Detection and genetic characterization of emerging and re-emerging viral enteric diseases in swine in Thailand

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การตรวจหาและลักษณะทางพันธุกรรมของโรคไวรัสอุบัติใหม่และอุบัติซ้ำของระบบทางเดินอาหารในสุกรในประเทศไทย

นายทวีศักดิ์ เจนธนกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ทวีศักดิ์ เจนธนกิจ : การตรวจหาและลักษณะทางพันธุกรรมของโรคไวรัสอุบัติใหม่และอุบัติซ้ำของระบบทางเดินอาหารในสุกรในประเทศไทย. (Detection and genetic characterization of emerging and re-emerging viral enteric diseases in swine in Thailand) อ.ที่ปรึกษาหลัก : ศ. น.สพ. ดร.อลงกร อมรศิลป์

โรคไวรัสอุบัติใหม่และอุบัติซ้ำของระบบทางเดินอาหารในสุกร ได้แก่ โรคติดเชื้อไวรัสพีอีดี (Porcine epidemic diarrhea virus) ไวรัสเดลตาโคโรนา (Porcine deltacoronavirus) และ ไวรัสเอนเทอโรชนิดจี (Enterovirus G) เป็นโรคที่สำคัญทางด้านความมั่นคงทางอาหารและทางสาธารณสุข ในประเทศไทยพบว่า ข้อมูลเกี่ยวกับอัตราปรากฏและสถานะของไวรัสพีอีดี ไวรัสเดลตาโคโรนา และไวรัสเอนเทอโรชนิดจี มีไม่เพียงพอ โดยเฉพาะไวรัสเอนเทอโรชนิดจี ยังไม่เคยพบรายงานในประเทศไทย การศึกษาวิจัยในวิทยานิพนธ์นี้มี 3 ขั้นตอน ประกอบด้วย ขั้นตอนที่ 1 สำรวจเชื้อไวรัสของระบบทางเดินอาหารในสุกรในฟาร์มสุกร ขั้นตอนที่ 2 วิเคราะห์รหัสพันธุกรรม และความหลากหลายทางสายพันธุ์ของเชื้อไวรัสของระบบทางเดินอาหารในสุกร ขั้นตอนที่ 3 พัฒนาชุดทดสอบอย่างรวดเร็วด้วยวิธี RT-LAMP ร่วมกับ Lateral flow และ DNA aptamer ในขั้นตอนที่ 1 ผลการสำรวจเชื้อไวรัสของระบบทางเดินอาหารในสุกรระหว่าง ธันวาคม 2557 ถึง มกราคม 2561 โดยเก็บตัวอย่างอุจจาระและลำไส้จำนวน 777 ตัวอย่าง จากฟาร์มสุกรจำนวน 73 ฟาร์ม ใน 20 จังหวัด ใน 7 เขตปศุสัตว์ พบอัตราปรากฏของเชื้อไวรัสพีอีดี เชื้อไวรัสเดลตาโคโรนา และ เชื้อไวรัสเอนเทอโรชนิดจี ในตัวอย่าง คือ ร้อยละ44.02 ร้อยละ3.47 และ ร้อยละ71.56 ตามลำดับ ในฟาร์มพบอัตราปรากฏของเชื้อไวรัสพีอีดี เชื้อไวรัสเดลตาโคโรนา และ เชื้อไวรัสเอนเทอโรชนิดจี คือ ร้อยละ50.68 ร้อยละ9.59 และ ร้อยละ69.86 ตามลำดับ สามารถพบเชื้อไวรัสพีอีดี และ เชื้อไวรัสเอนเทอโรชนิดจี ได้ทั่วประเทศไทย ส่วนเชื้อไวรัสเดลตาโคโรนาพบได้ในจังหวัดที่มีการเลี้ยงสุกรจำนวนมาก พบว่าอายุของสุกรมีผลต่อการติดเชื้อไวรัสพีอีดี และเชื้อไวรัสเอนเทอโรชนิดจี และฤดูกาลมีผลต่อการติดเชื้อไวรัสเดลตาโคโรนาและเอนเทอไวรัสชนิดจี นอกจากนี้พบการติดเชื้อร่วมของเชื้อไวรัสพีอีดี เชื้อไวรัสเดลตาโคโรนา และเชื้อไวรัสเอนเทอโรชนิดจี ในระดับต่ำ (ร้อยละ0.13) ในขั้นตอนที่ 2 พบว่าเชื้อไวรัสพีอีดีในไทย สามารถจำแนกเชื้อไวรัสพีอีดีจำนวน 39 เชื้อได้เป็น 3 จีโนไทป์ (genotypes) ได้แก่ โนเวลจี 1 (Novel G1) จี2เอ (G2a) และโนเวลจี 2 (Novel G2) ซึ่งยังไม่พบการรายงานเชื้อโนเวลจี 1 และโนเวลจี 2 ในประเทศไทยมาก่อน และพบการเปลี่ยนแปลงของลำดับกรดอะมิโนหลายตำแหน่งทั้ง 3 เอปิโทป (epitopes) ได้แก่ COE SS6 และ 2C10 และพบรูปแบบใหม่จำนวน 2 รูปแบบที่เอปิโทป SS6 (^{764}PQEGQVKI^{771}) และ 2C10 (^{1368}GPRFQPY^{1374}) สำหรับเชื้อไวรัสเดลตาโคโรนาในไทย สามารถจำแนกเชื้อไวรัสเดลตาโคโรนาจำนวน 16 เชื้อได้เป็นกลุ่มของประเทศไทย โดยมีความใกล้เคียงกับเชื้อไวรัสเดลตาโคโรนาในลาว พบการเปลี่ยนแปลงของลำดับกรดอะมิโนหลายตำแหน่งทั้ง 3 เอปิโทป (NTD CTD และ S2) สำหรับเชื้อไวรัสเอนเทอโรชนิดจี สามารถจำแนกเชื้อไวรัสเอนเทอโรชนิดจีจำนวน 34 เชื้อ ได้เป็น 6 จีโนไทป์ ได้แก่ จี1 จี3 จี4 จี8 จี9 และ จี10 โดยพบจีโนไทป์ จี3 มากที่สุด และยังพบว่าการติดเชื้อของแต่ละจีโนไทป์สัมพันธ์กับอายุของสุกร ในขั้นตอนที่ 3 สามารถพัฒนาชุดทดสอบโดยวิธี RT-LAMP ร่วมกับ Lateral flow ที่มีความไวและมีความจำเพาะสูง สามารถใช้จำแนกระหว่างการติดเชื้อพีอีดี และการติดเชื้อเดลตาโคโรนาในฟาร์มสุกร และสามารถค้นพบตัวแทนของ DNA aptamer จำนวน 2 ตัว ที่มีความจำเพาะและมีความสามารถในการจับกับโปรตีน NP ของเชื้อไวรัสพีอีดี ผลของการศึกษานี้ยืนยันว่าพบการปรากฏของเชื้อไวรัสของระบบทางเดินอาหารในสุกรในประเทศไทย ดังนั้นผลการสำรวจเชื้อไวรัสของระบบทางเดินอาหารในสุกรในฟาร์มสุกร และการวิเคราะห์รหัสพันธุกรรมเป็นประโยชน์สำหรับการป้องกันและควบคุมเชื้อไวรัสของระบบทางเดินอาหารในสุกร นอกจากนี้ชุดทดสอบอย่างรวดเร็วสามารถนำไปประยุกต์ใช้เพื่อตรวจหาเชื้อในระยะแรก และใช้จำแนกระหว่างการติดเชื้อไวรัสพีอีดี และเชื้อไวรัสเดลตาโคโรนาได้ในอนาคต

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
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Taveesak Janetanakit : Detection and genetic characterization of emerging and re-emerging viral enteric diseases in swine in Thailand. Advisor: Prof. Dr. ALONGKORN AMONSIN

Emerging and re-emerging important enteric viruses in pigs including porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV) and enterovirus G (EVG) are important pathogens of food security and public health concerns. In Thailand, the information on the occurrences and status of PEDVs, PDCoVs and EVGs is limited. Especially, Thai-EVGs have never been reported before. This thesis composes of three study phases. Phase 1 is surveillance of swine enteric viruses in pig farms. Phase 2 is genetic characterization and phylogenetic analysis of swine enteric viruses. Phase 3 is development of rapid diagnostic tests using RT-LAMP with lateral flow device (LFD) and DNA aptamer. For phase 1, surveillance of swine enteric viruses in pig farms was performed during December 2014 – January 2018. The fecal and intestinal samples (n=777) were collected from 73 pig farms from 20 provinces of 7 livestock regions. The occurrences of PEDVs, PDCoVs and EVGs by samples were 44.02%, 3.47% and 71.56%, respectively. By pig farms, the occurrences of PEDVs, PDCoVs and EVGs were 50.68%, 9.59% and 69.86%, respectively. Thai-PEDVs and Thai-EVGs were circulating throughout the country. While Thai-PDCoVs were only circulating in high density of pig production provinces of Thailand. Age groups of pigs associated with Thai-PEDVs and Thai-EVGs infections and seasonal patterns associated with Thai-PDCoVs and Thai-EVGs infections were observed. Moreover, the co-circulation of PEDVs, PDCoVs and EVGs with low rate (0.13%) were detected. For phase 2, for Thai-PEDVs, representative PEDVs (n=39) were classified into 3 genotypes (Novel G1, G2a and Novel G2). While Novel G1 and Novel G2 have never been reported in Thailand before. At least 3 epitopes (COE, SS6 and 2C10) showed multiple amino acid changes and 2 novel patterns at epitopes SS6 (^{764}PQEGQVKI^{771}) and 2C10 (^{1368}GPRFQPY^{1374}) were identified. For Thai-PDCoVs, representative PDCoVs (n=16) were grouped into Thailand cluster which closely related to Laos-PDCoVs. The multiple amino acid substitutions at 3 epitopes (NTD, CTD and S2) were observed. For Thai-EVGs, representative EVGs (n=34) were classified into 6 genotypes (G1, G3, G4, G8, G9 and G10) which the predominant genotype of Thai-EVGs was G3. The age groups of pigs were associated with genotypes of EVGs infection. For phase 3, The RT-LAMP with LFD kits with high sensitivity and specificity were developed to differentiate PEDVs and PDCoVs infections in field settings. The 2 candidate aptamers (N04 and N25) which had specific binding and high binding affinity to NP protein of PEDV were established. In summary, our results confirmed that swine enteric viruses are circulating in pig farms in Thailand. Therefore, the results of surveillance of swine enteric viruses in pig farms and genetic characterization of swine enteric viruses provided valuable information for prevention and control of swine enteric viruses. Moreover, the rapid diagnostic kits could be applied for early detection and distinguish between PEDVs and PDCoVs infections.

Field of Study: Veterinary Public Health Student's Signature .....

Academic Year: 2020 Advisor's Signature .....

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Taveesak Janetanakit

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bp	Base pair(s)
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
E gene	Envelop gene
ELISA	Enzyme-linked immune absorbance assay
EMSA	Electrophoretic mobility shift assay
et al.	Et alibi, and others
EVG	Enterovirus species G
FAM	Carboxyfluorescein
fmol	Femtomole
g	Gram(s)
G	Gravitational force unit
HEX	Hexachloro-fluorescein
hr	Hour(s)
H_2SO_4	Sulfuric acid
INDEL	Insertion and deletion
IPTG	Isopropyl β- d-1-thiogalactopyranoside
K_D	Dissociation constant
LB	Luria-Bertani
LC-MS/MS	Liquid chromatography – Mass spectrometry
LFD	Lateral flow device
M	Molar
M gene	Membrane gene
$MgSO_4$	Magnesium sulfate

$MgCl_2$	Magnesium chloride
mL	Milliliter(s)
mM	Millimolar
NA	Not available
NaCl	Sodium Chloride
ng	Nanogram(s)
nm	Nanometer(s)
NP gene	Nucleoprotein gene
NS	Non-structural protein
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffer saline
PBST	Phosphate buffer saline with 0.05%Tween® 20
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PDCoV	Porcine deltacoronavirus
PEDV	Porcine epidemic diarrhea virus
PEV	Porcine enterovirus
PRCV	Porcine respiratory coronavirus
PSV	Porcine sapelovirus
PTV	Porcine teschovirus
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rpm	Round per minute
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
S gene	Spike gene
SD	Standard deviation
SECV	Swine enteric coronavirus
SELEX	Systematic evolution of ligands by exponential enrichment
SIV	Swine influenza virus

ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris borate EDTA
$TCID_{50}$	50% Tissue culture infectious dose
TGEV	Transmissible gastroenteritis virus
TMB	3,3',5,5'-Tetramethylbenzidine
U	Unit
V	Volt(s)
VP1	Viral protein 1
WGS	Whole genome sequencing
X-Gal	5-bromo-4-chloro-3-indolyl-d-galactoside
3'UTR	3' untranslated region
5'UTR	5' untranslated region
°C	Degree celsius
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar

CHAPTER 1
INTRODUCTION

Swine industry trades more than 110 million pigs with production values of 23.4 billion dollars worldwide. Thailand is one of the export countries for pork and processed pork. In 2015, the department of livestock development of Thailand reported that Thailand exported pork and processed pork for 16,530 tons or 3.2 billion baht. However, Thai swine industry may decrease its production due to emerging and re-emerging swine diseases causing respiratory and enteric problems in pigs. For example, emerging and re-emerging important enteric viruses in pigs are porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV) and enterovirus G (EVG).

PEDV and PDCoV belong to family *Coronaviridae*. The *Coronaviridae* can be classified into 4 genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Woo *et al.*, 2012). Coronaviruses cause both respiratory and enteric diseases in several mammal and avian species. Porcine epidemic diarrhea virus (PEDV), a member of *Alphacoronavirus*, causes diarrhea, vomiting, dehydration and high mortality in piglets (Wang *et al.*, 2013; Zhang *et al.*, 2015).

Recently, PEDVs can be classified into 4 major groups based on S gene sequences including G1a, G1b, G2a and G2b. Firstly, G1a is classical PEDV which was discovered in Belgium 1978 (CV777). G1b is S-INDEL North America group which found in France, Germany, Japan, Portugal, South Korea and USA. G2a could be isolated from China in 2010 and G2b is non S-INDEL North America group causes high virulence of diseases and spreads throughout the world (Grasland *et al.*, 2015; Hanke *et al.*, 2015; Lee and Lee, 2014b; Mesquita *et al.*, 2015; Murakami *et al.*, 2015; Wang *et al.*, 2015).

Porcine deltacoronavirus (PDCoV), a member of *Deltacoronavirus*, was first identified in Hong Kong in 2012 (Woo *et al.*, 2012). In early 2014, PDCoVs were discovered in pig farms in the US and spread to many US states (Hu *et al.*, 2015; Li *et al.*, 2014; Marthaler *et al.*, 2014a; Marthaler *et al.*, 2013; Marthaler *et al.*, 2014b; Wang *et al.*, 2014a). In addition, PDCoVs have been reported in China and South Korea (Dong *et al.*, 2015; Lee and Lee, 2014a; Song *et al.*, 2015). Clinical signs of PDCoVs infection are similar to PEDVs infection in pigs but with lower mortality (Chen *et al.*, 2015; Jung *et al.*, 2015).

Enterovirus G (EVG) belong to the family *Piconaviridae*, genus *Enterovirus*. Enteroviruses cause varying diseases in several animal species and humans. For example, the important enterovirus is enterovirus EV71, causes hand foot and mouth diseases in children. Moreover, human enterovirus EV71 could infect pigs in an experimental study (Yang *et al.*, 2014). EV71 infected pigs showed symptoms similar to foot and mouth diseases virus infection. To date, EVGs consist of 20 genotypes. EVG genotype 1 (EVGs-G1) can infect and causes disease in pigs. While other genotypes could be reported in both healthy and diarrheic pigs of all ages. The clinical sign of EVGs-G1 infection is flaccid paralysis in piglets (Yang *et al.*, 2013). EVGs were documented in Brazil, China, USA and Vietnam (Anbalagan *et al.*, 2014; Boros *et al.*, 2012a; Boros *et al.*, 2011; Donin *et al.*, 2014; Moon *et al.*, 2012; Van Dung *et al.*, 2014; Yang *et al.*, 2013). In addition, the occurrences of inter- and intra-species recombination between enterovirus G and other genotypes have been reported (Sun *et al.*, 2014; Tapparel *et al.*, 2009; Van Dung *et al.*, 2014; Yozwiak *et al.*, 2010). Thus, EVGs infections are the important swine diseases of public health concerns.

In general, rapid viral detection is based on nucleic amplification technique or protein detection. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) based on nucleic amplification technique is one of the useful techniques to detect viruses and bacteria. RT-LAMP assay is a rapid technique (only 45 minutes) and requires less equipment (only water bath) to perform RT-LAMP reaction (Nagamine *et al.*, 2002; Notomi *et al.*, 2000). Moreover, RT-LAMP assay is inexpensive and easy to use. This technique is suitable for application of viral or bacterial

detection in field settings. Moreover, interpretation of RT-LAMP results can be performed by several techniques such as visual analysis, gel electrophoresis, turbidity analysis and/or lateral flow device (LFD). Lateral flow device (LFD) can be applied for RT-LAMP interpretation by using immunochromatographic principle. LFD utilizes antibody to capture the labeled RT-LAMP products. Hence, diagnostic kits using RT-LAMP with LFD has advantages in specificity, sensitivity, basic equipment requirement and ease of interpretation.

Protein based diagnostic kits have been developed since 1983. Most of the test kits base on antibody such as enzyme-linked immune absorbance assay (ELISA), lateral flow device (LFD). Recently, aptamer acting like antibody has been developed for multipurpose such as diagnostics and therapeutics (Zhang *et al.*, 2019c). Bounded specific-target aptamer could be nucleic acid (DNA and RNA) or peptide. Advantages of DNA aptamer are easier to synthesis, lower level of batch-to-batch variation, longer shelf-life storage, easier to modification for specific application, faster in development and detection small molecule (≥60 daltons) such as ampicillin (Kaiser *et al.*, 2018). An example of commercial diagnostic kit based on aptamer is OTA-sense® (Neoventures Biotechnology Inc.) which has been developed for Orchratoxin A detection in food and beverages. Moreover, DNA aptamer has been developed for aptamer real-time PCR in H9N2 avian influenza virus detection (Hmila *et al.*, 2017). Therefore, DNA aptamer could be a candidate for rapid diagnostic kit development for viral detection.

In Thailand, our concerns are raised since swine enteric viruses are significant to food security and public health. For example, PEDVs and PDCoVs outbreaks cause high mortality rate of piglets ranging from 40% to 100%. This problem obstructs pig production in pig farms and consequently leads to economic problem and food security for the country. Thus, early detection of emerging and re-emerging swine viral enteric diseases is important for prevention and control of diseases in pig farms and subsequently beneficial for public health. Since the information on the occurrences of PEDVs, PDCoVs, and EVGs is still limited. Especially, EVGs have never been reported in Thailand. The development of rapid diagnostic kits to detect and

differentiate swine enteric viruses, PEDVs and PDCoVs, in field settings is still in needed. Thus, this study will provide the information on the occurrences of these emerging and re-emerging enteric diseases in pigs, PEDVs, PDCoVs, and EVGs. Genetic characteristics and major genotypes of the viruses circulating in pig farms in Thailand were elucidated. Rapid diagnostic kits using RT-LAMP with LFD and potential DNA aptamer have been developed.

Research questions

1. What are the occurrences of PEDVs, PDCoVs and EVGs in outbreak prone pig farms in Thailand?

2. Which are the major strains of PEDVs, PDCoVs and EVGs in outbreak prone pig farms in Thailand?

3. Could emerging and re-emerging swine enteric viruses, PEDVs and PDCoVs be identified and differentiated in field settings?

Objectives of study

1. To determine the occurrences of PEDVs, PDCoVs and EVGs circulating in outbreak prone pig farms in Thailand during December 2014 to January 2018.

2. To characterize PEDVs, PDCoVs and EVGs isolated from pig farms in Thailand.

3. To develop rapid diagnostic kits for the detection of PEDVs and PDCoVs.

Hypotheses

Emerging and re-emerging viruses causing swine enteric diseases, PEDVs, PDCoVs and EVGs, are circulating in pig farms in Thailand. Genotypes of swine enteric viruses, PEDVs, PDCoVs and EVGs, that circulating in Thailand could be identified. Rapid diagnostic tests for the detection of PEDVs and PDCoVs could be developed and used in field settings.

CHAPTER 2

LITERATURE REVIEW

There are several emerging and re-emerging swine diseases causing respiratory and enteric problems in pigs that impact pig productions and public health. For example, porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV) and enterovirus G (EVG) are important emerging and re-emerging viral diseases of pigs. Porcine coronaviruses, PEDV and PDCoV, cause high mortality in piglets and lead to pig production losses. While EVG infections are the important swine disease of public health concerns.

2.1 Porcine coronaviruses

Coronavirus (CoV) is an enveloped, single-stranded positive RNA virus of the family *Coronaviridae*. CoVs can cause respiratory and enteric diseases in humans and animals. The viruses can be classified into 4 genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (Woo *et al.*, 2012). *Alphacoronavirus* and *Betacoronavirus* usually infect in humans and mammals. While *Gammacoronavirus* and *Deltacoronavirus* are usually found in avian species (Woo *et al.*, 2012).

In pigs, porcine coronaviruses cause both respiratory and enteric diseases of pigs such as porcine epidemic diarrhea viruses (PEDVs), transmissible gastroenteritis viruses (TGEVs) and porcine respiratory coronaviruses (PRCVs). The PEDVs and TGEVs are known as the causative agents of diarrhea in pigs. Both PEDVs and TGEVs could be named as swine enteric coronaviruses (SECVs). To date, PEDVs cause diarrhea in pigs in worldwide. While TGEVs are less common in pigs due to effective immunization. In 2012, novel deltacoronaviruses have been reported in diarrheic pigs in Hong Kong. Subsequently, the novel deltacoronaviruses in pigs spread to USA and renamed as porcine deltacoronaviruses (PDCoVs). Both PEDVs and PDCoVs

infections are similar in clinical signs and pathological changes in infected pigs (Jung *et al.*, 2015). Thus, PEDVs, TGEVs and PDCoVs are considered as SECVs group and important causative agents of diarrhea in pigs.

2.1.1 Porcine epidemic diarrhea viruses

Porcine epidemic diarrhea virus (PEDV) is a member of the family *Coronaviridae*, genus *Alphacoronavirus*. The classical PEDVs were first discovered in 1978 (Pensaert and de Bouck, 1978). PEDVs can infect in all ages of pigs. However, PEDVs cause severe diarrhea, vomiting, and dehydration in piglets with up to 100% mortality (Wood, 1977). PEDVs cause outbreaks and lead to economic losses in swine industry worldwide. In late 2010, the outbreaks of PEDVs genotype G2a were reported in China (Wang *et al.*, 2013). In 2013, the PEDVs emerged in USA and spread to several US states. The emerging PEDVs in USA have been classified, based on S gene sequences, into 2 groups including classical North America (Non S-INDEL NA or G2b) and S-INDEL North America (S-INDEL NA or G1b). It has been reported that S-INDEL NA group could be found in Belgium, France, Germany and South Korea (Grasland *et al.*, 2015; Hanke *et al.*, 2015; Lee and Lee, 2014b; Marthaler *et al.*, 2013; Wang *et al.*, 2014b). Recently, PEDVs have been classified into 4 genotypes including G1a (classical PEDV), G1b (S-INDEL NA), G2a and G2b (Non S-INDEL NA) (Lee, 2015). In Thailand, the Chinese-like PEDVs and classical PEDVs were first reported in 2007 and 2014 (Puranaveja *et al.*, 2009; Temeeyasen *et al.*, 2014). Whereas emerging North America groups including non-S-INDEL and S-INDEL have been reported in several countries (Chung *et al.*, 2016; Grasland *et al.*, 2015; Hanke *et al.*, 2015; Islam *et al.*, 2016; Mesquita *et al.*, 2015; Paraguison-Alili and Domingo, 2016; Wang *et al.*, 2016a).

PEDVs genome (28 kb) consists of 7 open reading frames (ORFs) including ORF 1a and ORF1b encoding 16 non-structural proteins, while ORF 2-6 encoding four structural and one accessory proteins (Spike, Membrane, Envelop, Nucleocapsid, and ORF3) (Figure 2.1). Spike gene of PEDVs contains high variable region. It is generally used for genetic diversity and evolution study and can be used for strain

differentiation among PEDVs. In addition, S gene contains 4 epitopes including COE, SS2, SS6 and 2C10 relating to induction of anti PEDV Neutralizing antibody (Chang *et al.*, 2002; Sun *et al.*, 2008). On the other hand, ORF3 gene is highly conserved gene. It is preferentially used for virulent study of the virus and can be used to distinguish between wild-type and vaccine strains (Park *et al.*, 2008; Song *et al.*, 2003).

2.1.2 Porcine deltacoronaviruses

The genus *Deltacoronavirus* has been reported in birds and mammals including pigs. Porcine deltacoronavirus (PDCoV) has been reported in China, Hong Kong, South Korea and USA (Lee and Lee, 2014a; Li *et al.*, 2014; Marthaler *et al.*, 2014a; Marthaler *et al.*, 2013; Marthaler *et al.*, 2014b; Song *et al.*, 2015; Wang *et al.*, 2014a; Wang *et al.*, 2015; Woo *et al.*, 2012). PDCoVs infected pigs showed clinical signs similar to PEDV infection including severe diarrhea and body weight losses. Gross lesions and histopathological lesions demonstrated thin intestinal wall and shortened of villi. It is noted that the clinical signs, gross lesions and histopathological lesions of PDCoVs are similar to PEDVs (Chen *et al.*, 2015; Jung *et al.*, 2015). Furthermore, Immunization against PEDVs could not protect PDCoVs infection in pigs and versa immunization against PDCoVs could not protect PEDVs infection in pigs. While ELISA testing showed that immunization against NP protein of PEDVs and PDCoVs can cross-react to each other (Ma *et al.*, 2016).

The PDCoVs genome (25kb) consists of 5’ untranslated region (5’ UTR), ORF 1a/b, spike (S), envelop (E), membrane (M), nonstructural 6 and 7 (NS 6 and NS 7), nucleocapsid (NP), and 3’untranslated region (3’ UTR) (Figure 2.1). In detail, the S protein contains with 2 subunits including S1 and S2 subunits which can be cleaved by protease during cell attachment and entry. S2 subunit can serve as membrane fusion activity. S1 subunit can interact with APN receptor for cell attachment. Moreover, S protein contains 3 epitopes including NTD, CTD and S2 which induce

production of neutralizing antibody against PDCoVs (Chen *et al.*, 2020; Li *et al.*, 2018a; Shang *et al.*, 2018; Zhang *et al.*, 2019b).

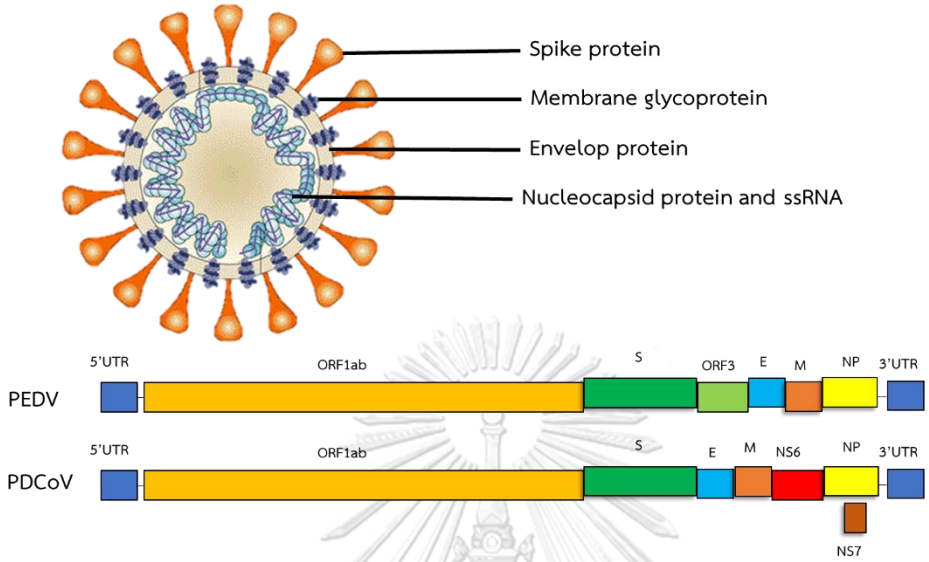

Figure 2.1 Schematic of porcine coronaviruses with their proteins and genome organization of PEDV and PDCoV (Adapted from Stadler et al., 2003).

2.2 Enteroviruses species G

Porcine enterovirus (PEV) belongs to the family *Picornaviridae*. PEV can be classified into 13 serotypes based on serology, while based on CPE pattern and tissue tropism classified PEV into 3 groups. Furthermore, taxonomical studies indicated that porcine enteroviruses can be classified into 3 genera including *Porcine teschovirus*, *Porcine sapelovirus*, and *Porcine enterovirus B* (PEV-B). Recently, PEV-B has been renamed as porcine enteroviruses G (EVGs) (Krumbholz *et al.*, 2002; Palmquist *et al.*, 2002). The prototypes of EVGs are PEV9 (EVGs-G1) and PEV10 (EVGs-G2). To date, EVGs have been further classified into 20 genotypes (Boros *et al.*, 2012a; Boros *et al.*, 2012b; Boros *et al.*, 2011; Moon *et al.*, 2012; Van Dung *et al.*, 2014; Zhang *et al.*, 2012).

Porcine enteroviruses can be found in both healthy and diarrheic pigs (Shan *et al.*, 2011; Zhang *et al.*, 2014a). Porcine teschoviruses (PTVs) and porcine

sapeloviruses (PSVs) normally cause various clinical conditions in pigs such as poliomyelitis, pneumonia and enteritis (Palmquist *et al.*, 2002; Pogranichniy *et al.*, 2003). While, EVGs have been found in healthy pigs in Brazil, China, Hungary, Japan, South Korea, USA, and also in wild boars in Hungary. In Vietnam, EVGs were first reported in diarrhea pigs. A study reported clinical signs of EVGs-G1 infection in 2 weeks-old pigs and the infected pigs showed flaccid paralysis and pneumonia signs. However, the clinical signs due to other genotypes of EVGs still unclarified (Anbalagan *et al.*, 2014; Boros *et al.*, 2012a; Boros *et al.*, 2011; Donin *et al.*, 2014; Moon *et al.*, 2012; Van Dung *et al.*, 2014; Yang *et al.*, 2013; Yang *et al.*, 2014; Zhang *et al.*, 2012).

EVG is a small non-enveloped, positive sense single strand RNA virus. EVG has only one open reading frame encoding viral polyproteins. EVGs genome organization and arrangement (7.4 kb) include 5'UTR for viral translation, P1 is a viral structural protein, P2, P3 and 3'UTR for transcription initiation. For post-translational and proteolytic processing, the P1 can be cleaved into 4 structural proteins including VP1, VP2, VP3, and VP4. Whereas the P2 and P3 can be cleaved into 7 nonstructural proteins including 2Apro, 2B, 2C, 3A, 3B(VPg), 3Cpro, and 3Dpol (RdRp) (Figure 2.2). It has been reported that the zoonotic potential of enteroviruses could be occurred since pigs are important mixing vessels for the EV71 and EVG. Enterovirus 71 (EV71), a member of enterovirus species A, causes hand foot and mouth disease in children in many countries (Kim *et al.*, 2016a; Tapparel *et al.*, 2013). Pigs could be infected with EV71 and developed clinical signs in experimental settings (Yang *et al.*, 2014). Although, Inter- and Intra-species recombination of genus *enterovirus* rarely occur. Novel recombinant enteroviruses in humans and animals have been reported (Sun *et al.*, 2014; Tapparel *et al.*, 2009; Van Dung *et al.*, 2014; Yozwiak *et al.*, 2010).

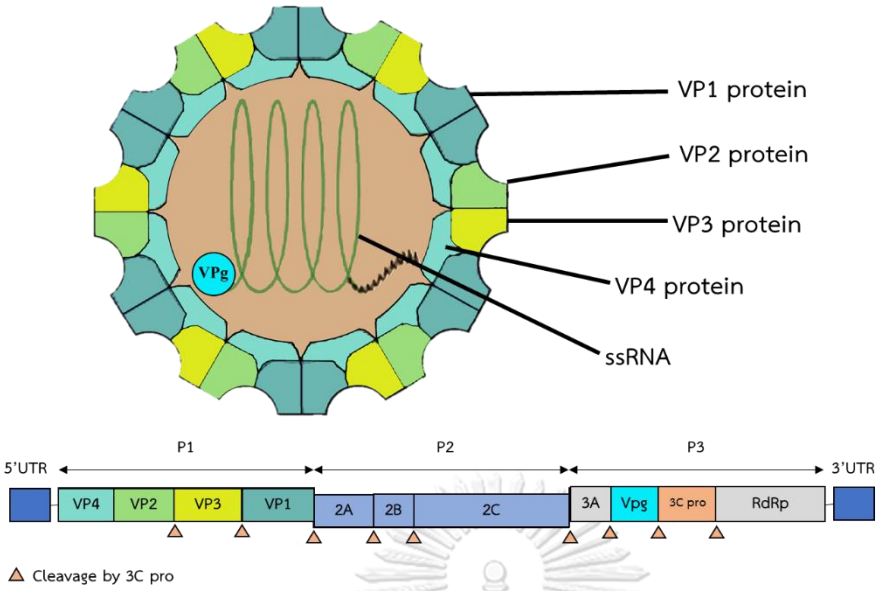

Figure 2.2 Schematic of enterovirus with their proteins and genome organization of EVG (Adapted from ViralZone 2008).

2.3 Rapid diagnostic kits

2.3.1 Loop-mediated isothermal amplification (LAMP)

Swine enteric diseases cause economic losses in swine industry. The clinical signs of PEDVs and PDCoVs infections are similar including diarrhea, vomiting and dehydration. Moreover, PEDVs and PDCoVs infections could not be distinguished by gross lesions and histopathological lesions (Chen *et al.*, 2015; Jung *et al.*, 2015). There are several methods to detect viruses such as electron microscope, viral isolation, immunohistrochemistry and nucleic acid amplification. In this thesis, nucleic amplification based assay is a useful technique to detect and differentiate emerging and re-emerging enteric viruses, PEDVs and PDCoVs, in the field settings.

The loop-mediated isothermal amplification (LAMP) technique based on nucleic amplification method was first developed in 2000. LAMP mixture contains 4-6 primers that recognized 6-8 distinct regions of target gene (F3, B3, FIP, BIP, loop F and loop B) and Bst DNA polymerase enzyme for stranded displacement during cDNA synthesis. The principle of LAMP reaction consists of 3 steps. First, starting material

producing step, dumbbell-liked DNA-form is generated by using LAMP primers. Example of eight distinct regions of six primers are shown in Figure 2.3. Second, cycling amplification step, dumbbell-liked DNA-forms are generated continuously. Third, Elongation step, the products from second step are generated into ladder sizes of LAMP amplicons (Nagamine *et al.*, 2002; Notomi *et al.*, 2000). Therefore, LAMP technique can amplify nucleotide sequences at one temperature within 45 - 60 minutes. The LAMP technique has been widely applied to detect viruses, bacteria, fungi and blood parasites in humans and animals due to the technique requires less equipment, only water bath or heat block to perform (Park *et al.*, 2016; Ravan *et al.*, 2016; Suebsing *et al.*, 2016; Suleman *et al.*, 2016; Tang *et al.*, 2016; Wang *et al.*, 2016b; Xu *et al.*, 2016; Yamazaki *et al.*, 2013; Yang *et al.*, 2016a; Yang *et al.*, 2016b; Zhou *et al.*, 2016).

To date, there are several methods to detect LAMP amplicons. The LAMP amplicons can be detected using turbidity analysis by insoluble white precipitate magnesium pyrophosphate (Parida *et al.*, 2006). Another method, LAMP amplicons can be detected, based on intercalation activity of fluorescent with double strand DNA, using gel-electrophoresis or visual analysis (Zhang *et al.*, 2014b). Another technique, lateral flow device based on immunochromatographic technique can be used to detect labeled-LAMP amplicons (Yamazaki *et al.*, 2013). Lateral flow device (LFD) has advantages in specificity, sensitivity, basic equipment requirement and ease to interpretation. Thus, LAMP technique combining LFD could provide rapid results and easy to use in the field settings.

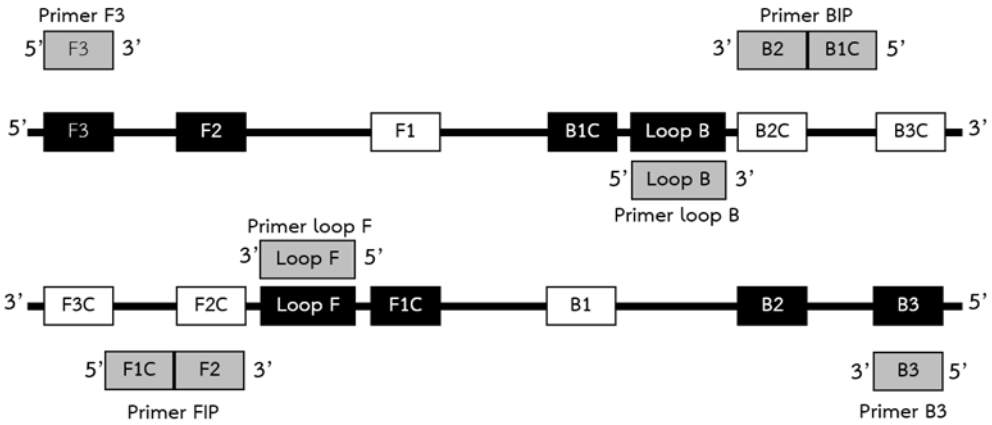

Figure 2.3 Eight distinct regions of six primers on target gene. Black boxes indicate 8 distinct regions for 6 primers designing. Grey boxes indicate arrangement of each primer for LAMP reaction. White boxes indicate important regions for primer annealing during LAMP amplification.

2.3.2 DNA aptamer based assay

Nucleic acid aptamer is short single-strand DNA or RNA binding to the target with high affinity and specificity (Ellington and Szostak, 1990; Tuerk and Gold, 1990). The intermolecular forces (e.g. hydrogen binding and Van der waals forces) and structure fitting are employed for the binding between nucleic acid aptamer and target which similar to antibody binding to the antigen (Gold, 1995). Nucleic acid aptamer has several advantages, such as shorter generation time, lower costs of manufacturing, no batch-to-batch variability, higher modifiability and better thermal stability. The comparison between aptamers and antibodies is described in Table 2.1 (Zhou and Rossi, 2017). Currently, the various applications of nucleic acid aptamer are diagnostic and therapeutic in various targets, such as metal, toxin, virus, bacteria and cancer.

Recently, commercial diagnostic kit based on aptamer is OTA-sense® (Neoventures Biotechnology Inc.) which has been developed for Orchratoxin A detection in food and beverages. Moreover, DNA aptamer has been developed for aptamer real-time PCR for the detection of avian influenza virus subtype H9N2 (Hmila

et al., 2017). Therefore, DNA aptamer could be a candidate for rapid diagnostic kit development for viral detection.

Table 2.1 Comparison of advantages and disadvantages of nucleic acid aptamers and antibodies. Modification from Zhou and Rossi, 2017.

	Nucleic acid aptamers	Antibodies
Stability	- Stable on each round of denaturation/renaturation - Stable at room temperature - Long shelf life (Can be lyophilized) - Resistant to proteases but degradable by nucleases	- Temperature sensitive and require refrigerated temperature - Limited shelf life - Degradable by proteases but resistant to nucleases
Synthesis	- In vitro synthesis (SELEX) takes only 2–8 weeks - No batch-to-batch variation - Cheap to synthesize	- In vivo production variable time up to 6 months - Batch-to-batch variation - Laborious and expensive
Target potential	- From ions and small molecules to whole cells and live animals	- Targets must cause a strong immune response for antibodies to be produced
Size	- Small molecules	- Relatively large
Modifiability	- Easy to modify without affinity loss	- Reduced activity when modifications
Affinity	- High affinity and increased affinity in multivalent aptamers	- Dependent on the number of epitopes on the antigen
Specificity	- Single point mutations Identifiable	- Different antibodies might bind the same antigen
Tissue uptake/kidney filtration	- Rapid	- Slow

CHAPTER 3

MATERIALS AND METHODS

This dissertation comprises 3 phases including: Phase 1, Surveillance of swine enteric viruses in pig farms; Phase 2, Genetic characterization and phylogenetic analyses of swine enteric viruses and Phase 3, Development of rapid diagnostic tests using RT-LAMP with lateral flow device and DNA aptamer. The conceptual framework of this study is shown in Figure 3.1.

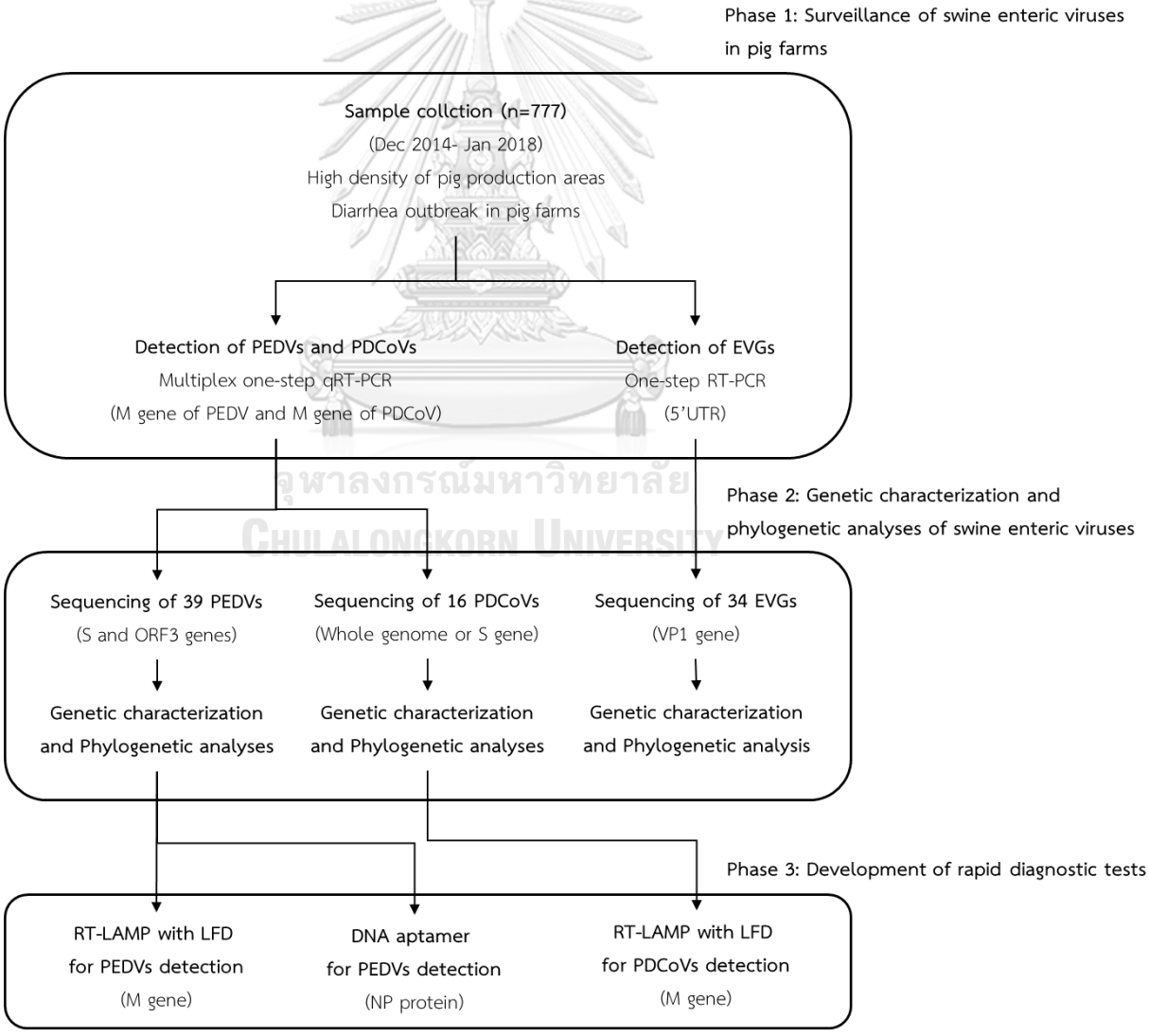

Figure 3.1 The conceptual framework of this thesis.

