GENETIC CHARACTERIZATION OF *PORCINE EPIDEMIC DIARRHEA VIRUS* (PEDV) ISOLATES FROM SOUTHERN VIETNAM DURING 2009- 2010 OUTBREAKS

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ลักษณะทางพันธุกรรมของไวรัสพีอีดีสายพันธุ์เวียดนามที่แยกได้จากภาคใต้ของประเทศเวียดนามระหว่างการระบาดในปี ค.ศ. 2009 - 2010

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โด เตียน ดุย : ลักษณะทางพันธุกรรมของไวรัสพีอีดีสายพันธุ์เวียดนามที่แยกได้จากภาคใต้ของประเทศเวียดนามระหว่างการระบาดในปี ค.ศ. 2009 - 2010. (GENETIC CHARACTERIZATION OF *PORCINE EPIDEMIC DIARRHEA VIRUS* (PEDV) ISOLATES FROM SOUTHERN VIETNAM DURING 2009- 2010 OUTBREAKS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, 84 หน้า.

ยีนของโปรตีนสไปค์และโปรตีนเมมเบรนของไวรัสพีอีดี เป็นยีนที่มีความหลากหลายทางพันธุกรรมซึ่งจะมีความแตกต่างกันในแต่ละพื้นที่ มีรายงานว่าลำดับทางพันธุกรรมของจีโนมที่ต่างกันในยีนดังกล่าวมีความเกี่ยวข้องกับพยาธิกำเนิดของโรค เมื่อทำการถอดรหัสและวิเคราะห์พันธุกรรมของไวรัสพีอีดีจำนวน 8 ตัวอย่าง ซึ่งแยกได้จากลูกสุกรในฟาร์มทางใต้ของประเทศเวียดนามที่แสดงอาการท้องเสีย ในช่วงที่มีการระบาดของโรคพีอีดีระหว่าง ปี ค.ศ. 2009 – 2010 พบว่าบางส่วนของยีนสไปค์และทั้งหมดของยีนเมมเบรน ของไวรัสพีอีดีดังกล่าว มีความเหมือนกันของร้อยละ 98.9-100 และ 99.7-100 ตามลำดับ โดยสามารถแบ่งออกเป็น 2 กลุ่มย่อยตามจังหวัดที่พบเชื้อ ซึ่งมีความแตกต่างจากเชื้อที่พบในทวีปยุโรป (Br1/87 และ CV777) และประเทศเกาหลี (Spk1, Chinju99, DR13 and KNU-0801) ผลการวิเคราะห์ความสัมพันธ์ของเชื้อแบบแผนภูมิต้นไม้พบว่าเชื้อที่แยกได้จากการศึกษานี้จัดอยู่ในกลุ่มเดียวกับเชื้อไวรัสที่ก่อโรคปัจจุบันในประเทศจีน (JS-2004-2 และ DX) ประเทศไทย (07NP01, 08NP02 และ 08CB01) และประเทศเกาหลี (KNU-0802 และ CPF299) จากผลการศึกษาในครั้งนี้พบว่าเป็นไปได้ว่าไวรัสพีอีดีที่ระบาดในเวียดนามครั้งนี้มีสายวิวัฒนาการมาจากไวรัสในประเทศจีน และจากการที่ไวรัสมีการเปลี่ยนแปลงลักษณะทางพันธุกรรมดังกล่าวหลังจากระบาดมาระยะเวลาหนึ่ง อาจทำให้เกิดไวรัสพีอีดีสายพันธุ์ใหม่ในเวียดนามได้

ภาควิชา....พยาธิวิทยา..... ลายมือชื่อนิสิต.....

สาขาวิชา...พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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Porcine epidemic diarrhea virus (PEDV) spike (S) protein and membrane (M) protein genes are believed to have genetic variation. The heterogeneity in those genomic sequences has been reported and is known essentially for the diverse PEDV pathogenicity. Eight southern Vietnamese PEDVs collected from watery diarrhea piglets of various farms of the recent emerging outbreaks (2009-2010) were sequenced and analyzed. The results revealed high homology of the partial S gene of the current isolates at 98.9-100% and 99.7-100% identity of full M gene among these isolates despite dividing into two clades of different provincial origins. It should be note that the Vietnamese PEDVs contained high differences on nucleotide sequence of partial S gene with other reference isolates in Europe (Br1/87, CV777) and Korea (Spk1, Chinju99, DR13 and KNU-0801). The phylogenetic relationship of both partial S and M protein genes indicated that the current Vietnamese PEDVs were in the same cluster with the recent isolates from China (JS-2004-2 and DX), Thailand (07NP01, 08NP02 and 08CB01) and Korea (KNU-0802 and CPF299). The results suggested that these Vietnamese PEDV isolates might have originated from the same Chinese ancestor and they have been undergoing genetic variation and forming a new PEDV genotype in Vietnam.

Department : Veterinary Pathology Student's Signature .....

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CONTENTS

Chapters Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	adenosine
ACE2	angiotensin I converting enzyme 2
APN	aminopeptidase N
BCoV	bovine Coronavirus
bp	base pairs
C	cytosine
CCoV	canine Coronavirus
C-terminus	carboxyl-terminus
CEACAM	carcinoembryonic antigen-related cell adhesion molecules
cDNA	complementary deoxyribonucleic acid
E	envelop protein
et al.	et alii (and others)
FECoV	feline Coronavirus
G	guanine
HCoV-229E	human Coronavirus 299E
HEV	Hemagglutinating encephalomyelitis virus
IBV	infectious bronchitis virus
IgG	immunoglobulin gamma
kDa	kilo dalton
M	membrane protein
$MgCl_2$	magnesium chloride
MHV	murine hepatitis virus
min	minute
ml	milliliter
mRNAs	messenger ribonucleic acid
N	nucleocapsid protein
N- terminus	amino(NH_2)-terminus

ORF	open reading frame
°C	degree celsius
PED (V)	porcine epidemic diarrhea (virus)
PRCoV	porcine respiratory Coronavirus
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
rpm	the number of rounds per minute
S	spike protein
SARS-CoV	severe acute respiratory syndrome Coronavirus
sec	second (s)
T	thymine
TCoV	turkey Coronavirus
TGE (V)	transmissible gastroenteritis (virus)
UV	ultraviolet
VN92S1	Vietnamese 92 PED isolate, S gene sequencing
VN92M1	Vietnamese 92 PED isolate, M gene sequencing
VN94S2	Vietnamese 94 PED isolate, S gene sequencing
VN97S3	Vietnamese 97 PED isolate, S gene sequencing
VN103S4	Vietnamese 103 PED isolate, S gene sequencing
VN103M2	Vietnamese 103 PED isolate, M gene sequencing
VN109S5	Vietnamese 109 PED isolate, S gene sequencing
VN109M3	Vietnamese 109 PED isolate, M gene sequencing
VN112S6	Vietnamese 112 PED isolate, S gene sequencing
VN112M4	Vietnamese 112 PED isolate, M gene sequencing
VN116S7	Vietnamese 116 PED isolate, S gene sequencing
VN116M5	Vietnamese 116 PED isolate, M gene sequencing
VN122S8	Vietnamese 122 PED isolate, S gene sequencing
VN122M6	Vietnamese 122 PED isolate, M gene sequencing
µl	micro liter
µM	micro mol

(+)	positive
%	percentage

Protein:

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	glutamic acid
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

CHAPTER I
INTRODUCTION

Porcine epidemic diarrhea (PED) is a devastating contagious disease caused by porcine epidemic diarrhea virus (PEDV) producing acute enteritis and fatal watery diarrhea as well as severe dehydration leading to death. This disease can cause very high mortality ratio in suckling pig reaching to 90% (Pensaert and Yeo, 2006). PED was first identified in England in 1971 (Oldham, 1972). Since then, it has been reported in Belgium and the United Kingdom in 1978 (Pensaert and Debouck, 1978). Currently, it has become a problematic disease causing massive economic losses in many countries, particularly in Europe and Asia (Pensaert and Yeo, 2006, Chen et al., 2008).

PEDV belongs to genus Coronavirus (family *Coronaviridae*), contributing to many major diseases in humans and animals. The virus consists of non-segmented, single-stranded, (+) sense RNA, approximately 27 to 30 kb long and synthesizing a nested set of multiple sub-genomic mRNAs (Cann, 2005). This virus possesses four main structural proteins consisting of the spike (S), membrane (M), envelope (E) and phosphorylated nucleocapsid (N) proteins (Duarte and Laude, 1994; Bos et al., 1996).

PEDV is related to various diarrhea outbreaks and variability in pathogenic features has occurred in either naïve or immunized pig herds. In particular, the heterogeneity in genomic sequences has been reported and is known essentially as the cause for the diverse PED pathogenicity. The S glycoprotein gene is genetically highly variable and is divided into two subunits S1 and S2. Many molecular investigations have been performed and revealed low to high variation of nucleotide sequences of PEDV, especially in the S gene (Duarte and Laude., 1994; Lee et al., 2010). Insertion or deletion found in the study of Lee et al. (2010) demonstrated the combined presence of notable 15 bp insertion and 6 bp deletion within the N-terminal region of S1 domain of the Korean isolates.

Phylogenetic analysis comparing PEDVs from various countries demonstrated that PEDVs were classified into three main groups based on nucleotide homology of the partial

S gene and M gene. For the partial S gene, group 1 contains previous isolates like CV777 (Belgium), Br1/87 (Britain), JS-2004-2 (China), parent DR13, KPED-9 (Korea) and recent Thai PEDV isolates (Park et al., 2007; Puranaveja et al., 2009). Group 2 includes Spk1 isolate (Korea) and group 3 includes Chinju99 isolate (Korea). The phylogenic features depend on the mutation of local isolated PEDV in China, Korea or Thailand (Park et al., 2007; Chen et al., 2008; Puranaveja et al., 2009; Lee et al., 2010). It should be noted that the research on heterogeneity in the spike protein gene of PEDV in Korea (Lee et al., 2010) demonstrated the tree topology based on the nucleotide sequences representing the S1 domain or S1 N-terminal region similar to the full S gene-based phylogenetic tree.

Recently, PED outbreaks have occurred and caused major impact on swine production showing economic losses in most of southern provinces of Vietnam since late 2008/ early 2009 (unpublished data). In order to effectively prevent the spread of PEDV, precise analysis on genomic characterization of the current PEDVs is needed to identify the origin of this emerging virus. Therefore, molecular characterization of the current Vietnamese PEDV isolates will elucidate the epidemiologic relationship among PEDV isolates in the Vietnamese neighboring countries. Such data can be used to plan for appropriated PED prevention and control in the future.

Objectives of the study

To genetically characterize the partial S and M genes of current southern Vietnamese PEDVs collected from PED-affected swine herds in three provinces of southern Vietnam for elucidation the epidemiologic relationship among PEDV isolates in PED-bearing countries.

Research questions:

1) What are the genetic characterizations of current southern Vietnamese PEDV isolates based on the partial S and M gene during the outbreaks 2009 – 2010?
2) Are there the differences of the current Vietnamese PEDV isolates with other reference strains?

CHAPTER II
LITERATURE REVIEWS

2.1. Porcine epidemic diarrhea in general

Porcine epidemic diarrhea (PED) is a devastating acute diarrhea in pigs of all ages especially in piglets causing high morbidity and mortality (Debouck and Pansaert, 1980; Pensaert and Yeo, 2006). The disease is caused by a Coronavirus called porcine epidemic diarrhea virus (PEDV). The virus damages the intestinal villi thus reducing the absorptive surface, causing quick loss of fluid and leading to severe dehydration (Figure 2.1). Acute outbreak of diarrhea occurred in susceptible herds first infected with PEDV. In those cases, up to 100% sows can be affected, showing mild to severe watery diarrhea. PED shares similarly important characteristic with transmissible gastroenteritis (TGE), rapidly spread of diarrhea to other pigs almost 100% morbidity within 7 to 14 days. PED divided into two pathogenic types (type1 and type 2) Type 1 only affects on growing pigs whereas type 2 revealed more severe clinical signs in all age groups of swine including suckling pigs and adult animals. The clinical signs and pathologic lesions are summarized in Table 2.1.

Table 2.1 The summarized PED clinical signs and pathologic lesions in pigs

Pig groups	Clinical signs	Pathologic lessons
Piglets	- Watery diarrhea, dehydration - Vomiting, - Morbidity (100%) - Mortality (30-80%), 100%[(a)]	- Intestinal watery gassy fluids - Thin intestinal wall - Villous blunting and atrophy
Weaners & growers	- Acute watery diarrhea - High morbidity (100%) - Low mortality (2-3%)	- Sudden death (a few case) with back muscle necrosis[(c)]
Adult/ sow	- Mild to high watery diarrhea - Reduced reproductive performance[(b)]	- No evidence

a) Puranaveja et al., 2009; b) Olanratmanee et al., 2010; c) Pensaert and Yeo, 2006

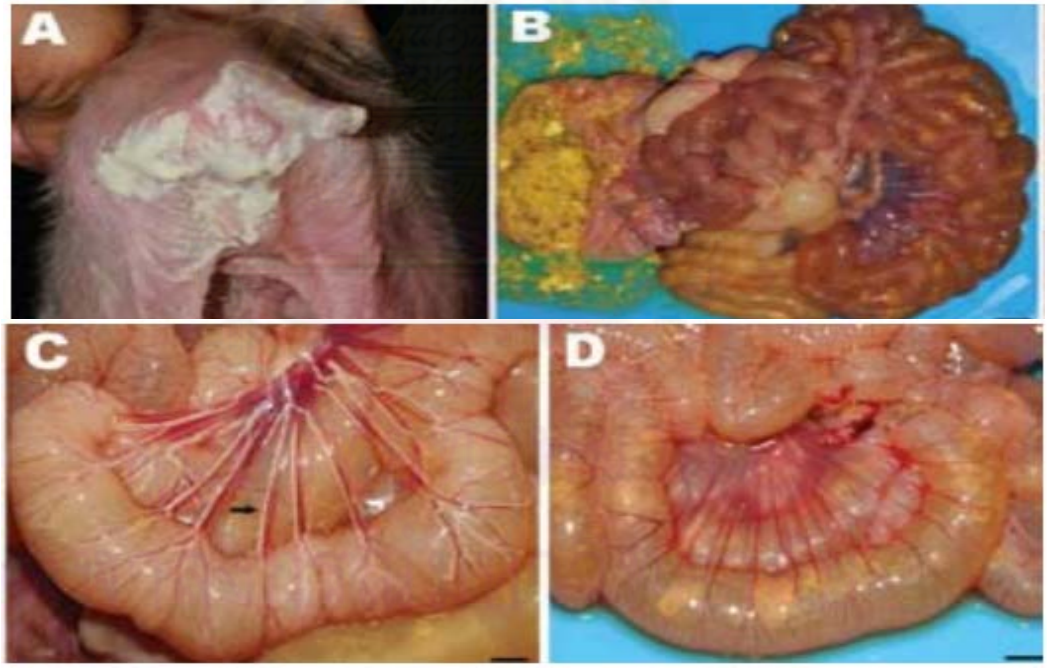


Figure 2.1 Clinical signs and pathological features in PEDV infection of piglet (Puranaveja et al., 2009). A, severe diarrhea and dehydration in piglet. B, severe catarrhal enteritis with congestion (scale bar = 1 cm). C, intestinal lacteals (arrow) grossly demonstrating normal absorption capacity of the intestinal villi in a normal piglet. D, disappearance of intestinal lacteals.

Following the observation of Pensaert (1999), the severity of clinical PED depends on the sow immune status of the affected herds. When the susceptible herd (with no immunity to PEDV) is infected, the clinical symptoms will occur similar to TGE, and mortality in neonatal piglets can reach up to 80%. In contrast, PEDV infections in herds previously exposed to PEDV consist of pigs with sub-clinical signs showing sporadic outbreaks and low mortality.

The mechanisms of viral replication and villous degeneration are similar to TGE and other viral enteritis (Figure 2.2) in which the affected epithelial cells will be seen in the small and large intestines around 12 to 18 hours and 5 days after inoculation, respectively (Pensaert, 1999). After PEDV pass through the defensive border of stomach fluid, virus attaches to specific receptor porcine aminopeptidase N (Weiss et al., 2005; Li et al., 2007) and causes the changes of morphology and function of intestinal tract characterized by villous atrophy. The atrophied intestinal tract lost the function of absorption due to reduction of digestive enzymatic activity causing changes in the digestion of lactose and cellular transportation and hydrolysis of nutrition. Finally, animals will drop into the condition of mal-absorption, diarrhea and dehydration, and died within few days particularly in suckling pigs (Pensaert, 1999; Sestak and Saif, 2002, Weiss et al., 2005).

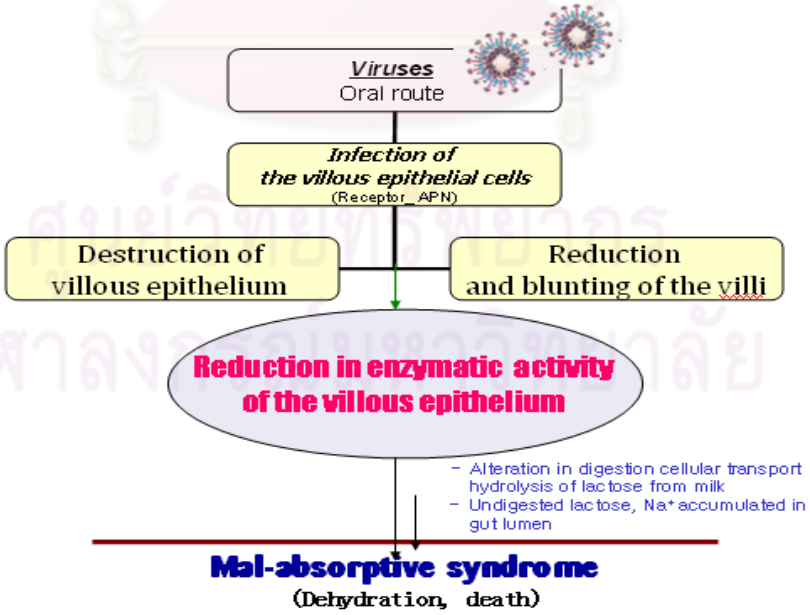


Figure 2.2 Mechanism of porcine epidemic diarrhea virus infection (Pensaert, 1999; Sestak and Saif, 2002, Weiss et al., 2005)

2.2. Geographical distribution of PEDV

PEDV has been isolated in most swine-raising countries in Europe and Asia such as United Kingdom/Belgium (Oldham., 1972; Pensaert and Debouck, 1978), China (Chen et al., 2008), Japan (Takahash et al., 1983), Korea (Hwang et al., 1994), Thailand (Puranaveja et al., 2009) and recently in southern Vietnam (unpublished data). However, no PEDV has yet been reported from North American countries (Sestak and Saif, 2002).

In Europe, acute watery diarrhea outbreak occurred in England (1971) on growing and finishing pigs except suckling pigs and considered to be type 1 (Oldham, 1972). TGE and other entero-pathologic agents were ruled out. After that, this disease rapidly spread to numerous European countries. Type 2 PED was called by the new outbreak (1976) like TGE observed in all age groups of animals including suckling pigs. Immediately, the virus was isolated, designated and experimental challenged to prove the pathogenicity differentiated from type 1 PED. Pansaert et al. (1982) proposed the name porcine epidemic diarrhea and generally has been used until now (Pensaert and Yeo, 2006). Genome of CV777 has been sequenced fully with 28033 nucleotides and based on decoded amino acid sequence. This PED strain closely related to human Coronavirus 229E and TGEV (Kocherhans et al., 2001). The investigation of sero-conversion of PEDV (1982-1990) revealed the PED widespread almost of European countries where the positive result was found in the swine populations in Belgium, England, Germany, France, Netherlands, Switzerland and Bulgaria (Pensaert and Yeo, 2006).

In Asia, an outbreak of acute diarrhea has occurred in pig populations of many countries, where epidemiologic patterns of severe PED outbreaks clinically resembling TGE were associated with important economic losses (Sueyoshi et al., 1995). The PED outbreaks gradually recorded in Japan (Takahash et al., 1983), China (Qinghua et al., 1992; cited by Pensaert and Yeo, 2006), Korea (Hwang et al., 1994), Thailand (Puranaveja et al., 2009) and recently (2008/2009) in southern Vietnam (unpublished data).

Currently, the first PED outbreaks in late 2008/ early 2009 confirmed by pathological features and RT-PCR method caused massive economic losses of swine production in most

of southern provinces of Vietnam (data not shown). The investigation of 2009-2010 PED outbreaks revealed that acute diarrhea syndrome occurred in all age groups in pig farms (Figure 2.3) and the affected animals showed acute watery diarrhea condition prolonged a few days to over one week in adult animals. Most pigs were recoverable with medical care excepting for suckling piglets becoming more severe watery diarrhea, dehydration and finally died in progression of disease in a few days. Morbidity in suckling piglets reached nearly 100%, but mortality varied among provinces ranging from 65% to 91%.

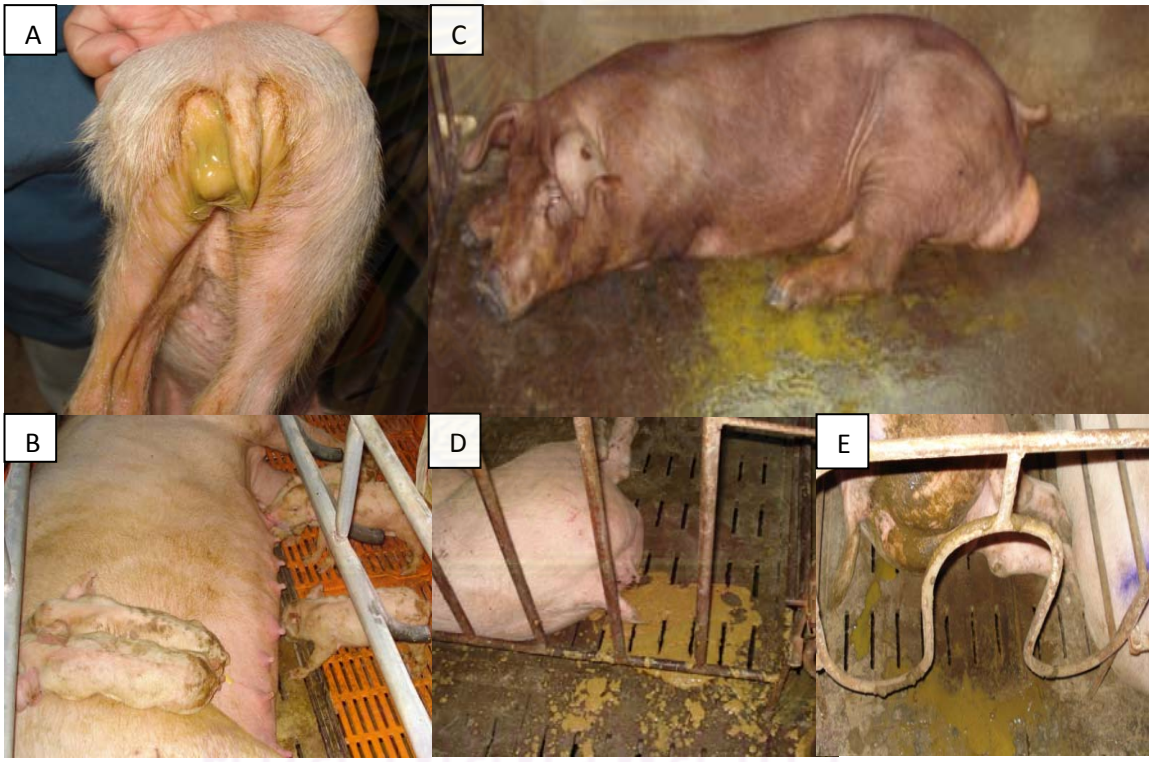


Figure 2.3 Clinical signs of diarrhea outbreaks in Vietnam, 2009-2010. A, severe watery diarrhea in piglet with dehydration; B, loss of heat in piglet lying on abdominal area of sow; C, watery diarrhea in boar; D and E, watery diarrhea in gilt and sow.

