ลำดับทางพันธุกรรมแบบสมบูรณ์ของไวรัส PORCINE EPIDEMIC DIARRHEA ในประเทศเวียดนาม

นางสาวดาม ถิ วุย

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COMPLETE GENOME SEQUENCE OF PORCINE EPIDEMIC DIARRHEA VIRUS IN VIETNAM

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ดาม ถิ วุย : ลำดับทางพันธุกรรมแบบสมบูรณ์ของไวรัส PORCINE EPIDEMIC DIARRHEA ในประเทศเวียดนาม (COMPLETE GENOME SEQUENCE OF PORCINE EPIDEMIC DIARRHEA VIRUS IN VIETNAM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. เดชฤทธิ์ นิลอุบล, 69 หน้า.

โรคพีอีดี (Porcine epidemic diarrhea, PED) เกิดจากเชื้อไวรัสพีอีดี ที่เป็นอาร์เอ็นเอไวรัสแบบมีเปลือกหุ้ม ในจีนัส *Alphacoronavirus* ตระกูล *Coronaviridae* ออเดอร์ *Nidovirales* ประเทศเวียดนามพบโรคพีอีดีเป็นครั้งแรกในปี ค.ศ. 2009 การศึกษานี้ได้นำเชื้อไวรัสพีอีดีจำนวน 3 ไอโซเลท ที่แยกได้จากลูกสุกรอายุ 3 สัปดาห์แสดงอาการท้องเสีย จากฟาร์มในประเทศเวียดนาม มาทำการถอดรหัสพันธุกรรมทั้งสาย โดยเชื้อ 2 ไอโซเลทมาจากฟาร์มในเขตภาคใต้ และอีก 1 ไอโซเลตมาจากฟาร์มในเขตภาคเหนือ สายพันธุกรรมของเชื้อไวรัสทั้ง 3 ไอโซเลท มีความยาว 28,035 นิวคลีโอไทด์ และมีโครงสร้างทางพันธุกรรมและการจัดเรียงตัวของจีโนมเหมือนไวรัสพีอีดีทั่วไป มีลำดับยีน 5'-ORF1a/1b-S-ORF3-E-M-N-3' เชื้อทั้ง 3 ไอโซเลท มีความเหมือนกันที่ 99.8% และ 99.6% ในระดับนิวคลีโอไทด์และกรดอะมิโนตามลำดับ ลักษณะสำคัญของเชื้อไวรัสพีอีดีทั้ง 3 ไอโซเลทนี้คือ มีการเติมของกรดอะมิโน 4 ตัว (GENQ) และ 1 กรดอะมิโน (N) ที่ตำแหน่ง 56-59 และ 140 และการขาดหายไปของกรดอะมิโน 2 ตัว (DG) ที่ตำแหน่ง 160 และ 161 จากการทำแผนภูมิต้นไม้วงศ์วานวิวัฒนาการจากสายพันธุกรรมทั้งสายพบว่า เชื้อไวรัสพีอีดีทั้ง 3 ไอโซเลทอยู่ในกลุ่ม New variants กลุ่มนี้เป็นเชื้อไวรัสพีอีดีสายพันธุ์ใหม่ ที่ก่อให้เกิดการระบาดครั้งใหญ่ในประเทศจีน ระหว่างปี ค.ศ. 2011-2012 และมีความแตกต่างทางพันธุกรรมจากเชื้อไวรัสพีอีดีสายพันธุ์ดั้งเดิม จากผลการศึกษาครั้งนี้สรุปได้ว่าเชื้อไวรัสพีอีดีที่แยกได้จากประเทศเวียดนาม เป็นเชื้อไวรัสพีอีดีสายพันธุ์ใหม่และมีลักษณะทางพันธุกรรมที่ใกล้เคียงกับเชื้อไวรัสพีอีดีสายพันธุ์ใหม่ของประเทศจีน ผลการศึกษาครั้งนี้ทำให้เกิดความเข้าใจเกี่ยวกับลักษณะทางพันธุกรรมของเชื้อไวรัสพีอีดีที่แยกได้จากประเทศเวียดนาม

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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DAM THI VUI: COMPLETE GENOME SEQUENCE OF PORCINE EPIDEMIC DIARRHEA VIRUS IN VIETNAM. ADVISOR: ASST. PROF. DR. DACHRIT NILUBOL, 69 pp.

Porcine epidemic diarrhea (PED) is caused by PED virus (PEDV), an enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales*. PEDV first emerged in Vietnam in 2009. In this study, the complete genomes of three Vietnamese PEDV isolates were characterized. These three isolates were isolated from 3-day-old pigs experiencing diarrhea. Two isolates were from swine farms in the south, and the other was from North Vietnam. The whole genome sequences of these isolates are 28,035 nucleotides in length and have a genome characterization similar to that of other PEDV isolates with gene order 5'-ORF1a/1b-S-ORF3-E-M-N-3'. All three Vietnamese PEDV isolates share 99.8% and 99.6% sequence identity at the nucleotide and amino acid levels, respectively and have characteristics including deletion and insertion in the spike gene, namely, the insertion of 4 amino acids (GENQ) and 1 amino acid (N) at positions 56-59 and 140, respectively, and one deletion of 2 amino acids (DG) at positions 160 and 161. Phylogenetic analysis based on the whole genome revealed that the three Vietnamese PEDV isolates are grouped together with new variants from China that were responsible for an outbreak of PEDV from 2011 to 2012, and genetically distinct from US isolates and the classical PEDV variant. The results suggest that Vietnamese PEDV isolates are new variants as evidenced by their unique genetic composition of insertions and a deletion in the spike gene and share high genetic similarity with the new variants of Chinese PEDV isolates. This study provides a better understanding of the molecular characteristics of PEDV in Vietnam.

Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	percentage
µl	Microliter
aa	Amino acid
DMEM	Dulbecco's modified Eagle'smedium
E gene/protein	Envelope gene/protein
GM	The growth medium
HCoV-229E	Human coronavirus 299E
IBV	Infectious bronchitis virus
M gene/protein	Membrane gene/protein
MHV	Murine hepatitis virus
min	Minute
N gene/protein	Nucleocapsid gene/protein
nt	Nucleotide
^{o}C	Degree Celsius
ORF gene	Open reading frame gene
PED(V)	Porcine epidemic diarrhea (virus)
RBD	Receptor-binding domain
S gen/protein	Spike gene/protein
TGEV	Transmissible gastroenteritis virus
UTR	Untranslated region

Protein:

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	phenylalanine
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

1.1 Importance and Rationale

Porcine epidemic diarrhea (PED) is a devastating enteric disease caused by PED virus. PED is characterized by vomiting and acute watery diarrhea leading to severe dehydration and death. The disease is very infectious. Pigs of all ages are susceptible to PED and display clinical diseases with 100% morbidity (Pospischil et al., 2002). The disease, however, causes high mortality up to 100% in suckling pigs under 7 days of age.

PED was first identified in England in 1971. The disease subsequently spread to many pig herds in European countries including Switzerland, Germany, France, The Netherlands, and Bulgaria (Pensaert and Yeo, 2006). The causative agent was not identified as PEDV until in 1978 when PEDV was first identified in Belgium (Chasey and Cartwright, 1978; Pensaert and De Bouck, 1978), approximately 7 years after the initial recognition of the clinical diseases.

In Asia, PED was first identified in Japan in 1982 (Takahashi et al., 1983), China in 1986 (Chen et al., 2008), India in 2003 (Pensaert and Yeo, 2006), Thailand in 2007 (Puranaveja et al., 2009). Unlike on the European continent, PED has continued to be a devastating enteric disease and has impacted the economies of many Asian countries since it first emerged. At the time, PEDV was considered as an endemic pathogen in South East Asia (Pensaert and Yeo, 2006). However, the emergence of PED in North America in 2013 has made PEDV one of the most serious enteropathogenic diseases threatening the global swine industry.

PED virus (PEDV), an enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales* is the causative agent of PED. The phylogenetic analysis based on the full-length genome sequences of PEDV showed that all PEDV strains were further evolved into two distinct genogroups. At present, two variants of PEDV, designated classical and new variants, have been recognized. CV777 isolate was the prototype of classical variant. The full-length genome sequence of PEDV isolate CV777 was characterized

and demonstrated that PEDV had a close relationship with the human coronavirus (HCoV)-229E and transmissible gastroenteritis virus (TGEV) (Kocherhans et al., 2001). Genome order is ORF1a/b-spike (S)-ORF3-E-matrix (M)-Nucleocapsid (N).

Interestingly, all PEDV strains isolated in recent years were classified in group 2, genetically distant from the classical (Huang et al., 2013). The new PEDV variant was found in Korea and Thailand, and was reported as the new PEDV variant that caused the outbreak of diarrhea in pig farms in China in 2011 (Li et al., 2012).

The full-length genome sequence of new variants were characterized and demonstrated that the genome of the new variant were similar to the prototype CV777 PEDV strain with similar gene order, 5'-ORF1a/1b-S-ORF3-E-M-N-3' (Chen et al., 2011; Park et al., 2012; Zhao et al., 2012; Li et al., 2013), characterized by the notable insertion of 4 amino acid at position 56-59 and one amino acid at position 140, and the notable deletion of two amino acid located between amino acid 159-160 (Lee et al., 2010; Temeeyasen et al., 2013).

In Vietnam, porcine epidemic diarrhea emerged in 2009. The disease caused massive economic losses in the Vietnamese swine industry, particularly in southern provinces. Soon after the emergence, the disease spread throughout the major swine producing regions in Vietnam including Northern, Middle and Southern Vietnam. Currently, PED in Vietnam has developed into an endemic stage causing sporadic outbreaks. A report suggested that Vietnamese PEDV isolated in farms in Southern provinces during 2009-2010 were closely related to Chinese and Thai isolates (Duy et al., 2011). However, the study focused on partial S and M genes. Therefore, in order to understand the overall genetic diversity and evolutionary characteristics of Vietnamese PEDV strains, the determination of the whole genome is needed. Such data will demonstrate the epidemiological relationship between the Vietnamese PEDV strains and the source of introduction, and can be used to plan an appropriate prevention and control PED program including vaccination in the future.

1.2 Objectives of Study

The objectives of the study were to characterize the whole genome sequence of PEDV isolates in Vietnam, and establish those genome sequence data as benchmark references for sequence data for further molecular studies.

1.3 Hypothesis

Vietnamese PEDV isolates are new variant, and possess owning genetic characteristics in the full-length sequences.

1.4 Research questions

What are the molecular characteristics of complete PEDV genome sequence in Vietnam?

What is the difference between Vietnamese PEDV and classical PEDV isolates?

What is the relationship between Vietnamese and global PEDV isolates?

CHAPTER II LITERATURE REVIEW

2.1 Characteristics of porcine epidemic diarrhea

Porcine epidemic diarrhea (PED), caused by PED virus (PEDV), is a contagious disease. The clinical symptoms include anorexia, vomiting, severe watery diarrhea and dehydration resulting in high mortality rates in sucking piglets under 7 days of age (Pensaert and Yeo, 2006) (Figure 1.A).

The small intestinal wall of infected pigs was congested with watery and undigested milk curd (Figure 1.B). Segmental enteritis was descripted by segmental disappearance of intestinal lacteal caused by malabsorption in intestinal parts affected (Puranaveja et al., 2009) (Figure 1.C and D).

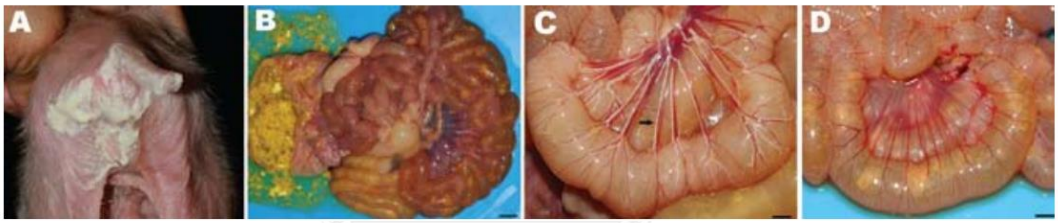

Figure 1. Clinical signs and gross pathological lesions in piglets infected by PEDV. A) Severe diarrhea and dehydration in the suckling piglet (Pensaert and Yeo, 2006). B) Severe catarrhal enteritis with congestion (scale bar = 1 cm). C) Intestinal lacteals (arrow) grossly demonstrating normal absorption capacity of the intestinal villi in a normal piglet. D) Disappearance of intestinal lacteals (Puranaveja et al., 2009).

Therefore, the mechanism of viral replication of PEDV and the degeneration of villous are similar to TGE and other viral enteritis. The affected epithelial cells will be seen in the small and large intestines, around 12 to 18 hours and 5 days after inoculation (Pensaert and Yeo, 2006). The transmission of PEDV is maintained via feces or by oral. After PEDV passed through the defensive border of stomach fluid, the virus then attached to specific receptor porcine aminopeptidase N (Weiss and Navas-Martin, 2005; Li et al., 2007) and caused the changes in morphology and the intestinal tract's function characterized by villous atrophy. The atrophied intestinal tract lost the function of absorption due to the reduction of the digestive enzymatic

activity. Consequently, that caused the changes in the digestion of lactose and cellular transportation and hydrolysis of nutrition. Finally, Pigs fell into the condition of mal-absorption, diarrhea and hydration, and sucking piglets died within few days (Sestak and Saif, 2002; Weiss and Navas-Martin, 2005; Pensaert and Yeo, 2006).

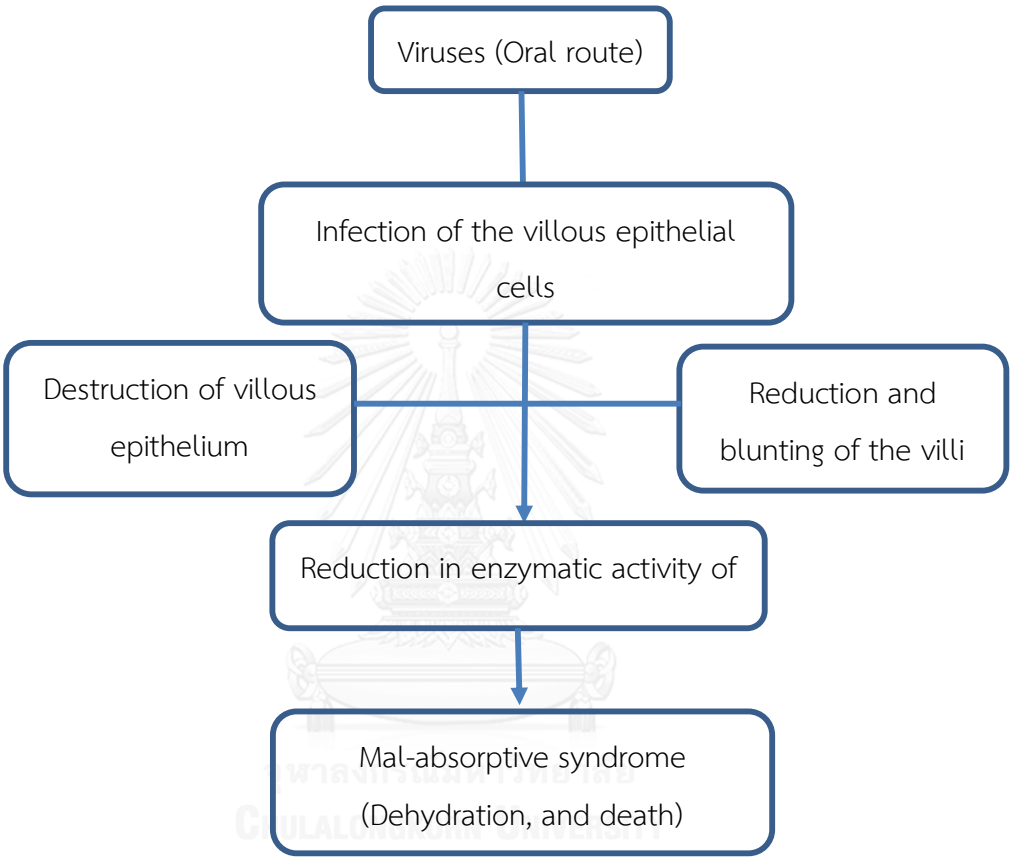

Figure 2. Mechanism of porcine epidemic diarrhea virus infection

2.2 Molecular and genetic characteristic of porcine epidemic diarrhea virus

The causative agent was identified as a coronavirus, PEDV (strains CV777) in Belgium in 1978 (Pensaert and De Bouck, 1978). PEDV is an enveloped, single-stranded, positive-sense RNA virus belonging to the order *Nidovirales*, the family *Coronaviridae*, subfamily *Corovirinae*, and genus *Alphacoronavirus*. PEDV genome is approximately 28 kb in length and includes 5' and 3' untranslated region (UTR) and 7 open reading frames (ORFs) (Kocherhans et al., 2001) that encode 4 structural

proteins [spike (S), envelope (E), membrane (M), and nucleocapsid (N)] and three non-structural proteins (replicase 1a and 1b, and ORF3). The genomic organization of PEDV is arranged in the order 5'- replicase (1a/1b)-S-ORF3-E-M-N-3'(Song and Park, 2012).

