CHAPTER III

MATERIALS AND METHODS

1. Sample collection

Twenty one samples from pigs with a history of PED infection in fifteen swine commercial farms located in the central part of Thailand (including Nakornpathom, Ratchaburi, Chachoengsao and Ayuthaya provinces) were submitted to The Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakornpathom province during May 2011- December 2012. A total of twenty one samples included TH/NP-156/11, TH/NP-795/11, TH/RB-1421/1, TH/RB-807/11, TH/RB-833/11, TH/RB-KHF/11, TH/CS-866/11, TH/CS-1019/11, TH/RB-15/12, TH/NP-68/12, TH/RB-123/12, TH/CS-65/12, TH/RB-236/12, TH/NP-79/12, TH/NP-657/12, TH/CS-80712/12, TH/RB-881/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/NP-1157/12 and TH/NP-1169/12. The fresh or frozen of whole small intestinal, also stool samples (at least 10 grams) were taken from dead piglets at age less than 4 weeks except 1 fecal sample was taken from a fattening pig (15-20 weeks of age). All cases showed clinical signs of watery diarrhea, dehydration and vomiting. The detail of sample collection is shown in Table 1.

Furthermore, intestinal mucosa was collected by scraping technique from duodenum and the upper part of jejunum, especially in the thin wall area or gas content inside the lumen. All samples were diluted with sterile phosphate buffered saline solution (PBS; 0.1 M, pH 7.2) to be 10% suspension (Kim et al., 2001; Song et al., 2006; Chen et al., 2008). The suspensions were vortexed and centrifuged at 10,000 rpm for 20 minutes. The supernatants were collected and filtrated through 0.8 um Millipore filters (Corning Inc., USA) to eliminate the residue tissue debris. The filtrates were kept at -80 °C until use.

Table1: The data of PEDV samples which were submitted during 2011-2012.

Number of isolates	Location	Date submitted	Sample origin	Age	Clinical sign	History taking			
						%morbidity	%mortality	Previous outbreak	Vaccine status
TH/NP-156/11	Nakornpathom	17-Aug-11	small intestine	3-4 days	watery diarrhea	80%	40%	-	vaccinated
TH/RB-1421/11	Ratchaburi	28-Sep-11	small intestine	4 days	watery diarrhea	NA	NA	-	-
TH/NP-795/11	Nakornpathom	12-Oct-11	small intestine	5 days	watery diarrhea / dehydration and MMA in lactating sows	50%	10%	-	vaccinated
TH/RB-807/11	Ratchaburi	13-Oct-11	small intestine	7 days	watery diarrhea	NA	NA	-	-
TH/RB-833/11	Ratchaburi	20-Oct-11	small intestine	7 days	watery diarrhea	NA	NA	-	-
TH/RB-KHF/11*	Ratchaburi	29-Oct-11	small intestine	5-7 days	watery diarrhea	NA	NA	Sep-11 (TH/RB-1421/11)	-
TH/CS-866/11	Chachoengsao	2-Nov-11	small intestine	5 days	watery diarrhea	NA	NA	-	-
TH/CS-1019/11	Chachoengsao	17-Dec-11	small intestine	5 days	watery diarrhea	NA	NA	-	-
TH/RB-15/12	Ratchaburi	10-Jan-12	small intestine	7 days	watery diarrhea	70%	-	-	-
TH/NP-68/12	Nakornpathom	26-Jan-11	small intestine	3-4 days	watery diarrhea / vomiting	100%	100%	2011	vaccinated
TH/RB-123/12*	Ratchaburi	16-Feb-12	small intestine	4-10 days	watery diarrhea	70%	70%	Oct-11 (TH/RB-KHF/12)	-
TH/CS-65/12*	Chachoengsao	10-May-12	small intestine	15 days	watery diarrhea	100%	100%	Dec-11 (TH/CS-1019/11)	-

(*Continue*)