2.3. Properties and pathogenicity of PEDV

PEDV belongs to genus Coronavirus, family *Coronaviridae*. The *Coronaviridae*, *Arteriviridae* and *Okaviridae* are in the order *Nidovirales*, sharing some common features with respect to genomic organization, replication and transcription (Cavanagh, 2005). Coronaviruses have largest RNA genome consisting of non-segmented, single-stranded, (+) sense RNA, approximately 27 to 30 kb long, and synthesizing a nested set of multiple sub-genomic mRNAs (Cann, 2005). This virus possesses four main structural proteins consisting of the Spike (S) glycoprotein, the membrane (M) glycoprotein, the envelope (E) protein and the phosphorylated nucleocapsid (N) protein (Duarte and Laude, 1994; Bos et al., 1996).

Infectious bronchitis virus (IBV) in the 1930s, mouse hepatitis virus (MHV) and TGEV in pigs in the 1940s were the early isolations of Coronaviruses (Beaudette, 1937; Doyle and Hutchings, 1946; Cheever et al., 1949*)*. The appearance of these viruses under electron microscope is crown-like, called Coronavirus. The viruses with identical appearances have been detected in a wide range of animals and in humans shown to have similar genome organization and replication strategies.

Coronaviruses are associated with economical loss of major diseases in cattle, poultry, and pigs as well as in cats (Lai et al., 2007). Coronaviruses can be divided into three distinctly serological groups (Table 2.2) in that serogroups I and II have been isolated from mammals and serogroup III from birds. Each group has separated cellular receptors (Murphy el al., 1999; Weiss et al., 2005; Lai et al., 2007). They are also distinguished to three groups based on phylogenetic analysis and antigenic cross reactivity. Some important veterinary viruses belong to group I including canine enteric Coronavirus (CECoV), transmissible gastroenteritis virus, porcine epidemic diarrhea virus, porcine respiratory Coronavirus (PRCoV) and Coronaviruses (FCoVs) (Weber and Schmidt, 2005).

Table 2.2 Coronaviruses, hosts, diseases, and specific receptors

Groups	Virus	Host	Diseases	Cellular receptor	Preferences
I	HCoV-229E	Human	Respiratory infection	Human APN	Weiss et al., 2005; Lai et al., 2007
	TGEV	Pig	Respiratory and enteric infection	Porcine APN	Weiss et al., 2005
	PEDV	Pig	Enteric infection	Porcine APN	Murphy et al., 1999
	PRCoV	Pig	Respiratory infection	Porcine APN	Weiss et al., 2005
	CCoV	Dog	Enteric infection	Canine APN	Weiss et al., 2005; Lai et al., 2007
	FeCoV	Cat	Enteric infection	Feline APN	Weiss et al., 2005
	FIPV	Cat	Respiratory, enteric, and neurologic infection, and hepatitis	Feline APN	Weiss et al., 2005
	HCoV-NL-63	Human	Respiratory infection	ACE2	Weiss et al., 2005; Lai et al., 2007
	RbCoV	Rabbit	Enteritis		Lai et al., 2007
II	HCoV-OC43	Human	Respiratory infection and possibly enteric infection	Neu5,9Ac2-containing moiety	Weiss et al., 2005; Lai et al., 2007
	MHV	Mouse	Enteric and neurologic infection and hepatitis	Murine CEACAM1	Weiss et al., 2005
	Sialodacryoadenitis Coronavirus	Rat	Neurologic infection	ND	Weiss et al., 2005
	HEV	Pig	Respiratory, enteric, and neurologic infection	Neu5,9Ac2-containing moiety	Weiss et al., 2005
	BCoV	Cow	Enteric infection	Neu5,9Ac2-containing moiety	Weiss et al., 2005
	HCoV-HKU1	Human	Respiratory infection	Neu5,9Ac2-	Weiss et al., 2005;

				containing moiety	Lai et al., 2007
	SARS-CoV	Human	Severe acute respiratory syndrome	ACE2	Weiss et al., 2005
III	IBV	Chicken	Respiratory infection, hepatitis, other	ND	Weiss et al., 2005
	TCoV	Turkey	Respiratory and enteric infection	ND	Weiss et al., 2005; Lai et al., 2007

ND, not determined. TGEV, transmissible gastroenteritis virus; BCoV, bovine Coronavirus; IBV, avian infectious bronchitis viruses; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome Coronavirus; PEDV, porcine epidemic diarrhea virus; FIPV, feline infectious peritonitis virus; FECoV, feline enteric Coronavirus; HCoV-229E, OC43, NL63 and HKU1, human Coronavirus; PRCoV, porcine respiratory Coronavirus; TCoV, turkey enteric Coronavirus; CCoV, canine Coronavirus; HEV, Hemagglutinating encephalomyocarditis virus; RbCoV, Rabbit Coronavvirus. CEACAM, carcinoembryonic antigen-cell adhesion molecule; ACE2, angiotensin-converting enzyme 2; APN, aminopeptidase N.

Evidently, many mutation positions in the genomes of Coronaviruses can lead to changes in pathogenicity (Ballesteros et al., 1997; Leparc-Goffart et al., 1997). For instances, replacement of the S protein gene of an attenuated strain of IBV with that from a pathogenic strain, the virus stills remained non-pathogenic (Hodgson et al., 2004). The structural protein (S protein) is considered a major determinant of antigenicity and pathogenicity. Likewise, Ballesteros et al. (1997) performed the production of enteropathogenic and non-enteropathogenic strains recombinants of TGEV which had extremely similar genomic sequences. The authors concluded that some of the viral recombinants with non-enteropathogenic had only one to two amino acid differences in the S protein. In addition, the role of the S protein in entero-pathogenicity using recombinant TGEV by targeted recombination was evident (Sanchez et al., 1999). S protein contains sequences responsible for binding to specific receptors on the surface of susceptible host cells. S1 sequences are variable, containing various degrees of deletions and substitutions in different coronaviral strains.

2.4. Genomic structure and function of PEDV

The observation of Lai et al. (2007) revealed the shape-pattern of Coronaviruses having a round structure around 100 to 160 nm in diameter with distinctive long, petal-shaped spikes on the surface (Figure 2.4).

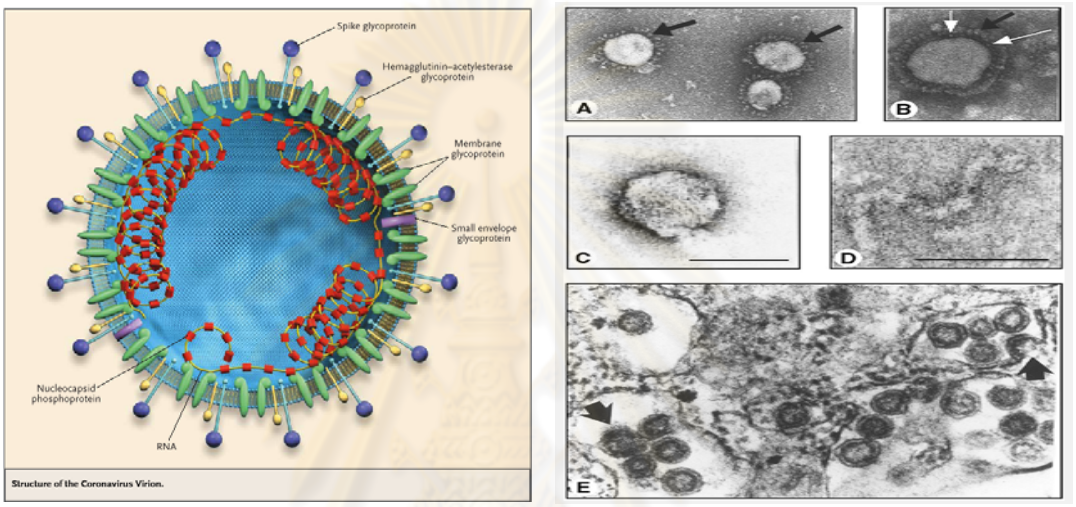


Figure 2.4 Left image, model of structure of the Coronavirus virion, showing structural enveloped proteins including Spike protein (S), Envelope protein (E), Membrane protein (M), Haemagglutinin-esterase (H) and phosphoprotein (N) (Holmes, 2003). Right image, morphology of Coronaviruses. Human respiratory Coronavirus HCoV-OC43 (A), and turkey enteric Coronavirus TCoV (B), showing the large, petal-shaped spikes (black arrows) in negatively stained preparations. Some Coronaviruses also exhibit a fringe of shorter spikes composed of the hemagglutinin-esterase glycoprotein (white arrows). (C) showing the internal core of porcine transmissible gastroenteritis virus (TGEV). (D) Helical nucleocapsid released from the TGEV core structure by Triton X-100 treatment. (E) Infection of Human cell with human respiratory Coronavirus HCoV-229E. The budding of viral particle (spherical virions) occurring at membranes of the endoplasmic reticulum and in smooth-walled vesicles (arrows) (Lai et al., 2007).

The genomic organization of PEDV (Figure 2.5) is typical of Coronavirus, having characteristic gene order (5'-polymerase [Orf1ab], spike [S], envelop [E], membrane [M], and nucleocapsid [N]-3'). Apparent molecular weight in SDS polyacrylamide gels (kDa) of structural proteins are 180-220, 23-35, 9-12, 65, and 50-60 kDa of spike glycoprotein, integral membrane protein, small envelope protein, haemagglutinin-esterase protein (group 2 Coronavirus) and nucleocapsid protein, respectively (Cavanagh, 2005; Lai et al., 2007). Lai et al. (2007) described that the S glycoprotein forms the large, petal-shaped spikes on the surface of the virion. S glycoprotein can be divided into three structural domains (from N- to C-terminus) as a large external domain divided into two subdomains, S1 and S2, a transmembrane domain and a short carboxylterminal cytoplasmic domain. The S1 subdomain includes the N-terminal half of the molecule and forms the globular portion of the spikes. It contains sequences responsible for binding to specific receptors on the surface of susceptible cells. S1 sequences are variable, containing various degrees of deletion and substitutions in different Coronavirus strains. Mutations in S1 sequences have been associated with altered antigenicity and pathogenicity of the virus (Ballesteros et al., 1997; Leparc-Goffart et al., 1997). In contrast, S2 sequence is more conserved and constitutes the stalk of the spikes. In most MHV strains and BCoVs, the 180-kd S protein is cleaved during or after virus maturation by a cellular protease to yield the S1 and S2 proteins remaining non-covalently associated in the viral spikes. However, in other Coronaviruses, such as severe acute respiratory syndrome Coronavirus (SARS-CoV), cleavage occurs as part of the viral entry process.

As describing of Lai et al. (2007), the S and M glycoproteins belonging to structural enveloped proteins have several important biological functions (Table 2.3). Monoclonal antibodies against S protein can neutralize virus infectivity, consistent with the observation that S protein binds to cellular receptors (Gallagher and Buchmeier, 2001). The receptor-binding domain of the S protein of MHV is localized within the N-terminal 330 amino acids of the S1 domain (Kubo et al., 1994), whereas, the binding domain of SARS-CoV S protein comprises residues 318 to 510 (Wong et al., 2004). Consequently, the amino acid sequences of the S1 domain are critical for establishing species specificity in animals. This

was particularly illustrated for the SARS-CoV S protein. It was shown that SARS-CoV virus crossed species from bats to palm civets and then to humans. The S glycoprotein must adapt to species-specific differences in the host cell receptor (angiotensin-converting enzyme 2-ACE2). The S protein from palm civets and humans differs in a few residues. Experiments in which specific amino acids within the receptor-binding domain of the two S proteins were swapped, coupled with crystallographic analysis, revealing the importance of these residues in enhancing binding to human ACE2 (Lai et al., 2007). In swine, the non-enteropathogenic variant of TGEV, called porcine respiratory Coronavirus is the respiratory pathogen considered emerging from deletions within the spike gene of TGEV. It is a strong example for the evolution of Coronavirus with altered tissue tropism and reduced virulence (Saif, 2004). The genome structure of TGEV and PRCoV are 96% identical except for the 5' region of the spike gene, and the difference in pathogenic outcome between the two strains is associated with deletions of various lengths at nucleotide 45 to 752, corresponding 225 of the first 300 amino acids deleted (Britton et al., 1991) within the 5' end of the spike protein gene of PRCoV (Weiss et al., 2005). For more instances, most IBV serotypes differ from each other by 20 to 25% of S1 amino acids (Adzhar et al., 1995; Kingham et al., 2000). A few amino acid differences (2 to 3%) of S1 amino acid residues result in a change of serotype, leading to lack of cross-neutralization (Cook et al., 1986; Cavanagh et al., 1992).

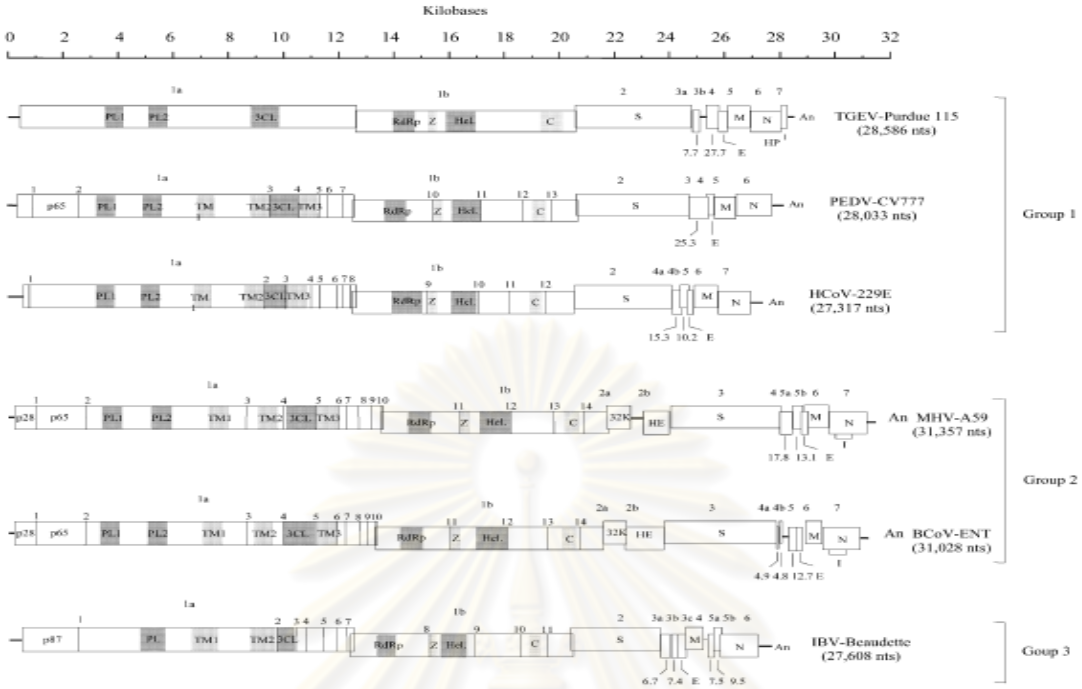


Figure 2.5 Genomes of the six sequenced species of Coronaviruses (Brian and Baric, 2005). The data are derived from GenBank in November 2002. The gene 1 (ORFs 1a and 1b) the predicted protease cleavage sites are indicated by numbers and domains of known or predicted function are shaded and identified (PL, papainlike protease; 3CL, poliovirus 3C-like protease; TM, transmembrane domain; RdRp, RNA-dependent RNA polymerase; Z, zinc finger (metal-binding) domain; Hel, helicase domain; C, conserved sequence domain). Genes 2–8 (or 9) are identified by their transcript name (1a, 1b, etc.) or their abbreviated name of the protein product (S, spike; E, envelope; M, membrane; N, nucleocapsid; HP, hydrophobic protein; HE, hemagglutinin-esterase; I, internal).

Table 2.3 Summarized coronaviral properties and functions of structural proteins

Nucleocapsid	Binds to viral RNA
phosphoprotein N	Forms nucleocapsid
	Elicits cell-mediated immunity
Membrane glycoprotein M	An integral membrane protein on the Golgi
	Determines virus budding site

	Triggers virus particle assembly Interacts with viral nucleocapsid Forms the shell of internal viral core (TGEV and MHV) Induces interferon
Envelope (small membrane) protein E (formerly sM)	Triggers virus particle assembly Associates with viral envelope May cause apoptosis
Spike glycoprotein S	Forms large spikes on virion surfaces Binds to specific cellular receptors Induces fusion of viral envelope with cell membranes (plasma membrane or endosomal membrane) May induce cell to cell fusion Binds Fc fragment of immunoglobulin (MHV and TGEV) Binds 9-*O*-acetylated neuraminic acid or *N*- glycolylneuraminic acid Induces neutralizing antibody Elicits cell-mediated immunity
Hemagglutinin-esterase glycoprotein HE	Forms small spikes on the virion surface of some coronaviruses Binds to 9-*O*-acetylated neuraminic acid Causes hemagglutination May cause hemadsorption Esterase cleaves acetyl groups from 9-*O*-acetyl neuraminic acid

TGEV, porcine transmissible gastroenteritis virus; MHV, murine hepatitis virus.

Several other biological activities have been associated with the S glycoprotein. It was shown that the S glycoprotein of several Coronaviruses is able to bind to the Fc

fragment of IgG (Oleszak et al., 1992). This molecular mimicry may play a role in viral pathogenesis by shielding the S protein from antivirus antibodies. In summary, S protein is a large, multifunctional protein playing a central role in the biology and pathogenesis of Coronavirus infections (Lai et al., 2007).

Briefly, S protein of Coronavirus species generally exhibits the greatest sequence variation where most of differences occur within the amino-terminal part of this molecule, equivalent to the N-terminal S1 glycopolypeptide. S2 domain reveals more conserve on the polypeptide sequence, involving in anchoring the protein in the membrane, forming the coiled-coil multimeric mature S protein and activating membrane fusion. Furthermore, the S1 subunit functions provide a receptor-binding domain (Cavanagh, 2005). The S protein of PEDV is a type I membrane glycoprotein composed of 1383 amino acids, containing a signal peptide including 1-24 amino acids, a large extra cellular region, a single transmembrane domain (1334–1356 acid amines), and a short cytoplasmic tail. The S protein divided into S1 (1–735 acid amines) and S2 (736–1383 acid amines) domains (Sturman and Holmes, 1984; Jackwood et al., 2001). Therefore, the S glycoprotein is considered the primary target for the development of suitable vaccines against PEDV and an important viral component for studying of genetic relationships among PEDV isolates and pursuing the epidemiological status of PEDV (Park et al., 2007; Puranaveja et al., 2009).

2.5. Sequencing of PEDV

Similar nucleotide sequences of two European isolates of PEDV derived from cases of PEDV infection in Belgium and Britain in 1977 and 1987, respectively, contained a 1323 nucleotide open reading frame (nucleocapsid gene) showing high identity to human Coronavirus 229E and porcine transmissible gastro-enteritis viruses, porcine respiratory Coronavirus, feline infectious peritonitis virus, canine Coronavirus and feline enteric Coronavirus (Bridgen et al., 1993). In 1994, the complete sequence of the spike glycoprotein (S) gene of the Br1/87 isolate of PEDV was analyzed to find out predicted polypeptide sequence with 1383 amino acids, containing 29 potential N-linked glycosylation

sites. This S gene had similarly structural features to those of the Coronavirus such as TGEV, lacking a proteolytic site to yield cleaved amino and carboxy subunits S1 and S2 (Duarte and Laude, 1994). The comparison of S sequence confirmed PEDV closely related to the human respiratory Coronavirus HCV 229E but S polypeptide sequence containing an additional 250 residue N-terminal domain lacks in HCV 229E and PRCV. Later, Kocherhans et al. (2001) has sequenced completed genome of PEDV (CV777) and found 28033 nucleotides in length.

The first report of S gene sequence of PEDV isolate (Chinju99) in Korea revealed the nucleotide sequence homology 94.5% with those of European strains, CV777 and Br1/87. The full S gene open reading frame of Chinju99 comprised 4125 bases long encoding 1383 amino acids, including adenine (24.1%), cytosine (20.4%), guanine 21.1%) and thymine (34.4%) residues (Yeo et al., 2003). The field Korean PEDV isolates have been used as an attenuated vaccine candidate of PEDV cultured in Vero cells at the 93rd passages, designated as PEDV-9. Efficiency of this particular attenuated vaccine was confirmed by reducing pathogenicity in neonatal piglets and delivered piglets were protected by PEDV wide-type. Comparing of M gene of cell culture attenuated virus showed the 98.97% nucleotide and 98.24% amino acid identity with previously reported PEDV strains (Kweon et al., 1999). Additional Korean isolate (Spk1) was also cloned, sequenced and expressed of its neutralizing epitope in 2005 for development of an edible vaccine (Kang et al., 2005). In the study of molecular biology of PEDV, M gene of the Chinese isolate LJB/03 has been cloned, sequenced and compared with other PEDV strains in Genbank database. The LJB/03 encompasses an open reading frame of 681 nucleotides, deduced a 226 amino acid sequence. The composition of encoding polypeptide comprised 152 adenines (22%), 153 cytosines (23%), 161 guanines (24%), and 214 thymines (31%) and shared 97.79% amino acid identities with CV777, 97.35% with Br1/87, 96.90% with KPEDV-9. The phylogenetics separated PEDVs (CV777, Br1/87, Jme2, KPEDV-09) to three distinct genetic groups in which LJB/03 branched in the independent group (Jinghui and Yijing, 2004). Continually, N gene of porcine epidemic diarrhea virus LJB/03 was cloning and sequence analysis in 2006 by Junwei et al. (2006). Genomic characterization was clarified and

compared with other PEDVs and revealed that LJB/03 N shared high nucleotide sequence homology to those of other PEDV isolates, 97.4% with JS-2004-2, 95.6% with chinju99, 96.6% with Br1/87, and 96.8% with CV777. For the encoded protein, it shared 96.4% identities with CV777, 96.1% with Br1/87, 98% with JS-2004-2 and 96.90% with Chinju99, respectively (Junwei et al., 2006). For the Chinese isolates (M gene analysis: CH/HLJH/06, CH/HNCH/06, CH/IMB/06, CH/IMT/06, CH/JSX/06, CH/SHH/06, and completed gene analysis: DX), those were cloned and sequenced and compared with other reference PEDV strains. The M gene of the Chinese isolates revealed highly conserved sequence homology ranged from 98.8 - 100% and encoding amino acid sequence identity ranged from 98.2-100% with each other. The nucleotide and amino acid identity of M gene of those isolates and other reference PEDV strains varied from 97.2 to 99.4% and 96.9 to 100%. The phylogenetic tree analysis revealed close relationship of M gene forming a separate cluster including other field Chinese isolate JS-2004-2 (Chen et al., 2008).

