CHAPTER IV

MATERIALS AND METHODS

1. Stool specimens

A total of 332 fecal specimens were collected from adult patients with diarrhea at Maharaj Nakorn Chaing Mai between January to December, 2008. All specimens were stored at −70 °C unil used.

2. Preparation of 10% stool suspension

The fecal specimens were made to 10% (w/v) suspensions in phosphate-buffered saline (PBS), pH 7.2 and centrifuged at 5,000 rpm for 5 min, and the supernatants were used for viral genome extraction.

3. Extraction of viral genome

The genomes of both RNA and DNA viruses were extracted from 10% fecal suspensions using Geneaid kit according to the manufacturer's protocol. Firstly, 200 µl of 10% fecal supernatant were mixed with LB lysis buffer containing carrier RNA by pulse-vortexing for 15 sec. The mixture was incubated at 25°C for 10 min and 450 µl of AD buffer was added. After that, 600 µl of the mixture was loaded onto the Geneaid spin column and centrifuged at 13,000 rpm for 1 min. Then, the column was placed into new collecting tube and the remaining mixture was applied onto column and repeated the step above. The column was placed into a new 2 ml collecting tube and 450 µl of W1 buffer was added. The column was centrifuged at 13,000 rpm for

30 sec to remove unbound materials, and washed by adding of wash buffer, centrifuged at 13,000 rpm for 3 min to dry the column matrix. Then, placed the collecting tube into a new 1.5 ml microcentrifuge tube. Finally, 50 µl of RNase free water was added directly onto the column to elute viral genome. After incubating at 25°C for 1 min, the column was centrifuged at 13,000 rpm for 1 min for collecting the eluted genome. The column was spun down and the eluted viral genome was stored at - 70 °C until used as a template for a reverse transcription reaction.

4. Reverse transcription (RT) reaction

For RT, the viral RNA was reverse transcribed by reverse transcriptase using protocol according to the manufactures's instruction (Fermentas, Glen Burnie, MD, 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. Then, complementary DNA (cDNA) was synthesized using random hexamer primers (Takara, Shiga, Japan) and RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas, Glen Burnie, MD, 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 65 °C for 5 min to denature the secondary structure of the template and chill on ice. After that, the mixture was mixed with 4.0 µl of 5X reaction buffer (Fermentas, Glen Burnie, MD, USA), 2 µl of 10 mM dNTP mix. RNase-free water was added to give a total volume of 18.5 µl. Then, 0.5 µl of RNase inhibitor (Ribolock ^{TM} RNase Inhibitor 40 unit/µl; Fermentas, Glen Burnie, MA, USA) and 1 µl of RevertAid ^{TM} M-MuLV revese transciptase (200 units/µl; Fermentas, Glen Burnie, MA, USA) was added and incubated at 25°C for 10 min. The reverse transcription reaction was carried out at

42 °C for 1 hr, followed by heating at 70 °C for 10 min to inactivate the enzyme, and cooling at 4 °C immediately. The cDNA was stored at -20 °C for further used as a template in the multiplex polymerase chain reaction (Multiplex-PCR) for the detection of 10 types of diarrheal viruses.

5. Multiplex polymerase chain reaction (Multiplex-PCR) for the detection of diarrheal viruses

Multiplex-PCR is the method designed for amplication of cDNA generated from RT with mixed- primers in a single tube (Khamrin et al., 2011). Oligonucleotide primers for the detection of each virus are shown in Table 4. This method targeted to detect Sapovirus (SaV), Aichi virus (AiV), Group C rotavirus (RCV), Human parechovirus (HPeV), Norovirus GI (NoV GI), Norovirus GII (NoV GII), Enterovirus (EV), Adenovirus (AdV), Group A rotavirus (RAV), and Astrovirus (AstV). The amplification reaction components contained 3.7 µl of RNA-free water, 2.5 µl of 5X Colorless GoTaq® Flexi buffer (Promega, Madison, USA), 1.25 µl of 25 mM $MgCl_2$ (Promega, Madison, USA), 1 µl of 2.5 mM dNTP mix (Promega, Madison, USA), 2.5 µl of 20 µM mixed primer, 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, USA), and 1.5 µl of cDNA template from the RT step, in a total volume of 12.5 µl. A reaction mixture without template was used as negative control. The amplification was performed for 40 cycles under the following thermal cycling condition, 94 °C for 3 min to initiate denaturation, 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The specific PCR products were detected by electrophoresis through 2.5% agarose gel in TAE buffer at 100 volts for 40 min. The gel was stained with ethidium bromide (0.5 µl/ml) and