3.1 Phase 1: Surveillance of swine enteric viruses in pig farms

In this thesis, pig farms were selected for sample collection based on the following criteria;

1) pig farm located in high density of pig production areas

2) pig farm had diarrhea outbreak in pigs at any age groups

3) cooperation of pig farm owner

During December 2014 to January 2018, 777 fecal or intestinal tissue samples were collected from 73 pig farms in 20 provinces including Ayutthaya, Burirum, Chachoengsao, Chaiyaphum, Chiang Rai, Chonburi, Kanchanaburi, Khon Kean, Mukdahan, Nakhon Nayok, Nakhon Pathom, Nakhon Ratchasima, Nakhon Si Thammarat, Prachinburi, Prachuap Khiri Khan, Ratchaburi, Saraburi, Suphanburi, Trang and Ubon Ratchathani. Pig farms are located in 7 livestock regions (Figure 3.2). Each pig farm was visited at least once or more. The details of sample collection are listed in Table 3.1 and Figure 3.2.

3.1.1 Sample collection from pig farms

The samples (n=777) were collected from pigs of difference age groups including suckling pigs (n=444), nursery pigs (n=169), fattening pigs (n=58) and breeder pigs (n=106). By type of sample, the samples were fecal (n=663) and intestinal samples (n=114). The samples were collected in plastic bag and kept at 4°C and transported to laboratory within 24 hours. All of the samples were stored at -80°C immediately until sample preparation.

3.1.2 Sample preparation

3.1.2.1 Fecal sample

The preparation of fecal samples, 1 g of fecal sample was diluted with 9 mL of 1X sterile PBS to be 10% fecal suspension. Then, the 10% suspension sample was centrifuged at 2,500 rpm at 4°C for 10 minutes. Later on, the supernatant was divided into 2 aliquots, 150 µL for RNA extraction, and the rested supernatant for stock. Stock samples were kept in -80°C.

3.1.2.2 Intestinal sample

The preparation of intestinal samples, 1 g of intestinal sample was homogenized with 9 mL of MEM to be 10% tissue homogenate suspension. Then, the 10% suspension sample was centrifuged at 2,500 rpm at 4°C for 10 minutes. The supernatant was divided into 2 aliquots, 150 µL for RNA extraction, and the rested supernatant for stock. Stock samples were kept in -80°C.

3.1.3 Detection of swine enteric viruses

3.1.3.1 RNA extraction from fecal and intestinal samples

For RNA extraction, 150 µL of supernatant samples from step 3.1.2.1 and 3.1.2.2 were subjected to RNA extraction by RNA extraction kit (QIAamp® Viral RNA mini, Qiagen, Germany). Briefly, 150 µL of supernatant was added with 560 µL of lysis buffer AVL with RNA carrier and incubated at room temperature for 10 minutes. Later on, 560 µL of 96% ethanol was added to the mixture. Then, the 630 µL of lysate was loaded into the column and centrifuged at 6000 x G for 1 minute and repeated this step with the rest of lysate. First wash, the column was washed with 500 µL of buffer AW1 and centrifuged at 6000 x G for 1 minute. Second wash, 500 µL of buffer AW2 was added to the column and centrifuged at 20,000 x G for 3 minutes. To dry the column, the column was placed in a new 2 mL collection tube and centrifuged at full speed for 1 minute. The column was placed in a 1.5 mL microcentrifuge tube. To elute the RNA, 50 µL of buffer AVE was added to the column and incubated at room temperature for 1 minute. Later on, the column was

centrifuged at 6000 x G for 1 minute. The Viral RNA was kept in -80°C for the detection of viruses.

3.1.3.2 Detection of PEDVs and PDCoVs by using multiplex one-step qRT-PCR

In this thesis, multiplex one-step qRT-PCR was used for the detection of PEDVs and PDCoVs. The specific primers and hydrolysis or TaqMan® probes for the detection of PEDVs and PDCoVs were designed for the specific detection of membrane gene of the viruses. The primer and TaqMan probe sequences are shown in Table 3.2.

In total, viral RNA samples (n=777) from step 3.1.3.1 were subjected to the detection of PEDVs and PDCoVs by using multiplex one-step qRT-PCR. In brief, 12.5 µL of reaction contained 4 µL of viral RNA, 0.4 µM of each forward and reverse primers of PEDVs and PDCoVs, 0.1 µM of each TaqMan® probes (FAM channel for PEDVs detection and HEX channel for PDCoVs detection), 1X reaction mixture contained 0.4 mM of each dNTP and 6 mM $MgSO_4$, 0.25 µL of SuperScript™ III RT/Platinum™ Taq Mix (Invitrogen, USA), 0.08 µL of 50mM $MgSO_4$ and nuclease-free water. Multiplex one-step qRT-PCR was performed on MyGo Pro real-time PCR instrument (MyGo PCR systems, IT-IS Life Science Ltd.). The reaction conditions for PEDVs and PDCoVs detection were reverse transcription at 50°C for 15 minutes; initial denaturation at 95°C for 2 minutes; 50 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 30 seconds. Data acquisition and analysis of the Multiplex one-step qRT-PCR were done through the MyGo Pro software, v. 3.4.8. Samples exhibiting cycle threshold value (ct) of less than 36 were interpreted as positive for both PEDVs (FAM channel) and PDCoVs (HEX channel) detection. While ct value greater than 36 were interpreted as negative.

3.1.3.3 Detection of EVGs by RT-PCR

Viral RNA samples from step 3.1.3.1 were subjected to EVGs detection. EVGs detection was conducted by using RT-PCR specific to 5'UTR as previously described

with modification (Table 3.2). In brief, 10 µL of PCR mixture contained 1 µL of viral RNA, 0.4 µM of each forward and reverse primers, 5 µL of 2X Reaction Mix containing 0.4 mM of each dNTP and 2.4 mM $MgSO_4$ (Invitrogen), 0.2 µL of RT/Platinum™ Taq Mix (Invitrogen) and nuclease-free water up to reaction. RT-PCR conditions for EVGs detection were reverse transcription at 55°C for 30 minutes; initial denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds and elongation at 72°C for 1 minute; and final elongation at 72°C for 7 minutes. PCR products were then visualized by gel electrophoresis on a 1.5% of agarose gel in 0.5X Tris borate EDTA (TBE). Expected amplification product size was 150 base pairs (bps).

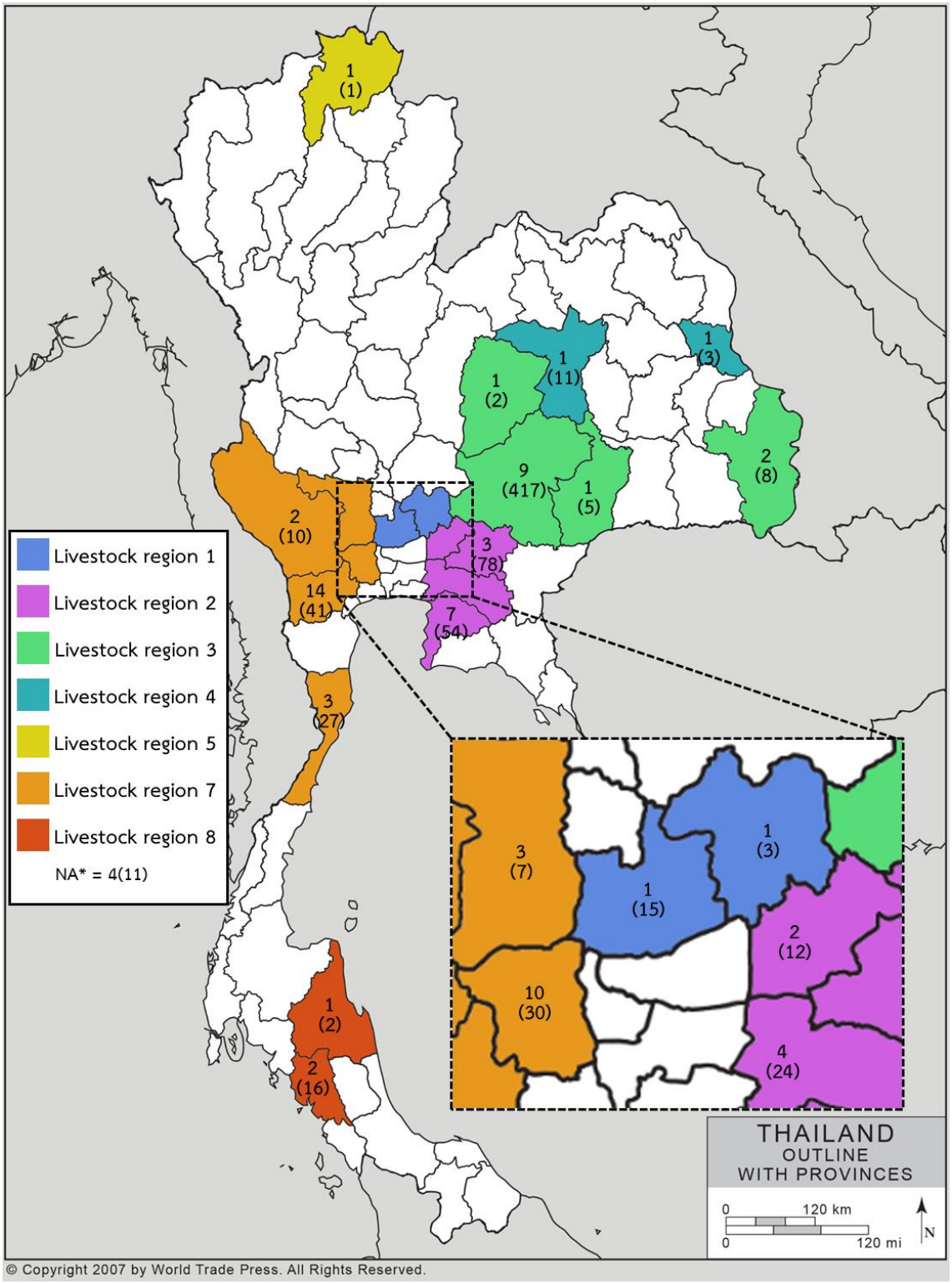

*Figure 3.2 Location of pig farms selected in this thesis and number of samples collected by provinces. The number of pig farms and number of collected samples (within blanket) are shown in picture. *NA; not available.*

Table 3.1 Swine sample collection by livestock regions, provinces, farms and pigs at age groups

Livestock region	Province	# of farm	# of sample				
			Suckling	Nursery	Fattening	Breeder	Total
1	Ayutthaya	1	0	15	0	0	15
	Saraburi	1	0	0	3	0	3
	Total	2	0	15	3	0	18
2	Chachoengsao	4	13	0	6	5	24
	Chonburi	7	45	5	0	4	54
	Nakhon Nayok	2	10	2	0	0	12
	Prachinburi	3	49	2	20	7	78
	Total	16	117	23	26	16	182
3	Burirum	1	0	5	0	0	5
	Chaiyaphum	1	0	0	0	2	2
	Nakhon Ratchasima	9	227	100	15	75	417
	Ubon Ratchathani	2	8	0	0	0	8
	Total	13	235	105	15	77	432
4	Khon Kean	1	2	1	8	0	11
	Mukdahan	1	3	0	0	0	3
	Total	2	5	1	8	0	14
5	Chiang Rai	1	0	1	0	0	1
	Total	1	0	1	0	0	1
7	Kanchanaburi	2	10	0	0	0	10
	Nakhon Pathom	10	25	2	0	3	30
	Prachuap Khiri Khan	3	13	14	0	0	27
	Ratchaburi	14	22	12	0	7	41
	Suphanburi	3	5	0	0	2	7
	Total	32	75	14	0	12	101
8	Nakhon Si Thammarat	1	2	0	0	0	2
	Trang	2	0	10	6	0	16
	Total	3	2	10	6	0	18
N/A	N/A	4	10	0	0	1	11
	Total	**73**	**444**	**169**	**58**	**106**	**777**

Table 3.2 Primer sets for detection of PEDVs, PDCoVs and EVGs in this thesis.

Primer name	Gene	Sequence (5'-3')	Product size (bps)	Reference
Multiplex one-step real-time RT-PCR for PEDVs and PDCoVs detection				
qRT-PED-F	M	AAGGTTGCTACTGGCGTACA	90	This study
qRT-PED-R		ACCAACACGTCCRTARACAA		
qRT-PED-P		FAM-GGCCTTGGCGACTGTGACGA-BHQ1		
qRT-PDCoV-F	M	ACCTGTGGAATCAAACAACGA	150	
qRT-PDCoV-R		GTGAAGTCCAGCGTTTGAAG		
qRT-PDCoV-P		HEX-TGTTCTGATATACCAGGGTGACCGC-BHQ1		
RT-PCR for EVGs detection				
EVG-F	5'UTR	CCCTGAATGCGGCTAAT	150	Beld *et al.*, 2004
EVG-R		ATTGTCACCATAAGCAGCC		

3.2 Phase 2: Genetic characterization and phylogenetic analyses of swine enteric viruses

3.2.1 Genetic characterization and phylogenetic analyses of Thai-PEDVs

To characterize the Thai-PEDVs, representative PEDVs (n=39) were selected and subjected to full-length S and ORF3 gene sequencing. S and ORF3 genes of PEDVs were amplified by using PCR and oligonucleotide primer sets previously described with modification (Diep *et al.*, 2017; Tian *et al.*, 2013). The list of primer sets for S and ORF3 genes sequencing is shown in Table 3.3.

3.2.1.1 cDNA synthesis of PEDVs

The representative PEDVs (n=39) were selected from positive samples from step 3.1.3.2 and 3.1.3.3. Criteria for the sample selections were 1) location of pig farms, 2) date of sample collection and 3) virus with low cycle threshold value (Ct) (high-RNA copies). The RNA was reverse transcribed to cDNA using SuperScript® III First-Strand System (Invitrogen,USA). In brief, one reaction of cDNA synthesis contained 2 µL of viral RNA, 3 µM of random hexamer, 1 mM of each dNTP and 6 µL of DEPC-treated water. The mixture was placed in thermocycler with the conditions at 65°C for 5 minutes and 4°C for 1 minute. The mixture was added with 2 µL of 10X RT buffer, 5 mM $MgCl_2$, 0.1 M DTT, 40 U RNase out™ and 200 U SuperScript® RT. Then, the mixture was placed in thermocycler with the conditions at 25°C for 1 minute, 50°C for 50 minutes, stop reaction at 85°C for 5 minutes and chill on ice for 1 minute. The mixture mixed with 1 µL of RNase H and incubated at 37°C for 20 minutes. The cDNA samples were kept at -20°C until further use for sequencing.

3.2.1.2 S and ORF3 genes of PEDVs sequencing

S and ORF3 genes of PEDVs were amplified by using PCR and oligonucleotide primer sets previously described with modification (Table 3.3). In detail, a total of 30 µL of PCR mixture contained 2 µL of cDNA from step 3.2.1.1, 4 µM of each forward and reverse primers, 15 µL of 2X TOPTaq Master Mix (QIAGEN), 3 µL of 10X Coral Load, and 8.1 µL of nuclease-free water. PCR conditions include initial denaturation

at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds and elongation at 72°C for 2 minutes; and final elongation at 72°C for 7 minutes. PCR amplicons were gel-purified and sequenced at 1st Base Laboratories, Kembangan, Malaysia. Nucleotide sequences were assembled and validated by using SeqMan software version 5.03 (DNASTAR Inc., Madison, WI, USA).

3.2.1.3 Genetic and phylogenetic analyses of S and ORF3 genes

For pairwise comparison and genetic analysis of PEDVs, nucleotide sequences and deduced amino acids of S and ORF3 genes of PEDVs were aligned with those of reference PEDVs including PEDVs of genotype G1a (classical), G1b (US-indel), G2a and G2b from Belgium, China, France, Japan, Netherland, Philippines, South Korea, Thailand, USA and Vietnam. The nucleotide sequences were aligned by using MEGA version 7.0.26 and MegAlign version 5.03 (DNASTAR Inc., Madison, WI, USA) software (Kumar *et al.*, 2016).

For phylogenetic analysis, full-length S and ORF3 genes of Thai-PEDVs were compared with those of reference PEDVs. Phylogenetic analyses were performed by using MEGA version 7.0.26 (http://www.megasoftware.net/) with the neighbor-joining algorithm and bootstrap analysis of 1,000 replications.

3.2.2 Genetic characterization and phylogenetic analyses of Thai-PDCoVs

To characterize the Thai-PDCoVs, representative PDCoVs were selected and subjected to whole genome sequencing (n=2) and S gene sequencing (n=14). PDCoV genomes were amplified by using PCR and oligonucleotide primer sets previously described or new primer sets designed by using the Primer3 program. The list of primer sets for S gene sequencing is shown in Table 3.3.

3.2.2.1 cDNA synthesis of PDCoVs

See 3.2.1.1

3.2.2.2 Whole genome and S gene of PDCoVs sequencing

Whole genome and S gene of PDCoVs were amplified by using PCR and oligonucleotide primer sets previously described or new primer sets (Table 3.3). In brief, a total of 30 µL of PCR mixture contained 2 µL of cDNA from step 3.2.2.1, 0.4 µM of each forward and reverse primers, 15 µL of 2X TOPTaq Master Mix (QIAGEN), 3 µL of 10X CoralLoad, and 8.1 µL of nuclease-free water. PCR conditions were initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 45 seconds and elongation at 72°C for 2 minutes; and final elongation at 72°C for 7 minutes. PCR amplicons were gel-purified and sequenced at 1st Base Laboratories, Kembangan, Malaysia. Nucleotide sequences were assembled and validated by using SeqMan software version 5.03 (DNASTAR Inc., Madison, WI, USA).

3.2.2.3 Genetic and phylogenetic analyses of whole genome and S gene

For pairwise comparison and genetic analysis of PDCoVs, nucleotide sequences and deduced amino acids of Thai-PDCoVs were aligned with those of reference PDCoVs from China, Japan, Laos, South Korea, Thailand, USA and Vietnam by MEGA version 7.0.26 and MegAlign version 5.03 (DNASTAR Inc., Madison, WI, USA) software (Kumar *et al.*, 2016).

For phylogenetic analysis, whole-genome sequences of Thai-PDCoVs (n=2) were compared with those of reference PDCoVs. Phylogenetic analysis was performed by using MEGA version 7.0.26 (http://www.megasoftware.net/) with the neighbor-joining algorithm and bootstrap analysis of 1,000 replications. Moreover, phylogenetic tree of S gene of Thai-PDCoVs (n=16) and those of reference PDCoVs from the GenBank database was constructed with the neighbor-joining algorithm and bootstrap analysis of 1,000 replications.

3.2.3 Genetic characterization and phylogenetic analysis of Thai-EVGs

To characterize the Thai-EVGs, representative EVGs (n=34) were selected and subjected to VP1 gene sequencing. VP1 gene were amplified by using PCR and

oligonucleotide primer sets previously described with modification (Dung et al., 2014). The list of primer sets is shown in Table 3.3.

3.2.3.1 cDNA synthesis of EVGs

See 3.2.1.1

3.2.3.2 VP1 gene of EVGs sequencing

Selected EVGs (n=34) were subjected to VP1 gene sequencing. VP1 gene were amplified by using PCR and oligonucleotide primer sets previously described with modification (Table 3.3). In brief, a total of 30 µL of PCR mixture contained 2 µL of cDNA from step 3.2.3.1, 0.4 µM of each forward and reverse primers, 15 µL of 2X TOPTaq Master Mix (QIAGEN), 3 µL of 10X Coral Load, and 8.1 µL of nuclease-free water. PCR conditions were initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds and elongation at 72°C for 2 minutes; and final elongation at 72°C for 7 minutes. PCR amplicons were gel-purified and sequenced at 1st Base Laboratories, Kembangan, Malaysia. Nucleotide sequences were assembled and validated by using SeqMan software version 5.03 (DNASTAR Inc., Madison, WI, USA).

3.2.3.3 Genetic and phylogenetic analyses of VP1 gene

For pairwise comparison and genetic analysis of EVGs, nucleotide sequences and deduced amino acids of Thai-EVGs were aligned with representative EVGs (20 genotypes) from China, Germany, Hungary, South Korea, United Kingdom and Vietnam by MEGA version 7.0.26 and MegAlign version 5.03 (DNASTAR Inc., Madison, WI, USA) software (Kumar *et al.*, 2016). The representative EVGs (20 genotypes) are listed in Table 3.4.

For phylogenetic analysis, VP1 gene sequences of Thai-EVGs were compared with those of reference EVGs. Phylogenetic analysis was performed by using MEGA version 7.0.26 (http://www.megasoftware.net/) with the neighbor-joining algorithm and bootstrap analysis of 1,000 replications.

Table 3.3 Primer sets for sequencing of PEDVs, PDCoVs and EVGs in this thesis.

Primer name	Gene	Sequence (5'-3')	Product size (bps)	Reference
PEDVs sequencing				
S1F	S	CCATTAGTGATGTTGTGTTAG	905	Diep *et al.*, 2017; Tian et *al.*, 2013
CS1R		CATGCACCAAAGTGGAATCATT		
CS2F	S	AATGGCCACATACCAGAAGG	918	
CS2R		GTGAAATGGTAAATTGTCTAGTGTCAA		
CS3F	S	GCATCTGACACTACTATCAATGG	822	
CS3R		TAACAGGCGTGTTGTAAAGCTG		
CS4F	S	GTATTCCCACCAACTTTAGTATGAG	854	
CS4R		CAATAGAACTAGAAATGGCTTGGAAG		
CS5F	S	CTTACCGTACAGCTGCAACAC	1,057	
CS5R		GACATCTTTGACAACTGTGT		
ORF3-F	ORF3	GTCCTAGACTTCAACCTTACGAAG	739	
ORF3-R		AACTACTAGACCATTATCATTCAC		
PDCoVs sequencing				
Dcor_17F	S	TTCGCTATCTCGTTGCATCA	920	This thesis
Dcor_17R		GCCTAGCTCTAACGGCAGATT		
Dcor_18F	S	CAGCGCTCTTCTCACGAAGT	912	
Dcor_18R		TGGTATTTCAACTTCGCCATC		
Dcor_19F	S	ACTCGACCATCCATAGTTTCA	925	
Dcor_19R		TGATGAGACGGTCAACTTGC		
Dcor_20F	S	ATCACACCTGACTGCACAGC	917	
Dcor_20R		CACAACAACCAAAGCAACCA		
Dcor_21F	S	CATTGCCCTGGCTCTTATTG	958	
Dcor_21R		TGGATCGTTGTTTGATTCCA		
EVGs sequencing				
EVGVP1F	Partial VP1	GCTGGKTATRTKACYGGDTGGTWYC	1000	Dung *et al.*, 2014
EVGVP1R		TCTTCCCAYTCDASRTTBTCCCA		

Table 3.4 The representative EVGs (20 genotypes).

Virus strain	Genotype	GenBank accession no.	Host
EVG/Swine/UKG/410/1973	G1	AF363453	Swine
EVG/Swine/Germany/PEV10_LP_54/2002	G2	AF363455	Swine
EVG/Swine/Hungary/K23/2008	G3	HQ702854	Swine
EVG/Wild_Boar/Hungary/WBD/2011	G4	JN807387	Wild boar
EVG/Ovine/Hungary/TB4-OEV/2009	G5	JQ277724	Sheep
EVG/Swine/Korea/PEV-B-KOR/2009	G6	JQ818253	Swine
EVG/Ovine/UK/990	G7	MG958646	Sheep
EVG/Swine/Vietnam/724118/2012	G8	KJ156437	Swine
EVG/Swine/Vietnam/724162/2012	G9	KJ156438	Swine
EVG/Swine/Vietnam/734123/2012	G10	KJ156446	Swine
EVG/Swine/Vietnam/744257/2012	G11	KJ156451	Swine
EVG/Swine/Vietnam/714036/2012	G12	KT265880	Swine
EVG/Swine/Vietnam/714270/2012	G13	KT265903	Swine
EVG/Swine/Vietnam/714405/2012	G14	KT265909	Swine
EVG/Swine/Vietnam/724307/2012	G15	KT265941	Swine
EVG/Swine/Vietnam/BS14-173H2/2014	G16	KT266010	Swine
EVG/Swine/USA/08NC/2015	G17	KY761948	Swine
EVG/Swine/Germany/F26-2/2013	G18	MF113370	Swine
EVG/Swine/Germany/F8-2/2013	G19	MF113372	Swine
EVG/Goat/China/JL14/2014	G20	KU297674	Goat

3.3 Phase 3: Development of rapid diagnostic tests using RT-LAMP with lateral flow device and DNA aptamer

3.3.1 Development of RT-LAMP with LFD for PEDVs detection

3.3.1.1 Quantitation of DNA standard

A DNA standard was constructed from M gene of PEDV, ligated into pGEM® (Promega, USA) and evaluated the quantity of DNA using Nanodrop 2000. In detail, the full-length M gene of PEDV was amplified with the primers listed in Table 3.4 (Kim *et al.*, 2015). The PCR reaction contained 2 µL of cDNA of the virus (PEDV/Swine/Thailand/S5001/2014), 2.5 µL of 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each forward and reverse primers, 2 U of Platinum™Taq DNA polymerase (Invitrogen, USA) and 18.15 µL of nuclease-free water. PCR conditions contained initial denaturation at 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes; and final extension at 72°C for 7 minutes. The PCR amplicons were purified and measured by using Nanodrop 2000 (ThermoFisher Scientific, USA).

For plasmid construction, one ligation reaction contained 75 ng of purified amplicons, 5 µL of 2X rapid ligation buffer, 1 µL of T4 DNA ligase, 50 ng of pGEM®-T easy vector (Promega, USA) and 1 µL of nuclease-free water. A reaction was incubated at room temperature for 1 hour prior incubated at 4°C overnight. Competent *E.coli* strain JM109 was used for transformation by using heat-shock according to manufacturer's instruction. In Brief, 2 µL of ligation reaction was mixed with 50 µL of competent cells and placed on ice for 20 minutes. Then, mixture was incubated at 42°C for 45 seconds and immediately placed on ice for 2 minutes. The mixture was added with 950 µL of LB broth and incubated at 37°C for 1 hour and 30 minutes with 150 rpm shacking. 100 µL of incubated transformant mixture was plated onto LB/amplicillin/IPTG/X-Gal plate and incubated at 37°C for 18 hours. White colonies were selected to culture in 10 mL of LB/ampicillin broth at 37°C for

18 hours. The cultured mixture was aliquoted into 1 mL of culture for plasmid DNA purification and the rested culture in 25% glycerol.

To quantitate the amount of DNA standard, 1 mL of transformant culture was subjected to plasmid DNA purification by using Nucleospin® Plasmid (Macherey-Nagel, Germany). In brief, transformant culture was centrifuged at 11,000 x G for 30 seconds and discarded the supernatant. To lyse the cell, the pellet was resuspended with 250 µL of buffer A1. Then, resuspended mixture was added with 250 µL of buffer A2 and mixed by inverting the tube 6-8 times prior incubated at room temperature for 5 minutes. Then, the mixture was centrifuged at 11,000 x G for 5 minutes. The clear supernatant was added to column and centrifuged at 11,000 x G for 1 minute for DNA binding. First wash, 500 µL of buffer AW was added to column and centrifuged at 11,000 x G for 1 minute. Second wash, 600 µL of buffer A4 was added to column and centrifuged at 11,000 x G for 1 minute. Then, the column was placed to a new collection tube and centrifuged at 11,000 x G for 2 minutes. To elute the DNA, 50 µL of buffer AE was added to the column and incubated at room temperature for 1 minute prior centrifuged at 11,000 x G for 1 minute. Plasmid DNA concentration was quantified by using Nanodrop 2000 and converted to number of copies per µL (Figure 3.3)

3.3.1.2 RT-LAMP with LFD for PEDVs detection

To perform RT-LAMP for PEDVs detection, primers (n=4) (PED-F3, PED-B3, PED-FIP and PED-BIP) and probes (n=2) (PED-loop F and PED-loop B) were designed by PrimerExplorer 4.0 (https://primerexplorer.jp/e/). Primers and probes were designed based on 8 conserve regions of M gene of PEDVs. The primer and probe sequences in this thesis are shown in Table 3.5. Detail to perform RT-LAMP, the reaction contained 2 µL of RNA sample, 1 U of AMV RT (NEB, USA), 8 U of Bst 2.0 DNA Polymerase (NEB, USA), 2.5 µL of 10X Isothermal amplification buffer, 6mM $MgSO_4$, 1.4 mM of each dNTP, 1.6 µM of each FIP and BIP primers, 0.2 µM of each F3 and B3 primers, 0.4 µM of each loopF and loopB probes and 11.4 µL of nuclease-free water.

RT-LAMP reaction was placed in thermocycler with RT-LAMP conditions at 63°C for 45 minutes and termination at 80°C for 5 minutes. The labeled LAMP amplicons were visualized by gel electrophoresis on a 1.5% of agarose gel in 0.5X Tris borate EDTA (TBE).

To visualized by lateral flow device (LFD), the labeled LAMP amplicons were applied to LFD strip (Milenia Biotec, Germany). Briefly, 10 µL of labeled LAMP amplicons were added to 100 µL of assay buffer. Then, the LFD strip was dipped into the mixture and the result could be interpreted within 10 minutes. For result interpretation, for positive result, control line and test line of LFD strip were present. While negative detection, only control line of LFD strip was observed. Example of positive and negative results of rapid diagnostic kits using RT-LAMP with LFD are shown in Figure 3.4.

3.3.1.3 Analytical sensitivity and specificity of RT-LAMP with LFD for PEDVs detection

For analytical sensitivity test or minimum detection limit of the assay, ten-fold dilution of DNA standard samples from step 3.3.1.1 were tested in triplicate with RT-LAMP with LFD. The copy numbers of set of DNA standard were $2X10^0$ or 2, $2X10^1$, $2X10^2$, $2X10^3$, $2X10^4$, $2X10^5$, $2X10^6$, $2X10^7$, $2X10^8$, $2X10^9$, and negative.

For analytical specificity test, the RT-LAMP with LFD was applied to test with other important swine pathogens including *E.coli*, PCV2, PDCoV, PRRSV strain EU and US, *Salmonella* Typhimurium and SIV subtypes H1N1 and H3N2. The RT-LAMP with LFD for PEDVs detection was prepared for other swine pathogens as previous described in step 3.3.1.2, to evaluate the specificity of the assay.

3.3.1.4 Diagnostic sensitivity, specificity and agreement of RT-LAMP with LFD for PEDVs detection

In this thesis, positive and negative RNA samples of PEDVs (n=80) were randomly selected and blinded. The blinded samples were tested by qRT-PCR for PEDVs detection and RT-LAMP with LFD. The results of qRT-PCR and RT-LAMP with LFD were compared by two-by-two table. Then, diagnostic sensitivity and specificity

were calculated by the equations in Figure 3.5 and 3.6, respectively. For agreement of RT-LAMP with LFD, Kappa statistic was performed with the equation in Figure 3.7. The two-by-two table to evaluate diagnostic sensitivity, specificity and agreement of the test in this thesis is shown in Table 3.7.

Table 3.5 Primer sets for development of RT-LAMP for PEDVs detection in this thesis.

Primer Name	Sequence (5'-3')	Product size (bps)	Reference
Amplification of M gene of PEDVs			
M-F	CTCAATGTAGTTCCAAT	1,496	Kim et al., 2015
M-R	TTTCTGTTTAGACTAAAT		
RT-LAMP for PEDVs detection			
PED-F3	ACTTTGTCAATAGCATTCGG	NA	This study
PED-B3	AGCCCTCTACAAGCAATG		
PED-FIP	ATCACAGAAGTAGTGAGAAGCGCCAGGACACATTCTTGGTGG		
PED-BIP	CGACAGGTCTGCATTCCAGTCACTAAGGAGTGTTAGCGT		
PED-loop F	Biotin-GTCTGTTTCAGGATTGAAAGA		
PED-loop B	FAM-CTTGGAGCACCAACTGG		

3.3.2 Development of RT-LAMP with LFD for PDCoVs detection

3.3.2.1 Quantitation of DNA standard

A DNA standard was constructed from M gene of PDCoV, ligated into pGEM® (Promega, USA) and evaluated the quantity of DNA using Nanodrop 2000. In detail, the full-length M gene of PDCoV (PDCoV/Swine/Thailand/S5022/2015) was amplified with the primers listed in Table 3.6. PCR reaction, plasmid construction and DNA quantitation were conducted as in 3.3.1.1.

3.3.2.2 RT-LAMP with LFD for PDCoVs detection

To perform RT-LAMP with LFD for PDCoVs detection, primers (n=4) (PDCoV-F3, PDCoV-B3, PDCoV-FIP and PDCoV-BIP) and probes (n=2) (PDCoV-loop F, and PDCoV-loop B) were designed by PrimerExplorer 4.0 (https://primerexplorer.jp/e/) based on 8 conserve regions of M gene of PDCoVs. The primer and probe sequences in this thesis are shown in Table 3.6. RT-LAMP with LFD assays for PDCoVs detection were prepared as previous described in step 3.3.1.2.

3.3.2.3 Analytical sensitivity and specificity of RT-LAMP with LFD for PDCoVs detection

Analytical sensitivity and specificity were performed as previous described in step 3.3.1.3

3.3.2.4 Diagnostic sensitivity, specificity and agreement of RT-LAMP with LFD for PDCoVs detection

Diagnostic sensitivity, specificity and agreement of the test were achieved as previous described in step 3.3.1.4.

Table 3.6 Primer sets for development of RT-LAMP for PDCoVs detection in this thesis.

Primer Name	Sequence (5'-3')	Product size (bps)	Reference
Amplification of M gene of PDCoVs			
DCor-21F	CATTGCCCTGGCTCTTATTG	1,645	This study
DCor-22R	GTGGTTGCTGGGGTATCATT		
RT-LAMP for PDCoVs detection			
PDCoV-F3	CATGGAGGTGCATTCCCA	NA	This study
PDCoV-B3	TCAGAACAGCGAATTCTGGA		
PDCoV-FIP	TTGCCCATGTAGCTTGAGCTTGGACCACATGGCTCCRATT		
PDCoV-BIP	AATCCGCCACAGGATATGGTGACGTTGTTTGATTCCACAGGT		
PDCoV-loop F	Biotin-TGCTTAACGACTGGTGTGAG		
PDCoV-loop B	FAM-AGTGACACCTTTCACTACACTT		

$$Number\ of copies\ per\ \mu L = \frac{Plasmid\ DNA\ concentration\ \ X\ 6.022X10^{23}}{Plasmid\ DNA\ length\ X\ 1X10^{9}X\ 650}$$

Figure 3.3 Equation for conversion of plasmid DNA concentration to copies/µL.

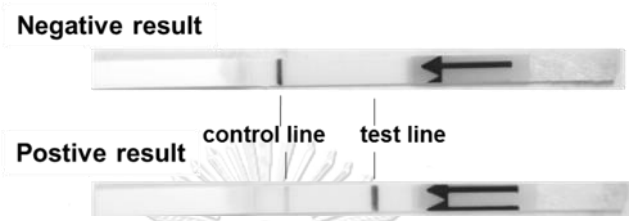

Figure 3.4 Result interpretation of rapid diagnostic kits using RT-LAMP with lateral flow device.

Table 3.7 Diagram of two-by-two table to evaluate diagnostic sensitivity and specificity.

		q RT-PCR result (reference test)	
		Positive	Negative
RT-LAMP result (developed test)	Postive	*a* *(tested positive)*	*b* *(false positive)*
	Negative	*c* *(false negative)*	*d* *(tested negative)*

$$Diagnostic\ sensitivity = \frac{tested\ positive}{tested\ positive + false\ negative} = \frac{a}{a+c}$$

Figure 3.5 Equation for diagnostic sensitivity.

$$Diagnostic\ specificity = \frac{tested\ negative}{tested\ negative + false\ positive} = \frac{d}{b+d}$$

Figure 3.6 Equation for diagnostic specificity.

$$Kappa = \frac{Observe\ agreement - Expected\ agreement}{1 - Expected\ agreement}$$

Where:

$$Observe\ agreement = \frac{a+d}{a+b+c+d}$$

$$Expected\ agreement = \frac{Expected(a)+Expected(d)}{a+b+c+d}$$

$$Expected\ (a) = \frac{(a+b)X(a+c)}{a+b+c+d}$$

$$Expected\ (d) = \frac{(b+d)X(c+d)}{a+b+c+d}$$

Figure 3.7 Equation for agreement of the test (Kappa statistic).

3.3.3 Establishment of DNA aptamer for PEDVs detection

3.3.3.1 Construction of full-length NP gene plasmid

Consensus full-length NP sequence were generated from full-length NP gene of reference PEDVs (n=611) from GenBank database. The consensus was archived codon optimization and cloned to pET-24b(+) by GenScript®. The constructed full-length NP plasmid was transformed to competent *E.coli* strain BL21DE3 (Novagen®, USA) by using heat shock. The protocols for transformation and PCR of T7 primers are shown in Appendix A.

3.3.3.2 NP protein preparation

Transformant of full-length NP gene was cultured in 100 mL of LB/Kanamycin broth and incubate at 37°C for 18 hours. The culture was induced with 200μM IPTG (NEB, USA) at 37°C for 18 hours for protein expression. Recombinant NP protein was extracted and measured by using B-PER® Bacterial Protein Extraction Reagent (ThermoFisher Scientific, USA) and Pierce™ BCA Protein Assay Kit (Invitrogen, USA). The methods for protein expression, protein purification and protein quantification are shown in appendix A.

3.3.3.3 Randomized single-stranded DNA library, aptamers and primers

The single-stranded DNA library (WAP40) comprising of 40 randomized nucleotide and constant DNA sequence of flanking regions for primer binding. The primers and aptamers in this thesis were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences of primers and ssDNA library are shown in Table 3.8.

3.3.3.4 Single round selection of aptamers from ssDNA library

Aptamer candidates against PEDV-NP protein were selected using onestep SELEX protocol (Arnold *et al.*, 2012). A single-stranded aptamer library (WAP40) (Lamont *et al.*, 2014), consisting of a 40-mer randomized regions sequence flanked by primer binding regions, was used (Integrated DNA Technologies, Inc., Coralville, IA).

The elution of binding aptamer was performed using gradient salt (NaCl) elution. The protocol for aptamer selection is shown in Appendix A.

Fifty of white colonies were randomly picked up and analyzed for the presence of the corrected insertion size by direct PCR amplification with M13 forward and M13 reverse primers. The selected sequences were sequenced and aligned, then classified into clusters using MEGA software (http://www.megasoftware.net/) and the similar motif sequences among those sequences were identified by MEME Suite (Bailey and Elkan, 1994)

3.3.3.5 EMSA and competitive EMSA

Biotinylated 2 selected aptamer sequences were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Protein binding assay was analyzed by using a LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Invitrogen, USA) according to the manufacturer's instructions (Appendix A).

3.3.3.6 DNase I footprinting assay

DNase I footprinting assay was performed to identify the binding site(s) of the 2 selected aptamers. The DNase I footprinting assay was performed as described in Appendix A. The binding region of aptamer was analyzed by Peak Scanner software (v.2.0; Applied Biosystems, Foster City, CA) with Liz500 as an internal size standard.

3.3.3.7 Binding affinity of selected aptamers

Enzyme-linked aptamer assay was performed to have an estimation of the affinity binding of each aptamer to recombinant NP protein. The protein was diluted in PBS to be 1 ng/µL and 100 µL of diluted protein samples were added to wells and the plate was stored at 4°C for 16 hours. To remove unbound protein, wells were washed three times with PBST (10 mM PBS, pH 7.2, 0.05% Tween-20). The plate was then blocked with 250 µL/well of 2% bovine serum albumin (BSA) for 1 hour at room temperature, and unbound BSA was removed. Subsequently, 100 µL of different concentrations of 5'biotinylated DNA aptamers in 2-fold dilutions (512–0.25 nM) were

added to each well and binding was allowed for 1 hour at room temperature. The unbound aptamers were removed by washing 5 times with PBST. Streptavidin-horseradish peroxidase conjugate, at a dilution of 1/5000 in PBS, was added and the plate was incubated at room temperature for another 30 minutes. Then, TMB (3,3',5,5'-Tetramethylbenzidine) was added to the plate before an incubation step for 20 minutes. The color reaction was stopped by adding 50 µL of 1 M H_2SO_4. Then, the absorbance was read at 450 nm and the dissociation constant (K_D) calculated, based on a nonlinear regression equation.

3.3.3.8 Limit of detection of selected aptamers against PEDV vaccine

Enzyme-linked aptamer assay was performed to define the limit of detection of 2 selected aptamers. 100 µL of difference concentration of PEDV vaccine (Zoetis, USA) in PBST including 2000, 1000, 500, 100, 50, 10, 5, 1, 0.5, 0.1, 0.01 and 0 $TCID_{50}$/µL were added to wells. The enzyme-linked aptamer assay was performed as previous described in step 3.3.3.7 and the limit of detection of each DNA aptamer was determine by using the 3 times standard deviation (SD) of OD value of negative control.

Table 3.8 Sequences of primers and ssDNA library in this thesis.

Name	Sequence (5'-3')
WAP40	AGTGCAAGCAGTATTCGGTC-N(40)-GGCATCACGCATCAGCTTTA
W20F	AGTGCAAGCAGTATTCGGTC
W20R	TAAAGCTGATGCGTGATGCC
Bio-W20F	Biotin-AGTGCAAGCAGTATTCGGTC
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
T7F	TAATACGACTCACTATAGGG
T7R	GCTAGTTATTGCTCAGCGG

CHAPTER 4

RESULTS

4.1 Surveillance of swine enteric viruses in pig farms

In this thesis, surveillance of swine enteric viruses in pig farms were retrospectively and prospectively performed during December 2014 - January 2018. The total number of samples was 777 including fecal (n=663) and intestinal samples (n=114). The samples were collected from 73 pig farms from 20 provinces of 7 livestock regions including livestock regions 1, 2, 3, 4, 5, 7 and 8. The provinces (n=20) for sample collection in this thesis were Ayutthaya, Burirum, Chachoengsao, Chaiyaphum, Chiang Rai, Chonburi, Kanchanaburi, Khon Kaen, Mukdahan, Nakhon Nayok, Nakhon Pathom, Nakhon Ratchasima, Nakhon Si Thammarat, Prachinburi, Prachuap Khiri Khan, Ratchaburi, Saraburi, Suphanburi, Trang and Ubon Ratchathani. Ages of pigs were classified into 4 age groups including suckling group (less than 4-week-old), nursery group (5 week-old - 8 week-old), fattening group (9 week-old – 20 week-old) and breeder group (boar, gilt and sow).

