ความหลากหลายทางพันธุกรรมของไวรัสพีอาร์อาร์เอสในประเทศไทย หลังจากการระบาดของ HP-PRRSV ในประเทศไทย ในปี พ.ศ. 2553

นายกรกฤต พูนสุข

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GENETIC VARIATIONS OF THAI PRRSV AFTER THE 2010 HP-PRRSV OUTBREAK IN THAILAND

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กรกฤต พูนสุข : ความหลากหลายทางพันธุกรรมของไวรัสพีอาร์อาร์เอสในประเทศไทยหลังจากการระบาดของ HP-PRRSV ในประเทศไทย ในปี พ .ศ. 2553 (GENETIC VARIATIONS OF THAI PRRSV AFTER THE 2010 HP-PRRSV OUTBREAK IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร.คมกฤช เทียนคำ, 87 หน้า

กลุ่มอาการพีอาร์อาร์เอสที่เกิดจากการติดเชื้อไวรัสพีอาร์อาร์เอส เป็นหนึ่งในสาเหตุหลักที่ทำให้เกิดความเสียหายอย่างรุนแรงต่ออุตสาหกรรมการผลิตสุกรในประเทศไทย นับตั้งแต่เกิดการระบาดของไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงในประเทศไทยในปี พ .ศ. 2553 ด้วยเหตุนี้ การศึกษาที่ทำการวิเคราะห์เปรียบเทียบ และศึกษาลักษณะเฉพาะทางพันธุกรรมของไวรัสพีอาร์อาร์เอสในประเทศไทยภายหลังการระบาดของไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงจึงมีความสำคัญ เพื่อเป็นข้อมูลพื้นฐานทางพันธุกรรมของไวรัสพีอาร์อาร์เอสในประเทศไทย และผลกระทบที่มีต่อลักษณะทางพันธุกรรมของไวรัสอันเกิดจากการระบาดของไวรัสสายพันธุ์ใหม่ การศึกษานี้ ทำโ ดยถอดรหัสพันธุกรรมของไวรัสพีอาร์อาร์เอสที่ได้จากฝูงสุกรที่เกิดโรคทั้งหมด 11 ฟาร์ม ที่อยู่ในพื้นที่ต่างๆกัน 10 จังหวัด ใน 4 ภาค ของประเทศไทย ลำดับพันธุกรรมที่ใช้ในการศึกษาได้แก่ บางส่วนของยีนเอ็นเอสพี 2 และโออาร์เอฟ 5 ซึ่งเป็นส่วนที่มีความหลากหลายทางพันธุกรรมสูงที่สุดของไวรัสพีอาร์อาร์เอส ผลจากการเปรียบเทียบลำดับพันธุกรรม และการวิเคราะห์ แผนภูมิต้นไม้พบว่าลำดับพันธุกรรม 10 สาย จาก 9 จังหวัดใน 4 ภาคของประเทศ มีการขาดหายไปของกรดอะมิโน รวม 30 กรดอะมิโน ซึ่งเป็นลักษณะจำเพาะของไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรง ในขณะที่ 1 ลำดับพันธุกรรมที่เหลือ มีความใกล้เคียงและจัดอยู่ในกลุ่มเดียวกันกับไวรัสพีอาร์อาร์เอสชนิดที่ 2 สายพันธุ์ประจำถิ่นที่เคยพบในประเทศไทย จาก ผลการศึกษานี้ สามารถสรุปได้ว่าไวรัสพีอาร์อาร์เอสสายพันธุ์รุ นแรง มีการระบาดเข้าสู่ประเทศไทยอย่างต่อเนื่องนับตั้งแต่ปี พ .ศ. 2553 และไวรัสที่ก่อให้เกิดความเสียหายจากกลุ่มอาการพีอาร์อาร์เอสส่วนใหญ่ เป็นไวรัสพีอาร์อาร์เอสชนิดที่ 2 ทั้งชนิดสายพันธุ์รุนแรงและไวรัสประจำถิ่น อย่างไรก็ตาม มีความจำเป็นอย่างยิ่งที่จะมีการศึกษาความหลากหลายทางพันธุกรรมของไวรัสอย่างต่อเนื่อง เ พื่อนำมาประยุกต์ใช้ในการควบคุมและป้องกันโรคในประเทศไทยต่อไป