visualized under UV light. The targeted viruses were identified based on PCR product size by comparing with reference viruses and 100 bp DNA Ladder Plus marker (GeneRulur™, Fermentas, Glen Burnie, MD, USA).

For the genetic characterization of the detected viruses found in this study, nucleotide sequence and phylogenetic analyses were performed. AiVs were analyzed based on sequence analysis of the 3CD junction region using another set of primers different from the primer used in screening process, whereas HPeVs were characterized by amplification of VP1 gene. For other viruses, their genetic characterizations were performed by direct sequencing of the PCR products with the same primers used in multiplex-PCR screening method.

Table 4 Oligonucleotide primers used for the detection of 10 types of diarrheal viruses

Virus	Primer	Sequences (5'-3')	Positions No.	Polarity	Length (bp)	Reference
SaV	SLV5317	CTCGCCACCTACRAWGCBTGGTT	5124-5146	+	100	Yan et al. (2003)
	SMP-R	CMWWCCCCTCCATYTCAAACAC	5202-5223	-		Khamrin et al. (2011)
AiV	C94b	GACTTCCCCGGAGTCGTCGTCT	6398-6419	+	158	Yamashita et al. (2000)
	AiMP-R	GCRGAGAATCCRCTCGTRCC	6536-6555	-		Khamrin et al. (2011)
RCV	GCMP-F	CAAATGATTCAGAATCTATTG	500-520	+	205	Khamrin et al. (2011)
	G8NA2	GTTTCTGTACTAGCTGGTGAA	684-704	-		Yan et al. (2004)
HPeV	Ev22(+)	CYCACACAGCCATCCTC	312-328	+	270	Joki-Korpela and Hyypia (1998)
	Ev22(-)	TRCGGGTACCTTCTGGG	565-581	-		Joki-Korpela and Hyypia (1998)
NoV GI	G1SKF	CTGCCCGAATTYGTAAATGA	5342-5361	+	330	Yan et al. (2003)
	G1SKR	CCAACCCARCCATTRTACA	5653-5671	-		Yan et al. (2003)

International Union of Biochemistry (IUB) base code

IUB code	R = A or G	B = C, G or T	K= G or T	H= A, C or T	M= A or C	V= A or C
	Y= C or T	D= A, G or T	S = G or C	W=A or T	N= A, T, C	

Table 4 (Continue)

Virus	Primer	Sequences (5'-3')	Positions No.	Polarity	Length (bp)	Reference
NoV GII	COG2F	CARGARBCNATGTTYAGRTGGATGAG	5003-5028	+	387	Yan et al. (2003)
	G2SKR	CCRCCNGCATRHCCRTTRTACAT	5367-5389	-		Yan et al. (2003)
EV	F1	CAAGCACTTCTGTTTCCCCGG	160-180	+	440	Zoll et al. (1992)
	R1	ATTGTCACCATAAGCAGCCA	580-599	-		Zoll et al. (1992)
AdV	Ad1	TTCCCCATGGCICAYAACAC	1834-1853	+	482	Yan et al. (2004)
	Ad2	CCCTGGTAKCCRATRTTGTA	2296-2315	-		Yan et al. (2004)
RAV	VP7'(F)	AAAGGATGGCCAACAGGATCAGT	373-395	+	569	Yan et al. (2004)
	End 9 (s)	GTATARAAHACTTGCCACCAT	921-941	-		Khamrin et al. (2011)
AstV	PreCAP1	GGACTGCAAAGCAGCTTCGTG	4235-4255	+	719	Yan et al. (2003)
	82b	GTGAGCCACCAGCCATCCCT	4934-4953	-		Yan et al. (2003)