Number of isolates	Location	Date submitted	Sample origin	Age	Clinical sign	History taking			
						%morbidity	%mortality	Previous outbreak	Vaccine status
TH/RB-236/12	Ratchaburi	15-May-12	feces	lactating sow	watery diarrhea	NA	NA	-	-
TH/RB-79/12	Ratchaburi	29-May-12	small intestine	3-4 days	watery diarrhea	20%	-	-	-
TH/NP-657/12	Nakornpathom	28-Aug-12	small intestine	2-3 days	watery diarrhea	30%	10%	2008	vaccinated
TH/CS-80712/12*	Chachoengsao	10-Oct-10	small intestine	< 7 days	watery diarrhea	NA	NA	Nov-11 (TH/CS-866/11)	-
TH/RB-881/12	Ratchaburi	30-Oct-12	small intestine	3-5 days	watery diarrhea	NA	NA	-	vaccinated
TH/AY-2.2/12	Ayutthaya	17-Oct-12	minced small intestine	3 days	watery diarrhea	NA	NA	Jan-11	-
TH/AY-2.7/12*	Ayutthaya	17-Oct-12	minced small intestine	3 days	watery diarrhea	NA	NA	Jan-11 (TH/AY-2.2/12)	-
TH/NP-1157/12	Nakornpathom	20-Dec-12	small intestine	14 days	watery diarrhea	NA	NA	-	-
TH/NP-1169/12*	Nakornpathom	25-Dec-12	feces	15-20 wks	watery diarrhea	NA	NA	Oct-11 (TH/NP-795/11)	vaccinated

*re-outbreak sample

2. RNA extraction and RT-PCR

2.1 RNA extraction

The viral RNA was extracted from fecal or intestinal samples using Invisorb® Spin Virus RNA Mini Kit (Stratec molecular, Germany) following the method of manufacture's recommendation. Briefly, approximately 200 ul of filtered supernatant was mixed with lysis buffer, proteinase K and carrier RNA. The binding solution will be added to bind the viral RNA. Washing buffer was used to remove the residue contamination. After that, the viral RNA was eluted by using elution buffer. Finally, viral RNA volume was prepared in a total of 100 ul. The viral RNA was kept at -20°C until use.

2.2 Reverse-transcription polymerase chain reaction (RT-PCR)

The RT-PCR was performed in a final volume of 50 µl using one-step RT-PCR system kit (Access quick™, Promega, USA). PEDV primer sets for N gene amplification were designed based on PEDV strain S (accession number DQ355223.1). Primer set of S gene was obtained from Song et al. (2006). The primer sequences and PCR product sizes are shown in Table 2.

Table2. Primer sequences of S and N gene.

Primers	Protein gene sequences	Location	PCR product size
S gene (Song et al., 2006)	5' -TTCTGAGTC ACGAACAGCCA-3'	1466-1485 [A]	651 bp
	5'-CATATGCAGCCTGCTCTGAA-3'	2097-2116 [A]	
N gene	N1 : 5'-TGCGGTTCTCACAGATAGTG-3'	40-59 [B]	738 bp
	5'-ATCCTTGACAGCAGCCACC-3'	759-777 [B]	
	N2 : 5'-CACAGAATCGTGGAAATAACC-3'	617-636 [B]	845 bp
	5'- ACTACCCTGGAACATAGCC-3'	1443-1461 [B]	
	N3 : 5'- CTAAACAGAAACTTTATGGCTT-3'	79-100 [B]	760 bp
	5'- ATGTCTTTGAGGTCACGTTC-3'	907-926 [B]	
	N4 : 5'- CTTCTCAGAACAGAGGAGG-3'	650-668 [B]	848 bp
	5'- GTGTCACCACCATCAACAG-3'	1358-1376 [B]	

[A] Porcine epidemic diarrhea virus strain DR13 spike protein gene (accession number DQ862099.1)

[B] Porcine epidemic diarrhea virus strain S nucleocapsid protein (N) gene (accession number DQ355223.1)

The amplification steps of S gene were the following conditions: Reverse transcription at 48°C for 45 minutes, initial step at 95°C for 2 minutes, followed by 30 cycles of amplification step including denaturation at 94°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes (Song et al., 2003). The amplification step of the most N gene primer sets were similar to S gene, except the annealing temperature of the N1 primer set which was 60°C for 1 minute.