The polymerase gene is composed of the two largest ORFs, 1a and 1b, covering two-thirds of the full-length that is located at the 5' of the coronavirus genome, and encodes the non-structural replicase polyproteins (replicases 1a and 1b). The replicase gene, ORF1a and ORF1b has an overlapping region at a potential ribosomal frame shift site which was called "slippery sequence". It consists of a specific seven nucleotide (UUUAAAC) and a pseudoknot structure (Kocherhans et al., 2001; Chen et al., 2011). This region is required for the translation sequence of ORF1b (Chen et al., 2011). According to Kocherhans and colleagues, the amino acid sequence of ORF1b is more conserved than ORF1a compared to corresponding sequences of other coronavirus (Kocherhans et al., 2001). When comparison between amino acid sequence, ORF1a and 1b of PEDV had a close relationship with HCoV-229E and TGEV and less related to MHV and IBV (Kocherhans et al., 2001). The amino acid sequence of ORF1a is similar to the sequence of ORF1a of HCoV-229E and TGE that are 59.4% and 52.1%, respectively. The amino acid sequence of ORF1b shared 83.2 % and 80.3% identity to HCoV-229E and TGEV amino acid, respectively. Whereas, the similarity of amino acid sequence of ORF1a are 39.4% and 38.7%, respectively, compared to HCoV-229E and TGEV (Kocherhans et al., 2001).

The PEDV S protein is a type I glycoprotein. It consists of 1,383 amino acid. S protein is classed into sub-domain 1 and sub-domain 2 (Figure 3) (Woo et al., 2010; Shirato et al., 2011). Sub-domain 1 or N-terminal S1 subunit, signal peptide and regions of mapped receptor-binding domains (RBDs), is located between 1-789 amino acid (aa) positions (Song and Park, 2012). Sub-domain 2 or C- Terminal transmembrane S2 subunit (TM) is located between 790-1,383 amino acid (Woo et al., 2010). The S2 subunit is a transmembrane domain and an endodomain with cysteine-rich cytoplastic tail or CT domain (Masters, 2006; Woo et al., 2010; Shirato et al., 2011). In addition, S protein can be separated into 4 domains. It contains a signal

peptide (1–18 aa), neutralizing epitopes (499–638, 748–755, 764–771, and 1,368–1,374 aa), a transmembrane domain (1,334–1,356 aa), and a short cytoplasmic domain.

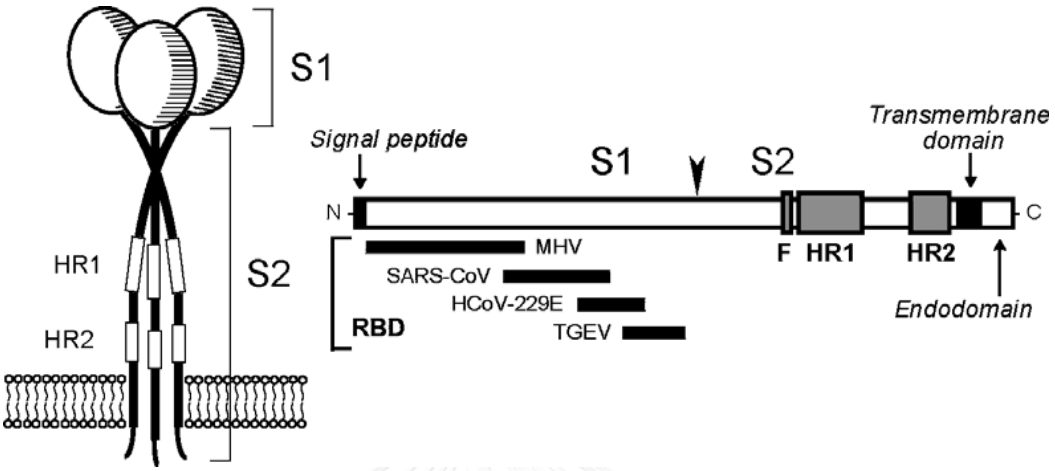

Figure 3. The spike (S) protein structure of coronavirus

Like other coronavirus S protein, PEDV S protein is a glycoprotein peplomer (surface antigen) on the viral surface. It has the important role in receptor binding activity (by S1 subunit) and membrane fusion (by S2 subunit) between PEDV and specific host cell (Masters, 2006). So the spike gene of PEDV is divided into S1 and S2. It is associated with tissue tropism and production of neutralizing antibodies (Kim et al., 2001; Chang et al., 2002). Thus, the S glycoprotein could be a primary target for the development of effective vaccines against PEDV (Song and Park, 2012).

S protein is required for promoting the viral budding into intercellular vesicles within the infected host cell (Shirato et al., 2011). Moreover, it is associated with growth adaptation in vitro, and attenuation of virulence in vivo (Song and Park, 2012). Therefore, S protein is an important target for studying the genetic relationships between, and the diversity of PEDV isolates, the PEDV epidemiology in the field, and the association between genetic mutations and viral function (Song and Park, 2012)

The E protein (or called sM) is a small polypeptide, about 8.4 to 12 kDa (76–109 amino acids), this is a minor constituent of virions (Masters, 2006). E gene is

conserved among all members of coronaviruses, but it is not used as a target for phylogenetic studies because it contains a short sequence (Masters, 2006).

The membrane gene (M gene) is coded for membrane protein (M protein), a structural membrane glycoprotein and the most abundant envelope component (Utiger et al., 1995). Furthermore, the M protein not only performs an important role in the viral assembly process (Nguyen and Hogue, 1997; de Haan et al., 1998), but also consists of antibodies that neutralize virus in the presence of complement (Saif, 1993). It has been suggested that the M protein may play a role in α-interferon (α-IFN) induction (Laude et al., 1992). Furthermore, Co-expression of E and M proteins permitted the pseudoparticles to form, which exhibit an interferogenic activity in the same way as complete virions (Baudoux et al., 1998).

The N protein is encoded by nucleocapsid gene, which could be important for induction cell-mediated immunity in the host (Saif, 1993). The N protein also plays a role in transcription of the viral genome, the package of viral RNA, and the formation of the viral core (Li et al., 2007; Lee et al., 2008). Moreover, since N protein is highly conserved, it is the best candidate protein used as an antigen for early diagnosis reagent and vaccine development (Duarte et al., 1993; Tobler et al., 1993).

The ORF3 gene is an accessory gene that is located between spike and envelope genes (Masters, 2006). It encodes for accessory proteins whose functions are less well known. It also is one of the pathogenicity factors and might be a marker for monitoring of nucleotide sequence varied during higher passage adaptation (Kweon et al., 1999; Song et al., 2003). Recently, the ORF3 gene has received much attention in the virulence of PEDV because the virulence can be reduced by altering this gene through cell culture adaptation (Park et al., 2007b; Park et al., 2008). The ORF3 gene has been used in several reports to differentiate between field and vaccine-derived isolates (Park et al., 2008)

2.3 Distribution of porcine epidemic diarrhea disease

The first outbreak of diarrhea was observed in feeder and fattening pigs in England in 1971. The clinical sign is the same as the observation of transmissible gastroenteritis virus (TGEV) infection, but the suckling piglets did not become sick. So

the TGEV and other enteropathogenic infectious agents were elided from these outbreaks. This disease spread to other countries in Europe and the name “epidemic viral diarrhea” (EVD) was designated (Pensaert and Yeo, 2006). In 1976, the TGE-like outbreaks of acute diarrhea recurred in pigs of all ages including suckling pigs. The name “EVD type 2” was used to differentiate with the outbreak observed in 1971.

In 1978, the experiment pig with the strain designated CV777 was observed, and caused pathogenesis to pigs of all ages which associated to EVD type 1 and 2. Thus, the disease was called Porcine epidemic diarrhea (PED)” (Pensaert et al., 1982). During 1982-1990, some reports were published on the antibodies against PEDV in Belgium, England, Germany, France, the Netherlands, Switzerland, Bulgaria, and Taiwan (Debouck et al., 1982; Hofmann and Wyler, 1987). The PEDV was identified in many countries in Europe. For example, in the 1990s, PED caused the small epizootic of watery diarrhea in seven farms in Spain (Carvajal et al., 1995a), and the specific antibodies to PEDV were detected in 1513 of 5052 sows, and seropositive animals were identified in 55.0% of 803 breeding farms (Carvajal et al., 1995b). In the Netherlands, an acute outbreak of PEDV was found in breeding and finishing pigs (Pijpers et al., 1993). It was also found in the Czech Republic in 1993 (Song and Park, 2012). Subsequently, the outbreak of PED occurred in Hungary in 1995 (Nagy et al., 1995), in Great Britain in 1999 (Pritchard et al., 1999). In Italy, the observed outbreak of PEDV occurred in all ages of pigs, and contained piglets between May 2005 and June 2006 (Martelli et al., 2008). Presently, PEDV outbreaks are rarely found in European countries (Martelli et al., 2008).

In Asia, PED outbreaks are more acute and severe than those observed in Europe. This disease is of serious concern in Asian countries. PED was first identified in Japan in 1982 (Takahashi et al., 1983), China in 1986 (Chen et al., 2008), India in 2003 (Pensaert and Yeo, 2006), In Korea, PEDV was first isolated in 1992 (Kweon et al., 1993). Since then, it has spread to many provinces and become one of the most devastating viral enteric diseases in this country. In Thailand, the PED outbreak was first reported in 1995 on a farm located in the southern region of Thailand (Srinuntapunt S et al., 1995). The disease did not spread to other swine producing

areas until late 2007. The disease then reoccurred in Nakorn Pathom province (Puranaveja et al., 2009). Since its reemergence, it has evolved into a pandemic PED outbreak in Thailand. As a result, more than 90% of Thai swine farms have been infected. In 2008, PEDV isolates were achieved and the data reported in complete M gene sequence by the Department of Livestock Development, National Institute of Animal Health, Thailand (NCBI dataset). A well-described disease characterization of Thai PED situation based on molecular epidemiology was first published by Puranaveja and colleagues in 2009 (Puranaveja et al., 2009).

In Vietnam, the outbreak of PEDV was reported in 2009. The disease caused massive economic losses in the swine industry in most of the southern provinces. At present, PED has occurred throughout the country and is the single most important factor affecting the swine industry. The infected pigs revealed severe watery diarrhea, dehydration and died within a few days in suckling piglets. Adult pigs showed an acute watery diarrhea condition and recovered after several days. According to Duy et al, 2011 Vietnamese PEDV in the southern provinces during 2009-2010 were closely related to the Chinese and Thai isolates. Vietnamese PEDV isolates could share the same origin with Chinese ancestor undergoing genetic variation and possibly forming a new PEDV genotype in Vietnam (Duy et al., 2011).

Up until late 2013, the emergence of PEDV in Taiwan was not reported. However, at the beginning of December 2013, a remarkable increase in outbreaks of PEDV occurred in the country (Lin et al., 2014).

Prior to April or May 2013, PED was not reported in North America. However, the disease was first identified in the United States in May 2013 (Huang et al., 2013). Since then, PEDV has spread to 27 or more states (Stevenson et al., 2013; Chen et al., 2014). In late January 2014, the first case of PED was confirmed in a swine herd in South Western Ontario, Canada (Pasick et al., 2014).

Despite the history of PED outbreaks in Europe, little is known about current virus strains and their effect. A case of PED occurred in a swine-fattening farm in Germany in May 2014. The causative virus was fully characterized by using conventional methods and next-generation sequencing (Hanke et al., 2015).

Presently, PED is one of the most serious enteropathogenic diseases and has had a major impact on the swine industry worldwide. PEDV has been considered as an endemic pathogen in South East Asia and the United States (Wang et al., 2014). And the virus is most often found in suckling piglets less than 10 days of age (Pensaert and Yeo, 2006) .

2.4 The study of porcine epidemic diarrhea virus

2.4.1 The study of PEDV across the world

To investigate the molecular epidemiology of PEDV, genetic and phylogenetic analysis based on the S, M and ORF3 genes has been used to determine the genetic relationship among PEDV isolates. In Korea 2007, the study of phylogenetic analysis of PEDV isolates between January 2002 and August 2005 based on partial S gene consists of an epitope region, shows that Korean isolates share a similarity to the Chinese strain (JS-2004-2) in one subgroup (Park et al., 2007a). In another report, Lee et al (2010) showed that the phylogenetic tree based on the entire S gen of all Korea PEDV isolates in 2008-2009 were divided in the same group as Korean parent strains, and were distinct from PEDV references (CV777 and Br 1/87), Korean vaccine strains (SM98P and DR13) and all Chinese strains (DX, LJB, LZC, and JS-2004-2) with low nucleotides identities (Lee et al., 2010). In 2011, a separate study reported that the sequence and phylogenetic analysis based on the complete M and ORF3 genes of Korean PEDV field strains from 2003 – 2007 revealed prevalent Korean field isolates have close relationships to Chinese field strains and differ genetically from European strains and vaccine strains used in Korea (Park et al., 2011).

In China, the first PEDV strain (CH/S) was isolated in 1986 (Chen et al., 2008). The phylogenetic relationship of Chinese PEDV strains in 2006 based on M gene revealed higher homology to Chinese strain (JS-2004-2) than CV777, Br1/87. Chinju99 and JMe2 (Chen et al., 2008). The sequence insertions and mutations were found in the high pathogenicity of the new PEDV variant that caused the outbreak of diarrhea in pig farms in China in 2011 (Li et al., 2012). In 2010, the study of phylogenetic analysis of PEDV strains during January 2006 – August 2009 based on ORF3 gen

showed that the Chinese PEDV field strains have a close relationship to Korean strains and are genetically different from PEDV vaccine strains. (Chen et al., 2010).

In Thailand, the PED outbreak first emerged in Nakornpathom province with the highest pig densities in late 2007. The partial S gene and full M gene were immediately cloned and sequenced. The study showed that the recent Thai PEDV strains indicated in the same clade as the Chinese isolates (JS-2004-2) identified in 2004, and differed genetically from previous Thai isolates (Puranaveja et al., 2009). Recently, a study of the PEDV in Thailand during 2008-2012 based on the partial and complete S gene, and ORF3 gene was reported. Temeeyasen et al (2013) showed that Thai isolates responsible for outbreaks in Thai during the period 2008-2012 were a new variant of PEDV with the insertion and deletion in S gene that was previously reported in China and Korea (Temeeyasen et al., 2013).

In this case, the molecular epidemiology of PEDV was studied based on whole genome sequence. The full-length sequence of PEDV was first completed in 2001 (Kocherhans et al., 2001), Subsequently, the entire genome of PEDV were reported in many countries such as China, Korea, the United States (Chen et al., 2011; Bi et al., 2012; Chen et al., 2012a; Huang et al., 2013; Marthaler et al., 2013; Cho et al., 2014; Yang et al., 2014).

In China, the first PEDV strain, isolated in a pig breeding farm in Shanghai in 1986, had its genome sequence completed in 2011 (Chen et al., 2011). Then, the whole genome of PEDV was reported in 2011- 2013 (Bi et al., 2012; Chen et al., 2012b; Fan et al., 2012; Luo et al., 2012; Wei et al., 2012; Zhao et al., 2012; Li et al., 2013). The new variant of PEDV was confirmed in China in 2011 (Li et al., 2012). A report demonstrated that the recent Chinese PEDV isolates belong to the same group distant from the CV777 vaccine strain and other foreign isolates (Fan et al., 2012; Pan et al., 2012).

In Korea, PED outbreaks first occurred in 1992. A virulent strain, DR13, was identified from suckling piglets suspected of having porcine epidemic diarrhea in 1999. The entire genome was thus first completed for the virulent and its attenuated strains in 2012 (Park et al., 2012). However, the PED outbreaks have reemerged and

spread rapidly across the country since December 2013. Determination of the origin and diversity of PEDV strains are ongoing in South Korea, PEDV strains were sequenced for the whole genome and analyzed. The reports showed that the complete genome sequence of recent Korean strains were most genetically similar to those of the US PEDV strains isolated in 2013, and distinct from previous endemic Korean PEDV strains (Lee; Choi et al., 2014).

In the United States, PEDV outbreaks suddenly emerged in May, 2013. The complete genome of this PEDV strain was sequenced and analyzed (Marthaler et al., 2013). Several reports showed that PEDV strains were clustered into two distinct genogroups, and the genetic and phylogenetic analyses of the US strains were genetically closely related to a Chinese PEDV strain (AH2012) isolated in 2012 (Huang et al., 2013; Chen et al., 2014). A recent report showed that the new variant of PEDV was different compared to the current PEDV strains (OH851) in the United States based on phylogenetic analysis of the complete genome. In addition, phylogenetic analysis of the full-length of spike gene also indicated OH851 strain was separated with other isolates of PEDV in China and had a close relationship with a Chinese strain, CH/HBQX/10. However, it was distinct from the recent PEDV isolated in the United States and AH2012 strain (Wang et al., 2014).