In this thesis, some pigs with clinical signs were subjected to gross lesions and histopathological examination. The piglet and sow with diarrhea clinical signs in this thesis are shown in figure 4.1A and 4.1B. Emaciated piglet with yellowish feces was examined (Figure 4.1C). Gross lesions showed transparent intestinal wall with watery fluid containing due to swine enteric coronavirus infection (Figure 4.1D). Histopathological lesions showed shortened villi and vacuolated enterocytes (Figure 4.1E and 4.1F).

For the detection of swine enteric viruses, the fecal and intestinal samples were tested with multiplex one-step qRT-PCR for porcine epidemic diarrhea viruses (PEDVs) and porcine deltacoronaviruses (PDCoVs) detection and RT-PCR for

Enteroviruses G (EVGs) detection. Our results showed that the occurrences of PEDVs, PDCoVs and EVGs by samples were 44.02% (342/777), 3.47% (27/777) and 71.56% (556/777), respectively. By pig farms, the occurrences of PEDVs, PDCoVs and EVGs in pig farms were 50.68% (37/73), 9.59% (7/73) and 69.86% (51/73), respectively. By livestock regions, PEDVs could be detected from 5 out of 7 livestock regions including livestock regions 1, 2, 3, 4 and 7. PDCoVs were detected from 2 out of 7 livestock regions including livestock regions 2 and 7, while EVGs could be detected in all livestock regions (Figure 4.2 and 4.3). By ages of pigs, PEDVs could be detected highest in suckling group (48.42%) and followed by nursery group (46.75%) but PDCoVs could be detected mostly in fattening group (6.90%). EVGs mostly detected in nursery group (89.35%) and fattening group (89.66%) (Table 4.5). By time, PEDVs and EVGs could be detected in almost every month and every year but PDCoVs could only be detected in January, June, July 2015; January, November 2016; March, June 2017; and January 2018 with low occurrences (ranging 0%-100%). In this thesis, the representative viruses for PEDVs (n=39), PDCoVs (n=16) and EVGs (n=34) were selected for genetic characterization and phylogenetic analyses. The viruses were selected with the following criteria including 1) location of pig farms, 2) date of sample collection and 3) virus with low cycle threshold value (Ct) (high-RNA copies). The representative viruses for PEDVs, PDCoVs and EVGs selected for genetic characterization in this thesis are shown in Table 4.7, 4.12 and 4.17, respectively.

4.1.1 The occurrences of PEDVs, PDCoVs and EVGs by locations

In this thesis, the samples were collected from 20 provinces of 7 livestock regions. In total, seventy-three farms were included for sample collection. It is noted that 7 out of 9 livestock regions in Thailand were included in this thesis except livestock regions 6 and 9. Eleven samples from 4 farms had no record of provinces and livestock regions.

4.1.1.1 The occurrences of PEDVs

By pig farms, the occurrences of PEDVs showed that 5 out of 7 livestock regions were tested positive for PEDVs. The highest occurrence of PEDVs was observed in livestock region 1 (100%; 2/2) and followed by livestock regions 2 (62.50%; 10/16), 3 (61.54%; 8/13), 7 (53.13%; 16/32) and 4 (50.00%; 1/2) (Figure 4.2 and Table 4.1). By samples, the occurrence of PEDVs was highest in livestock region 4 (71.43%; 10/14) and followed by livestock regions 7 (52.17%; 49/115), 3 (47.45%; 205/432), 2 (36.31%; 61/168) and 1 (33.33%; 6/18) (Figure 4.3 and Table 4.1). By provinces, PEDVs could not be found in 5 provinces including Chiang Rai, Mukdahan, Nakhon Si Thammarat, Suphanburi and Trang (Figure 4.4 and Table 4.1).

4.1.1.2 The occurrences of PDCoVs

By pig farms, the occurrences of PDCoVs showed that 2 livestock regions, including livestock regions 2 and 7, were tested positive for PDCoVs. The highest occurrence of PDCoVs was found in livestock region 2 (25%; 4/16) (Figure 4.2 and Table 4.1). By samples, the highest occurrence of PEDVs was also found in livestock region 2 (10.12%; 17/168) (Figure 4.3 and Table 4.1). By provinces, the PDCoVs could be detected in 5 provinces including 3 provinces (Chachoengsao, Chonburi and Prachinburi) from livestock region 2 and 2 provinces (Nakhon Pathom and Ratchaburi) from livestock region 7 (Figure 4.5 and Table 4.1).

4.1.1.3 The occurrences of EVGs

By pig farms, the occurrences of EVGs showed that all livestock regions were positive for EVGs ranging from 50% to 100% (Figure 4.2 and Table 4.1). Two livestock regions (1 and 5) showed 100% positive for EVGs (Figure 4.3 and Table 4.1). By provinces, our results showed that six provinces, including Ayutthaya, Burirum, Chiang Rai, Khon Kaen, Saraburi, and Trang, showed 100% positive for EVGs (Figure 4.6 and Table 4.1).

In summary, the occurrences of PEDVs, PDCoVs and EVGs by pig farms and samples are shown in graphs in Figure 4.2 and 4.3. The details of sample collection and occurrences of PEDVs, PDCoVs and EVGs from each livestock region and each

province by pig farms and samples are shown in Table 4.1. The distributions of PEDVs, PDCoVs and EVGs by pig farms and samples in each province are shown in Figure 4.4, 4.5 and 4.6.

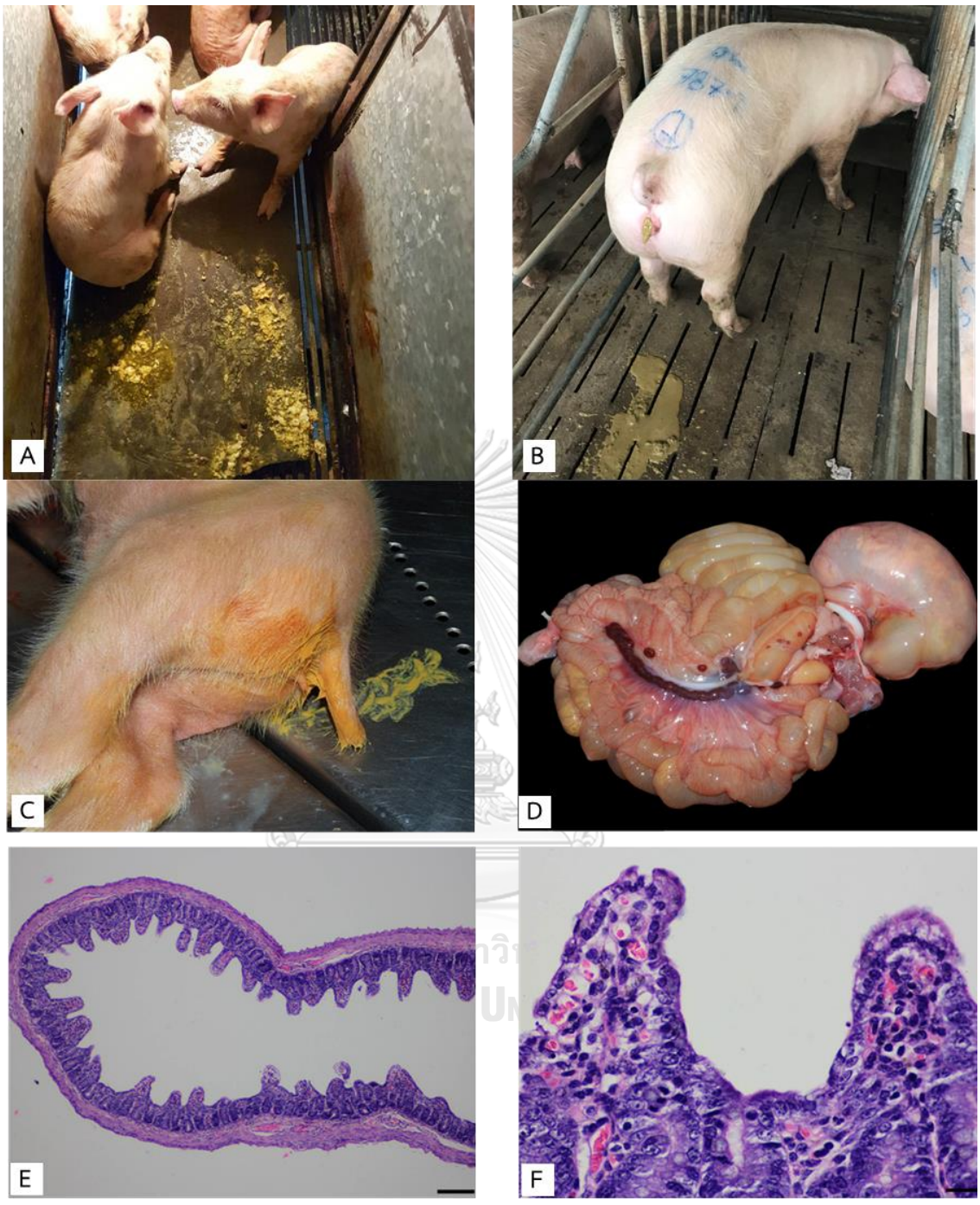

Figure 4.1 Gross lesions and histopathological lesions of swine enteric coronavirus infection. A) Diarrheic piglet in farm. B) Diarrheic sow in farm. C) Emaciated piglet showing yellowish feces staining around anal areas. D) Curdled milk in gastric lumen and transparent intestinal wall. E) Histopathological lesion showing shortened villi. Scale bar = 400 μm. F) Histopathological analysis showing vacuolated cytoplasm of enterocytes. Scale bar = 40 μm (Adapted from Janetanakit et al., 2016)

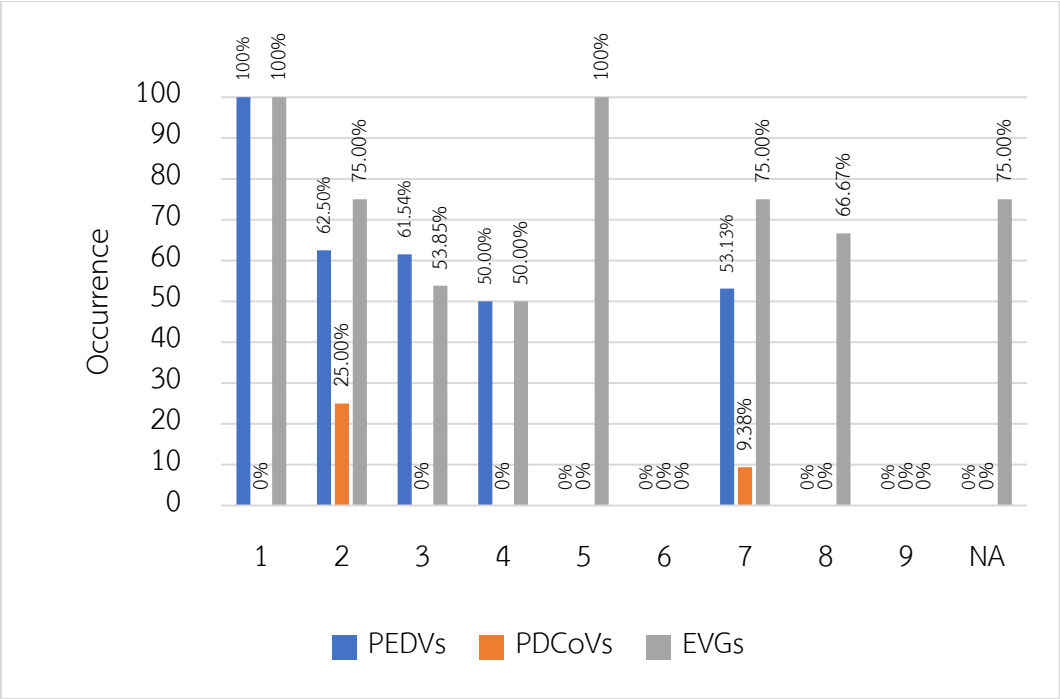

Figure 4.2 Occurrences of PEDVs, PDCoVs and EVGs by farms and livestock regions.

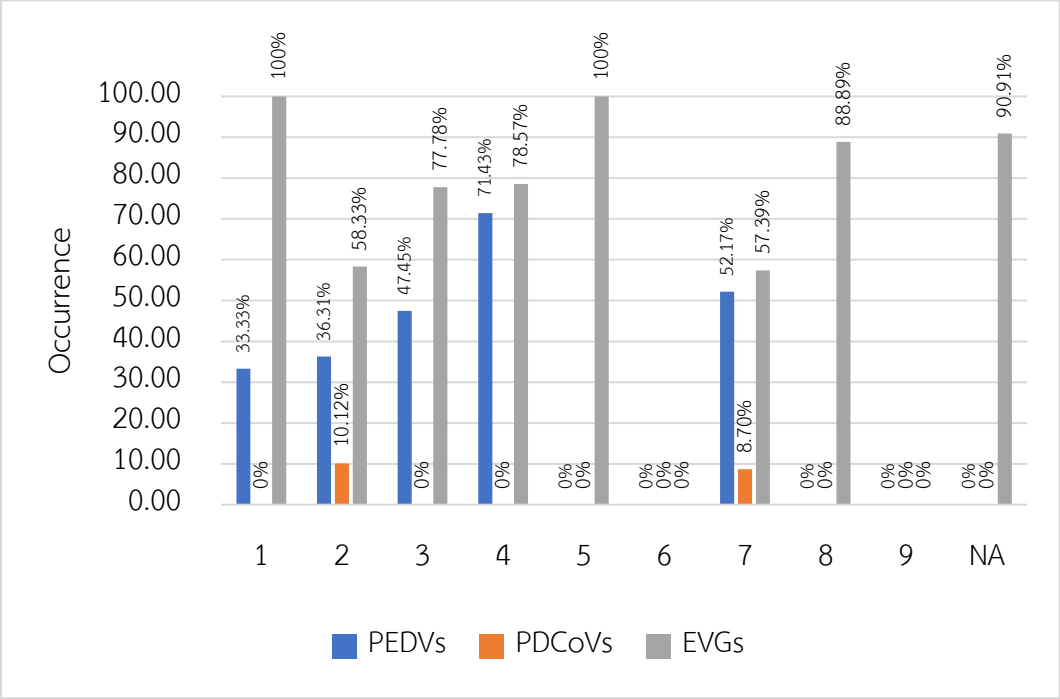

Figure 4.3 Occurrences of PEDVs, PDCoVs and EVGs by samples and livestock regions.

Table 4.1 Occurrences of PEDVs, PDCoVs and EVGs by livestock regions and provinces.

Livestock region	Province	PEDVs		PDCoVs		EVGs	
		Positive farm (%)	Positive sample (%)	Positive farm (%)	Positive sample (%)	Positive farm (%)	Positive sample (%)
1	Ayutthaya	1/1 (100%)	3/15 (33.33%)	0/1 (0%)	0/15 (0%)	1/1 (100%)	15/15 (100%)
	Saraburi	1/1 (100%)	3/3 (100%)	0/1 (0%)	0/3 (0%)	1/1 (100%)	3/3 (100%)
	Total	**2/2** (100%)	**6/18 (33.33%)**	**0/2** (0%)	**0/18 (0%)**	**2/2** (100%)	**18/18 (100%)**
2	Chachoengsao	3/4 (75%)	8/24 (33.33)	1/4 (25%)	3/24 (12.5%)	4/4 (100%)	18/24 (75%)
	Chonburi	4/7 (57.14%)	38/54 (70.37%)	2/7 (28.57%)	4/54 (7.41%)	4/7 (57.14%)	24/54 (44.44%)
	Nakhon Nayok	1/2 (50%)	1/12 (8.33%)	0/2 (0%)	0/12 (0%)	1/2 (50%)	1/12 (8.33%)
	Prachinburi	1/3 (33.33%)	14/78 (17.95%)	1/3 (33.33%)	10/78 (12.82%)	3/3 (100%)	55/78 (70.51%)
	Total	**10/16 (62.50%)**	**61/168 (36.31%)**	**4/16 (25%)**	**17/168 (10.12%)**	**12/16 (75%)**	**98/168 (58.33%)**
3	Burirum	1/1 (100%)	5/5 (100%)	0/1 (0%)	0/5 (0%)	1/1 (100%)	5/5 (100%)
	Chaiyaphum	1/1 (100%)	2/2 (100%)	0/1 (0%)	0/2 (0%)	0/1 (0%)	0/2 (0%)
	Nakhon Ratchasima	4/9 (44.44%)	193/417 (46.28%)	0/9 (0%)	0/417 (0%)	5/9 (55.56%)	330/417 (79.14%)
	Ubon Ratchathani	2/2 (100%)	5/8 (62.50%)	0/2 (0%)	0/8 (0%)	1/2 (50%)	1/8 (12.5%)
	Total	**8/13 (61.54%)**	**205/432 (47.45%)**	**0/13 (0%)**	**0/432 (0%)**	**7/13 (53.85%)**	**336/432 (77.78%)**
4	Khon Kaen	1/1 (100%)	10/11 (90.91%)	0/1 (0%)	0/11 (0%)	1/1 (100%)	11/11 (100%)
	Mukdahan	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/3 (0%)
	Total	**1/2 (50%)**	**10/14 (71.43%)**	**0/2 (0%)**	**0/14 (0%)**	**1/2 (50%)**	**11/14 (78.57%)**
5	Chiang Rai	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	1/1 (100%)	1/1 (100%)
	Total	**0/1 (0%)**	**0/1 (0%)**	**0/1 (0%)**	**0/1 (0%)**	**1/1 (100%)**	**1/1 (100%)**
7	Kanchanaburi	1/2 (50%)	1/10 (10%)	0/2 (0%)	0/10 (0%)	2/2 (100%)	6/10 (60%)
	Nakhon Pathom	7/10 (70%)	14/30 (46.67%)	2/10 (20%)	8/30 (2.67%)	7/10 (70%)	11/30 (36.67%)
	Prachuap Khiri Khan	3/3 (100%)	24/27 (88.89%)	0/3 (0%)	0/27 (0%)	3/3 (100%)	26/27 (96.30%)
	Ratchaburi	6/14 (42.86%)	21/41 (51.22%)	1/14 (7.14%)	2/41 (4.88%)	10/14 (71.43%)	20/41 (48.78%)
	Suphanburi	0/3 (0%)	0/7 (0%)	0/3 (0%)	0/7 (0%)	2/3 (66.67%)	3/7 (42.86%)
	Total	**16/32 (50%)**	**49/115 (42.61%)**	**3/32 (9.38%)**	**10/115 (8.70%)**	**23/32 (71.88%)**	**52/115 (45.22%)**
8	Nakhon Si Thammarat	0/1 (0%)	0/2 (0%)	0/1 (0%)	0/2 (0%)	0/1 (0%)	0/2 (0%)
	Trang	0/2 (0%)	0/16 (0%)	0/2 (0%)	0/16 (0%)	2/2 (100%)	16/16 (100%)
	Total	**0/3 (0%)**	**0/18 (0%)**	**0/3 (0%)**	**0/18 (0%)**	**2/3 (66.67%)**	**16/18 (88.89%)**
N/A	N/A	0/4 (0%)	0/11 (0%)	0/4 (0%)	0/11 (0%)	3/4 (75%)	10/11 (90.90%)
Total		**37/73 (50.68%)**	**342/777 (44.02%)**	**7/73 (9.59%)**	**27/777 (3.47%)**	**51/73 (69.86%)**	**556/777 (71.56%)**

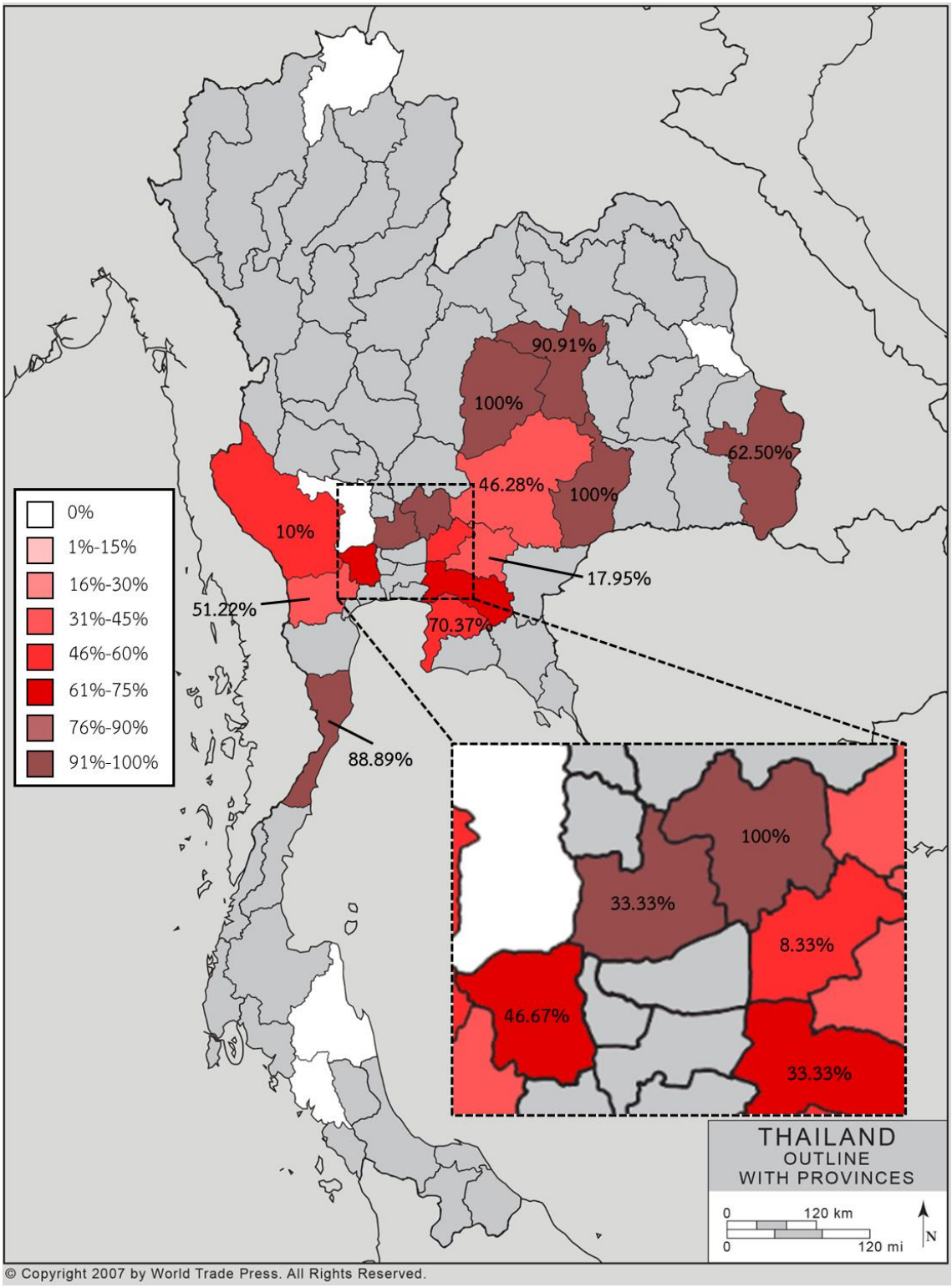

Figure 4.4 Distribution of PEDVs by provinces. The highlighted provinces represent the occurrence of PEDVs by farms and the number represent the occurrence of PEDVs by samples in each province.

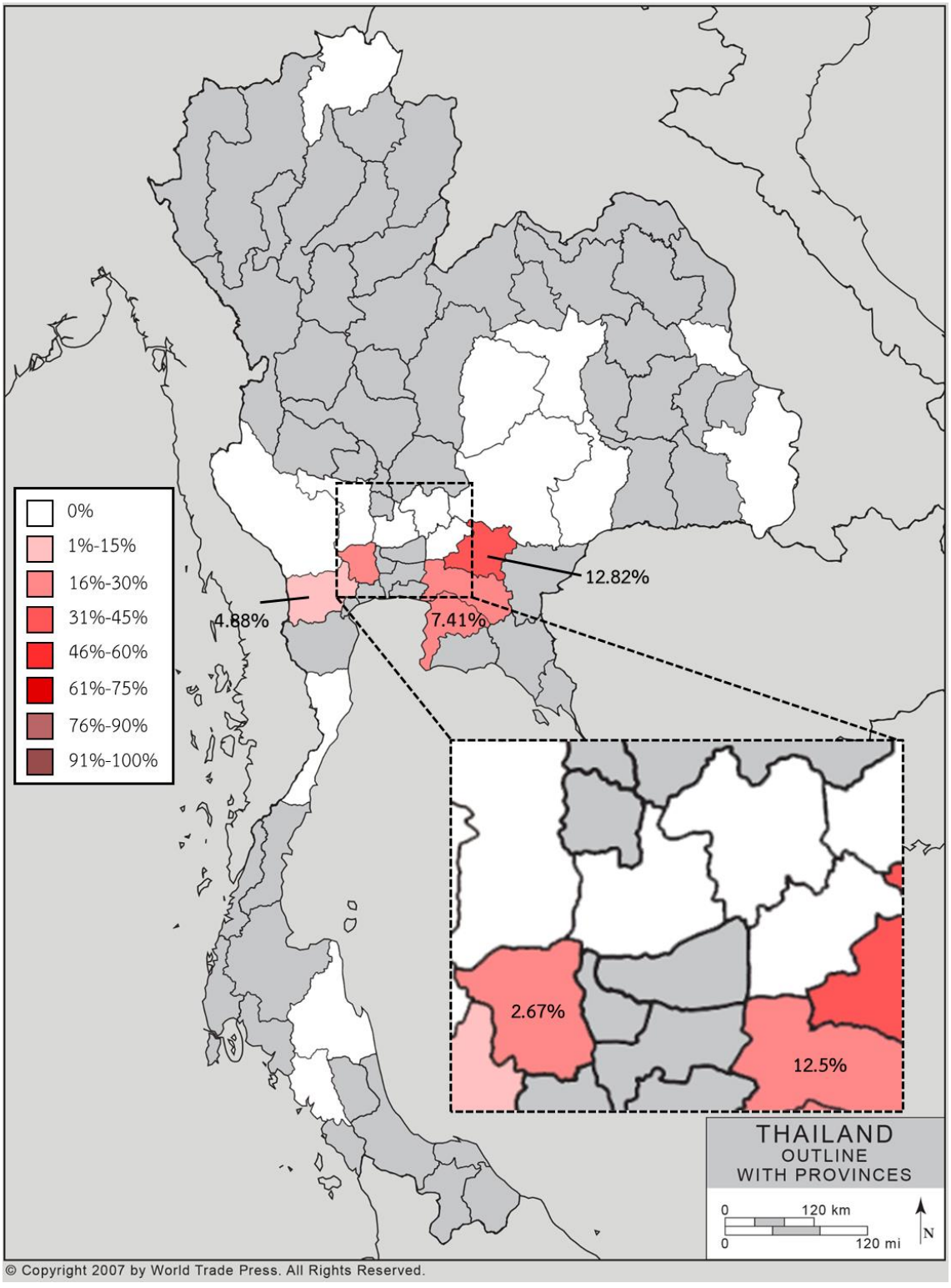

Figure 4.5 Distribution of PDCoVs by provinces. The highlighted provinces represent the occurrence of PDCoVs by farms and the number represent the occurrence of PDCoVs by samples in each province.

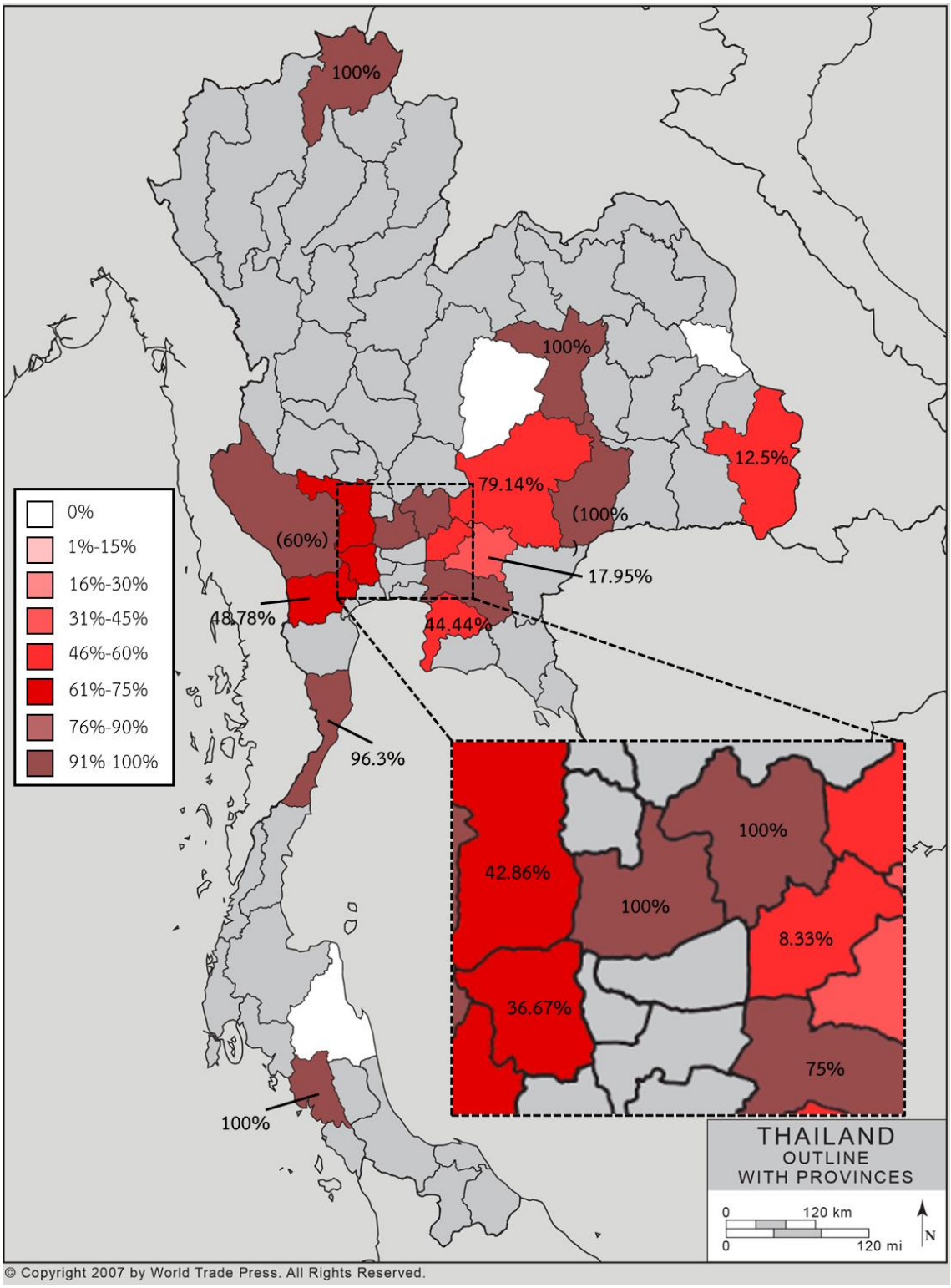

Figure 4.6 Distribution of EVGs by provinces. The highlighted provinces represent the occurrence of EVGs by farms and the number represent the occurrence of PDCoVs by samples in each province.

4.1.2 The occurrences of PEDVs, PDCoVs and EVGs by age groups of pigs

Pigs are classified into 4 groups, including suckling group, nursery group, fattening group and breeder group, based on pig age and type of pig. In this thesis, the samples were collected from suckling group (less than 4-week-old) (n=444), nursery group (5 week-old - 8 week-old) (n=169), fattening group (9 week-old - 20 week-old) (n=58) and breeder group (boar, gilt and sow) (n=106).

4.1.2.1 The occurrences of PEDVs

The occurrence of PEDVs was highest in suckling group (48.42%; 215/444) and followed by nursery group (46.75%; 79/169), fattening group (32.76%; 19/58) and breeder group (27.36%; 29/106) (Figure 4.7 and Table 4.2). Statistical analysis by chi-square test, the suckling and nursery groups showed statistically significant higher than breeder group, but only suckling group show statistically significant higher than fattening group (Table 4.3).

4.1.2.2 The occurrences of PDCoVs

The occurrence of PDCoVs was highest in fattening group (6.90%; 4/58) and followed by suckling group (3.83%; 17/444), breeder group (2.83%; 3/106) and nursery group (1.78%; 3/169) (Figure 4.7 and Table 4.2). Statistical analysis by chi-square test, there is no statistically significant difference among age groups (Table 4.3).

4.1.2.3 The occurrences of EVGs

the occurrence of EVGs was highest in fattening group (89.66%; 52/58) and followed by nursery group (89.35%; 151/169), suckling group (64.86%; 288/444) and breeder group (61.32%; 65/106) (Figure 4.7 and Table 4.2). Statistical analysis by chi-square test, the nursery and fattening groups showed statistically significant higher than suckling and breeder groups and no statistically significant difference between nursery and fattening groups (Table 4.3).

In summary, the comparisons of occurrences of PEDVs, PDCoVs and EVGs among age groups are shown in graphs in Figure 4.7 and Table 4.2. The statistical analysis results by chi-square test among age groups is shown in Table 4.3.

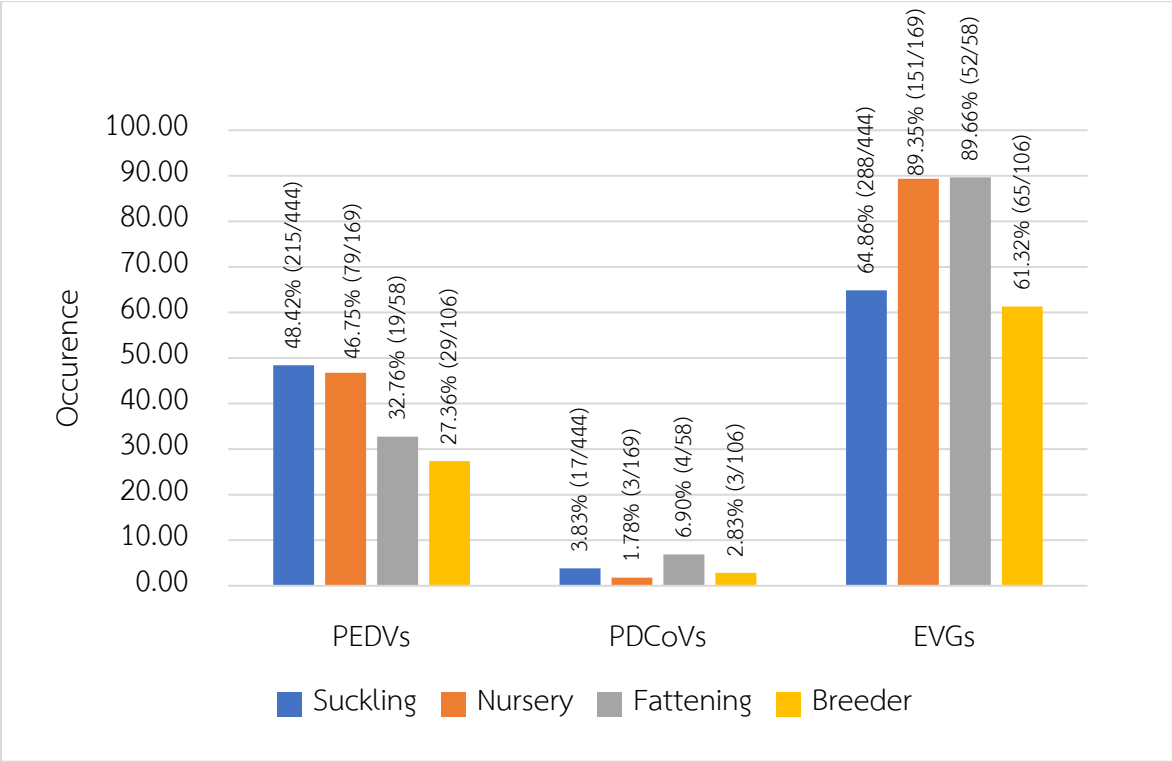

Figure 4.7 Occurrences of PEDVs, PDCoVs and EVGs by age groups of pigs.

Table 4.2 Occurrences of PEDVs, PDCoVs and EVGs by age groups of pigs.

Age group of pigs	Age	PEDVs	PDCoVs	EVGs
Suckling (n=444)	< 4 weeks	215/444 (48.42%)*	17/444 (3.83%)	288/444 (64.86%)
Nursery (n=169)	5 - 8 weeks	79/169 (46.75%)*	3/169 (1.78%)	151/169 (89.35%)*
Fattening (n=58)	9 - 20 weeks	19/58 (32.76%)	4/58 (6.90%)	52/58 (89.66%)*
Breeder (n=106)	boar, gilt and sow	29/106 (27.36%)	3/106 (2.83%)	65/106 (61.32%)
Total (n=777)		**342/777 (44.02%)**	**27/777 (3.47%)**	**556/777 (71.56%)**

* indicate the statistical significance (p≤0.05)

Table 4.3 Statistical analysis (p-value) by Chi-square test of the occurrences of PEDVs, PDCoVs and EVGs among age groups of pigs.

Comparison of age groups	PEDVs	PDCoVs	EVGs
All age groups	0.000	0.287	0.000
Suckling and Nursery	0.718	0.308	0.000
Suckling and Fattening	0.017	0.287	0.000
Suckling and Breeder	0.000	0.778	0.501
Nursery and Fattening	0.067	0.073	1.000
Nursery and Breeder	0.001	0.679	0.000
Fattening and Breeder	0.478	0.245	0.000

Number in grey boxes are P-value ≤0.05

4.1.3 The occurrences of PEDVs, PDCoVs and EVGs by seasonal patterns

Thai meteorological department (TMD) has classified Thai weather into 3 seasons including summer or hot season starting from mid-February to mid-May, rainy season starting from mid-May to mid-October and winter or cold season starting from mid-October to mid-February. In this thesis, samples from diarrheic pigs (n=777) were collected during December 2014 to January 2018 including sample collection during summer season (n=167), rainy season (n=451) and winter season (n=159).

4.1.3.1 The occurrences of PEDVs

The occurrences of PEDVs by time of sample collection showed that 31 out of 36 months were positive for PEDVs in ranging from 25% to 100% (by pig farms) and 15.38% up to 100% (by samples) (Table4.4). It should be noted that PEDVs were mostly detected in summer season (46.11%; 77/167) but no statistically significant higher than rainy (44.79%) and winter season (36.72%) (Figure 4.8, Table 4.5 and Table 4.6).

4.1.3.2 The occurrences of PDCoVs

The occurrences of PDCoVs by time of sample collection showed that PDCoVs could be found in January, June and July in 2015; January and November in 2016; March and June in 2017; and January in 2018. The occurrences of PDCoVs were ranging from 20% to 100% (by pig farms) and 7.32% to 100% (by samples). The highest occurrence was found in July in 2015 while the lowest occurrence was found in March in 2017 (Table 4.4). Furthermore, statistical analysis by chi-square test showed that the occurrence of PDCoVs during winter season was statistically significant higher than summer and rainy seasons (Figure 4.8, Table 4.5 and Table 4.6).

4.1.3.3 The occurrences of EVGs

The occurrences of PDCoVs by time of sample collection showed that EVGs could be detected in almost every month (except May in 2015). The occurrences of EVGs were ranging from 0% to 100% (by pig farms and by samples) (Table 4.4).

Statistical analysis by chi-square test suggested that the occurrence of EVGs during rainy season was statistically significant higher than summer and winter seasons.

In summary, the occurrences of PEDVs, PDCoVs and EVGs by months and years are shown Table 4.4. The comparisons of occurrences of PEDVs, PDCoVs and EVGs among seasons are shown in Figure 4.8 and Table 4.5. The statistical analysis results by chi-square test among seasons are shown in Table 4.6.

Table 4.4 Occurrences of PEDVs, PDCoVs and EVGs by months and years.

