CHAPTER IV

MATERIALS AND METHODS

1. Fecal specimens

Two hundred and ninety-six fecal samples were collected from children hospitalized with diarrhea in four different hospitals and one private clinic in Chiang Mai province between May 2000 and March 2002. Of these, 168 were from McCormick Hospital, 77 from Maharaj Nakorn Chiang Mai Hospital, 33 from Nakornping Hospital, 10 from Sanpatong Hospital and 8 from a private clinic. The ages of the subjects were ranged from neonate up to 5 years old. All specimens were stored at -20 ^{O}C until use.

2. Preparation of 10% fecal suspension

An approximately 10% fecal suspension was prepared in 10 mM PBS, pH 7.4 and clarified by centrifugation at 5,000 rpm for 5 min. Then, the supernatant was collected and used for an extraction of viral RNA.

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3. Viral RNA extraction

Viral RNA was extracted from the 10% fecal supernatant by a spin column technique using the QIAamp viral RNA Mini Kit (QIAGEN, Germany). One hundred and forty µl of 10% fecal supernatant was mixed with 560 µl of AVL viral lysis buffer containing carrier RNA by pulse-vortexing for 15 sec. The mixture was

incubated at room temperature for 10 min and 560 µl of 95% ethanol was added. Then, the mixture was applied to the spin column and centrifuged at 8,000 rpm for 1 min. The column was placed into a new 2 ml collection tube and 500 µl of AW1 buffer was added. The column was centrifuged at 8,000 rpm for 1 min to remove unbound materials, and washed by addition of 500 µl of AW2 buffer. Then, the column was centrifuged at full-speed (about 14,000 rpm) for 3 min, and placed into a new 1.5 ml microcentrifuge tube. Finally, 60 µl of AVE buffer was added directly onto the column to elute RNA. After incubating at room temperature for 1 min, the column was centrifuged at 8,000 rpm for 1 min. The viral RNA was spun down into the collecting tube and used as a template for the reverse transcription polymerase chain reaction (RT-PCR).

4. Reverse transcription (RT) reaction

The viral RNA was reverse transcribed by reverse transcriptase using protocol according to the manufacturer's instruction (Fermentas, Lithuania). Briefly, 10 µl of extracted viral RNA was mixed with 0.8 µl (0.25 µg/µl) of random hexamer primer (Takara, Shiga, Japan) and Rnase-free water was added to give a total volume of 13.0 µl. Then, the mixture was heated at 70 ^{O}C for 5 min and cooled on ice. After cooling, the mixture was mixed with 4.0 µl of 5X reaction buffer (Fermentas) and 2.0 µl of 10 mM dNTP Mix (10 mM of each dNTP; Promega, USA). Rnase-free water was added to give a total volume of 19.0 µl. The mixture was incubated at 25 ^{O}C for 5 min, and then 1 µl of 200 units/µl of RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas) was added and incubated the mixture at 25 ^{O}C for 10 min. The reverse

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transcription step was carried out at 42 ^{O}C for 1 hr, followed by heating at 70 ^{O}C for 10 min to inactivate the enzyme, and cooling at 4 ^{O}C immediately.

5. Viral target genes and specific-primer pairs binding regions

A diagram of capsid genes with nucleotide position of primer binding regions of NVGI, NVGII, SV, and HAstV is shown in Figure 3 (Yan et al., 2003). Two sets of primers G1-SKF/G1-SKR and COG2F/G2-SKR were used for amplifying the partial capsid gene of norovirus genogroup I (NVGI) and genogroup II (NVGII), which generated a 330 bp and a 387 bp PCR product, respectively. The SLV5317 and SVL5749 primers, which generated a 434 bp PCR product, were used for amplifying the partial capsid gene of all genogroups of sapovirus (SV). For human astrovirus (HAstV), the primers PreCAP1 and 82b were used to generate a 719 bp PCR product of its partial capsid gene. The primer sequences and relative locations of the primers binding regions are shown in Table 1 (Yan et al., 2003).

