cooled absolute ethanol and washed with 70% ethanol and then dried the pellet in heat box for10 min at 50ºC. Finally, the sequencing product was analyzed on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems, Foster city, CA, USA).

4.4.3 Sequence analysis and construction of phylogenetic tree

The obtained nucleotide sequences of group A rotaviruses were manually assembled and analyzed by using ClustalX and BioEdit softwares. The obtained sequences were searched for a close genetic relationship with reference sequences by using BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Those reference sequences obtained from BLAST serch, the viruses from each genotype (VP7 (Table 4.4), VP4 (Table 4.5), VP6 (Table 4.6), NSP4 (Table 4.7), and NSP5 (Table 4.8)) and the sequences of rotavirus strains detected in this study were aligned using ClustalX software. Phylogenetic

analysis was performed with MEGA software version 5.05 based on the neighbor-joining method (Tamura et al., 2011).

Table 4.4 The GenBank accession numbers of VP7 gene of group A rotavirus reference strains

Reference strains of VP7				
AU007 (AB081799) G1	KU (D16343) G1	CMH015/05 (GU288622) G1	CMH032/05 (GU288623) G1	OH3625/2012 (AB796448) G1
CMH008/05 (GU288621) G2	MMC6 (EU839923) G2	CMH134/05 (GU288625) G2	J-4777 (DQ904510) G2	AU-1 (D86271) G3
L621 (EU708588) G3	CMP039 (AY707788) G3	CMP213 (DQ786576) G3	CMH055/07 (JQ043273) G3	Gottfried (X06759) G4
Hochi (AB039035) G4	CMP77 (DQ683521) G4	E931 (EU708602) G4	HV157 (FR822302) G4	CMP204 (DQ683523) G5
CMP178 (DQ515961) G5	LL3354 (EF159575) G5	OSU (X04613) G5	IAL28 (EF672588) G5	RF (X65940) G6
CH2 (X56784) G7	69M (EF672560) G8	86 (GU984762) G8	68 (GU984760) G8	04-97s379 (JX156636) G8
UP (JX442786) G8	A5 (D01054) G8	Mc323 (D38053) G9	Mc345 (D38055) G9	RMC321 (AF501578) G9
CMH022/03 (EF199738) G9	CMH035/04 (EF199728) G9	CMP054/10 (JX102482) G9	CMP066/09 (JX102481) G9	PRG9121 (JF796739) G9
Hokkaido-14 (AB176677) G9	JP35-7 (AB176683) G9	WI61 (AB180969) G9	A64 (EF672567) G10	KTM368/2004 (GU199497) G11

Table 4.4 (continued)

Reference strains of VP7				
ZJhz13-3 (JX498966) G11	DK12572S (JN410645) G11	CAU12-2 (KC140587) G11	CAU-1 (HQ198807) G11	Dhaka6 (AY773003) G11
L26 (M58290) G12	L338 (JF712560) G13	E403 (JF712582) G14	Hg18 (AF237666) G15	EHP (U08425) G16
Ty-1 (S58166) G17	PO-13 (D82979) G18	02V0002G3 (FJ169861) G19	Ecu534 (EU805775) G20	AzuK-1 (AB454421) G21
Tu-03V0002E10 (EU486973) G22	Phea14246-Hun-08 (FN393054) G23	Dai-10 (AB513837) G24	KE4852 (GU983676) G25	TJ4-1 (AB605258) G26
SG33 (AB621363) G27				

Table 4.5 The GenBank accession numbers of VP4 gene of group A rotavirus reference strains

Reference strains of VP4				
RF (U65924) P[1]	SA11-H96 (DQ841262) P[2]	K9 (D14725) P[3]	DS-1 (AJ540227) P[4]	LB2744 (HM467941) P[4]
TB-Chen (AY787644) P[4]	KUN (AB733131) P[4]	L26 (EF672591) P[4]	UK (M22306) P[5]	ST3 (L33895) P[6]
OSU (M33516) P[7]	CK00088 (JX027876) P[8]	Wa (L34161) P[8]	VA70 (AJ540229) P[8]	CK00011 (JF490189) P[8]
RMC100 (AF531911) P[8]	RMC437 (AY603158) P[8]	DRC88 (DQ005111) P[8]	AU-1 (D10970) P[9]	69M (M60600) P[10]
116E/AG (FJ361204) P[11]	E30 (JF712575) P[12]	CMP29/08 (HQ268857) P[13]	CMP178 (DQ536362) P[13]	CMP213 (DQ786578) P[13]
A64 (EF672563) P[14]	Lamb-NT (FJ031027) P[15]	ETD_822 (GQ479950) P[16]	PO-13 (AB009632) P[17]	L338 (JF712558) P[18]
CMP029 (AY689219) P[19]	CMP138/10 (JX102490) P[19]	CMP066/09 (JX102489) P[19]	mani-97/06 (GQ240618) P[19]	CMP099 (AY689208) P[19]
NIV929893 (DQ887060) P[19]	Mc323 (D38052) P[19]	Mc345 (D38054) P[19]	RMC321 (AF523677) P[19]	EHP (U08424) P[20]
Hg18 (AF237665) P[21]	308/01 (AF526373) P[22]	CMP40/08 (HQ268851) P[23]	TUCH (AY596189) P[24]	Dhaka6 (AY773004) P[25]