PED outbreaks have rarely been found in European countries for many years, though a case of PED was reported on a swine fattening farm in Germany in May, 2014. The full-length of this virus was characterized by using conventional methods and next generation sequencing. The study showed that German isolates share a very high (99.5%) identity with the new variant OH851 (GenBank accession no. KJ399978) recently reported from United States in 2014.

Today, there are more than 150 complete genome sequences of PEDV isolates available in the GenBank database. And the PED virus has changed in the genotype, compared to the classical PEDV strains. The prevention and control programs for this disease did not generate successful results. Thus, PED is one of the most serious enteropathogenic diseases to impact the swine industry and is fast becoming a major concern throughout the world.

2.4.2 The study of PEDV in Vietnam

Porcine epidemic diarrhea was identified in South Vietnam in 2009. Subsequently, the disease spread to other provinces and has become a major factor affecting the swine industry. The study of PEDV strains is still limited in Vietnam. According to Duy et al., 2011, the genetic relationship between Vietnamese isolates and reference isolates were analyzed based on partial S and M gene sequences. Vietnamese PEDV in the southern provinces during 2009-2010 were related closely to the Chinese isolate (JS-2004-2), Korea and Thai isolates (Figure 4). Vietnamese PEDV isolates may have originated from the same Chinese ancestor undergoing genetic variation and possibly forming a new PEDV genotype in Vietnam (Duy et al., 2011). Therefore, in order to understand the overall genetic diversity and evolutionary characteristics of Vietnamese PEDV strains and provide the data references for the further studies, the determination of PEDV is necessary.

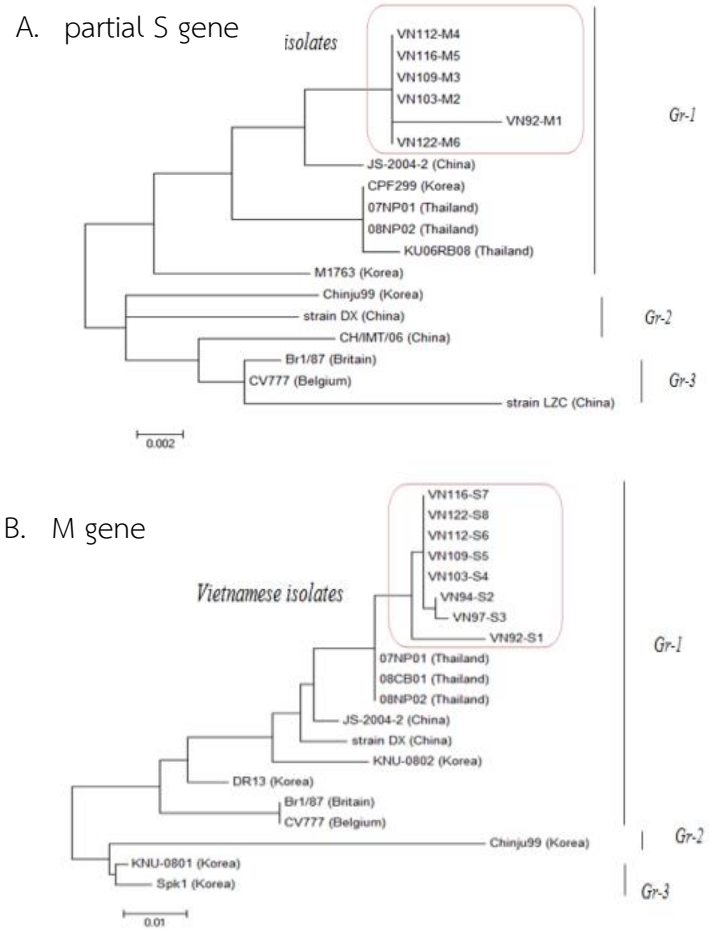

Figure 4. Phylogenetic tree based on the nucleotide sequences of the partial S and M gene of southern Vietnamese PEDV with those of other reference strains (Duy et al., 2011)

CHAPTER III MATERIALS AND METHODS

3.1 Conceptual framework

This study was completed within the following framework (Figure 5). To define the molecular characteristics of PEDV isolates in Vietnam, the nucleotide and deduced amino acid sequences of Vietnamese PEDV isolates were analyzed. The genetic and phylogenetic analyses of whole genome, ORF1, S, ORF3, E, M and N gene were compared with other PEDV strains available in the Genbank database.

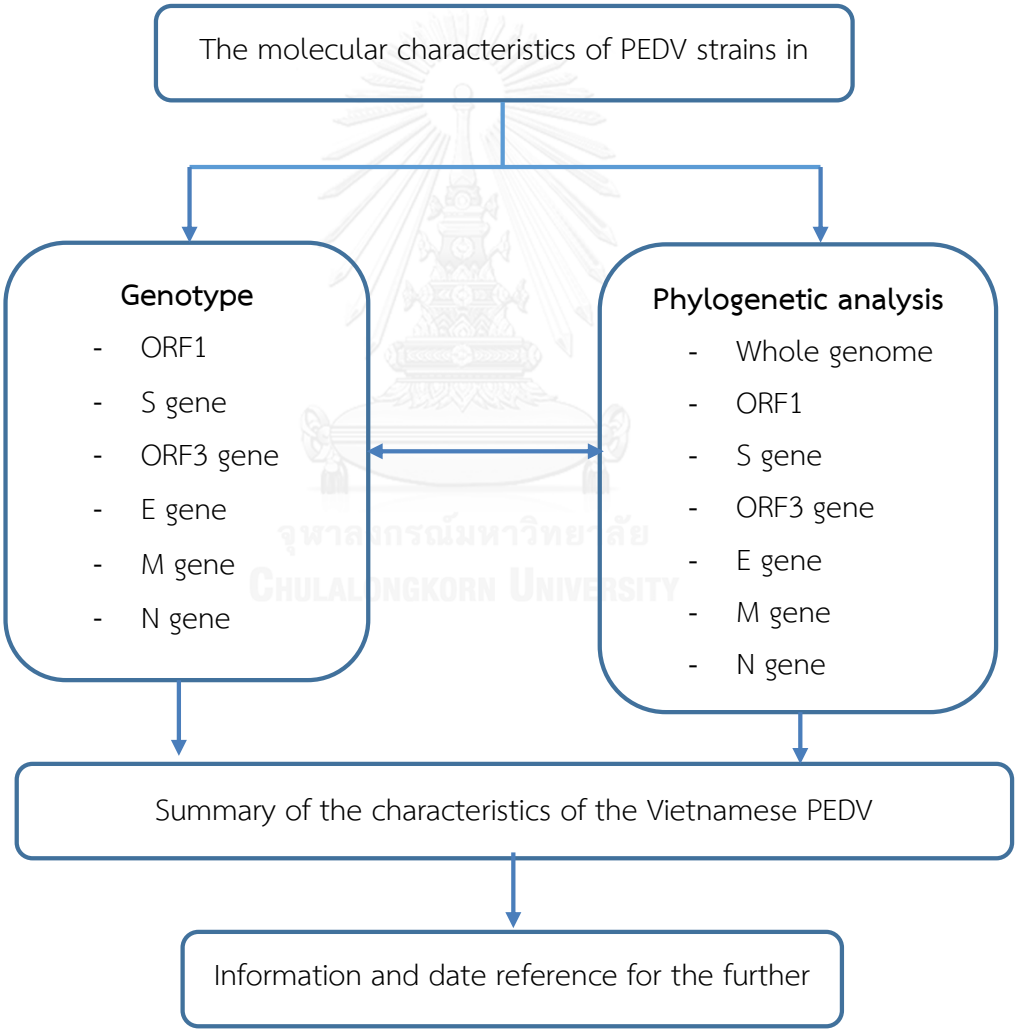

Figure 5. Conceptural framework

3.2 Virus and viral isolation

Three Vietnamese PEDV isolates were isolated from intestinal samples of 3 day-old pigs displaying severe watery diarrhea and dehydration. One isolate was taken from the collection samples of Virology section at the National Center for Veterinary Diagnosis (Hanoi Vietnam) in January 2013, and another two isolates were from two different affected farms in Southern Vietnam in October 2013.

To isolate PEDV, intestinal samples were minced into small pieces and homogenized with phosphate buffer saline (PBS; 0.1M, pH 7.2) to 10% suspension. Homogenized samples were then vortexed and clarified by centrifugation at 3000g for 30 minutes. Supernatant were collected and filtrated through a 0.45-μm filters. The filtrates were stored at -80 C until used for virus isolation in Vero cell (Hofmann and Wyler, 1988).

Vero cells were grown in a 25-cm^2 flask with the growth medium (GM) (Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplements with 5% heat-inactivated fetal bovine serum and antibiotics until a full confluence monolayer at 37^0C in 5% CO_2 was reached. The GM was removed from confluent monolayer Vero cells, which were washed twice with TM (consisting DMEM and 10μg/ml trypsin (Dibco, USA)). And then the cells were infected with 0.5 ml per flask of the clarified diluted virus suspension at 37^0C in 5% CO_2 for 1 h. After adsorption for 1 hour at 37°C, the maintenance media was added (10 ml per flask) without removing the viral inoculum. The Vero cell cultures were incubated again for 5-7 days for observing the cyto-pathic effect (CPEs). The flasks were frozen and thawed twice and centrifuged at 3000g for 10 minutes to collect the supernatant, which were subjected to a polymerase chain reaction.

3.3 Reverse transcription-polymerase chain reaction

Viral RNAs of three strains were extracted from the infected Vero culture supernatant using the Nucleospin® viral RNA isolation kit (Macherey-Nagel Inc, Germany), according to the manufacturer's instructions and were dissolved in

nuclease-free water. cDNA were synthesized from the extracted RNA using M-MuLV Reverse Transcriptase (BioLabs Inc., MA.USA).

Twelve pairs of oligonucleotide primers were used to amplify the different regions of the Vietnamese PEDV strains, and were designed based on the sequence of PEDV CV777 strain (Pan et al., 2012). PCR amplification was performed on the cDNA using Gotap® Green Master mix (Promega, MA, USA). The primer sequences and PCR product sizes are shown in Table 1.

3.4 Cloning, plasmid purification and sequence determination

The PCR products were purified using the Nucleospin® Gel extraction kit (Macherey-Nagel Inc, Germany), and cloned into pGEM-T® Easy Vector vectors for the subsequent transformation of *Escherichia coli* (JM109) by using a commercial kit (Promega, WI, USA). Bacterial transformant colonies were randomly selected from each sample for plasmid purification using Nucleospin® Plasmid purification kit (Macherey-Nagel, Germary) in accordance with the manufacturer's instructions, and sequenced in both directions in triplicate by Biobasic Inc (Ontario) using an ABI Prism 3730XL sequencer. The 5' terminal sequences are determined by 5' rapid amplification of cDNA ends (RACE) (Sambrook and Russell, 2006).

Cloning DNA process using the qGem®- T Easy vector:

Ligation protocol

1. Briefly centrifuge the pGEM®-T or pGEM ®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.

2. Set up ligation reactions as described below.

Note: Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat # 20170-310).

Vortex the 2X Rapid Ligation Buffer vigorously before each use.

3. Mix the reactions by pipet. Incubate the reactions for 1 hour at room temperature.

Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Reaction component	Standard reaction
2X Rapid Ligation Buffer, T4 DNA ligation	5µl
pGEM®-T or pGEM ®-T Easy Vector (50ng)	1µl
T4 DNA Ligation (3 Weiss units/µl)	1µl
PCR product	3µl

Transformation protocol

Material: - Competent *Escherichia coli JM109*

- LB agar plates with ampicillin/IPTG (50µl)/ X-gal (20µl)

Transformation protocol:

1. Prepare LB/ampicillin/IPTG/X-Gal plates and incubate at 37°C for 30 minutes
2. Carefully transfer 10 µl each purified PCR product into a 200 µl competent cell. Gently flick the tubes to mix and keep on ice for 30 minutes.
3. The DNA- competent cell mixes were changed and placed in a water bath at 42°C for 1 minute.
4. Then the DNA-competent cell mixes were returned to the ice for 2 minutes.
5. After, 800 µl LB broth was added into the mix of DNA and competent, which were incubated in the shaker incubator at 37°C/ 1 hour.
6. The mixes were centrifuged at 8000g/minute for 1 minute. The supernatants were discarded. The 100µl of each transformation was cultured in a LB agar/ ampicillin/IPTG/X-Gal plates, and incubated at 37°C for 16-24 hours.
7. The white colonies were selected to culture on LB agar/ampicillin plates at 37°C/16-24 hours. Six or eight colonies were selected from each sample. Bacterial transformants colonies were randomly selected from each sample for plasmid purification using Nucleospin® Plasmid kit (Macherey-Nagel, Germary) in accordance with the manufacturer's instructions.

Table 1. Primers used for sequencing reaction.

Primer pairs	Oligonucleotides	Location*	Sequences
1	1F	190-209	GCGTTCCGTCGCCTTCTACA
	1R	2751-2729	CAGGAATCTGGAAGACACTTGCA
2	2F	2663-2684	GTATTATGCCACCAGTGTCCCA
	2R	4957-4938	CAGTTGCCAGCAGGCACTGT
3	3F	4887-4906	ACCAGCGGTGCATTGCTTGA
	3R	7475-7453	CAATGTGCTCTTGCAATCCTGCA
4	4F	7327-7350	CTGTTAAGTTAGTGGACTCAGCGT
	4R	9875-9856	ACTAGCGCCTTCAACTTGCA
5	5F	9712-9731	GCGCTTGTGGTTCACCTGGT
	5R	12259-12240	GGATCCACAGCGAAAGCGCA
6	6F	12182-12202	ACGCTTGCAGGCTGGTAAACA
	6R	14462-14442	TGGGCAGTGCTCTATCGCACT
7	7F	14322-14341	ATACTAGGGGCGCTTCGGTT
	7R	16780-16760	GTCAGGGTGCACAGGAATGAA
8	8F	16662-16684	GTATGTGTGCCCTTAAGCCTGAT
	8R	19002-18980	GTAAGTGGACGTTCGGCTTCATA
9	9F	18874-18898	CGTAGCTTTTGAGTTGTATGCCA
	9R	21330-21309	GCAATTAGCTGTACAGGGTTCA
10	10F	21080-21101	CCATTCCAGCTTATATGCGTGA
	10R	23487-23456	GTACATGTGAAGCTTCTCAGCGT
11	11F	23272-23292	GTGTACGATCCTGCAAGTGGC
	11R	25715-25694	TCACCTCATCAACGGGAATAGA
12	12F	25535-25557	TCGTCCAATTGGTTAATCTGTGC
	12R	27840-27820	TACCGTTGTGTGCAAGACCAA
	5’ RACE primer	307-288	TTGCTAGCCATAGCCGGCAG
	3’ RACE primer	27725-27747	CTATGTTCCAGGGTAGTGCCATT

*Location corresponds to position within the CV777 (AF353511) genome.

3.5 Sequence analysis

The nucleotide and deduces amino acid sequence alignments were performed by using the CLUSTALW program (Thompson et al., 1994). Phylogenetic analyses were performed using the MEGA version 5.2 (phylogenetic and molecular evolutionary analyses software) by the neighbor-joining method, and bootstrap analysis with 1,000 replicates based on the nucleotide sequence of the entire genome, ORF1, complete S, ORF3, E, M, and N genes of three PEDV strains in this study with others PEDV isolates that have been previously published in NCBI Genbank database (Tamura et al., 2011). The nucleotide sequence of PEDV reference strains of whole genome sequence are showed in Table 2.

Table 2. The reference PEDV strains for the full-length genomic nucleotide sequences used for sequence analysis and phylogenetic analysis were compared with current Vietnamese PEDV strains.