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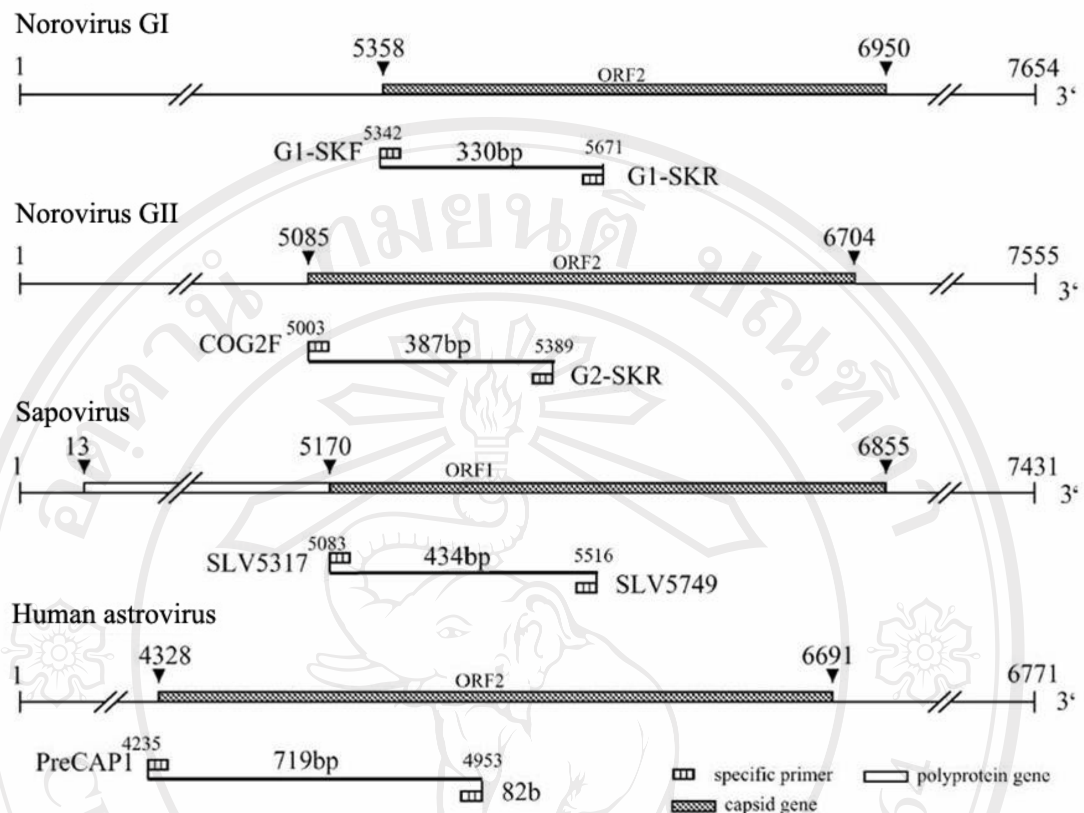

Figure 3. Diagram illustrating the amplification of partial capsid genes of NVGI, NVGII, SV, and HAstV by PCR (Yan et al., 2003). The diagram indicated the positions of the primer pairs relative to the plus sense strand of RNA genome are shown for representative strains Norwalk/68 (GenBank accession no. M87661), Lordsdale/93 (GenBank accession no. X86557), Manchester/93 (GenBank accession no. X86560), and human astrovirus serotype 1 Oxford (GenBank accession no. L23513) in NVGI, NVGII, SV, and HAstV, respectively. Arrows denote location of first AUG in predicted ORF encoding viral capsid protein. Capsid gene sites in ORF2 of norovirus GI and GII and human astrovirus genome are also indicated. In sapovirus genome, capsid gene is fused with polyprotein gene in a single ORF (ORF1) as described in the text.

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Table 1. Specific primers for the detection of NVGI, NVGII, SV, and HAstV

Virus	Primer	Polarity	Sequence (5′ to 3′)	Position No.
Norovirus Genogroup I	G1-SKF*	+	CTGCCCGAATTYGTAAATGA	5342-5361
(NVGI)	G1-SKR*	-	CCAACCCARCCATTRTACA	5653-5671
Norovirus Genogroup II	COG2F*	+	CARGARBCNATGTTYAGRTGGATGAG	5003-5028
(NVGII)	G2-SKR*	-	CCRCCNGCATRHCCRTTRTACAT	5367-5389
Sapovirus	SLV5317*	+	CTCGCCACCTACRAWGCBTGGTT	5083-5105
(SV)	SLV5749*	-	CGGRCYTCAAAVSTACCBCCCCA	5494-5516
Human astrovirus	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	4235-4255
(HAstV)	82b	-	GTGAGCCACCAGCCATCCCT	4934-4953

(From Yan et al., 2003)

*IUB codes: B = C, G or T; H = A, C or T; N = any base; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

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6. Polymerase chain reaction (PCR) for the detection of Norovirus genogroup I (NVGI)