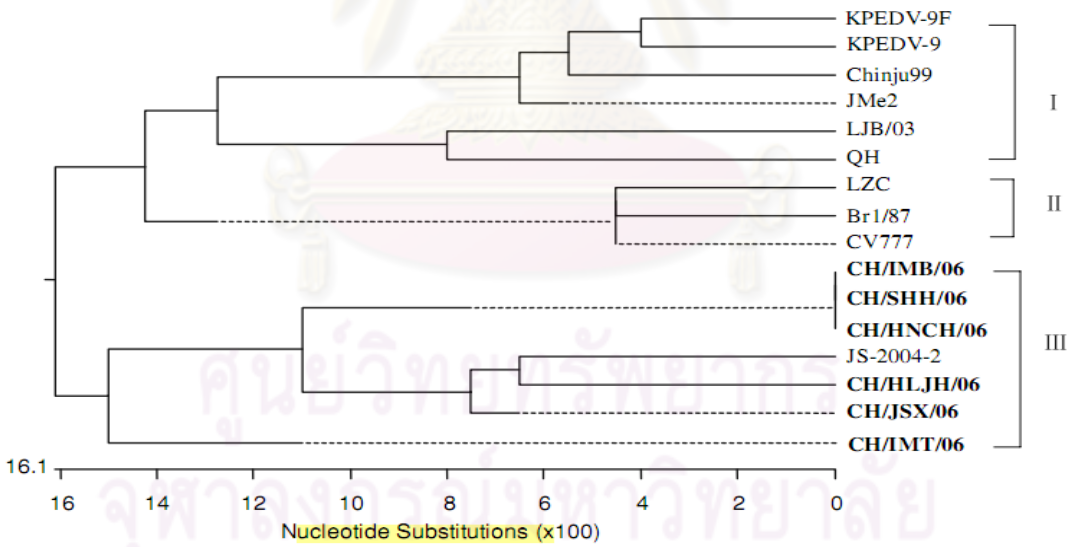


Figure 2.6 Dendrogram of genetic relationship on M gene of Chinese isolates and those of other strains. Chinese isolates are in bold in phylogenetic tree (Chen et al., 2008)

The DX isolate was sequenced in completed genome including structure genes spike (S), nucleocapsid (N), membrane (M), small membrane (sM) and found closely related with LJB/03, JS-2004-2 and CH/HLJH/06 (Figure 2.6) isolated from China. These findings indicated that the prevalence of some PEDV isolates in China were widespread from the same ancestor since the JS-2004-2 isolate originated from the south of the China and LJB/03 and CH/HLJH/06 from northeast China (Li et al., 2009).

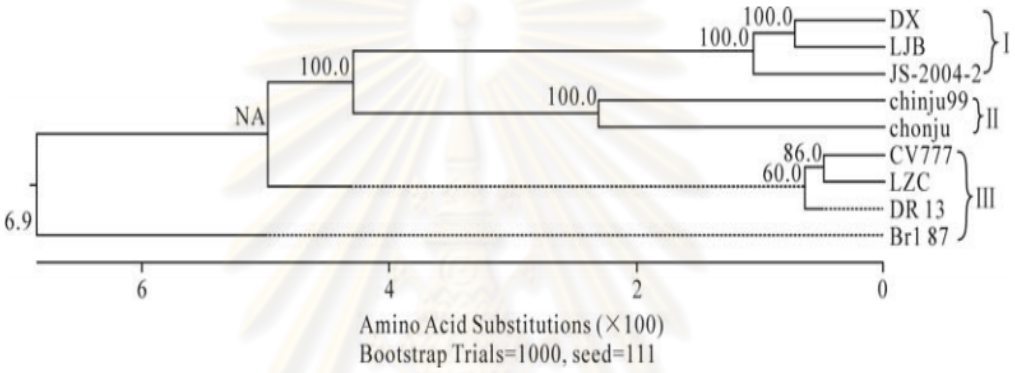


Figure 2.7 Phylogenetic relationship of PEDV DX and other PEDV strains based on a comparison of S amino acid sequences: Chonju (Korean), Chinju99 (Korean), JS-2004-2 (China), CV777 (England), from Genbank database reference (Li et al., 2010).

The study on sequence analysis of the partial spike glycoprotein gene of porcine epidemic diarrhea viruses was first reported by Korean authors (Park et al., 2007) that could be a representative section of gene for the whole genomic analysis. Based on the partial S gene, Korean PEDV isolates were divided into three distinct groups. Group 1, Korean PEDV isolates were highly homologous to CV777, Br1/87, JS-2004-2, KPED-9, P-5V, parent DR13, and attenuated DR13. Group 2 composed of Spk1, and Chinju99 was grouped in group 3 (Figure 2.8).

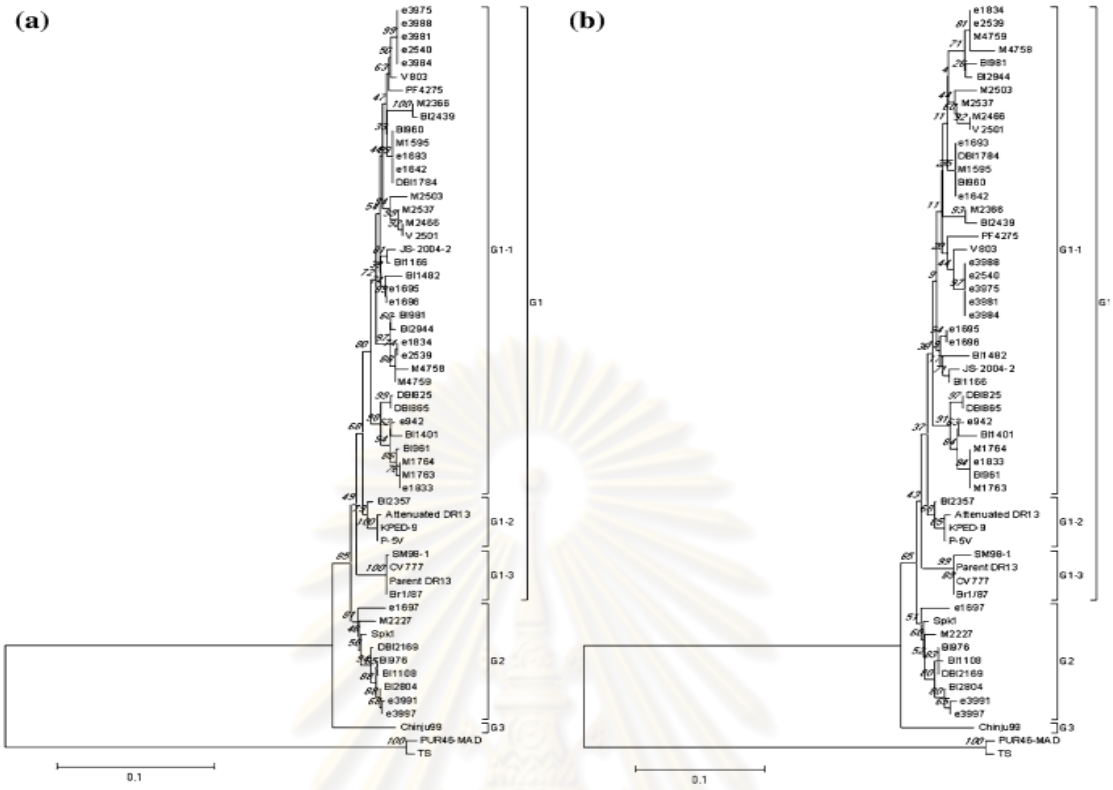


Figure 2.8 Dendrogram making on the basis of (a) nucleotide and (b) encoded amino acid sequences of the partial S glycoprotein genes of Korean PEDV isolates with reference PEDV strains constructed with neighbor-joining method (Park et al., 2007).

In Korea, Lee et al. (2010) additionally described the heterogeneity in spike protein genes of porcine epidemic diarrhea virus strains in Korea (KNU-0801, KNU-0802, KNU-0901, KNU-0902, KNU-0903, KNU-0904, KNU-0905). The homology analysis demonstrated that Korean PEDV isolates underwent the genetic diversity in their S glycoprotein genes. Interestingly, the finding of Lee et al. (2010) was quite different from the previous study of Park et al. (2007). As describing by Part et al. (2007), PEDV isolates were divided into three distinct groups based on the C-terminal region of S1 domain of the partial S gene and were distantly related to other previously identified Korean field isolates Spk1 and Chinju99. However, this study revealed only two distinct groups based on full S gene phylogenetic analysis. PEDV reference strains including the Chinese isolates and the Korean vaccine

strains belonged to group 1 whereas the Korean field isolates (Spk1 and Chinju99) were in group 2. Nevertheless, Lee et al. (2010) described that genetic analysis based on the C-terminal part of the S1 domain is not enough for being a representative to full S gene based analysis but based on the S1 domain and the S1 N-terminus.

In Thailand, re-emerging PED outbreak has begun since late 2007. Immediately, the partial gene and full M gene were cloned and sequenced. The phylogenetic analysis determined that all current Thai PEDV isolates during the outbreaks were in the same clade as the Chinese isolate JS-2004-2 and were clustered into group 1 (Puranaveja et al., 2009).

In summary, a number of researches on genetic characterization and phylogenetics of PEDV in N gene, M gene, full S gene, partial S gene, and complete genome were done and clarified S gene or partial S gene particularly S1 domain having hypervariable region with mutants (nucleotide/ amino acid substitution, deletion or/and insertion) of nucleotide sequence. Besides that, M gene and other structural protein genes are more conserved during viral evolution.

2.6. Molecular epidemiology in viral research

Molecular epidemiology is a type of epidemiological research investigating geographical distribution of pathogenic agents. In veterinary field, scientists interested in understanding the global epidemiology of important pathogens would use cross-sectional and longitudinal studies on both sero-epidemiology and molecular epidemiology. The molecular techniques are employed to monitor the evolution of the viruses (Murphy et al., 1999).

In general, pathogenic viruses undergo an infinitely long series of replication cycles when transmitted from host to host. Therefore, the mutational process continually generates and creates different biological properties from the parental virus (Schleif, 1993; Murphy et al., 1999). Mutation includes point mutations, substitution, deletions, insertions, and/or damage one or more nucleotides as well as amino acid sequence. The mutation of virus altered in the nucleotide sequence of the DNA or in the case of RNA viruses may alter

the amino acid sequences of proteins and may have the potential for changing the expression of genes (Schleif, 1993). Some properties important in the survival and evolutionary progression of various viruses in nature include the capacity to replicate rapidly, the capacity to replicate to high titer, the capacity to shed for long periods of time, the capacity to evade host defenses, the capacity to survive after being shed into the environment and the capacity to transmit vertically (Murphy et al., 1999).

Murphy et al. (1999) described molecular epidemiology becoming available since the 1980s in many veterinary and zoonotic virology laboratories when molecular cloning and nucleotide sequencing methods were applied after the introduction of the polymerase chain reaction. The potential of these methods contributed to clarify and understand the viral genetics. This approach also help finding out on route of virus transmission and understanding on prevention and control strategies (Page and Holmes, 1998). Traditionally, tracing the transmission of infectious diseases is performed by serologic investigation but not possible for pathogens strains alteration. Therefore the replaceable powerful approach is using the comparison of genetic sequencing (Page and Holmes, 1998). The comparison of nucleotide or protein sequences from the same or different organisms is able to demonstrate homology among sequences and can infer genomic function of newly sequenced genes, predict new members of gene families and additionally explore evolutionary relationships. The phylogenetic tree analysis can also illustrate the origin of the agents (Page and Holmes, 1998; Madden, 2002). The field of molecular epidemiology has blossomed, based on sequencing of large numbers of isolates for investigation disease outbreaks, epidemics, or endemic viral disease problems. For instances, porcine epidemic diarrhea virus isolates in European countries including CV777, Br1/87 (Duarte and Laude, 1994; Kocherhans et al., 2001), in China including JS-2004-2, DX, LJB/03, CHIMT06 (Chen el al., 2010), in Korea including Chinju99, Spk1, DR13, CPF299, M1763 (Yeo el al., 2003; Park el al., 2007; Lee et al., 2010) and in Thailand including 07NP01, 08NP02, 08CB01 (Puranaveja et al., 2009) and so on were cloned, sequence to clarify the genomic characterization and observed the molecular epidemiology.

Generally, viral RNAs are more mutational alterations on genomic sequence than viral DNA because of replication errors due to absence of a cellular proofreading mechanism (Moya et al., 2004). Moreover, nonlethal mutations in the genome of RNA viruses accumulate very rapidly. Therefore, the term “Viral Quasispecies”, as a model of RNA virus evolution (Eigen and Schuster, 1997; Moya et al., 2004) revealed the individual viral genomes differ in one or more nucleotides from the consensus or average sequence of the population. Relatively, short times genotypic drift occurs as particular variants may gain advantage. This term describe such diverse, rapidly evolving and competing viral populations. The process would be most obvious in large RNA genomic viruses. Indeed, Coronaviruses are a great candidate because they possess the largest RNA genomes. At the mutation rates, each one out of 30000 nucleotides coronaviral genome will be changed in each circle of replication. Furthermore, coronaviral genomes undergo other more substantial mutations, including massive deletions, which will affect their pathogenicity (Murphy et al., 1999; Moya et al., 2004).

CHAPTER III

MATERIALS AND METHODS

The research was done as the following framework (Figure 3.1). Firstly, the history of the PED-affected herds was recorded and intestinal samples were collected. RNA isolation and RT-PCR were performed to amplify genetic materials of the field PED virus isolates and sequenced the partial S and full M genes. The nucleotide and deduced amino acid sequence of current PEDV isolates were compared with other selected PEDV strains in Genbank database.

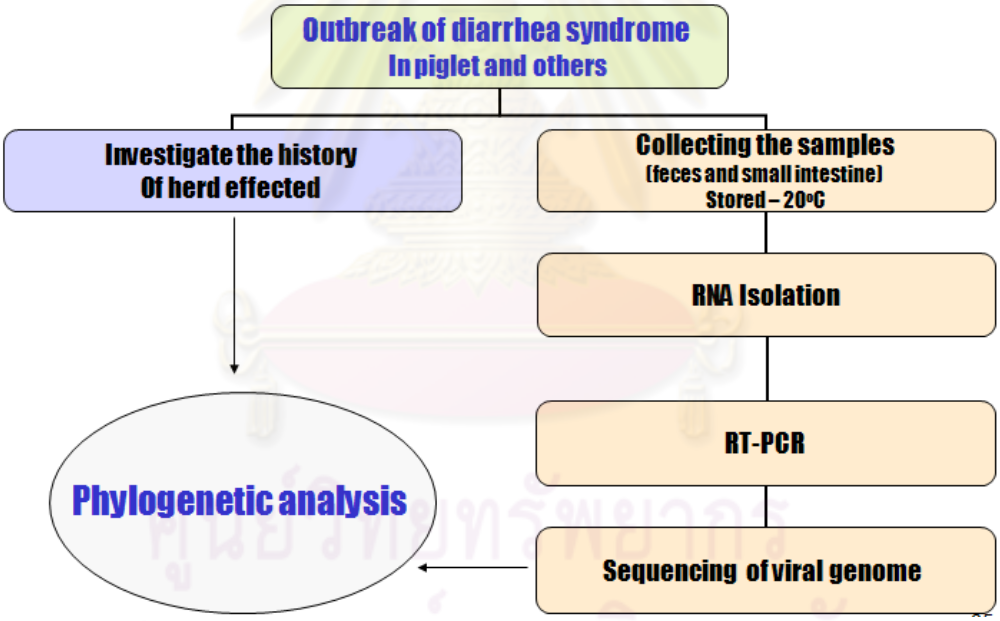


Figure 3.1 Conceptual framework, composing of six steps of the study: history taking, sampling, RNA isolation, RT-PCR, sequencing of viral genome, and phylogenetics.

3.1 Herd History

Observation of the PED outbreaks began in the initially occurred province and the adjacent provinces of industrialized pig production in southern Vietnam. History of affected herds was recorded with the questionnaire including pig production system, sources of replacement stocks, clinical manifestation, and some epidemiologic features such as morbidity and mortality rate.

3.2. Sample collection

Three provinces in southern Vietnam were selected (Dongnai, Binhduong and Hochiminh). The intestinal samples were collected from 3-10 days old piglets suffered from PED in affected farms. Totally, sixty samples of acute watery diarrhea piglets were collected in five commercial swine farms of those provinces (Table 3.1 and Figure 3.2). These provinces have adjacent geographically where Dongnai was considered the first place dealing with PED outbreak in early 2009 (unpublished data). The acute watery diarrhea outbreak widely spread to adjacent provinces of Hochiminh, Binhduong, Longan and throughout southern area of Vietnam. The clinical signs of disease appeared in all age groups of pigs with the current state of severe acute diarrhea especially in suckling piglets with morbidity reaching nearby 100% and mortality varying from 65-91%.

At necropsy, small intestine was collected in three parts separately including duodenum, jejunum, and ileum in each piglet. Mixed intestinal part of each piglet was separated in sterile plastic bags and kept in -20^{o}C until processed.

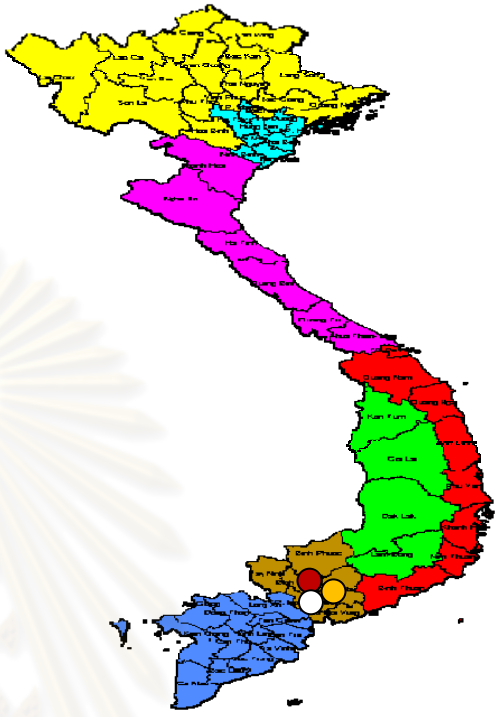


Figure 3.2 Schematic revealed the sample origin in three provinces of southern Vietnam. The white circle indicates position of Hochiminh city, yellow one is Dongnai and the last one is Binhduong.

Table 3.1 Geographic origin, numbers of farms and PED isolates

Geographic areas	Provinces	Farms	Pigs	Samples	Collected isolates
Southern area	DONG NAI	1	Piglets	20	1[a]
	HO CHI MINH	2		25	4[c]
	BINH DUONG	2		15	3[b]
Total	03	5		60	8

a) Isolate VN109S5 (Farm 3, Dongnai), b) isolates VN92S1 (Farm 1, Binhduong); VN94S2, VN97S3 (Farm 2, Binhduong), and c) isolates VN103S4, VN112S6 (Farm 4, Hochiminh), VN116S7, VN122S8 (Farm 5, Hochiminh)

3.3. RNA isolation

3.3.1. Sample preparation

Suspected individual pig infected with PED virus was collected (duodenum, jejunum and ileum). To ensure having sufficient amount of tissue samples for RNA analysis, at least 1 gram for each site was collected. The samples were processed as soon as possible to maximize the chance of obtaining good quality viral RNA. When processing procedure could not take place immediately, the samples were stored at 4°C with extra care. The tissue samples were homogenized by adding PBS, sand into mortar and well grinded by pestle. After that sample suspension was centrifuged at 3000 rpm, 10-15 minutes at 4°C. The supernatant of 1-3 ml is collected into the centrifuged sterile tubes and stored at -70°C until RNA isolation.

3.3.2. Viral RNA isolation procedures

RNA extraction was performed in a clean, separated area to minimize the chance of cross-contamination. The procedures of total viral RNA isolation are performed according to the protocol of commercial kit's instruction (SV Total RNA Isolation System, Promega, Madison, WI, USA). The SV Total RNA Isolation System provides a fast and simple technique for preparing purified and intact total RNA from tissues, cultured cells and white blood cells. The successful isolation of intact RNA requires four main steps including effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and removal of contaminating DNA and proteins. The most important step is the immediate inactivation of endogenous RNases released from membrane-bound organelles upon cell disruption based on the properties of guanidine thiocyanate and β-mercaptoethanol. Dilution of cell extracts in the presence of high concentrations of guanidine thiocyanate causes selective precipitation of cellular proteins, while the RNA remains in solution. After centrifugation to clear the lysate of precipitated proteins and cellular debris, the RNA is selectively precipitated with ethanol

and bound to the silica surface of the glass fibers found in the spin basket. By effectively clearing the lysate of precipitated proteins and cellular debris, these cleared lysates may be bound to the spin baskets by a centrifugation. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the silica. RNase-free Dnase was applied directly to the silica membrane to digest contaminating genomic DNA. The bound total RNA is further purified from contaminating salts, proteins and cellular impurities by simple washing steps. Finally, the total RNA is eluted from the membrane by the addition of nuclease-free water.

Sample homogenization and RNA extraction were done in the Animal Biotech Laboratory of Molecular Biology and Bio-chemistry Institute, Nong Lam University, Vietnam. The extracted RNA was preserved in 0.5 ml absolute ethanol and submitted to the Veterinary Diagnostic Laboratories, Faculty of Veterinary Sciences, Chulalongkorn University, Thailand. Therefore, extracted RNA must be separated from absolute ethanol prior to running the RT-PCR (Bogner and Killeen, 2006; Li et al., 2008). The suspensions were centrifuged for 5-10 min at 14,000 rpm (4^{o}C) then the RNA pellets were washed with 1 ml ethanol 75%, centrifuged again for 5-10 min at 14,000 rpm (4^{o}C), and finally dried in room temperature before re-suspension in 30 µl of diethyl-pyrocarbonate (DEPC)-treated deionized water.

3.4. RT-PCR

3.4.1. Primers used for RT-PCR

The two specific pairs of primers used for amplification of partial S gene and full M gene of PEDV were selected from previous publications (Park et al., 2007; Chen et al., 2008). The nucleotide strands of the partial S gene primers are:

5’-TTCTGAGTCACGAACAGCCA-3’ (PS1, forward)

5’-CATATGCAGCCTGCTCTGAA-3’ (PS2, backward)

and of full M gene primers are:

5’-CCCCAGTACTGTTATTGACGTATAAAC-3’ (PM1, forward)

5'-GTTTAGACTAAATGAAGCACTTTC-3' (PM2, backward)

The sizes of specifically amplified products are 651bp and 715bp, respectively.

3.4.2. RT-PCR one-step protocols

This study used one tube reaction to amplify RNA of PEDV (one step RT-PCR). The reverse-transcribed into cDNA and PCR of the partial of S and M glycoprotein genes were performed in the one-tube reaction of total 25 µl in volume. The RT-PCR kit being used is AccessQuick™ RT-PCR System (Promega, Madison, WI, USA) containing AMV reverse transcriptase, the thermostable Tfl DNA polymerase provided a combined reverse transcription (transcription of RNA template to standard first-strand cDNA) and PCR. Exactly, 4 µl RNA template mixed with a reaction mixture containing 10 µl of 2X AccessQuick™ Master Mix (Promega, Madison, WI, USA), and 1 µl of each specific primer (10 µM), 0.5 µl of $MgCl_2$ (25 µM), 0.5 µl AMV reverse transcriptase (10u/µl), and add 8 µl nuclease-free water reach to total volume reaction of 25 µl . The RT-PCR reactions run in Thermal hybrid PCR machine (USA). The RT-PCR cycling procedure divided into three stages (Figure 3.3). Firstly, reverse transcription reaction was incubated at 48°C for 45 min to make the first strand cDNA dynthesis. Then, the second strand cDNA synthesis and PCR amplification was denatured at 95°C for 2 min (01 cycle) and 30 cycles of repeated denaturation at 94°C 30 sec, annealing 53°C 60 sec, extension at 72°C 60 sec. Additional step is the final extension at 72°C for 5 min. The last stage is to held PCR product at 4°C.

PCR products were recognized by agarose gel electrophoresis of 1.5% that readily visible in UV transillumination of an ethidium bromide-stained gel. The correct size of PCR product bands (651 bp for partial S gene, 715 bp for M gene) were selected and purified by using NucleoSpin® Extract II Kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions. These purified PCR products of partial S and full M gene were submitted for sequencing.