IUB code R = A or G B = C, G or T K= G or T H= A, C or T M= A or C V= A or C

Y= C or T D= A, G or T S = G or C W=A or T N= A, T, C

6. Polymerase chain reaction for identification of Aichi virus genotypes

The detected AiVs were futher characterized for their genotypes using specific primers 6261 and 6779, which targeted to amplify the C terminus of 3C and the N terminus of 3D of regions of 519 bp product as described previously by Yamashita et al. (2000). The specific primers that used for AiV genotype identification are shown in Table 5. The amplification reaction components contained 5.75 µl of RNA-free water, 2.5 µl of 5X Colorless GoTaq® Flexi buffer (Promega, Madison, USA), 1.25 µl of 25 mM $MgCl_2$ (Promega, Madison, USA), 1 µl of 2.5 mM dNTP mix (Promega, Madison, USA), 0.5 µl each of 20 µM primers 6261 (forward) and 6779 (reverse), 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, USA), and 1 µl of cDNA template from the RT step, in a total volume of 12.5 µl. A reaction mixture without template was used as negative control. The amplification was perfomed for 40 cycles under the following thermal cycling condition, 94 °C for 3 min followed by, 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The specific PCR products were detected by electrophoresis through 1.5% agarose gel in TAE buffer at 100 volts for 40 min. The expected PCR product size of partial 3CD region was 519 bp. The PCR product was purified and further subjected to nucleotide sequencing and phylogenetic analyses.

7. Nested polymerase chain reaction (Nested-PCR) for the confirmation of human parechoviruses (HPeV)

HPeV was screened using primer Ev22(+) and Ev22(-) which are specific for amplification of 270 bp PCR product of the 5'untranslated region (5' UTR) described previously by Joki-Korpela and Hyypia et al. (1998). For classification of HPeV, genotype identification was done based on the sequence of VP1 region. Amplification of VP1 was done by nested-PCR. The first round PCR reaction mixture contained 2.5 µl of 5X Colorless GoTaq® Flexi buffer (Promega, Madison, USA), 1.25 µl of 25 mM $MgCl_2$ (Promega, Madison, USA), 1 µl of 2.5 mM dNTP mix (Promega, Madison, USA), 0.05 µl of GoTaq® Flexi DNA polymerase (Promega, Madison, USA), and 1 µl of cDNA with external primers (VP1-parEchoFand VP1-parEchoR) as shown in Table 5 at a concentration of 20 µM of each primer, in a total volume of 12.5 µl. The thermal cycling conditions were as follows ; 94 °C for 3 min to initiate denaturation, 94 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The amplification was performed for 40 cycles. The expected PCR product size was 760 bp.

First round PCR products were used as a template in second round PCR reactions with internal primers (VP1-parEchoF1 and VP1-HPeV-R) as shown in Table 5 at a concentration of 20 µM of each primer. The nested PCR reaction and cycling conditions were the same as those for external PCR. The amplicons were visualized by 1.5% agarose gel electrophoresis. The expected PCR product size was 477 bp.

Table 5 Specific primers used for the confirmation of Aichi viruses and human parechoviruses

Primer	Sequence (5'-3')	Position No.	Polarity	Length (bp)	Reference
Polymerase chain reaction					
6261	ACACTCCCACCTCCCGCCAGTA	6261-6282	+	519	Yamashita et al. (2000)
6779	GGAAGAGCTGGGTGTCAAGA	6779-6760	-		Yamashita et al. (2000)
Nested-PCR 1st PCR					
VP1-parEchoF	CCAAAATTCRTGGGGTTC	2332-2349	+	760	Pham et al. (2010)
VP1-parEchoR	AAACCYCTRTCTAAATAWGC	3090-3071	-		Pham et al. (2010)
Nested-PCR 2nd PCR					
VP1-parEchoF1	CCAAAATTCRTGGGGTTC	2332-2349	+	477	Benschop et al. (2006)
VP1-HPeV-R	GTCATYTGYTGYTCHCCWGCNGG	2789-2808	-		Thongprachum (unpublished)

IUB code R = A or G K= G or T H= A, C or T M= A or C Y= C or T W=A or T

N= A, T, C

8. Nucleotide sequencing and phylogenetic analyses of sapoviruses, Aichi viruses, human parechoviruses, noroviruses GI, noroviruses GII, enteroviruses, adenoviruses and astroviruses

