CHAPTER 4

Materials and Methods

4.1 Stool sample collection

Stool specimens were collected from pediatric patients admitted to the hospitals with acute gastroenteritis at Maharaj Nakorn Chiang Mai Hospital, Sriphat Medical Center, Nakornping Hospital, and Sanpatong Hospital during 2015 to 2016. The age of the patients included in this study ranged from 2 months to 15 years. The symptom of acute gastroenteritis is defined by watery diarrhea for less than 14 days, nausea or vomiting, abdominal pain, and fever (Green et al., 2013). All samples were stored at -20 ^{o}C until use. In addition, the archival stool specimens from 2005 to 2014 together with the specimens collected in this study were used for NoV recombination analysis. The present study was conducted with the approval of the ethical committee for human rights related to human experimentation, Faculty of Medicine, Chiang Mai University (MIC-2557-02710).

4.2 Preparation of 10% stool suspension

The 10% stool suspension was prepared in phosphate buffered saline (PBS), pH 7.2 and centrifuged at 5,000 rpm for 5 min. The obtained supernatant was used for viral genome extraction.

4.3 Extraction of viral genome

The 10% stool suspension from 4.2 was extracted by using Geneaid viral genomic extraction kit (Geneaid Biotech, Taiwan) according to the manufacturer's instructions. Two hundred microliters (µl) of stool supernatant were added to a 1.5 ml microcentrifuge tube containing 400 µl of lysis buffer and incubated at room temperature for 10 min. The 450 µl of AD buffer was added to the mixture and mixed by vortexing. Then, the tube was briefly centrifuged to remove droplet from inside of the lid. Six hundred microliters of the mixture were added to spin column.

After that, the spin column was centrifuged at 13,000 rpm for 1 min. The flow-through liquid was discarded, then, placed the column back into the 2 ml collection tube. Remaining lysate mixture was transferred to the column then centrifuged at 13,000 rpm for 1 min and the flow-through was discarded. The column was transferred to a new 2 ml collection tube and 400 µl of W1 buffer were added to the column, then, centrifuged at 13,000 rpm for 30 sec. The flow-through liquid was removed and placed the column back to the new collection tube. To remove the entire remained W1 buffer, the column was centrifuged at 13,000 rpm for 3 min and placed the column in a clean 1.5 ml microcentrifuge tube. The 50 µl of RNase-free water was added to the center of the column matrix and the column was stood for 3 min. Finally, to elute the purified viral nucleic acid, the column was centrifuged at 13,000 rpm for 1 min. The eluted viral nucleic acid was stored at -70 ^{o}C until use.

4.4 Reverse transcription (RT) reaction

Reverse transcription (RT) was performed by using RevertAid RT Reverse Transcription Kit (Thermo Scientific, USA). Random hexamer primers were used to synthesize complementary DNA (cDNA). Ten microliters of extracted viral RNA genome were added to mixture of 1 µl of random hexamer primers and 1 µl of MilliQ water, and heated at 65 ^{o}C for 5 min. Then, the mixture was immediately chilled on ice. After that, the mixture was mixed with a reaction mixture containing 4 µl of 5x reaction buffer, 2 µl of 10 mM dNTP mix, 1 µl of RNase inhibitor and 1 µl of reverse transcriptase enzyme. The RT step was carried out at 25 ^{o}C for 5 min, followed by 42 ^{o}C for 1 hr and 72 ^{o}C for 5 min. The obtained cDNA was kept at -20 ^{o}C for further use as the template for diarrheal virus detection.

4.5 Detection of norovirus and sapovirus by multiplex-PCR and semi-nested PCR

The multiplex PCR using three sets of primers that specific for NoV GI, GII, and SaV was performed for screening of NoV GI, GII, and SaV, respectively, in a single tube reaction. The primer sets including G1SKF and G1SKR primers for NoV GI detection, COG2F and G2SKR primers for NoV GII detection (Kojima et al., 2002), and SLV5317 and SLV5749 primers for SaV detection (Yan et al., 2003) are shown in Table 4.1. Expected PCR products size for NoV GI, GII, and SaV were 330 bp,