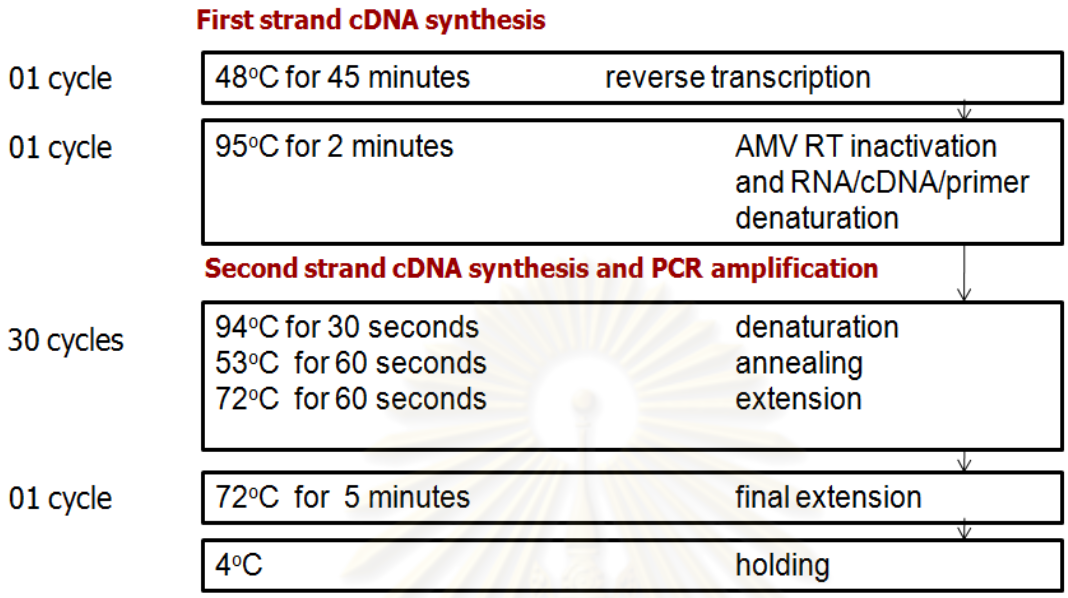


Figure 3.3 Reverse transcription and PCR cycling procedure

3.5. Sequencing of DNA

Purified PCR products corresponding to the partial S glycoprotein gene and full M gene were sequenced by 1st BASE Pte Ltd (Singapore). All sequencing reactions were carried out in duplicate and all sequences were determined by sequencing both strands (forward and backward strands).

The DNA sequencing reaction used BigDye Terminator v3.1 cycle sequencing kit chemistry in 96-well plate. The steps involved (1) cycle sequencing reaction setup, (2) post sequencing clean-up (purifying extension products), (3) electrophoresis in DNA Analyzer of 3730XL, and (4) basecalling using KB basecaller (according to BigDye® Terminator v3.1 Cycle Sequencing Kit' protocol, 2002).

3.6. Sequencing analysis

Nucleotide sequences of the current Vietnamese PEDV isolates and other selected isolates see on Table 3.2 (Genbank: http://www.ncbi.nlm.nih.gov) were aligned (pairwise and multi-alignment), edited, and analyzed with Chromas 2.33, Bioedit v7.0.5.3 and ClustalX 2.0.11 program. A phylogenetic trees were then generated comparing those of partial S glycoprotein and M glycoprotein gene nucleotide and deduced amino acid sequences with some reference PEDV strains based on the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 4.1 program (Tamura et al,. 2007). The relative support for each branch the bootstrap value of 1000 replicates was computed. The selected strains used for sequence alignment, sequence analysis, and phylogenetic analysis with selected reference PEDV isolates are described in Table 3.2.

Table 3.2 The reference strains used for sequence analysis and phylogenetic analysis were compared with current Vietnamese PEDV isolates

Order	Strains	Countries and year of sampling	Accession number	References
1	CV777 [S/M]	Belgium, 1977	AF353511.1[S/M]	Kocherhans et al., 2001
2	Br1/87[S/M]	Britain, 1987	Z25483[S]; Z24733.1[M]	Duarte and Laude, 1994
3	JS-2004-2 [S/M]	China, 2004	AY653204.1[S]; AY653205.1[M]	Unpublished
4	LZC [M]	China, 2006	EF185992.1[M]	Unpublished
5	DX[S/M]	China, 2007	EU031893.1[S/M]	Unpublished
6	CHIMT06 [M]	China, 2006	EU033965.1 [M]	Chen et al., 2010
7	Spk1 [S]	Korea, 2002	AF500215.1[S]	Kang et al., 2005
8	Chinju99 [S/M]	Korea, 1999	AY167585.1 [S/M]	Yeo et al., 2003
9	DR13 [S]	Korea, 2006	DQ462404.2 [S]	Park et al., 2007
10	KNU-0801 [S]	Korea, 2008	GU180142.1 [S]	Lee et al., 2010
11	KNU-0802 [S]	Korea, 2008	GU180143.1 [S]	Lee et al., 2010
12	M1763 [M]	Korea, 2003	FJ687455.1[M]	Unpublished

13	CPF299 [M]	Korea, 2007	FJ687467.1 [M]	Unpublished
14	KPEDV-09 [M]	Korea, 1997	AF015888.1 [M]	Kweon et al., 1999
15	07NP01 [S/M]	Thailand, 2007	FJ196196.1 [S/M]	Puranaveja et al., 2009
16	08NP02 [S/M]	Thailand, 2008	FJ196204.1 [S/M]	Puranaveja et al., 2009
17	08CB01 [S]	Thailand, 2008	FJ196197.1 [S]	Puranaveja et al., 2009
18	KU06RB08 [M]	Thailand, 2008	FJ196194.1 [M]	Puranaveja et al., 2009
19	VN92 [S/M]	Vietnam, 2010	-	This study
20	VN94 [S]	Vietnam, 2010	-	This study
21	VN97 [S]	Vietnam, 2010	-	This study
22	VN103 [S/M]	Vietnam, 2010	-	This study
23	VN109 [S/M]	Vietnam, 2010	-	This study
24	VN112 [S/M]	Vietnam, 2010	-	This study
25	VN116 [S/M]	Vietnam, 2010	-	This study
26	VN122 [S/M]	Vietnam, 2010	-	This study

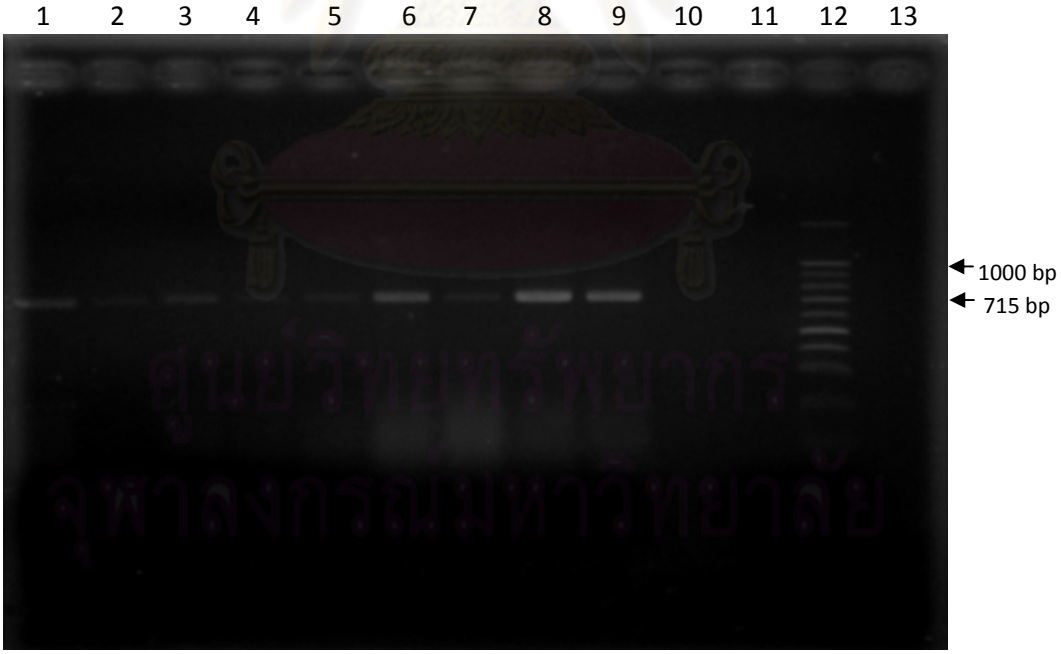
[S] strain used for sequence analysis of the partial S gene

[M] strain used for sequence analysis of the full M gene

CHAPTER IV
RESULTS

Eight southern Vietnamese PEDVs were from the PED affected commercial farms during 2009-2010 in three provinces. These isolates were confirmed by one-step RT-PCR amplification to recognize the specific products of partial S gene and full M gene (Figure 4.1), and named as VN92 (S1/M1) (Farm 1, Binhduong); VN94S2, VN97S3 (Farm 2, Binhduong); VN109 (S5/M3) (Farm 3, Dongnai); and VN103(S4/M2), VN112(S6/M4) (Farm 4, Hochiminh); VN116 (S7/M5), VN122 (S8/M6) (Farm 5, Hochiminh). These isolates were sequenced and analyzed genomic characterization by comparing with selected corresponding strains in Genbank database (http://www.ncbi.nlm.nih.gov).

A



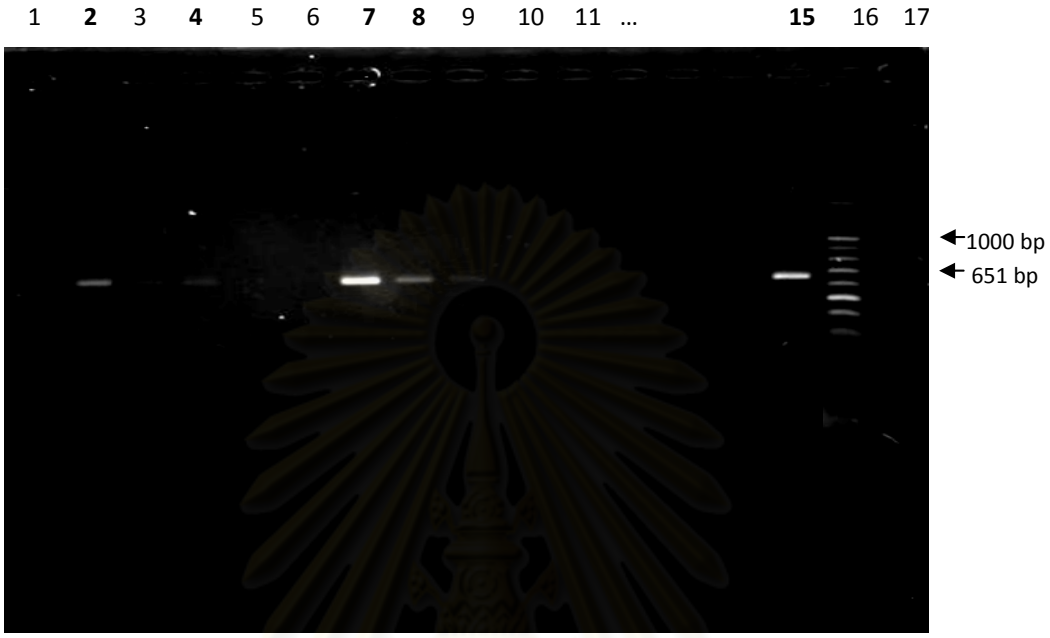


Figure 4.1 One-step RT-PCR for amplification of specific products of field PEDVs in this study. A, RT-PCR products of field isolates on M gen (lane 1~VN92M1, lane 3~VN109M3, lane 5~VN103M2, lane 7~VN112M4, lane 8~VN116M5, lane 9~VN122M6, lane 6~positive control, lane 11~negative control, lane 12~DNA marker, lane 13~blank, remain others were negative). B, RT-PCR products of field isolates on S gen (lane 2~VN92S1, lane 4~VN94S2, lane 8~VN97S3, lane 7 and 15~positive controls, lane 17~negative control, lane 16~marker, lane 17~blank, and remain others were negative).

4.1. Sequence homology

The partial S gene

The pairwise alignment of each couple of nucleotide sequences of southern Vietnamese isolates showed high nucleotide homology together (98.9-100%). VN92S1 had minor different nucleotide and encoded amino acid sequence with the remaining isolates (VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) at 98.9% and

97.3% identity, respectively. However, the current PEDV isolates contained variable difference on nucleotide sequences of the partial S gene with other reference strains (Table 4.1). These current isolates shared 95.6-96.4% nucleotide identity with the PED prototype strains CV777, Br1/87 (European strains) and 91.4-97.8% identity with the Korean isolates (Chinju99, Spk1, DR13, KNU-0801, KNU-0802). Interestingly, the current Vietnamese isolates shared high nucleotide homology with the Chinese isolates (JS-2004-2, DX) and the Thai isolates (07NP01, 08NP02, 08CB01) at 97.7-98.5% and 98.8–99.5%, respectively. These results revealed the current Vietnamese PEDV isolates closely related to those mentioned as well as the particular Korean isolate (KNU-0802). Based on phylogenetics of the partial S gene, PEDVs are divided into three distinct genetic groups (Gr1, Gr2, Gr3). The mean of nucleotide identity percentage was computed for elucidating the genetic relationship among the Vietnamese isolates with other selected isolates from PED outbreaks of the neighboring countries. In each group average (%) of nucleotide identity was calculated following bearing countries. The average of group 1 (CV777, Br1/87, DR13, KNU-0802, JS-2004-02, DX, 07NP01, 08NP02, 08CB01, and 8 current Vietnamese PEDV isolates) shared 92.72% nucleotide identity with group 3 (Chinju99), 96.20% with group 2 (Spk1, KNU-0801) and group 2 shared 95.50% nucleotide identity with group 3. Mainly, PEDV isolates belonged to Gr1 and shared high homology in nucleotide sequence alignment together. In those Vietnamese isolates shared 96.24% nucleotide identity with European strains (CV777, Br1/87), 97.51% with the Korean isolates (DR13, KNU-0802), 98.18% with the Chinese isolates (JS-2004-2, DX) and closest related to the Thai isolates (07NP01, 08NP02, 08CB01) at 99.35% nucleotide homology. However, they were highly different from the strains in Gr2 and Gr3. The European strains (CV777, Br1/87) and the Chinese isolates (JS-2004-2, DX) shared average 96.85% nucleotide identity. However, the Chinese PEDV isolates and the Korean PEDV isolates (DR13, KNU-0802) shared 98.10% identity. These Korean isolates shared 97.8% nucleotide homology with the current Thai isolates (07NP01, 08NP02, 08CB01), and 97.21% with the European strains. The Thai isolates shared 96.4% and 98.85% nucleotide homology with the European strains and the Chinese isolates, respectively.

Table 4.1 Comparison homology (%) of nucleotide and encoded amino acid sequences of the partial S gene of southern Vietnamese PEDV isolates with PEDV reference strains

	Br1/87	CV777	Spk1	Chinju99	DR13	KNU-0801	KNU-0802	JS-2004-2	DX	07NP01	08CB01	08NP02	VN92-S1	VN94-S2	VN97-S3	VN103-S4	VN109-S5	VN112-S6	VN122-S8	VN116-S7
Br1/87	-	100	91,6	87,1	92,3	91,6	87,9	87,9	90,9	87,1	87,1	87,1	85,6	87,1	86,4	87,9	87,9	87,9	87,9	87,9
CV777	100	-	91,6	87,1	92,3	91,6	87,9	87,9	90,9	87,1	87,1	87,1	85,6	87,1	86,4	87,9	87,9	87,9	87,9	87,9
Spk1	96,9	96,9	-	90,9	95,9	100	93,1	91,6	93,1	90,1	90,1	90,1	88,6	91,6	90,9	90,9	90,9	90,9	90,9	90,9
Chinju99	94,2	94,2	95,3	-	86,4	90,9	83,2	81,6	84,8	81,6	81,6	81,6	80,0	83,2	82,4	82,4	82,4	82,4	82,4	82,4
DR13	97,9	97,9	97,5	93,8	-	95,9	93,8	94,5	95,9	92,3	92,3	92,3	90,9	92,3	91,6	93,1	93,1	93,1	93,1	93,1
KNU-0801	97,1	97,1	99,5	95,7	97,9	-	93,1	91,6	93,1	90,1	90,1	90,1	88,6	91,6	90,9	90,9	90,9	90,9	90,9	90,9
KNU-0802	96,5	96,5	97,2	92,9	97,6	97,4	-	94,5	95,9	94,5	94,5	94,5	91,6	93,1	92,3	93,8	93,8	93,8	93,8	93,8
JS-2004-2	96,6	96,6	96,2	92,0	97,9	96,3	98,2	-	97,3	95,9	95,9	95,9	93,1	94,5	93,8	95,2	95,2	95,2	95,2	95,2
DX	97,1	97,1	96,4	92,5	98,1	96,5	98,1	99,1	-	95,2	95,2	95,2	92,3	93,1	93,1	94,5	94,5	94,5	94,5	94,5
07NP01	96,4	96,4	95,8	92,2	97,5	95,9	98,1	99,1	98,6	-	100	100	97,3	98,6	98,0	99,3	99,3	99,3	99,3	99,3
08CB01	96,4	96,4	95,8	92,2	97,5	95,9	98,1	99,1	98,6	100	-	100	97,3	98,6	98,0	99,3	99,3	99,3	99,3	99,3
08NP02	96,4	96,4	95,8	92,2	97,5	95,9	98,1	99,1	98,6	100	100	-	97,3	98,6	98,0	99,3	99,3	99,3	99,3	99,3
VN92-S1	95,6	95,6	95,0	91,4	96,8	95,1	97,1	97,8	97,7	98,8	98,8	98,8	-	97,3	98,0	98,0	98,0	98,0	98,0	98,0
VN94-S2	96,2	96,2	95,9	92,4	97,4	96,0	97,6	98,4	97,9	99,3	99,3	99,3	98,9	-	99,3	99,3	99,3	99,3	99,3	99,3
VN97-S3	96,1	96,1	95,7	92,2	97,2	95,9	97,5	98,2	97,8	99,2	99,2	99,2	99,1	99,9	-	98,6	98,6	98,6	98,6	98,6
VN103-S4	96,4	96,4	95,8	92,2	97,5	95,9	97,8	98,5	98,1	99,5	99,5	99,5	99,1	99,9	99,7	-	100	100	100	100
VN109-S5	96,4	96,4	95,8	92,2	97,5	95,9	97,8	98,5	98,1	99,5	99,5	99,5	99,1	99,9	99,7	100	-	100	100	100
VN112-S6	96,4	96,4	95,8	92,2	97,5	95,9	97,8	98,5	98,1	99,5	99,5	99,5	99,1	99,9	99,7	100	100	-	100	100
VN122-S8	96,4	96,4	95,8	92,2	97,5	95,9	97,8	98,5	98,1	99,5	99,5	99,5	99,1	99,9	99,7	100	100	100	-	100
VN116-S7	96,4	96,4	95,8	92,2	97,5	95,9	97,8	98,5	98,1	99,5	99,5	99,5	99,1	99,9	99,7	100	100	100	100	-

Nucleotide identity (%) in lower triangle of table; Decoded amino acid identity (%) in upper triangle of table

For deduced amino acid sequence analyses of the partial S glycoprotein gene, the current Vietnamese PEDV isolates also showed high amino acid sequence homology with 97.3–100% together. These isolates closely related to the Chinese isolates (JS-2004-2, DX), and the Thai isolates (07NP01, 08NP02, 08CB01) at 92.3–95.2% and 97.3–99.3% encoded amino acid sequence identity, respectively. In contrast, these current Vietnamese isolates had quite difference in genetic distance of amino acid sequences with previous isolates in Europe (CV777, Br1/87) and in Korea (Chinju99, Spk1, DR13, KNU-0801, KNU-0802) (85.6-87.9% and 80.0–93.8%, respectively). The comparison in deduced amino acid sequence homology of partial S gene among PEDV groups and within each group was revealed in Figure 4.2. Gr1 shared 82.59% amino acid identity with Gr3, 91.58% with Gr2 and 90.90% amino acid sequence identity between Gr2 and Gr3. However, Gr1 contained most current PEDV strains of affected countries. The Vietnamese PEDV isolates shared 87.31% deduced amino acid identity of partial S gene with European strains (CV777, Br1/87), 92.87% with Korean isolates (DR13, KNU-0802) and these Vietnamese isolates considerably closed to the Chinese isolates (JS-2004-2, DX) and the Thai isolates (07NP01, 08NP02, 08CB01) at 94.30% and 98.81 %, respectively. In addition the homology of other strains in different countries also revealed in Figure 4.2.

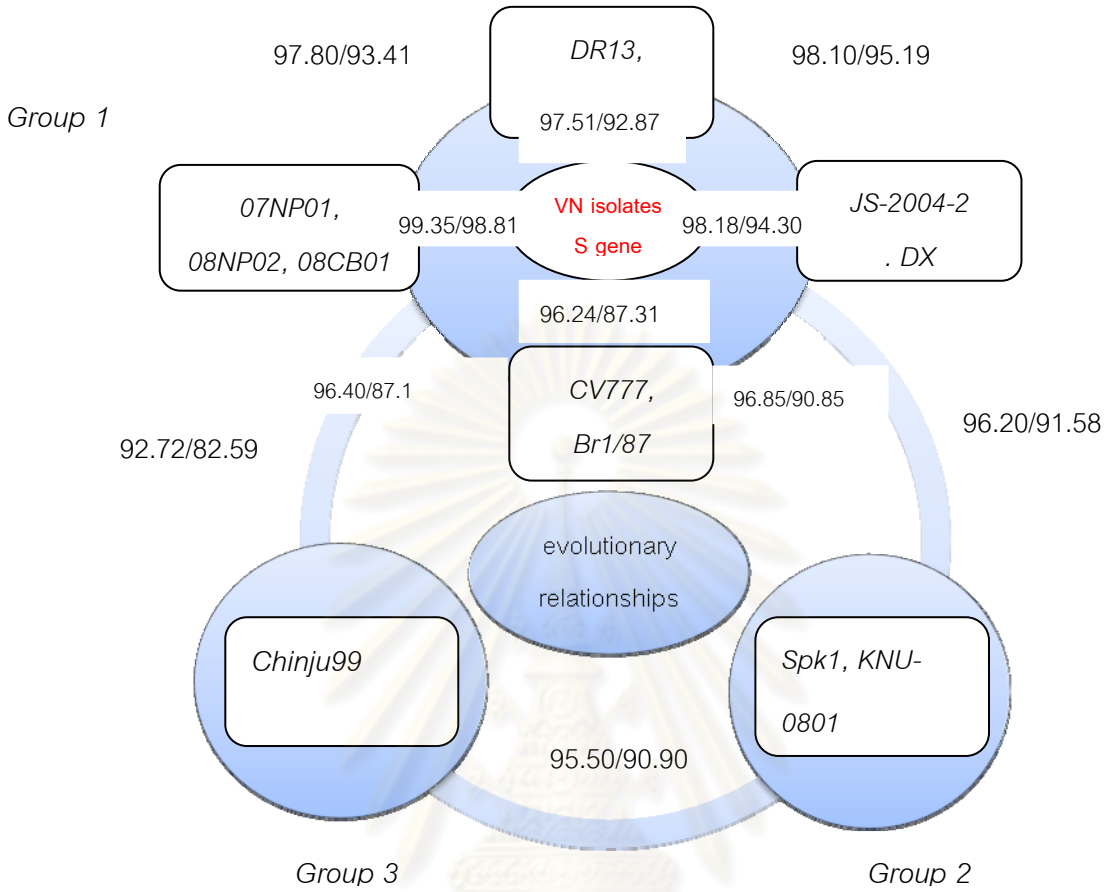


Figure 4.2 Comparing genetic distance (% identity average) of nucleotide/deduced amino acid sequences of the partial S gene among southern Vietnamese PEDV isolates with reference strains (pairwise distance computed by Mega 4.1). European strains including CV777, Br1/87; Chinese isolates, JS-2004-2 and DX; Korean isolates, Chinju99, Spk1, DR13, KNU0801 and KNU0802; Thai isolates, 07NP01, 08NP02, 08CB01 and Vietnamese isolates in this study were VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8

The full M gene

Homology of nucleotide and deduced amino acid sequence in full M gene of Vietnamese PEDV isolates shared considerably high similarity (99.7-100% and 99.0-100%) in this study. However, VN92M1 had minor different nucleotide and amino acid sequence with other Vietnamese isolates (VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) at

99.7% and 99% identity (Table 4.2). Similar to partial S gene, the average percentages of nucleotide and amino acid sequence homology were computed to make genetic relationship between groups and within each group. Group 1 (JS-2004-2, DX, CPF299, M1763, 07NP01, 08NP02, KU06RB08, and 6 current Vietnamese PEDV isolates) shared 97.89% and 96.18% in nucleotide and deduced peptide sequence identity, respectively with group 2 and 97.33% and 95.09% with group 3. Moreover, group 2 shared 99.54% and 97.15% identity with group 3. Within group 1, the Vietnamese PEDV isolates shared 98.89% and 97.07% of nucleotide and amino acid sequence identity with the Thai isolates (07NP01, 08NP02, KU06RB08), 98.53% and 97.49% identity with the Chinese isolates (JS-2004-2, DX), and 98.56% and 96.71% identity with the Korean isolates (CPF299, M1763). Furthermore, those Thai isolates shared 98.24% and 96.97% of nucleotide and peptide sequence homology with the Chinese isolates, 98.95% and 97.74% identity with the recent Korean isolates. In particular, the Thai isolates had absolute homology of M gene with the only Korean isolate (CPF299) at 100% identity of nucleotide and deduced amino acid sequence. The Chinese isolates and the recent Korean isolate shared 98.06% and 96.35% of nucleotide and decoded amino acid sequence identity, respectively.