Strain	Year	Place of isolation	Accession #
AH2012	2012	China/Anhiu	KC210145
BJ-2011-1	2011	China/Beijing	JN825712
GD-B	2012	China/Guangdong	JX088695
JS-HZ2012	2012	China/Jiangsu	KC210147
CH/ZMDZY/11	2011	China/Henan	KC196276
CH/FJND-3/2011	2011	China/Fujian	JQ282909
CH/FJZZ-9/2012	2012	China/Fujian	KC140102
AJ1102	2011	China/Hubei	JX188454
LC	2011	China/Guangdong	JX489155
ZJCZ4	2011	China/Jiangsu	JX524137
CHGD-01	2011	China/Guangdong	JX261936
GD-A	2012	China/Guangdong	JX112709
GD-1	2011	China/Guangdong	JX647847
CH/GDGZ/2012	2012	China/Guangdong	KF384500

Strain	Year	Place of isolation	Accession #
DR13/virulent	1999	Korea	JQ023161
CH/S	1986	China/Shanghai	JN547228
CV777	1978	Belgium	AF353511
LZC	<2006	China/Gansu	EF185992
SM98	1998	Korea	GU937797
JS2008	2008	China/Jiangsu	KC109141
DR13/attenuated	Cell adapted/2003	Korea	JQ023162
SD-M	2012	China/Shandong	JX560761
Attenuated vaccine	Cell adapted/2012	China/Hubei	KC189944
MN	2013	USA/Minnesota	KF488752
IA1	2013	USA/Iowa	KF488753
IA2	2013	USA/Iowa	KF488754
USA/Colarado/2013	2013	USA/Colorado	KF272920
USA/Iowa/18984/2013	2013	USA/Iowa	KF804028
USA/Indiana/17846/2013	2013	USA/Indiana	KF452323
USA/Iowa/16465/2013	2013	USA/Iowa	KF452322
13-019349	2013	USA/Co	KF267450
KNU-1305	2013	Korea	KJ662670
KUIDL-PED-2014-007	2014	Korea	KJ588062
KUIDL-PED-2014-002	2014	Korea	KJ588063
KUIDL-PED-2014-001	2014	Korea	KJ588064
VN/JFP1013-1/2013	2013	This study	KJ960178
VN/VAP1113-1/2013	2013	This study	KJ960179
VN/KCHY-311013/2013	2013	This study	KJ960180

CHAPTERS IV RESULTS

4.1 Viral isolation

Three PEDV isolates were isolated from intestine samples of 3-day-old pigs displaying severe watery diarrhea and dehydration using Vero cells. One isolate designated VN/KCHY-310113 (accession number KJ960180) was taken from the collection samples in the Virology section at the National Center for Veterinary Diagnosis and two strains designated VAP1113-1 (accession number KJ960179) and JFP1013-1 (accession number KJ960178) were taken from two swine farms in Southern Vietnam in October 2013. The cytopathic effect characterized by cell fusion and syncytia formation was observed following the second blind passages in Vero cells (Figure 6).

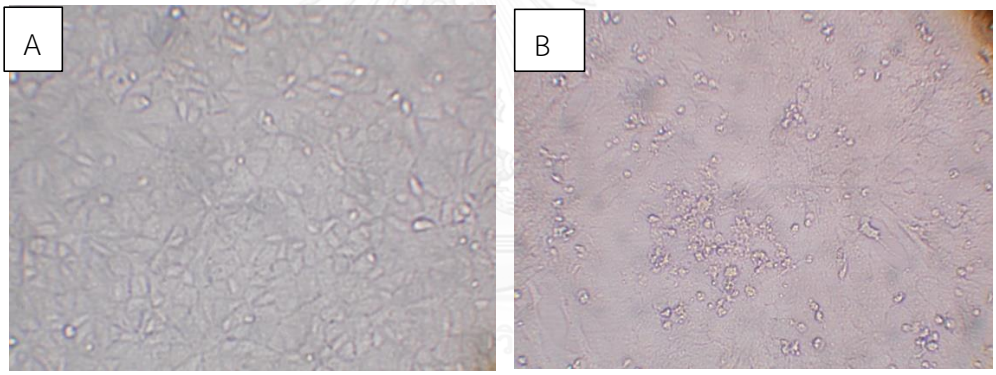

Figure 6. The cytopathic effect in Vero cells infected with Vietnamese PEDV strains. (A) Cell control. (B) Infected cells: showing fusion and syncytia formation.

4.2 Sequencing results and phylogenetic analyses

The relationship between Vietnamese and other PEDV isolates available in Genbank were analyzed based on full-length, ORF1, spike, ORF3, envelope (E), membrane (M) and nucleocapsid (N) gene sequences. The phylogenetic tree was constructed by using the neighbor-joining method (bootstrapping for 1000replicase) in Mega version 5.

4.2.1 Whole genome sequence

Genome of three Vietnamese PEDV isolates are 28,035 nucleotides (nt) in length and have the genome organization resembling other PEDV strains previously reported (Kocherhans et al., 2001; Song and Park, 2012), characterized by a gene order of 5'-ORF1a/1b-S-ORF3-E-M-N-3' (Figure 7).

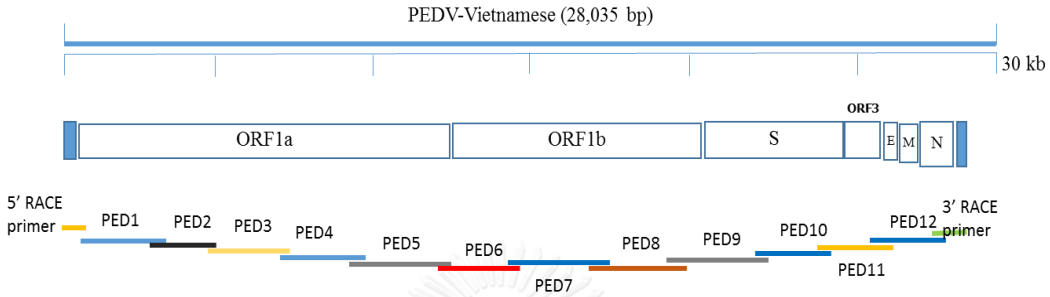

Figure 7. Schematic diagram of genomic structure, and twelve overlapping cDNA fragments covering the entire PEDV genome for each of the three Vietnamese strains.

To investigate the genetic relationship of these viruses, a phylogenetic tree was constructed based on the whole genome of the nucleotide sequences of the three Vietnam PEDV isolates along with 35 PEDV isolates sequences from other countries available in Genbank. The phylogenetic tree demonstrated that all PEDV isolates evolved into two distinct groups, designated groups G1 and G2 (Figure 8). Group G1 consisted of 9 isolates including the prototype CV777 along with three isolates from Korea (SM98, and virulent and attenuated DR13), and five isolates from China (SD-M, LZC, CH/S, attenuated vaccine, and JS2008). Group G2 consisted of isolates from Vietnam, Korea, China and USA and was further divided into two subgroups consisting of G2-1 and G2-2. Three Vietnamese PEDV were clustered in subgroup G2-1 along with 7 isolates from China (LC, AJ1102, ZJCZ4, GD-1, GD-A, CHGD-01 and CH/GDGZ/2012). Interestingly, these 7 isolates were reported to be a new variant responsible for 2011-2012 PEDV outbreaks in China. Subgroup G2-2 includes 7 isolates from China (2011-2012), 8 isolates from the United Stated (2013), and 4 isolates from Korea during 2013-2014 (KNU-1305, KUIDL-PED-2014-007, KUIDL-PED-2014-002, and KUIDL-PED-2014-001) that were reported to be US-like PEDV.

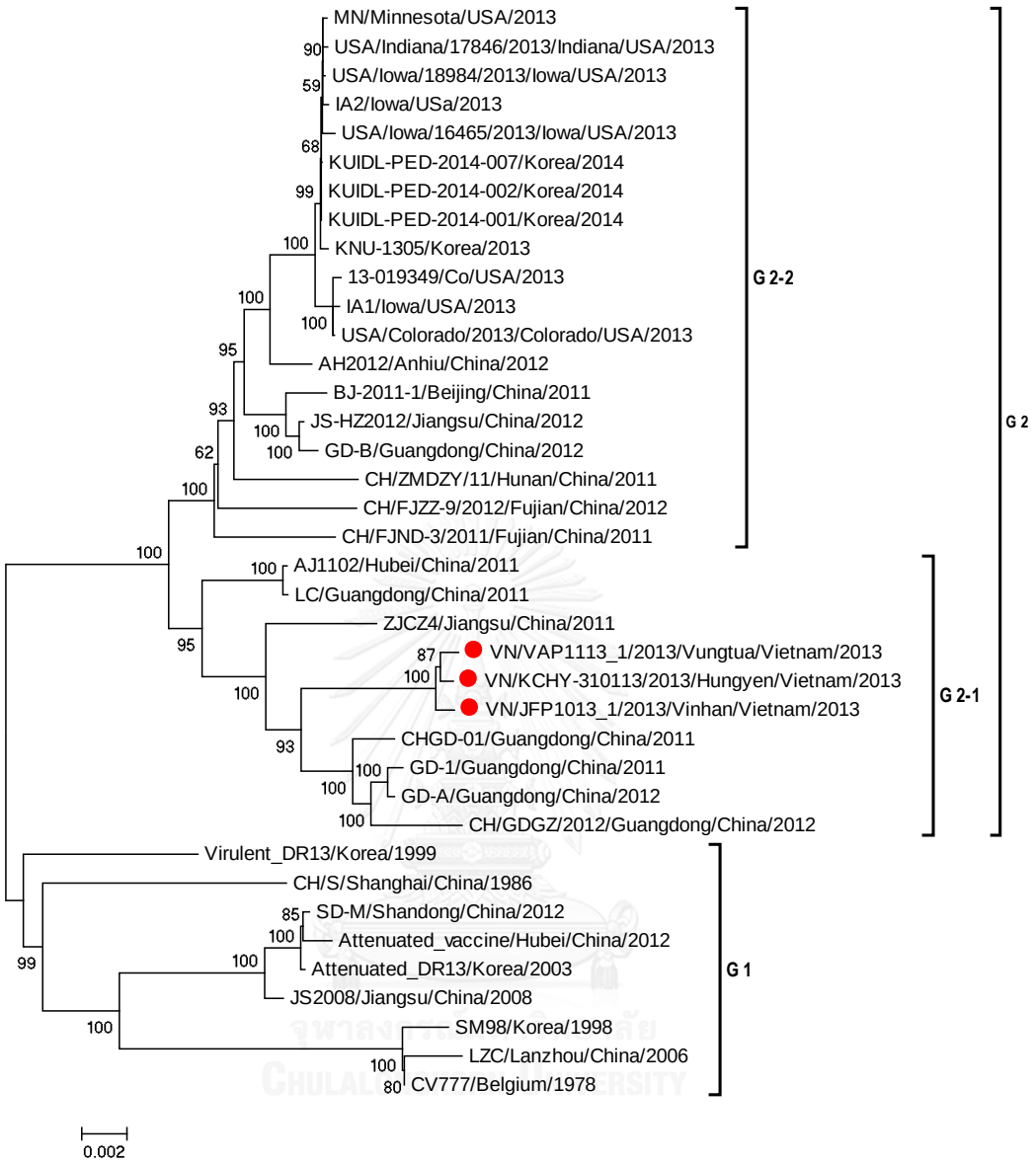

Figure 8. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of whole genome of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

To analyze the genetic diversity, the percentage of similarities between isolates based on the complete genome at nucleotide and amino acid level were calculated. Three PEDV isolates from Vietnam shared the highest genetic similarities with Chinese isolates in subgroup G2-1 (LC, ZJCZ4, CHGD-01, CH/GDGZ/2012, GD-1, GD-A, and AJ1102) with nucleotide and amino acid identities ranging 98.4%-98.9% and 95.9%-97.1%, respectively. The highest similarity is with GD-1 and GD-A isolates at 98.9% and 97.1% at nucleotide and amino acid levels, respectively. It is noteworthy that Chinese PEDV isolates in the sub-cluster 2-1 were reported for being responsible for outbreaks in 2011-2012. Three PEDV isolates from Vietnam share only 97.0%-98.1% and 93.9%-95.3% identities at nucleotide and amino acid level with subgroup G2-2. In addition, the genetic similarity between three isolates from Vietnam and isolates in group G1 were 96.0%-97.3% and 90.2%-93.3% similarity in nucleotide and amino acid levels, respectively (Table 3).

Table 3. The nucleotide and amino acid identities of the three Vietnamese PEDV strains compare with other isolates available in Genbank based on whole genome sequence.

Groups	Nucleotide identity (%)	Amino acid identity (%)
GD-1 and GD-A isolates	98.9	97.1
G1	96.0 – 97.3	90.2 – 93.3
G 2-1	98.4 – 98.9	95.9 – 97.1
G 2-2	97.0 – 98.1	93.9 – 95.3

4.2.2 ORF1 replicase sequence

The nucleotide and deduced amino acid sequences of the complete genome of the three Vietnamese PEDV isolates along with that of 35 PEDV isolates were aligned. The sequence alignments demonstrated that the three PEDV isolates from Vietnam have a genome organization similar to other PEDV isolates previously

reported (Kocherhans et al., 2001; Song and Park, 2012), which are characterized by the gene order of ORF1a/1b-S-ORF3-E-M-N. The ORF1a/1b is 20,344 nucleotides in length, accounting for the largest ORF. Genes ORF1a (nt 297- 12602) and ORF1b (nt 12601-20637) cover two-thirds of the genome. The ORF1 of the three Vietnamese PEDV isolates has 91.4%-97.1% amino acid homology compared to other isolates, exhibiting the highest identity (96.0%-97.1%) with 7 Chinese strains (CH/GDGZ2012, GD-1, GD-A, ZJCZ4, AJ1102, CHGD-01 and LC)* (Table 4),. There are no insertions or deletions in the sequence of this ORF1. Compared to other PEDV strains, the amino acid identities of ORF1a and ORF1b of all Vietnamese PEDV isolates are 91.2%-96.7% and 91.6%-97.9%, respectively (Table 4).

Table 4. The amino acid identity of the ORF1 gene the Vietnamese PEDV strains compare to other isolates available in Genbank

Genes	Amino acid identity (%)
ORF1	91.4 – 97.1
ORF1a	91.2 – 96.7
ORF1b	91.6 – 97.9
ORF1 (7 Chinese isolates)*	96.0 – 97.1

The phylogenetic tree based on the nucleotide of ORF1 gene sequence demonstrated that all three PEDV isolates along with other 35 PEDV isolates evolved into two groups, namely, G1 and G2 (Figure 9). Group G1 consisted of the prototype CV777, 4 isolates from China (SD-M, JS2008, LZC and attenuated vaccine) and 2 from Korea (SM98, and attenuated DR13). Three Vietnamese isolates were classified into group G2 along with 15 isolates from China, 8 from the US and 5 Korean isolates with the nucleotide and amino acid homology of 97.3%-98.9% and 94.1%-97.1%, respectively.

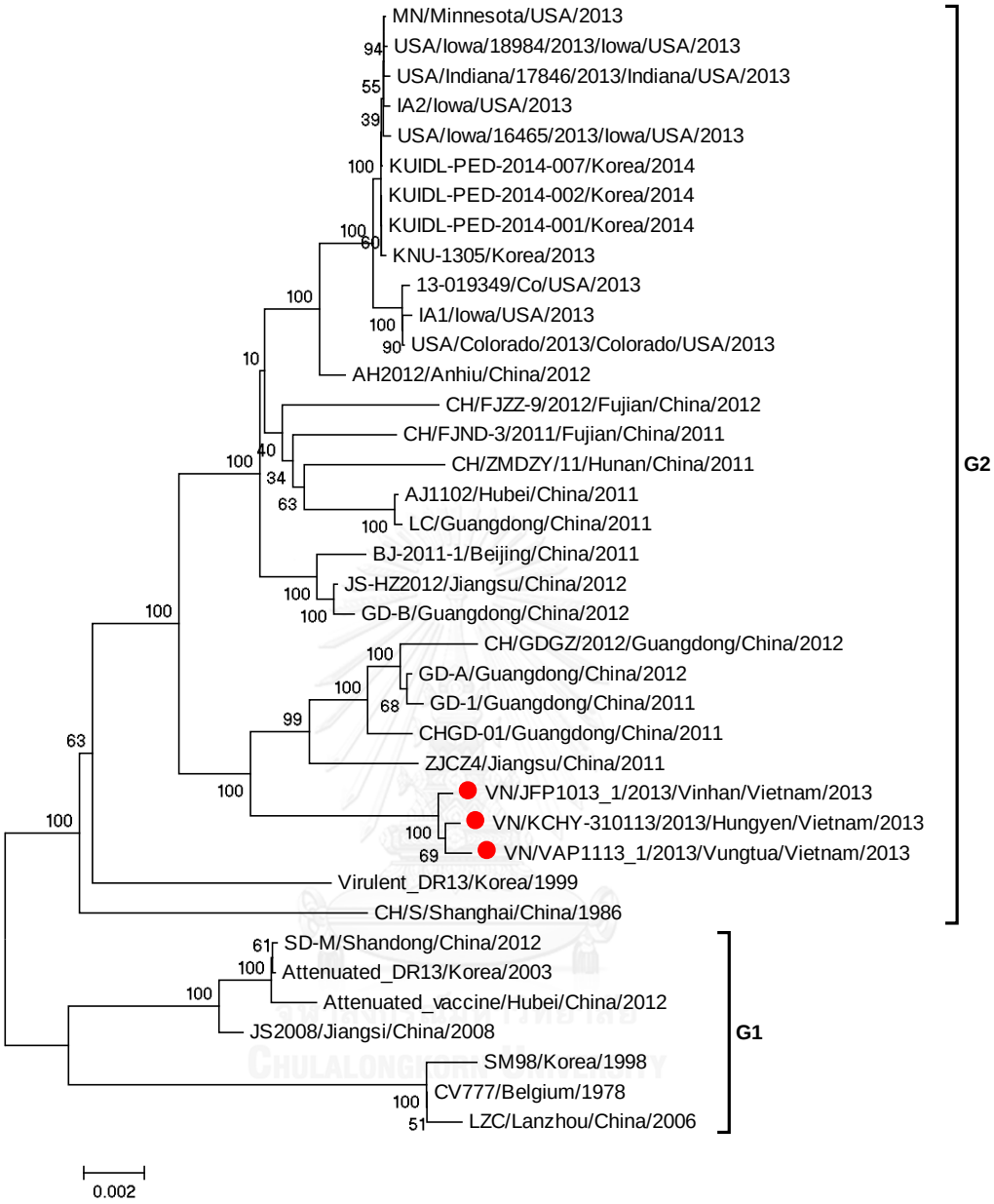

Figure 9. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of ORF1 replicase of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

4.2.3 The spike (S) gene sequence

The S gene follows ORF1 in sequence. The S gene encodes for S protein. The S gene of the three PEDV isolates from Vietnam is 4,158 nucleotides in length, encoding for 1,386 amino acids. The phylogenetic tree based on the complete S gene sequence demonstrated that all three PEDV isolates along with another 35 PEDV isolates evolved into two groups, namely, G1 and G2 (Figure 10), similar to the classification of the whole genome. Group G1 consisted of CV777, 5 isolates from China (CH/S, SD-M, JS2008, LZC and attenuated vaccine) and 3 from Korea (SM98, virulent and attenuated DR13). Group G2 was further divided into three subgroups designated G2-1, 2-2, and 2-3. As noted above, the PEDV isolates in Group G2 have unique characteristics, including 2 insertion position and 1 deletion positions in the spike genes, making them genetically distinct from isolates in Group G1. The three PEDV isolates from Vietnam clustered into subgroup G2-3 along with eight isolates from China that were isolated during the period from 2011 to 2012 with a nucleotide and amino acid homology of 97.1%-98.6% and 96.9%-98.2% (Table 5), respectively. When compared to subgroup G2-2, the nucleotide and amino acid identities of subgroup G2-3 are 96.7%-98.7% and 96.4%-99.0%, respectively. When compared to Group G1, the nucleotide and amino acid identities of subgroup G2-3 are 92.2%-94.6% and 90.9%-94.0%, respectively (Table 6). In addition, the three PEDV isolates from Vietnam clustered distinctly from the US-PEDV isolates.