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

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

ปีการศึกษา.....2556..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: NUCLEOTIDE SEQUENCE/ PIGS/ PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME/ THAILAND

KORAKRIT POONSUK: GENETIC VARIATIONS OF THAI PRRSV AFTER THE 2010 HP-PRRSV OUTBREAK IN THAILAND. ADVISOR: ROONGROJE THANAWONGNUWECH, Ph.D., CO-ADVISOR: KOMKRICH TEANKUM, Dr. med. vet., 87pp.

Since multiple HP-PRRSV outbreaks occurred in Thailand in 2010, PRRSV infection was one of major reasons for economic losses in the Thai swine industry. To improve knowledge about the disease and effects of novel PRRSV isolates introduced in Thailand, comparative analysis and genetic characteristics of the hyper-variable regions, NSP2 and ORF5, of PRRSV collected from the clinically-affected herds between February and October 2012 were studied. Partial NSP2 and ORF5 sequences of PRRSV obtained from clinically infected pigs from 11 different herds originated from different geographic regions of Thailand were aligned and used for phylogenetic tree constructions. Eleven sequences were successfully sequenced in this study. Ten sequences collected from 9 provinces in 4 parts of Thailand showed 30 amino acids deletions, closely related to the newly introduced HP-PRRSV while 1 sequence from the central part of Thailand had the similarity to the local Thai type 2 PRRSV. The studied Thai PRRSVs were located separately in these 2 groups in the partial NSP2 and ORF5 based phylogenetic tree. The results indicate that the novel virus were reintroduced in Thailand after the 2010 outbreaks, simultaneously with the local virus evolution. It should be noted that most clinical cases of PRRS during this study were caused by HP-PRRSV infection with a local Thai type 2 PRRSV infection. However, further study is needed to reveal more knowledge on the newly introduced virus in the Thai pig population.

Department .....Veterinary Pathology..... Student's signature.....
Field of Study.....Veterinary Pathobiology..... Advisor's Signature.....
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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	=	base pair (s)
˚C	=	degree Celcius (centrigrade)
ELISA	=	Enzyme-linked immunosorbent assay
GP	=	glycoprotein
Ig	=	immunoglobulin
kb	=	kilobase
kD	=	kilodalton
MEM	=	minimum essential medium eagle
NSP	=	Non-structural protein
nm	=	nanometer
ORF	=	Open Reading Frame
pp	=	polyprotein
rpm	=	revolutions per minute
RT-PCR	=	reverse transcriptase polymerase chain reaction
TBE	=	tris-borate-EDTA buffer
µl	=	microliter
µM	=	micromolar

Chapter I

INTRODUCTION

1.1 Importance and rationale

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV). PRRS outbreaks in the finishing farms can cause damages due to the disease and culling pigs because of low growth rate and higher mortality rate, especially in the case of the 2006 outbreaks of the highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) in China. This particular HP-PRRSV has caused severe damages in most swine industries in China with extremely high morbidity and mortality rates of the infected herds, 50-100 and 20-100 percent, respectively (Tong et al., 2007). According to the severity of the disease in China, there were more than 2,000,000 pigs infected and more than 243,000 pigs died in 2007 (Tian et al., 2007; Xiao et al., 2010).

Following to the first HP-PRRSV outbreak in China, the virus spread to other vicinity countries such as Vietnam in 2008. More than 65,000 pigs in Vietnam were affected (Feng et al., 2008). After that, the HP-PRRSV spread into many countries in the region, including Thailand. Multiple HP-PRRSV outbreaks were found since August 2010 (Nuntawan Na Ayudhya et al., 2011). The HP-PRRSV outbreak was one of the reasons for the increased of Thailand pork price in 2010 due to more than 30 percent losses in the sow herds. The previous study described that there were 2 or more subclusters of the Chinese HP-PRRSV introduced into Thailand (Nuntawan Na Ayudhya et al., 2011; Nuntawan Na Ayudhya et al., 2012). To encounter with the situation, the Thai Swine Veterinary Association (TSVA) has established the Clinical Practice Guideline (CPG) for PRRS in Thailand: 3rd Revision (3rd CPG) for Thai swine practitioners. According to the guideline, RT-PCR and specific nucleotide sequencing was established as the major diagnostic technique for the HP-PRRSV nucleic acid detection. This method is implemented for the prevention, surveillance

and eradication procedures of the Thai PRRSV and also for the current HP-PRRSV outbreaks.