Polymerase chain reaction for identification of NVGI was carried out in a separate reaction in order to avoid an interference from nonrelevant primer sets. After RT step, 3 µl of cDNA template was added to a PCR reaction mixture containing 17.25 µl of RNase-free water, 2.5 µl of 10X PCR buffer (Invitrogen), 0.75 µl of 50 mM $MgCl_2$ (Invitrogen), 0.5µl of 10 mM dNTP mix, 0.4 µl of each 20 µM of forward and reverse primers (G1-SKF and G1-SKR; Pacific science, Canada), 0.2 µl of *Taq* DNA polymerase (5 units/µl; Invitrogen), in total volume of 25 µl. Then, the amplification was performed for 40 cycles under the following thermal cycling conditions: 94°C for 2 min to initiate a denaturation of cDNA template and then denaturing at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was detected by electrophoresis through 1.5% agarose gel in TBE buffer at 100 volts for 40 min. After electrophoresis, the gel was stained with 0.5 µl/ml ethidium bromide and then visualized under ultraviolet light source and documented by using the molecular analysis software (Bio-Rad). Negative control was also concurrently included along with the tested samples in order to monitor if there was any possible contamination that might occur in the PCR process. The size of amplification product generated by NVGI specific primer was identified by comparing with GeneRuler™ 100bp DNA Ladder Plus marker (Fermentas) and reference strains of NVGI (kindly provided by Professor Hiroshi Ushijima, the University of Tokyo, Japan).

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7. Multiplex polymerase chain reaction (Multiplex PCR) for the detection of Norovirus genogroup II (NVGII), Sapovirus (SV), and Human astrovirus (HAstV)

Multiplex PCR was conducted using the cDNA from RT step as template with specific mixed-primers (NVGII-, SV-, and HAstV-specific primers, Table 1) based on the modified method of Yan *et al*., 2003. The amplification reaction components contained 15.93 µl of RNase-free water, 2.5 µl of 10X PCR buffer (Invitrogen), 0.75 µl of 50 mM $MgCl_2$ (Invitrogen), 0.5µl of 10 mM dNTP mix (Promega), 0.2 µl of 20 µM COG2F primer, 0.2 µl of 20 µM G2-SKR primer, 0.66 µl of 20 µM SLV5317 primer, 0.66 µl of 20 µM SLV5749 primer, 0.2 µl of 20 µM PreCAP1 primer, 0.2 µl of 20 µM 82b primer, 0.2 µl of *Taq* DNA polymerase (5 units/µl; Invitrogen), and 3 µl of cDNA template from RT step, in a total volume of 25 µl. Then, the amplification was performed for 40 cycles under the following thermal cycling conditions: 94°C for 2 min to initiate denaturation, 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR product was detected by electrophoresis through 1.5% agarose gel in TBE buffer at 100 volts for 40 min. After staining with ethidium bromide, the size of amplification products generated by NVGII, SV, and HAstV specific primers were identified by comparing with GeneRuler™ 100bp DNA Ladder Plus marker (Fermentas) and reference strains of NVGII, SV, and HAstV (kindly provided by Professor Hiroshi Ushijima, the University of Tokyo, Japan).

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8. Nucleotide sequencing of partial capsid genes

The PCR products of partial capsid genes from NVGI, NVGII, SV, or HAstV were purified by QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's protocol. Initially, the PCR product was electrophoresed in 1.5% agarose gel containing 0.5 µl/ml ethidium bromide, followed by excision the DNA fragment from the agarose gel under UV transluminator. After weighing the gel slice, 3 volumes of QG buffer were added into 1 volume (mg~µl) of the gel slice and incubated at $50^{O}C$ for 10 min until the gel slice had completely dissolved. DNA fragment containing less than 500 bp or more than 4 kb, 1 volume of isopropanol was added into the mixture for increasing the yield of DNA. Then, the mixture was applied onto a QIAquick spin column, centrifuged at 14,000 rpm for 1 min. The flow-through filtrate was discarded and the QIAquick column was placed back into the same tube. Next, 0.75 ml of PE buffer was added to the column and centrifuged for 1 min. The flow-through filtrate was discarded again, and the column was centrifuged for an additional 1 min. After that, the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. The DNA bound to the column was eluted with 30 µl of EB buffer or H_2O. Finally, the amount of eluted DNA was measured at 260 nm by using spectrophotometer (BECKMAN, DU Series 500).

Nucleotide sequencing was performed using the BigDye Terminator Cycle Sequencing kit (PE Biosystem). Dideoxy-termination sequencing reaction was conducted according to the manufacturer's instruction. The cycle sequencing reaction was performed in 20 µl of the mixture containing 6 µl of terminator ready reaction mix, 100 ng of purified PCR product and 5 pmol of sequencing primer (Table 2). The cycle sequencing reaction was performed for 25 cycles in the thermal cycler

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(Eppendorf) as follows: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Then, the DNA product was purified by ethanol precipitation, washed with 70% ethanol and dried the pellet in a vacuum centrifuge. Finally, the nucleotide sequence of the DNA product was analyzed by using an automated DNA sequencer (ABI PRISM Model 3100 version 3.7, PE Biosystem) at the National Science and Technology Development Agency (NSTDA), Thailand.