387 bp, and 434 bp, respectively, as shown in Figure 4.1. The amplification reaction components consisted of 1.67 µl of 5X Green GoTaq® Reaction Buffer (Promega, Medison, WI, USA), 0.67 µl of 2.5 mM dNTP mix (Thermo Scientific™, England), 0.13 µl each of specific-primer (20 µM), 0.03 µl of GoTaq® DNA polymerase (Promega, Madison, WI, USA), 0.4 µl of cDNA template, and adjusted to a final volume of 8.4 µl with RNase-free water. The amplification reaction was performed under the following thermal cycling condition; 94 ºC for 3 min, 35 cycles of 94 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 1 min, and the final extension step at 72 ºC for 10 min in thermal cycler machine (GeneAmp® PCR System 2700 thermal cycler, Applied Biosystems, California, USA). To avoid any possible contaminations, NoV and SaV positive controls and a negative control was included in the system throughout the experiments. For the semi-nested of NoV GII, first round PCR products of NoV GII negative were used as a template in second round PCR reactions with primers (G2SKF and G2SKR) (Figure 4.1) at a concentration of 20 µM of each primer. The nested PCR reaction and cycling conditions were the same as those for the first round PCR. The expected PCR products size of 344 bp was evaluated by electrophoresis on 1.5% agarose gel containing RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, INC., Korea) and visualized under ultraviolet (UV) light.

Table 4.1 List of primers used for the detection of norovirus and sapovirus in stool samples by multiplex-PCR and semi-nested PCR

Virus	Primer	Sequence	Position	Polarity	Reference
SaV	SLV5317	5’-CTCGCCACCTACRAWGCBTGGTT-3’	5083-5105	+	Yan et al., 2003
	SLV5749	5’-CGGRCYTCAAAVSTACCBCCCCA-3’	5516-5494	-	
NoV GI	G1SKF	5’-CTGCCCGAATTYGTAAATGA-3’	5342-5361	+	Kojima et al., 2002
	G1SKR	5’-CCAACCCARCCATTRTACA-3’	5671-5653	-	
NoV GII	COG2F	5’-CARGARBCNATGTTYAGRTGGATGAG-3’	5003-5028	+	Kojima et al., 2002
	G2SKR	5’-CCRCCNGCATRHCCRTTRTACAT-3’	5389-5367	-	
	G2SKF	5’-CNTGGGAGGGCGATCGCAA-3’	5046-5389	+	