Table 5. The nucleotide and amino acid homology of spike gene of the three Vietnamese PEDV isolates compared with Chinese strains in subgroup G2-3.

Isolates	Nucleotide identity (%)			Amino acid identity (%)		
	VN/JFP1013_1/2013	VN/VAP1113_1/2013	VN/KCHY-310113/2013	VN/JFP1013_1/2013	VN/VAP1113_1/2013	VN/KCHY-310113/2013
CH/GDGZ/2012/China/2012	98.2	98.4	98.4	97.5	97.9	97.9
CHGD-01/China/2011	98.1	98.3	98.3	97.6	98.0	97.9
GD-A/China/2012	98.4	98.6	98.6	97.8	98.2	98.1
GD-1/China/2011	98.4	98.6	98.6	97.8	98.2	98.1
LC/China/2011	97.9	98.1	98.1	97.8	98.2	98.1
AJ1102/China/2011	97.9	98.1	98.1	97.8	98.2	98.1
CH/FJZZ-9/2012/China/2012	97.1	97.3	97.3	96.9	97.4	97.3
ZJCZ4/Jiangsu/China/2011	97.7	97.9	97.8	97.4	97.8	97.7

Table 6. The homology of the nucleotide and deduce amino acid sequence of spike gene sequence between groups.

Groups		G1	G2		
			G2-1	G2-2	G2-3
G1		ID	91.7-94.1	91.6-94.2	90.9-94.0
G2	G2-1	93.1-94.9	ID	96.7-97.4	95.8-96.9
	G2-2	92.7-94.7	97.4-97.7	ID	96.4-99.0
	G2-3	92.2-94.6	96.5-97.5	96.7-98.7	ID

Nucleotide identity (%) in lower triangle

Deduced amino acid identity (%) in upper triangle

The deduced amino acid sequence demonstrated that all three PEDV isolates from Vietnam have unique characteristics in the S gene, including deletion and insertion mutations in the spike gene. The S protein of three Vietnamese PEDV isolates possesses 2 notation insertions of 4 (^{56}GENQ^{59}) and 1 (^{140}N) amino acids at positions 56-59 and 140, respectively, and 1 deletion of 2 amino acids (^{160}DG^{161}) at positions 160 and 161 (Figure 11). The insertion and deletion markers are located in the hypervariable domain in the N-terminus of S1 region, indicating that the Vietnamese isolates are genetically similar to isolates considered new variants that were responsible for the 2011 to 2012 outbreaks in China.

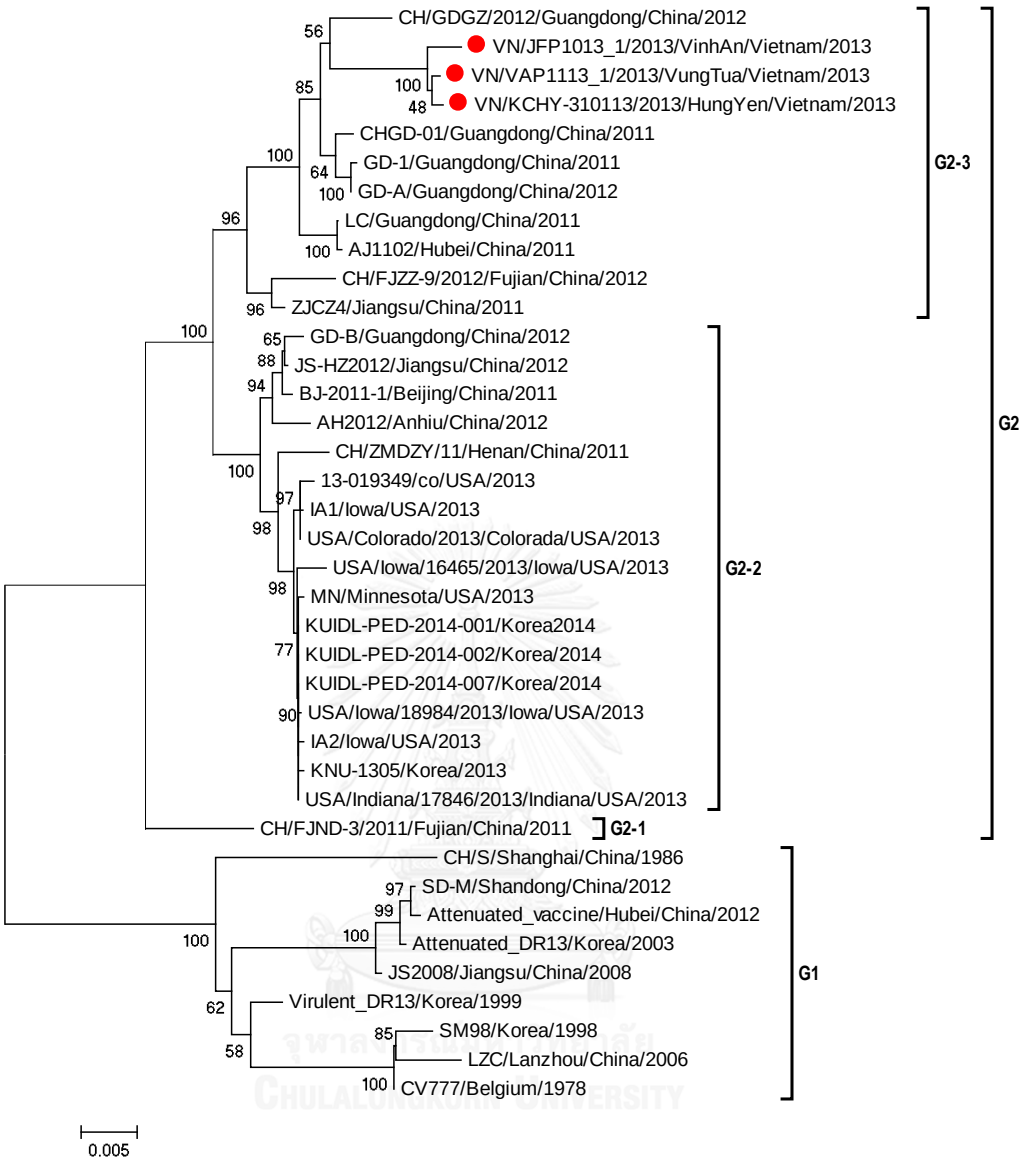

Figure 10. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of spike gene of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

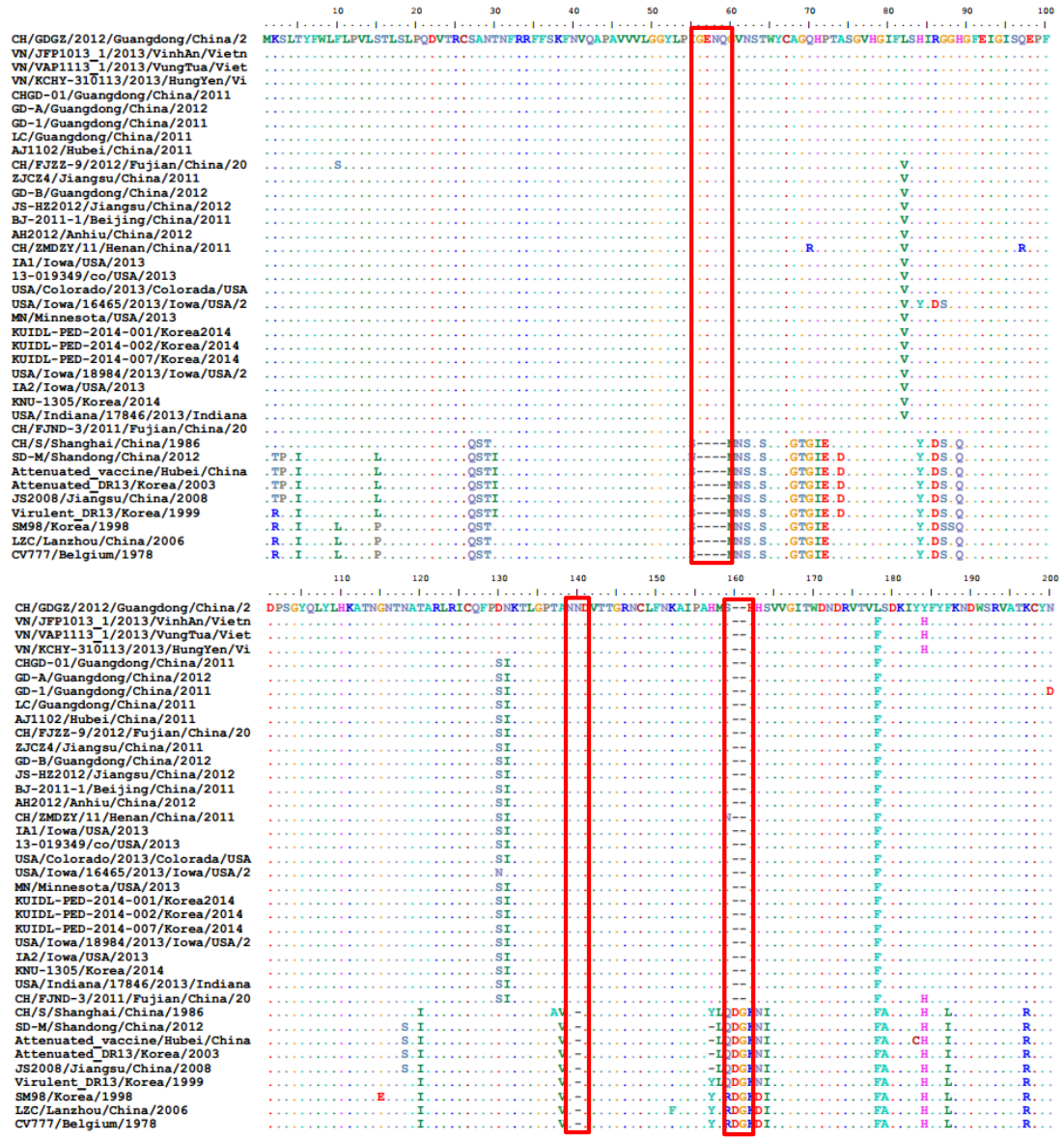

Figure 11. Multiple amino acid sequences alignments of spike protein of Vietnamese PEDV isolates with other isolates available in Genbank.

4.2.4 The ORF3 gene sequence

The ORF3 of Vietnamese PEDV isolates is 675 nucleotides in length and encodes for 225 amino acids. The phylogenetic analysis based on the ORF3 gene revealed that all PEDV isolates were divided into four groups, designated G1, G2, G3 and G4 (Figure 12). Group G1 consisted of CV777, SM98 and LZC. Group G2 contained 3 Chinese isolates (JS2008, SD-M and attenuated vaccine) and attenuated DR13, which is a vaccine isolate from Korea. Isolates in Group G2 have the unique characteristic of a 17-amino-acid deletion at positions 82-98 (Figure 13). Group G3 included isolates from the United States and China as well as 5 Korean field isolates (Virulent-DR13, KNU-1305, KUIDL-PED-2014-007, KUIDL-PED-2014-002, KUIDL-PED-2014-001). All three PEDV isolates from Vietnam were in Group G4 along with seven Chinese isolates (CH/GDGZ/2012, GD-A, GD-1, LC, AJ1102, CHGD-01 and ZJCZ4). Isolates in Group G4 possess four unique amino acid substitutions ($^{L}25^{S}$, $^{I}70^{V}$, $^{C}107^{F}$ and $^{D}168^{N}$) compared to isolates in the other groups. The VN/KCHY-310113/2013 isolate has two more amino acid changes ($^{F}57^{L}$ and $^{C}100^{R}$) and an amino acid substitution ($^{I}203^{V}$) in JFP10113-1 and VAP1113-1 compared to the other isolates (Figure 13). Sequence analysis showed that the three Vietnamese PEDV isolates share 98.5%-99.4% and 98.2%-99.5% nucleotide and amino acid identity with other strains in Group G4. The homology of nucleotides and amino acids is 95.5%-97.0% and 95.9%-97.3%, 89.3%-89.7% and 89.2%-90.1%, and 91.5%-96.2% and 89.7%-95.0% compared to groups G3, 2 and 1, respectively (Table 7).

Table 7. The nucleotide and amino acid identities of ORF3 gene of the three Vietnamese PEDV strains compare with other isolates available in Genbank.

Groups	Nucleotide identity (%)	Amino acid identity (%)
G 4	98.5 – 99.4	98.2 – 99.5
G 3	95.5 – 97.0	95.9 – 97.3
G 2	89.3 – 89.7	89.2 – 90.1
G 1	91.5 – 96.2	89.7 – 95.0

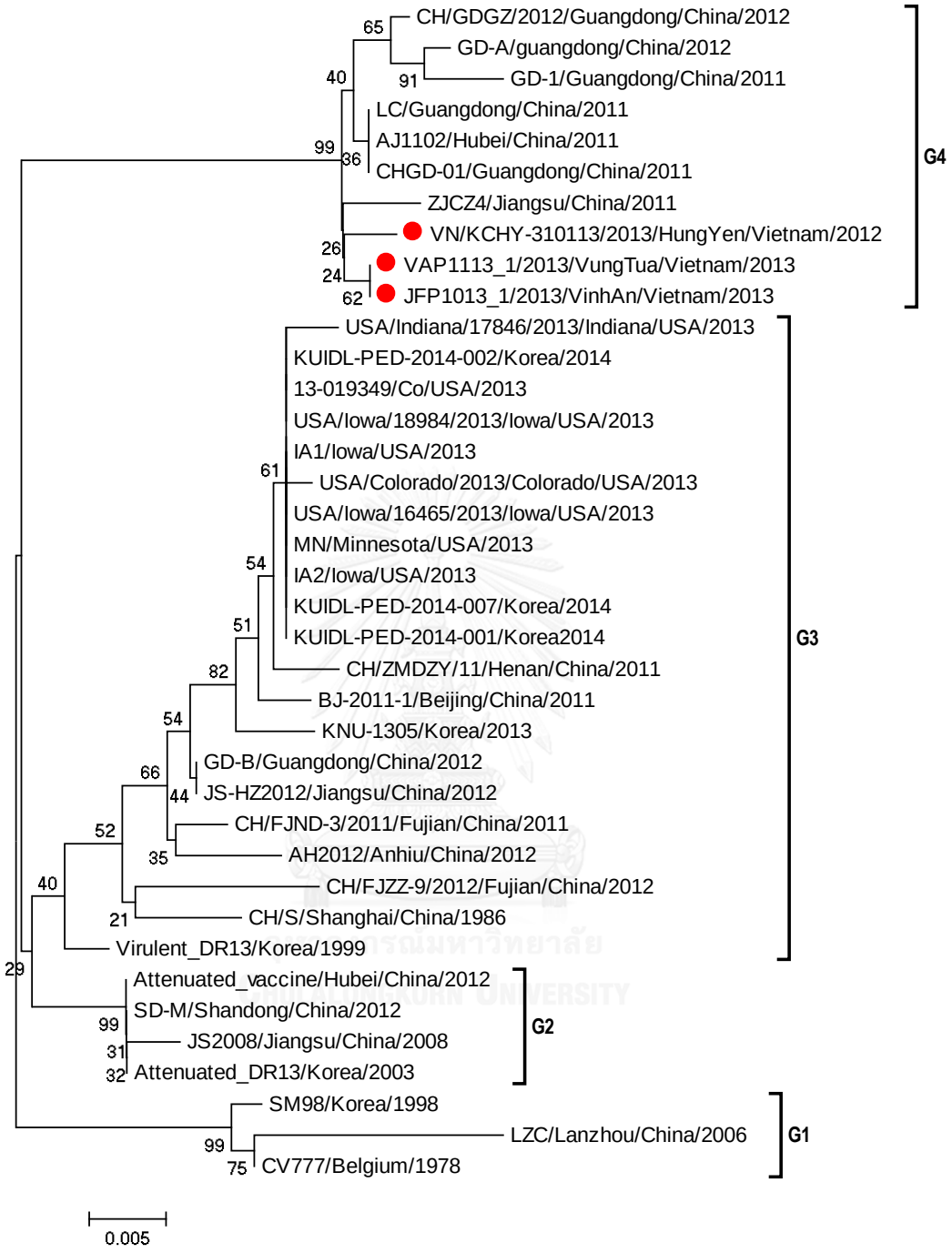

Figure 12. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of ORF3 gene of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