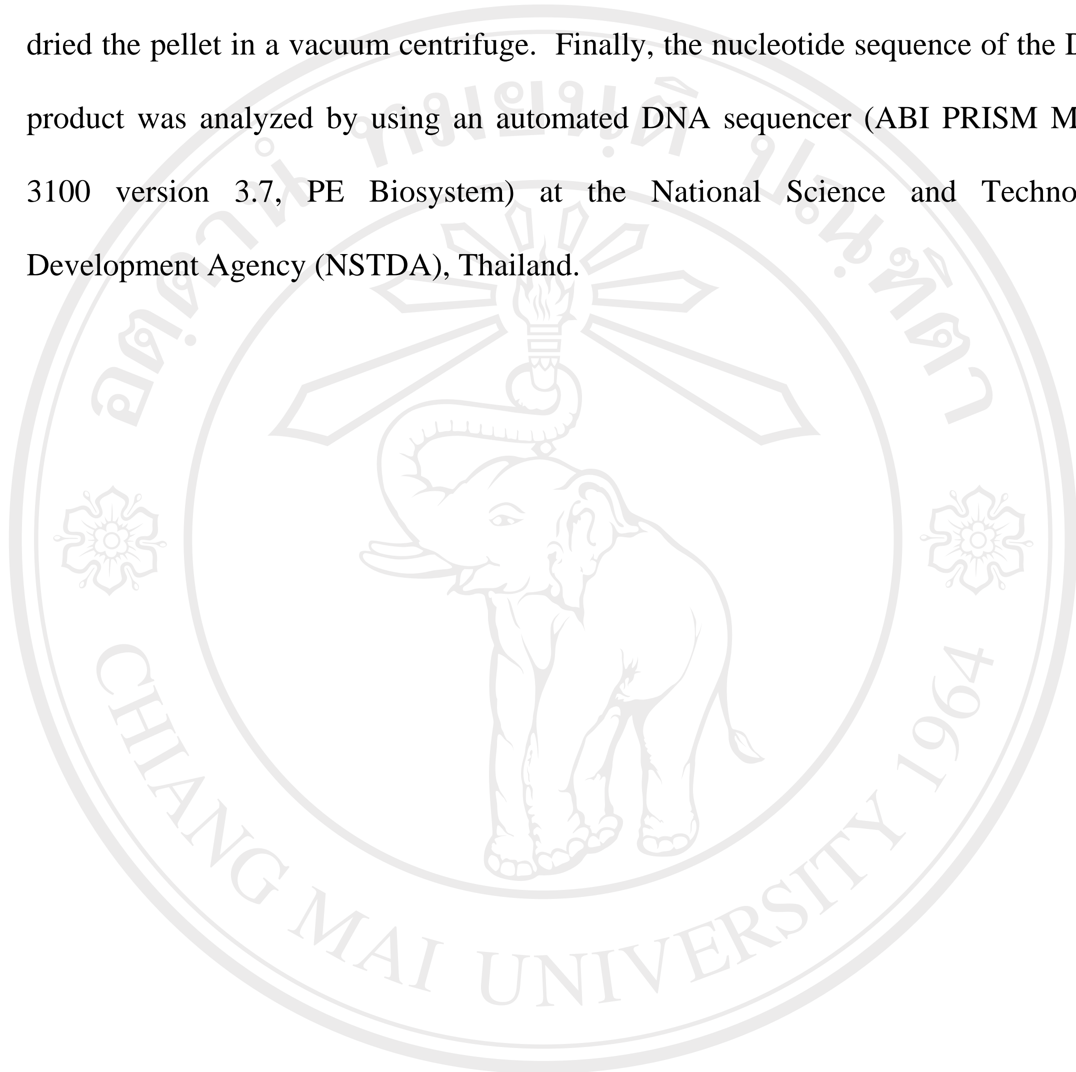

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Table 2. Oligonucleotide primers for nucleotide sequencing of partial capsid genes of NVGI, NVGII, SV, and HAstV

Primer	Polarity	Sequence (5′ to 3′)	Position No.
G1-SKF* for NVGI	+	CTGCCCGAATTYGTAAATGA	5342-5361
COG2F* for NVGII	+	CARGARBCNATGTTYAGRTGGATGAG	5003-5028
SLV5317* for SV	+	CTCGCCACCTACRAWGCBTGGTT	5083-5105
PreCAP1 for HAstV	+	GGACTGCAAAGCAGCTTCGTG	4235-4255

*IUB codes: B = C, G or T; H = A, C or T; N = any base; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

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9. Amino acid sequences and phylogenetic analysis of partial capsid genes

Nucleotide sequences of partial capsid genes of NVGI, NVGII, SV, and HAstV were translated into amino acid sequences using GeneDoc version 2 software. Amino acid sequences were imported to the BioEdit Sequence Alignment Editor, and multiple alignment was performed using the Clustal W algorithm. The phylogenetic trees were constructed based on amino acid sequences and were generated by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA version 3.1) software.