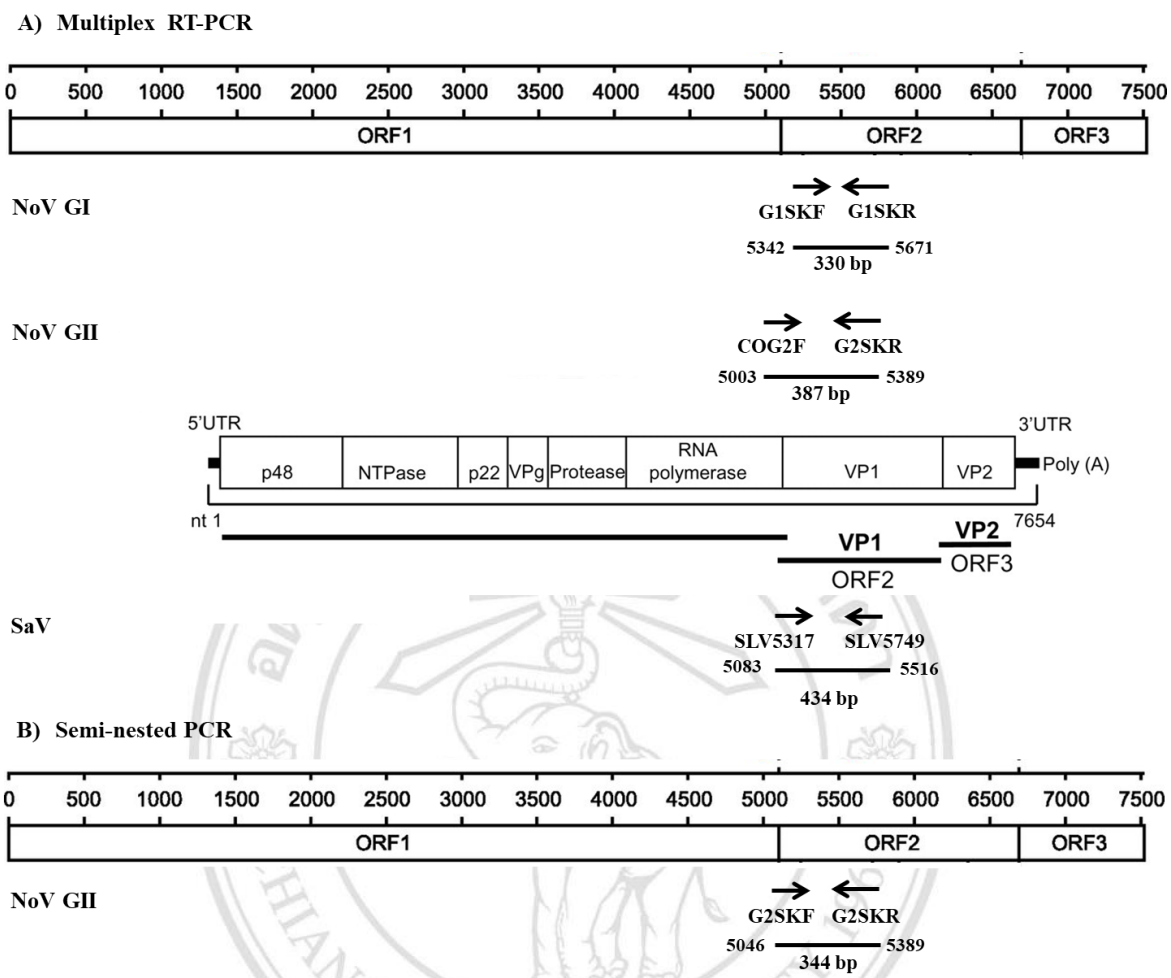

Figure 4.1 Diagram representing the annealing positions of the primers used for the screening of NoV GI and GII by (A) multiplex-PCR and (B) semi-nested PCR. The diagram demonstrates the position and directions of amplification relative to the plus sense strand of genomic RNA of NoV GI, GII and SaV by using mixed primer G1SKF, COG2F, and SLV5317 as forward primers and G1SKR, G2SKR, and SLV5749 as reverse primers for multiplex-PCR, respectively, and G2SKF (forward) and G2SKR (reverse) for semi-nested PCR. The expected PCR product sizes in multiplex PCR for NoV GI was 330 bp, NoV GII was 387 bp, and SaV was 434 bp. The expected PCR product size of semi-nested PCR for NoV GII was 344 bp.

4.6 Norovirus and sapovirus genotyping by nucleotide sequencing and phylogenetic analyses

The positive samples for NoV and SaV obtained from multiplex PCR and semi-nested PCR screening methods were characterized further for their genotypes by nucleotide sequencing. The PCR products were purified by using GenepHlow™ Gel/PCR kit (Geneaid Biotech, Taiwan) according to the manufacturer's protocol. The PCR products were electrophoresed in 1.5% agarose gel containing RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, INC., Korea), followed by excision of the gel containing the specific DNA fragment and put the gel into 1.5 ml microcentrifuge tube. After that 500 µl of Gel/PCR buffer were added to the sample and incubated at 60 °C for 10 min until the gel piece completely dissolved. The sample mixture was added to DFH column that already placed into a 2 ml collection tube then centrifuged at 13,400 rpm for 30 sec. The flow-through fluid was discarded and the column was placed back to the collection tube. In washing step, wash buffer was added into the column and standed for 1 min, then centrifuged at 13,400 rpm for 30 sec. The flow-through fluid was discarded and the column was placed back to the collection tube. To dry the column matrix, the column was centrifuged at 13,400 rpm for 3 min. Finally, the purified DNA was eluted by adding 30 µl of elution buffer to the center of column matrix that placed on a new 1.5 ml microcentrifuge tube. The column matrix was standed for 2 min to allow elution buffer to be completely absorbed and centrifuged at 13,400 rpm for 2 min. The quantity and quality of the purified PCR products were assessed by agarose gel electrophoresis.

The purified PCR products and specific reverse primers (G1SKR for NoV GI, G2SKR for NoV GII, and SLV5317 for SaV) were used for nucleotide sequencing by sending to 1st Base laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia). The obtained nucleotide sequence was aligned with reference sequences using Bioedit Alignment Editor Programs. Furthermore, the obtained NoV genotypes were then confirmed using a NoroNet typing tool (http://www.rivm.nl/mpf/norovirus/typingtool). Multiple alignments of nucleotide sequences were performed using ClustalX. The phylogenetic trees were constructed by MEGA7 (Kumar et al.,

2016). The trees were constructed by neighbor-joining method with Kimura 2-parameter model with 1000 bootstrap replicates.