The study about genetic data of PRRSV is not only useful in the control and prevention of the disease, but it also can be used in the disease investigation. In term of different geographic locations, PRRSV isolates frequently contain different genotypic characteristics (Yoshii et al., 2005). Thus, specific genetic characteristics of any PRRSV, especially in hyper-variable regions of the virus, are beneficial for an epidemiological study. For this reason, the studies on the high variable genes including NSP2 and ORF5 of the Thai PRRSV are needed. Regarding to the previous genetic diversity studies of NSP2 gene, current Thai PRRSV tended to evolve and change their genetics from the original PRRSV (Kedkovid et al., 2010). However, no studies have been made on the variation of the NSP2 genes after the outbreak of HP-PRRSV in Thailand. Therefore, this study may be useful on the comparative analysis study of the recent Thai PRRSV genetic changes in NSP2 gene after the introduction of the HP-PRRSV. The information can also be used as a database of NSP2 gene of the recent Thai PRRSV. Another variable region of PRRSV is the ORF5 gene. This gene is encoded for a GP5 protein, the most critical structural protein for the development of neutralizing antibodies of the infected pigs. ORF5 is known as the most variable structural protein gene, due to the necessity for a survival of the virus. Similar to the NSP2, the variation of ORF5 sequences compared to the native PRRSV isolates were found in the Chinese HP-PRRSV isolates (Tong et al., 2007). This discovery was supported the previous studies of both NSP2 and ORF5 that known as the most variable regions. However, the evolution of the NSP2 and ORF5 of the Thai PRRSV after the HP-PRRSV outbreak is still in doubt, a study with these 2 genes is needed for the update information.

The goal of this study is to obtain genetic sequences of both NSP2 and ORF5 genes as an epidemiological data on the recent Thai PRRSV after the introduction of the HP-PRRSV in 2010. The benefits of the information obtained from this study can be applied for the prevention of PRRSV outbreaks in the future and can be used for management

strategies. This is of our interest and also rationale to study on the Thai PRRSV genetic diversity for development of a suitable method for diagnosis of both PRRSV and HP-PRRSV on NSP2 and ORF5 genes using RT-PCR technique. The information obtained from this study is useful, not only in epidemiology, but also in diagnosis, surveillance, control and eradication in local and regional levels.

Keywords

Genetic variation, NSP2, ORF5, Porcine Reproductive and Respiratory Syndrome virus (PRRSV)

Objectives of study

1. To investigate the genetic characteristics of the current Thai PRRSV isolates.
2. To determine the current molecular epidemiology of Thai PRRSV.
3. To provide the understanding of Thai PRRSV genetic characteristics for future prevention and control as well as for a better vaccine selection.

Chapter II

Literature Review

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV). The disease is one of the infectious diseases in pig industries occurring worldwide and causing impact on both microeconomics and macroeconomics levels. PRRS has become a trade barrier for live pigs and swine products such as boar semen (Petry et al., 2006; Zimmerman, 2008). The impact of the disease causes several damages including death loss, over growth rate compare to the non-infected farm, the risk of other infections and complications of the disease (Pejsak et al., 1997). In general, despite of other primary or secondary infections, PRRSV does not cause severely effects or lesions. However, PRRSV infection in farms level always complicated with co-infection due to several swine-based pathogens including porcine circovirus type 2 (PCV2) (Harms et al., 2001; Burgara-Estrella et al., 2012), *Streptococcus suis* (Thanawongnuwech et al., 2000; Xu et al., 2010; Hao et al., 2013), *Salmonella* Cholerasuis (Wills et al., 2000), *Mycoplasma hyopneumoniae* (Thanawongnuwech et al., 2000; Thanawongnuwech et al., 2004[b]), swine influenza virus (SIV) (Dorr et al., 2007) and also *Actinobacillus pleuropneumoniae* (Pol et al., 1995). Subsequently, increased costs are needed for disease prevention and control by vaccines and drugs treatment to reduce the disease complications.