	PEDVs		PDCoVs		EVGs	
Collection date	Positive farm (%)	Positive sample (%)	Positive farm (%)	Positive sample (%)	Positive farm (%)	Positive sample (%)
Dec 2014	1/1 (100.00%)	1/2 (50.00%)	0/1(0.00%)	0/2 (0.00%)	1/1 (100.00%)	2/2 (100.00%)
Jan 2015	1/2 (50.00%)	3/4 (75.00%)	1/2 (50.00%)	1/4 (25.00%)	2/2 (100.00%)	2/4 (50.00%)
Feb 2015	2/4 (50.00%)	3/7 (42.86%)	0/4 (0.00%)	0/7 (0.00%)	3/4 (75.00%)	5/7 (71.43%)
Mar 2015	1/2 (50.00%)	2/4 (50.00%)	0/2 (0.00%)	0/4 (0.00%)	2/2 (100.00%)	2/4 (50.00%)
Apr 2015	1/2 (50.00%)	1/2 (50.00%)	0/2 (0.00%)	0/2 (0.00%)	1/2 (50.00%)	1/2 (50.00%)
May 2015	1/1 (100.00%)	1/1 (100.00%)	0/1 (0.00%)	0/1 (0.00%)	0/1 (0.00%)	0/1 (0.00%)
Jun 2015	2/4 (50.00%)	5/15 (33.33%)	1/4 (25.00%)	6/15 (66.67%)	2/4 (50.00%)	6/15 (66.67%)
Jul 2015	0/1 (0.00%)	0/4 (0.00%)	1/1 (100.00%)	4/4 (100%)	1/1 (100.00%)	4/4 (100.00%)
Aug 2015	1/2 (50.00%)	1/5 (20.00%)	0/2 (0.00%)	0/5 (0.00%)	1/2 (50.00%)	4/5 (80.00%)
Sep 2015	NA	NA	NA	NA	NA	NA
Oct 2015	NA	NA	NA	NA	NA	NA
Nov 2015	0/2 (0.00%)	0/11 (0.00%)	0/2 (0.00%)	0/11 (0.00%)	1/2 (50.00%)	10/11 (90.91%)
Dec 2015	0/1 (0.00%)	0/2 (0.00%)	0/1 (0.00%)	0/2 (0.00%)	1/1 (100.00%)	1/2 (50.00%)
Jan 2016	2/3 (66.66%)	9/11 (81.82%)	1/3 (33.33%)	1/11 (9.09%)	2/3 (66.67%)	3/11 (27.27%)
Feb 2016	4/5 (80.00%)	12/15 (80.00%)	0/5 (0.00%)	0/15 (0.00%)	1/5 (20.00%)	3/15 (33.33%)
Mar 2016	2/4 (50.00%)	5/12 (41.67%)	0/4 (0.00%)	0/12 (0.00%)	1/4 (25.00%)	1/12 (8.33%)
Apr 2016	1/4 (25.00%)	10/21 (47.62%)	0/4 (0.00%)	0/21 (0.00%)	4/4 (100.00%)	20/21 (95.24%)
May 2016	2/4 (50.00%)	11/43 (25.58%)	0/4 (0.00%)	0/43 (0.00%)	4/4 (100.00%)	33/43 (76.74%)
Jun 2016	0/3 (0.00%)	0/18 (0.00%)	0/3 (0.00%)	0/18 (0.00%)	2/3 (66.67%)	12/18 (66.67%)
Jul 2016	2/3 (66.66%)	11/44 (25.00%)	0/3 (0.00%)	0/44 (0.00%)	2/3 (66.67%)	39/44 (88.64%)
Aug 2016	1/2 (50.00%)	10/38 (26.32%)	0/2 (0.00%)	0/38 (0.00%)	2/2 (100.00%)	34/38 (89.47%)
Sep 2016	2/4 (50.00%)	10/27 (37.04%)	0/4 (0.00%)	0/27 (0.00%)	4/4 (100.00%)	21/27 (77.78%)
Oct 2016	2/2 (100.00%)	13/16 (81.25%)	0/2 (0.00%)	0/16 (0.00%)	2/2 (100.00%)	16/16 (100.00%)
Nov 2016	4/5 (80.00%)	20/61 (32.79%)	1/5 (20.00%)	7/61 (11.48%)	4/5 (80.00%)	20/61 (32.79%)
Dec 2016	0/3 (0.00%)	0/6 (0.00%)	0/3 (0.00%)	0/6 (0.00%)	3/3 (100.00%)	4/6 (66.67%)
Jan 2017	1/2 (50.00%)	5/7 (71.43%)	0/2 (0.00%)	0/7 (0.00%)	2/2 (100.00%)	6/7 (85.71%)
Feb 2017	3/5 (60.00%)	7/29 (24.14%)	0/5 (0.00%)	0/29 (0.00%)	3/5 (60.00%)	12/29 (41.38%)
Mar 2017	3/9 (33.33%)	23/41 (56.10%)	1/9 (11.11%)	3/41 (7.32%)	6/9 (66.67%)	18/41 (43.90%)
Apr 2017	4/6 (66.66%)	16/25 (80.00%)	0/6 (0.00%)	0/25 (0.00%)	5/6 (83.33%)	14/25 (56.00%)
May 2017	3/3 (100%)	25/55 (45.45%)	0/3 (0.00%)	0/55 (0.00%)	3/3 (100.00%)	51/55 (92.73%)
Jun 2017	2/3 (66.66%)	5/12 (41.67%)	1/3 (33.33%)	3/12 (25.00%)	3/3 (100.00%)	10/12 (83.33%)
Jul 2017	1/3 (33.33%)	9/26 (34.62%)	0/3 (0.00%)	0/26 (0.00%)	2/3 (66.67%)	21/26 (80.77%)
Aug 2017	1/1 (100.00%)	14/32 (43.75%)	0/1 (0.00%)	0/32 (0.00%)	1/1 (100.00%)	32/32 (100.00%)
Sep 2017	1/1 (100.00%)	76/118 (64.41%)	0/1 (0.00%)	0/118 (0.00%)	1/1 (100.00%)	100/118 (84.75%)
Oct 2017	1/2 (50.00%)	25/38 (65.79%)	0/2 (0.00%)	0/38 (0.00%)	2/2 (100.00%)	26/38 (68.42%)
Nov 2017	1/1 (100.00%)	2/13 (15.38%)	0/1 (0.00%)	0/13 (0.00%)	1/1 (100.00%)	12/13 (92.31%)
Dec 2017	1/1 (100.00%)	1/5 (20.00%)	0/1 (0.00%)	0/5 (0.00%)	1/1 (100.00%)	4/5 (80.00%)
Jan 2018	1/1 (100.00%)	6/7 (85.71%)	1/1 (100.00%)	2/7 (28.57%)	1/1 (100.00%)	7/7 (100.00%)

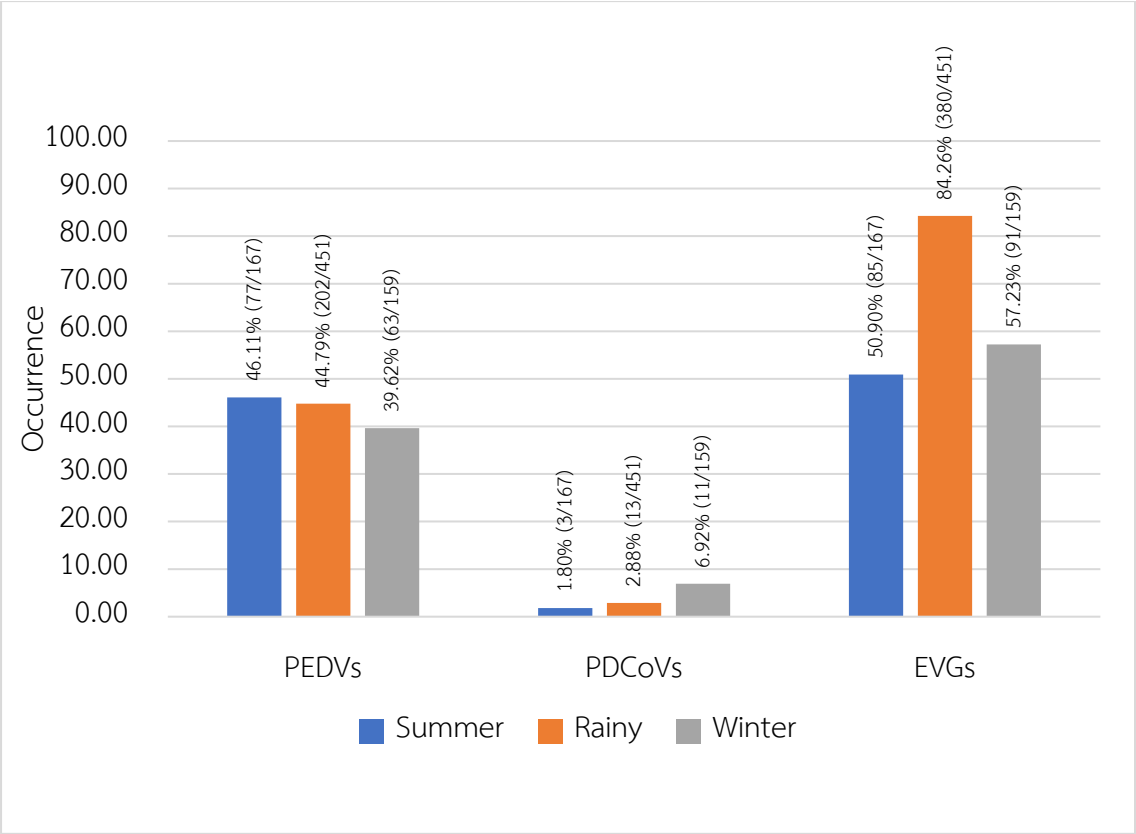

Figure 4.8 Occurrences of PEDVs, PDCoVs and EVGs by seasons.

Table 4.5 Occurrences of PEDVs, PDCoVs and EVGs by seasons.

Season (date)	PEDVs	PDCoVs	EVGs
Summer (16 Feb – 15 May)	77/167 (46.11%)	3/167 (1.80%)	85/167 (50.90%)
Rainy (16 May – 15 Oct)	202/451 (44.79%)	13/451 (2.88%)	380/451 (84.26%)*
Winter (16 Oct – 15 Feb)	63/159 (39.62%)	11/159 (6.92%)*	91/159 (57.23%)
Total	**342/777 (44.02%)**	**27/777 (3.47%)**	**556/777 (71.56%)**

* indicate the statistical significance (p≤0.05)

Table 4.6 Statistical analysis (p-value) by Chi-square test of the occurrences of PEDVs, PDCoVs and EVGs among seasons.

Comparison of seasons	PEDVs	PDCoVs	EVGs
All seasons	0.438	0.024	0.000
Summer and Rainy	0.785	0.577	0.000
Summer and Winter	0.264	0.028	0.268
Rainy and Winter	0.266	0.032	0.000

Number in grey boxes are P-value ≤0.05

4.1.4 Analysis of co-circulation of PEDVs, PDCoVs and EVGs

In this thesis, co-circulation of PEDVs, PDCoVs and EVGs was analyzed. The co-circulation can be found as 8 patterns including positive samples to PEDVs, PDCoVs and EVGs (0.13%); positive samples to PDCoVs and EVGs (1.67%); positive samples to PEDVs and EVGs (30.37%); positive samples to PEDVs and PDCoVs (0%); positive samples to EVGs (39.38%); positive samples to PDCoVs (1.67%); positive samples to PEDVs (13.51%); and negative samples to all viruses (13.26%). The details of co-circulation patterns are shown in Figure 4.9.

4.1.4.1 Co-circulation of PEDVs, PDCoVs and EVGs in suckling, nursery, fattening and breeder groups

For suckling group (n=444), infection of EVGs was highest detected (34.23%; 152/444). Co-circulation of PEDVs and EVGs and co-circulation of PDCoVs and EVGs were observed (Figure 4.10).

For nursery group (n=169), infection of EVGs was highest detected (48.52%) and followed by sample positive to both PEDVs and EVGs (39.05%); sample positive to PEDVs (7.69%); sample negative to all viruses (2.96%); and sample positive to PDCoVs and EVGs (1.78%) (Figure 4.11).

For fattening group (n=58), the highest detection was EVGs accounting for 53.45% (31/58). The co-circulation of PEDVs and PDCoVs was not observed (Figure 4.12).

For breeder group (n=106), the highest detection was EVGs group. It is noted that 1 pig from a farm in Ratchaburi was positive for co-circulation of PEDVs, PDCoVs and EVGs (Figure 4.13).

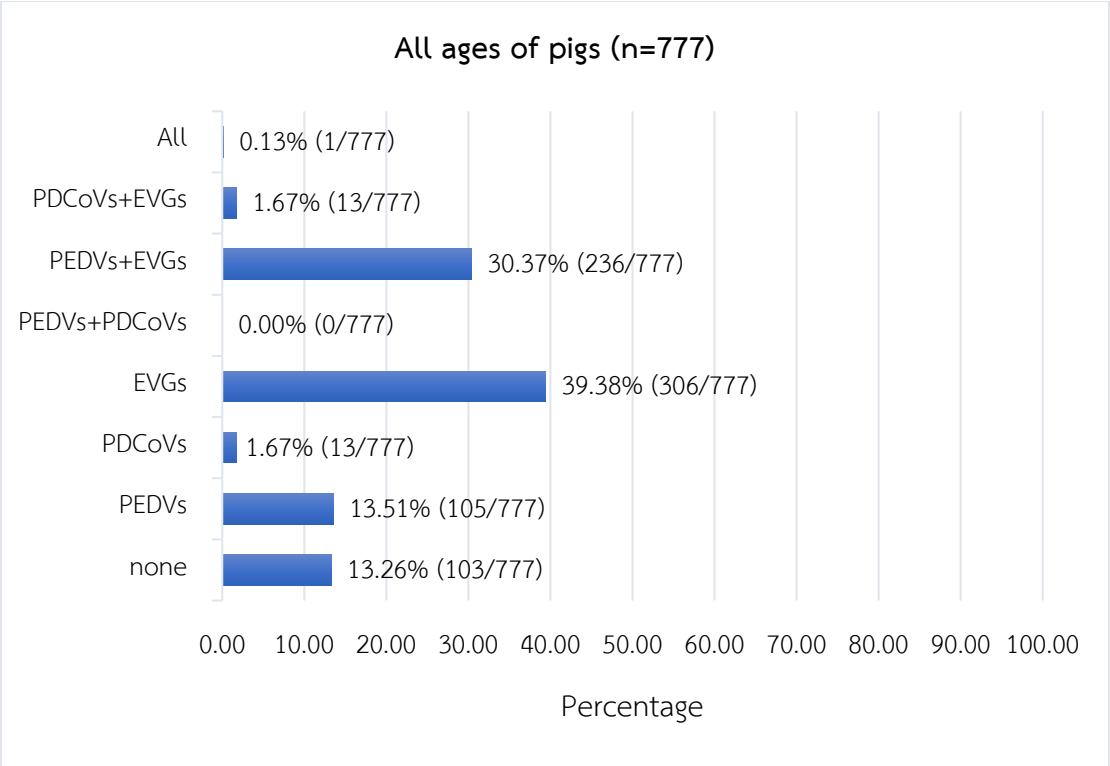

Figure 4.9 Percentage of co-circulation of swine enteric viruses in all ages of pigs.

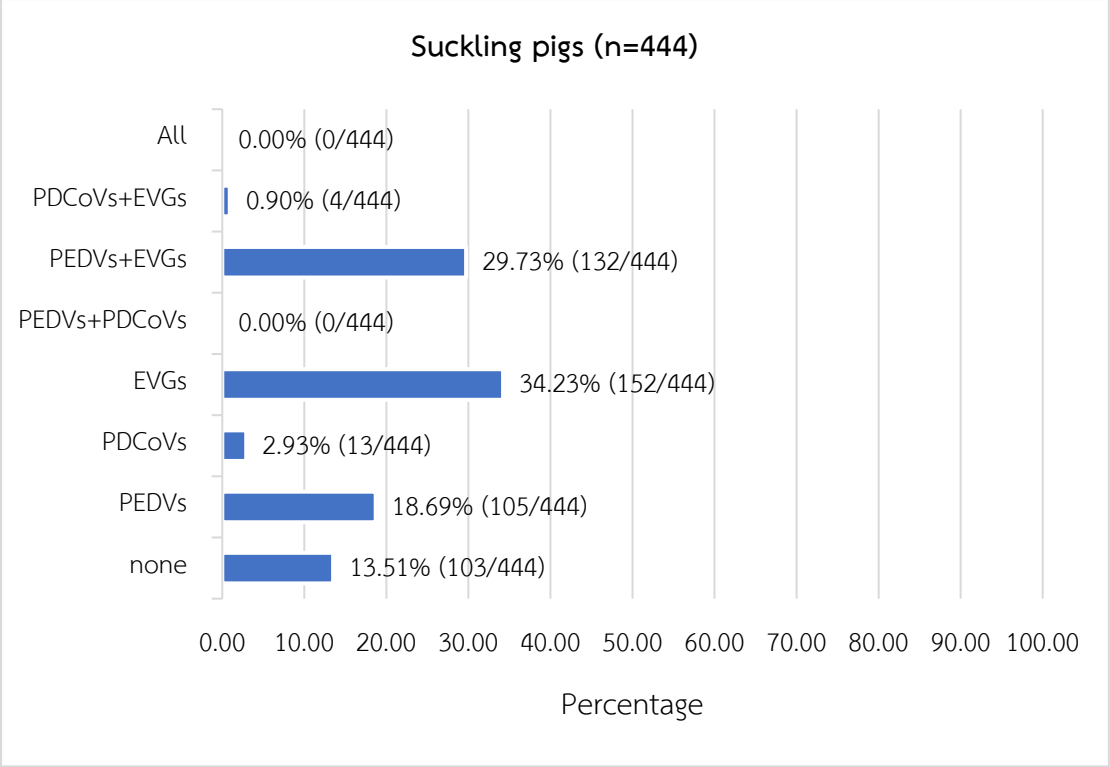

Figure 4.10 Percentage of co-circulation of swine enteric viruses in suckling pigs.

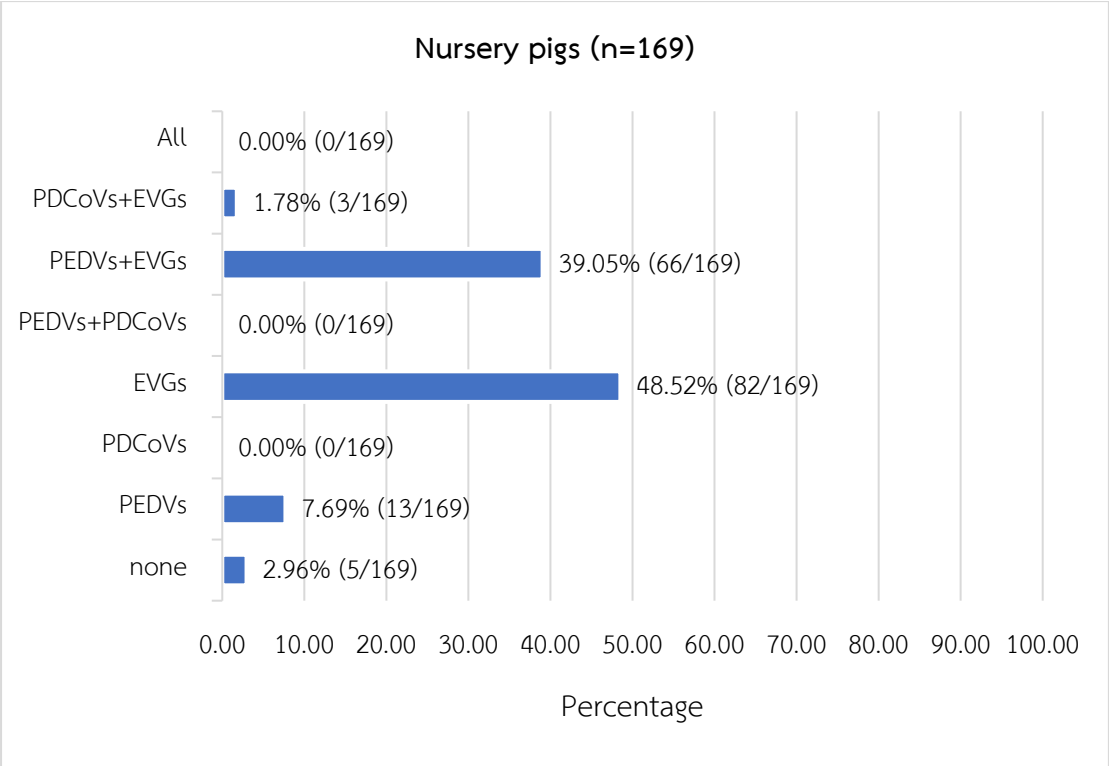

Figure 4.11 Percentage of co-circulation of swine enteric viruses in nursery pigs.

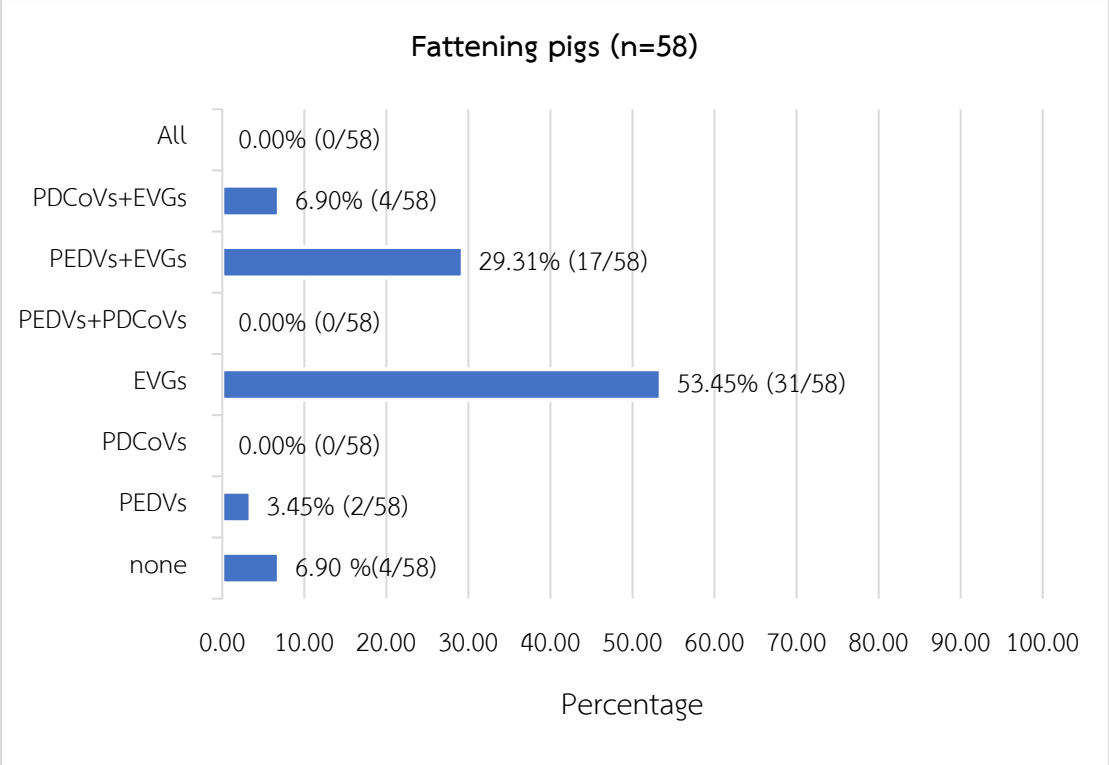

Figure 4.12 Percentage of co-circulation of swine enteric viruses in fattening pigs.

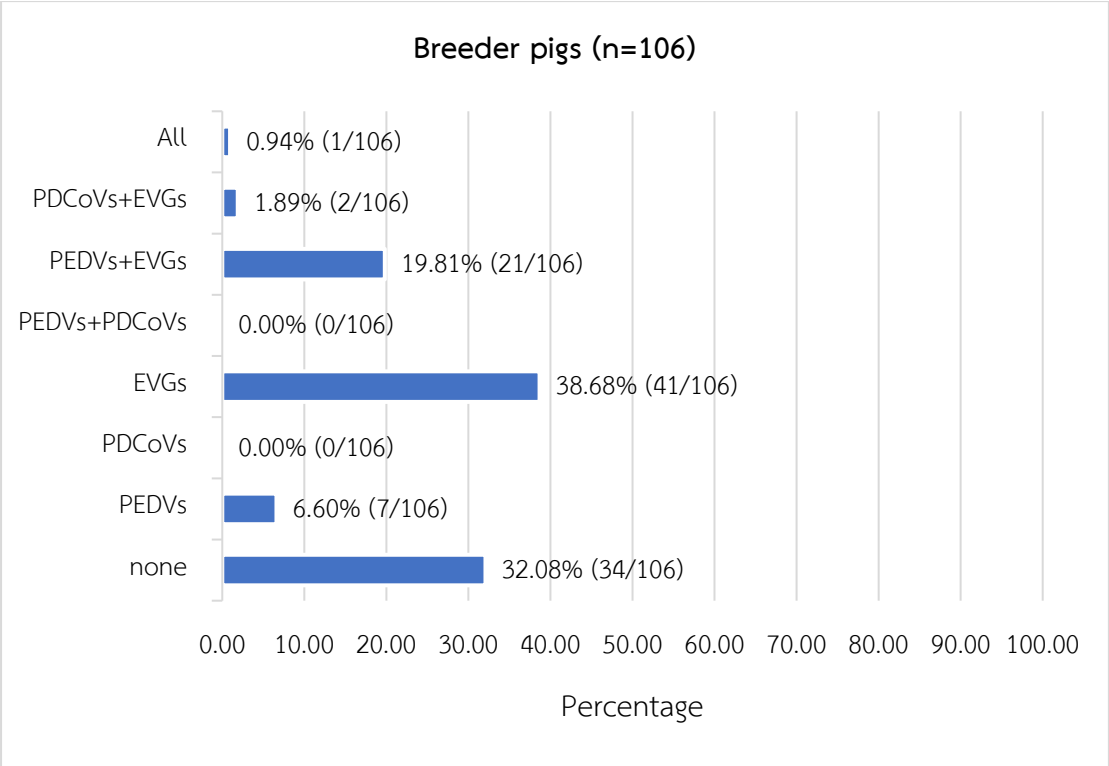

Figure 4.13 Percentage of co-circulation of swine enteric viruses in breeder pigs.

4.2 Genetic characterization and phylogenetic analyses of swine enteric viruses

In this thesis, representative PEDVs (n=39) were selected from 342 positive samples. Thai-PEDVs were subjected to Spike (S) and ORF3 genes sequencing. For genetic characterization, the S gene sequences of Thai-PEDVs were aligned and compared with reference PEDVs including 4 genotypes (G1a, G1b, G2a and G2b). To analyze the genetic diversity of PEDVs, the phylogenetic trees of S and ORF3 genes were constructed with MEGA software version 7.0.26 (Kumar *et al.*, 2016).

Representative Thai-PDCoVs (n=16) were selected from 27 positive samples. Whole genome sequencing was performed in 2 samples with low cycle threshold value (Ct) (high RNA copies) whereas other 14 samples were subjected to S gene sequencing. For genetic characterization, the whole genome and S gene sequences of Thai-PDCoVs were aligned and compared with reference PDCoVs from China, Laos, South Korea, USA and Vietnam. To analyze the genetic diversity of PDCoVs, the phylogenetic trees of whole genome and S gene were constructed with MEGA software version 7.0.26

Representative Thai-EVGs (n=34) were selected from 556 positive samples. For genetic characterization, VP1 gene sequences of Thai-EVGs were aligned and compared with that of 20 genotypes of reference EVGs from the GenBank database. To reveal the genetic diversity of EVGs, the phylogenetic tree of VP1 gene was constructed with MEGA software version 7.0.26

4.2.1 Genetic characterization and phylogenetic analyses of Thai-PEDVs

Thai-PEDVs (n=39) were selected from 19 farms in 12 provinces including Ayutthaya, Chachoengsao, Chaiyaphum, Chonburi, Khon Kaen, Nakhon Pathom, Nakhon Ratchasima, Prachinburi, Prachuap Khiri Khan, Ratchaburi, Saraburi and Ubon Ratchathani. Thai-PEDVs characterized in this thesis were recovered from pigs in 2014 (n=2), 2015 (n=2), 2016 (n=19), 2017 (n=15) and 2018 (n=1) (Table 4.7).

All S gene sequences of Thai-PEDVs were aligned with reference PEDVs of 4 genotypes including G1a (PEDV strains CV777 and EAS1), G1b (PEDV strains IOWA106 and 001), G2a (PEDV strains GD01 and CBR1) and G2b (PEDV strains ZMDZY and OKN-2). The nucleotide and amino acid identities were performed with MegAlign software version 5.03 (DNASTAR Inc., Madison, WI, USA). The results showed that nucleotide and amino acid identities of Thai-PEDVs (vs PEDV strain CV777) were 91.63%-93.59% and 91.14%-93.57%, respectively (Table 4.8).

For genetic analysis of S gene of PEDVs, at least 8 patterns of insertions and deletions have been observed. The details of 8 patterns of insertions and deletions of representative Thai-PEDVs are shown in Table 4.9. In details, there are 3 insertion and deletion regions (12 nucleotide insertion at position 171-172, 3 nucleotide insertion at position 402-403 and 6 nucleotide deletion at position 472-477). This pattern shares the same across with other 6 patterns, but those 6 patterns have additional insertion and/or deletion in other regions of S gene. Interestingly, the last pattern shows insertion and deletion in 2 regions including 3 nucleotide insertion at position 36-37 and 27 nucleotide deletion at position 2635-2661.

For phylogenetic analysis of S gene, full-length S gene sequences of Thai-PEDVs (n=39) were aligned with 73 reference PEDVs isolated from Belgium (n=1), China (n=10), France (n=1), Japan (n=5), Netherland (n=1), Philippines (n=2), South Korea (n=7), Thailand (n=29), USA (n=11) and Vietnam (n=6). The phylogenetic tree of S gene showed that the diversity of PEDVs could be classified into 6 groups including G1a, G1b, Novel G1, G2a, G2b and Novel G2. Thai-PEDVs were grouped in Novel G1 (n=2), G2a (n=17) and Novel G2 (n=20). Members of novel G2 group were all Thai-PEDV isolates in 2008-2018. Interestingly, there are two pig farms showing co-circulation between G2a and Novel G2 groups in this thesis (Figure 4.14). Moreover, full-length ORF3 gene sequences of Thai-PEDVs were aligned with all available Thai isolates (n=63) from the GenBank database and vaccine strains including PEDV strains calaf 14, CV777 and DR13. The results showed that all Thai-PEDVs have no deletion on ORF3 gene, but PEDV vaccines show deletion on ORF3 gene. The phylogenetic tree of ORF3 gene suggested that Thai-PEDVs could be grouped into 5 groups and

none of those were grouped with vaccine strains. The phylogenetic analysis of ORF3 gene is shown in Figure 4.15.

In this thesis, we found that genotype Novel G1 was circulating in Saraburi. While genotype G2a was circulating in 5 provinces including Chachoengsao, Khon Kaen, Prachinburi, Prachuap Khiri Khan, and Ubon Ratchathani. Genotype Novel G2 could be found in 2 provinces including Ayutthaya and Chaiyaphum. The co-circulation between G2a and Novel G2 was found in 4 provinces including Chonburi, Nakhon Pathom, Nakhon Ratchasima and Ratchaburi. The co-circulation of genotypes of PEDVs is shown in Figure 4.16.

For S gene analysis at epitopes, there are 4 important epitopes including COE, SS2, SS6 and 2C10 relating to PEDV antibody induction. At COE epitope, multiple amino acid substitutions in various positions were observed. Three out of 39 Thai-PEDVs (PEDV strain S5052, S5054 and S5843) showed 12-14 amino acid substitutions while the other 36 Thai-PEDVs have number of amino acid substitutions ranging from 7-10 positions (Table 4.10). Although, there is one pattern (^{748}YSNIGVCK^{755}) showing on SS2 epitope, 3 patterns (^{764}SQSGQVKI^{771}, ^{764}PQEGQVKI^{771} and ^{764}PQDGQVKI^{771}) were observed at SS6 epitope. One Thai-PEDV (PEDV strain S5074) showed 1 amino acid substitution at position 1371 at 2C10 epitope (Table 4.11).

Table 4.7 Summary of Thai-PEDVs characterized in this thesis.

Farm #	Virus ID	Collection date	Province	Age group	Sample type	Gene characterized
1	S5001	Jun-14	Ratchaburi	Suckling	Small intestine	S, ORF3 genes
	S5003	Jun-14	Ratchaburi	Suckling	Small intestine	S, ORF3 genes
	S5005	Apr-15	Ratchaburi	Suckling	Small intestine	S, ORF3 genes
3	S5032	Aug-15	Ratchaburi	Suckling	Small intestine	S, ORF3 genes
7	S5466	Mar-17	Ratchaburi	Nursery	Feces	S, ORF3 genes
14	S5843	Jan-18	Ratchaburi	Sow	Feces	S, ORF3 genes
19	S5321	Oct-16	Nakhon Pathom	Suckling	Small intestine	S, ORF3 genes
21	S5413	Feb-17	Nakhon Pathom	Suckling	Feces	S, ORF3 genes
	S5414	Feb-17	Nakhon Pathom	Suckling	Feces	S, ORF3 genes
	S5415	Feb-17	Nakhon Pathom	Suckling	Feces	S, ORF3 genes
25	S5386	Nov-16	Chonburi	Suckling	Feces	S, ORF3 genes
	S5450	Mar-17	Chonburi	Suckling	Small intestine	S, ORF3 genes
34	S5034	Jan-16	Prachinburi	Suckling	Feces	S, ORF3 genes
	S5036	Jan-16	Prachinburi	Suckling	Feces	S, ORF3 genes
	S5037	Jan-16	Prachinburi	Suckling	Small intestine	S, ORF3 genes
	S5039	Jan-16	Prachinburi	Suckling	Small intestine	S, ORF3 genes
	S5297	Sep-16	Prachinburi	Suckling	Feces	S, ORF3 genes
37	S5043	Feb-16	Nakhon Ratchasima	Suckling	Small intestine	S, ORF3 genes
	S5044	Feb-16	Nakhon Ratchasima	Suckling	Small intestine	S, ORF3 genes
	S5045	Feb-16	Nakhon Ratchasima	Suckling	Small intestine	S, ORF3 genes
38	S5051	Feb-16	Nakhon Ratchasima	Suckling	Small intestine	S, ORF3 genes
41	S5324	Nov-16	Nakhon Ratchasima	Nursery	Feces	S, ORF3 genes
	S5412	Jan-17	Nakhon Ratchasima	Nursery	Feces	S, ORF3 genes
	S5495	Apr-17	Nakhon Ratchasima	Nursery	Small intestine	S, ORF3 genes
	S5530	May-17	Nakhon Ratchasima	Nursery	Feces	S, ORF3 genes
	S5598	Jul-17	Nakhon Ratchasima	Suckling	Feces	S, ORF3 genes
	S5726	Sep-17	Nakhon Ratchasima	Suckling	Feces	S, ORF3 genes
	S5765	Oct-17	Nakhon Ratchasima	Suckling	Feces	S, ORF3 genes
	S5799	Oct-17	Nakhon Ratchasima	Suckling	Feces	S, ORF3 genes
46	S5074	Apr-16	Khon Kaen	Fattening	Feces	S, ORF3 genes
47	S5102	May-16	Prachuap Khiri Khan	Suckling	Feces	S, ORF3 genes
	S5317	Oct-16	Prachuap Khiri Khan	Nursery	Feces	S, ORF3 genes
60	S5057	Mar-16	Ubon Ratchathani	Suckling	Small intestine	S, ORF3 genes
64	S5489	Apr-17	Chachoengsao	Suckling	Small intestine	S, ORF3 genes
65	S5519	May-17	Chachoengsao	Fattening	Feces	S, ORF3 genes
67	S5381	Nov-16	Chaiyaphum	Sow	Feces	S, ORF3 genes
71	S5556	May-17	Ayutthaya	Nursery	Feces	S, ORF3 genes
72	S5052	Feb-16	Saraburi	Fattening	Feces	S, ORF3 genes
	S5054	Feb-16	Saraburi	Fattening	Feces	S, ORF3 genes

Table 4.8 Nucleotide and amino acid identities of S gene of Thai-PEDVs and reference PEDVs.

					S gene		
	GenBank				size	% nucleotide	% aa
Virus	accession No.	genotype	country	year	(bp)	identity	identity
CV777	AF353511	G1a	Belgium	1978	4149	100.00%	100.00%
EAS1	KR610991	G1a	Thailand	2014	4149	98.96%	97.51%
IOWA106	KJ645695	G1b	USA	2013	4149	95.62%	96.17%
001	KR011756	G1b	France	2014	4149	95.67%	95.94%
GD01	JX261936	G2a	China	2011	4155	93.78%	93.41%
CBR1	KR610993	G2a	Thailand	2014	4155	93.17%	92.48%
ZMDZY	KC196276	G2b	China	2011	4158	93.62%	93.18%
OKN-2	LC063816	G2b	Japan	2014	4158	93.71%	93.42%
S5001	This study	Novel G2	Thailand	2014	4158	93.30%	93.03%
S5003	This study	Novel G2	Thailand	2014	4158	93.33%	93.03%
S5005	This study	Novel G2	Thailand	2015	4158	93.17%	92.87%
S5032	This study	G2a	Thailand	2015	4155	93.51%	93.33%
S5466	This study	G2a	Thailand	2017	4155	93.59%	93.41%
S5843	This study	Novel G2	Thailand	2018	4158	91.63%	91.14%
S5321	This study	G2a	Thailand	2016	4155	93.43%	93.49%
S5413	This study	Novel G2	Thailand	2017	4155	92.73%	92.87%
S5414	This study	Novel G2	Thailand	2017	4155	92.73%	92.87%
S5415	This study	Novel G2	Thailand	2017	4155	92.73%	92.87%
S5386	This study	Novel G2	Thailand	2016	4164	92.93%	92.71%
S5450	This study	G2a	Thailand	2017	4158	93.25%	92.48%
S5034	This study	G2a	Thailand	2016	4155	93.49%	93.41%
S5036	This study	G2a	Thailand	2016	4155	93.46%	93.41%
S5037	This study	G2a	Thailand	2016	4155	93.43%	93.33%
S5039	This study	G2a	Thailand	2016	4155	93.49%	93.41%
S5297	This study	G2a	Thailand	2016	4155	93.40%	93.41%
S5043	This study	Novel G2	Thailand	2016	4167	93.54%	93.42%
S5044	This study	Novel G2	Thailand	2016	4167	93.54%	93.42%
S5045	This study	Novel G2	Thailand	2016	4167	93.54%	93.42%
S5051	This study	G2a	Thailand	2016	4155	93.56%	93.57%
S5324	This study	Novel G2	Thailand	2016	4167	93.49%	93.26%
S5412	This study	Novel G2	Thailand	2017	4167	93.44%	93.18%
S5495	This study	G2a	Thailand	2017	4152	93.32%	92.71%
S5530	This study	Novel G2	Thailand	2017	4167	93.30%	93.03%
S5598	This study	Novel G2	Thailand	2017	4167	93.49%	93.34%
S5726	This study	Novel G2	Thailand	2017	4167	93.38%	93.11%
S5765	This study	Novel G2	Thailand	2017	4167	93.38%	93.11%
S5799	This study	Novel G2	Thailand	2017	4167	93.28%	92.95%
S5074	This study	G2a	Thailand	2016	4155	93.51%	93.41%
S5102	This study	G2a	Thailand	2016	4155	93.54%	93.41%
S5317	This study	G2a	Thailand	2016	4155	93.49%	93.26%
S5057	This study	G2a	Thailand	2016	4155	93.51%	93.02%
S5489	This study	G2a	Thailand	2017	4155	93.33%	92.71%
S5519	This study	G2a	Thailand	2017	4152	93.35%	92.71%
S5381	This study	Novel G2	Thailand	2016	4167	93.46%	93.26%
S5556	This study	Novel G2	Thailand	2017	4167	93.25%	93.03%
S5052	This study	Novel G1	Thailand	2016	4125	91.92%	92.84%
S5054	This study	Novel G1	Thailand	2016	4125	91.92%	92.99%

Table 4.9 Genetic analysis of nucleotide sequences of Thai-PEDVs and reference PEDVs of 4 genotypes.

			S1 subunit (position 1-2178)						S2 subunit (position 2179-4149)		
Genotype, Virus	GenBank accession No.	Year isolated	3nt insertion at position 36-37	12nt insertion at position 171-172	3nt deletion at position 202-204	3nt insertion at position 402-403	6nt deletion at position 472-477	9nt insertion at position 690-691	27nt deletion at position 2635-2661	3nt insertion at position 2673-2674	3nt deletion at position 3580-3582
G1a											
CV7777	AF353511	1978	No	No	No	No	No	No	No	No	No
EAS1	KR610991	2014	No	No	No	No	No	No	No	No	No
G1b											
IOWA106	KJ645695	2013	No	No	No	No	No	No	No	No	No
001	KR011756	2014	No	No	No	No	No	No	No	No	No
G2a											
GD01	JX261936	2011	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	TAT
CBR1	KR610993	2014	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	TAT
G2b											
ZMDZY	KC196276	2011	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	No
OKN-2	LC063816	2014	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	No
S5001	NA	2014	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	No
S5032	NA	2015	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	No	TAT
S5450	NA	2017	No	AACCAGGGTGTC	No	AAT	GATATT	No	No	AAG	TAT
S5043	NA	2016	No	AACCAGGGTGTC	No	AAT	GATATT	AGGGAATAT	No	No	No
S5386	NA	2016	No	AACCAGGGTGTC	No	AAT	GATATT	AGGGACTAT	No	No	TAT
S5495	NA	2017	No	AACCAGGGTGTC	GAA	AAT	GATATT	No	No	No	TAT
S5556	NA	2017	No	AAACAGGGTGTC	GAA	AAT	GATATT	AGGGAATAT	No	No	No
S5052	NA	2016	GTA	No	No	No	No	No	GTGTACGATCCTGCAAGTGGCAGGGTG	No	No

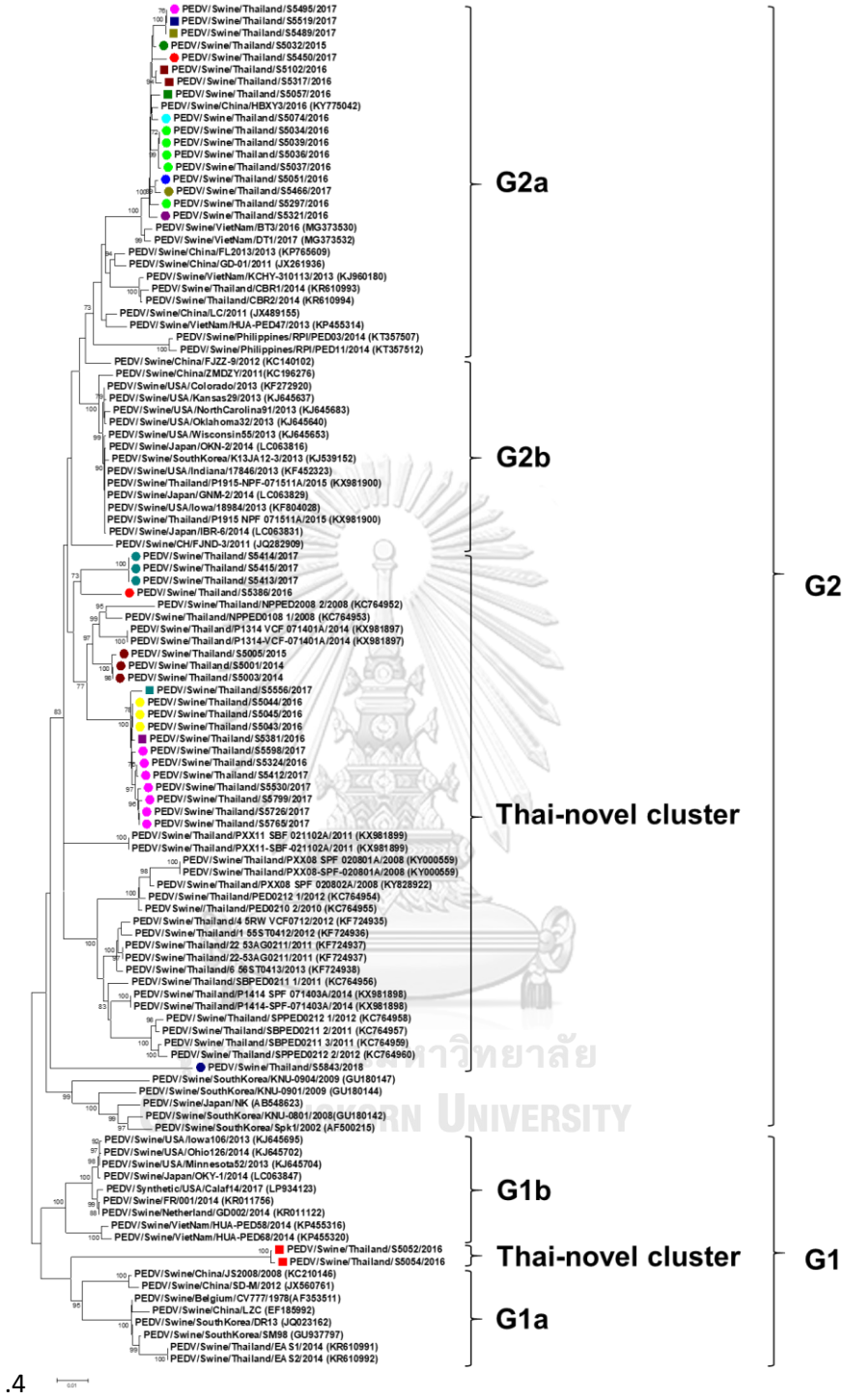

Figure 4.14 Phylogenetic analysis of S gene of the representative PEDVs and reference PEDVs. The representative viruses are highlighted by circle and square with difference colors. Each color and each shape indicate the representative viruses isolated from the same farm. The scale bar represents the distance unit between sequence pairs.

Figure 4.15 Phylogenetic analysis of ORF3 gene of the representative PEDVs and reference PEDVs. The representative viruses are highlighted by circle and square with difference colors. Each color and each shape indicate the representative viruses isolated from the same farm. The scale bar represents the distance unit between sequence pairs.