8.1 Purification of PCR products

All PCR products of SaV, AiV, HPeV, NoV GI, NoV GII, EV, AdV or AstV were purified by Geneaid gel Extraction kit (Geneaid, Taipei, Taiwan) according to the manufacturer's protocol. Firstly, the PCR products were electrophoresed in 1.5% agarose gel containing 0.5 μl/ml ethidium bromide. Then, the DNA fragment of expected size was excised under the UV transluminator. The gel slice was transfered to a 1.5 ml microcentrifuge tube, 500 μl of DF buffer were added to the gel slice, and mixed by vortex and followed by incubation at 60 °C for 10 min or until the gel slice was completely dissolved. Then, the mixture was applied onto a spin column and centrifuged at 13,400 rpm for 30 sec. The flow-through filtrate was discarded. Six hundred μl of W1 buffer was added and centrifuged for 30 sec, followed by adding of 600 μl of wash buffer and centrifuged again for 30 sec. The flow-through filtrate was discarded, and the column was centrifuged for an additional 1 min to remove residual ethanol. After that, the spin column was placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted with 30 μl of elution buffer. The column was standed for 1 min and then centrifuged for 1 min. Finally, the quantity and quality of the purified DNA was assessed by electrophoresis in 1.5% agarose gel (Amresco, Cochran, USA).

8.2 Nucleotide sequencing

The purified PCR products were sequenced directly by fluorescence based cycle sequencing method using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA). Each sequencing reaction consisted of 8.0 µl of premix, 1.0 µl of 5 µM sequencing primer. List of sequencing primers for each virus is shown in Table 6. One hundread ng of purified PCR product, and H_2O were added in order to adjust the final volume to 20 µl. Cycle sequencing was performed in a thermocycler using the following profiles: initial denaturation at 96°C for 1 min, and 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min. Then, the sequencing DNA product was precipitated in cooled absolute ethanol and washed with 70% ethanol and then dried the pellet at 50 °C for 10 min. Finally, the sequencing product was analyzed on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems, Carlsbad, USA) at the Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University.

8.3 Phylogenetic analysis

The obtained nucletide sequences of NoV GII, AdV, HPeV, EV, and AiV were edited manually by the FinchTV (Geospiza, Seattle, USA) software. Multiple sequence alignments were created using ClustalX (version 1.81), and SeaView Sequence Alignment Editor Program. Phylogenetic trees for each type of virus were constructed using the neighbor-joining method by MEGA (version 4) software. The nucleotide sequences deposited in the GenBank database were used for phylogenetic analysis, virus strains and accession numbers are shown in Table 7.

Table 6 Oligonucleotide primers for nucleotide sequencing of Aichi viruses, noroviruses GII, adenoviruses, enteroviruses, and human parechoviruses

Primer	**Target region**	**Polarity**	**Sequence (5' to 3')**	**Position No.**
6779 for AiV	3CD	-	GGAAGAGCTGGGTGTCAAGA	6779-6760
G2-SKR for NoV GII	Capsid	-	CCRCCNGCATRHCCRTTRTACAT	5367-5389
R1 for EV	5'-UTR	-	ATTGTCACCATAAGCAGCCA	599-580
Ad2 for AdV	VP1	-	CCCTGGTAKCCRATRTTGTA	2296-2315
VP1-HPeV-R for HPeV	VP1	-	GTCATYTGYTGYTCHCCWGCNGG	4390-4409

IUB code R = A or G K= G or T H= A, C or T Y= C or T N= A, T, C

Table 7 List of the virus reference strains and accession numbers used in the phylogenetic analyses of adenoviruses, Aichi viruses, noroviruses GII, human parechoviruses, and enteroviruses