2.1 Etiology

PRRS virus (PRRSV) is classified as a member of the Nidovirales order, within the Arteriviridae family and Arteriviruus genus. The virus is categorized within the same family as equine arterivirus (EAV) in horses, lactate dehydrogenase-elevating virus (LDV) in mice and simian hemorrhagic fever virus (SHFV) in monkeys (Plagemann and Moennig, 1992; Meulenberg et al., 1993). PRRSV currently is classified into two genotypes. Type 1 PRRSV is

classified as the European PRRSV having Lelystad (LV) virus as a prototype. Type 2 PRRSV is classified as the North American PRRSV having VR2332 virus as a prototype. These two PRRSV genotypes are different in several aspects including antigenicity, genetically and virulence (Allende et al., 1999; Nelsen et al., 1999).

Viral genome contains 15.4 kb of a positive single-stranded RNA with 48-83 nm in size (Dea et al., 2000; Meng, 2000). In structure, PRRSV diameter is 50-65 nm with nucleocapsid and envelope protein covering (Meulenberg et al., 1993). Genome of the virus can be classified into 9 Open reading frames (ORF) composing of ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6 and ORF7, respectively (Wootton and Yoo, 2003; Lee and Yoo, 2006).

2.1.1 Genetic and proteins

1) ORF1

More than 80% of PRRSV genome is ORF1. This region encoded Non-structural proteins (NSP) of the virus. Divided into ORF1a and ORF1b, these parts of the viral genome encoded pp1a polyprotein and pp1b polyprotein, respectively (Meulenberg et al., 1993; Den Boon et al., 1995). Function of the protein encoded by ORF1a is proteolytic activity and the protein encoded by ORF1b is act as replicase-related activity. For examples, RNA dependent RNA polymerase and NTPase/RNA helicase (Gorbalenya et al., 1989; Godeny et al., 1993; Meulenberg et al., 1993; van Dinten et al., 1996). Both pp1a and pp1b polyprotein are the origins of non-structural proteins including nsp1α, nsp1α, nsp2, nsp3, nsp4, nsp5, nsp6, nsp7, nsp8, nsp9, nsp10, nsp11 and nsp12 (Meulenberg et al., 1997; Snijder, 1998; Ziebuhr et al., 2000).

2) ORF2

ORF2 encoded 2 minor envelope protein, GP2a and E protein from ORF2a and ORF2b, respectively. GP2a is composes of 168 amino acids ectodomain with transmembrane chain and 20 amino acids ectodomain (Wissink et al., 2004). On the other

hand, E protein encoded by ORF2b is transmembrane glycoprotein that is function as oligomeric ion cahnnel (Lee ans Yoo, 2006).

3) ORF3

GP3 encoded by the ORF3 is the largest PRRSV protein. This protein is minor envelope protein, consisting of 265 amino acids or 254 amino acids in type 1 and type 2 PRRSV, respectively (Gonin et al., 1998; Wieringa et al., 2002). With 6 glycosylation sites, size of the protein is 42-50 kDa when measure by SDS-PAGE technique and 27-28 kDa by molecular weight per amino acid (Mardassi et al., 1995; Meulenberg et al., 1995; Gonin et al., 1998).

4) ORF4

ORF4 encoded GP4 protein. This minor envelope transmembrane protein consists of 183 amino acids and 178 amino acids in type 1 and type 2 PRRSV, respectively. This glycoprotein contains signal peptide at the position 1-21 and contains 4 glycosylation sites (Wieringa et al., 2002).