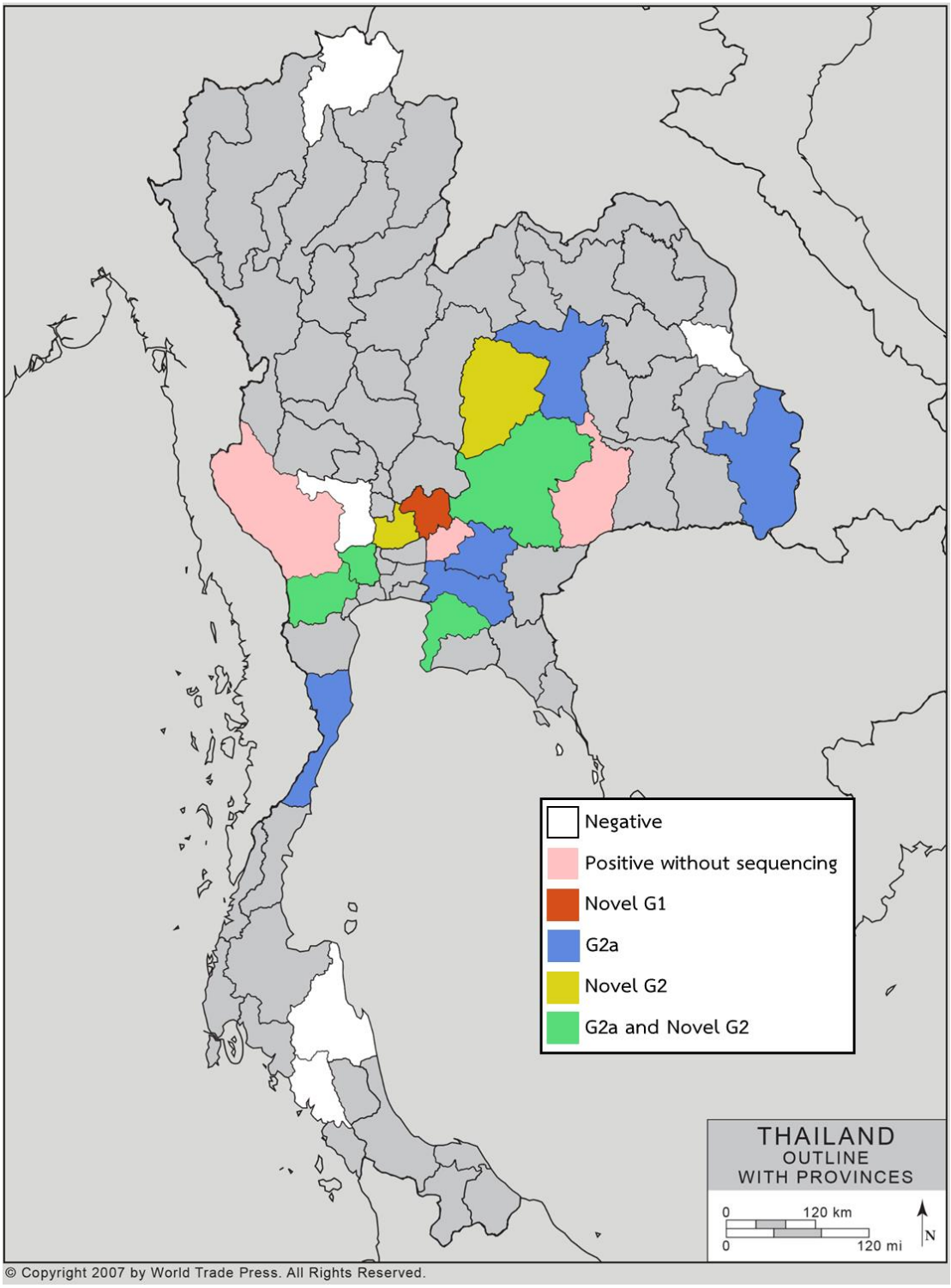

Figure 4.16 Distribution of the representative PEDVs by genotypes. The highlight colors are provinces and genotypes.

Table 4.10 Amino acid substitutions at the epitope COE of Thai-PEDVs.

Epitrope	COE (490-615)																												
Position	492	496	513	517	520	521	523	525	527	528	536	549	551	563	566	568	590	591	594	595	597	603	605	606	608	609	610	612
PEDV/Belgium/CV777/1978(AF353511)	H	I	T	A	G	L	S	N	V	A	F	T	F	K	D	N	S	L	G	A	T	Y	A	F	S	G	V	L
PEDV/SouthKorea/DR13 (JQ023162)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/USA/calaf14 (LP934123)	.	.	.	S	.	H	G	.	I	.	.	S	.	.	.	.	N	.	S	D	.	.	E	.	.	.	.	F
PEDV/Thailand/S5001/2014	.	.	.	S	.	H	G	.	I	.	.	S	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5003/2014	.	.	.	S	.	H	G	.	I	.	.	S	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5005/2015	.	.	.	S	.	H	G	.	I	.	.	S	.	N	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5032/2015	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5034/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5036/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5037/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	R	.	.	F
PEDV/Thailand/S5039/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5043/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5044/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5045/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5051/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5052/2016	Q	.	.	.	D	A	G	.	.	.	L	K	V	.	.	K	.	.	.	.	V	H	Q	S	.	.	.	.
PEDV/Thailand/S5054/2016	Q	.	.	.	D	A	G	.	.	.	L	K	V	.	.	K	.	.	.	.	V	H	Q	S	.	.	.	.
PEDV/Thailand/S5057/2016	.	T	.	.	D	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5074/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5102/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5297/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5317/2016	.	T	.	.	.	R	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5321/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5324/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5381/2016	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5386/2016	.	.	.	S	D	H	G	.	I	.	.	S	.	T	Y	.	.	.	S	.	.	.	D	.	.	.	.	.
PEDV/Thailand/S5412/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5413/2017	.	.	.	S	.	H	G	.	I	.	.	S	.	N	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5414/2017	.	.	.	S	.	H	G	.	I	.	.	S	.	N	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5415/2017	.	.	.	S	.	H	G	.	I	.	.	S	.	N	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5450/2017	.	T	S	.	.	R	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5466/2017	.	T	.	.	.	R	G	D	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5489/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5495/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5519/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5530/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5556/2017	.	T	.	.	.	H	G	.	I	V	.	.	.	T	.	.	.	.	S	.	.	.	E	.	.	A	.	F
PEDV/Thailand/S5598/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5726/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5765/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5799/2017	.	T	.	.	.	H	G	.	I	.	.	.	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	F
PEDV/Thailand/S5843/2018	Q	.	.	.	D	A	G	.	.	.	L	.	V	N	.	K	.	F	.	.	V	H	Q	T	.	.	F	.

. indicate no amino acid substitution

Table 4.11 Amino acid substitutions at the epitopes SS2, SS6 and 2C10 of Thai-PEDVs.

Epitope	SS2 (748-755)								SS6 (764-771)								2C10 (1368-1374)							
Position	748	749	750	751	752	753	754	755	764	765	766	767	768	769	770	771	1368	1369	1370	1371	1372	1373	1374
PEDV/Belgium/CV777/1978(AF353511)	Y	S	N	I	G	V	C	K	S	Q	Y	G	Q	V	K	I	G	P	R	L	Q	P	Y
PEDV/SouthKorea/DR13 (JQ023162)	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/USA/calaf14 (LP934123)	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5001/2014	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5003/2014	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5005/2015	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5032/2015	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5034/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5036/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5037/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5039/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5043/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5044/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5045/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5051/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5052/2016	.	.	.	.	.	.	.	.	P	.	E	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5054/2016	.	.	.	.	.	.	.	.	P	.	E	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5057/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5074/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	F	.	.	.
PEDV/Thailand/S5102/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5297/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5317/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5321/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5324/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5381/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5386/2016	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5412/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5413/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5414/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5415/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5450/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5466/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5489/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5495/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5519/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5530/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5556/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5598/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5726/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5765/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5799/2017	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
PEDV/Thailand/S5843/2018	.	.	.	.	.	.	.	.	P	.	D	.	.	.	.	.	.	.	.	.	.	.	.

. indicate no amino acid substitution

4.2.2 Genetic characterization and phylogenetic analyses of Thai-PDCoVs

Thai-PDCoVs (n=16) were selected from 6 farms in 4 provinces including Chonburi, Nakhon Pathom, Prachinburi and Ratchaburi. Thai-PDCoVs characterized in this thesis were recovered from pigs in 2015 (n=11), 2016 (n=3), 2017 (n=1) and 2018 (n=1) (Table 4.12).

In this thesis, all S gene sequences of Thai-PDCoVs (n=16) were aligned with those reference PDCoVs isolated from China (n=3), Laos (n=1), South Korea (n=3), USA (n=3), and Vietnam (n=2). The nucleotide and amino acid identities were performed with MegAlign software version 5.03 (DNASTAR Inc., Madison, WI, USA). Those S gene sequences of Thai-PDCoVs were compared with PDCoV strain HKU15-155. The results showed that nucleotide and amino acid identities among Thai-PDCoVs were ranging 95.63%-96.41% and 96.48%-97.90%, respectively (Table 4.13).

For genetic analysis, the nucleotide positions were numbered based on nucleotide sequence of PDCoV strain HKU15-155. In this thesis, whole genome sequences of 2 Thai-PDCoVs was compared with PDCoVs strain HKU15-155. There are 6 insertion and deletion regions in whole genome (25,404 bps) including 2 deletion regions of 5'UTR with 3 and 1 nucleotide deletion, 2 deletion regions of ORF1a gene with 6 and 9 nucleotide deletion, 3 nucleotide insertion of S gene and 4 nucleotide insertion of 3'UTR. The S gene sequences of 14 Thai-PDCoVs were compared with S gene of HKU15-155. The results show that all Thai-PDCoVs have 3 nucleotide insertion of S gene. Genetic characterization of 2 whole genome and 14 S gene of Thai-PDCoVs is shown in Table 4.14.

For phylogenetic analysis, two whole genome sequences of Thai-PDCoVs were aligned with 98 whole genome sequences of reference PDCoVs. Reference PDCoVs were isolated from China (n=49), Japan (n=6), South Korea (n=4), Laos (n=1), Thailand (n=4), USA (n=32) and Vietnam (n=2). The phylogenetic analysis results showed that the diversity of PDCoVs could be classified into 4 clusters based on geographic locations including China cluster, Thailand cluster, USA and Korea cluster, and Vietnam cluster. 2 Thai-PDCoVs were grouped in Thailand cluster. The phylogenetic

analysis of whole genome of 2 representative PDCoVs is shown in Figure 4.17. Moreover, the phylogenetic tree of S gene of Thai-PDCoVs and reference PDCoVs (n=109) was analyzed. The phylogenetic analysis of S gene suggested that PDCoVs could be classified into 4 groups based on geographic locations which similar to the result of phylogenetic analysis of whole genome. All Thai-PDCoVs were grouped in Thailand cluster. The phylogenetic analysis of S gene is shown in Figure 4.18.

For the distribution analysis of PDCoVs, PDCoVs could be detected in 5 provinces. 4 out of 5 provinces including Chonburi, Nakhon Pathom, Prachinburi and Ratchaburi had Thai-PDCoVs in Thailand cluster circulation while the other clusters were not observed in this thesis (Figure 4.19).

For S gene analysis at epitopes, position of amino acid was numbered based amino acid sequence of PDCoV strain HKU15-155. There are 3 important epitopes including NTD, CTD and S2 on S gene of PDCoVs relating to PDCoV antibody induction. At NTD epitope, 16 out of 236 positions showed amino acid substitution. Amino acid substitutions of Thai-PDCoVs at NTD epitope were ranging from 8 to 12 positions. PDCoV strain S5841 has the most amino acid substitutions. At CTD epitope, there are 3-5 positions showed amino acid substitution. The amino acid substitutions at NTD and CTD epitopes of PDCoVs is shown in Table 4.15. Moreover, amino acid changes at S2 epitope were detected. The most amino acid changes were observed in PDCoV strain S5841. The amino acid changes at S2 epitope are shown in Table 4.16.

Table 4.12 Summary of Thai-PDCoVs characterized in this thesis.

Farm #	Virus ID	Collection date	Province	Age group	Sample type	Gene characterized
14	S5841	Jan-2018	Ratchaburi	Sow	Feces	S gene
17	S5192	Jan-2015	Nakhon Pathom	Suckling	Feces	S gene
20	S5396	Nov-2016	Nakhon Pathom	Suckling	Feces	S gene
	S5397	Nov-2016	Nakhon Pathom	Suckling	Feces	S gene
27	S5444	Jan-2016	Chonburi	Suckling	Small intestine	S gene
29	S5475	Mar-2017	Chonburi	Nursery	Feces	S gene
34	S5011	Jun-2015	Prachinburi	suckling	Small intestine	WGS
	S5012	Jun-2015	Prachinburi	suckling	Small intestine	S genes
	S5013	Jun-2015	Prachinburi	suckling	Feces	S genes
	S5014	Jun-2015	Prachinburi	suckling	Small intestine	S genes
	S5015	Jun-2015	Prachinburi	suckling	Small intestine	WGS
	S5016	Jun-2015	Prachinburi	suckling	Feces	S genes
	S5022	Jul-2015	Prachinburi	fattening	Feces	S genes
	S5023	Jul-2015	Prachinburi	fattening	Feces	S genes
	S5024	Jul-2015	Prachinburi	fattening	Feces	S genes
	S5025	Jul-2015	Prachinburi	fattening	Feces	S genes

Table 4.13 Nucleotide and amino acid identities of S gene of Thai-PDCoVs and reference PDCoVs.

					S gene		
virus	GenBank accession No.	Cluster	country	year	size (bp)	% nucleotide identity	% aa identity
HKU15-155	JQ065043	China	China	2010	4149	100.00%	100.00%
GD16-06	KY078909	China	China	2016	4146	98.54%	98.96%
GD16-03	KY363867	China	China	2016	4152	96.73%	97.81%
Illinois133	KJ601777	USA and Korea	USA	2014	4152	98.60%	98.96%
Minnesota140	KX022603	USA and Korea	USA	2014	4152	98.60%	98.87%
Michigan447	KR265849	USA and Korea	USA	2016	4152	98.51%	98.96%
KNU14-04	KM820765	USA and Korea	South Korea	2014	4152	98.69%	99.04%
KNU16-08	KY926511	USA and Korea	South Korea	2016	4149	97.89%	97.90%
KNU16-11	KY926512	USA and Korea	South Korea	2016	4149	97.83%	97.81%
Binh21	KX834352	Vietnam	Vietnam	2015	4152	96.86%	97.81%
HaNoi6	KX834351	Vietnam	Vietnam	2015	4152	96.76%	97.72%
P1_16_BTL_0115	KX118627	Thailand	Laos	2016	4152	96.52%	97.54%
TT_1115	KU984334	Thailand	Thailand	2015	4149	96.31%	97.45%
S5481	This study	Thailand	Thailand	2018	4152	95.63%	96.48%
S5192	This study	Thailand	Thailand	2015	4152	96.41%	97.90%
S5396	This study	Thailand	Thailand	2016	4152	96.10%	97.46%
S5397	This study	Thailand	Thailand	2016	4152	96.10%	97.46%
S5444	This study	Thailand	Thailand	2016	4152	96.26%	97.37%
S5475	This study	Thailand	Thailand	2017	4152	96.16%	97.55%
S5011	This study	Thailand	Thailand	2015	4152	96.32%	97.37%
S5012	This study	Thailand	Thailand	2015	4152	96.32%	97.37%
S5013	This study	Thailand	Thailand	2015	4152	96.32%	97.46%
S5014	This study	Thailand	Thailand	2015	4152	96.29%	97.28%
S5015	This study	Thailand	Thailand	2015	4152	96.29%	97.28%
S5016	This study	Thailand	Thailand	2015	4152	96.32%	97.37%
S5022	This study	Thailand	Thailand	2015	4152	96.29%	97.37%
S5023	This study	Thailand	Thailand	2015	4152	96.29%	97.37%
S5024	This study	Thailand	Thailand	2015	4152	96.32%	97.28%
S5025	This study	Thailand	Thailand	2015	4152	96.35%	97.37%

Table 4.14 Genetic analysis of nucleotide sequences of Thai-PDCoVs and reference PDCoVs.

				5'UTR		ORF1a		S gene			3'-UTR	
								S1 subunit at position 19327-20979		S2 subunit at position 20980-22,803		
Country, Virus	GeneBank accession no.	Year isolated	Genome size, bp	3-nt deletion at position 116–118	1-nt deletion at position 302	6-nt deletion at position 1737-1742	9-nt deletion at position 2808–2816	3nt insertion at position 19473-19474	3nt deletion at position 19804-19806	3nt deletion at position 22750-22752	3-or 4-nt insertion at position 25043–25044	1-nt deletion at position 25258
China												
HKU15-44	JQ065042	2009	25,421	No	No	No	No	AAT	No	No	GTT	T
HKU15-155	JQ065043	2010	25,416	No	No	No	No	No	No	No	No	No
S27/2012	KT266822	2012	25,404	No	No	AGTTTG	GAGCCAGTC	No	No	No	GTT	No
JXNI	KR131621	2015	25,419	No	No	No	No	No	No	No	TT	No
GD16-06	KY078909	2016	S gene	NA	NA	NA	NA	No	TTT	No	NA	NA
USA												
IA8734	KJ567050	2014	25,422	No	No	No	No	AAT	No	No	GTT	No
South Korea												
KNU14-04	KM820765	2014	25,422	No	No	No	No	AAT	No	No	GTT	No
KNU16-08	KY926511	2016	S gene	NA	NA	NA	NA	AAT	No	AAG	NA	NA
Vietnam												
Binh21	KX834352	2015	25,406	TCT	No	AGTTTG	GAGCCAGTC	AAT	No	No	CTGT	No
Laos												
P1_16_BTL_0115	KX118627	2016	25,405	TCT	A	AGTTTG	GAGCCAGTC	AAT	No	No	CTCT	No
Thai												
S5011	NA	2015	25,404	TCT	A	AGTTTG	GAGCCAGTC	AAT	No	No	CTCT	No
S5013	NA	2015	S gene	NA	NA	NA	NA	AAT	No	No	NA	NA
S5192	NA	2015	S gene	NA	NA	NA	NA	AAT	No	No	NA	NA
S5396	NA	2016	S gene	NA	NA	NA	NA	AAT	No	No	NA	NA
S5475	NA	2017	S gene	NA	NA	NA	NA	AAT	No	No	NA	NA
S5481	NA	2018	S gene	NA	NA	NA	NA	AAT	No	No	NA	NA

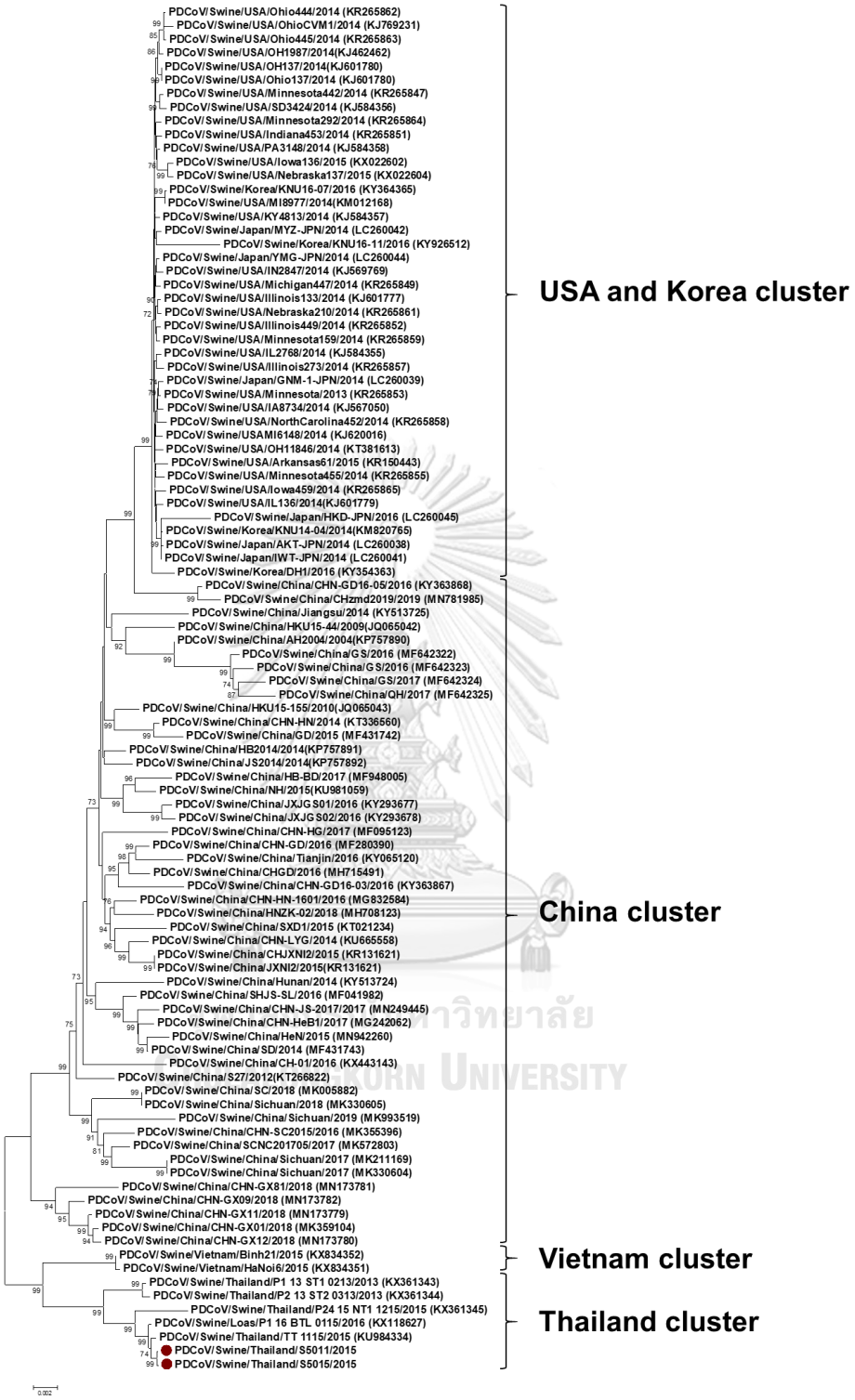

Figure 4.17 Phylogenetic analysis of whole genome of the representative PDCoVs and reference PDCoVs. The representative viruses are highlighted by circle with difference colors. Each color indicates the representative viruses isolated from the same farm. The scale bar represents the distance unit between sequence pairs.

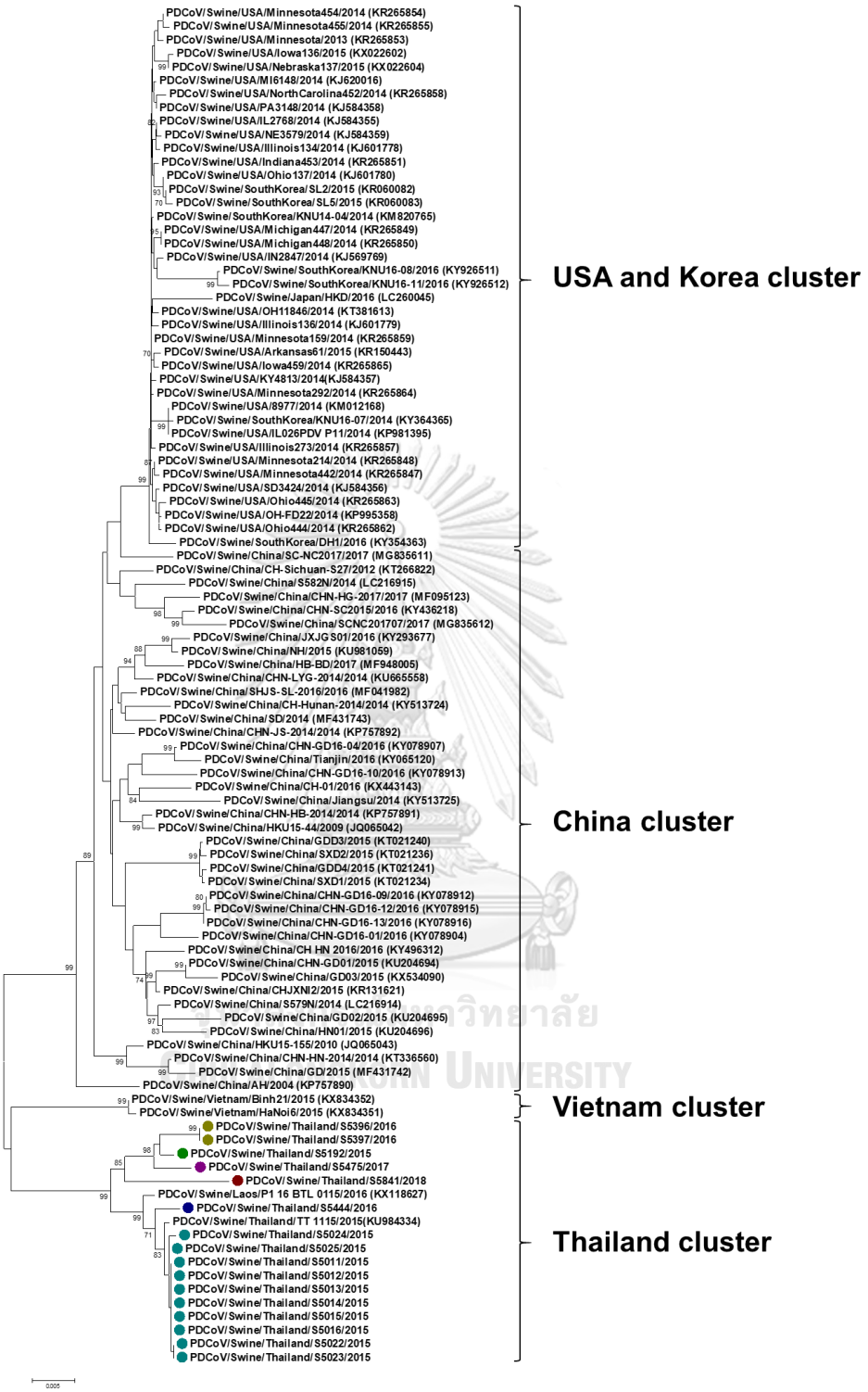

Figure 4.18 Phylogenetic analysis of S gene of the representative PDCoVs and reference PDCoVs. The representative viruses are highlighted by circle with difference colors. Each color indicates the representative viruses isolated from the same farm. The scale bar represents the distance unit between sequence pairs.

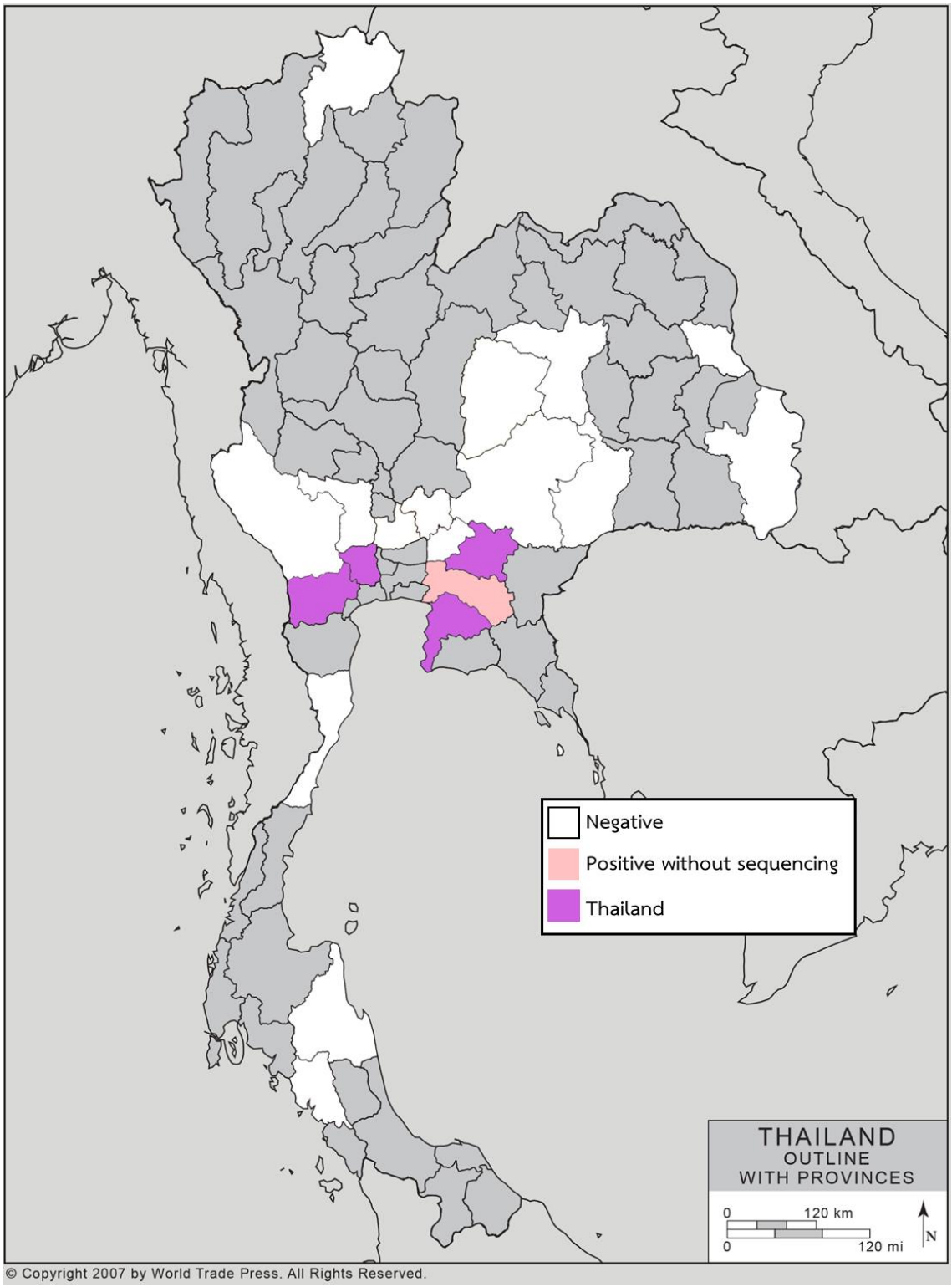

Figure 4.19 Distribution of the representative PDCoVs by genotypes. The highlighted colors are provinces and genotypes.

Table 4.15 Amino acid substitutions at the epitopes NTD and CTD of Thai-PDCoVs.

Epitope	NTD (51-286)															CTD (287-552)												
Position	57	100	111	122	123	136	140	149	159	168	178	219	229	231	236	276	318	348	350	355	486	489	517	530	543	551
PDCoV/Swine/China/HKU15-155/2010 (JQ065043)	V	H	I	K	Y	T	H	H	E	H	E	T	H	A	H	E	F	M	T	N	T	Q	V	F	S	A
PDCoV/Swine/Thailand/S5011/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5012/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5013/2015	.	.	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5014/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	D	L	.	.
PDCoV/Swine/Thailand/S5015/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	D	L	.	.
PDCoV/Swine/Thailand/S5016/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5022/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5023/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5024/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5025/2015	.	Q	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	.	L	.	.
PDCoV/Swine/Thailand/S5192/2015	.	.	V	.	H	I	R	A	D	Q	.	.	Q	.	.	Q	.	L	R	.	.	.	.	.	.	V
PDCoV/Swine/Thailand/S5396/2016	.	.	.	.	H	I	R	A	D	Q	.	.	Q	.	.	Q	.	L	R	.	.	L	.	.	N	V
PDCoV/Swine/Thailand/S5397/2016	.	.	.	.	H	I	R	A	D	Q	.	.	Q	.	.	Q	.	L	R	.	.	L	.	.	N	V
PDCoV/Swine/Thailand/S5444/2016	.	.	.	.	.	I	R	A	D	.	Q	.	Q	S	.	Q	.	L	R	.	.	.	A	.	.	.
PDCoV/Swine/Thailand/S5475/2017	.	Q	V	.	H	I	R	A	D	Q	.	.	Q	.	.	Q	.	L	R	.	N	.	.	.	.	V
PDCoV/Swine/Thailand/S5841/2018	I	.	.	R	H	I	R	A	D	Q	.	I	Q	.	Y	Q	Y	L	R	K	.	.	.	.	.	V

. indicate no amino acid substitution

Table 4.16 Amino acid substitutions at the epitope S2 of Thai-PDCoVs.

Epitope	S2 (553-1087)																														
Position	553	571	590	624	632	639	642	668	670	687	698	702	706	799	805	865	867	870	879	887	907	909	934	948	1011	1016	1080	1084	1087
PDCoV/Swine/China/HKU15-155/2010 (JQ065043)	T	V	S	A	L	N	Q	T	L	S	A	D	A	A	E	L	T	L	Q	V	S	Y	A	A	N	V	N	D	W
PDCoV/Swine/Thailand/S5011/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5012/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5013/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5014/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5015/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5016/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5022/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5023/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5024/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	V	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5025/2015	.	I	A	V	.	S	K	S	V	.	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5192/2015	.	.	A	.	.	.	.	P	V	.	S	.	.	.	.	.	.	.	R	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5396/2016	.	.	A	.	.	.	R	P	V	.	S	.	.	.	.	.	N	M	.	I	P	.	.	T	.	.	.	.	.
PDCoV/Swine/Thailand/S5397/2016	.	.	A	.	.	.	R	P	V	.	S	.	.	.	.	.	N	M	.	I	P	.	.	T	.	.	.	.	.
PDCoV/Swine/Thailand/S5444/2016	.	I	A	V	V	S	K	S	V	T	S	N	.	.	.	.	N	.	.	I	P	.	.	.	.	.	.	.	.
PDCoV/Swine/Thailand/S5475/2017	.	.	A	.	.	.	.	S	V	T	S	.	.	.	.	.	.	.	.	I	P	.	.	.	.	.	D	.	.
PDCoV/Swine/Thailand/S5841/2018	A	I	A	.	.	.	.	.	V	.	S	.	T	V	D	I	N	.	.	I	P	H	.	.	S	I	.	N	L

. indicate no amino acid substitution

4.2.3 Genetic characterization and phylogenetic analysis of Thai-EVGs

Thai-EVGs (n=34) were selected from 21 farms in 13 provinces including Chachoengsao, Chiang Rai, Chonburi, Kanchanaburi, Khon Kaen, Nakhon Nayok, Nakhon Pathom, Nakhon Ratchasima, Prachinburi, Prachuap Khiri Khan, Ratchaburi, Suphanburi and Trang. Thai-EVGs characterized in this thesis were recovered from pigs in 2015 (n=4), 2016 (n=23) and 2017 (n=7) (Table 4.17).

For genetic analysis, all VP1 sequences of Thai-EVGs were aligned with reference EVGs of 20 genotypes. The details of 20 genotypes of EVGs were listed in Table 3.4. Nucleotide comparisons of 34 Thai-EVGs and 20 reference genotypes showed 55.05%-79.95% nucleotide identities. We found that 34 Thai-EVGs showed the highest similarities to G1 (n=7), G3 (n=22), G4 (n=1), G8 (n=1), G9 (n=1) and G10 (n=2). The nucleotide identities of VP1 gene of Thai-EVGs compared with reference EVGs of 20 genotypes are shown in Table 4.18.

For phylogenetic analysis, all VP1 gene sequences of Thai-EVGs were aligned with reference EVGs of 20 genotypes (n=69). The phylogenetic analysis showed that all Thai-EVGs were grouped in 6 groups including G1 (n=7), G3 (n=22), G4 (n=1), G8 (n=1), G9 (n=1) and G10 (n=2) (Figure 4.20). Interestingly, 2 pig farms in 2 provinces, including Nakhon Ratchasima and Ratchaburi, contained more than 1 genotype of EVGs circulation.

In this thesis, EVGs could be detected in 13 out of 17 provinces. Genotype 1 was circulating in 3 provinces including Chiang Rai, Nakhon Pathom and Trang. Genotype 3 was circulating in 6 provinces including Chonburi, Kanchanaburi, Khon Kaen, Nakhon Nayok, Prachinburi and Suphanburi. Genotype 10 was discovered in Prachuap Khiri Khan. Co-circulation of more than 1 genotype of EVGs were observed in 3 provinces, including Chachoengsao, Nakhon Ratchasima and Ratchaburi. The distribution of genotypes of EVGs is shown in Figure 4.21.

Table 4.17 Summary of Thai-EVGs characterized in this thesis.

Farm #	Virus ID	Collection date	Province	Age group	Sample type	Gene characterized
1	S5400	Dec-2016	Ratchaburi	Nursery	Feces	VP1
	S5401	Dec-2016	Ratchaburi	Nursery	Feces	VP1
18	S5193	Feb-2015	Nakhon pathom	Suckling	Feces	VP1
20	S5327	Nov-2016	Nakhon pathom	Nursery	Feces	VP1
25	S5391	Nov-2016	Chonburi	Suckling	Feces	VP1
32	S5430	Feb-2017	Kanchanaburi	Suckling	Feces	VP1
33	S5080	Apr-2016	Kanchanaburi	Suckling	Small intestine	VP1
34	S5129	Jun-2016	Prachinburi	Suckling	Feces	VP1
	S5268	Aug-2016	Prachinburi	Suckling	Feces	VP1
35	S5078	Apr-2016	Prachinburi	Suckling	Feces	VP1
36	S5273	Sep-2016	Prachinburi	Suckling	Feces	VP1
40	S5083	May-2016	Nakhon Ratchasima	Breeder	Feces	VP1
	S5087	May-2016	Nakhon Ratchasima	Suckling	Feces	VP1
41	S5109	May-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5137	Jun-2016	Nakhon Ratchasima	Breeder	Feces	VP1
	S5244	Aug-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5409	Jan-2017	Nakhon ratchasima	Suckling	Feces	VP1
	S5410	Jan-2017	Nakhon ratchasima	Suckling	Feces	VP1
	S5412	Jan-2017	Nakhon ratchasima	Suckling	Feces	VP1
	S5617	Aug-2017	Nakhon ratchasima	Nursery	Feces	VP1
43	S5338	Nov-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5342	Nov-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5346	Nov-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5350	Nov-2016	Nakhon Ratchasima	Suckling	Feces	VP1
	S5353	Nov-2016	Nakhon Ratchasima	Suckling	Feces	VP1
46	S5082	Apr-2016	Khon kaen	Suckling	Small intestine	VP1
51	S5196	Feb-2015	Suphanburi	Suckling	Feces	VP1
53	S5215	Dec-2015	Suphanburi	Suckling	Feces	VP1
58	S5221	Nov-2015	Trang	Nursery	Feces	VP1
63	S5308	Oct-2016	Prachuap Khiri Khan	Nursery	Feces	VP1
65	S5517	May-2017	Chachoengsao	Fattening	Feces	VP1
66	S5568	Jun-2017	Chachoengsao	Suckling	Feces	VP1
68	S5404	Dec-2016	Nakhon nayok	Suckling	Feces	VP1
70	S5405	Dec-2016	Chiang Rai	Nursery	Feces	VP1

Table 4.18 Nucleotide identities of VP1 gene of Thai-EVGs and 20 genotypes of reference EVGs.