Virus	Type	Accession number	Strain name
AdV	AdV1	AF534906	-
	AdV2	J01917	-
	AdV3	X76549	-
	AdV3	GQ477371	A546
	AdV4	X84646	-
	AdV4	GQ477377	G123
	AdV7	Z48571	-
	AdV8	AB500121	Sapporo-1994
	AdV12	X73487	-
	AdV12	GQ477372	B456
	AdV15	DQ149617	-
	AdV15	AB562586	CH38
	AdV17	AF108105	-
	AdV18	DQ149610	-
	AdV24	DQ149622	-
	AdV24	AB330105	AV-3153
	AdV25	DQ149623	-
	AdV25	AB330106	BP-1
	AdV26	AB330107	BP-2
	AdV32	AB330113	HH

Table 7 (continue)

Virus	Type	Accession number	Strain name
AdV	AdV40	GQ477379	123
	AdV41	X51783	-
		EU603438	Lopburi057
		EU603441	Lopburi078
	AdV51	AB330132	Bom
	AdV52	DQ923112	T03-2244
Bovine adenovirus (outgroup)	-	AY388617	-
AiV	Genotype A	AB010145	-
	Genotype A	AB040749	-
	Genotype A	AB034652	A848/88
	Genotype A	EF079158	B-370/05
	Genotype A	EF079161	VN-636/03
	Genotype A	AB034655	N128/91
	Genotype A	EF466013	JPN-4383
	Genotype A	AY747174	BAY/1/03
	Genotype A	AB092828	494/97
	Genotype A	EU159251	E1859
	Genotype A	DQ145762	R380
	Genotype A	FJ872481	Monasti/20/2004
	Genotype A	AB034650	A1471/96
	Genotype A	AB092826	364/96
	Genotype A	EF079154	J-4827/02

Table 7 (continue)

Virus	Type	Accession number	Strain name
AiV	Genotype A	EF079160	T-132/02
	Genotype B	FJ890517	Chshc6
	Genotype B	FJ890522	Chshc4
	Genotype B	FJ890521	Chshc3
	Genotype B	GU339099	58118
	Genotype B	GU339099	58174
	Genotype B	EU715251	Qld/2008
	Genotype B	GU459258	Fujian/1021/2008
	Genotype B	AB092830	139/96
	Genotype B	AB034662	P880/91
	Genotype B	AB092833	488/97
	Genotype B	DQ028632	GO/03/01
	Genotype B	AB092834	700/98
	Genotype B	EF079157	B-171/05
	Genotype B	EF079159	B-5/05
	Genotype B	EF079159	B-1055/05
	Genotype B	EF079156	B-101/05
	Genotype B	AB034654	N1277/91
	Genotype B	AB034657	M166/92
	Genotype C (outgroup)	DQ145759	RN48

Table 7 (continue)

Virus	Type	Accession number	Strain name
NoV GII	GII/1	U07611	Hawaii
	GII/2	X81879	Melsham
	GII/3	AB058582	Saitama KU80aGII
	GII/4	AB067542	U201 GII
	GII/4	AB541234	Chiba 5/2007
	GII/4	EU703755	Beijing/2007
	GII/4	AB541344	Sakai4/2007
	GII/4	EU703746	Beijing 74/2007
	GII/4	GU991353	Shanghai/SH2/2007
	GII/4	AB447455	Ehime 5/2006
	GII/4	EU703750	Beijing/162/2007
	GII/4	HM635147	Seoul/2071/2008
	GII/4	AB541303	Nagano2/2007
	GII/4	AB541324)	Osaka3/2008
	GII/4	AY587958	SAkaeo-14
	GII/4	AB541267	Hokkaido5/2007
	GII/4	E187497F)	N7327/2006
	GII/4	DQ078814	Hunter 504D/040
	GII/4	AB220926	Chiba/2004
	GII/4	X767716	Bristol
	GII/5	AJ277607	Hillingdon
	GII/6	AB039776	Saitama U3
	GII/7	AJ277608	Leed
	GII/8	AB067543	U25

Table 7 (continue)