5) ORF5

GP5 encoded by ORF5 is the major envelope protein of PRRSV. This protein is an important protein in viral replication. This 25 kDa structural protein is associated with the identification of the swine neutralizing antibodies (Shen et al., 2000; Gao et al., 2004; Ropp et al., 2004; Han et al., 2006).

6) ORF6

ORF6 encoded matrix protein (M protein). This non-glycoprotein is 19 kDa in size that is one of the major envelope proteins (Wissink et al., 2005).

7) ORF7

PRRSV nucleocapsid protein (N protein) encoded by ORF7. This base protein is cover genome of PRRSV. More than 20-40% of PRRSV structural protein is nucleocapsid protein (Nelson et al., 1993; Mardassi et al., 1994; Bautista et al., 1996).

As the virion formation, minor virion components including E protein, GP2a, GP3 and GP4 assemble as complex structure via non-covalent interactions. On the other hand, M and GP5 protein complexes are forming with disulfide-linked heterodimer of the cysteine in the envelope. Major structural proteins complex and minor virion components complex bind together via GP4 and GP5 connection, forming a structure of envelope virion particle (Wissink et al., 2005; Breedam et al., 2010; Das et al., 2010; Das et al., 2011).

2.1.2 PRRSV entry and replication

PRRSV need limited way of cell tropism. The virus exhibits a preference for only the monocyte or macrophage lineage. The viral target cells in vivo are specific subset of swine differentiated macrophages located in only lungs, lymphoid tissues and placenta whereas other macrophages precursor cells including bone marrow cells, peripheral blood monocytes and also peripheral macrophages are not susceptible. Since this very narrow cell tropism, porcine alveolar macrophages (PAMs) are the first choice used for in vitro study of PRRSV infection. Additionally, besides of the PAMs culture, African green monkey kidney cell line (MA-104) and its derivatives especially MARC-145 and CL2621 are being used to sustain PRRSV infection. However, PAMs are majority used as the target cells to explore the PRRSV replication cycle.

Among the PRRSV limited cell tropism, various cellular molecules important to the viral infection have been explored. Heparan sulphate, sialoadhesin and CD163 were identified as the essential PRRSV entry mediators. Mostly, these molecular factors have been shown to simplify the virus to bind and infect the target cells or act as port of viral-receptor complexes as well as viral genome releasing stimulators. However, many other

entry mediators and its made of action are still unknown and need further research to be elucidated.

At the beginning, PRRSV contact with the heparin sulphate GAGs on the macrophage cell surface initially. Then, more stable interaction occurs via an engagement between sialoadhesin (pSn) and the virus at the N-terminal part of pSn and the viral M/GP5 complex. Two sialic acid binding domains at the N terminus of pSn and on the virion surface play an important role in this interaction. PRRSV attach with the pSn mediator encourage a clathrin-mediated endocytosis process. By this process, viral-receptor complex absorption occurs and viral genome is released into the target cells cytoplasm. The viral particle is presented to the endosome, reacting with a scavenger receptor CD163. Reaction between GP2 and GP4 glycoprotein of the virus and SRCR domain 5 of the CD163 is required to enhance the reaction of viral genome releasing. Moreover, protease cathepsin E and trypsin-like serine protease are essential to this process. However, many PRRSV alternative entry pathways are needed to find out the overall processes.

Beyond the infection of PRRSV into the target cell, non-structural polyprotein encoded by ORF1a and ORF1b is translated. Minus strand RNA translated by this region is the original pattern of the viral genomic RNA transcription. Subgenomic replicative intermediates (RIs) are produced and act as the origin of the viral subgenomic RNAs of ORF2-7. Afterward, viral genome is covered by the nucleocapsid protein in smooth endoplasmic reticulum and golgi complex membrane and the novel viral particles are budding out of the target cells (Snijder and Meulenberg, 1998; Pasternak et al., 2006).