4.7 Detection and characterization of norovirus recombination

To characterize NoV GII recombinant genotypes, all the NoV GII positive samples of pediatric patients which collected during 2005-2015 were selected and analyzed for the similarity to reference recombinant strains available in the GenBank databases. Then, the sequences which similar to the reference recombinant strains were characterized for the partial ORF1 (RdRp)/ORF2 (capsid) regions by semi-nested PCR. The primers JV12Y (Vennema et al., 2002) and G2SKR were used for the first PCR reaction. Semi-nested PCR was then conducted using the primer P289IUB (Puustinen et al., 2012) and G2SKR. All the primers used for NoV GII recombinant strain amplifying are listed in Table 4.2. The amplified fragment was 1,095 bp, which covered the partial sequence of 3' RdRp and 5' capsid regions (Figure 4.2). The amplification reaction components contained 7.05 µl of RNA-free water, 2.5 µl of 5X Green GoTaq® Reaction buffer (Promega, Madison, USA), 1 µl of 2.5 mM dNTP mix (Thermo Scientific™, England), 0.2 µl each of 20 µM each primer, 0.05 µl of GoTaq® DNA polymerase (Promega, Madison, USA), and 1.5 µl of cDNA template, in a total volume of 12.5 µl. A reaction mixture without template was used as negative control. The amplification was performed for 35 cycles under the following thermal cycling condition, 94 ºC for 3 min followed by, 94 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 2 min, and the final extension step at 72 ºC for 10 min. First round PCR products were used as a template in second round PCR reactions with internal primers (P289IUB and G2SKR) (Figure 4.2) at a concentration of 20 µM of each primer. The nested PCR reaction and cycling conditions were the same as those for the first round PCR. The purification of the PCR product was conducted and then sequenced. The sequences were analyzed separately for the partial ORF1 (RdRp) and ORF2 (capsid) by phylogenetic analysis. In addition, the recombination point of detected recombinant strains was predicted by using Simplot software v.3.5.1 with the nucleotide position of Lordsdale (X86557) strain as reference. Moreover, the potential recombination events were confirmed by Maximum Chi-Square test using RDP4 program. The

obtained maximum chi-square value that less than the *p*-value ($p<0.01$) indicated the significant recombination point.

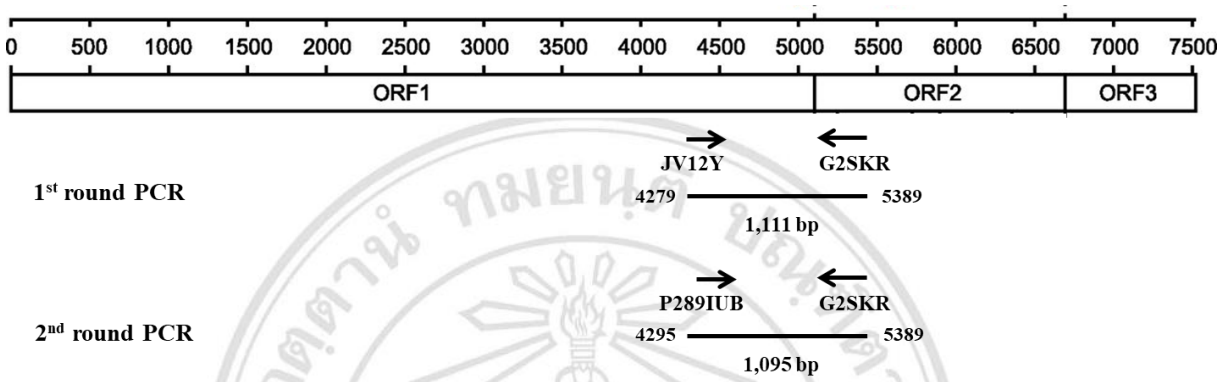

Figure 4.2 Diagram representing the annealing positions of the primers used for the detection of norovirus GII recombinant strains by semi-nested PCR. The diagram demonstrates the position and directions of amplifications relative to the plus sense strand of genomic RNA of NoV GII recombinant strain by using primer JV12Y and G2SKR as forward and reverse primer, respectively, for the first round PCR reaction and P289IUB (forward) and G2SKR (reverse) for semi-nested PCR. The expected PCR product sizes of the first round PCR reaction and semi-nested PCR were 1,111 bp and 1,095 bp, respectively.

Table 4.2 List of primers used for the detection of norovirus recombinant strains

Primer	Sequence	Position	Polarity	Reference
JV12Y	5’ - ATACCACTATGATGCAGAYTA - 3’	4279-4299	+	Vennema et al., 2002
P289IUB	5’- GATTACTCCARGTGGGAYTCMAC -3’	4295-4317	+	Puustinen et al., 2012
G2SKR	5’-CCRCCNGCATRHCCRTTRTACAT-3’	5389-5367	-	Kojima et al., 2002