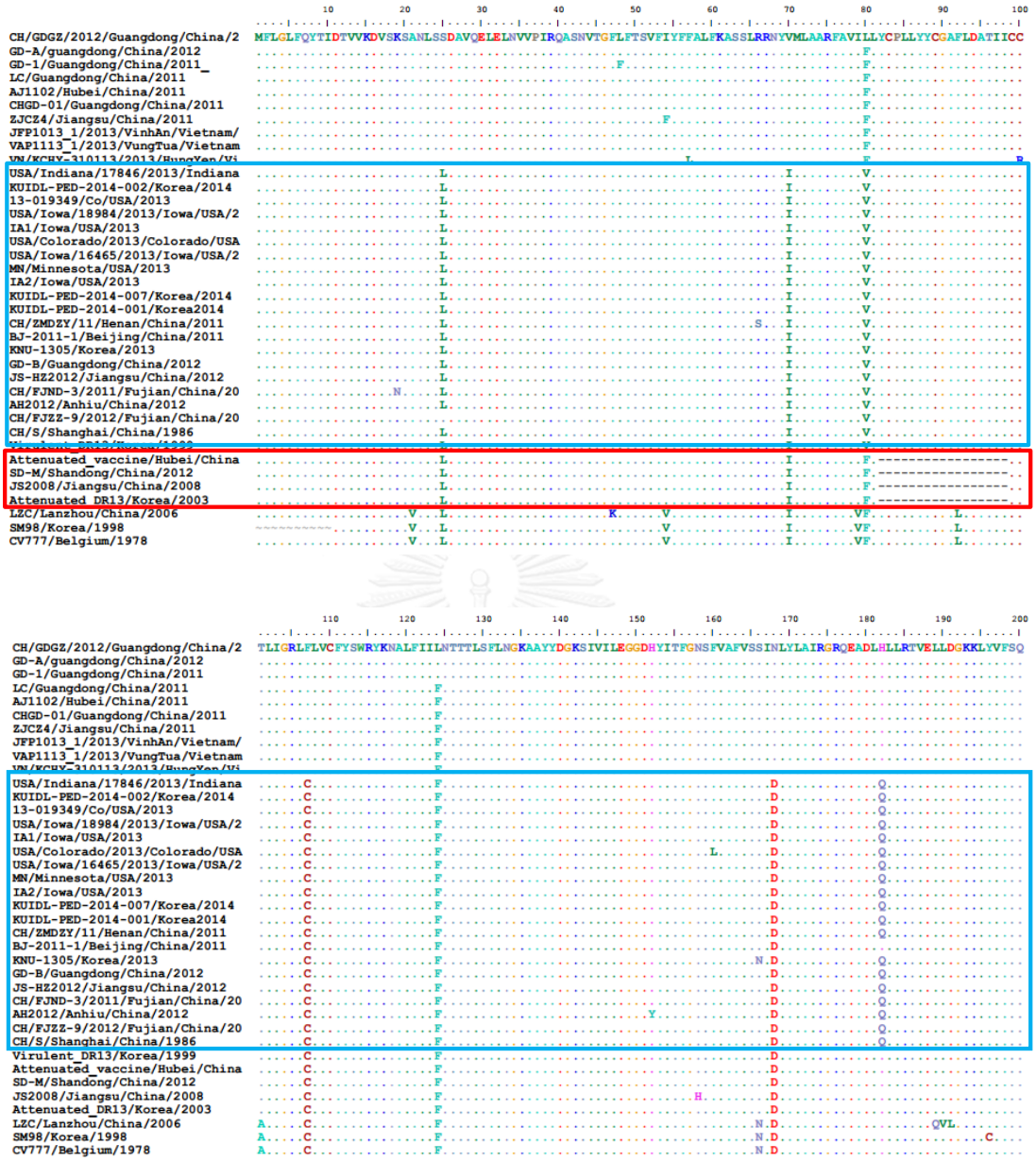

Figure 13. Multiple amino acid sequences alignments of ORF3 protein of Vietnamese PEDV isolates with other isolates available in Genbank.

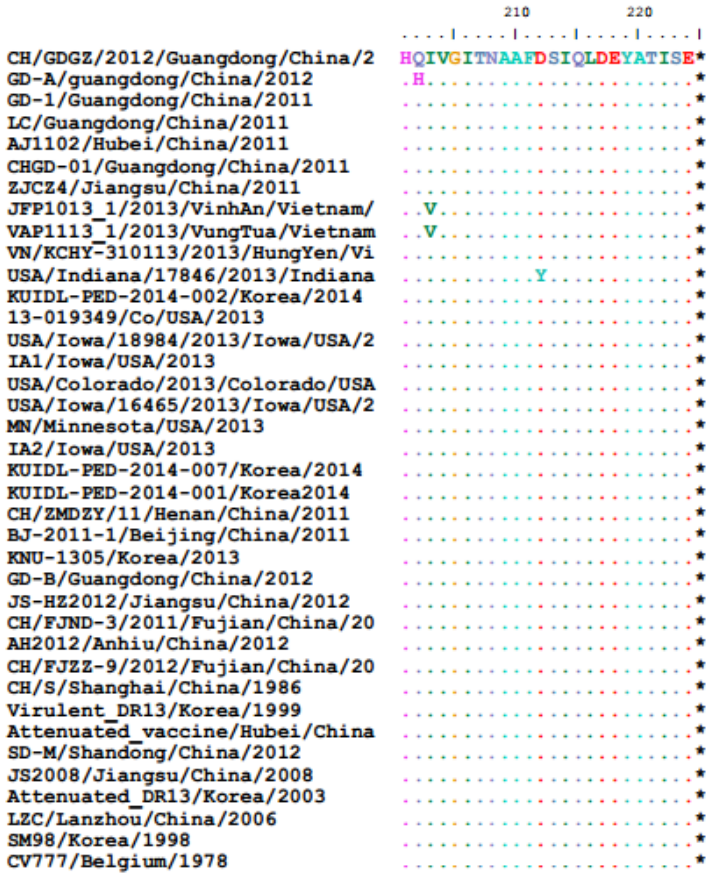

Figure 13. Multiple amino acid sequences alignments of ORF3 protein of Vietnamese PEDV isolates with other isolates available in Genbank (continued).

4.2.5 The E gene sequence

The E gene of PEDV isolates from Vietnam has 231 nucleotides, encoding for 77 amino acids. A phylogenetic analysis based on E protein demonstrated that all PEDV isolates were classified into two distinct groups, designated G1 and G2. PEDV isolates from Vietnam were in group 2 along with the recent PEDV from China and the United States (Figure 14). All 3 PEDV isolates from Vietnam showed an amino acid change ($^{T}51^{I}$) compared to other isolates (Figure 15). The nucleotide and amino acid homologies of Vietnamese PEDV strains are 97.8%-99.5% and 97.3%-98.6% compared to other isolates in Group G2. When the Vietnamese strains are compared to Group G1, the nucleotide and amino acid identities are 88.3%-97.8% and 85.5%-97.3%, respectively (Table 8).

Table 8. The nucleotide and amino acid homology of envelope gene of the three Vietnamese PEDV strains compare with other isolates.

Groups	Nucleotide homology (%)	Amino acid homology (%)
G 2	97.8 – 99.5	97.3 – 98.6
G 1	88.3 – 97.8	85.5 – 97.3

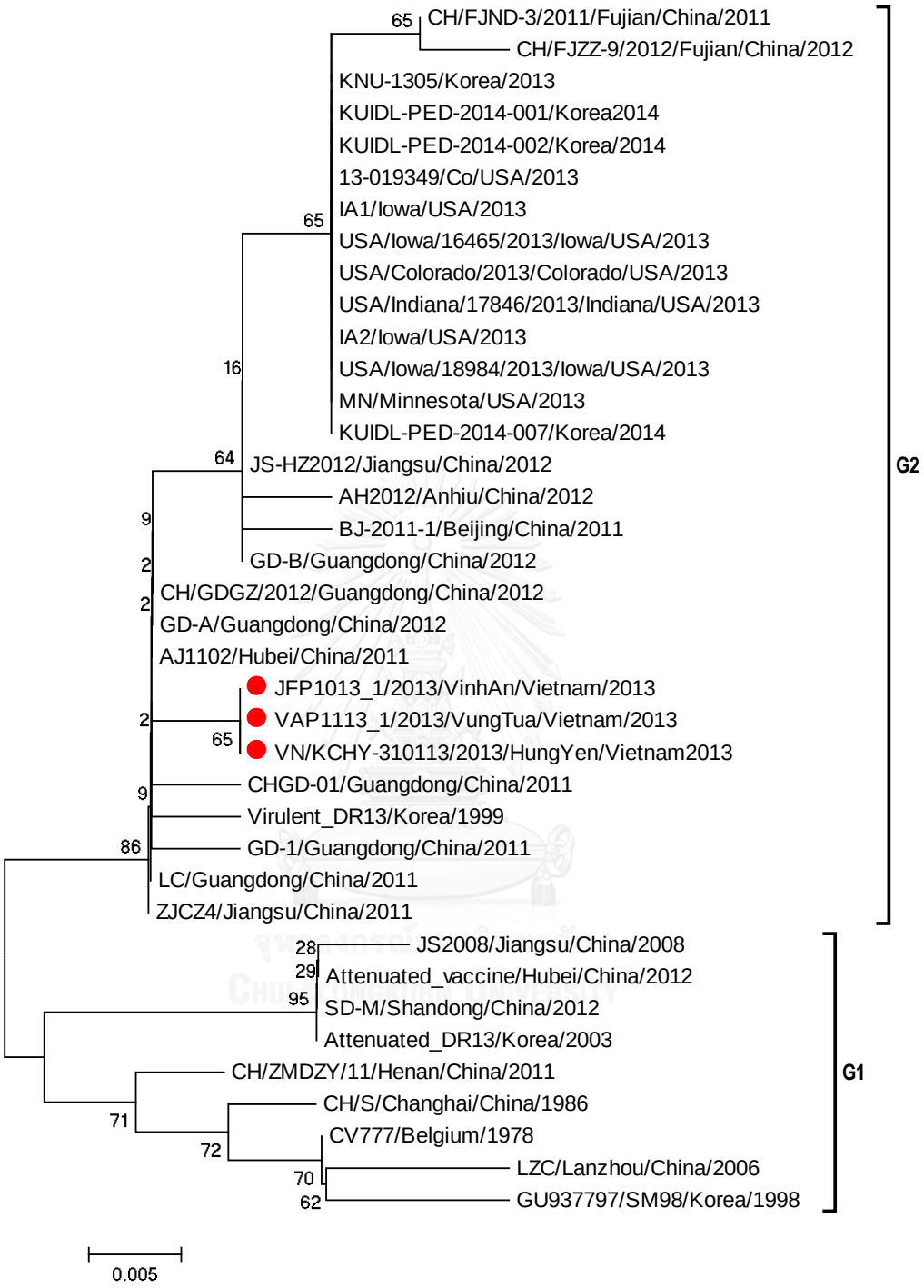

Figure 14. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of envelope gene of three Vietnamese PEDV with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name

Figure 15. Multiple amino acid sequences alignments of envelope protein of Vietnamese PEDV isolates with other isolates available in Genbank.

4.2.6 The M gene sequence

The M gene of all Vietnamese PEDV isolates is 681 nucleotides in length and codes for 226 amino acids. No insertion or deletion mutations were found in the M gene of these isolates. Phylogenetic analysis indicated that all PEDV isolates were separated into two groups, designated G1 and G2 (Figure 16). Group 1 consisted of CV777, 5 isolates from China (SD-M, LZC, CH/S, attenuated vaccine and JS2008) and 2 isolates from Korea (SM98 and attenuated DR13). Three Vietnamese PEDV isolates were in subgroup 2-1 along with 7 Chinese isolates (CH/GDGZ/2012, CHGD-01, GD-A, GD-1, LC, ZJCZ4 and AJ1102) with 99.1%-100% amino acid identity. These isolates also have an amino acid change ($^{G}192^{S}$) when compared to other groups. Group 2-2 contains all the US isolates, 6 isolates from China (CH/FJZZ-9/2012, GD-B, JS-HZ2012, AH2012, BJ-2011-1 and CH/FJND-3/2011) and 4 isolates from Korea (KNU-1305, KUIDL-PED-2014-007, KUIDL-PED-2014-002, KUIDL-PED-2014-001). This group was characterized by 2 amino acid changes ($^{E}13^{Q}$ and $^{A}214^{S}$). All PEDV isolates in Group 2 shared an amino acid mutation from V to A at amino acid position 42. The virulent isolate DR13 from Korea clustered separately in subgroup 2-3, as it possessed an additional amino acid change ($^{F}43^{L}$) (Figure17). Sequence analysis revealed that the Vietnamese PEDV strains shared 99.5%-99.8% and 99.1%-100% identity in nucleotide and amino acid levels, respectively, with Chinese strains in subgroup G2-1. When the Vietnamese PEDV strains were compared with those in Group G2-2, the homologies of nucleotides and amino acids were 98.3%-98.8% and 98.2%-98.6%, respectively. When these strains were compared with those in Group G1, the homologies of nucleotides and amino acids were 96.6%-98.6% and 96.5%-99.1%, respectively (Table 9).

Table 9. The nucleotide and amino acid homology of membrane gene of the three Vietnamese PEDV strains compare with other isolates.

Groups	Nucleotide homology (%)	Amino acid homology (%)
G 2-2	98.3 – 98.8	98.2 – 98.6
G 2-1	99.5 – 99.8	99.1 - 100
G 1	96.6 – 98.6	96.5 – 99.1

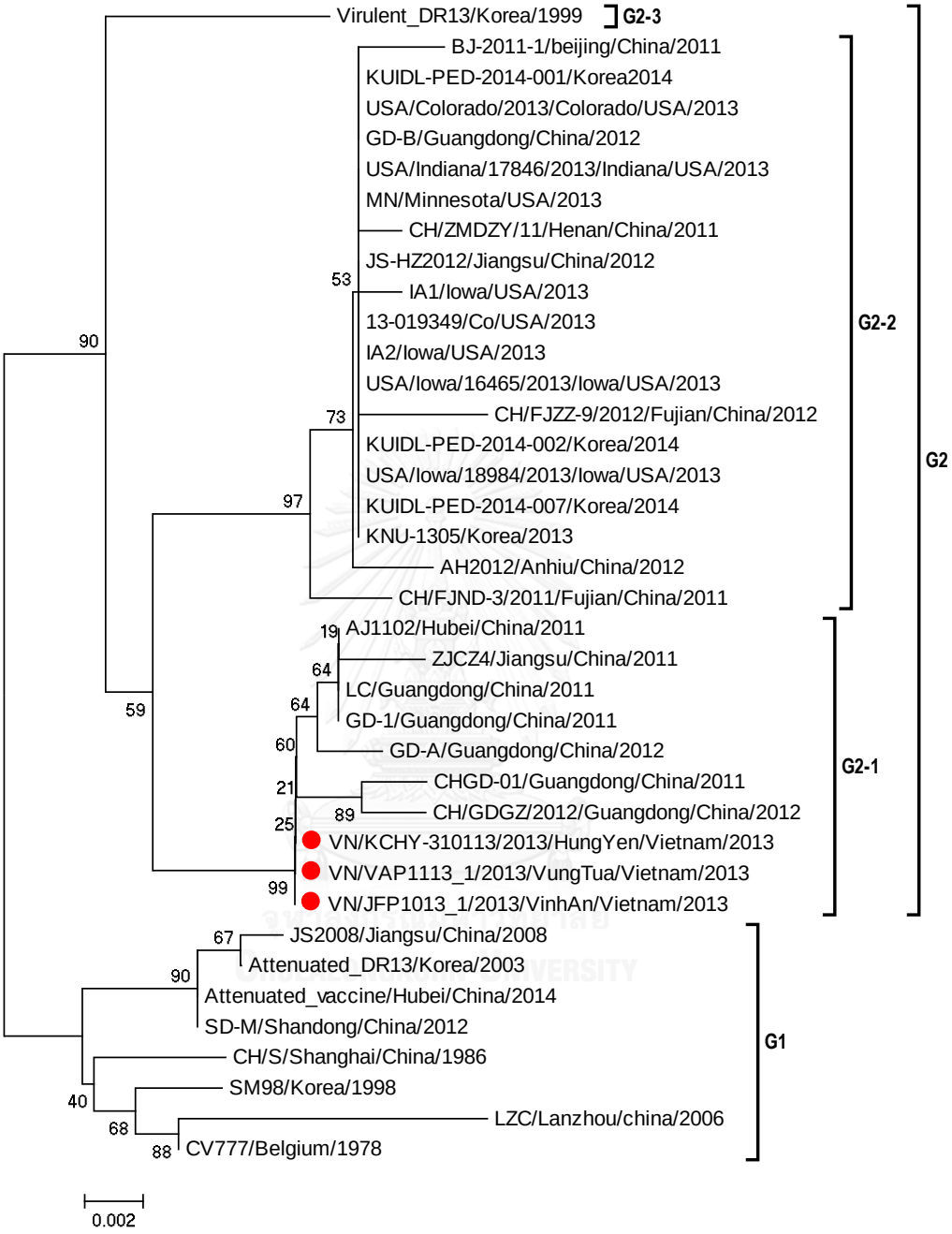

Figure 16. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of membrane gene of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

Figure 17. Multiple amino acid sequences alignments of membrane protein of Vietnamese PEDV isolates with other isolates available in Genbank.