2.2 Clinical signs

Pigs infected with PRRSV exhibits various clinical signs depending on many factors such as the age of the infected pigs, the genotype or virulence of the virus, the herd size and herd type, immune status of the infected herd, host susceptibility, co-infection and also the factors of farm management (Halbur et al., 1995[b]; Thanawongnuwech et al., 1997; Thanawongnuwech and Suradhat, 2010). Basically, PRRSV causes many problems with the

reproductive system in sows as well as respiratory system in piglets and nursery pigs. The clinical signs of infected nursery pigs include anorexia, pyrexia (39-41°C), lethargy, pneumonia, discoloration or cyanosis on extremities and subocular edema. An infected sow herd frequently exhibit poor reproductive performance characterized by increasing of abortions and decreasing of farrowing rate in all or some ages. Abortion is one of the marked observable problems resulting from PRRSV infection in sow herd. Variable between 10-50%, abortion can occur at any gestation period especially at the late stage. Premature farrowing, increase number of stillbirths and /or mummified fetuses, weak born piglets and increase number of preweaning mortality cases are also associated with the PRRSV infection as well as return to estrus and increase of non-productive days in sows (Benfield et al., 1992; Done et al., 1996; Cho and Dee, 2006; Zimmerman et al., 2006).

2.3 Host range and pathological change

As other members of the Arteriviridae family, PRRSV infectivity is limited in a single host. Pigs, including wild boars and domesticated hogs, are primary host of PRRSV. Type 1 and type 2 PRRSV can cause diseases in similar patterns. Mostly in the respiratory system, major macroscopic lesions due to the infection include diffuse pneumonia and generalized lymphadenopathy (Halbur et al., 1995a; Rossow et al., 1995). In addition, other localized lesions such as subcutaneous edema and ocular edema are also shown (Rossow et al., 1995). Microscopic lesions associated with PRRSV infection include interstitial pneumonia and germinal center lymphoplasia in some lymph nodes (Halbur et al., 1995[a]; Rossow et al., 1995).

As the virus shows a predilection for immune cells of the activated monocytes and macrophages, infected cells can distribute the virus into many internal organs such as lungs, spleen, lymph nodes, placenta and also umbilical cord (Duan et al., 1997; Lawson et al., 1997; Murtaugh et al., 2002). Moreover, PRRSV can exist as prolonged viremia and persistent replication in some lymphoid tissues and tonsils to extend its infection period (Allende et al., 2000). This mechanism is one of the modes that accelerated intra-host

population of the virus, increase viral genetic diversity and vary the viral phenotypes differ to the original one over the time (Rowland et al., 1999; Goldberd et al., 2003).

2.4 Immunological responses

Firstly, after infected with PRRSV, immunoglobulin responses are developed. Viremia and high viral load in tissues were found in this acute phase, lasting for more than 30 days and persisting in some tissues for at least five months (Albina et al., 1998[a]; Allende et al., 2000). After the first infection, PRRSV specific antibody detected by Enzymed-linked immunosorbent assay (ELISA) will be presented within 7-9 days (Yoon et al., 1995; Lopez and Osorio, 2004). The immunoglobulin M (IgM) is the first antibodies response to the infection within 5-7 dpi and lasting until 14-21 dpi with the highest level at 14 dpi (Loemba et al., 1996; Joo et al., 1997). On the other hand, PRRSV specific IgG is response later but lasts up for a longer period. IgG response to the infection can be detected firstly within 7-10 dpi (Nelson et al., 1994; Loemba et al., 1996) and lasts up to 10 months after infection (Nelson et al., 1994; Murtaugh et al., 2002). Unfortunately, these early response antibodies are non-neutralizing antibodies (Yoon et al., 1994; Labarque et al., 2000). The early-stage antibody plays an important role in the antibody-dependent enhancement (ADE) phenomenon that is one of the mechanisms enhancing an effectiveness of PRRSV infection (Yoon et al., 1996; Yoon et al., 1997). PRRSV-specific neutralizing antibody occurs later within 28 dpi (Yoon et al., 1994; Lopez and Osorio, 2004).

Mostly, this neutralizing antibody is specific to the GP5 protein (Nelson et al., 1994; Pirzadeh and Dea, 1997; Gonin et al., 1999). The viremia is reduced within 4 weeks post-infection, almost the same time as neutralizing antibody is developed (Nilubol et al., 2004). However, low level of the PRRSV or persistent infection in some tissues is still found for more than 150 days after infection in postnatally infected pigs (Allende et al., 2000) and as long as 210 dpi in congenitally infected piglets (Cho and Dea, 2006).