Virus	Type	Accession number	Strain name
NoV GII	GII/9	AY054229	Idaho
	GII/10	AY237415	Mc37
	GII/11	AB112221	Saitama
	GII/12	AB039775	Saitama U1
	GII/13	AY130761	M7/1999
	GII/14	AB078334	Kashiwa 47
	GII/16	AB112260	Saitama
	GII/17	AF195847	Alphatron/98-2
NoV GI (outgroup)		M87661	
NoV GII/4 variant	Hunter	DQ078794	Hunter284E/040
	Fermington Hill	AB541267	Hokkaido5
	Fermington Hill	AY587985	Oxford/B4S6/2002
	Fermington Hill	DQ658413	MD/200
	2006a	EF187497	NZ327/2006
	2006b	AB541324	Osaka3/2008
	2006b	GU991354	SH5/2009
	2006b	AB541303	Nagano2/2007
	2006b	AB543808	FUMI/2010
	2006b	AB447440	Hokkaido3/2006
	2006b	AB447456	Saga1/2006
	2006b	FJ514242	CUK-3/2008
	2008a	GQ303445	Mannheim131/2009
	2008a	HM738973	NSW892U/2009
	2008a	GQ845367	NSW001P/2008

Table 7 (continue)

Virus	Type	Accession number	Strain name
NoV GII/4 variant	2008a	AB541272	Iwate3/2008
	2008a	AB541310	Nigata1/2008
	2008a	AB541320	Osaka1/2008
	2008a	AB541202	Achi1/2008
	2008b	AB492092	Stockholm/19865
	2007 (outgroup)	AJ844470	Chiba/04502
HPeV	HPeV1	EU024634	BNI-R21
	HPeV1	AB443809	03-0812
	HPeV1	FJ373135	677008
	HPeV1	FJ648762	T-141
	HPeV1	GQ183025	K63-94
	HPeV1	AB112487	A10987-00
	HPeV1	HQ163882	JP-8275
	HPeV1	GQ149453	T-90
	HPeV1	FJ648761	T-69
	HPeV1	FJ648757	T-96
	HPeV1	FJ646759	T-103
	HPeV2	AJ005695	-
	HPeV2	NC001897	-
	HPeV3	FJ648756	T-86
	HPeV3	FJ648758	T-102
	HPeV3	GQ183028	-
	HPeV3	AJ889918	Can82853-01
	HPeV4	AM235750	T-75-4077

Table 7 (continue)

Virus	Type	Accession number	Strain name
HPeV	HPeV4	GU125398	R15492/1999/HUN
	HPeV4	DQ315670	K251176-02
	HPeV5	AM23549	T-92-15
	HPeV5	HQ696576	BR/77/2006
	HPeV6	AB252582	NII561-2000
	HPeV6	FJ888592	SH6
	HPeV6	EU077518	2005-823
	HPeV7	EU556224	-
	HPeV8	EU716175	BR/217/2006
Saffold virus (outgroup)		EF165067	-
EV	CV-A2	AY421760	Fleetwood
	CV-A3	AY421761	Olson
	CV-A13	AF465511	
	CV-A14	AY421769	G-14
	CV-A17	AF329688	G-12
	CV-A17	DQ995645	BAN01-10577
	CV-A20	EF015014	BAN01-10618
	CV-A20	EF015015	BAN01-10529
	CV-A20	EF015017	BAN01-10447
	CV-A20	AF412354	-
	CV-A20	EF015020	MOR83-10617
	CV-A24	D90457	IH-35
	CV-A24	EF026081	Joseph

Table 7 (continue)

Virus	Type	Accession number	Strain name
EV	Poliovirus 2	M12197	Lansing
	Poliovirus 2	EF628441	V2-Sak
	POliovirus 3	FJ859192	-
	Poliovirus 3	AJ783739	MF1
	Poliovirus 3	K01392	Leon/37
	Poliovirus 3	FJ460227	31974
	Echovirus 9	X84981	Hill
	Echovirus 9	AF524866	Barty
	Echovirus 9	DQ530416	Seoul-21
	Echovirus 11	GQ126866	-
	Echovirus 25	AY302549	JV-4
	Echovirus 30	AF311938	Bastianni
	Echovirus 25	HM031191	HN-2
	Echovirus 30	AM237034	-
	Enterovirus 68	AY426531	Fermon
	Enterovirus 96	FJ571915	FIN05-2
	Enterovirus 99	EF015010	BAN04-01679
	Enterovirus 99	EF555644	USA-GA84
Bovine enterovirus (outgroup)	-	NC001859	-