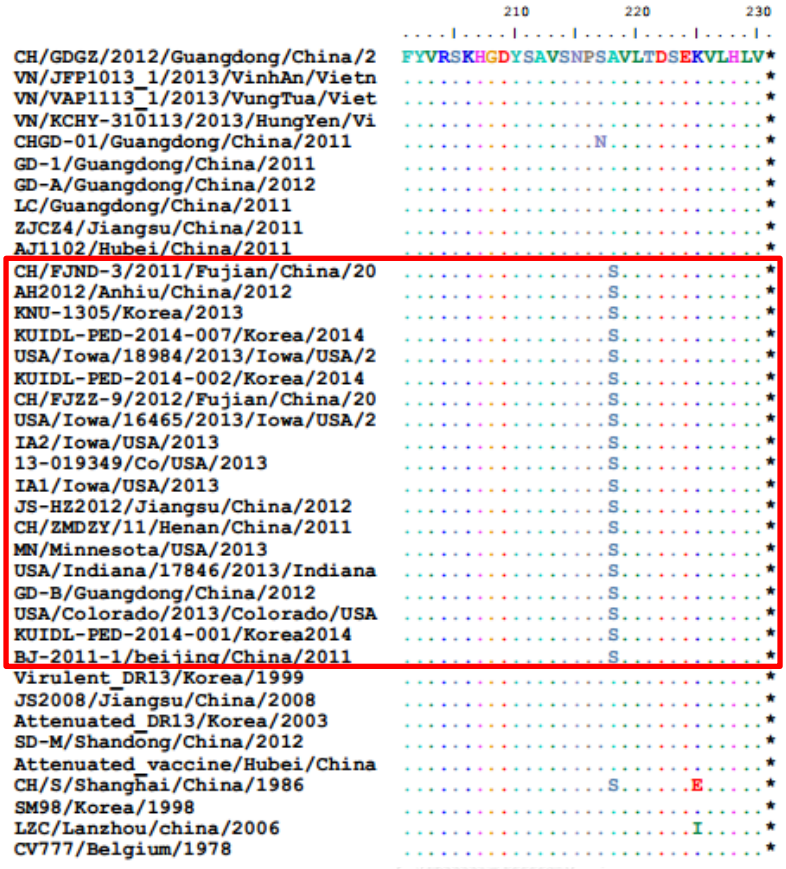

Figure 17. Multiple amino acid sequences alignments of membrane protein of Vietnamese PEDV isolates with other isolates available in Genbank (continued)

4.2.7 The N gene sequence

Sequence analyses of the N gene revealed that all Vietnamese PEDV isolates are 1,326 nucleotides in length and encode 441 amino acids. These isolates have 95.6%-98.7% nucleotide and 95.0%-97.9% amino acid identity with other isolates, and the highest homology is with CHGD-01 from China at the nucleotide (98.7%) and amino acid (97.7%-97.9%) levels. The phylogenetic tree based on the nucleotide sequences of the N gene indicated that all PEDV isolates were classified into two groups, designated G1 and G2 (Figure 18). The three Vietnamese PEDV isolates were in subgroup G2 along with 6 Chinese isolates (CH/GDGZ2012, CHGD-01, GD-1, LC, AJ1102, and GD-A) with nucleotide and amino acid identities ranging from 97.4%-98.7% and 95.0%-97.9% (Table 10). When compared to Group G1, these strains shared 95.6%-97.5% and 95.9%-97.9% nucleotide and amino acid homology, respectively (Table 10). There was no insertion or deletion in this region. Compared to other isolates, all Vietnamese PEDV isolates possessed 5 amino acid substitutions ($^{N}146^{S}$, $^{P}248^{L}$, $^{K}312^{R}$, $^{G}313^{V}$, and $^{T}356^{I}$). And one amino acid change from G to P at amino acid position 84 compared to the CV777 strain (except VN/KCHY-310113) (Figure 19).

Table 10. The nucleotide and amino acid homology of nucleocapsid gene of the three Vietnamese PEDV strains compare with other isolates.

Groups	Nucleotide homology (%)	Amino acid homology (%)
G 2	97.4 - 98.7%	95.0 - 97.9
G 1	95.6 - 97.5	95.9 - 97.9

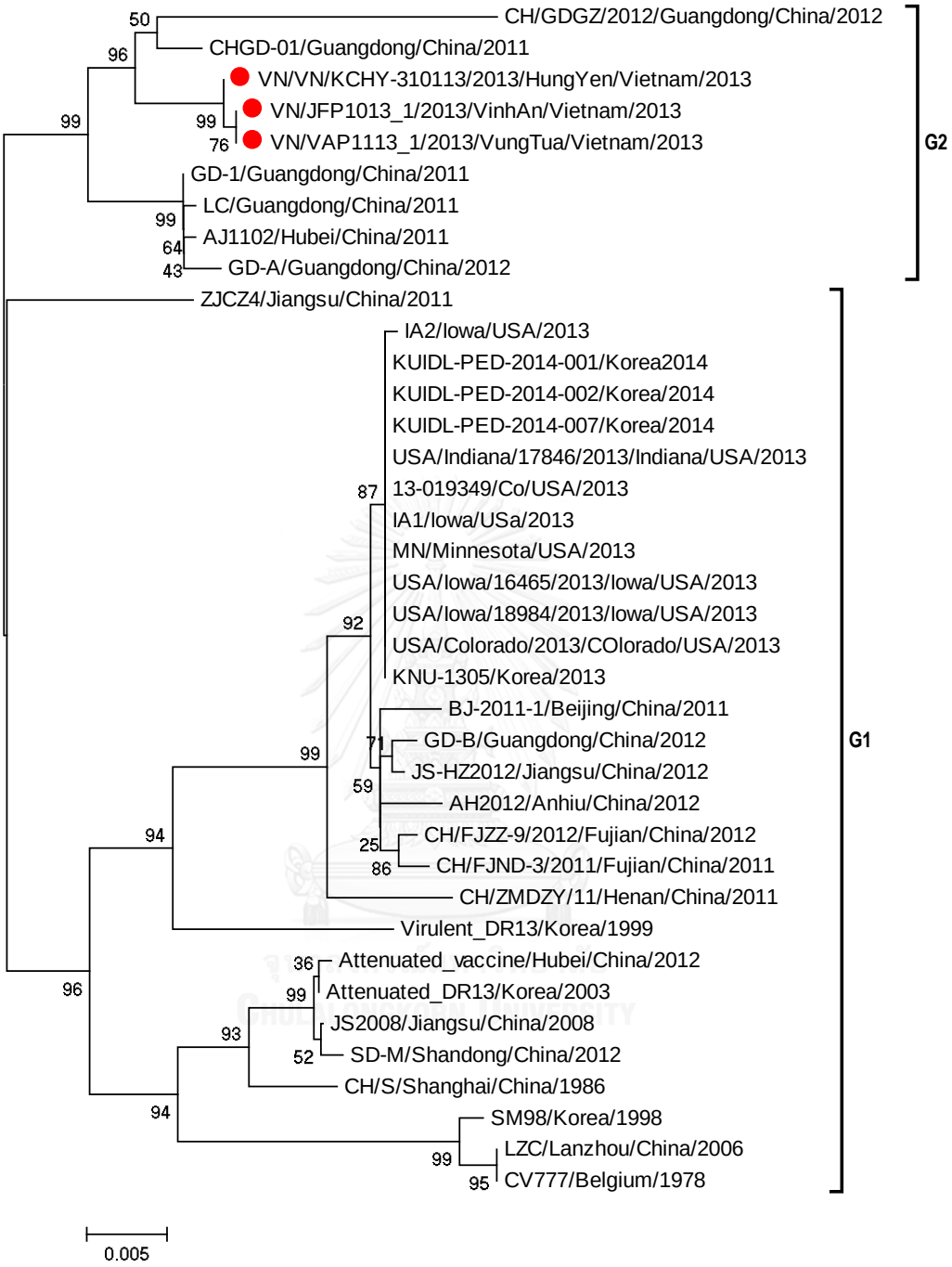

Figure 18. Phylogenetic analysis by neighbor- joining method (bootstrapping for 1000 replicase) based on the nucleotide sequences of nucleocapsid gene of three Vietnamese PEDV strains with other isolates available in Genbank. The Vietnamese PEDV isolates were in the red circle symbols in front of their name.

Figure 19. Multiple amino acid sequences alignments of nucleocapsid protein of Vietnamese PEDV isolates with other isolates available in Genbank.

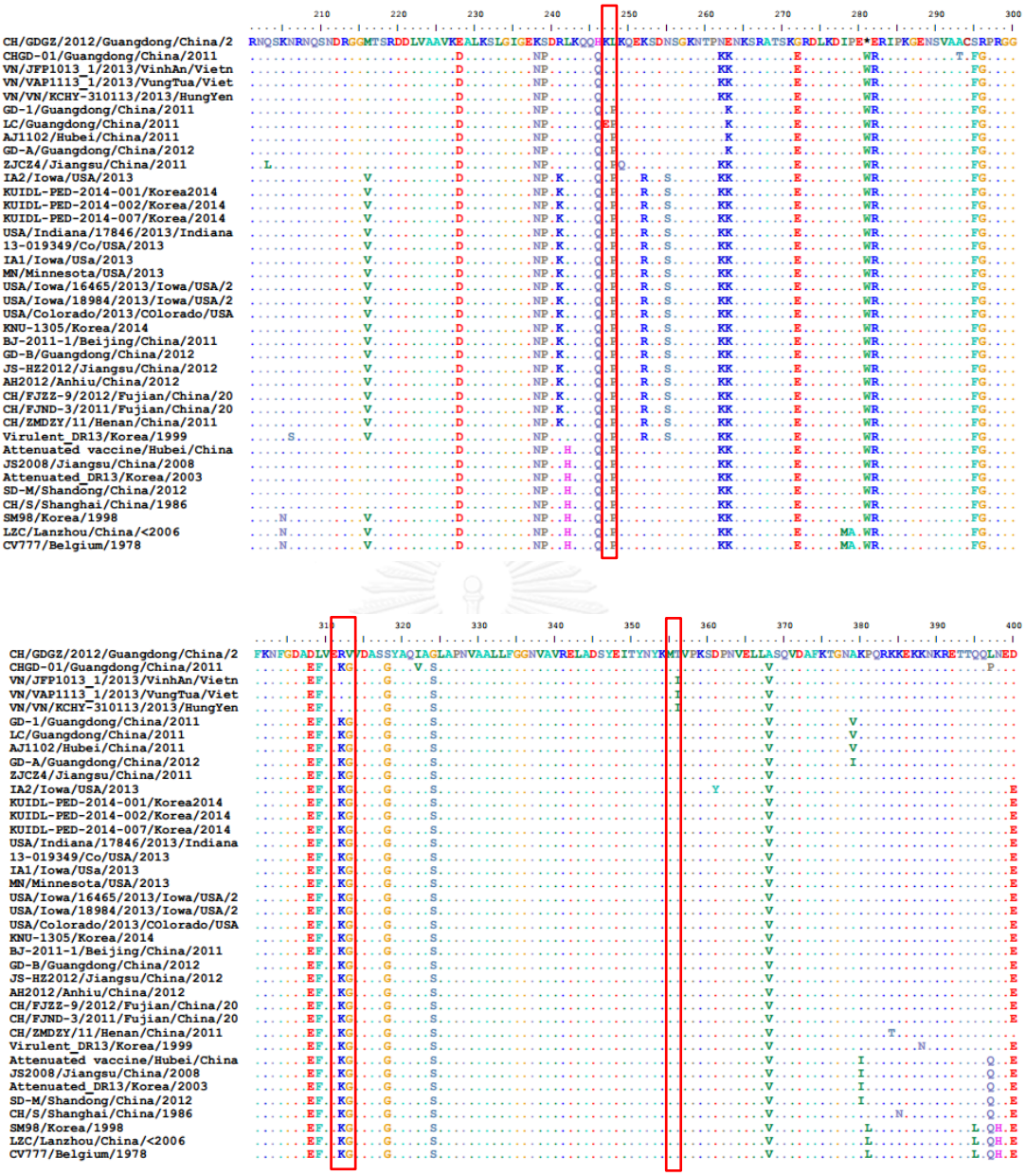

Figure 19. Multiple amino acid sequences alignments of nucleocapsid protein of Vietnamese PEDV isolates with other isolates available in Genbank (Continued).

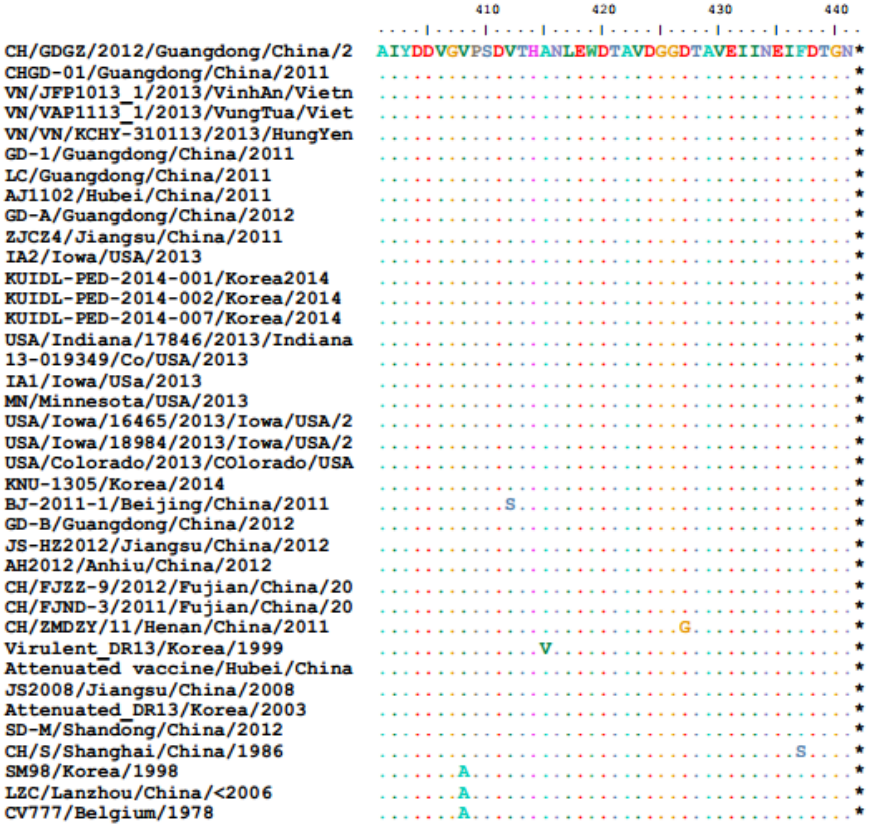

Figure 19. Multiple amino acid sequences alignments of nucleocapsid protein of Vietnamese PEDV isolates with other isolates available in Genbank (Continued).