Table 4.2 Comparison homology of nucleotide and encoded amino acid sequences of full M gene of southern Vietnamese PEDV isolates with PEDV reference strains

	Br1/87	CV777	Chinju99	CPF299	M1763	LZC	DX	CHIMT06	JS-2004-2	07NP01	KU06RB08	08NP02	VN092-M1	VN103-M2	VN109-M3	VN112-M4	VN116-M5	VN122-M6
Br1/87	-	99,5	97,9	95,3	96,4	96,9	96,4	97,4	95,8	95,3	94,8	95,3	94,8	95,8	95,8	95,8	95,8	95,8
CV777	100	-	98,5	95,8	96, 9	97,4	96, 9	97,9	96,4	95,8	95,3	95,8	95,3	96,4	96,4	96,4	96,4	96,4
Chinju99	99,7	99,7	-	95,3	96,4	95,8	96,4	97,4	95,83	95,3	94,8	95,3	94,76	95,8	95,8	95,8	95,8	95,8
CPF299	97,2	97,2	97,5	-	95,8	93,1	95,8	95,8	98,5	100	99,5	100	96,4	97,4	97,4	97,4	97,4	97,4
M1763	98,6	98,6	98,3	97,9	-	94,2	94,8	95,8	96,4	95,8	95,3	95,8	95,3	96,4	96,4	96,4	96,4	96,4
LZC	99,3	99,3	99,0	96,4	97,9	-	94,2	95,3	93,7	93,1	92,6	93,1	92,6	93,7	93,7	93,7	93,7	93,7
DX	98,6	98,6	99,0	97,2	97,2	97,9	-	97,9	97,4	95,8	95,3	95,8	95,3	96,4	96,4	96,4	96,4	96,4
CHIMT06	99,7	99,7	99,3	97,5	98,3	99,0	99,0	-	97,4	95,8	95,3	95,8	96,4	97,4	97,4	97,4	97,4	97,4
JS-2004-2	97,9	97,9	98,3	99,3	98,6	97,2	97,9	98,3	-	98,5	97,9	98,5	97,9	99,0	99,0	99,0	99,0	99,0
07NP01	97,2	97,2	97,5	100	97,9	96,4	97,2	97,5	99,3	-	99,5	100	96,4	97,4	97,4	97,4	97,4	97,4
KU06RB08	97,2	97,2	97,5	100	97,9	96,4	97,2	97,5	99,3	100	-	99,5	95,8	97	96,9	96,9	96,9	96,9
08NP02	97,2	97,2	97,5	100	97,9	96,4	97,2	97,5	99,3	100	100	-	96,4	97,4	97,4	97,4	97,4	97,4
VN092-M1	97,2	97,2	97,5	98,6	97,9	96,4	97,2	97,5	99,3	98,6	98,6	98,6	-	99,0	99,0	99,0	99,0	99,0
VN103-M2	97,5	97,5	97,9	99,0	98,3	96,8	97,5	97,9	99,7	99,0	99,0	99,0	99,7	-	100	100	100	100
VN109-M3	97,5	97,5	97,9	99,0	98,3	96,8	97,5	97,9	99,7	99,0	99,0	99,0	99,7	100	-	100	100	100
VN112-M4	97,5	97,5	97,9	99,0	98,3	96,8	97,5	97,9	99,7	99,0	99,0	99,0	99,7	100	100	-	100	100
VN116-M5	97,5	97,5	97,9	99,0	98,3	96,8	97,5	97,9	99,7	99,0	99,0	99,0	99,7	100	100	100	-	100
VN122-M6	97,5	97,5	97,9	99,0	98,3	96,8	97,5	97,9	99,7	99,0	99,0	99,0	99,7	100	100	100	100	-

Nucleotide identity (%) in lower triangle of table; decoded amino acid identity (%) in upper triangle of table

4.2. Genomic characterization

The partial S gene

The nucleotide sequence in partial S gene of the southern Vietnamese PEDV isolates composed of average 600 nucleotides (encoded about 200 amino acids), located from position 1530 to 2130 based on full length of S gene of prototype strain CV777 (Figure 4.3a). The nucleotide sequence revealed variability sites from nucleotides 1561 to 1600 and 1821–1851. Totally, there were 25 nucleotides substituted changes in sequence, from G to A (1574/position), C to T (1575), T to A (1577), G to T (1578) A to G (1582), G to A (1594), G to T(1596), C to G (1661), G to A (1711), T to C (1713), G to A (1795), C to G (1825), C to A (1829), G to A (1830), A to G (1837), C to T (1849), G to T (1851), A to G (1918), T to G (1920), G to C (1948), A to C (1978), A to G (1979), A to T (1980), A to T (2014), and finally C to T (2098). In detail, VN92S1, VN94S2, and VN97S3 contained individual changes. For the nucleotide substitution of VN92S1, substitution included G to A (1711), T to C (1713), C to G (1825), A to G (1837), G to C (1948). VN94S2 and VN97S3 contained individual changes from G to A (1574), C o T (1575) and C to G (1825), respectively, comparing with other isolates. These Vietnamese PEDV isolates showed nucleotide changes mainly substitution pattern of scattered nucleotide in sequence. Multiple alignment of these Vietnamese isolates had similar sequence changes to the Chinese isolates (JS-2004-2, DX), and the Thai isolates (07NP01, 08NP02, 08CB01) at most positions in nucleotide sequence (1577, 1578, 1582, 1594, 1596, 1661, 1795, 1829, 1830, 1849, 1851, 1918, 1920, 2014, and 2098).

Encoded amino acid sequence alignment of the partial S gene had corresponding changes at position of those in nucleotide sequence (Figure 4.3b). At position 525, individual differences of amino acid occurred in VN94S2, VN97S3 (change from G to D), and position 609, from P to A comparing with the prototype strains (CV777). Furthermore, VN92S1 had more individual changes than other Vietnamese isolates, from D to N (571), P to A (609), S to G (613) and V to L (650). The remaining Vietnamese PEDV isolates shared similar homology of deduced amino acid sequence changes to the Chinese isolates (JS-

2004-2, DX), the Thai isolates (07NP01, 08NP02, 08CB01) as the following from L to H (526/position), S to G (528), V to I (532), T to S (554), G to S (599), A to E (610), L to F (617), I to V (640), I to F (672), H to Y (700). However, at position 660 of the Vietnamese PEDV isolates contained different change (K to R) from the Chinese and Thai isolates having similar amino acid to CV777. Likewise, the Chinese isolates (JS-2004-2, DX), and the Thai isolates (07NP01, 08NP02, 08CB01) contained a few difference amino acids with the Vietnamese isolates. In summary, there were total 15 amino acid changes within corresponding 25 nucleotides substitution in the partial S gene of the Vietnamese PEDV isolates.

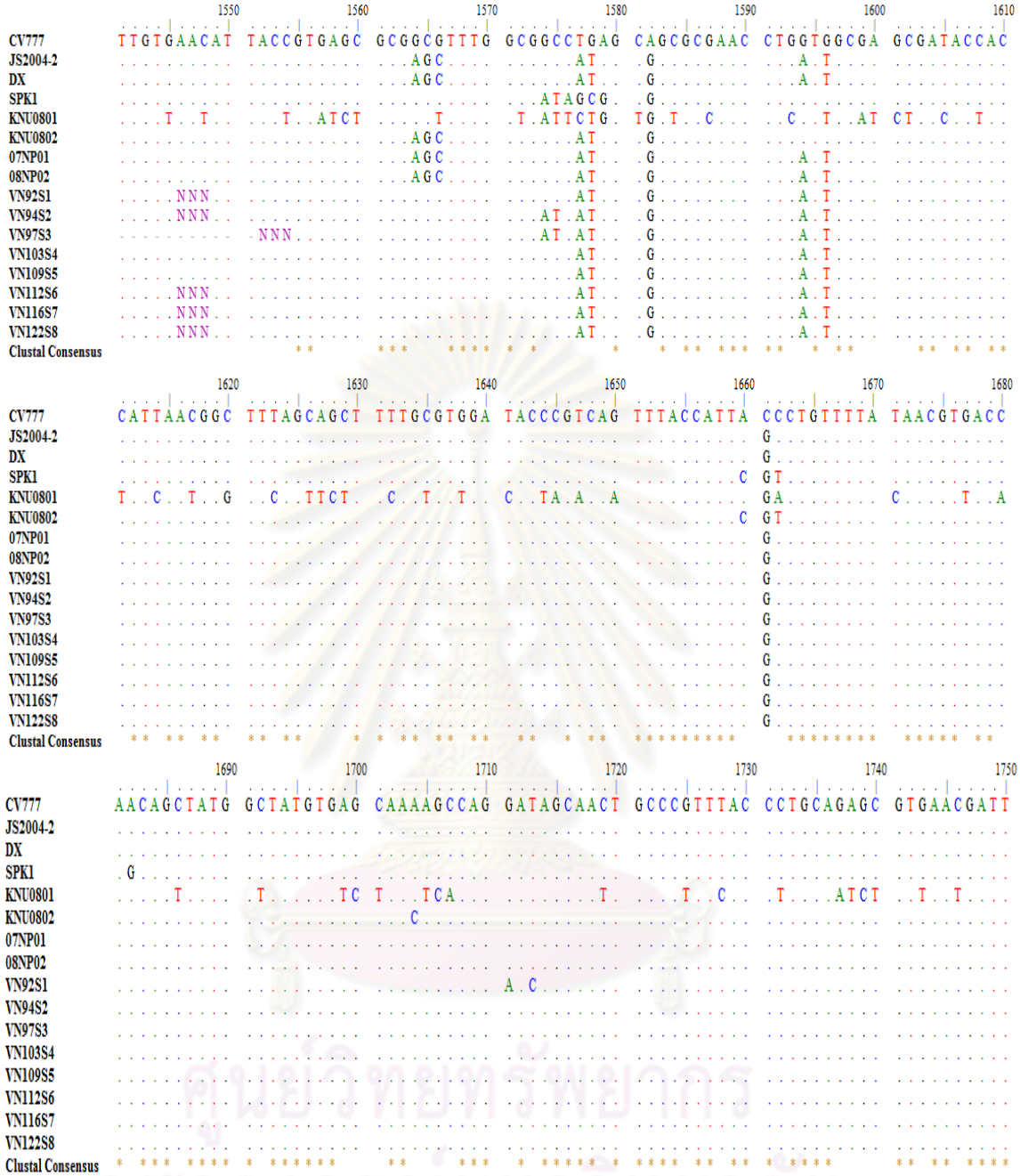
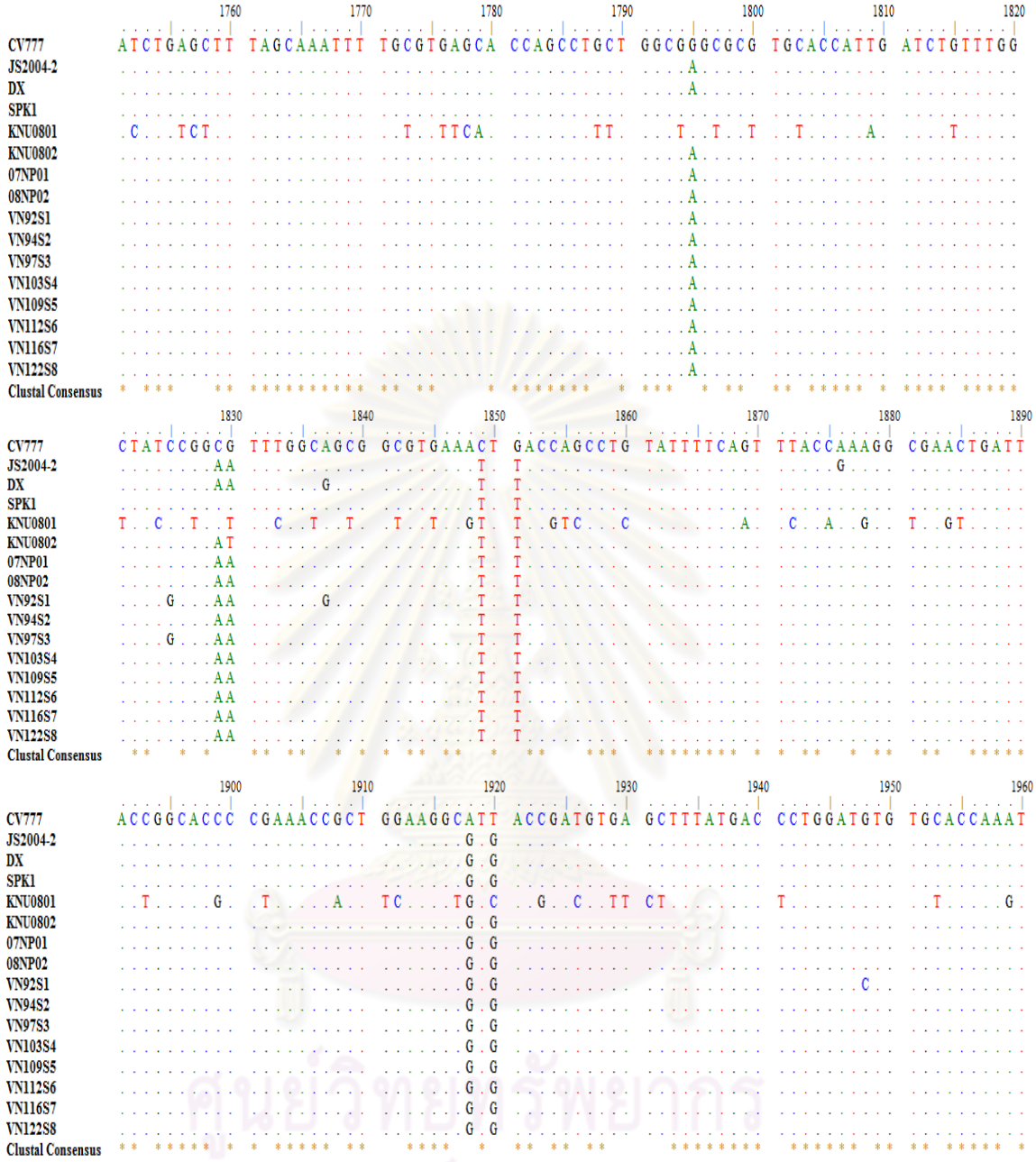
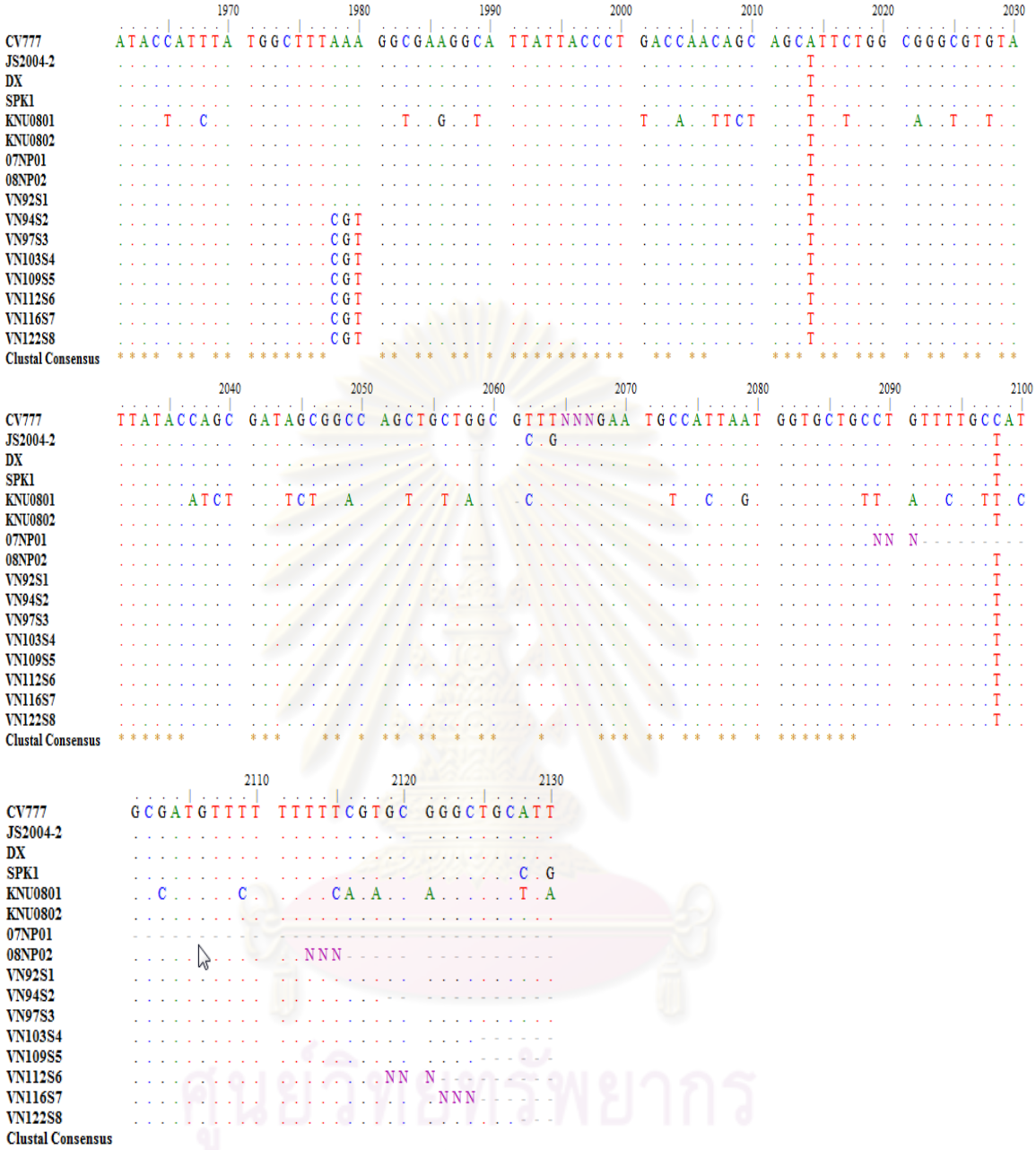


Figure 4.3 (a), nucleotide sequence alignment of the partial S gene of eight Vietnamese PEDV isolates (VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) and selected reference PEDV strains (European strains, CV777; Chinese isolates, JS-2004-2 and DX; Korean isolates, Spk1, KNU-0801, KNU-0802; Thai isolates, 07NP01, 08NP02). Dash (.) reveals the nucleotide identity of isolates compare with prototype strain (CV777). There were some sites in sequence with different nucleotides due to substitution of nucleotides comparing with CV777.



(Figure 4.3a continued)



(Figure 4.3a continued)

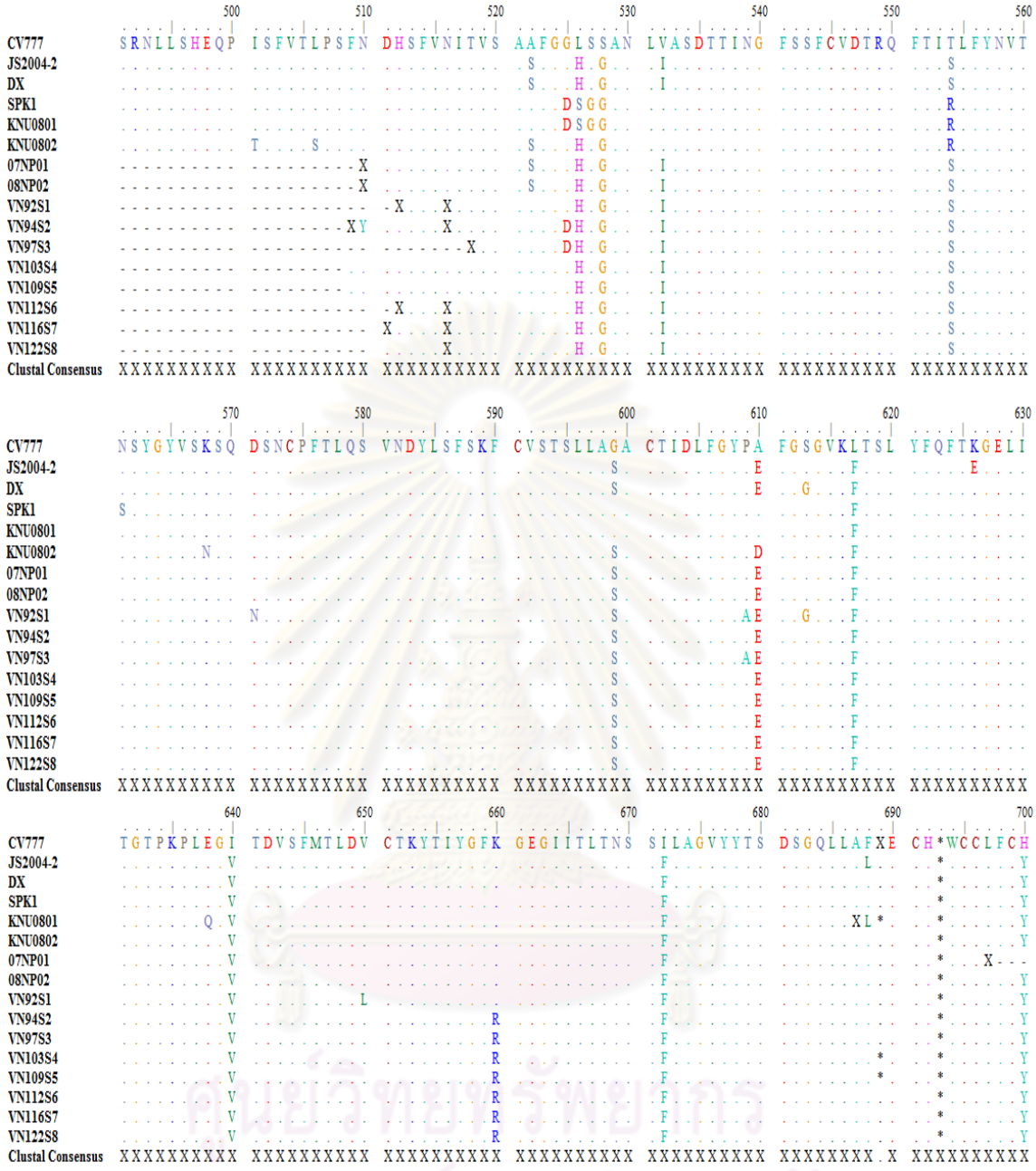


Figure 4.3 (b), encoded peptide sequence alignment of the partial S gene of eight Vietnamese PEDV isolates (VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) and selected reference PEDV strains (European strains, CV777; Chinese isolates, JS-2004-2 and DX; Korean isolates, Spk1, KNU-0801, KNU-0802; Thai isolates, 07NP01, 08NP02). Dash (.) reveals the amino acid identity of isolates compare with prototype strain (CV777). There were some sites in sequence with different encoded amino acids due to substitution of amino acids comparing with CV777.