2.4.1 Immune evasion of PRRSV

PRRSV is well-known as one of the most effective virus that utilized multiple tactics for its immune evasion. Several researches have proved that the virus may evade from both innate and adaptive specific immunity by its heterogeneity, modifying the cytokine production of macrophages and/or dendritic cells, altering the molecular expression of the antigen presentation process and also delaying neutralizing antibody production as well (Kimman et al., 2009).

1) Strain heterogeneity

Since PRRSV originated from two different genotypes, type 1 and type 2 PRRSV, heterogeneity in genetic and antigenicity between PRRSV strains were found. High variation, in approximately 40%, between type 1 and type 2 PRRSV at the genomic level was reported (Kim and Yoon, 2008). Similarly, within each genotype, PRRSV contains up to 20% genetic diversity (Nelsen et al., 1999; Ment et al., 2000; Plagemann, 2003; Han et al., 2006). Random mutation and also intragenic recombination plays an important role in this heterogeneity of the virus. As a basic of RNA virus and the virus within the Arteriviridae family, nucleotide substitution rates of the virus is as high as $1\text{-}3x10^{-2}$ per site per year (Prieto et al., 2009). This heterologous strains of the PRRSV is interfering and incomplete the protective immunity.

2) Apoptosis-induced cell death

Necrosis due to the PRRSV infection is evident. Additionally, infected cells and also bystander cells adjacent to the PRRSV infected cells are inducible to apoptosis (Miller and Fox, 2004). This mechanism is incomplete explored and consequences of the apoptosis are still in doubt. It has been believed that both GP5 and apoptogenic inflammatory cytokines including TNF-α may play an important role in this process (Miller and Fox, 2004; Fernandez et al., 2002) and no correlation between the viral load and severity of the lesions were observed (Kimman et al., 2009).

3) Innate immunity interferences due to PAMs infection

As innate immunity is an important mean to prevent the virus in an early stage of infection. PRRSV use one of its immune evasion strategies in PAMs interference. PAMs infected with PRRSV failed to express several inflammatory cytokines including interleukin-1 (IL-1), tumor-necrosis factor-α (TNF- α) and interferon-α (IFN- α) (Van Reeth et al., 1999; Thanawongnuwech et al., 2001). The process was described completely that down-regulation of these set of cytokines especially type I interferon may gain a profit for the virus since IFN-α is one of type I interferon inhibiting the virus replication (Albina et al., 1998b). Moreover, these interferences may also alter the induction of a specific helper T-cell as well as specific humoral immune response of the infected pig.

4) Innate immunity interferences due to dendritic cells infection and delay the DCs maturation

One of the consequences associated to PRRSV infected PAMs is the alteration of the IFN-α expression. This consequence affects the DCs maturation as well since the DCs need major stimulation by IFN-α from PAMs. Moreover, the virus can infect both mature and immature porcine DCs directly and interfering many DCs-related functions as well as in infected PAMs (Loving et al., 2007; Chalerntantanakul et al., 2006; Wang et al., 2007). Infected monocyte-derived DCs induced many abnormality expressions including apoptosis-induced cell death, down regulation of CD11b/c, CD14 and CD80/86 and also decreasing expression of both major histocompatibility complex (MHC) class I and class II. In addition, PRRSV infected DCs elicit expression of IL-10, IL-12 and TNF-α as well as reduced the allogenetic stimulation of T-cells similar to the infected PAMs (Wang et al., 2007; Genini et al., 2008).

5) Lack of viral protein expression

Similar to other Arteriviruses, PRRSV particles especially nucleocapsid protein assemble into the ER and/or golgi apparatus lumens. Viral antigens are retained and accumulated only in the compartments of its budding sites. This process helps the virus evasion since the infected cells present only limited viral proteins into its plasma membrane and remain invisible to the PRRSV specific antibody as well (Costers et al., 2006).