Viruses	genotype	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20
S5400	G1	77.59%	62.52%	63.3%	65.27%	60.42%	67.37%	58.32%	61.07%	64.74%	67.1%	66.32%	67.76%	67.5%	67.37%	62.25%	66.06%	61.86%	60.81%	66.71%	57.8%
S5401	G4	64.88%	65.53%	67.37%	78.9%	62.39%	73.53%	58.85%	64.22%	66.45%	65.27%	70.25%	66.84%	66.97%	68.68%	67.76%	65.53%	64.61%	64.88%	67.63%	61.34%
S5193	G1	77.2%	62.25%	63.3%	65.27%	60.03%	67.23%	58.32%	60.55%	65.53%	66.71%	65.4%	67.1%	66.58%	66.71%	62.65%	66.06%	61.07%	61.34%	67.23%	57.67%
S5327	G1	77.33%	61.86%	63.04%	64.74%	61.21%	67.1%	57.93%	61.34%	63.96%	65.92%	67.1%	66.58%	65.92%	67.37%	61.86%	66.06%	61.07%	60.42%	65.66%	58.32%
S5391	G3	64.48%	62.78%	74.97%	66.58%	61.47%	69.33%	57.54%	63.56%	72.48%	70.77%	69.2%	70.38%	67.23%	65.53%	71.04%	64.74%	62.65%	63.17%	68.15%	62.39%
S5430	G3	62.12%	60.55%	79.95%	65.79%	60.16%	68.15%	55.96%	60.16%	72.35%	69.72%	67.89%	67.76%	65.66%	65.79%	70.38%	66.06%	60.94%	61.21%	65.53%	62.12%
S5080	G3	61.6%	61.21%	79.29%	66.97%	60.42%	67.89%	55.83%	60.29%	71.69%	70.12%	67.5%	66.06%	65.01%	66.06%	69.59%	63.3%	59.76%	61.47%	65.14%	61.34%
S5129	G3	62.25%	62.39%	79.29%	65.79%	60.16%	65.92%	55.83%	60.55%	72.61%	70.77%	68.55%	67.89%	66.71%	64.48%	70.25%	64.88%	61.6%	60.81%	66.06%	60.29%
S5268	G3	60.81%	61.86%	77.85%	65.92%	59.76%	66.06%	56.62%	60.55%	73%	71.17%	68.28%	68.15%	65.92%	65.27%	70.38%	65.66%	61.47%	61.86%	65.01%	60.42%
S5078	G3	62.12%	61.07%	79.82%	65.53%	59.63%	67.23%	56.62%	60.81%	72.61%	71.17%	69.07%	67.5%	65.92%	65.27%	69.86%	65.01%	62.39%	60.55%	65.27%	60.29%
S5273	G3	62.25%	61.07%	80.08%	65.27%	59.5%	67.23%	56.62%	60.81%	72.61%	71.17%	69.2%	67.23%	65.79%	64.48%	70.64%	64.88%	62.39%	60.55%	64.61%	60.68%
S5083	G3	61.6%	64.74%	77.2%	68.68%	59.11%	68.94%	56.62%	63.83%	71.95%	72.08%	69.86%	67.1%	67.37%	66.71%	69.86%	64.61%	62.25%	60.81%	65.66%	60.16%
S5087	G3	62.25%	61.07%	80.08%	65.27%	59.5%	67.23%	56.62%	60.81%	72.61%	71.17%	69.2%	67.23%	65.79%	64.48%	70.64%	64.88%	62.39%	60.55%	64.61%	60.68%
S5109	G3	62.25%	64.74%	78.77%	68.41%	58.98%	68.02%	57.54%	63.96%	71.95%	70.9%	68.68%	68.02%	65.53%	67.63%	68.28%	65.14%	62.52%	61.21%	63.83%	60.16%
S5137	G1	78.51%	62.78%	62.52%	64.35%	61.21%	66.71%	58.98%	62.65%	64.09%	66.71%	65.53%	66.19%	66.06%	66.45%	62.65%	66.45%	61.47%	61.47%	65.66%	59.37%
S5244	G3	61.86%	64.61%	77.72%	68.81%	59.76%	68.68%	56.75%	63.96%	72.08%	71.69%	69.59%	67.23%	67.1%	66.32%	70.12%	64.22%	62.25%	60.68%	65.66%	59.63%
S5409	G9	64.22%	61.34%	72.35%	66.97%	60.55%	67.76%	56.09%	61.86%	76.28%	70.25%	68.81%	69.07%	66.06%	68.81%	70.77%	63.7%	62.52%	63.3%	66.06%	59.9%
S5410	G3	59.9%	63.7%	76.93%	68.55%	60.68%	68.68%	56.75%	62.39%	72.48%	71.56%	69.72%	67.63%	65.14%	66.71%	69.86%	64.22%	62.78%	61.73%	65.79%	60.03%
S5412	G1	77.33%	62.65%	63.17%	63.43%	60.55%	66.58%	58.85%	61.73%	64.22%	66.19%	65.79%	66.58%	65.4%	66.71%	63.56%	65.53%	60.42%	60.29%	67.1%	58.72%
S5617	G10	63.7%	64.09%	68.68%	69.07%	59.24%	67.89%	55.31%	60.81%	73.39%	79.16%	68.94%	67.37%	64.22%	67.37%	69.2%	66.58%	63.96%	62.91%	66.19%	60.94%
S5338	G3	62.52%	63.3%	79.55%	66.19%	60.16%	65.14%	55.7%	61.21%	72.61%	68.55%	68.41%	67.63%	66.19%	66.58%	71.82%	64.61%	63.43%	60.81%	66.97%	59.9%
S5342	G3	63.17%	62.78%	79.42%	66.32%	60.16%	65.53%	56.62%	61.34%	72.48%	68.68%	68.15%	67.76%	66.32%	66.32%	71.3%	64.74%	63.17%	60.81%	66.71%	59.37%
S5346	G3	62.65%	63.3%	79.55%	66.58%	61.07%	65.53%	56.23%	61.21%	73%	69.07%	68.15%	67.89%	66.84%	66.58%	71.56%	64.35%	63.3%	61.07%	66.58%	59.76%
S5350	G3	62.52%	62.91%	79.42%	66.32%	60.03%	65.53%	56.62%	60.81%	72.87%	68.55%	68.81%	67.89%	66.19%	66.71%	71.69%	64.61%	63.43%	60.68%	66.84%	59.76%
S5353	G3	62.91%	63.17%	79.82%	66.32%	60.42%	65.66%	56.36%	61.21%	72.87%	68.55%	68.55%	68.15%	66.58%	66.58%	71.69%	64.74%	63.43%	60.94%	66.84%	59.63%
S5082	G3	61.34%	61.73%	78.77%	66.19%	59.24%	68.02%	56.09%	62.25%	71.43%	70.38%	70.25%	68.28%	66.45%	65.79%	70.12%	62.65%	61.47%	62.52%	65.27%	61.07%
S5196	G3	61.6%	61.34%	79.42%	66.32%	60.94%	68.41%	55.57%	60.03%	71.43%	69.59%	67.23%	67.23%	63.96%	65.79%	70.12%	64.61%	59.63%	61.34%	65.4%	61.73%
S5215	G3	61.47%	60.42%	78.11%	66.71%	60.94%	66.19%	55.05%	61.73%	71.3%	69.07%	68.02%	67.23%	64.88%	65.27%	69.46%	64.22%	60.68%	60.81%	64.88%	60.42%
S5221	G1	77.2%	61.73%	63.17%	64.48%	60.29%	66.58%	56.36%	61.47%	64.09%	66.84%	67.89%	66.97%	67.37%	68.28%	62.52%	65.14%	62.25%	61.21%	66.32%	59.37%
S5308	G10	62.65%	63.7%	68.41%	68.81%	59.37%	66.97%	55.31%	61.34%	72.48%	77.59%	68.28%	66.71%	65.01%	66.71%	69.33%	66.19%	63.04%	62.65%	66.97%	58.45%
S5517	G8	62.25%	65.92%	61.07%	63.83%	64.09%	64.88%	61.6%	78.11%	61.21%	61.86%	65.92%	65.79%	63.04%	65.92%	62.91%	61.07%	67.89%	66.97%	65.79%	61.73%
S5568	G3	62.78%	60.55%	77.98%	66.58%	61.6%	67.1%	55.96%	60.29%	73.13%	69.46%	66.58%	68.02%	66.06%	65.4%	69.46%	62.39%	62.39%	60.55%	66.19%	61.86%
S5404	G3	61.47%	63.3%	79.03%	66.71%	60.03%	68.68%	56.75%	61.99%	72.35%	69.2%	69.07%	67.5%	67.1%	67.89%	67.63%	65.53%	62.25%	62.12%	64.74%	60.42%
S5405	G1	74.57%	62.25%	65.14%	66.97%	60.42%	66.06%	56.75%	62.91%	68.15%	67.1%	71.04%	68.55%	66.06%	69.72%	63.56%	66.32%	64.48%	62.65%	66.84%	61.07%

Number in grey boxes are the highest similarity with reference EVGs

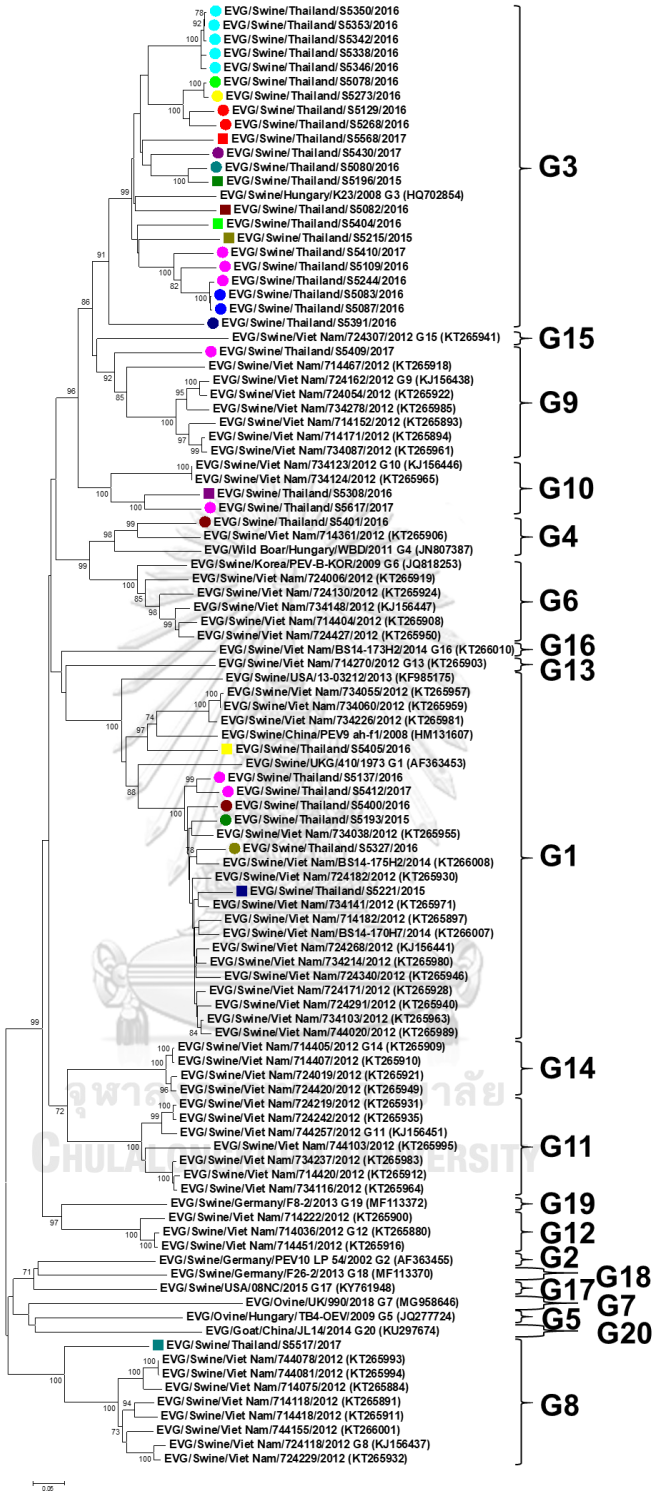

Figure 4.20 Phylogenetic analysis of VP1 gene of the representative EVGs and reference EVGs. The representative viruses are highlighted by circle and square with difference colors. Each color indicates the representative viruses isolated from the same farm. The scale bar represents the distance unit between sequence pairs.

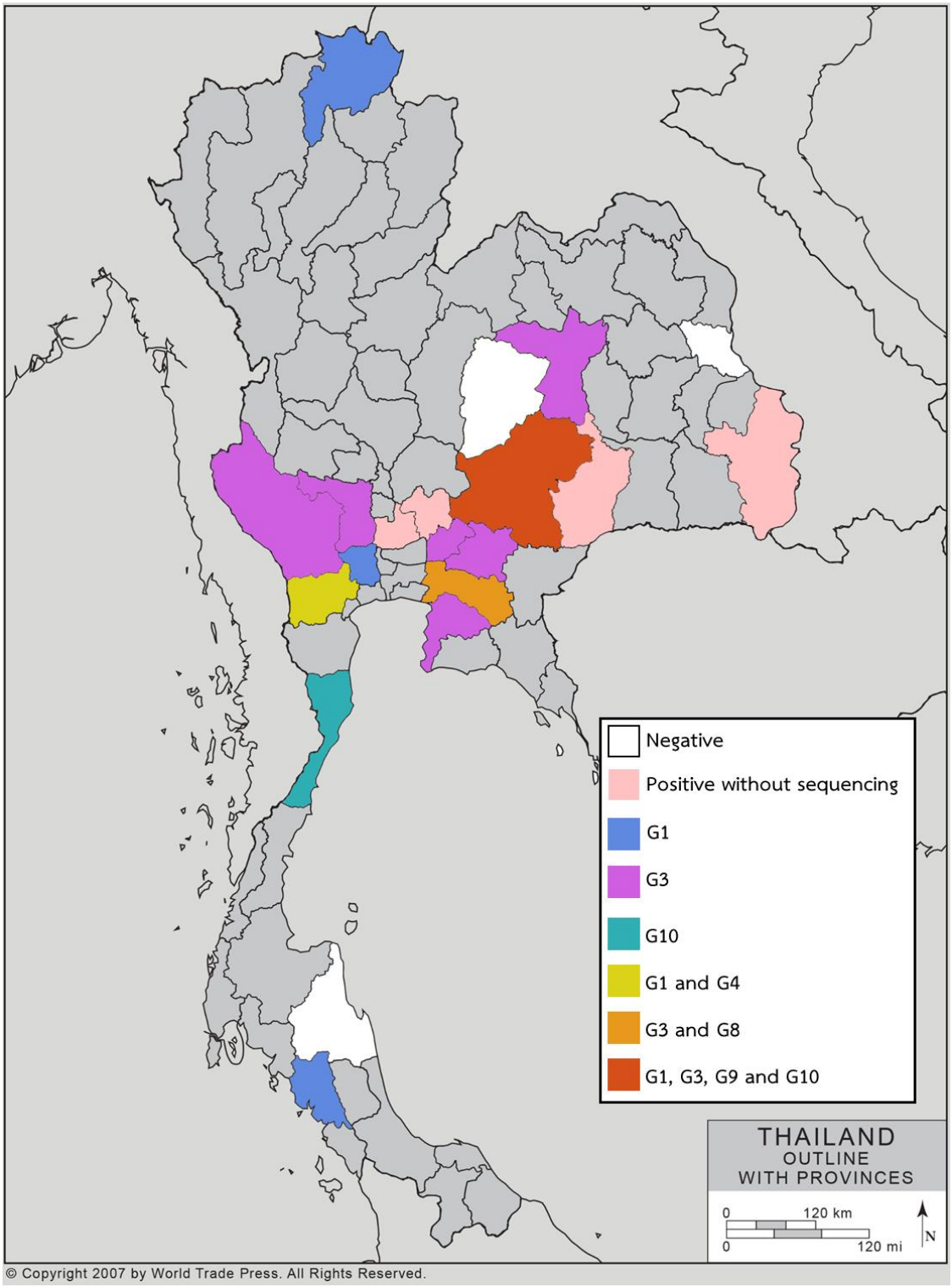

Figure 4.21 Distribution of the representative EVGs by genotypes. The highlight colors are provinces and genotypes.

4.3 Development of rapid diagnostic tests using RT-LAMP with lateral flow device and DNA aptamer

4.3.1 Development of RT-LAMP with LFD for PEDVs detection

For RT-LAMP with LFD for PEDVs detection, the development and evaluation of the assay were carried out for analytical sensitivity and specificity, diagnostic sensitivity and specificity and agreement of the test.

4.3.1.1 Analytical sensitivity and specificity of RT-LAMP with LFD for PEDVs detection

For analytical sensitivity, each DNA standard set contained varied amount of DNA from $2X10^0$-$2X10^9$ copies and negative. The DNA standard sets were tested by using RT-LAMP with LFD for PEDVs detection in triplicate. All three sets of DNA standard showed the consistent results with minimum limit of detection as $2X10^0$ or 2 copies. The results of analytical sensitivity of RT-LAMP with LFD for PEDVs detection are shown in Figure 4.22.

For analytical specificity, The RT-LAMP with LFD reactions were performed in triplicate of bacteria and viruses (n=9) including PEDV, PDCoV, PCV2, *E.coli*, *Salmonella* Thyphimurium, PRRSV strain EU, PRRSV strain US, SIV subtype H1N1 and SIV subtype H3N2. The results showed that RT-LAMP with LFD for PEDVs detection is specific to only PEDVs while the other pathogens are negative. The results of analytical specificity of RT-LAMP with LFD for PEDVs detection are shown in Figure 4.23.

4.3.1.2 Diagnostic sensitivity and specificity and agreement of RT-LAMP with LFD for PEDVs detection

For diagnostic sensitivity, PEDVs positive (n=25) and negative (n=55) samples by qRT-PCR were subjected to RT-LAMP with LFD for PEDVs detection. Then, the comparison between qRT-PCR and RT-LAMP with LFD results was evaluated (Table 4.19). Both diagnostic sensitivity and specificity of RT-LAMP with LFD are 100%. For agreement of the test, the results show 100% agreement between qRT-PCR and RT-

LAMP with LFD. The details of samples (n=80) and results of qRT-PCR and RT-LAMP with LFD for PEDVs detection are shown in Appendix B.

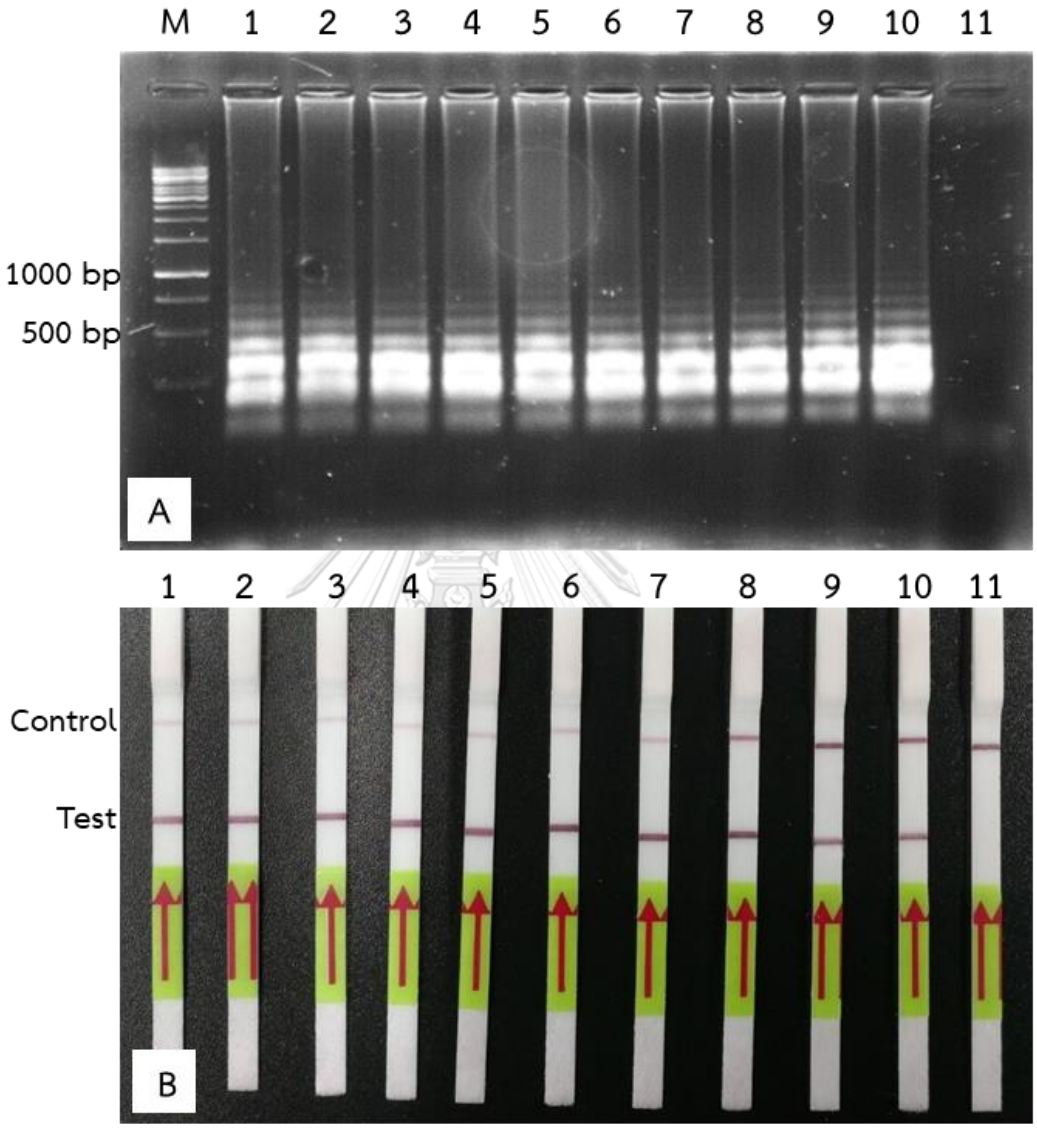

Figure 4.22 Analytical sensitivity of RT-LAMP with LFD for PEDVs detection. A) Visualization of LAMP amplicons by gel electrophoresis. B) Visualization of LAMP amplicons by LFD. M: marker 1kb, 1:2X10^{9} DNA copies, 2:2X10^{8} DNA copies, 3:2X10^{7} DNA copies, 4:2X10^{6} DNA copies, 5:2X10^{5} DNA copies, 6:2X10^{4} DNA copies, 7:2X10^{3} DNA copies, 8:2X10^{2} DNA copies, 9:2X10 DNA copies, 10:2 DNA copies, and 11:negative.

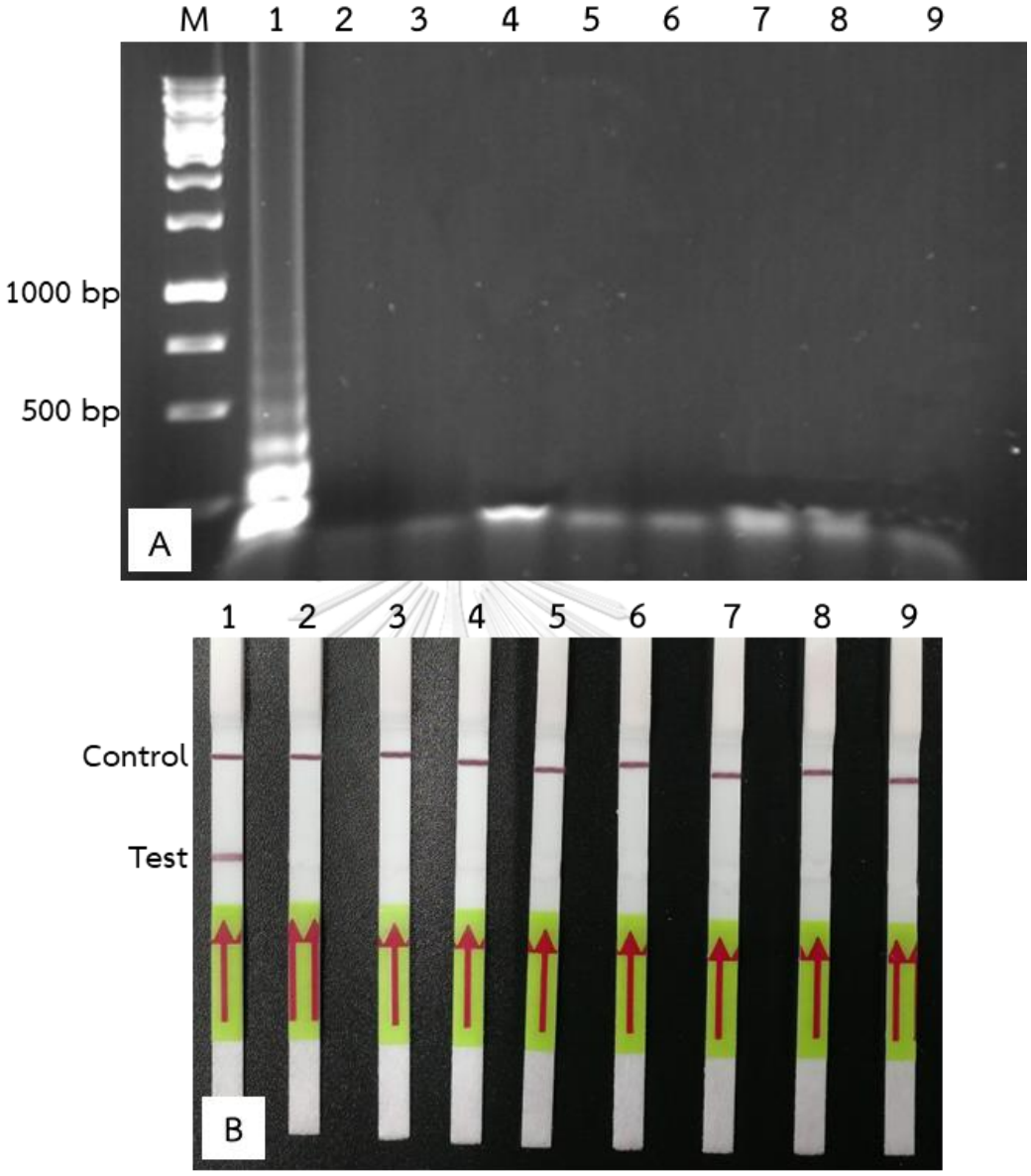

Figure 4.23 Analytical specificity of RT-LAMP with LFD for PEDVs detection. A) Visualization of LAMP amplicons by gel electrophoresis. B) Visualization of LAMP amplicons by LFD. M: marker 1kb, 1:PEDV, 2:PDCoV, 3:PCV2, 4:E.coli, 5:Salmonella Thyphimurium*, 6:PRRSV strain EU, 7:PRRSV strain US, 8:SIV subtype H1N1, 9: SIV subtype H3N2.*

Table 4.19 Diagnostic sensitivity and specificity and agreement of RT-LAMP with LFD for PEDVs detection.

		qRT-PCR (reference)	
		Positive	Negative
RT-LAMP (test)	Positive	25	0
	Negative	0	55

4.3.2 Development of RT-LAMP with LFD for PDCoVs detection

For RT-LAMP with LFD for PDCoVs detection, the development and evaluation of the assay were carried out for the analytical sensitivity and specificity, the diagnostic sensitivity and specificity and agreement of the test.

4.3.2.1 Analytical sensitivity and specificity of RT-LAMP with LFD for PDCoVs detection

For analytical sensitivity, each DNA standard set contained varied amount of DNA from $2X10^0$-$2X10^9$ copies and negative. The DNA standard sets were tested by using RT-LAMP with LFD for PDCoVs detection in triplicate. All three sets of DNA standard showed the consistent results with minimum limit of detection as $2X10^0$ or 2 copies. The results of analytical sensitivity of RT-LAMP with LFD for PDCoVs detection are shown in Figure 4.24.

For analytical specificity, The RT-LAMP with LFD reactions were performed in triplicate of bacteria and viruses (n=9) including PEDV, PDCoV, PCV2, *E.coli*, *Salmonella* Thyphimurium, PRRSV strain EU, PRRSV strain US, SIV subtype H1N1 and SIV subtype H3N2. The results show that RT-LAMP with LFD for PDCoVs detection is specific to only PDCoVs while the other pathogens are negative. The results of analytical specificity of RT-LAMP with LFD for PDCoVs detection are shown in Figure 4.25.

4.3.2.2 Diagnostic sensitivity and specificity and agreement of RT-LAMP with LFD for PDCoVs detection

For diagnostic sensitivity, PDCoVs positive (n=20) and negative (n=60) samples by qRT-PCR were subjected to RT-LAMP with LFD for PDCoVs detection. The comparison between qRT-PCR and RT-LAMP with LFD results is shown in Table 4.20. Both diagnostic sensitivity and specificity of RT-LAMP with LFD for PDCoVs detection are 100%. For agreement of the test, the results showed 100% agreement between qRT-PCR and RT-LAMP with LFD. The details of samples (n=80) and results of qRT-PCR and RT-LAMP with LFD for PDCoVs detection are shown in Appendix B.

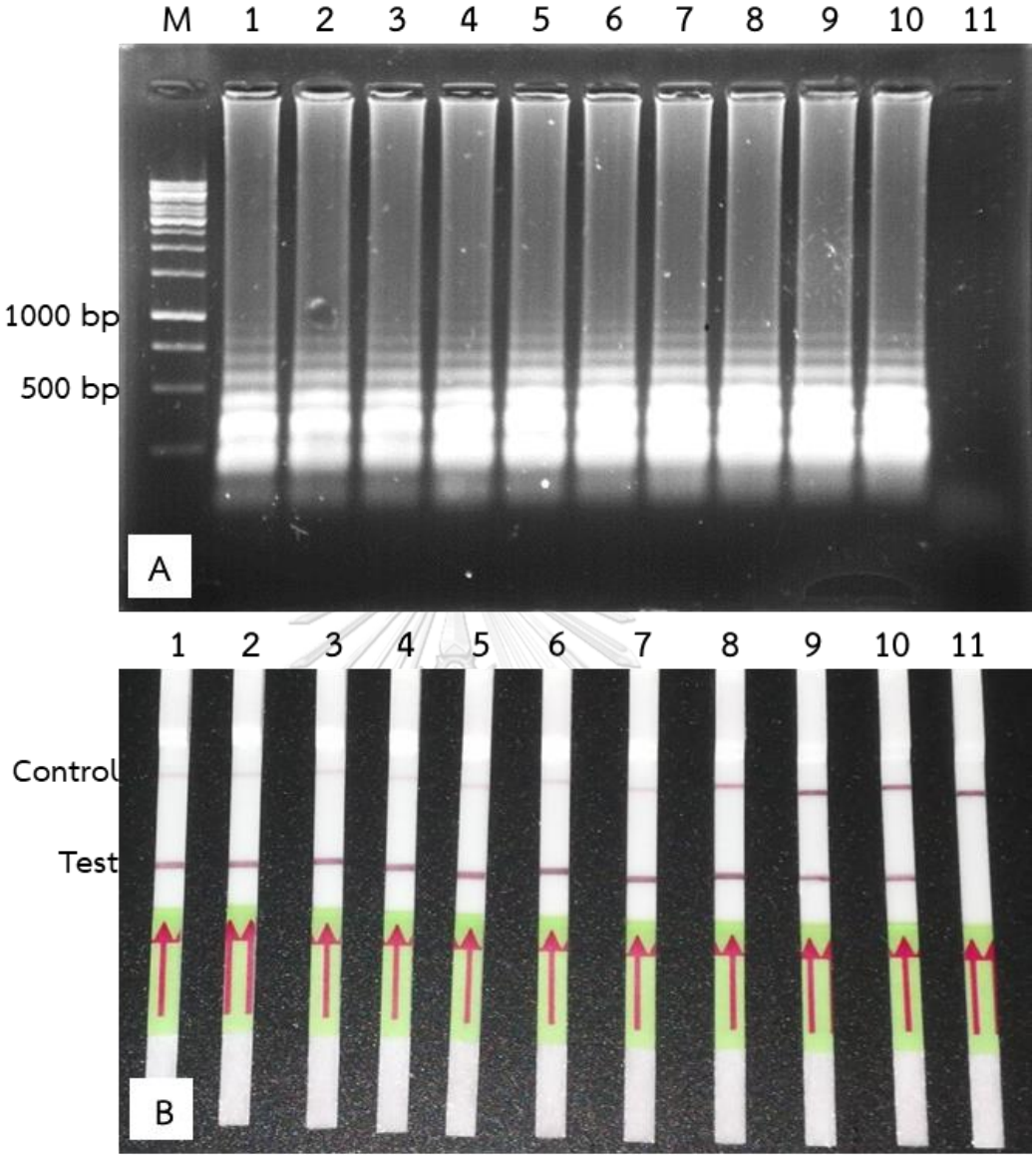

Figure 4.24 Analytical sensitivity of RT-LAMP with LFD for PDCoVs detection. A) Visualization of LAMP amplicons by gel electrophoresis. B) Visualization of LAMP amplicons by LFD. M: marker 1kb, 1:2X10^{9} DNA copies, 2:2X10^{8} DNA copies, 3:2X10^{7} DNA copies, 4:2X10^{6} DNA copies, 5:2X10^{5} DNA copies, 6:2X10^{4} DNA copies, 7:2X10^{3} DNA copies, 8:2X10^{2} DNA copies, 9:2X10 DNA copies, 10:2 DNA copies, and 11:negative.

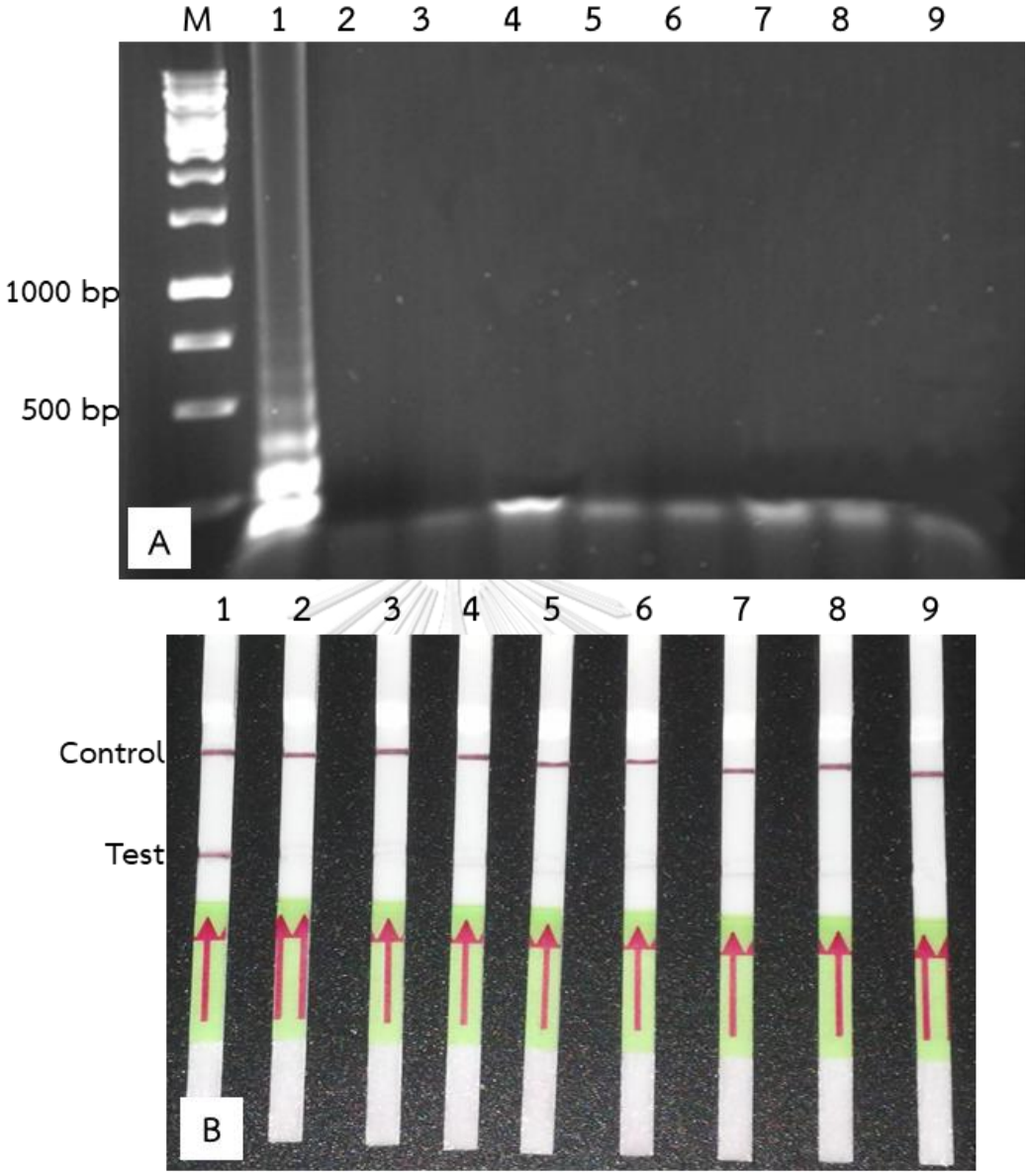

Figure 4.25 Analytical specificity of RT-LAMP with LFD for PDCoVs detection. A) Visualization of LAMP amplicons by gel electrophoresis. B) Visualization of LAMP amplicons by LFD. M: marker 1kb, 1:PDCoV, 2:PEDV, 3:PCV2, 4:E.coli, 5:Salmonella Thyphimurium*., 6:PRRSV strain EU, 7:PRRSV strain US, 8:SIV subtype H1N1, 9: SIV subtype H3N2.*

Table 4.20 Diagnostic sensitivity and specificity and agreement of RT-LAMP with LFD for PDCoVs detection.

		qRT-PCR (reference)	
		Positive	Negative
RT-LAMP (test)	Positive	20	0
	Negative	0	60

4.3.3 Establishment of DNA aptamer for PEDVs detection

4.3.3.1 Preparation of NP protein of PEDV

In this thesis, the consensus NP protein of PEDV (441 amino acid) was retrieved from the reference PEDVs (n=661) from the GenBank database. To evaluate amino acid identities, the consensus sequence and reference PEDVs were aligned and analyzed by using MEGA software version 7.0.26. The results showed that the consensus share amino acid identities with reference PEDVs as 94.78-100%. It is noted that the amino acid identity between consensus and porcine epidemic diarrhea virus vaccine (Zoetis) was 99.32%.

For protein preparation, 58 kDa of recombinant NP based on *E.coli* system including full-length NP and his_6-tagged was expressed. The expressed NP protein and purified NP protein was analyzed and visualized in SDS-PAGE and Coomassie staining. LC/MS-MS of recombinant NP protein show 48% coverage along the full-length NP amino acid. SDS-PAGE and Coomasie staining of expressed NP and purified NP and LC/MS-MS results are shown in Figure 4.26. The quantification of recombinant NP protein is 320 ng/µL.

4.3.3.2 Aptamer preparation and selection

For aptamer preparation and selection, 50 sequences of transformants containing aptamer insertion were examined. None of 50 sequences showed similarities in a Clustal W alignment using MEGA 7 software. Conserved motif sequences among 50 sequences were performed by using MEME analysis. Seven out of 50 candidate aptamers shared the same motif sequences. Seven candidate aptamers with and without primer-binding area were then selected and subjected to process for secondary structure prediction. After analysis, two candidate aptamers (N04 and N25) showed the same stem-loop structure of aptamers.

To evaluate aptamer for specific binding, N04 and N25 aptamers were subjected to EMSA for evaluating that the aptamers recognized recombinant NP protein in dose-dependent form. Our results showed that the competitive binding of EMSA of both aptamers showed specific recognition of aptamers and recombinant NP protein. EMSA and competitive EMSA are shown in Figure 4.27.

To describe the strength of the binding affinity between aptamer and recombinant NP protein, dissociation constant (K_D) of each aptamer were evaluated using enzyme linked aptamer assay based on a nonlinear regression analysis. A concentration of each aptamer that produce 50% of aptamer-protein complex was referred as K_D. The results of binding curve were fitted to a modified equation as follows: AB= ABmax[A]/(K_D+[A]). Where; AB represents aptamer-protein complex as measured by OD at 450 nm, [A] is a concentration of aptamer, and K_D is a dissociation constant. The affinity of N04 and N25 were 3.7 and 6.3 nM respectively. The dissociation constants of each aptamer are shown in Figure 4.28.

To identify the binding region, DNase I footprinting assay was performed. For N04, the binding region between aptamer and recombinant NP protein is aptamer residue between 17 and 20 where the motif sequence is AAGT. While the binding of N04 aptamer and other proteins including bovine serum albumin, recombinant S2 subunit of PEDV and without protein showed signal at residues 17 and 20. For N25, the binding region between aptamer and recombinant NP protein is aptamer residue between 16 and 19 where the motif sequence is AACT. While the binding of N25 aptamer and other proteins including bovine serum albumin, recombinant S2 subunit of PEDV and without protein showed signal at residues 16 and 19. The binding regions of N04 and N25 aptamers are shown in Figure 4.29. In addition, N04 and N25 were achieved site-direct mutations at aptamer residue 19 and 18 respectively for confirmation the binding region area. In theorical, the dissociation constant is changed either increase or decrease the affinity when the sequence of binding region is changed. The dissociation constants of delta N04 and delta N25 were 0.9 and 69.2 nM respectively.

For the detection limit, N04 and N25 aptamers were tested in triplicate with various titers of PEDV vaccine (calaf14) by using enzyme-linked aptamer assay. The titers of the PEDV vaccine were ranging from 0-2X10^5 $TCID_{50}$. The results showed that the limit of detections of N04 and N25 were 1X10^4 and 5X10^4 $TCID_{50}$, respectively. The results of enzyme-linked aptamer assay to identify the detection limit are shown in Figure 4.30.

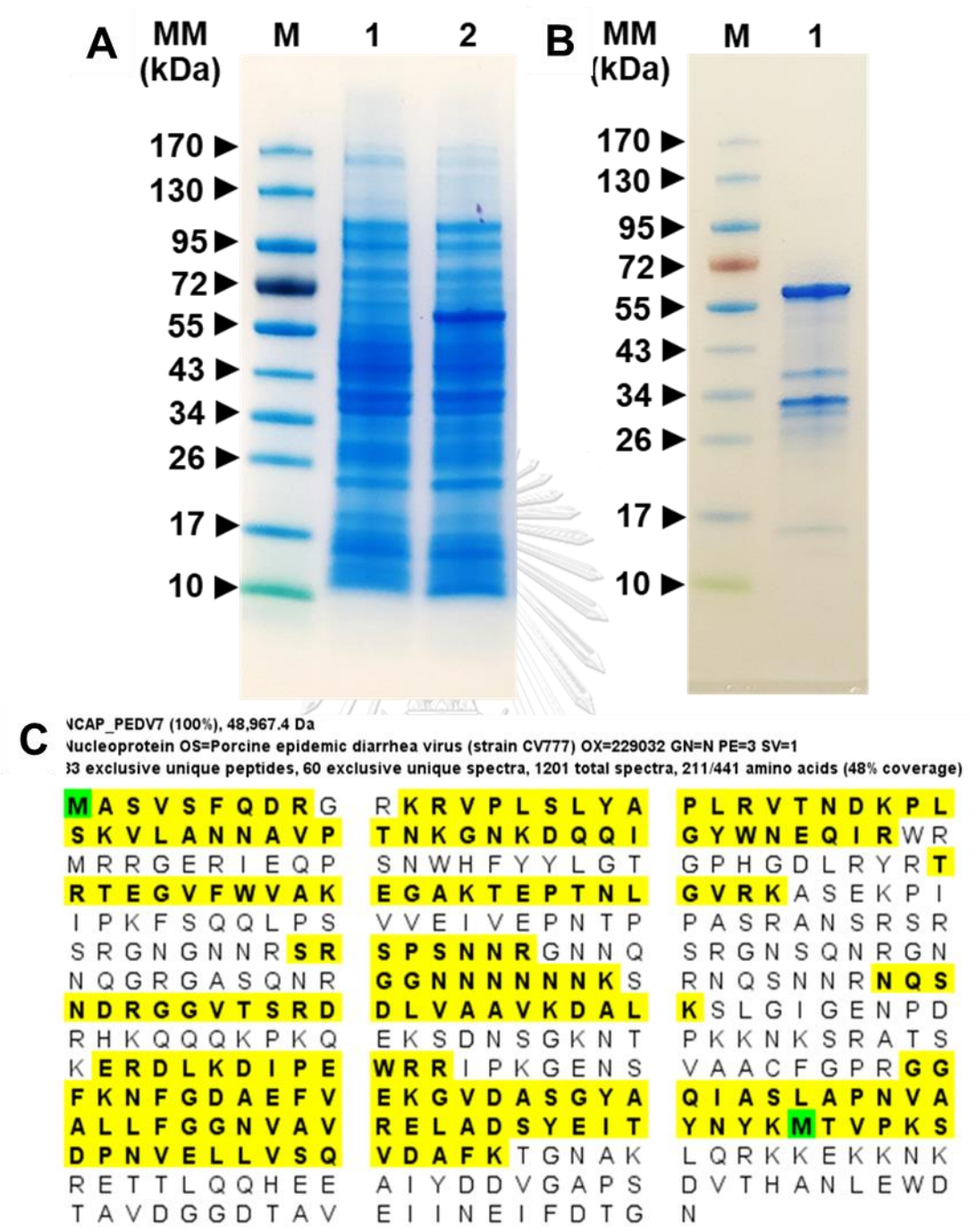

Figure 4.26 Recombinant NP protein preparation. A) NP protein expression. M: Protein marker, 1: pET-24b+ and 2: NP-pET-24b+. B) NP protein purification. M: protein marker and 1: purified NP protein. C) LC-MS/MS of purified recombinant NP protein.

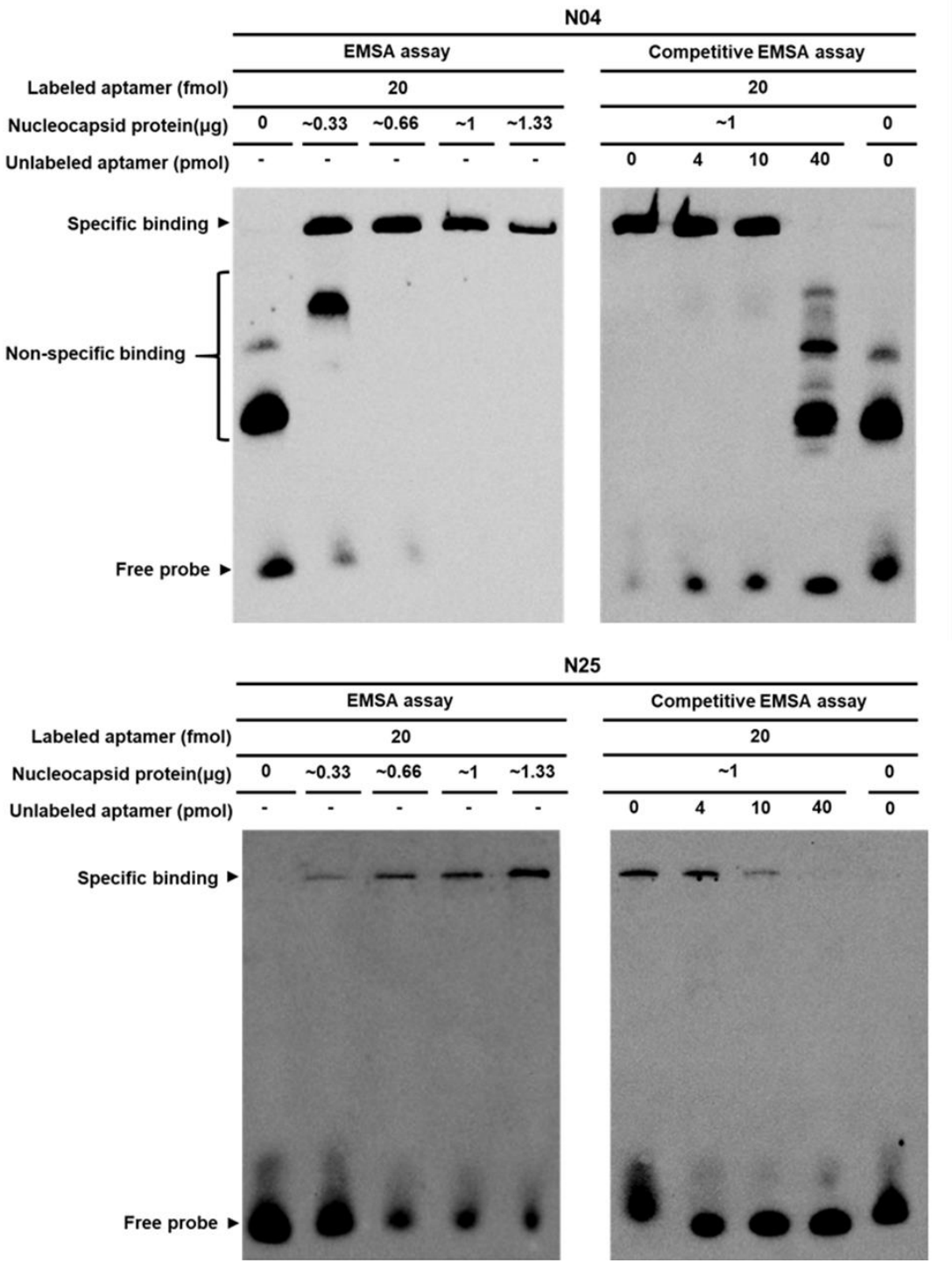

Figure 4.27 EMSA and competitive EMSA of N04 and N05 aptamers.