The full M gene

The nucleotide sequence of full M gene of the Vietnamese PEDV isolates composed about 687 nucleotides encoding about 225 amino acids (Figure 4.4a,b). Six current Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) and reference European prototype (CV777, Br1/87) shared high homology, together having only 13 nucleotide changes including from T to C (123), T to C (125), G to T (186), C to T (198), C to T (213), C to T (234), T to C (285), T to A (348), C to T (537), C to T (540), G to A (574), T to C (618), and G to T (621). Interestingly, VN92M1 had a few changes compared with the other Vietnamese PEDV isolates at position 123 (from T to C), 537 (from C to T), and 621 (from G to T). The Vietnamese isolates shared high similarity to the Chinese isolates (JS-2004-2, DX), the Korean isolates (CPF299, M1763) and the Thai isolates (07NP01, 08NP02, KU06RB08) at those mentioned above position changes compared with the European prototypes. However, PEDV isolates from China, Korea, and Thailand shared high similarity to each other.

Similar to nucleotide sequence alignment, deduced amino acid of M gene shared high homology together except at the position 42, 192 of the Vietnamese isolates containing the substitution of V to A, G to S, respectively.

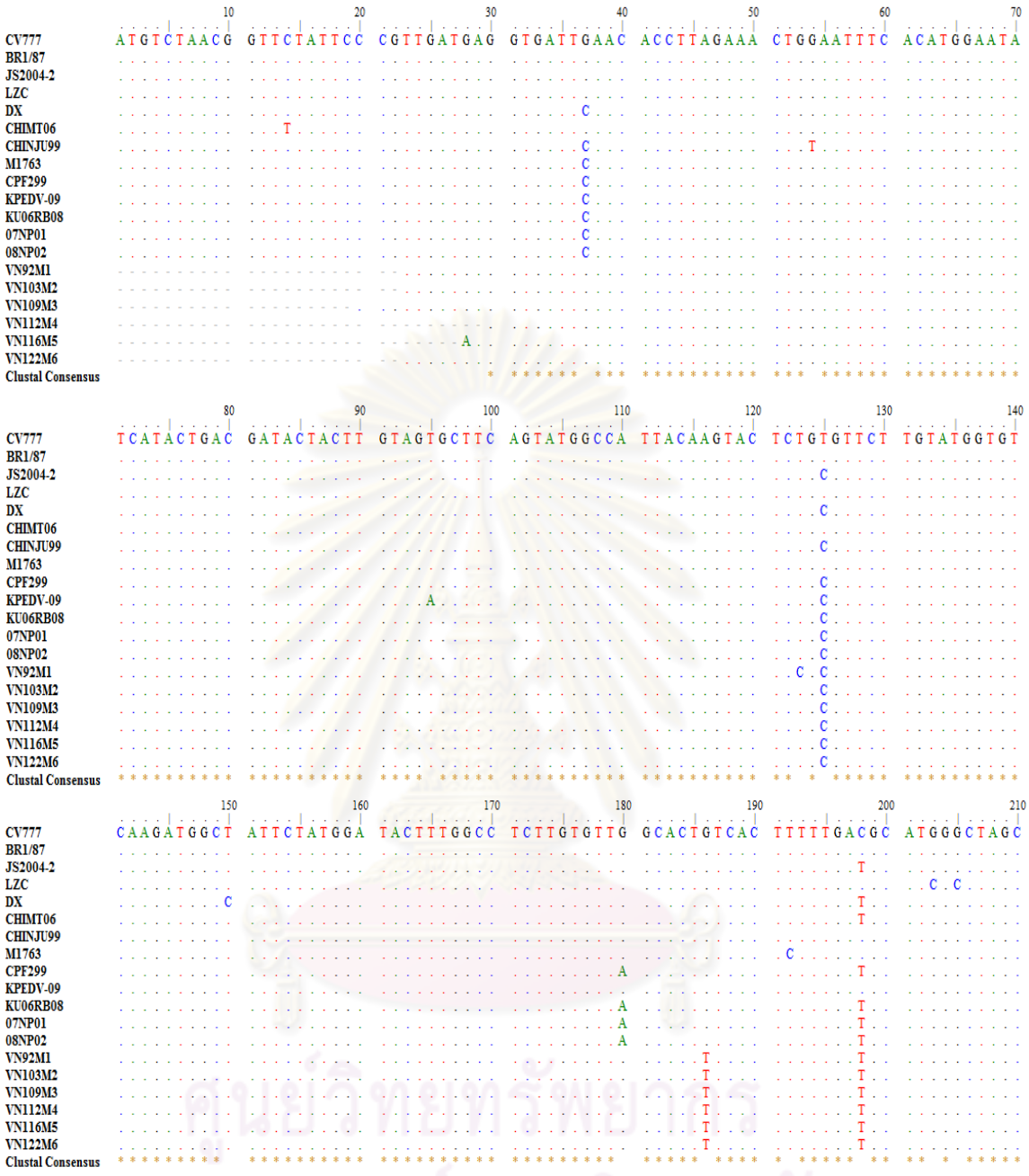
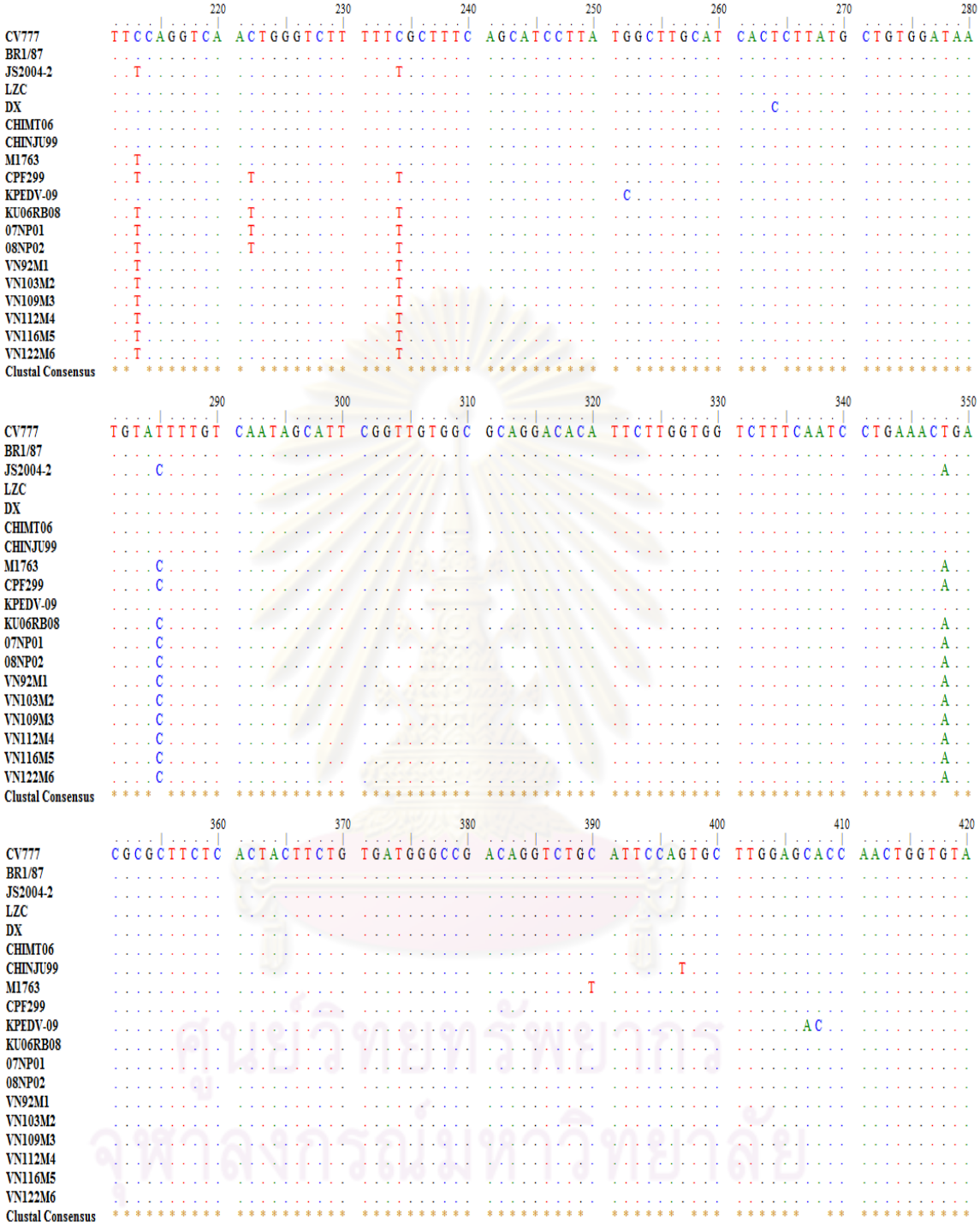
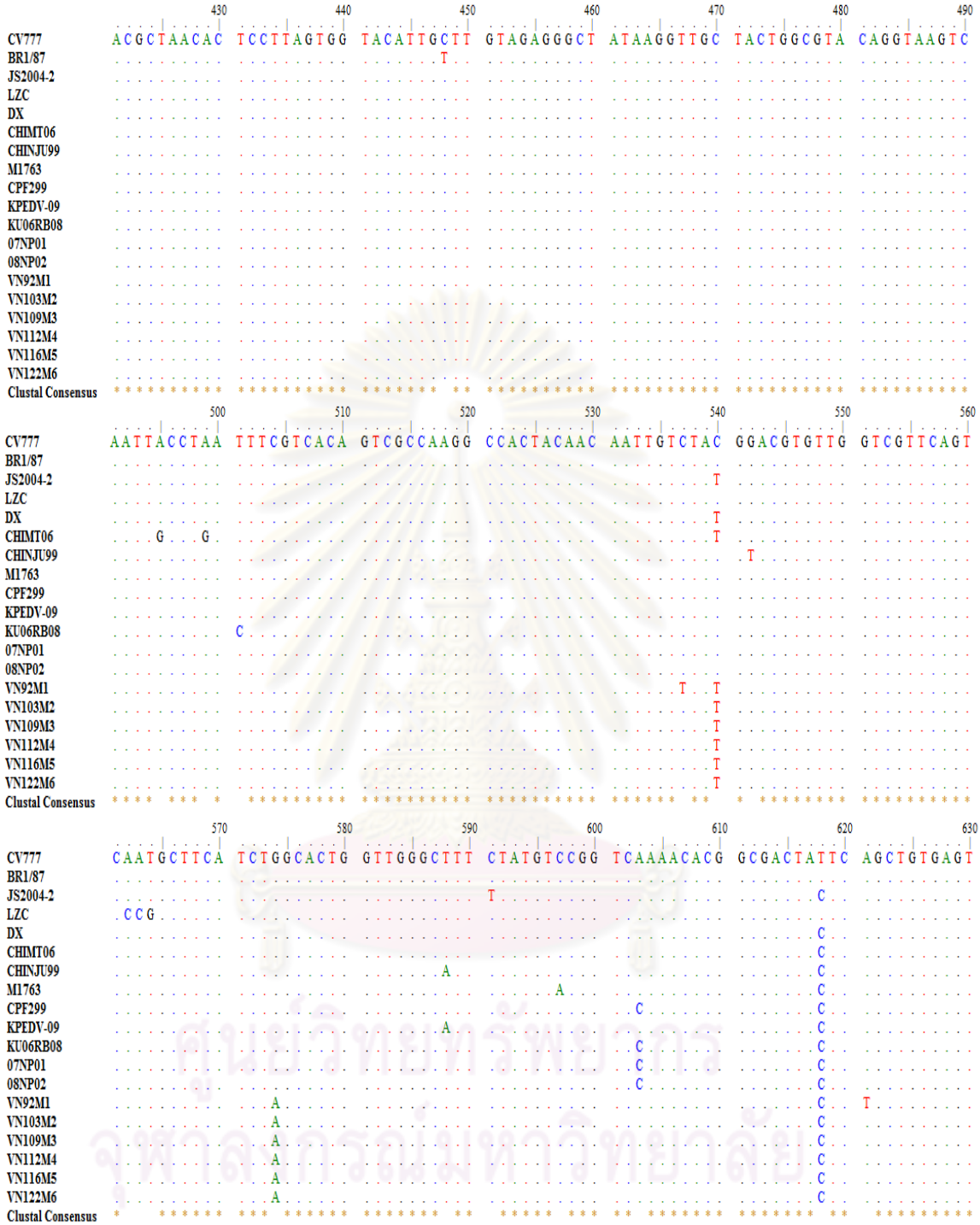


Figure 4.4 (a), comparison of nucleotide sequence of the full M gene of six Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) and selected reference PEDV strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2, LZC, DX and CHIMT06; Korean isolates, Chinju99, M1763, CPF299, KPEDV-09); Thai isolates, 07NP01, 08NP02 and KU06RB08). Dash (.) reveals the nucleotide identity of isolates compare with prototype strains (CV777 and Br1/87). There were some sites in sequence with different nucleotides due to substitution of nucleotides comparing with CV777, Br1/87.



(Figure 4.4 a continued)



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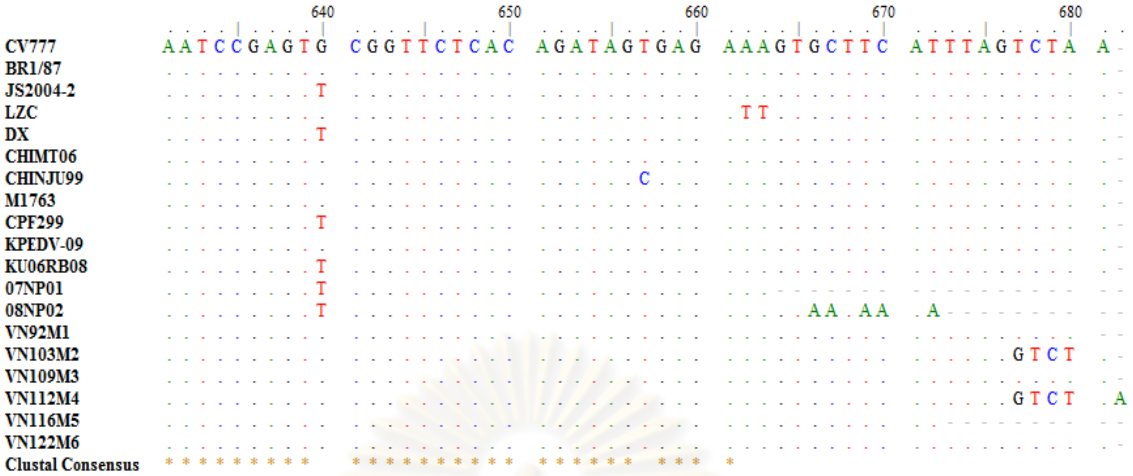

(Figure 4.4 a continued)

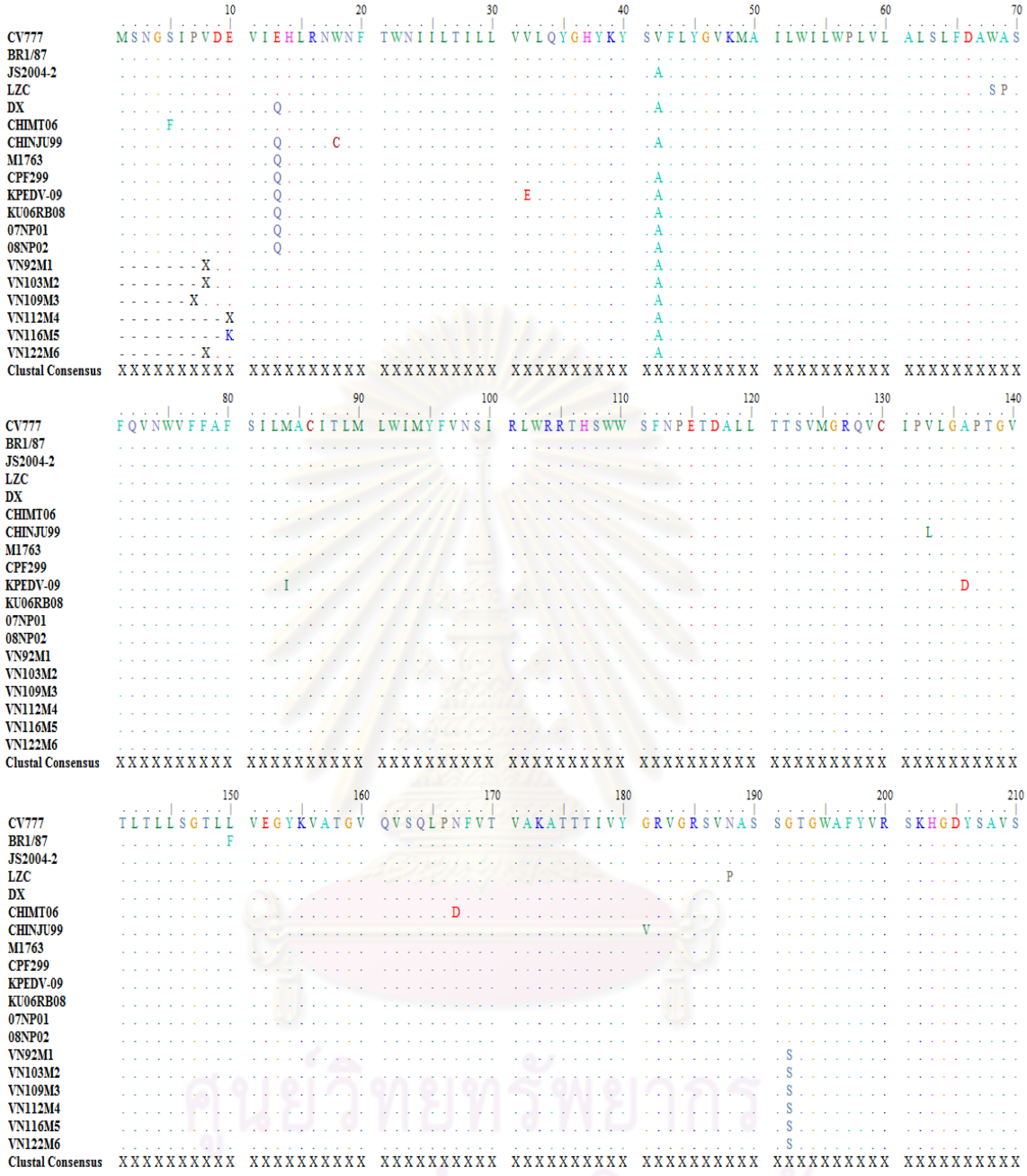

Figure 4.4 (b), comparison of deduced amino acids sequence of the full M gene of six Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) and selected reference PEDV strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2, LZC, DX and CHIMT06; Korean isolates, Chinju99, M1763, CPF299, KPEDV-09); Thai isolates, 07NP01, 08NP02 and KU06RB08). Dash (.) reveals the amino acid identity of isolates compare with prototype strains (CV777 and Br1/87). There were some sites in sequence with different amino acids due to substitution of amino acids comparing with CV777, Br1/87.

4.3. Phylogenetics

The partial S gene

Phylogenetic analysis based on nucleotide and encoded amino acid sequence of the partial S gene revealed PEDVs strains dividing into three groups (Figure 4.5a, b). Group 1 contained most PED strains in those affected countries included Europe (CV777/1977, Belgium; Br1/87/1987, Britain), China (JS-2004/2004, DX/2007), Korea (DR13/2006, KNU-0802/2008), Thailand (07NP01/2007, 08NP02/2008, 08CB01/2008) and Vietnam (current isolates in this study). Group 2 comprised of Spk1, KNU-0801 (Korean isolates) and group 3 comprised of the first Korean isolate (Chinju99). The current Vietnamese PEDV isolates had high homology of alignment together. However, they divided into two separated clades (clade 1: VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8; clade 2: VN92S1) with 98.9% sequence identity. Actually, Vietnamese PEDV isolates contained high differences on the nucleotide sequence of partial S gene with other reference strains in Europe (CV777, Br1/87), and Korea (Chinju99, Spk1, KNU-0801). Interestingly, the current Vietnamese isolates closely related to the Thai isolates (07NP01, 08NP02, 08CB01), the Chinese isolates (JS-2004-2, DX) and the recent Korean isolate (KNU-0802).

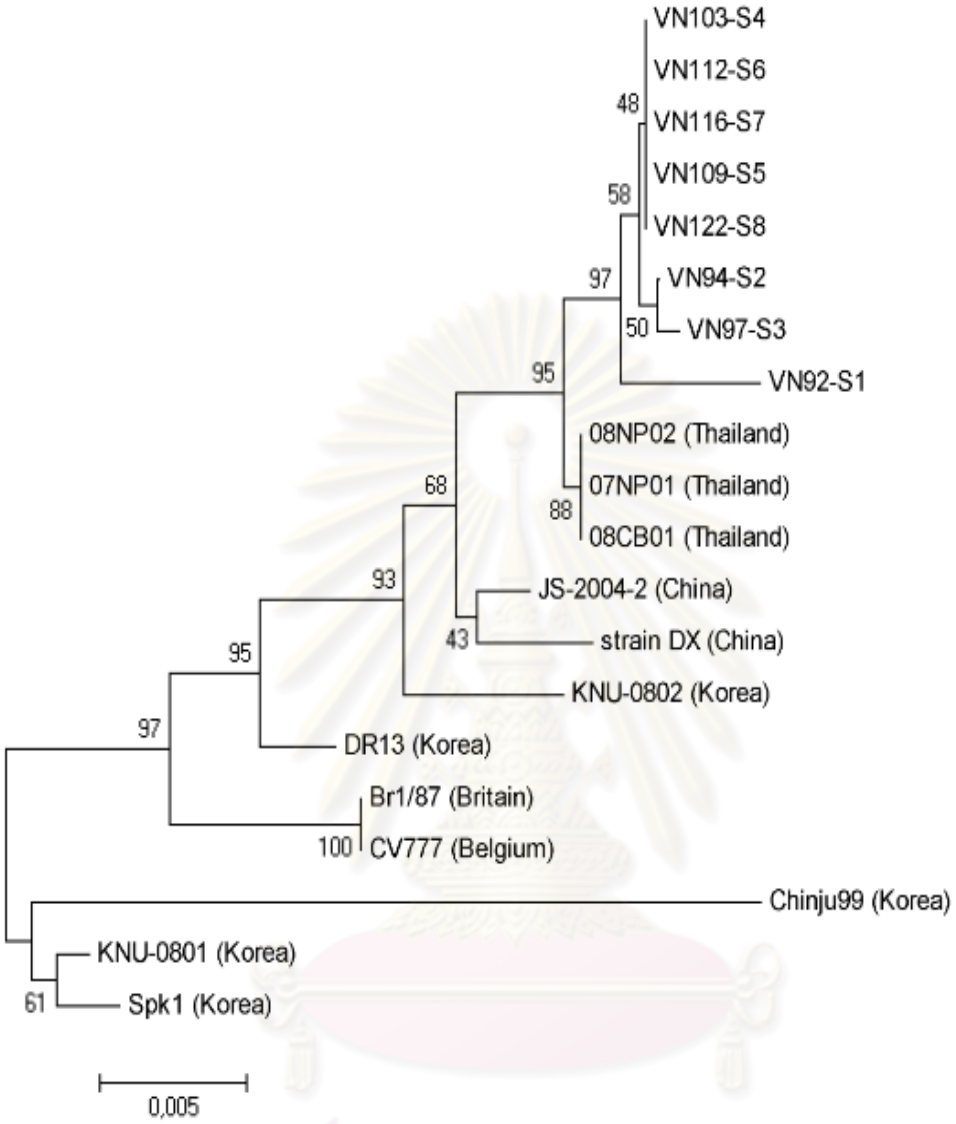

Figure 4.5 (a), dendrogram based nucletotide sequence of the partial S gene amongst eight southern Vietnamese PEDV isolates (VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) with those of other reference strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2 and DX; Korean isolates, Chinju99, DR13, Spk1, KNU-0801, KNU-0802; Thai isolates, 07NP01, 08NP02, and 08CB01). Multiple alignment method performed by using ClustalX program. The phylogenetic tree was constructed by using neighbor-joining algorithm. The number on each branch presents the bootstrap values of 1000 replicates. The scale bars indicate the number of 0,005 estimate evolutionary time.