The amplicon were separated in 1% agarose gels (Vivantis technologies,USA.) which was immersed in tris-acetic acid-EDTA (TAE) buffer, stained with nucleic acid gel stain (GelStar® Nucleic Acid Gel Stain, Lonza, USA), and visualized by UV transilluminator.

3. Sequence analysis

Gel purification was performed as the manufacturing protocol by using NucleoSpin Extract II (Macherey Nagel, Düren, Germany). Then, the products were submitted for genetic sequencing at 1st BASE Pte Ltd., in Singapore. The nucleotide sequences were compared with other PEDV strains that have been previously published in NCBI Genbank database using the MEGA version 5 (phylogenetic and molecular evolutionary analyses software). The nucleotide sequence of PEDV reference strains of S gene and N gene are showed in Table 3 and Table 4, respectively.

Table3: The reference PEDV isolates used for partial S gene comparisons.

Countries origin	Strains and accession numbers
Thailand	07NP01 (FJ196196.1), 08CB01 (FJ196197.1), 08CB02 (FJ196198.1), 08CB03 (FJ196199.1), 08CB04 (FJ196200.1), 08CB05 (FJ196201.1), 08CB06 (FJ196202.1), 08CC01 (FJ196203.1), 08NP03 (FJ196205.1), 08NP04 (FJ196206.1), 08NP05 (FJ196207.1), 08NP06 (FJ196208.1), 08NP07 (FJ196209.1), 08NP08 (FJ196210.1), 08PB01 (FJ196211.1), 08PC01 (FJ196212.1), 08RB01 (FJ196213.1), 08RB02 (FJ196214.1), 08RB03 (FJ196215.1), 08RB04 (FJ196216.1), 08RB05 (FJ196217.1), 08RB06 (FJ196218.1), 08RB07 (FJ196219.1), 08UB01 (FJ196220.1), KU01CB08 (FJ196221.1), KU02NK08 (FJ196222.1), KU03CB08 (FJ196223.1), KU04RB08 (FJ196224.1), KU05CB08 (FJ196225.1), KU06RB08 (FJ196226.1), KU07RB08 (FJ196227.1), KU08RB08 (FJ196228.1)