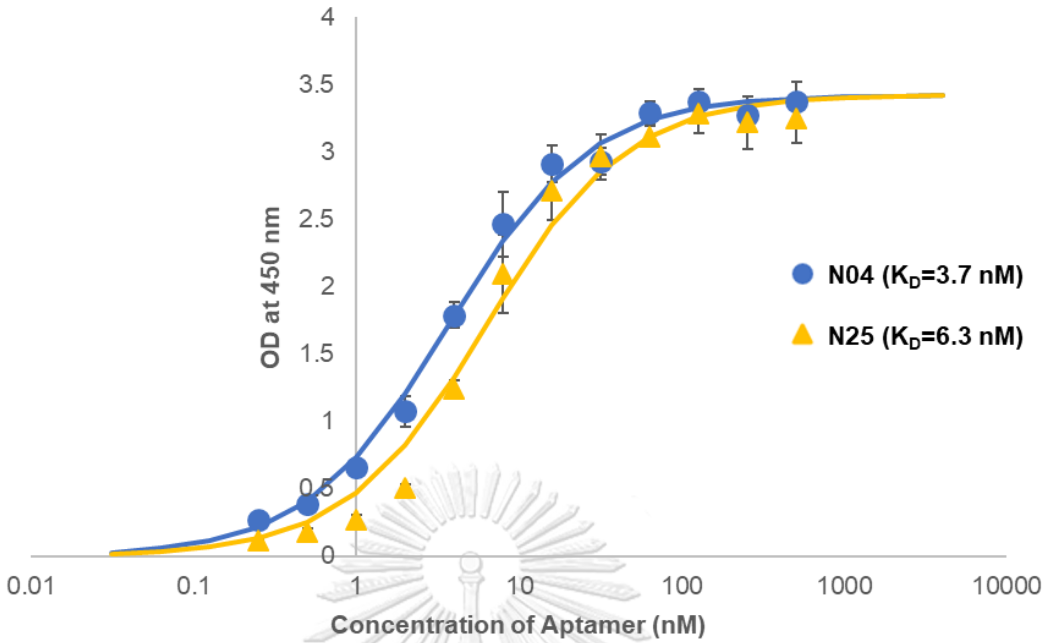

Figure 4.28 Dissociation constants of N04 and N25 aptamers by using nonlinear regression analysis.

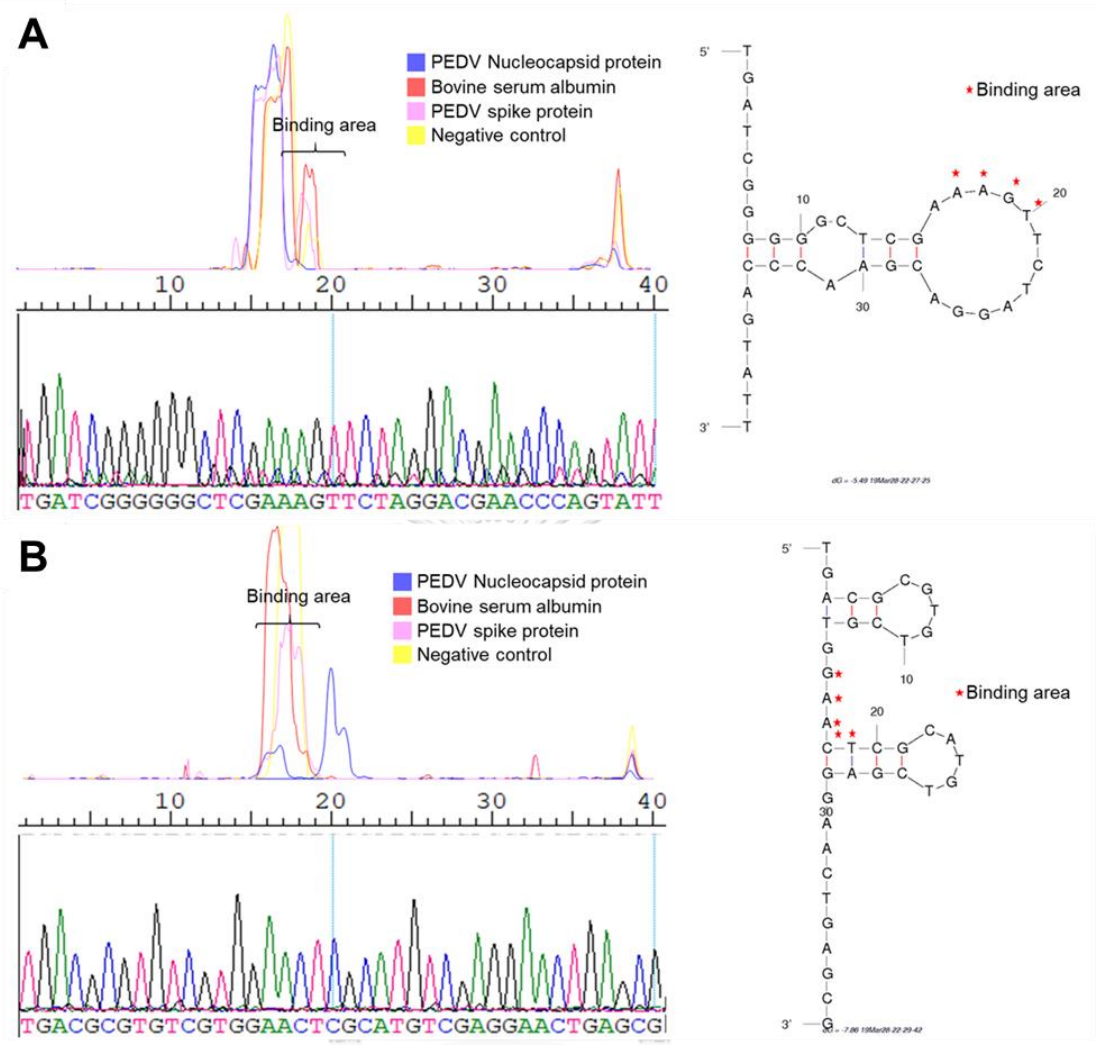

Figure 4.29 DNase I footprinting and secondary structures of N04 and N25 aptamers. A) Binding area of N04 aptamer. B) Binding area of N25 aptamer.

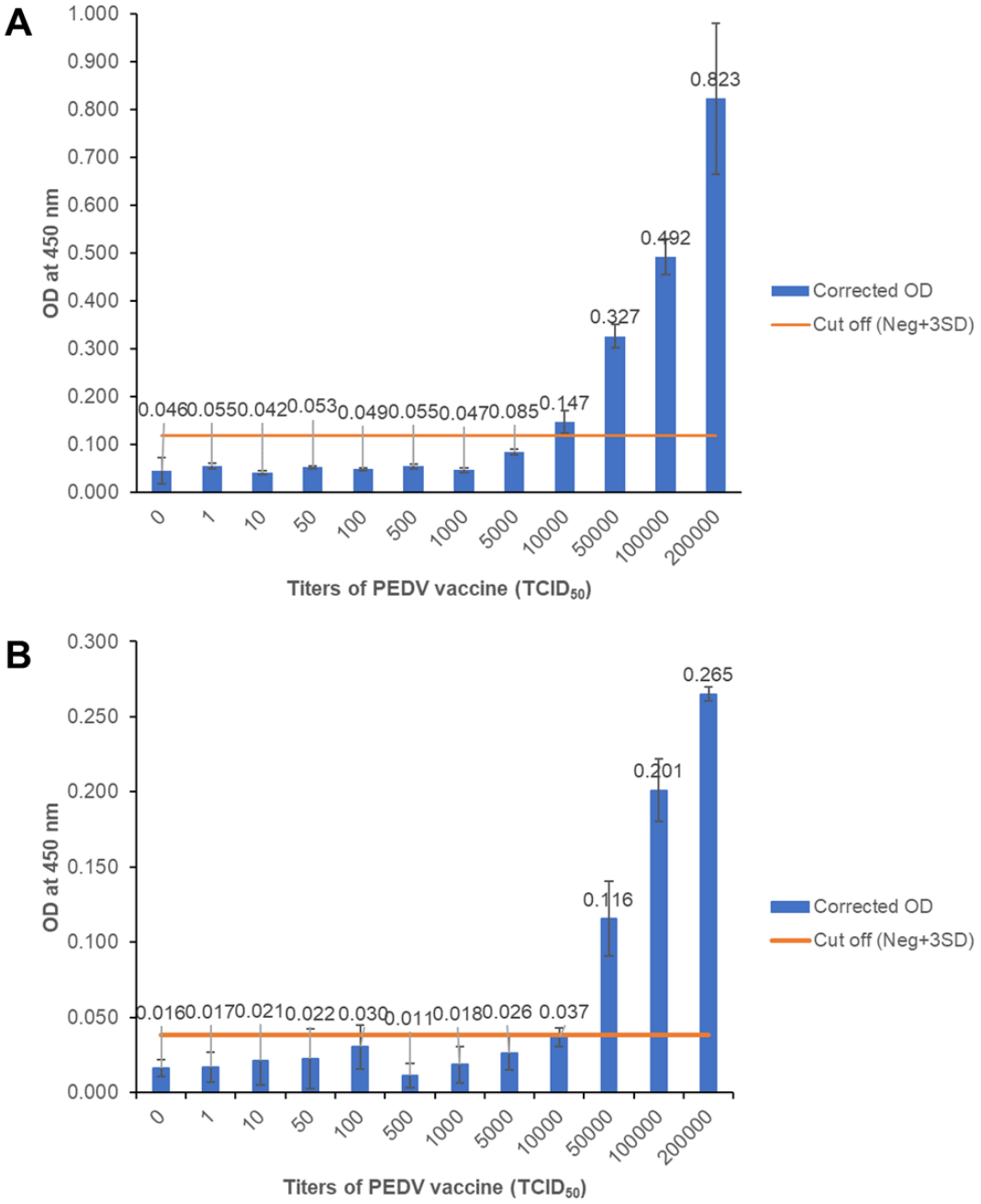

Figure 4.30 Detection limit of N04 and N25 aptamers. A) Results of enzyme-linked aptamer assay of N04 aptamer. B) Results of enzyme-linked aptamer assay of N25 aptamer.

CHAPTER 5

DISCUSSION

5.1 Surveillance of swine enteric viruses in pig farms

Since the emerging of PEDVs in Thailand, pig productions have been suffered due to continuous circulation of PEDVs in pig farms. In 2007, Thai-PEDVs were isolated form the provinces in western and eastern parts of Thailand. The PEDVs in 2007 were characterized and classified as genotype G2 (Chinese-like). Then during 2008-2014, the surveillance of Thai-PEDVs has been performed and the results showed that PEDVs genotype G1 and G2 were circulating throughout the country (Puranaveja *et al.*, 2009; Stott *et al.*, 2017; Temeeyasen *et al.*, 2014). It has been reported that PEDVs can infect in pigs of all age groups. However, PEDVs cause severe diarrhea, vomiting, and dehydration in piglets with up to 100% mortality (Wood, 1977).

In 2012, the first identification of PDCoVs was reported in pigs in Hong Kong. Later on, PDCoVs were discovered in worldwide. Co-circulation of PEDVs and PDCoVs was reported in China and some countries (Lee and Lee, 2014a; Marthaler *et al.*, 2014a; Saeng-Chuto *et al.*, 2017; Song *et al.*, 2015; Woo *et al.*, 2012; Zhang *et al.*, 2019a). The comparisons of clinical signs, gross lesions and histopathological lesions between PEDVs and PDCoVs infections are similar (Wang *et al.*, 2016c).

Recently, 20 genotypes of EVGs have been discovered in China, England, German, Hungary, Japan, Scotland, South Korea, USA and Vietnam (Boros *et al.*, 2012a; Boros *et al.*, 2012b; Boros *et al.*, 2011; Moon *et al.*, 2012; Van Dung *et al.*, 2014; Zhang *et al.*, 2012). In infected pigs, various conditions including diarrhea, poliomyelitis, pneumonia and enteritis have been observed (Palmquist *et al.*, 2002; Pogranichniy *et al.*, 2003). Inter- and Intra-species recombination of genus *enterovirus* rarely occur but novel recombinant enteroviruses in humans and animals

have been reported (Sun *et al.*, 2014; Tapparel *et al.*, 2009; Van Dung *et al.*, 2014; Yozwiak *et al.*, 2010).

Since, PEDVs, PDCoVs and EVGs are important pathogens of food security and public health concerns. The limit information on the occurrences and status of PEDVs, PDCoVs and EVGs in Thailand lead us to carry out the surveillance of swine enteric viruses in pig farms during December 2014 – January 2018 from 73 pig farms from 20 provinces of 7 livestock regions. Subsequently, the recommendations for the prevention and control strategies in pig farms have been developed.

5.1.1 The occurrences of PEDVs

Our results showed that the occurrences of PEDVs by samples (44.02%; 342/777) and by pig farms (50.68%; 37/73) were high. Comparing with the previous study in Thailand, the occurrence of PEDVs was higher than previous report (19.90%; 153/769). Previous studies in China reported that the occurrences of PEDVs were ranging from 32.18% to 57.32% which comparable to the result of this thesis. By pig farms, the occurrences of PEDVs in this thesis was lower than the previous study in China which almost all pig farms were positive (96.43%). However, the study in the US showed that the occurrence of PEDVs in pig farms was 40.5% which similar to our results (Beam *et al.*, 2015; Chen *et al.*, 2019; Feng *et al.*, 2020; Puranaveja *et al.*, 2009; Stott *et al.*, 2017; Temeeyasen *et al.*, 2014; Tuanthap *et al.*, 2019; Zhang *et al.*, 2019a).

5.1.1.1 The occurrences of PEDVs by locations

Our results showed that Thai-PEDVs could be detected in 5 livestock regions but not in 2 livestock regions of the North and South of Thailand. Thai-PEDVs were recovered from pigs in the high density of pig production provinces such as Chachoengsao, Chonburi, Nakhon Ratchasima, Nakhon Pathom, Prachinburi and Ratchaburi. Our results showed that Thai-PEDVs were recovered from pig farms in the same geographic locations as previous studies in Thailand (Puranaveja *et al.*, 2009; Stott *et al.*, 2017; Temeeyasen *et al.*, 2014; Tuanthap *et al.*, 2019).

5.1.1.2 The occurrences of PEDVs by age groups of pigs

Our results showed that Thai-PEDVs were detected in all age groups of pigs which were similar to previous studies of PEDVs in the US (Chen *et al.*, 2014; Stevenson *et al.*, 2013). The highest occurrence of PEDVs was in suckling pigs (48.42%). Moreover, PEDVs infection in suckling and nursery pigs was statistically significant higher than in fattening and breeder pigs. Our findings agreed with the previous study on PEDV epidemiological survey by using meta-analysis which indicated that the prevalence of PEDVs in piglets was statistically significant higher than in fattening and sow pigs (Chen *et al.*, 2019).

5.1.1.3 The occurrences of PEDVs by seasonal patterns

Our results showed that Thai-PEDVs were recovered from pigs in almost every month and every year. PEDVs were mostly detected in summer season (46.11%) but no statistically significant difference from the other seasons. Our results contradict with the meta-analysis study in China, which PEDVs were mostly detected in spring and winter seasons.

5.1.2 The occurrences of PDCoVs

Our results showed that the occurrences of PDCoVs by samples (3.47%; 27/777) and by pig farms (9.59%; 7/73) were lower than the occurrences of PEDVs. Our findings were in agreement with previous studies in China, which the prevalence of PDCoVs (13.25% and 27.22%) was lower than the prevalence of PEDVs (32.18% and 57.32%) (Feng *et al.*, 2020; Zhang *et al.*, 2019a).

5.1.2.1 The occurrences of PDCoVs by locations

Our results showed that Thai-PDCoVs were detected in pigs from livestock regions 2 and 7. According to 3 year-reports of Thai-DLD (2014, 2015 and 2017), those livestock regions are top 2 of pig production areas in Thailand. We found that PDCoVs were circulating in 5 provinces including Chachoengsao, Chonburi, Nakhon Pathom, Prachinburi and Ratchaburi. These results were similar to previous study in

Thailand that PDCoVs were detected from the same geographic locations (Saeng-Chuto *et al.*, 2017).

5.1.2.2 The occurrences of PDCoVs by age groups of pigs

Our results showed that the occurrence of Thai-PDCoVs was highest in fattening pigs while the lowest occurrence in nursery pigs. There is no statistically significant difference of PDCoVs infection among age groups of pigs. Previous studies reported that PDCoVs were mostly detected in suckling pigs and sows more than in fattening pigs. Another study in Japan revealed that PDCoVs most likely be detected in older pigs than suckling pigs (Feng *et al.*, 2020; Zhang *et al.*, 2019a).

5.1.2.3 The occurrences of PDCoVs by seasonal patterns

Our results showed that PDCoVs could be detected in all seasons but often detected during the cold weather more than the other seasons. These findings are the same as previous studies in China, which PDCoVs were detected in spring and winter seasons more than summer season (Feng *et al.*, 2020).

5.1.3 The occurrences of EVGs

Our results showed that the occurrences of EVGs by samples (71.56%; 556/777) and by pig farms (69.86%; 51/73)) were high. Our findings of EVGs infection were lower than the previous study in Vietnam (81.6%) (Van Dung *et al.*, 2016). In contrast, our results in Thailand were higher than those in the previous studies in China (8.3%), Czech Republic (50.2%), Italy (7.5%) and Spain (0%) (Buitrago *et al.*, 2010; Prodelalova, 2012; Sozzi *et al.*, 2010; Yang *et al.*, 2013).

5.1.3.1 The occurrences of EVGs by locations

Our results showed that Thai-EVGs could be detected from pigs in all livestock regions. There are only 3 provinces (Chaiyaphum, Nakhon Si Thammarat and Mukdahan) were not detected. These could be the effect of sample size in those provinces. Our findings indicated that EVGs might be circulating throughout the country.

5.1.3.2 The occurrences of EVGs by age groups of pigs

Our results showed that Thai-EVGs could be recovered from fattening (89.66%) and nursery (89.35%) pigs statistically significant higher than from breeder (61.32%) and suckling (64.86%) pigs. Our results in consistent with the previous study that EVGs were mostly detected in weaned pigs more than in older pigs (>1 year old) (Van Dung *et al.*, 2014).

5.1.3.3 The occurrences of EVGs by seasonal patterns

Our results showed that Thai-EVGs infection were high during rainy season. There is no report of seasonal pattern study of EVGs. However, a report of human enterovirus showed that human enterovirus infection was increased in rainy season (Puenpa *et al.*, 2018; Puenpa *et al.*, 2013).

5.1.4 Analysis of co-circulation of PEDVs, PDCoVs and EVGs

In this thesis, the infection of EVGs (single infection) was the highest in all age groups of pigs. Co-circulation of PEDVs and EVGs and co-circulation of PDCoVs and EVGs were observed in all age groups. While the co-circulation of PEDVs, PDCoVs and EVGs (0.13%) was only detected in breeder groups. In contrast with previous study in China showed that the co-circulation of PEDVs and PDCoVs (12.72%) was higher than our findings. It is noted that EVGs were excluded from their study (Zhang *et al.*, 2019a).

In summary, in phase 1 of this thesis, the occurrences of PEDVs by samples and by pig farms were high. Thai-PEDVs were circulating in 15 provinces of 5 livestock regions. One of risk factors related to PEDVs outbreak was age of pigs which mostly affected in suckling and nursery pigs. While seasonal patterns did not associate with PEDVs outbreak in this thesis. The occurrences of PDCoVs by samples and by pig farms were lower than the occurrences of PEDVs. Thai-PDCoVs were detected in 5 provinces of top 2 of pig production areas in Thailand. However, the occurrences of PDCoVs were increased during winter season, there is no relation between age of pigs and the occurrences of PDCoVs. The occurrences of EVGs by samples and by pig

farms in pigs were the highest of viral enteric detection in this thesis. Thai-EVGs were detected throughout the country. The rainy season and weaned pigs (nursery and fattening pigs) were risk factors for the EVGs infection. In addition, the co-circulation of PEDVs, PDCoVs and EVGs was low in this thesis.

5.2 Genetic characterization and phylogenetic analyses of swine enteric viruses

In phase 2, representative Thai-PEDVs (N=39) were selected from 342 positive samples for S and ORF3 genes sequencing. S and ORF3 genes of Thai-PEDVs and reference PEDVs (G1a, G1b, G2a and G2b) were aligned and analyzed for genetic characterization and the patterns of 4 epitopes (COE, SS2, SS6 and 2C10) of S gene were observed. To analyze genetic diversity, phylogenetic analyses of S and ORF3 genes were performed. In this thesis, the phylogenetic tree suggested that Thai-PEDVs could be grouped into novel G1, G2a and novel G2.

Representative Thai-PDCoVs (n=16) were selected from 27 positive samples for genetic characterization. Two viruses with high RNA copies were subjected to whole genome sequencing and 14 other viruses were subjected to S gene sequencing. Thai-PDCoVs sequences and reference PDCoVs were aligned and analyzed for genetic characterization and the patterns of 3 epitopes (NTD, CTD and S2) on S gene were observed. To analyze genetic diversity, phylogenetic analyses of WGS and S gene were performed. In this thesis, the phylogenetic tree of WGS and S gene showed consistent results that PDCoVs could be classified into 4 groups based on geographic locations.

Representative Thai-EVGs (n=34) were selected from 556 positive samples for VP1 gene sequencing. VP1 gene of Thai-EVGs and reference EVGs (20 genotypes) were aligned and analyzed for nucleotide identities. To analyzed genetic diversity, phylogenetic analysis of VP1 gene of viruses was performed. The phylogenetic tree and nucleotide identities results revealed that Thai-EVGs were classified into 6 genotypes (G1, G3, G4, G8, G9 and G10).

5.2.1 Genetic characterization and phylogenetic analyses of Thai-PEDVs

Our results showed that the nucleotide (91.63%-93.59%) and amino acid (91.14%-93.57%) identities among Thai-PEDVs (vs PEDVs strain CV777) were low which indicated that Thai-PEDVs could be distinct from PEDVs (CV777) lineage. In S gene characterization, at least 8 patterns of insertions and deletions of Thai-PEDVs were observed. It is noted that 3 out of 9 regions (12 nt insertion at position 202-204, 3 nt insertion at position 402-403 and 6 nt deletion at position 472-477) of S gene are genetic signature to differentiate between PEDVs genotypes G1 and G2 (Lee *et al.*, 2010). The unique insertion and deletion patterns of Thai-PEDVs (S5052 and S5054) that have not been reported were identified in this thesis. Recently, there are several variant PEDVs has been reported worldwide. A previous study in China reported that 7 amino acid deletion at S2 subunit of PEDVs (G2b) is related to mild diarrhea in piglets (Chen *et al.*, 2016a; Masuda *et al.*, 2015; Oka *et al.*, 2014; Park *et al.*, 2014; Zhang *et al.*, 2015). Moreover, PEDVs (G1) causing milder diarrhea or less virulence than PEDVs (G2) was reported (Chen *et al.*, 2016b).

Since the first outbreak of PEDVs in 2007, Thai pig farms faced economic losses from PEDVs infection. Circulating Thai-PEDVs were PEDVs genotypes G1a and G2 (unclassified subgroup) (Puranaveja *et al.*, 2009; Stott *et al.*, 2017; Temeeyasen *et al.*, 2014; Tuanthap *et al.*, 2019). In this thesis, the result of phylogenetic tree of S gene suggested that Thai-PEDVs could be classified into Novel G1, G2a and Novel G2. PEDVs genotypes Novel G1 and Novel G2 have not been reported before. While PEDVs genotype G2a were recovered from China, South Korea and South East Asia (Lee, 2015). All representative Thai-PEDVs (n=39) were not grouped with vaccine strain suggesting Thai-PEDVs are possible the field strains with no deletion on ORF3. Less pathogenicity in pigs and deletion on ORF3 gene of all attenuated PEDV vaccines have been observed due to cell adaptation (Park *et al.*, 2008; Si *et al.*, 2020).

PEDVs genotype 2 (G2a (n=17) and Novel G2 (n=20)) might be the predominant genotypes recently responsible for PEDVs outbreaks in Thailand. On the other hand,

very low occurrence of PEDVs (Novel G1 (n=2)) was observed. In details, the consistent genotypes of PEDVs were recovered from the same farms at different date of sample collection. There are 2 pig farms showed PEDVs recovered from the farms showed different genotypes. Our finding suggested that either new introduction of PEDVs strains or persistent circulating PEDVs strains are responsible for Thai-PEDVs outbreaks. To understand the Thai-PEDVs outbreak, the routes of transmission are important factors. Main routes of PEDVs transmission are fecal-oral route and indirect contact via contaminated fomites including transport trailers, farm workers, boots and cloths, feed, feed ingredients and additives (Jung *et al.*, 2020). Moreover, the aerosol transmission route was documented (Alonso *et al.*, 2015; Beam *et al.*, 2015; Gallien *et al.*, 2018; Li *et al.*, 2018b). PEDVs could be survive at room temperature for 35 days while cooler temperature increases the PEDVs viability time (Scott *et al.*, 2016). A previous study has been described that infectability PEDVs could survive in swine manure lagoon up to 9 months. Therefore, the swine manure lagoon might be another sources of maintain PEDVs in pig farms (Tun *et al.*, 2016).

At least 10 patterns at COE epitope, 1 pattern at SS2 epitope, 3 patterns at SS6 epitope and 2 patterns of 2C10 epitope could be observed. Moreover, 1 pattern (^{764}PQEGQVKI^{771}) at SS6 epitope of Novel G1 PEDVs (S5052 and S5054) and 1 pattern at 2C10 epitope (^{1368}GPRFQPY^{1374}) of Novel G2 PEDVs (S5843) have not been identified in Thailand before (Kim *et al.*, 2016b). The variations of 4 epitopes might lead to partial protection of gut feedback or vaccine. There are several studies reported that G2a PEDVs based vaccine is the candidate vaccine for protection of PEDVs infection (genotypes G1a, G1b, G2a and G2b) (Chen *et al.*, 2016c; Liu *et al.*, 2019; Opriessnig *et al.*, 2017; Sato *et al.*, 2018; Zhang *et al.*, 2020b). The efficacy of G2a PEDVs based vaccine should be evaluated for the protection of Novel G1 and Novel G2 PEDVs infection. Thus, the prevention and control strategies for PEDVs are enhance biosecurity (herd and farm managements and farm sanitation) and immunoprophylaxis (gut feedback and vaccination) (Jung *et al.*, 2020).

5.2.2 Genetic characterization and phylogenetic analyses of Thai-PDCoVs

Our results showed that the nucleotide (95.63%-96.41%) and amino acid (96.48%-97.90%) identities among Thai-PDCoVs (vs PDCoVs strain HKU15-155) were low indicating that Thai-PDCoVs could be distinct from PDCoVs (HKU-15-155) lineage. In WGS characterization, multiple insertions and deletions of Thai-PDCoVs were observed. Our findings suggested that Thai-PDCoVs could be form a new cluster of PDCoVs which were similar to the viruses from Vietnam and Laos (Le *et al.*, 2018; Saeng-Chuto *et al.*, 2017). In S gene characterization, all 16 Thai-PDCoVs contain consistent insertion (AAT) on S gene.

Phylogenetic trees of WGS and S gene showed consistent results which PDCoVs could be classified into 4 groups based on geographic locations (China, Thailand, USA and Korea, and Vietnam clusters). Our findings indicated that S gene could be used for genetic diversity study in comparable with previous study of other coronaviruses (Liu *et al.*, 2017; Maurel *et al.*, 2011). Thai-PDCoVs were belonging to Thailand cluster which were grouped with Laos-PDCoVs (Janetanakit *et al.*, 2016; Saeng-Chuto *et al.*, 2017). Moreover, our results indicated that only Thailand cluster is circulating in Thailand. The associations between severity and PDCoVs lineages have not been reported.

Our results showed that multiple amino acid changes were observed at 3 epitopes (NTD, CTD and S2). These variations could be led to the failure of vaccination with the other lineages. A previous study in China showed that the successful immunization of inactivated PDCoVs vaccine in experimental study reduce the clinical severity and viral shedding from challenging with the same strain of PDCoVs (Zhang *et al.*, 2020a). The routes of transmission of PDCoVs have not been documented but It might be fecal-oral route and indirect contact which are the same as PEDVs transmission. Interestingly, PDCoVs have been reported in wide host range (human, clave, chicken and turkey) in vivo and vitro studies which infected clave, chicken and turkey showed diarrhea signs and seroconversion. These studies indicated that avian and clave could be a reservoir of PDCoVs (Boley *et al.*, 2020;

Jung *et al.*, 2017; Li *et al.*, 2018a; Liang *et al.*, 2019). Therefore, the prevention and control strategies for PDCoVs are enhance biosecurity (herd and farm managements and farm sanitation) and immunoprophylaxis. However, there is no commercial PDCoVs vaccine available.

5.2.3 Genetic characterization and phylogenetic analysis of Thai-EVGs

Our results showed that at least 6 genotypes (G1, G3, G4, G8, G9 and G10) of Thai-EVGs were circulating in Thailand. The nucleotide similarities of VP1 gene were used for genotype classification which were based on > 25% nucleotide divergence (Oberste *et al.*, 1999). EVGs genotype G3 was predominant genotype in this thesis which were similar to a previous surveillance study in Japan (Tsuchiaka *et al.*, 2018). While a previous study in Vietnam reported that G1 and G6 were the most detected (Van Dung *et al.*, 2016). Moreover, our results showed that G1 infected in suckling, nursery and breeder pigs, G3 infected in suckling pigs and breeder, G9 only infected in suckling pigs, G10 and G4 only infected in nursery pigs and G8 only infected in fattening pigs. Thus, the genotypes of EVGs infection could be associated with ages of pigs which were in agreement with a previous study in Vietnam (Van Dung *et al.*, 2014).

EVGs-G1 were known as PEV-9 or PEV-B which caused diarrhea and flaccid paralysis of the hind limbs. EVGs-G3, G4, G8, G9 and G10 were recovered from healthy pigs in Germany, Hungary, Japan and Vietnam (Boros *et al.*, 2012a; Bunke *et al.*, 2018; Knutson *et al.*, 2017; Lee and Lee, 2019; Sekiguchi *et al.*, 2020; Tsuchiaka *et al.*, 2018; Van Dung *et al.*, 2014; Van Dung *et al.*, 2016; Yang *et al.*, 2013). Moreover, the recombinant of EVGs have been reported that insertion of papain-like cysteine protease (PL-CP) of torovirus between 2C and 3A genes have been observed. Those EVGs were genotype G1, G2, G8, G10 and G17 which were recovered from diarrhea pigs in China, Belgium, Germany, Japan, South Korea and USA (Sekiguchi *et al.*, 2020). Although, most EVGs were detected in healthy pigs, some virulent EVGs could be detected in infected pigs with clinical signs. Therefore, the continuous surveillance on inter- and intra-species recombination should be carried out.

In summary, in phase 2 of this thesis, Thai-PEDVs could be classified into 3 groups (Novel G1, G2a and Novel G2) which PEDVs G2a were circulating in China, South Korea and South East Asia. While Novel G1 and Novel G2 have not been reported. Most Thai pig farms could be suffered from PEDVs (G2) infection which either from introduction of new strains or persistent circulating PEDVs strains. At least 3 epitopes showed multiple amino acid changes and 2 novel patterns at SS6 and 2C10 epitopes were observed in this thesis. Thus, the efficacy of commercial PEDVs vaccine should be evaluated. PDCoVs could be classified into 4 groups based on geographic locations. The phylogenetic analysis of S gene of PDCoVs could be used for genetic diversity study instead of phylogenetic analysis of WGS. All Thai-PDCoVs were belonging to Thailand cluster. In addition, PDCoVs is a potential zoonoses. For EVGs, at least 6 genotypes of Thai-EVGs were circulating in Thailand. The predominant genotype was G3. The genotype of EVGs associated with ages of pigs. Although, most EVGs were detected in healthy pigs, some virulent EVGs could be observed in infected pigs with clinical signs.

5.3 Development of rapid diagnostic tests using RT-LAMP with lateral flow device and DNA aptamer

Infected pigs with PEDVs and PDCoVs showed similar in clinical signs, gross lesions and histopathological lesions (diarrhea, vomiting, transparency intestinal wall and shortened villi) (Chen *et al.*, 2015; Jung *et al.*, 2015; Wang *et al.*, 2016c). Even though, the morbidity and mortality rate of PDCoVs infection lower than of PEDVs infection were reported. The distinguish between PEDVs and PDCoVs infections in field settings is still difficult (Koonpaew *et al.*, 2019). Consequently, the rapid diagnostic kits in field settings to differentiate PEDVs and PDCoVs infections are required for prevention and control strategies for PEDVs and PDCoVs.

There are several methods for nucleic acid based detection including conventional RT-PCR, qRT-PCR and nested RT-PCR. Those methods are inappropriate assays in field settings because of required time consuming, specialized equipment

and experience scientists. Since LAMP technique was established in 2000, an application of nucleic based detection in field settings could be possible. Moreover, the varieties of result interpretation including turbidity analysis, visual analysis and lateral flow device are available for LAMP technique (Nagamine *et al.*, 2002; Notomi *et al.*, 2000; Parida *et al.*, 2006; Yamazaki *et al.*, 2013; Zhang *et al.*, 2014b).

5.3.1 Development of RT-LAMP with LFD for PEDVs and PDCoVs detection

Our results showed that those 2 test kits for PEDVs and PDCoVs detection were high in analytical sensitivity which could be detected $2X10^0$ or 2 DNA copies. In analytical specificity, the 2 test kits had high specificity which the other important swine pathogens could not be detected. In diagnostic sensitivity and specificity, there are no false negative and false positive were observed in clinical samples (n=80). Both test kits showed high diagnostic sensitivity (100%) and high specificity (100%). Moreover, 100% agreements of both tests were observed.

Although, the RT-LAMP with LFD for PEDVs and PDCoVs detection are high in analytical sensitivity and specificity, diagnostic sensitivity and specificity, agreement of the test. The limitation of the RT-LAMP with LFD is the requirement of nucleic acid extraction prior testing.

5.3.2 Establishment of DNA aptamer for PEDVs detection

Another possible technique is protein based or antibody detection such as ELISA and immunochromatography. The limitation of protein based assay are time consuming, expensive cost and batch to batch variability. On the other hand, DNA aptamer is bound to specific target with intermolecular force as same as antibody and has advantages on less time consuming, no batch to batch variability. Thus, prior to develop the test kit, the establishment of DNA aptamer for PEDVs detection will be performed. In this thesis, our results showed that the 2 candidate aptamers (N04 and N25) were selected based on modified one round SELEX. In term of aptamers selection, systemic evolution of ligands by exponential enrichment (SELEX) has been employed in 1990 for RNA aptamer to bind T4 DNA polymerase (Tuerk and Gold, 1990). Later on, modified one round SELEX has been succeeded by using

gradient salt solution which reduce time consuming to discover the aptamer candidates (Arnold *et al.*, 2012). The 2 candidate aptamers showed specific binding and high binding affinity (range in nanomolar) to recombinant NP protein. Moreover, the binding region of those 2 candidate aptamers showed the consistent sequence with the predicted motif sequence with MEME analysis. The limit of detection of two candidate aptamers were $1X10^4$ and $5X10^4$ $TCID_{50}$ which were evaluated with enzyme-linked aptamer assay which in comparable with immunochromatography in previous study (Lyoo *et al.*, 2017). Thus, these 2 candidate aptamers could be useful for development of the protein based test kit in the future.

In summary, in phase 3 of this thesis, the 2 RT-LAMP based test kits for PEDVs and PDCoVs detection were developed. These 2 test kits showed high analytical sensitivity and specificity; high diagnostic sensitivity and specificity; and 100% agreement of the test. However, the nucleic acid extraction should be performed before testing. These 2 test kits could be performed in field settings due to less time consuming, basic equipment and easy to interpretation. Moreover, the candidate aptamers were established for test kit development in the future. Thus, this thesis showed the directions to differentiate between PEDVs and PDCoVs in field settings which will lead us to prevent and control swine enteric diseases.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

Swine enteric viruses including PEDVs, PDCoVs and EVGs are important pathogens of food security and public health concerns. In this thesis, surveillance of swine enteric viruses in pig farms in Thailand and development of rapid diagnostic tests using RT-LAMP with LFD and DNA aptamer were carried out.

In phase 1, surveillance of swine enteric viruses in pig farms was performed during December 2014 – January 2018. The samples were collected from 73 pig farms from 20 provinces of 7 livestock regions in Thailand. The findings from this phase of the thesis are as following:

1.1 The fecal and intestinal samples (n=777) from various ages of pigs with diarrhea including suckling pigs (n=444), nursery pigs (n=169), fattening pigs (n=58) and breeder pigs (n=106) were subjected to PEDVs, PDCoVs and EVGs detection.

1.2 The occurrences of PEDVs, PDCoVs and EVGs by samples were 44.02%, 3.47% and 71.56%, respectively. By pig farms, the occurrences of PEDVs, PDCoVs and EVGs were 50.68%, 9.59% and 69.86%, respectively.

1.3 Thai-PEDVs and Thai-EVGs were circulating throughout the country. While Thai-PDCoVs were only circulating in high density of pig production provinces of Thailand.

1.4 Our results showed that one of the risk factors related to PEDVs outbreak is ages of pigs. While seasonal patterns did not associate with PEDVs outbreak.

1.5 Our results showed that PDCoVs mostly detected during winter season and affected in all age groups of pigs.

1.6 Our results showed that age groups of pigs and seasonal patterns are implicated in EVGs infection.

1.7 The co-circulation of PEDVs, PDCoVs and EVGs with low rate (0.13%) were observed in this thesis.

1.8 A part of the results from this phase is published in "Porcine deltacoronavirus, Thailand, 2015", Emerging Infectious Diseases, 2016; Volume 22, Issue 4, Page 757-759

In phase 2, representative Thai-PEDVs (n=39) were subjected to S and ORF3 genes sequencing. Representative Thai-PDCoVs (n=16) were subjected to whole genome sequencing (n=2) and S gene sequencing (n=14). Representative Thai-EVGs (n=34) were subjected to VP1 gene sequencing. The genetic characterization and phylogenetic analyses of Thai-PEDVs, PDCoVs and EVGs were generated and analyzed. The findings from this phase of the thesis are as following:

2.1 Our results showed that at least 3 genotypes of PEDVs (Novel G1, G2a and Novel G2) were circulating in Thailand. While Novel G1 and Novel G2 have never been reported before. The multiple amino acid changes of Thai-PEDVs at epitopes COE, SS6 and 2C10 were observed. The unique patterns of epitopes SS6 and 2C10 were identified.

2.2 Our results showed that Thai-PDCoVs have multiple insertion and deletion regions which were similar to the viruses from Laos and Vietnam. Only the viruses of Thailand cluster were circulating in Thailand. The multiple amino acid changes of Thai-PDCoVs at epitopes NTD, CTD and S2 were observed.

2.3 Our results showed that at least 6 genotypes (G1, G3, G4, G8, G9 and G10) of Thai-EVGs were circulating in Thailand. The genotypes of EVGs infection associated with ages of pigs.

In phase 3, the RT-LAMP with LFD based test kits to differentiate between PEDVs and PDCoVs infections were developed. The 2 candidate aptamers (N04 and N25) against NP protein of PEDVs were established to further develop the protein based test kit in the future. The findings from this phase of the thesis are as following:

3.1 Our results showed that the 2 developed RT-LAMP with LFD kits for PEDVs and PDCoVs detection had high analytical sensitivity and specificity; high diagnostic sensitivity and specificity; and 100% agreement of the test. These 2 kits could be applied to differentiate PEDVs and PDCoVs infections in field settings.

3.2 Our results showed that 2 candidate aptamers (N04 and N25) which specific binding and high binding affinity to NP of PEDV were established. These 2 candidate aptamers could be utilized for the development of test kit in the future.

In conclusion, the results in this thesis provided useful information and status of swine enteric viruses and successful of development of rapid diagnostic test kits to distinguish between PEDVs and PDCoVs infections in field settings. The information and rapid diagnostic kit will help to develop the prevention and control strategies for swine enteric viruses in Thailand. The significant findings are

1 Swine enteric viruses including PEDVs, PDCoVs and EVGs were circulating in Thailand during December 2014 – January 2018. The co-circulation of PEDVs, PDCoVs and EVGs was low.

2 There are 3 genotypes of Thai-PEDVs (Novel G1, G2a and Novel G2), 1 genotype of Thai-PDCoVs (Thailand cluster) and 6 genotypes (G1, G3, G4, G8, G9 and G10) of Thai-EVGs were identified in this thesis.

3 Successful development of test kits with high sensitivity and specificity to differentiate PEDVs and PDCoVs infections in field settings.

Our findings confirmed that swine enteric viruses (PEDVs, PDCoVs and EVGs) which are pathogens of food security and public health concerns are circulating in pig farms in Thailand. There are 3 genotypes of Thai-PEDVs, 1 genotype of Thai-PDCoVs and 6 genotypes of Thai-EVGs are circulating in Thailand. According to the results of this thesis, the recommendations for prevention and control of swine enteric viruses including

1 Surveillance of swine enteric viruses in pig farms should be routinely performed to determine the status of swine enteric viruses in pig farms.

2 Enhance biosecurity including herd and farm managements and sanitation can help decrease opportunity for transmission of swine enteric viruses.

3 Herd health immunity management (gut feedback and vaccination) in pig farms should be conducted for control the outbreak of swine enteric viruses.

4 Rapid diagnostic kits should be applied for early detection of the swine enteric viruses.

5 The information from this thesis could be used to develop prevention and control strategies for swine enteric viruses in pig farms in Thailand.

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REFERENCES

Alonso C, Raynor PC, Davies PR and Torremorell M 2015. Concentration, Size Distribution, and Infectivity of Airborne Particles Carrying Swine Viruses. PLoS One. 10(8): e0135675.

Anbalagan S, Hesse RA and Hause BM 2014. First identification and characterization of porcine enterovirus G in the United States. PLoS One. 9(5): e97517.

Arnold S, Pampalakis G, Kantiotou K, Silva D, Cortez C, Missailidis S and Sotiropoulou G 2012. One round of SELEX for the generation of DNA aptamers directed against KLK6. Biol Chem. 393(5): 343-353.

Bailey TL and Elkan C 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol. 2: 28-36.

Beam A, Goede D, Fox A, McCool MJ, Wall G, Haley C and Morrison R 2015. A Porcine Epidemic Diarrhea Virus Outbreak in One Geographic Region of the United States: Descriptive Epidemiology and Investigation of the Possibility of Airborne Virus Spread. PLoS One. 10(12): e0144818.

Beld M, Minnaar R, Weel J, Sol C, Damen M, van der Avoort H, Wertheim-van Dillen P, van Breda A and Boom R 2004. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J Clin Microbiol. 42(7): 3059-3064.