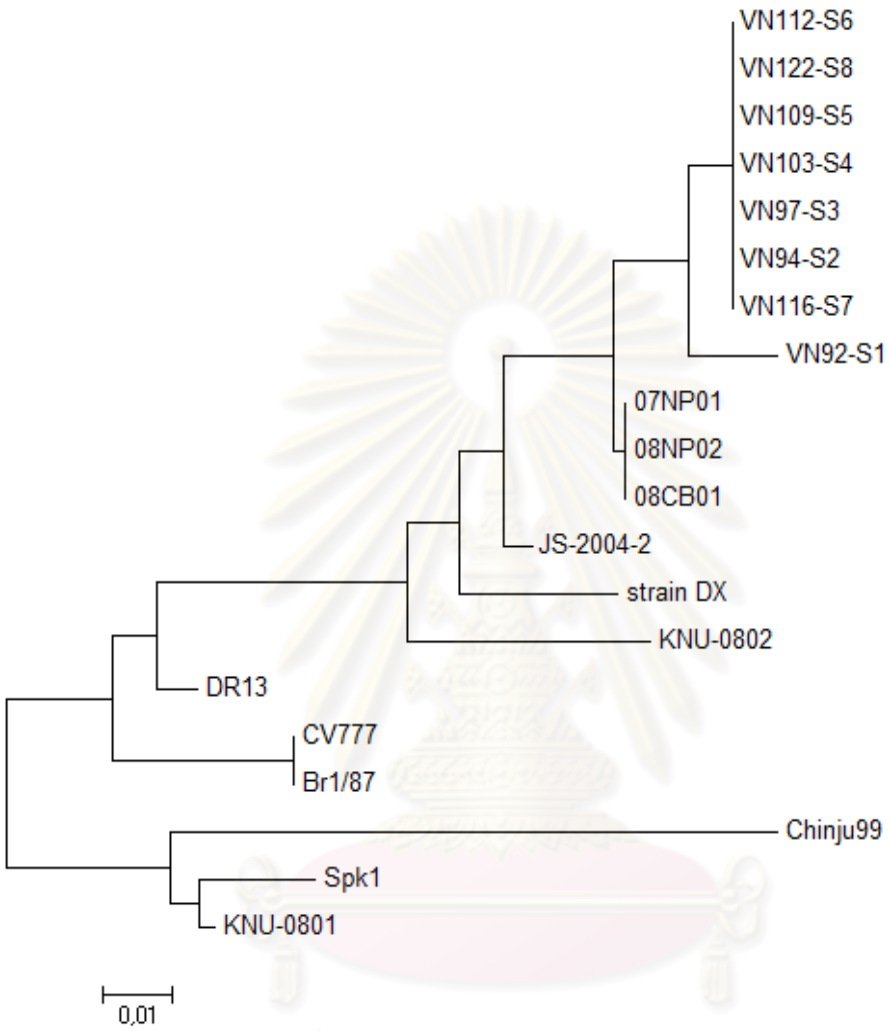

Figure 4.5 (b), dendrogram based on encoded amino acid sequence of the partial S gene amongst eight southern Vietnamese PEDV isolates (VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) with those of other reference strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2 and DX; Korean isolates, Chinju99, DR13, Spk1, KNU-0801, KNU-0802; Thai isolates, 07NP01, 08NP02, and 08CB01). Multiple alignment method performed by using ClustalX program. The phylogenetic tree was constructed by using neighbor-joining algorithm. The number on each branch presents the bootstrap values of 1000 replicates. The scale bars indicate the number of 0,01 estimate evolutionary time.

The full M gene

Based on M gene-based phylogenetic tree, PEDVs also divided into three distinct genetic groups (Figure 4.6a, b). Group 1 comprised of the Vietnamese isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6), the Chinese isolates (JS-2004-2, DX), the Korean isolates (CPF299, M1763) and the Thai isolates (07NP01, 08NP02, KU06RB08). The current Vietnamese PEDV isolates revealed close relationship with those isolates from China, Thailand, and Korea. However, those PEDV isolates were divided into subgroups (subgroup 1, including JS-2004-2, CPF299, KU06RB08, 08NP02, 07NP01 and the currents Vietnamese PEDV isolates; subgroup 2, including M1763 and DX). Group 2 comprised of Chinju99 (Korea) and CHIMT06 (China) and group 3 composed of CV777, Br1/87 (Europe) and LZC (China). Similar to S gene, phylogenetics of M gene had similar characteristics in genetic variation of the current Vietnamese isolates and VN92M1 possessed higher variable sequence than other remaining isolates.

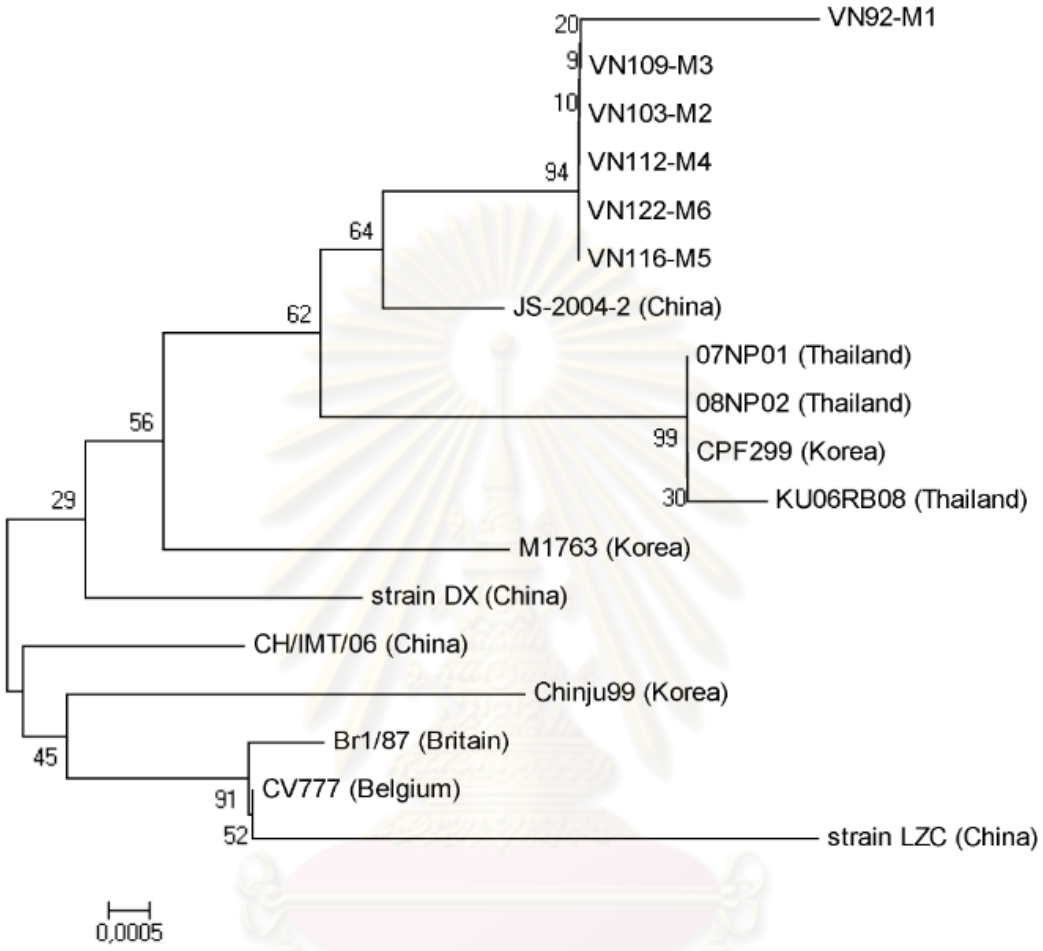

Figure 4.6 (a), dendrogram based on nucleotide sequence of the full M gene amongst six southern Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) with those of other reference strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2, LZC, DX and CHIMT06; Korean isolates, Chinju99, M1763, CPF299; Thai isolates, 07NP01, 08NP02 and KU06RB08). Multiple alignment method performed by using ClustalX program. The phylogenetic tree was constructed by using neighbor-joining algorithm. The number on each branch presents the bootstrap values of 1000 replicates. The scale bars indicate the number of 0.0005 estimate evolutionary time.

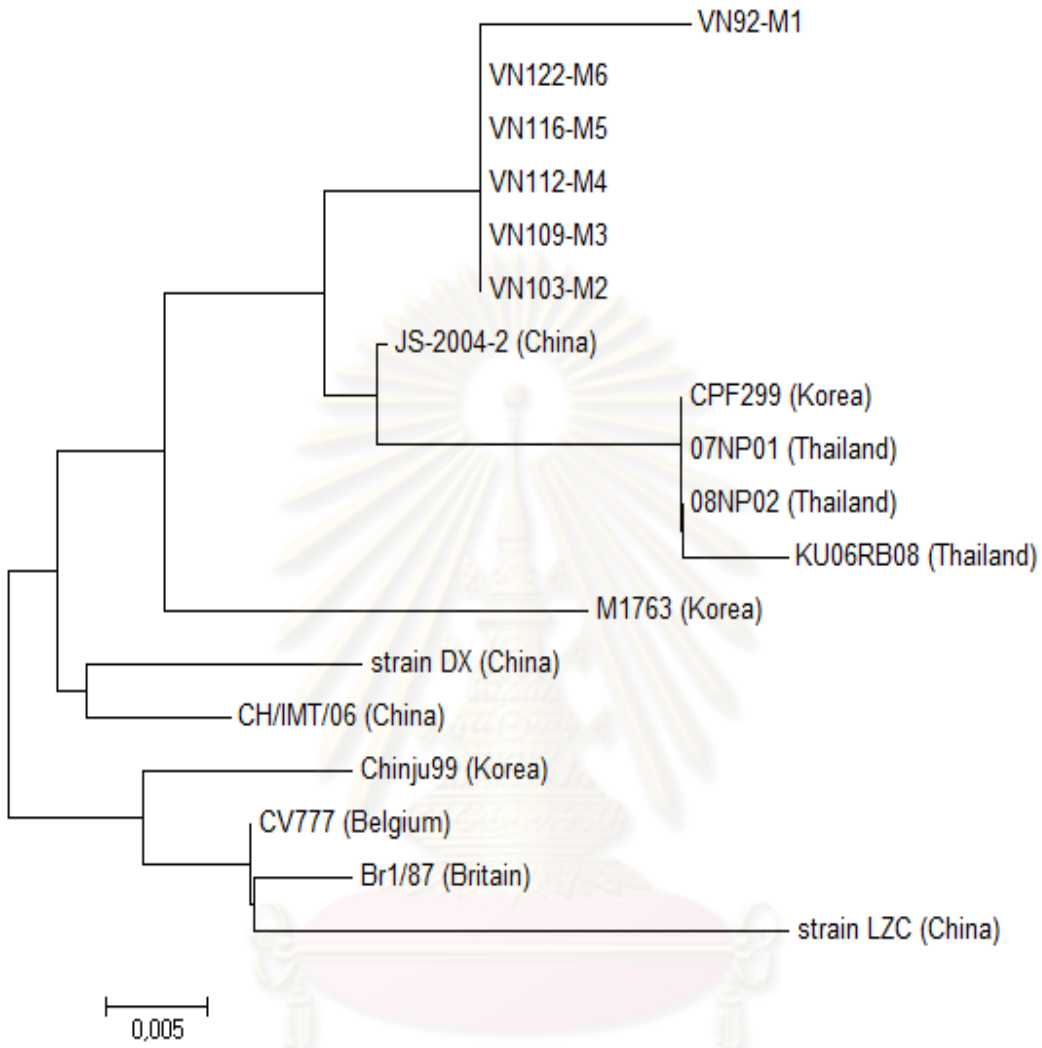

Figure 4.6 (b), dendrogram based on encoded amino acid sequence of the full M gene amongst six southern Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) with those of other reference strains (European strains, CV777 and Br1/87; Chinese isolates, JS-2004-2, LZC, DX and CHIMT06; Korean isolates, Chinju99, M1763, CPF299; Thai isolates, 07NP01, 08NP02 and KU06RB08). Multiple alignment method performed by using ClustalX program. The phylogenetic tree was constructed by using neighbor-joining algorithm. The number on each branch presents the bootstrap values of 1000 replicates. The scale bars indicate the number of 0.005 estimate evolutionary time.

CHAPTER V
DISCUSSION

Porcine epidemic diarrhea virus (PEDV) is genetically classified into group 1 Coronaviruses. Spike glycoprotein and membrane glycoprotein genes are believed to have genetic variation geographically (Cook et al., 1986; Britton et al., 1991; Cavanagh et al., 1992; Adzhar et al., 1995; Ballesteros et al., 1997; Leparc-Goffart et al., 1997; Kingham et al., 2000; Saif, 2004; Weiss et al., 2005). The heterogeneity in those genomic sequences has been reported and is known essentially for the diverse PED pathogenicity (Lai et al., 2007; Lee et al., 2010). Genomic PEDV is considered widely diversity based on numbers of molecular sequence analyses (Kweon et al., 1999; Kocherhans et al., 2001; Yeo et al., 2003; Jinghui and Yijing, 2004; Junwei et al., 2006; Park et al., 2007; Li et al., 2009; Puranaveja et al., 2009; Lee et al., 2010). The genetic region of nucleotide sequence contained highest variation is C-terminal and N-terminal regions of S1 domain (Park et al., 2007; Lee et al., 2010). Ballesteros et al. (1997) described two amino acid changes at the N-terminus of transmissible gastroenteritis Coronavirus spike glycoprotein resulting in the loss of enteric tropism. The N-terminus region contained specific receptor binding of Coronaviruses (Kubo et al., 1994; Wong et al., 2004) and porcine aminopeptidase N receptor of PEDV (Li et al., 2007).

M glycoprotein gene of Coronaviruses seems to be more conservable than S glycoprotein gene (Lai et al., 2007; Chen et al., 2008; Wongserepipatana and Nunthaprasert, 2008; Puranaveja et al., 2009). M protein has a triple-spanning viral membrane protein characterized as the short amino-terminal domain on the outside of the virus and a long carboxy-terminal domain inside. The important role of M gene contributes to assembly process of viral nucleocapsid and membrane of internal structure. It stimulates interferon secretion. Therefore, the M protein gene can be an ideal candidate for the development of genetically engineered vaccine for PED prevention or specific antigen for PEDV diagnosis in serology because it is on the exterior surface of viral envelope (Wongserepipatana and Nunthaprasert, 2008).

In these Vietnamese PEDV isolates, genomic characterization revealed highly conserved in M gene than partial S gene. However, the variability of nucleotide sequences of partial S and full M genes were not much different comparing with other isolates in neighboring countries (JS-2004-2, DX, 07NP01, 08NP02, 08CB01, CPF299 and M1763) that have the same ancestor (revealed on phylogenetics). The phylogenetic analysis showed the close relationship among isolates of group 1 as in China (JS-2004-2, DX), in Korea (KNU-0802, CPF299, and M1763), in Thailand (07NP01, 08NP02, 08CB01, KU06RB08) and in Vietnam. The European prototypes (CV777, Br1/87) and the Korean isolate (DR13) were in a separated subgroup. Interestingly, the current Thai isolates absolutely were closely related to the Korean isolate (CPF299) sharing 100% nucleotide and amino acid identity of M gene. This finding will need further study to clearly make on the similarity. Within eight southern Vietnamese PEDV isolates, there were a few differences in the isolate VN92S1 compared with other isolates because it came from different farm and province (Farm 1, Binhduong). Two other isolates (VN94S4, VN97S3) collected from other farms in Binhduong also revealed minor differences in nucleotide and amino acid sequence with the remaining isolates. This particular isolates from Binhduong belonged to its distinct clade from other Vietnamese PEDV isolates showing genetic diversity of PEDV population in Vietnam. However, the genetic differences among those were not much. The isolates in Dongnai (VN109S5/VN109M3) shared similarly in nucleotide and amino acid identity to Hochiminh isolates (VN103S4/VN103M2, VN112S6/VN112M4, VN116S7/VN116M5, VN122S8/VN122M6).

The southern Vietnamese PEDVs contained high differences on the nucleotide and deduced amino acid sequence of partial S gene with other reference isolates in Europe (Br1/87, CV777) and Korea (Spk1, Chinju99, DR13 and KNU-0801). Chronologically, the field PEDV strains might have evolved and spread geographically based on epidemiology. The first isolate, CV777 (Belgium, 1977) was recognized to be similar (100% sequence identity) to Br1/87 (Britain, 1987) and shared similar pathological features. Since then, PEDVs have spread widely to the swine farms of other geographical areas (Pensaert and Yeo, 2006). In China, there was a report confirming PED outbreaks in the past but the PEDV molecular genome was not available until LJB/03, JS-2004-2, and DX (isolated 2003, 2004,

2007) were sequenced and submitted to Genbank. Interestingly, these isolates revealed close relationship with European strains (Junwei et al., 2006). Likewise, Korean PEDVs (Kweon et al., 1999; Kang et al., 2005; Park et al., 2007), Thai PEDVs (Puranaveja et al., 2009) and current Vietnamese PEDVs were sequenced and showed clearly these isolates came from the same ancestor.

However, recent finding (Lee et al., 2010) was quite different from the previous study of Park et al. (2007) in phylogenetic analysis. As described by Park et al. (2007), PEDV isolates were divided into three distinct groups based on the C-terminal region of S1 domain and were distantly related to other previous field Korean isolates Spk1 and Chinju99 (group 2 and group 3). However, only two distinct groups based on full S gene phylogenetic analysis were documented including the Chinese and the Korean vaccine isolates belonged to group 1 and the Korean field isolates (Spk 1 and Chinju99) belonged to group 2. Since, genetic analysis based on the C-terminal part of the S1 domain was not enough to represent the full S gene (Lee et al., 2010). The S1 domain and the S1 N-terminus were suggested. The S1 sub-domain includes the N-terminal half of the molecule forming the globular portion of the spikes. It contains sequences responsible for binding to specific receptors on the surface of susceptible cells (Cavanagh, 2005; Li et al., 2007). In addition, S1 sequences are variable, containing various degrees of deletion and substitutions in different Coronavirus strains. Mutations in S1 sequences have been associated with altered antigenicity and pathogenicity of the virus (Ballesteros et al., 1997; Leparc-Goffart et al., 1997). These results could provide further researches on full S gene of PEDVs to analyze the powerful molecular epidemiology for tracing the outbreak transmission.

Moreover, viral RNAs are considered high mutational alterations on genomic sequence due to errors in replication from absence of a cellular proofreading mechanism (Moya et al., 2004). In addition, they were recognized as “Viral Quasispecies” (Eigen and Schuster, 1997; Moya et al., 2004). Coronaviruses revealed individual viral genomes differing in one or more nucleotides from the consensus or average sequence of the population and over relatively short times genotypic drift occuring as particular

variants. Since Coronaviruses possess largest RNA genome, mutation rates are always high. For example, each one out of 30000 nucleotides of coronaviral genome will change in each replication cycle. Furthermore, coronaviral genomes may undergo more substantial mutations, including massive gene deletions, which can possibly affect their pathogenicity (Murphy et al., 1999; Moya et al., 2004).

CONCLUSIONS

The phylogenetic analysis indicated that these current Vietnamese isolates have originated from the same Chinese ancestor as the recent Thai and Korean isolates and have been undergoing genetic variation to form a new PEDV sub-cluster in Vietnam. Based on the phylogenetic tree of the partial S gene, the Chinese isolates (JS-2004-2, DX), the Thai isolates (08NP02, 07NP01, 08CB01) and the Vietnamese isolates were grouped in the same cluster with the Thai and the Vietnamese sub-clusters. Therefore, these results suggested that the Chinese isolates could be a candidate virus origin before transmitting to other neighboring countries (Puranaveja et al., 2009). The PED epidemiology of outbreaks related to geographical influence among neighboring countries. Normally, animal movement and human transportation among neighboring countries are risk factors of disease transmission. Moreover, elucidation of origin and transmitted route of the emerging virus can be a powerful tool to control the disease.

Since emerging in Europe, PED outbreaks have been worldwide spread to many geographical areas but not in the US continents excepting an old report of PED-induced outbreak in Canada (Turgeon et al., 1980). The reasons explained for absence PED in Americas might be the geographical separation from the arising places of emerging virus and availability of good preventive strategies in these countries. In addition, no animal movement from the PEDV endemic areas to the north American continent. This disease has caused massive economic losses in the swine production, especially in Europe and Asia. Although numerous approaches have applied to control this disease such as gilt acclimatization, administration of yolk-derived immunoglobulin, and using attenuated vaccines, these strategies have shown no or partial protection. The appropriate vaccine strains are needed to resolve current problems in many swine raising countries since provoking specific immune response in sows before transmitting to suckling piglets is a key of success on PED control.