The following capsid sequences published in the GenBank were used in the phylogenetic analysis: NVGI: Appalachicola Bay/318/95/US (AF414406), Boxer/01/US (AF538679), Chiba/00/JP (AB042808), Chiba/000520/00/JP (AJ865481), Chiba/000782/00/JP (AJ865484), DSV395 (U04469), Hesse (AF093797), Koblenz/433/00/DE (AF394960), Musgrove/89/UK (AJ277614), Norwalk/68 (M87661), Saitama T36GI/01/JP (AB112133), Saitama T53aGI/02/JP (AB112134), Southampton (L07418), SRSV-KY-89/89/J (L23828), Stav/95/Nor (AF145709), Valetta/95/Malta (AJ277616), Whiterose/96/UK (AJ277610), Winchester/94/UK (AJ277609), WUG1 (AB081723)

NVGII: 416/97003156/96/LA (AF080559), Amsterdam/98-18/98/NET (AF195848), Arg320 (AF190817), Bristol (X76716), Chiba/000325/00/JP (AJ865524), Chiba/021050/02/JP (AJ865554), CS-E1/02/US (AY502009), Erfurt/546/00/DE (AF427118), Fayetteville/98/US (AY113106), Hawaii (U07611), Hillingdon/90/UK (AJ277607), J23/99/US (AY130762), Leeds/90/UK (AJ277608), Lordsdale/93 (X86557), M7/99/US (AY130761), Mex 7076/99 (AF542090), NongKhai-22/Thai (AY646866), NongKhai-51/Thai (AY646867), Oberhausen

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455/01/DE (AF539440), Saitama U25 (AB039780), Saitama U3 (AB039776), Sakaeo-14/Thai (AY646868), Seacroft/90/UK (AJ277620), Snow Mountain virus (AY134748), Tak-62/Thai (AY646877), Tiffin/99/US (AY502010), Toronto (U02030), VA97207/97 (AY038599), Vietnam 026 (AF504671), Westover/302/94/US (AF414418), Wortley/90/UK (AJ277618)

SV: 4408/Maizuru/JP (AB180209), 8/DCC/Tokyo/JP/44 (AB236377), Arg39 (AY289803), Chanthaburi-74/Thai (AY646854), Chiba/000671T/99/JP (AJ412805), Cruise/US (AY289804), Hou7-1181/90 (AF435814), Houston/90 (U95644), Karachi/874/92 (AB181129), Karachi/876/93 (AB181132), Karachi/877/90 (AB181133), Karachi/878/90 (AB181228), Karachi/938/94 (AB181248), Karachi/1021/92 (AB181230), Karachi/1026/92 (AB181134), London/92 (U95645), Lyon/598/97/F (AJ271056), Manchester/93 (X86560), Mex340/90 (AF435812), Moscow/2196/02/RF (AY538722), Moscow/4536/02/RF (AY538716), NongKhai-24/Thai (AY646856), NongKhai-50/Thai (AY646853), PEC (Porcine Enteric Calicivirus) (AF182760), Sapporo/82/JP (U65427), Songkhla-6/Thai (AY646857), Stockholm/318/97/SE (AF194182), Tak-69/Thai (AY646864)

HAstV: 816/93 (AB000286), CHN198 (AB037274), Dresden (AY720891), Goiania/GO/12/94/Brazil (DQ028633), HAstV-2 (L06802), HAstV-2 (2) (L13745), HAstV-3 (AF117209), HAstV-3 (2) (AF292074), HAstV-5 (AF292072), HAstV-6 (AF292077), HAstV-6 (2) (Z46658), HAstV-7 (AF248738), HAstV-7 (2) (Y08632), HAstV-8 (AF260508), HAstV-8 (2) (Z66541), O-15/88-532/Ehime1988 (AB025802), Oxford (L23513), and P437/91 (AB000285). Outgroups of NV, SV, and HAstV were Sapporo/82/JP (U65427), Southampton (L07418), and Turkey AstV (NC002470), respectively.

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