Table 4.5 (continued)

Reference strains of VP4				
134/04-15 (DQ061053) P[26]	CMP034 (DQ534016) P[27]	Ecu534 (EU805773) P[28]	AzuK-1 (AB454420) P[29]	02V0002G3 (FJ169856) P[30]
Ch-06V0661 (EU486962) P[31]	61/07/Ire (FJ492835) P[32]	Dai-10 (AB513836) P[33]	FGP51 (AB571047) P[34]	Tu-03V0002E10 (EU486958) P[35]
SG385 (AB823215) P[36]	10V0112H5 (JX204814) P[37]			

Table 4.6 The GenBank accession numbers of VP6 gene of group A rotavirus reference strains

Reference strains of VP6				
Wa (K02086) I1	mani-253/07 (HM348745) I1	DC2109 (FJ947378) I1	Hosokawa (DQ870492) I1	Matlab13/2003 (DQ146675) I1
CMP100/01 (EU372755) I1	CMH185/01 (EU372749) I1	CMH032/05 (GU288639) I1	CMH060/07 (JQ043297) I1	CMH110/07 (JQ043299) I1
US1205 (AF079357) I2	CMH030/07 (JQ043294) I2	CMH070/07 (JQ043298) I2	CMH190/01 (EU372726) I2	Chubut (FJ347104) I2
AU-1 (DQ490538) I3	PO-13 (D16329) I4	A411 (AF317125) I5	GD (FJ617209) I5	LL3354 (EU330646) I5
RMC321 (AF531913) I5	CMP034 (DQ534018) I5	CMP31/01 (EU372786) I5	CMP52/01 (EU372758) I5	CMP45/08 (HQ268858) I5
CMP12/03 (EU372798) I5	L338 (JF712559) I6	ETD_822 (GQ479952) I7	CMH222 (DQ288659) I8	TUCH (AY594670) I9
Lamb-NT (FJ031028) I10	02V0002G3 (DQ096805) I11	KTM368 (GU199496) I12	Ecu534 (EU805774) I13	CE-M-06-0003 (GU183245) I14
KE4852 (GU983675) I15	B10 (HM627557) I16			

Table 4.7 The GenBank accession numbers of NSP4 gene of group A rotavirus reference strains

Reference strains of NSP4				
134/04-7 (DQ186199) E1	RU172 (DQ204740) E1	PORV4 (HQ840946) E1	OSU (D88831) E1	PRG942 (JF796721) E1
CMH146/05 (GU288650) E1	CMP40/08 (HQ268838) E1	CMH014/07 (JQ043300) E1	CMP48/08 (HQ268841) E1	KUN (D88829) E2
CMH008/05 (GU288642) E2	CMH028/07 (JQ043301) E2	CMH054/05 (GU288648) E2	CMH070/07 (JQ043310) E2	DS-1 (AF174305) E2
RRV (L41247) E3	CMH222 (DQ288660) E3	CMH120/04 (DQ923799) E3	CMH134/04 (DQ923803) E3	CMH079/05 (EU791925) E3
AU-1 (D89873) E3	PO-13 (AB009627) E4	B4106 (AY740732) E5	RV176 (DQ490560) E6	ETD_822 (GQ479956) E7
PP-1 (AF427521) E8	CMP034 (DQ534017) E9	02V0002G3 (FJ169862) E10	Ty-3 (AB065286) E11	Chubut (FJ347109) E12
B10 (HM627562) E13	L338 (JF712564) E14			

Table 4.8 The GenBank accession numbers of NSP5 gene of group A rotavirus reference strains

Reference strains of NSP5				
KTM368 (GU199502) H1	Wa (AB091726) H1	LB2719 (HM467916) H1	LS00008 (KJ659441) H1	ES51 (DQ189249) H1
BP271 (KF835969) H1	Ryukyu-1120 (AB741659) H1	SB1A (EU169874) H1	LB2744 (HM467919) H2	DS-1 (HQ650126) H2
TB-Chen (AY787651) H2	BA222 (GU827416) H3	PAH136 (GU296418) H3	AU-1 (AB008656) H3	WC3 (EF990702) H3
B4106 (AY740731) H3	Tu-03V0002E10 (EU486980) H4	B10 (HM627563) H5	HCR3A (EU708911) H6	E30 (JF712576) H7
Ch-02V0002G3 (EU486978) H8	ETD_822 (GQ479957) H9	KE4852 (GU983680) H10	L338 (JF712565) H11	