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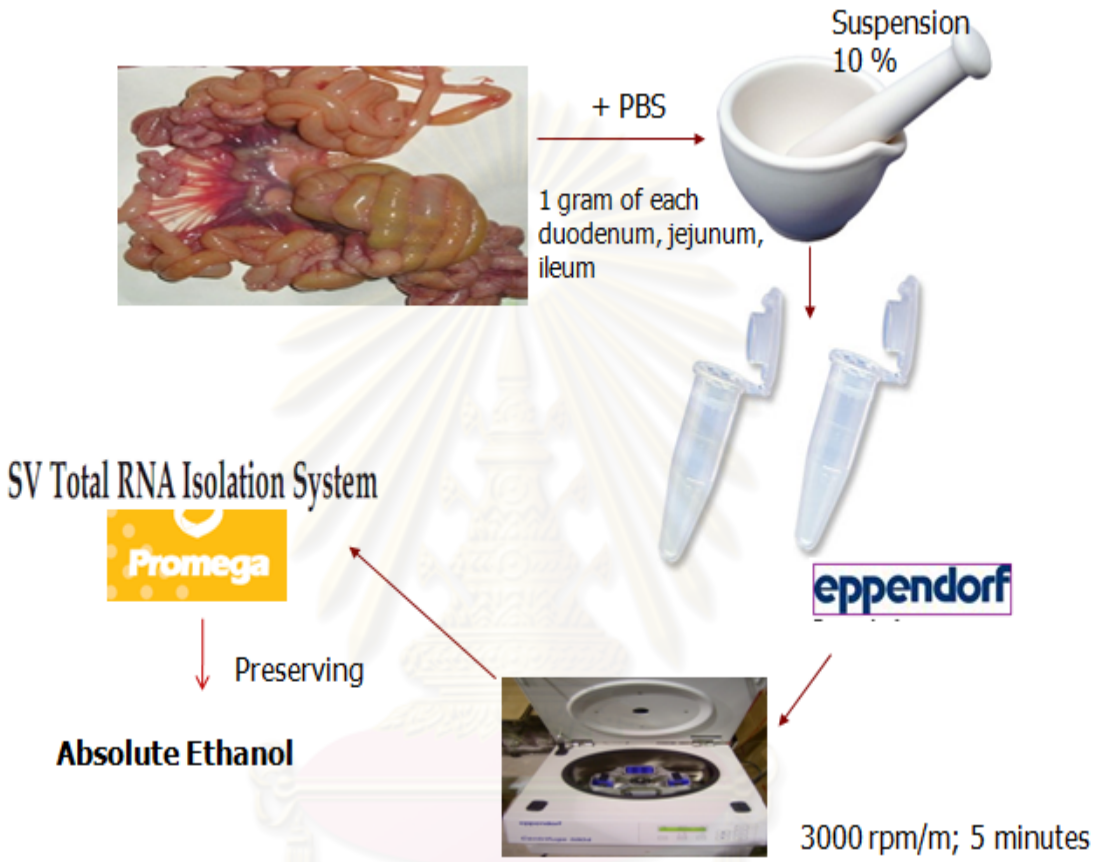
APPENDICES

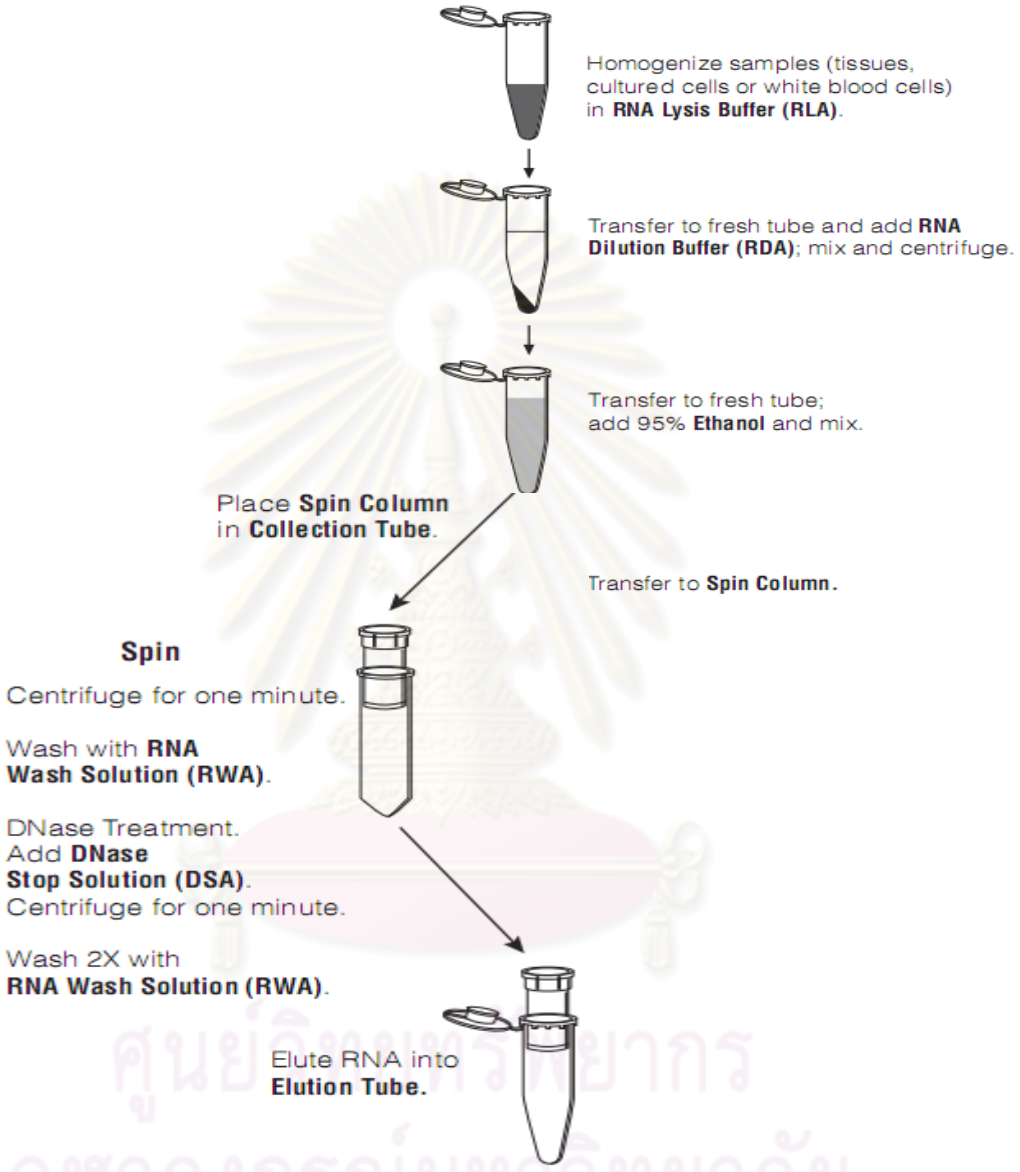
APPENDIX A

Sample analysis



Tissue dissociation: The tissue samples was homogenized by adding PBS (10/90, sample/PBS), put sand into mortar and well grinded. After that suspension of sample will be centrifuged at 3000 rpm per minute in 5-10 minutes at 4°C. The supernatant of 1-3 ml is collected into the tubes. Stored the supernatant at -70°C until RNA extraction

RNA extraction

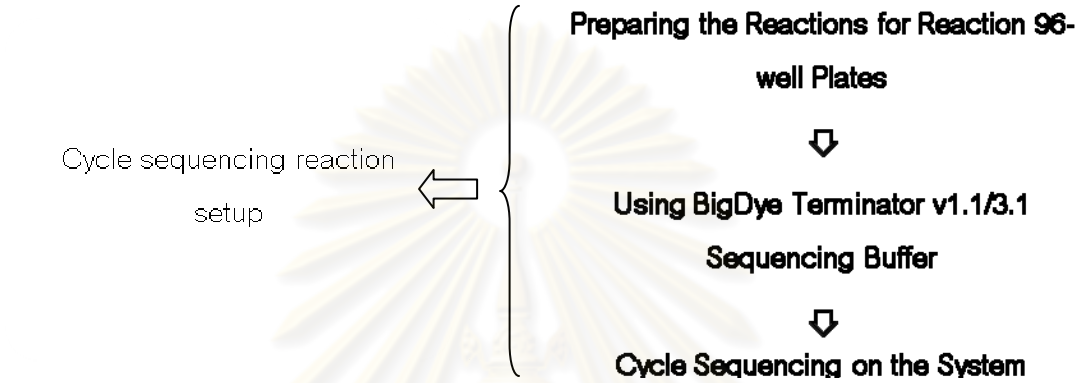


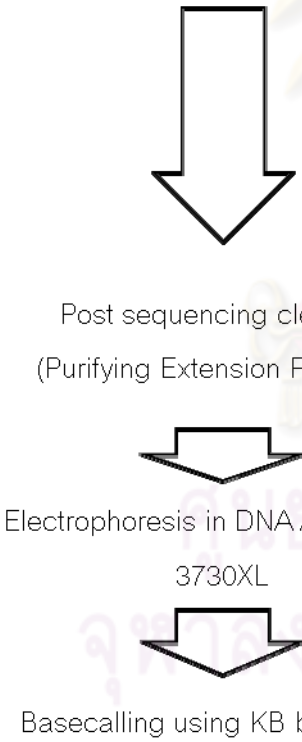
Schematic representation of the SV Total RNA Isolation System (protocol of total RNA isolation system, Madison, WI, USA)

APPENDIX B

Protocol of BigDye Terminator v3.1 cycle sequencing kit

(Applied Systems, 2002)





Preparing for Reaction 96-well Plates		
1	Each reaction: add following reagents a separate tube	
	Reagent	Volume
	Terminator ready reaction Mix	8.0 µl
	Template	5-20 ng
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µl
2	Mix well and spin briefly	

Cycle Sequencing on the System	
1	Place the tubes in a thermal cycler
2	Perform an initial denaturation a) Rapid thermal ramp to 96°C b) 96°C for 1 min
3	Repeat the following for 25 cycles - Rapid thermal ramp to 96°C - 96°C for 10 sec - Rapid thermal ramp to 50°C - 50°C for 5 sec - Rapid thermal ramp to 60°C - 60°C for 4 min
4	Rapid thermal ramp to 4°C and hold until ready to purify
5	Spin down the contents of tubes in microcentrifuge
6	Purifying extension products

APPENDIX C

Nucleotide sequences

Partial S gene

VN92S1

TGCATCATTCTTTTGTTATATTACTGTCTCTGCTGCTTTTGGTGGTCATAGTGGTGCCAACCTTATT
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TCAAGAATGTCACTAGTGGTGCTGTTTATTCTGTTACGCCATGTTCTTTTTCAGAGCAGGCTGCAT
ATGAGA

VN94S2

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VN97S3

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VN103S4

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VN109S5

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VN112S6

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VN116S7

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VN122S8

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Full M gene

VN92M1

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VN103M2

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VN109M3

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VN112M4

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VN116M5

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VN122M6

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CTATTCTATGGATACTTTGGCCTCTTGTGTTGGCACTTTCACTTTTTGATGCATGGGCTAGCTTTCA
GGTCAACTGGGTCTTTTTTGCTTTCAGCATCCTTATGGCTTGCATCACTCTTATGCTGTGGATAAT
GTACTTTGTCAATAGCATTCGGTTGTGGCGCAGGACACATTCTTGGTGGTCTTTCAATCCTGAAA
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ACACGGCGACTACTCAGCTGTGAGTAATCCGAGTGCGGTTCTCACAGATAGTGAGAAAGTGCTT
CATTTAGTCTAAACA

BIOGRAPHY

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วิทยานิพนธ์ที่ได้รับการตีพิมพ์เผยแพร่

ชื่อ-นามสกุลนิสิต...Mr.Do Tien Duy.....สาขาวิชา....พยาธิชีววิทยาทางสัตวแพทย์.....

ระดับปริญญา [/] โท ☐ เอก ปีที่จบการศึกษา.....พ.ศ.2553.....

หัวข้อวิทยานิพนธ์..... Genetic characterization of southern Vietnamese *porcine epidemic diarrhea virus (*PEDV) Isolates during 2009-2010 outbreaks

อาจารย์ที่ปรึกษาวิทยานิพนธ์.....ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช.....

อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม (ถ้ามี).....

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ชื่อเรื่อง.....

ชื่อวารสาร..... ☐ impact factor.....

ฉบับที่.....เล่มที่.....ปีที่พิมพ์.....หน้า.....ประเทศ.....

วารสารที่ตีพิมพ์ระดับนานาชาติ [ ] มี Peer Review ☐ ไม่มี Peer Review

ชื่อเรื่อง.....

ชื่อวารสาร.....

☐ impact factor.....

ฉบับที่.....เล่มที่.....ปีที่พิมพ์.....หน้า.....ประเทศ.....

การประชุมทางวิชาการระดับชาติ ☐ มี Peer Review ☐ ไม่มี Peer Review

ชื่อการประชุม.....

ชื่อเรื่อง.....

วัน/เดือน/ปีของการประชุม.....ปีที่พิมพ์.....หน้า.....

สถานที่จัดประชุม.....

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ชื่อการประชุม. The 36th Thai Veterinary Medical Association International Conference on Veterinary Science 2010: *"ASEAN+3, ASEAN+6 – Impact on Veterinary Roles*

ชื่อเรื่อง.....Genetic characterization of southern Vietnamese *porcine epidemic diarrhea virus (*PEDV) Isolates during 2009-2010 outbreaks

วัน/เดือน/ปีของการประชุม.....2-5 November 2010.....ปีที่พิมพ์....2010.....หน้า 131-135

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วิทยานิพนธ์ที่ได้รับรางวัลในระดับชาติหรือระดับนานาชาติ

ชื่อรางวัล.....

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GENETIC CHARACTERIZATION OF SOUTHERN VIETNAMESE *PORCINE EPIDEMIC DIARRHEA VIRUS* (PEDV) ISOLATES DURING 2009 – 2010 OUTBREAKS

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Abstract

Porcine epidemic diarrhea virus (PEDV) spike (S) protein and membrane (M) protein genes are believed to have genetic variation geographically. The heterogeneity in those genomic sequences has been reported and is known essentially for the diverse PED pathogenicity. Eight southern Vietnamese PEDVs from the recent outbreaks (2009-2010) were sequenced and analyzed. The results revealed high homology of the partial S gene of total 8 current isolates at 98.9-100% (despite dividing into two clades of different provincial origins) and 99.7-100% identity on full M gene among these isolates. However, the Vietnamese PEDVs contained high differences on the nucleotide sequence of partial S gene with other reference isolates in Europe (Br1/87, CV777) and Korean (Spk1, Chinju99, DR13 and KNU-0801). The phylogenetic relationship of both S and M genes indicated that the current Vietnamese PEDVs showed high homology with the Chinese isolates (JS-2004-2 and DX), the Thai isolates (07NP01, 08NP02 and 08CB01) and the Korean isolates (KNU-0802 and CPF299). The results demonstrated that the eight Vietnamese isolates of this study may originate from the same ancestor undergoing genetic variation and forming a new PEDV genotype in Vietnam.

Keywords: phylogenetic analysis, pigs, Porcine epidemic diarrhea virus, Vietnam

Introduction

Porcine epidemic diarrhea (PED) is a devastating contagious disease caused by a *Coronavirus*, porcine epidemic diarrhea virus (PEDV), producing acute enteritis and fatal watery diarrhea with high mortality ratio in suckling pig up to 90% (Pensaert and Yeo, 2006). PED was first identified in England in 1971 (Oldham, 1972). Currently, it has become a problematic disease causing massive economic losses in many countries, particularly in Europe and Asia (Chen et al., 2008; Pensaert and Yeo, 2006). Numerous molecular investigations have been performed and revealed low to high variation of nucleotide sequences of PEDV, especially in the S glycoprotein gene which is genetically highly variable and is divided into two subunits S1 and S2 (Duarte and Laude., 1994; Kocherhans et al., 2001; Yeo et al., 2003; Kang et al., 2005; Park et al., 2007; Lee et al., 2010).

The emerging PED outbreaks in early 2009 confirmed by pathological features and RT-PCR caused massive economic losses of swine production in most of southern provinces of Vietnam (unpublished data). Therefore, the objective of this study was to genetically characterize the current Vietnamese PEDV isolates for elucidation the epidemiologic relationship among PEDV strains.

Materials and methods

Sample collection: Total 8 PEDV isolates from porcine intestinal samples had been taken from piglets showing very watery diarrhea and dehydration on different affected 5 farms of three provinces in southern Vietnam. **RNA isolation:** The total RNA was extracted according to the protocol of commercial kit's (Promega, Madison, WI, USA). **RT-PCR:** Two different genomic regions, 651bp fragment of partial S glycoprotein gene and 715bp fragment of full M gene were amplified (Park et al., 2007; Chen et al., 2008). **RT-PCR one-step protocols:** Exactly, 4 µl RNA template was mixed with a reaction mixture containing 10 µl of 2X AccessQuick™ Master Mix (Promega, Madison, WI, USA), 1 µl of each specific primer (10 µM), 0.5 µl of $MgCl_2$ (25 µM), 0.5 µl AMV reverse transcriptase (10u/ µl), and add 8 µl nuclease-free water reach to total volume reaction of 25 µl. Firstly, reverse transcription reaction incubated at 48°C for 45 min to make the first strand cDNA synthesis. Then, the second strand cDNA synthesis and PCR amplification was denatured at 95°C for 2 min (01 cycle) and 30 cycles of repeated denaturation at 94°C for 30 sec, annealing at 53°C for 60 sec, extension at72°C for 60 sec. Additional step is the final extension at 72°C for 5 min. Analyze the PCR products by agarose gel electrophoresis of 1.5% was readily visible by UV transillumination of an ethidium bromide-stained gel. **Sequencing:** Purified PCR products were sequenced by 1st BASE Pte Ltd (Singapore). **Sequencing analysis:** Nucleotide sequences of current Vietnamese PEDV isolates and other selected isolates (Genbank: http://www.ncbi.nlm.nih.gov) were aligned, edited, and analyzed with Bioedit v7.0.5.3, ClustalX 2.0.11 program. The phylogenetic trees were then generated based on the neighbor-joining method in the MEGA 5.0. The relative support for each branch and the bootstrap value of 1000 replicates were computed.

Results

Sequence homology

The partial S gene, the pairwise alignment nucleotide sequences of southern Vietnamese isolates showed high homology together at 98.9 - 100%. However, the current PEDV isolates contained variable difference on nucleotide sequences with other reference strains. In reality, these current isolates shared 95.6 - 96.4% nucleotide identity with parentage European strains (CV777, Br1/87) and 91.4 – 97.8% identity with Korean strains (Chinju99, Spk1, DR13, KNU-0801, KNU-0802). Interestingly, Vietnamese isolates shared high nucleotide homology with Chinese strains (JS-2004-2, DX) and Thai strains (07NP01, 08NP02, 08CB01) at 97.7 – 98.5% and 98.8 – 99.5%, respectively.

The full M gene, homology of nucleotide sequence in full M gene of Vietnamese PEDV isolates shared considerably high together (9.7-100%) in this study. Within group 1, current Vietnamese PEDV isolates shared 98.6 – 99.0% of nucleotide sequence identity with Thai strains (07NP01, 08NP02, KU06RB08), 97.2 – 99.7% identity with Chinese strains (JS-2004-2, DX), and 97.9 – 99.0% identity with Korean strains (CPF299, M1763).

Phylogenetics

The partial S gene phylogenetic analysis based on nucleotide sequence revealed PEDVs strains were divided into three groups (Figure 3.1a). Group 1 contained PED strains in suffered countries included Europe (CV777, Belgium; Br1/87, Britain), China (JS-2004, DX), Korea (DR13, KNU-0802), Thailand (07NP01, 08NP02, 08CB01) and Vietnam (current isolates in this study). Group 2 comprised Spk1, KNU-0801 (Korean strains) and group 3 comprised the first Korean strain (Chinju99). It should be noted that the Vietnamese isolates contained high

differences on the nucleotide sequence of partial S gene with other referent strains in Europe (CV777, Br1/87), and Korea (Chinju99, Spk1, KNU-0801). Interestingly, current Vietnamese isolates closely related to Thai strains (07NP01, 08NP02, 08CB01), Chinese strains (JS-2004-2, DX) and Korean strain (KNU-0802).

In the full M gene, the PEDVs also divided into three distinct genetic groups (Figure 3.1b). Group 1 comprised Vietnamese isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6), Chinese strains (JS-2004-2, DX), Korean strains (CPF299, M1763) and Thai strains (07NP01, 08NP02, KU06RB08). Current Vietnamese PEDV isolates revealed closely relationship with above Chinese, Thai, and Korean strains. Group 2 comprised Chinju99 (Korea) and CHIMT06 (China) and group 3 included CV777, Br1/87 (Europe) and LZC (China).

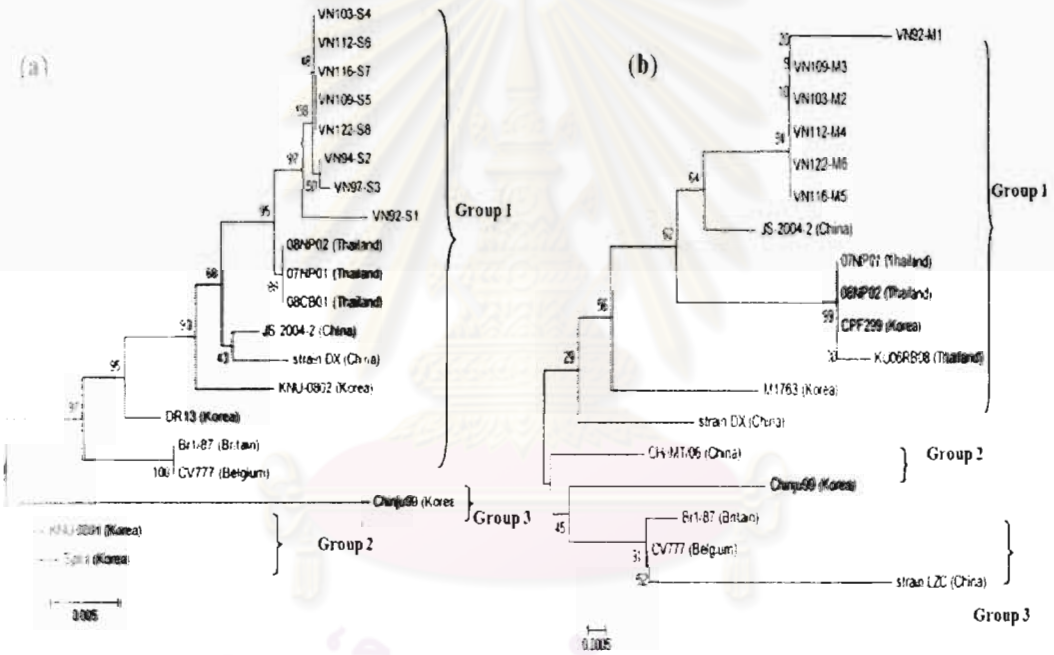

Figure 3.1 Dendrogram based-nucletotide sequence. (a) of the partial S gene amongst eight southern Vietnamese PEDV isolates (VN92S1, VN94S2, VN97S3, VN103S4, VN109S5, VN112S6, VN116S7, VN122S8) with those of other referent strains (European strains, CV777 and Br1/87; Chinese strains, JS-2004-2 and DX; Korean strains, Chinju99, DR13, Spk1, KNU-0801, KNU-0802; Thai strains, 07NP01, 08NP02, and 08CB01). (b) of full M gene amongst six southern Vietnamese PEDV isolates (VN92M1, VN103M2, VN109M3, VN112M4, VN116M5, VN122M6) with those of other referent strains (European strains, CV777 and Br1/87; Chinese strains, JS-2004-2, LZC, DX and CHIMT06; Korean strains, Chinju99, M1763, CPF299); Thai strains, 07NP01, 08NP02 and KU06RB08). Multiple alignment method performed by using ClustalX program. The phylogenetic tree was constructed by using neighbor-joining algorithm. The number on each branch presents the bootstrap values of 1000 replicates. The scale bars indicate the number of 0,005 estimate evolutionary time.

Discussion

Porcine epidemic diarrhea virus (PEDV) can be distinguished into genetic group 1 of Coronaviruses that spike (S) glyprotein and membrane (M) protein genes are believed to have genetic variation geographically (Cavanagh et al., 1992; Cook et al., 1986; Kingham et al., 2000; Lepare Goffart et al., 1997, Weiss et al., 2005). The heterogeneity in those genomic sequences has been reported and is known essentially for the diverse PED pathogenicity (Lai et al., 2007; Lee et al., 2010). The M protein gene of Coronaviruses seems to be more conservable than the S gene (Chen et al., 2008; Lai et al., 2007; Puranaveja et al., 2009). In addition, M protein gene can be an ideal candidate for the development of genetically engineered vaccine for PED prevention or specific antigen for PEDV diagnosis in serology because it is the exterior of viral envelope (Wongserepipatana and Nunthaprasert, 2008).

In the selected Vietnamese PEDV isolates, genomic characterization revealed highly more conserved in the M gene than the partial S gene but the variability of nucleotide sequences of partial S and full M genes were not much comparing with other isolates in neighboring countries (JS-2004-2, DX, 07NP01, 08NP02 08CB01, CPF299 and M1763) considered to derive from the same PEDV ancestor responsible in recent outbreaks. Within eight southern Vietnamese PEDV isolates, there were minor differences in nucleotide sequence of VN92S1, VN94S4 and VN97S3 isolates collected from two farms. These isolates belonged to distinct clade compared with other Vietnamese PEDV isolates showing that the current isolates have begun to create diversity of the PEDV population in Vietnam. In conclusion, the results demonstrated that the eight Vietnamese isolates of this study may be originated from the same ancestor undergoing genetic variation and form a new PEDV genotype in Vietnam.

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