Boley PA, Alhamo MA, Lossie G, Yadav KK, Vasquez-Lee M, Saif LJ and Kenney SP 2020. Porcine Deltacoronavirus Infection and Transmission in Poultry, United States(1). Emerg Infect Dis. 26(2): 255-265.

Boros A, Nemes C, Pankovics P, Biro H, Kapusinszky B, Delwart E and Reuter G 2012a. Characterization of a novel porcine enterovirus in wild boars in Hungary. Arch Virol. 157(5): 981-986.

Boros A, Pankovics P, Knowles NJ and Reuter G 2012b. Natural interspecies recombinant bovine/porcine enterovirus in sheep. J Gen Virol. 93(Pt 9): 1941-1951.

Boros A, Pankovics P and Reuter G 2011. Characterization of a novel porcine enterovirus in domestic pig in Hungary. Infect Genet Evol. 11(5): 1096-1102.

Buitrago D, Cano-Gomez C, Aguero M, Fernandez-Pacheco P, Gomez-Tejedor C and Jimenez-Clavero MA 2010. A survey of porcine picornaviruses and adenoviruses in fecal samples in Spain. J Vet Diagn Invest. 22(5): 763-766.

Bunke J, Receveur K, Oeser AC, Fickenscher H, Zell R and Krumbholz A 2018. High genetic diversity of porcine enterovirus G in Schleswig-Holstein, Germany. Arch Virol. 163(2): 489-493.

Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW, Laude H, Yang MS and Jang YS 2002. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. Mol Cells. 14(2): 295-299.

Chen F, Ku X, Li Z, Memon AM, Ye S, Zhu Y, Zhou C, Yao L, Meng X and He Q 2016a. Genetic characteristics of porcine epidemic diarrhea virus in Chinese mainland, revealing genetic markers of classical and variant virulent parental/attenuated strains. Gene. 588(1): 95-102.

Chen Q, Gauger P, Stafne M, Thomas J, Arruda P, Burrough E, Madson D, Brodie J, Magstadt D, Derscheid R, Welch M and Zhang J 2015. Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. Virology. 482: 51-59.

Chen Q, Gauger PC, Stafne MR, Thomas JT, Madson DM, Huang H, Zheng Y, Li G and Zhang J 2016b. Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. J Gen Virol. 97(5): 1107-1121.

Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER, Harmon KM, Main RG and Zhang J 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 52(1): 234-243.

Chen Q, Thomas JT, Gimenez-Lirola LG, Hardham JM, Gao Q, Gerber PF, Opriessnig T, Zheng Y, Li G, Gauger PC, Madson DM, Magstadt DR and Zhang J 2016c. Evaluation of serological cross-reactivity and cross-neutralization between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains. BMC Vet Res. 12: 70.

Chen R, Fu J, Hu J, Li C, Zhao Y, Qu H, Wen X, Cao S, Wen Y, Wu R, Zhao Q, Yan Q, Huang Y, Ma X, Han X and Huang X 2020. Identification of the immunodominant neutralizing regions in the spike glycoprotein of porcine deltacoronavirus. Virus Res. 276: 197834.

Chen X, Zhang XX, Li C, Wang H, Wang H, Meng XZ, Ma J, Ni HB, Zhang X, Qi Y and Sun D 2019. Epidemiology of porcine epidemic diarrhea virus among Chinese pig populations: A meta-analysis. Microb Pathog. 129: 43-49.

Chung HC, Lee JH, Nguyen VG, Huynh TML, Lee GE, Moon HJ, Park SJ, Kim HK and Park BK 2016. New emergence pattern with variant porcine epidemic diarrhea viruses, South Korea, 2012-2015. Virus Res. 226: 14-19.

Diep NV, Norimine J, Sueyoshi M, Lan NT and Yamaguchi R 2017. Novel Porcine Epidemic Diarrhea Virus (PEDV) Variants with Large Deletions in the Spike (S) Gene Coexist with PEDV Strains Possessing an Intact S Gene in Domestic Pigs in Japan: A New Disease Situation. PLoS One. 12(1): e0170126.

Dong N, Fang L, Zeng S, Sun Q, Chen H and Xiao S 2015. Porcine Deltacoronavirus in Mainland China. Emerg Infect Dis. 21(12): 2254-2255.

Donin DG, de Arruda Leme R, Alfieri AF, Alberton GC and Alfieri AA 2014. First report of Porcine teschovirus (PTV), Porcine sapelovirus (PSV) and Enterovirus G (EV-G) in pig herds of Brazil. Trop Anim Health Prod. 46(3): 523-528.

Ellington AD and Szostak JW 1990. In vitro selection of RNA molecules that bind specific ligands. Nature. 346(6287): 818-822.

Feng Y, Xu Z and Zhu L 2020. Prevalence and phylogenetic analysis of porcine deltacoronavirus in Sichuan province, China. Arch Virol. 165(12): 2883-2889.

Gallien S, Andraud M, Moro A, Lediguerher G, Morin N, Gauger PC, Bigault L, Paboeuf F, Berri M, Rose N and Grasland B 2018. Better horizontal transmission of a US non-InDel strain compared with a French InDel strain of porcine epidemic diarrhoea virus. Transbound Emerg Dis. 65(6): 1720-1732.

Gold L 1995. Oligonucleotides as research, diagnostic, and therapeutic agents. J Biol Chem. 270(23): 13581-13584.

Grasland B, Bigault L, Bernard C, Quenault H, Toulouse O, Fablet C, Rose N, Touzain F and Blanchard Y 2015. Complete genome sequence of a porcine epidemic

diarrhea s gene indel strain isolated in france in december 2014. Genome Announc. 3(3): e00535-00515.

Hanke D, Jenckel M, Petrov A, Ritzmann M, Stadler J, Akimkin V, Blome S, Pohlmann A, Schirrmeier H, Beer M and Hoper D 2015. Comparison of porcine epidemic diarrhea viruses from Germany and the United States, 2014. Emerg Infect Dis. 21(3): 493-496.

Hmila I, Wongphatcharachai M, Laamiri N, Aouini R, Marnissi B, Arbi M, Sreevatsan S and Ghram A 2017. A novel method for detection of H9N2 influenza viruses by an aptamer-real time-PCR. J Virol Methods. 243: 83-91.

Hu H, Jung K, Vlasova AN, Chepngeno J, Lu Z, Wang Q and Saif LJ 2015. Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. J Clin Microbiol. 53(5): 1537-1548.

Islam MT, Kubota T, Ujike M, Yahara Y and Taguchi F 2016. Phylogenetic and antigenic characterization of newly isolated porcine epidemic diarrhea viruses in Japan. Virus Res. 222: 113-119.

Janetanakit T, Lumyai M, Bunpapong N, Boonyapisitsopa S, Chaiyawong S, Nonthabenjawan N, Kesdaengsakonwut S and Amonsin A 2016. Porcine Deltacoronavirus, Thailand, 2015. Emerg Infect Dis. 22(4): 757-759.

Jung K, Hu H, Eyerly B, Lu Z, Chepngeno J and Saif LJ 2015. Pathogenicity of 2 porcine deltacoronavirus strains in gnotobiotic pigs. Emerg Infect Dis. 21(4): 650-654.

Jung K, Hu H and Saif LJ 2017. Calves are susceptible to infection with the newly emerged porcine deltacoronavirus, but not with the swine enteric alphacoronavirus, porcine epidemic diarrhea virus. Arch Virol. 162(8): 2357-2362.

Jung K, Saif LJ and Wang Q 2020. Porcine epidemic diarrhea virus (PEDV): An update on etiology, transmission, pathogenesis, and prevention and control. Virus Res. 286: 198045.

Kaiser L, Weisser J, Kohl M and Deigner HP 2018. Small molecule detection with aptamer based lateral flow assays: Applying aptamer-C-reactive protein cross-recognition for ampicillin detection. Sci Rep. 8(1): 5628.

Kim HJ, Hyeon JY, Hwang S, Lee YP, Lee SW, Yoo JS, Kang B, Ahn JB, Jeong YS and Lee JW 2016a. Epidemiology and virologic investigation of human enterovirus 71

infection in the Republic of Korea from 2007 to 2012: a nationwide cross-sectional study. BMC Infect Dis. 16(1): 425.

Kim SH, Lee JM, Jung J, Kim IJ, Hyun BH, Kim HI, Park CK, Oem JK, Kim YH, Lee MH and Lee KK 2015. Genetic characterization of porcine epidemic diarrhea virus in Korea from 1998 to 2013. Arch Virol. 160(4): 1055-1064.

Kim YK, Cho YY, An BH, Lim SI, Lim JA, Cho IS, Le VP and An DJ 2016b. Molecular characterization of the spike and ORF3 genes of porcine epidemic diarrhea virus in the Philippines. Arch Virol. 161(5): 1323-1328.

Knutson TP, Velayudhan BT and Marthaler DG 2017. A porcine enterovirus G associated with enteric disease contains a novel papain-like cysteine protease. J Gen Virol. 98(6): 1305-1310.

Koonpaew S, Teeravechyan S, Frantz PN, Chailangkarn T and Jongkaewwattana A 2019. PEDV and PDCoV Pathogenesis: The Interplay Between Host Innate Immune Responses and Porcine Enteric Coronaviruses. Front Vet Sci. 6: 34.

Krumbholz A, Dauber M, Henke A, Birch-Hirschfeld E, Knowles NJ, Stelzner A and Zell R 2002. Sequencing of porcine enterovirus groups II and III reveals unique features of both virus groups. J Virol. 76(11): 5813-5821.

Kumar S, Stecher G and Tamura K 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 33(7): 1870-1874.

Lamont EA, Wang P, Enomoto S, Borewicz K, Abdallah A, Isaacson RE and Sreevatsan S 2014. A combined enrichment and aptamer pulldown assay for Francisella tularensis detection in food and environmental matrices. PLoS One. 9(12): e114622.

Le VP, Song S, An BH, Park GN, Pham NT, Le DQ, Nguyen VT, Vu TTH, Kim KS, Choe S and An DJ 2018. A novel strain of porcine deltacoronavirus in Vietnam. Arch Virol. 163(1): 203-207.

Lee C 2015. Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. Virol J. 12: 193.

Lee DK, Park CK, Kim SH and Lee C 2010. Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. Virus Res. 149(2): 175-182.

Lee S and Lee C 2014a. Complete Genome Characterization of Korean Porcine

Deltacoronavirus Strain KOR/KNU14-04/2014. Genome Announc. 2(6): e01191-01114.

Lee S and Lee C 2014b. Outbreak-related porcine epidemic diarrhea virus strains similar to US strains, South Korea, 2013. Emerg Infect Dis. 20(7): 1223-1226.

Lee S and Lee C 2019. First detection of novel enterovirus G recombining a torovirus papain-like protease gene associated with diarrhoea in swine in South Korea. Transbound Emerg Dis. 66(2): 1023-1028.

Li G, Chen Q, Harmon KM, Yoon KJ, Schwartz KJ, Hoogland MJ, Gauger PC, Main RG and Zhang J 2014. Full-Length Genome Sequence of Porcine Deltacoronavirus Strain USA/IA/2014/8734. Genome Announc. 2(2): e00278-00214.

Li W, Hulswit RJG, Kenney SP, Widjaja I, Jung K, Alhamo MA, van Dieren B, van Kuppeveld FJM, Saif LJ and Bosch BJ 2018a. Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility. Proc Natl Acad Sci U S A. 115(22): E5135-E5143.

Li Y, Wu Q, Huang L, Yuan C, Wang J and Yang Q 2018b. An alternative pathway of enteric PEDV dissemination from nasal cavity to intestinal mucosa in swine. Nat Commun. 9(1): 3811.

Liang Q, Zhang H, Li B, Ding Q, Wang Y, Gao W, Guo D, Wei Z and Hu H 2019. Susceptibility of Chickens to Porcine Deltacoronavirus Infection. Viruses. 11(6).

Liu P, Shi L, Zhang W, He J, Liu C, Zhao C, Kong SK, Loo JFC, Gu D and Hu L 2017. Prevalence and genetic diversity analysis of human coronaviruses among cross-border children. Virol J. 14(1): 230.

Liu X, Zhang L, Zhang Q, Zhou P, Fang Y, Zhao D, Feng J, Li W, Zhang Y and Wang Y 2019. Evaluation and comparison of immunogenicity and cross-protective efficacy of two inactivated cell culture-derived GIIa- and GIIb-genotype porcine epidemic diarrhea virus vaccines in suckling piglets. Vet Microbiol. 230: 278-282.

Lyoo KS, Yeom M, Kim J, Kim D, Ha G, Na W, Le VP and Song D 2017. Development of rapid immunochromatographic strip test for the detection of porcine epidemic diarrhoea virus. Vet Rec. 181(22): 596.

Ma Y, Zhang Y, Liang X, Oglesbee M, Krakowka S, Niehaus A, Wang G, Jia A, Song H and Li J 2016. Two-way antigenic cross-reactivity between porcine epidemic diarrhea

virus and porcine deltacoronavirus. Vet Microbiol. 186: 90-96.

Marthaler D, Jiang Y, Collins J and Rossow K 2014a. Complete Genome Sequence of Strain SDCV/USA/Illinois121/2014, a Porcine Deltacoronavirus from the United States. Genome Announc. 2(2): e00218-00214.

Marthaler D, Jiang Y, Otterson T, Goyal S, Rossow K and Collins J 2013. Complete Genome Sequence of Porcine Epidemic Diarrhea Virus Strain USA/Colorado/2013 from the United States. Genome Announc. 1(4): e00555-00513.

Marthaler D, Raymond L, Jiang Y, Collins J, Rossow K and Rovira A 2014b. Rapid detection, complete genome sequencing, and phylogenetic analysis of porcine deltacoronavirus. Emerg Infect Dis. 20(8): 1347-1350.

Masuda T, Murakami S, Takahashi O, Miyazaki A, Ohashi S, Yamasato H and Suzuki T 2015. New porcine epidemic diarrhoea virus variant with a large deletion in the spike gene identified in domestic pigs. Arch Virol. 160(10): 2565-2568.

Maurel S, Toquin D, Briand FX, Queguiner M, Allee C, Bertin J, Ravillion L, Retaux C, Turblin V, Morvan H and Eterradossi N 2011. First full-length sequences of the S gene of European isolates reveal further diversity among turkey coronaviruses. Avian Pathol. 40(2): 179-189.

Mesquita JR, Hakze-van der Honing R, Almeida A, Lourenco M, van der Poel WH and Nascimento MS 2015. Outbreak of Porcine Epidemic Diarrhea Virus in Portugal, 2015. Transbound Emerg Dis. 62(6): 586-588.

Moon HJ, Song D, Seon BH, Kim HK, Park SJ, An DJ, Kim JM, Kang BK and Park BK 2012. Complete genome analysis of porcine enterovirus B isolated in Korea. J Virol. 86(18): 10250.

Murakami S, Miyazaki A, Takahashi O, Hashizume W, Hase Y, Ohashi S and Suzuki T 2015. Complete Genome Sequence of the Porcine Epidemic Diarrhea Virus Variant Tottori2/JPN/2014. Genome Announc. 3(4): e00877-00815.

Nagamine K, Hase T and Notomi T 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 16(3): 223-229.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N and Hase T 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28(12):

E63.

Oberste MS, Maher K, Kilpatrick DR and Pallansch MA 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol. 73(3): 1941-1948.

Oka T, Saif LJ, Marthaler D, Esseili MA, Meulia T, Lin CM, Vlasova AN, Jung K, Zhang Y and Wang Q 2014. Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. Vet Microbiol. 173(3-4): 258-269.

Opriessnig T, Gerber PF, Shen H, de Castro A, Zhang J, Chen Q and Halbur P 2017. Evaluation of the efficacy of a commercial inactivated genogroup 2b-based porcine epidemic diarrhea virus (PEDV) vaccine and experimental live genogroup 1b exposure against 2b challenge. Vet Res. 48(1): 69.

Palmquist JM, Munir S, Taku A, Kapur V and Goyal SM 2002. Detection of porcine teschovirus and enterovirus type II by reverse transcription-polymerase chain reaction. J Vet Diagn Invest. 14(6): 476-480.

Paraguison-Alili R and Domingo CY 2016. Phylogenetic tracking of current porcine epidemic diarrhea virus (PEDV) strains in the Philippines. Arch Virol. 161(9): 2601-2604.

Parida MM, Santhosh SR, Dash PK, Tripathi NK, Saxena P, Ambuj S, Sahni AK, Lakshmana Rao PV and Morita K 2006. Development and evaluation of reverse transcription-loop-mediated isothermal amplification assay for rapid and real-time detection of Japanese encephalitis virus. J Clin Microbiol. 44(11): 4172-4178.

Park JY, Park S, Park YR, Kang DY, Kim EM, Jeon HS, Kim JJ, Kim WI, Lee KT, Kim SH, Lee KK and Park CK 2016. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for the visual detection of European and North American porcine reproductive and respiratory syndrome viruses. J Virol Methods. 237: 10-13.

Park S, Kim S, Song D and Park B 2014. Novel porcine epidemic diarrhea virus variant with large genomic deletion, South Korea. Emerg Infect Dis. 20(12): 2089-2092.

Park SJ, Moon HJ, Luo Y, Kim HK, Kim EM, Yang JS, Song DS, Kang BK, Lee CS and Park

BK 2008. Cloning and further sequence analysis of the ORF3 gene of wild- and attenuated-type porcine epidemic diarrhea viruses. Virus Genes. 36(1): 95-104.

Pensaert MB and de Bouck P 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol. 58(3): 243-247.

Pogranichniy RM, Janke BH, Gillespie TG and Yoon KJ 2003. A prolonged outbreak of polioencephalomyelitis due to infection with a group I porcine enterovirus. J Vet Diagn Invest. 15(2): 191-194.

Prodelalova J 2012. The survey of porcine teschoviruses, sapeloviruses and enteroviruses B infecting domestic pigs and wild boars in the Czech Republic between 2005 and 2011. Infect Genet Evol. 12(7): 1447-1451.

Puenpa J, Auphimai C, Korkong S, Vongpunsawad S and Poovorawan Y 2018. Enterovirus A71 Infection, Thailand, 2017. Emerg Infect Dis. 24(7): 1386-1387.

Puenpa J, Chieochansin T, Linsuwanon P, Korkong S, Thongkomplew S, Vichaiwattana P, Theamboonlers A and Poovorawan Y 2013. Hand, foot, and mouth disease caused by coxsackievirus A6, Thailand, 2012. Emerg Infect Dis. 19(4): 641-643.

Puranaveja S, Poolperm P, Lertwatcharasarakul P, Kesdaengsakonwut S, Boonsoongnern A, Urairong K, Kitikoon P, Choojai P, Kedkovid R, Teankum K and Thanawongnuwech R 2009. Chinese-like strain of porcine epidemic diarrhea virus, Thailand. Emerg Infect Dis. 15(7): 1112-1115.

Ravan H, Amandadi M and Sanadgol N 2016. A highly specific and sensitive loop-mediated isothermal amplification method for the detection of Escherichia coli O157:H7. Microb Pathog. 91: 161-165.

Saeng-Chuto K, Lorsirigool A, Temeeyasen G, Vui DT, Stott CJ, Madapong A, Tripipat T, Wegner M, Intrakamhaeng M, Chongcharoen W, Tantituvanont A, Kaewprommal P, Piriyapongsa J and Nilubol D 2017. Different Lineage of Porcine Deltacoronavirus in Thailand, Vietnam and Lao PDR in 2015. Transbound Emerg Dis. 64(1): 3-10.

Sato T, Oroku K, Ohshima Y, Furuya Y and Sasakawa C 2018. Efficacy of genogroup 1 based porcine epidemic diarrhea live vaccine against genogroup 2 field strain in Japan. Virol J. 15(1): 28.

Scott A, McCluskey B, Brown-Reid M, Grear D, Pitcher P, Ramos G, Spencer D and

Singrey A 2016. Porcine epidemic diarrhea virus introduction into the United States: Root cause investigation. Prev Vet Med. 123: 192-201.

Sekiguchi Y, Nagata A, Sunaga F, Oi T, Imai R, Madarame H, Katayama Y, Oba M, Okabayashi T, Misawa N, Oka T, Mizutani T and Nagai M 2020. Multiple genotypes of enterovirus G carrying a papain-like cysteine protease (PL-CP) sequence circulating on two pig farms in Japan: first identification of enterovirus G10 carrying a PL-CP sequence. Arch Virol. 165(12): 2909-2914.

Shan T, Li L, Simmonds P, Wang C, Moeser A and Delwart E 2011. The fecal virome of pigs on a high-density farm. J Virol. 85(22): 11697-11708.

Shang J, Zheng Y, Yang Y, Liu C, Geng Q, Tai W, Du L, Zhou Y, Zhang W and Li F 2018. Cryo-Electron Microscopy Structure of Porcine Deltacoronavirus Spike Protein in the Prefusion State. J Virol. 92(4).

Si F, Hu X, Wang C, Chen B, Wang R, Dong S, Yu R and Li Z 2020. Porcine Epidemic Diarrhea Virus (PEDV) ORF3 Enhances Viral Proliferation by Inhibiting Apoptosis of Infected Cells. Viruses. 12(2).

Song D, Zhou X, Peng Q, Chen Y, Zhang F, Huang T, Zhang T, Li A, Huang D, Wu Q, He H and Tang Y 2015. Newly Emerged Porcine Deltacoronavirus Associated With Diarrhoea in Swine in China: Identification, Prevalence and Full-Length Genome Sequence Analysis. Transbound Emerg Dis. 62(6): 575-580.

Song DS, Yang JS, Oh JS, Han JH and Park BK 2003. Differentiation of a Vero cell adapted porcine epidemic diarrhea virus from Korean field strains by restriction fragment length polymorphism analysis of ORF 3. Vaccine. 21(17-18): 1833-1842.

Sozzi E, Barbieri I, Lavazza A, Lelli D, Moreno A, Canelli E, Bugnetti M and Cordioli P 2010. Molecular characterization and phylogenetic analysis of VP1 of porcine enteric picornaviruses isolates in Italy. Transbound Emerg Dis. 57(6): 434-442.

Stadler K, Masignani V, Eickmann M, Becker S, Abrignani S, Klenk HD and Rappuoli R 2003. SARS--beginning to understand a new virus. Nat Rev Microbiol. 1(3): 209-218.

Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, Cooper VL, Pillatzki A, Gauger P, Schmitt BJ, Koster LG, Killian ML and Yoon KJ 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs,

lesions, and viral genomic sequences. J Vet Diagn Invest. 25(5): 649-654.

Stott CJ, Temeeyasen G, Tripipat T, Kaewprommal P, Tantituvanont A, Piriyapongsa J and Nilubol D 2017. Evolutionary and epidemiological analyses based on spike genes of porcine epidemic diarrhea virus circulating in Thailand in 2008-2015. Infect Genet Evol. 50: 70-76.

Suebsing R, Pradeep PJ, Jitrakorn S, Sirithammajak S, Kampeera J, Turner WA, Saksmerprome V, Withyachumnarnkul B and Kiatpathomchai W 2016. Detection of natural infection of infectious spleen and kidney necrosis virus in farmed tilapia by hydroxynapthol blue-loop-mediated isothermal amplification assay. J Appl Microbiol. 121(1): 55-67.

Suleman E, Mtshali MS and Lane E 2016. Investigation of false positives associated with loop-mediated isothermal amplification assays for detection of Toxoplasma gondii in archived tissue samples of captive felids. J Vet Diagn Invest. 28(5): 536-542.

Sun D, Feng L, Shi H, Chen J, Cui X, Chen H, Liu S, Tong Y, Wang Y and Tong G 2008. Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. Vet Microbiol. 131(1-2): 73-81.

Sun G, Yi M, Tian H, Shao C, Yang S, Han J, Wang X, Wang Y, Shen Q, Wang H, Shao S, Yang Y and Zhang W 2014. A novel human enterovirus recombinant from a child with diarrhea. Arch Virol. 159(2): 333-337.

Tang Q, Tian S, Yu N, Zhang X, Jia X, Zhai H, Sun Q and Han L 2016. Development and Evaluation of a Loop-Mediated Isothermal Amplification Method for Rapid Detection of Aspergillus fumigatus. J Clin Microbiol. 54(4): 950-955.

Tapparel C, Junier T, Gerlach D, Van-Belle S, Turin L, Cordey S, Mühlemann K, Regamey N, Aubert JD, Soccal PM, Eigenmann P, Zdobnov E and Kaiser L 2009. New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses. Emerg Infect Dis. 15(5): 719-726.

Tapparel C, Siegrist F, Petty TJ and Kaiser L 2013. Picornavirus and enterovirus diversity with associated human diseases. Infect Genet Evol. 14: 282-293.

Temeeyasen G, Srijangwad A, Tripipat T, Tipsombatboon P, Piriyapongsa J, Phoolcharoen W, Chuanasa T, Tantituvanont A and Nilubol D 2014. Genetic

diversity of ORF3 and spike genes of porcine epidemic diarrhea virus in Thailand. Infect Genet Evol. 21: 205-213.

Tian Y, Yu Z, Cheng K, Liu Y, Huang J, Xin Y, Li Y, Fan S, Wang T, Huang G, Feng N, Yang Z, Yang S, Gao Y and Xia X 2013. Molecular characterization and phylogenetic analysis of new variants of the porcine epidemic diarrhea virus in Gansu, China in 2012. Viruses. 5(8): 1991-2004.

Tsuchiaka S, Naoi Y, Imai R, Masuda T, Ito M, Akagami M, Ouchi Y, Ishii K, Sakaguchi S, Omatsu T, Katayama Y, Oba M, Shirai J, Satani Y, Takashima Y, Taniguchi Y, Takasu M, Madarame H, Sunaga F, Aoki H, Makino S, Mizutani T and Nagai M 2018. Genetic diversity and recombination of enterovirus G strains in Japanese pigs: High prevalence of strains carrying a papain-like cysteine protease sequence in the enterovirus G population. PLoS One. 13(1): e0190819.

Tuanthap S, Vongpunsawad S, Phupolphan C, Duang-In A, Wattanaphansak S, Assavacheep P, Theamboonlers A, Luengyosluechakul S, Amonsin A and Poovorawan Y 2019. Analysis of the spike, ORF3, and nucleocapsid genes of porcine epidemic diarrhea virus circulating on Thai swine farms, 2011-2016. PeerJ. 7: e6843.

Tuerk C and Gold L 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 249(4968): 505-510.

Tun HM, Cai Z and Khafipour E 2016. Monitoring Survivability and Infectivity of Porcine Epidemic Diarrhea Virus (PEDv) in the Infected On-Farm Earthen Manure Storages (EMS). Front Microbiol. 7: 265.

Van Dung N, Anh PH, Van Cuong N, Hoa NT, Carrique-Mas J, Hien VB, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE and Simmonds P 2014. Prevalence, genetic diversity and recombination of species G enteroviruses infecting pigs in Vietnam. J Gen Virol. 95(Pt 3): 549-556.

Van Dung N, Anh PH, Van Cuong N, Hoa NT, Carrique-Mas J, Hien VB, Sharp C, Rabaa M, Berto A, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE and Simmonds P 2016. Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam. J Gen Virol. 97(2): 378-388.

Wang E, Guo D, Li C, Wei S, Wang Z, Liu Q, Zhang B, Kong F, Feng L and Sun D 2016a.

Molecular Characterization of the ORF3 and S1 Genes of Porcine Epidemic Diarrhea Virus Non S-INDEL Strains in Seven Regions of China, 2015. PLoS One. 11(8): e0160561.

Wang F, Li R, Shang Y, Wang C, Wang GQ, Zhou DX, Yang DH, Xi W, Wang KQ, Bao J, Kang Y and Gao ZC 2016b. A Pilot Study of Quantitative Loop-mediated Isothermal Amplification-guided Target Therapies for Hospital-acquired Pneumonia. Chin Med J (Engl). 129(2): 181-186.

Wang J, Zhao P, Guo L, Liu Y, Du Y, Ren S, Li J, Zhang Y, Fan Y, Huang B, Liu S and Wu J 2013. Porcine epidemic diarrhea virus variants with high pathogenicity, China. Emerg Infect Dis. 19(12): 2048-2049.

Wang L, Byrum B and Zhang Y 2014a. Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. Emerg Infect Dis. 20(7): 1227-1230.

Wang L, Byrum B and Zhang Y 2014b. New variant of porcine epidemic diarrhea virus, United States, 2014. Emerg Infect Dis. 20(5): 917-919.

Wang L, Hayes J, Sarver C, Byrum B and Zhang Y 2016c. Porcine deltacoronavirus: histological lesions and genetic characterization. Arch Virol. 161(1): 171-175.

Wang YW, Yue H, Fang W and Huang YW 2015. Complete Genome Sequence of Porcine Deltacoronavirus Strain CH/Sichuan/S27/2012 from Mainland China. Genome Announc. 3(5): e00945-00915.

Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, Bai R, Teng JL, Tsang CC, Wang M, Zheng BJ, Chan KH and Yuen KY 2012. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J Virol. 86(7): 3995-4008.

Wood EN 1977. An apparently new syndrome of porcine epidemic diarrhoea. Vet Rec. 100(12): 243-244.

Xu C, Wang H, Jin H, Feng N, Zheng X, Cao Z, Li L, Wang J, Yan F, Wang L, Chi H, Gai W, Wang C, Zhao Y, Feng Y, Wang T, Gao Y, Lu Y, Yang S and Xia X 2016. Visual detection of Ebola virus using reverse transcription loop-mediated isothermal amplification combined with nucleic acid strip detection. Arch Virol. 161(5):

1125-1133.

Yamazaki W, Mioulet V, Murray L, Madi M, Haga T, Misawa N, Horii Y and King DP 2013. Development and evaluation of multiplex RT-LAMP assays for rapid and sensitive detection of foot-and-mouth disease virus. J Virol Methods. 192(1-2): 18-24.

Yang JF, Zhao CZ and Lu KX 2016a. Development and application of a rapid detection system for human papillomavirus and Herpes simplex virus-2 by loop-mediated isothermal amplification assay. Microb Pathog. 97: 178-182.

Yang S, Wang Y, Shen Q, Zhang W and Hua X 2013. Prevalence of porcine enterovirus 9 in pigs in middle and eastern China. Virol J. 10: 99.

Yang X, Li G, Wen K, Bui T, Liu F, Kocher J, Jortner BS, Vonck M, Pelzer K, Deng J, Zhu R, Li Y, Qian Y and Yuan L 2014. A neonatal gnotobiotic pig model of human enterovirus 71 infection and associated immune responses. Emerg Microbes Infect. 3(5): e35.

Yang Y, Li Q, Wang S, Chen X and Du A 2016b. Rapid and sensitive detection of Babesia bovis and Babesia bigemina by loop-mediated isothermal amplification combined with a lateral flow dipstick. Vet Parasitol. 219: 71-76.

Yozwiak NL, Skewes-Cox P, Gordon A, Saborio S, Kuan G, Balmaseda A, Ganem D, Harris E and DeRisi JL 2010. Human enterovirus 109: a novel interspecies recombinant enterovirus isolated from a case of acute pediatric respiratory illness in Nicaragua. J Virol. 84(18): 9047-9058.

Zhang B, Tang C, Yue H, Ren Y and Song Z 2014a. Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China. J Gen Virol. 95(Pt 7): 1603-1611.

Zhang F, Luo S, Gu J, Li Z, Li K, Yuan W, Ye Y, Li H, Ding Z, Song D and Tang Y 2019a. Prevalence and phylogenetic analysis of porcine diarrhea associated viruses in southern China from 2012 to 2018. BMC Vet Res. 15(1): 470.

Zhang J, Chen J, Liu Y, Da S, Shi H, Zhang X, Liu J, Cao L, Zhu X, Wang X, Ji Z and Feng L 2020a. Pathogenicity of porcine deltacoronavirus (PDCoV) strain NH and immunization of pregnant sows with an inactivated PDCoV vaccine protects 5-day-old neonatal piglets from virulent challenge. Transbound Emerg Dis. 67(2):

572-583.

Zhang J, Chen J, Shi D, Shi H, Zhang X, Liu J, Cao L, Zhu X, Liu Y, Wang X, Ji Z and Feng L 2019b. Porcine deltacoronavirus enters cells via two pathways: A protease-mediated one at the cell surface and another facilitated by cathepsins in the endosome. J Biol Chem. 294(25): 9830-9843.

Zhang SQ, Tan B, Li P, Wang FX, Guo L, Yang Y, Sun N, Zhu HW, Wen YJ and Cheng SP 2014b. Comparison of conventional RT-PCR, reverse-transcription loop-mediated isothermal amplification, and SYBR green I-based real-time RT-PCR in the rapid detection of bovine viral diarrhea virus nucleotide in contaminated commercial bovine sera batches. J Virol Methods. 207: 204-209.

Zhang W, Yang S, Shen Q, Ren L, Shan T, Wei J, Cui L and Hua X 2012. Complete genome sequence of a novel porcine enterovirus strain in China. J Virol. 86(12): 7008-7009.

Zhang X, Pan Y, Wang D, Tian X, Song Y and Cao Y 2015. Identification and pathogenicity of a variant porcine epidemic diarrhea virus field strain with reduced virulence. Virol J. 12: 88.

Zhang Y, Chen Y, Yuan W, Peng Q, Zhang F, Ye Y, Huang D, Ding Z, Lin L, He H, Wu Q, Song D and Tang Y 2020b. Evaluation of Cross-Protection between G1a- and G2a-Genotype Porcine Epidemic Diarrhea Viruses in Suckling Piglets. Animals (Basel). 10(9).

Zhang Y, Lai BS and Juhas M 2019c. Recent Advances in Aptamer Discovery and Applications. Molecules. 24(5).

Zhou J and Rossi J 2017. Aptamers as targeted therapeutics: current potential and challenges. Nat Rev Drug Discov. 16(3): 181-202.

Zhou S, Gao ZX, Zhang M, Liu DY, Zhao XP and Liu Y 2016. Development of a quadruplex loop-mediated isothermal amplification assay for field detection of four Vibrio species associated with fish disease. Springerplus. 5(1): 1104.

APPENDICES

APPENDIX A

Protocol for Establishment of DNA aptamer for PEDVs detection

Protocol for Transformation by using heat-shock.

1) Added 200 ng of constructed plasmid to 50 µL of competent cell
2) Incubated on ice for 5 minutes
3) Incubated at 42 ^{O}C for 30 seconds
4) Incubated on ice for 2 minutes
5) Added SOC medium to mixture and incubated at 37^{O}C while shacking at 250 rpm for an hour.
6) 50 µL of mixture was plating on LB/Kanamycin agar.
7) Culture plate was incubated at 37^{O}C for 18 hours.
8) Collected 5 colonies of transformant and culture in LB/Kanamycin broth and stored in 25% glycerol stock at -80^{O}C.
9) The remnant of 5 colonies were subjected to amplify and sequencing with T7 primers

Protocol for PCR of T7 primers

the remnant of the colonies was placed in 20 µL of Nuclease-free water. The whole bacterial DNA was extracted using the boiling method.

Reagents	Volume	Final conc.
DNA	5 µL	
10 µM T7F and T7R primers	1 µL each	0.2 µM
10X PCR Buffer	5 µL	1X
25 mM $MgCl_2$	3 µL	1.5 mM
10 mM of each dNTP	1 µL	200 µM of each dNTP
HotstarTaq DNA polymerase	0.25 µL	2.5 U
Nuclease-free water	33.75 µL	
Final volume	**50 µL**	

PCR condition

Initial denature: 95°C for 15 minutes follow by 30 cycles of amplification

Denaturation: 95°C for 1 minute

Annealing: 50°C for 45 seconds

Elongation: 72°C for 1 minute and 45 seconds

final elongation: 72°C for 10 minutes

The amplicon size approximately 1,300 base pairs was visualized by agarose gel electrophoresis. The amplicons were purified using QIAquick PCR Purification Kit (Qiagen®, Germany) and sanger sequencing was performed by GENEWIZ®.

Protocol for protein purification and quantification

1) 100 ml of culture broth was centrifuged at 5000 x G for 10 minutes and discarded the supernatant
2) The pellet was added 8 ml of B-PER® mixture containing 16 µL of lysozyme and 16 µL of DNase I
3) Incubated at room temperature for 15 minutes.
4) Lysate was centrifuged at 15,000 x G for 5 minutes to separate the soluble protein.
5) The denaturing soluble protein was subjected to visualized using 4-20% Mini-PROTEAN® TGX™ Precast Protein gel and Coomasie staining.
6) The supernatant was subjected purified using HisPur™ Cobalt Resin (ThermoFisher Scientific, USA) and visualized using 4-20% Mini-PROTEAN® TGX™ Precast Protein gel and Coomasie staining.
7) Purified recombinant protein was performed Liquid chromatography-Mass spectrometry (LC-MS/MS) by MSU genomic core for protein confirmation.
8) Purified recombinant NP protein was quantified using Pierce™ BCA Protein Assay Kit (Invitrogen, USA).

Protocol for aptamer selection

1) Immobilization of the protein to the 96-well ELISA plate.
2) The well was dry blotted and 100 µL of single-stranded aptamer library (10 µM) was added and incubated for 1 hour.
3) 100 µL of PBS was added, incubated for 5 minutes,
4) The solution was collected, labeled, and stored at -20°C.
5) This step was repeated using 0.5, 0.75, 1.0, 1.25 and 1.5 M NaCl, with an incubation for 5 minutes each time.
6) The collected 1.5 M NaCl solution was amplified by using PCR with the W20F and W20R primers. The PCR reaction was prepared as previous described in protocol for PCR of T7. PCR conditions were 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and a final elongation at 72°C 7 minutes.
7) Pools of the PCR amplicons were purified with MiniElute PCR purification kit (Qiagen)
8) Cloned purified PCR amplicons into a cloning vector using the TA Cloning® Kits with pCR 2.1 Vector (Thermo Fisher Scientific)
9) transformed into *E. coli* strain Top10 by using heat-shock according to the manufacture instructions.
10) *E. coli* transformant cells were plated on LB/Ampicillin/X-Gal agar.

Protocol for EMSA

1) Selected aptamers (20 fmol) were added to recombinant NP protein in 1Xbinding buffer and nuclease-free water (final volume, 20 µL),
2) Incubated at room temperature for 30 minutes.
3) The samples were loaded onto an 6% native polyacrylamide gel (Bio-Rad, Hercules, CA) in 0.5XTBE buffer.
4) Electrophoresis was performed at 80V for 60 minutes,
5) the samples were electro transferred to a positively charged nylon membrane (Biodyne B; 0.45-um pore size; Biodyne, Pensacola, FL).
6) The membrane was processed and developed with a chemiluminescent nucleic acid detection module (Pierce) according to the manufacturer's instructions.
7) Reaction on the membrane was then visualized and imaged with the CHEMIDOC™ MP image system (Bio-rad, USA).

Protocol for DNase I footprinting

1) 5 µg of recombinant NP protein was mixed with 1 pmol of the selected aptamers labeled at 5' with 6-carboxyfluorescein (FAM) in 1X binding buffer (Light Shift; Pierce)
2) nuclease-free water was added to a final volume 50 µL,
3) Incubated at room temperature for 1 hour.
4) Added 0.2 U of DNase I (amplification grade; Invitrogen) to the mixture
5) Incubated at 37°C for 5 minutes.
6) Added 2 mM EDTA to each sample for DNase I inactivation
7) Incubated at 70°C for 10 minutes.
8) The samples were purified by using a MiniElute PCR purification kit (Qiagen) and eluted with 30 µL of nuclease-free water.
9) Approximately 12 µL of purified samples were submitted to GENEWIZ® for fragment analysis on a 3130XL genetic analyzer.

APPENDIX B

Results of RT-LAMP with LFD for PEDVs and PDCoVs detection

	PEDVs detection		PDCoVs detection	
Sample ID	qRT-PCR	RT-LAMP	qRT-PCR	RT-LAMP
S5001	positive	Positive	Negative	Negative
S5002	positive	Positive	Negative	Negative
S5003	positive	Positive	Negative	Negative
S5004	positive	Positive	Negative	Negative
S5005	positive	Positive	Negative	Negative
S5011	Negative	Negative	positive	positive
S5012	Negative	Negative	positive	positive
S5013	Negative	Negative	positive	positive
S5014	Negative	Negative	positive	positive
S5015	Negative	Negative	positive	positive
S5016	Negative	Negative	positive	positive
S5017	Negative	Negative	positive	positive
S5018	Negative	Negative	positive	positive
S5019	Negative	Negative	positive	positive
S5020	Negative	Negative	positive	positive
S5021	Negative	Negative	positive	positive
S5022	Negative	Negative	positive	positive
S5023	Negative	Negative	positive	positive
S5024	Negative	Negative	positive	positive
S5025	Negative	Negative	positive	positive
S5026	Negative	Negative	positive	positive
S5027	Negative	Negative	positive	positive
S5028	Negative	Negative	positive	positive
S5029	Negative	Negative	positive	positive
S5030	Negative	Negative	positive	positive
Sample ID	PEDVs detection		PDCoVs detection	

	qRT-PCR	RT-LAMP	qRT-PCR	RT-LAMP
S5032	positive	positive	Negative	Negative
S5033	positive	positive	Negative	Negative
S5034	positive	positive	Negative	Negative
S5036	positive	positive	Negative	Negative
S5037	positive	positive	Negative	Negative
S5038	positive	positive	Negative	Negative
S5039	positive	positive	Negative	Negative
S5040	positive	positive	Negative	Negative
S5041	positive	positive	Negative	Negative
S5042	positive	positive	Negative	Negative
S5043	positive	positive	Negative	Negative
S5044	positive	positive	Negative	Negative
S5045	positive	positive	Negative	Negative
S5046	positive	positive	Negative	Negative
S5058	Negative	Negative	Negative	Negative
S5059	Negative	Negative	Negative	Negative
S5060	Negative	Negative	Negative	Negative
S5061	Negative	Negative	Negative	Negative
S5062	Negative	Negative	Negative	Negative
S5063	Negative	Negative	Negative	Negative
S5064	Negative	Negative	Negative	Negative
S5065	Negative	Negative	Negative	Negative
S5066	Negative	Negative	Negative	Negative
S5067	Negative	Negative	Negative	Negative
S5077	Negative	Negative	Negative	Negative
S5078	Suspect	Negative	Negative	Negative
S5079	Negative	Negative	Negative	Negative
S5080	Negative	Negative	Negative	Negative

	PEDVs detection		PDCoVs detection	
Sample ID	qRT-PCR	RT-LAMP	qRT-PCR	RT-LAMP
S5081	positive	positive	Negative	Negative
S5082	Negative	Negative	Negative	Negative
S5083	Negative	Negative	Negative	Negative
S5084	Negative	Negative	Negative	Negative
S5085	Negative	Negative	Negative	Negative
S5086	Negative	Negative	Negative	Negative
S5087	Negative	Negative	Negative	Negative
S5088	Negative	Negative	Negative	Negative
S5089	Negative	Negative	Negative	Negative
S5090	Negative	Negative	Negative	Negative
S5091	Negative	Negative	Negative	Negative
S5092	Negative	Negative	Negative	Negative
S5093	Negative	Negative	Negative	Negative
S5094	Negative	Negative	Negative	Negative
S5095	Negative	Negative	Negative	Negative
S5096	Negative	Negative	Negative	Negative
S5097	Negative	Negative	Negative	Negative
S5098	positive	positive	Negative	Negative
S5099	positive	positive	Negative	Negative
S5100	positive	positive	Negative	Negative
S5101	positive	positive	Negative	Negative
S5102	positive	positive	Negative	Negative
S5103	positive	positive	Negative	Negative
S5104	positive	positive	Negative	Negative
S5105	positive	positive	Negative	Negative
S5106	positive	positive	Negative	Negative
S5107	Negative	Negative	Negative	Negative

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