Vietnam	VN103S4 (HQ883488.1), VN109S5 (HQ883489.1), VN112S6 (HQ883490.1), VN116S7 (HQ883491.1), VN122S8 (HQ883492.1), VN92S1 (HQ883485.1), VN94S2 (HQ883486.1), VN97S3 (HQ883487.1)
China	AJ1102 (JX188454.1), BJ-2011-1 (JN825712.1), BJ-2011-2 (JN825706.1), BJ-2011-3 (JX435298.1), BJ-2012-1 (JX435299.1), BJ-2012-2 (JX435300.1), CH/AHHF/2012 (JX018181.1), CH/AHHF-2/2012 (JX018182.1), CH/AY/11 (JQ627653.1), CH/CG/11 (JQ627654.1), CH/CY/12 (JX501317.1), CH/FJND-1/2011 (JN543367.1), CH/FJND-2/2011 (JN315706.1), CH/FJND-3/ 2011 (JN381492.1), CH/FJXM-1/2012 (JX070671.1), CH/FJXM-2/2012 (JX070672.1), CH/GXNN/2012 (JX018179.1), CH/HBQX/10 (JX501318.1), CH/HBSN/2012 (JX018183.1), CH/HBXX2/11 (JX501319.1), CH/HBXX3/11 (JX501320.1), CH/KF/11 (JQ257005.1), CH/S (JN547228.1), CH/TY/12 (JX501321.1), CH/XC/12 (JX501322.1), CH/XCYL/11 (JX501323.1), CH/YNKM/2012 (JX018180.1), CH/YY/11 (JQ257006.1), CH/ZMDZY/11 (KC196276.1), CH/ZY/11 (JQ257007.1), CH1 (JQ239429.1), CH13-GX (JQ979288.1), CH17-GZ (JQ979289.1), CH18-Hainan (JQ979291.1), CH2 (JQ239430.1), CH22-JS (JQ979290.1), CH3 (JQ239431.1), CH4 (JQ239432.1), CH5 (JQ239433.1), CH6 (JQ239434.1), CH7 (JQ239435.1), CH8 (JQ239436.1), CH9-FJ (JQ979287.1), CHGD-01 (JN980698.1), CH-HKC-08-2011 (JX242462.1), LC (JX489155.1), CH-SHT-12-2011 (JX242464.1), FQ/FJ/2012 (JX258672.1), GD-1 (JX647847.1), GD-A (JX112709.1), GD-B (JX088695.1), HB-2011-1 (JN825707.1), HB-2011-3 (JN825709.1), HB-2011-4 (JX435301.1), HB-2012-1 (JX435302.1), HB-2012-2 (JX435303.1), HB-2012-3 (JX435304.1), HB-2012-4 (JX435305.1), HBMC2012 (JX163294.1), HLJ-2012 (JX512907.1), HuN (JQ517274.1), JS-2004-2 (AY653204.1), ZJ-2011-1 (JN825710.1), ZJ-2011-2 (JN825711.1), ZJCZ4 (JX524137.1), LZC (EF185992.1), SD-M (JX560761.1), CV777 Chinese (JN599150.1), LJB/03 (DQ985739.1), DX (JN104080.1)
Japan	KH (AB548622.1), MK (AB548624.1), NK 9 (AB548623.1), 83P-5 (AB548618.1), 83P-5_100th-passaged (AB548621.1), 83P-5_34th-passaged (AB548619.1), 83P-5_61st-passaged (AB548620.1)
Korea	CNU-091222-01 (JN184634.1), CNU-091222-02 (JN184635.1), KNU-0801 (GU180142.1), KNU-0802 (GU180143.1), KNU-0901 (GU180144.1), KNU-0902 (GU180145.1), KNU-0903 (GU180146.1), KNU-0904 (GU180147.1), KNU-0905 (GU180148.1), virulent_DR13 (JQ023161.1), attenuated_DR13 (JQ023162.1), DQ462404.2_DR13 (DQ462404.2), DR13 (DQ862099.1), SM98 (GU937797.1), Spk1 (AF500215.1), Chinju99 (AY167585.1)

Britain	Br1/87 (Z25483.1)
Belgium	CV777 (AF353511.1)

Table4: The reference isolates of PEDV used for partial N gene comparisons.