CHAPTER V DISCUSSION AND CONCLUSION

PED first emerged in Vietnam in 2009. Since its emergence, the disease has developed into an endemic stage continuing to cause economic damage to the Vietnamese swine industry. A previous report suggested that PEDV isolates in Vietnam were closely related to Chinese isolates (Duy et al., 2011). The genetic analyses of the study, however, were based on partial S and M gene sequences only. An analysis based on partial S gene sequences alone might skew the epidemiological investigation and evolution studies of the virus. A previous study from China reported that the emergence of a new variant of PEDV was responsible for the recent outbreak in China from 2010 to 2011 (Li et al., 2012), suggesting that there have been 2 variants of PEDV in China. The new variant is genetically distinct from the old variant in the spike gene regions, with two insertions of 4 (^{56}GENQ^{59}) and 1 (^{140}N) amino acids at positions 56-59 and 140, respectively, and a deletion of 2 amino acids (^{160}DG^{161}) at positions 160 and 161. The analysis of partial S gene sequences using the primers reported would not have covered the insertion and deletion regions that would allow for differentiation between classical and new variants. Thus, the question remains as to what type of PEDV currently exists in Vietnam. The results reported in this study reveal the complete genome sequences of three PEDV strains isolated from pigs exhibiting severe diarrhea in Vietnam swine herds in the northern and southern regions of Vietnam. The results reported herein can provide a reference genome regarding sequence data of PEDV in Vietnam, and the sequence information can provide guidance for PEDV control in Vietnam.

For the characterization of PEDV isolates, a phylogenetic tree was constructed based on the complete genome of PEDV isolated from Vietnam and 35 other isolates whose sequencing data were available in GenBank. Deduced amino acids of the complete genome were also investigated. Based on the phylogenetic analysis of the complete genome sequence, three PEDV isolates from Vietnam were grouped together in group G2 along with PEDV isolates from China (isolated after 2010), Korea and the United States. This cluster is genetically distinct from cluster 1,

into which CV777, attenuated vaccine isolates and isolates from China (isolated before 2010) and Korea were grouped. The differences between these two clusters are based primarily at the spike gene region (Lee et al., 2010), in which isolates in cluster G2 possess a unique genetic characteristic that involves the insertion of 4 amino acids (GENQ) and 1 amino acid (N) at positions 56-59 and 140, respectively, and a deletion of 2 amino acids (DG) at positions 160 and 161 in the spike gene. Cluster 2 was further divided into 2 sub-clusters designated 2-1 and 2-2. Three PEDV isolates from Vietnam were grouped into sub-cluster 2-1. This sub-cluster includes, in addition to Vietnam isolates, isolates from China that were reported to be responsible for outbreaks between 2011 and 2012. These isolates were separated from Chinese (AH2012), US and US-like Korean isolates, which were included in sub-cluster 2-2. These findings suggest that the PEDV strains isolated in Vietnam are new variants. In addition, the results of genetic analyses suggest a close relationship between Vietnamese and variants of Chinese PEDV isolates that were responsible for the outbreak from 2011 to 2012.

In addition, sequences analyses indicated three Vietnamese PEDV isolates possess some mutation in ORF3, E, M, and N genes. The ORF3 gene is located between the S and E genes, but its function is unknown. Previous studies found that a continuous 49-51 nucleotide region was deleted in the ORF3 gene when PEDV was passaged in cell culture, and this may influence the pathogenicity of PEDV. As such, we can use it for differentiation of wild- and attenuated-type PEDVs (Park et al., 2008; Chen et al., 2010). Sequence analysis described there are ten amino acid changes in the ORF3 gene of Vietnamese isolates. Six of them were found in other PEDV from China and were isolated in 2011-2012 ($^{V}21^{A}$, $^{V}54^{I}$, $^{V}79^{I}$, $^{L}92^{F}$, $^{A}101^{T}$, and $^{N}166^{S}$) (Pan et al., 2012), Korean (Park et al., 2011) and US strains in groups G2, 3 and 4 compare to CV777, SM98, and LZC isolates. Four amino acids substitution only occurred in Vietnamese and Chinese strains in group 4 ($^{L}25^{S}$, $^{I}70^{V}$, $^{C}107^{F}$, and $^{D}168^{N}$). Significantly, there is an amino acid change ($^{I}203^{V}$) in JFP10113-1 and VAP1113-1, and two in VN/KCHY-310113/2013 strain ($^{F}57^{L}$, and $^{C}100^{R}$). These mutations may play an important role in the classification of the groups. All PEDV were classified into four groups based on phylogenetic analysis of the ORF3 gene. Vietnamese PEDV strains

were divided in group G4 including seven Chinese isolates (ZJCZ4, GD-1, GD-A, CH/GDGZ/2012, LC, AJ1102, and CHGD-01) with the high amino acid identity up to 98.2%-99.5%. Whereas three Chinese strains (JS2008, SM-D and vaccine attenuated) and attenuated DR13 vaccine strain form Korea, characterized by a 17 amino acid deletion, were separated in group 2.

The N protein also plays the role in transcription of the viral genome, packaging of viral RNA, and the formation of the viral core (Li et al., 2007; Lee et al., 2008), and induction cell-mediated immunity in the host (Saif, 1993). Moreover, since N protein is highly conserved, it is the best candidate protein to be used as an antigen for early diagnosis reagent and vaccine development (Duarte et al., 1993; Tobler et al., 1993). The N gene of Vietnamese PEDV strains has 1326 nucleotide in length, coding for a protein 441 amino acid. None of the Vietnamese PEDV strains were found to have notable insertions or deletions in the N gene. Further, Vietnamese PEDV possess five amino acid substitutions ($^{N}146^{S}$, $^{P}248^{L}$, $^{K}312^{R}$, $^{G}313^{V}$, and $^{T}356^{I}$) compared to other PEDV strains in this study, and has more one amino acid change from G to P located at amino acid position 84 (except VN/KCHY-310113 isolate) compared to CV777 strain in N gene sequence. In addition, three Vietnamese PEDV isolates was divided in the group 2 (G2) with six isolates in China (CH/GDGZ2012, CHGD-01, GD-1, LC, AJ1102, and GD-A) with nucleotide and amino acid identities ranging from 97.4%-98.7% and 95.0%-97.9%.

The membrane gen (M gene) coded for membrane protein (M protein), a structural membrane glycoprotein and the most abundant envelope component (Utiger et al., 1995). Furthermore, the M protein not only has an important role in the viral assembly process (Nguyen and Hogue, 1997; de Haan et al., 1998), but also consists of antibodies that neutralize virus in the presence of complement (Saif, 1993). It has also been suggested that the M protein plays a role in **α**-interferon (**α**-IFN) induction (Laude et al., 1992). In the M gene of Vietnamese PEDV strains, no insertions and deletions were found. The M gene of Vietnamese PEDV strains is highly conserved in the genome sequence. There is one amino acid substitution shared with the recent Chinese and US PEDV isolates ($^{V}42^{A}$) (Chen et al., 2008), and

Vietnamese PEDV also has one change at amino acid 192 (from G to S) compared to other PEDV references.

In 2014, several reports from Asian countries, including Korea, Taiwan and Japan (Choi et al., 2014; Lin et al., 2014), suggested that there has been an emergence of US-like PEDV in Asian countries. The results of our study, however, demonstrate that at present, PEDV in Vietnam is not influenced by US-like PEDV strains. PED is endemic in Korea, Japan and Taiwan. The emergence of US-like PEDV in those countries resulting in severe disease outbreaks warrants serious consideration as to whether immunity induced by previously existing PEDV strains could provide protection from other exotic isolates. The results of disease outbreaks in those countries suggest that cross-protection might not be completely effective. Therefore, in Vietnam, where the emergence of US-like PEDV strains has not yet been reported, intensive surveillance control programs, including the implementation of more surveys and obtaining more isolates to sequence, should be considered. The risk of virus introduction into the country should also be determined, and control programs should be implemented accordingly.

To successfully control PEDV, planned exposure of sows with minced intestines of PEDV-infected pigs has long been suggested (D. Nilubol, 2012b). However, side effects of this management protocol, including an increased percentage of mummified fetuses and risks of contamination by other pathogens, have been recognized (D. Nilubol, 2012a). Vaccination has been implemented as an alternative tool. The degree of successful control, however, is controversial. The route of vaccination and genetically distinct isolates that are used to manufacture vaccines have contributed to the unsuccessful results. Because mucosal immunity, especially secretory Immunoglobulin A, plays an important role in controlling PEDV infection (D. Nilubol, 2012a), intramuscular vaccination with PEDV vaccine might not be completely effective. In addition, genetic differences between the available vaccines and the isolates responsible for outbreaks is of concern. Several studies from China have reported severe PED outbreaks even though vaccination had been extensively used in the herd (Sun et al., 2012). However, the isolates responsible for outbreaks in China from 2011 to 2012 were new variants. The vaccines available are

manufactured from isolates known as classical variants, which are genetically distinct from the new variants. Thus, vaccines manufactured from classical variants may not provide an adequate solution for successfully controlling PEDV in Vietnam. A vaccine manufactured from new-variant PEDV in conjunction with the appropriate route of administration could be used to successfully control PED in Vietnam.

Conclusion

The results of the present study demonstrate that PEDV isolates from Vietnam are new variants, share high genetic similarity with the new variants of Chinese PEDV isolates, and possessing a unique characteristic in the spike gene defined by two notation insertions and one deletion markers that are genetically the same as those of isolates responsible for the outbreaks from 2010 to 2012 in China. A new generation of vaccines based on new variant isolates are being developed, planned exposure with PEDV-infected intestines might be an effective prevention strategy in spite of the risk of side effects. This is the first report of the entire genome of PEDV in Vietnam. We hope that this data will provide a better understanding of the molecular epidemiology and genetic diversity of PEDV field isolates in Vietnam and will help to improve the prevention strategy for controlling the disease in the future.

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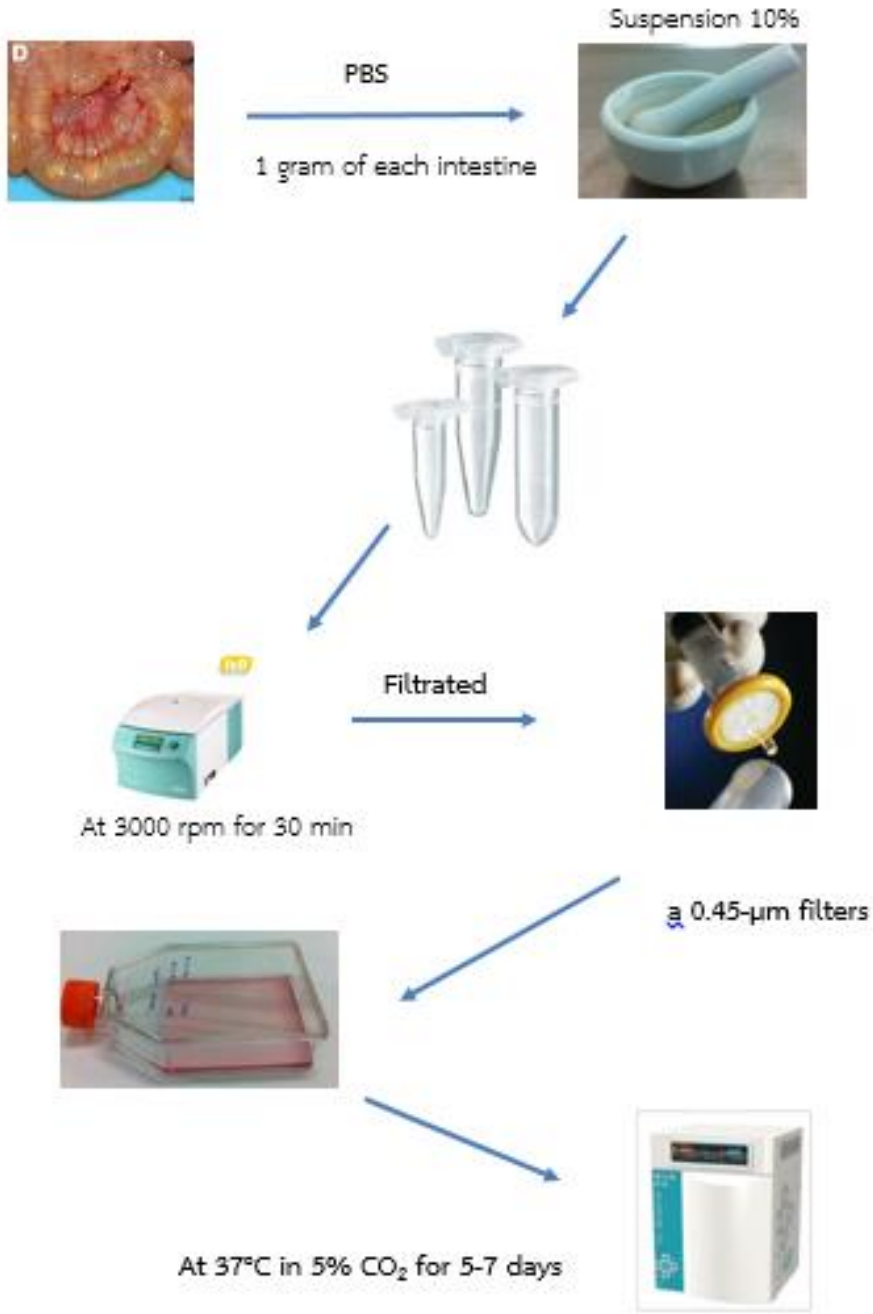
APPENDIX

APPENDIX A

Isolation PEDV



APPENDIX B

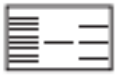
RNA isolation

		Mini	Funnel
		NucleoSpin® RNA Virus	**NucleoSpin® RNA Virus F**
1	**Lysis of viruses**	 150 µL sample volume 600 µL RAV1 70 °C, 5 min	 1 mL sample volume 4 mL RAV1 70 °C, 5 min
2	**Adjust binding conditions**	 600 µL ethanol	 4 mL ethanol
3	**Bind viral RNA**	 Load sample stepwise  8,000 x *g*, 1 min	 Load sample  3,000 x *g*, 3–5 min
4	**Wash and dry silica membrane**	 1st wash 500 µL RAW 2nd wash 600 µL RAV3 3rd wash 200 µL RAV3  1st and 2nd 8,000 x *g*, 1 min  3rd 11,000 x *g*, 5 min	 1st wash 5 mL RAW 2nd wash 8 mL RAV3 3rd wash 2 mL RAV3  1st and 2nd 3,000 x *g*, 3 min  3rd 3,000 x *g*, 10 min
5	**Elute highly pure RNA**	 50 µL RNase-free H_2O (70 °C) RT, 1–2 min  11,000 x *g*, 1 min	 50–100 µL RNase-free H_2O (70 °C) RT, 1–2 min  3,000 x *g*, 3 min

Total Viral RNAs of three strains were extracted from the infected Vero culture supernatant using the Nucleospin® viral RNA isolation kit (Macherey-Nagel Inc, Germany).

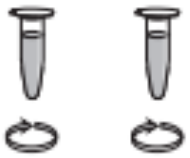
APPENDIX C

Protocol for PCR clean up, gel extraction of the Nucleospin® Gel extraction kit (Macherey-Nagel Inc, Germany).

	PCR clean-up	Gel extraction	DNA clean-up (with SDS)	Single stranded DNA clean-up
1	PCR clean-up, DNA clean-up, or single stranded DNA clean-up: Adjust binding condition Gel extraction: Excise DNA fragment / solubilize gel slice			
	200 µL NTI/ 100 µL PCR	200 µL NTI/ 100 mg gel 50 °C 5–10 min	500 µL NTB/ 100 µL sample	200 µL NTC/ 100 µL sample
2	Bind DNA	 11,000 x *g* 30 s		
3	Wash silica membrane	 700 µL NT3 11,000 x *g* 30 s Recommended: 2nd wash 700 µL NT3 11,000 x *g* 30 s		
4	Dry silica membrane	 11,000 x *g* 1 min		
5	Elute DNA	 15–30 µL NE RT 1 min 11,000 x *g* 1 min		

APPENDIX D

Protocol for plasmid purification of the Nucleospin® Plasmid purification kit (Macherey-Nagel, Germany)

	NucleoSpin® Plasmid	NucleoSpin® Plasmid (NoLid)	NucleoSpin® Plasmid QuickPure
1 Cultivate and harvest bacterial cells	 11,000 x *g* 30 s		 11,000 x *g* 30 s
2 Cell lysis	 250 µL Buffer A1 250 µL Buffer A2 RT, 5 min 300 µL Buffer A3		 250 µL Buffer A1 250 µL Buffer A2 RT, 5 min 300 µL Buffer A3
3 Clarification of the lysate	 11,000 x *g* 5 – 10 min		 11,000 x *g* 5 min
4 Bind DNA	 Load supernatant 11,000 x *g* 1 min		 Load supernatant 11,000 x *g* 1 min
5 Wash silica membrane	 *(Optional: 500 µL Buffer AW)* 600 µL Buffer A4 11,000 x *g* 1 min		 450 µL Buffer AQ 11,000 x *g* 3 min
6 Dry silica membrane	 11,000 x *g* 2 min		Drying is performed during centrifugation of the single washing step
7 Elute DNA	 50 µL Buffer AE RT, 1 min 11,000 x *g* 1 min		 50 µL Buffer AE RT, 1 min 11,000 x *g* 1 min

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2. An announcement in the title “Complete genome sequence of porcine epidemic diarrhea virus in Vietnam” on the GenomeA (Journals.ASM.org)