Countries origin	Strains and accession number
China	AJ1102 (JX188454.1), BJ-2011-1 (JN825712.1), YT12-4 (JX406145.1), WS12-2 (JX406143.1), JY12-3 (JX406139.1), JN12-2 (JX406137.1), DZ12-1 (JX406135.1), TA12-3 (JX406142.1), LY12-3 (JX406140.1), JN12-3 (JX406138.1), DZ12-3 (JX406136.1), CQ12-2 (JX406134.1), HB/GY (JQ934948.1), CH/FJND/2011 (JN601055.1), CH/FJND-3/2011 (JQ282909.1), CH/GXWM/2011 (JQ743656.1), CH/GXQZ/2011 (JQ743654.1), CH/GXWP/2011 (JQ743652.1), CH/HLJHRB/2011 (JQ743650.1), CH/BJSY/2011 (JQ735953.1), CH/ZJHZ/2011 (JQ743655.1), CH/XIUrumqi/2011 (JQ743653.1), CH/HLJHH/2011 (JQ743651.1), CH/GXNN/2011 (JN601062.1), CH/SDRZ-1/2011 (JN601060.1), CH/BJYQ-1/2011 (JN601053.1), CH/BJYQ-2/2011 (JN601054.1), CH/HNZZ/2011 (JN601052.1), CH/SDRZ-2/2011 (JN601061.1), CH/HLJHG/2011 (JN601059.1), CH/GDQY-1/2011 (JN601056.1), CH/GDQY-2/2011 (JN601057.1), CH/GDQY-3/2011 (JN601058.1), CH/IMB/06 (FJ473387.1), CH/HNCH/06 (FJ473388.1), CH/JSX/06 (FJ473389.1), CH/HLJH/06 (FJ473390.1), CH/IMT/06 (FJ473391.1), CH/SHH/06 (FJ473392.1), CH/HLJM/07 (FJ473393.1), CH/HNH/07 (FJ473394.1), CH/GSJ/07 (HM210880.1), CH/JL/09 (HM210881.1), CH/GDS/09 (HM210882.1), CH/ZMDZY/11 (KC196276.1), CH/S (DQ355224.1), CH/HLJHG/2010 (HQ455346.1), CH/HLJQ/2010 (HQ455345.1), CHGD-01 (JX261936.1), GD-1 (JQ081273.1), GD-A (JX112709.1), GD-B (JX088695.1), GDDWC (JN173274.1), GDLC 9 (JN173276.1), GDXS1 (JN255975.1), GDXS2 (JN255976.1), GDXS4 (JN173285.1), GDXS5 (JN173286.1), GDXS6 (JN255977.1), GDXS7 (JN173287.1), GDXS8 (JN255978.1), GDYA (JN173288.1), GDYB (JN173289.1), GDYD (JN173290.1), GDYE (JN173291.1), GDYG (JN173295.1), GDCB6 (JN255973.1), GDYE4 (JN173292.1), GDYE6 (JN255979.1), GDYE7 (JN173293.1), GDYE8 (JN255980.1), GDYE9 (JN255981.1), GDYE10 (JN255982.1), GDYE11 (JN255983.1), GDYE67 (JN173294.1), GDYE70 (JN255984.1), GDSTY (JN173284.1), GDST1 (JN173277.1), GDST2 (JN173278.1), GDST3 (JN173279.1), GDST4 (JN255974.1), GDST5 (JN173280.1), GDST6 (JN173281.1), GDST7 (JN173282.1), GDST8 (JN173283.1), GDHSY (JN173275.1), GDCB9 (JN173273.1), DWA1 (JN173272.1), QY1 (JN173297.1), QYA1 (JN173299.1),

	JN173270.1_Chinese (JN173270.1), FJ473395.1_Chinese (FJ473395.1), HuN (JN243758.1), XG1 (JN173302.1), XG2 (JN173303.1), QY2 (JN173298.1), JKA1 (JN173296.1), DBA1 (JN173271.1), BJ2010 (JF690780.1), HB/HS (JF700126.1), JS-2004-2 (AY653206.1), HLJBY (GU321197.1), CV777_Chinese (DQ355221.1), Strain S (DQ355223.1), SD-M (JX560761.1), ZJCZ4 (JX524137.1), LC (JX489155.1), LZC (EF185992.1), DX (EU031893.1), LJB/03 (DQ072726.1), SWK1 (JN173300.1), SWK2 (JN255985.1), SWK3 (JN255986.1), SWK4 (JN255987.1), SWK5 (JN255988.1), SWK6 (JN173301.1)
Japan	83P-5_parent (AB618619.1), 83P-5_34th-passaged (AB618620.1), 83P-5_61st-passaged (AB618621.1), 83P-5_100th-passaged (AB618622.1)
Korea	attenuated_DR13 (JQ023162.1), Virulent DR13 (JQ023161.1), Chinju99 (AF237764.1), SM98 (GU937797.1)
Britain	Br1_87 (Z14976.1)
Belgium	CV777 (AF353511.1)

4. Phylogenetic analysis

All nucleotide sequences of both genes of current Thai PEDV isolates in 2011-2012 were compared with the reference PEDV taken from NCBI Genbank database. The sequences were aligned with program package, Clustral X multiple alignment versions 2.0.11 (multiple alignments of nucleic acid and protein sequences; GNU Lesser General Public License) and BioEdit sequence alignment editor version 7.1.3.0. Phylogenetic analysis of nucleotide sequences were conducted with MEGA version 5. The Maximum Likelihood algorithm was used to construct the phylogenetic tree. The bootstrap method used 1000 replicates for standard error calculation. For phylogenetic relationship, the current Thai PEDV isolates in 2011-2012 were compared with the reference PEDV taken from NCBI Genbank database.