6) Decoy epitope on PRRSV GP5 protein

Decoy epitope is the strong immunodominant epitope adjacent to the major neutralizing epitope in GP5 of the PRRSV. Located at the amino acids position 27-30, the non-protective antibodies response to this epitope may produce strong and prior to the major neutralizing one. This masking effect is resulting in delay, weakness and impairment of the major PRRSV neutralizing epitope (Ostrowski et al., 2002).

7) Glycan-shielding phenomenon of the GP5 protein

PRRSV contains glycosylation modification on its envelope glycoprotein to evade pig immune system. Due to this glycan blocking, few neutralizing antibodies are produced after the PRRSV infection as well as most PRRSV neutralizing antibodies of an infected pig could not react with the major neutralizing epitope on GP5 of the virus. Many researches provided data about the locations of these N-linked glycosylation on GP5 including N30, N33, N44 and N51 of the type 2 PRRSV (Jiang et al., 2007) and N46, N37 and N53 of the type 1 PRRSV (Kimman et al., 2009). Moreover, recent report had raising up the topic that some N-linked glycosylation sites located in the GP3 protein may play a significant role in the PRRSV glycan shielding phenomenon as well as the GP5 (Kimman et al., 2009). However, the mechanisms of these processes are still needed further studies.

8) Antibody-mediated enhancement (ADE)

Since the PRRSV-specific murine monoclonal antibodies could be categorized into 3 groups including enhancing the viral infection, neutralizing the virus and neither activity. Some PRRSV-specific antibodies occurred by natural infection, maternal derived and also vaccination can act as ADE to sustain the viral infection. Many researches explored that some epitopes located within N and GP5 proteins might play an important role in this phenomenon (Kimman et al., 2009).

2.5 Epidemiology of PRRSV

Since late 1980s, outbreaks of unrecognized swine diseases occurred in North America and Europe. The diseases affected a lot of swine herds, causing severe reproductive loses. The presence of clinical signs associated with reproductive failure such as increase in the number of week live-born piglets and respiratory disease such as severe pneumonia in neonatal and nursery pigs were reported in North America (Keffaber, 1989; Loula, 1991; Hill, 1990). Similarly, the diseases with the same problems also occurred in many swine herds in Europe. Affected herds were suppressed with poor reproduction, growth performances and also increased mortality (Lunney et al., 2010). The disease, named “Swine mystery disease” in USA and “Blue-ear pig disease” or “porcine epidemic abortion and respiratory syndrome” in Europe, spread into other countries in North America and Europe in the next few years and got its accepted name as “porcine reproductive and respiratory syndrome or PRRS” until the present.

It was not until 1991, a small RNA virus fulfilling the Koch’s postulates of the disease was completely isolated in the Netherland and named as Lelystad virus (Terpstra et al., 1991; Wensvoort et al., 1991). Shortly after, the North American PRRSV prototype was isolated and named VR2332 (Collins et al., 1992). Both prototypes were fully described as the causative agent of the porcine reproductive and respiratory syndrome and were

categorized as a member of family Arteriviridae and genus Arterivirus (Benfield et al., 1992; Dea et al., 1992).

After the first outbreak in the North America and Europe, PRRSV also spread and caused damages to the pig production globally (Allende et al. 1999) and caused the first outbreak in Thailand in 1989 (Damrongwatanapokin et al., 1996b). This Thai evidence could be verified by a retrospective study using the serum bank and the viral isolation of the first type 2 PRRSV-challenged experiment was done in Thailand since 1996 (Damrongwatanapokin et al., 1996a). Later, both two types of PRRSV were widely distributed in different areas in Thailand (Thanawongnuwech et al., 2004a).

Nowadays, PRRSV emerged as one of the most prevalent disease in swine worldwide containing several genetic characteristics variable to its geographic origins (Lunney et al., 2010). The recent pandemic "Swine high fever syndrome" virus which occurred in China in 2006 is also one of the adapted PRRSV (Tong et al., 2007; An et al., 2010) as well as the highly virulent 1-18-2 strain emerged in USA in 2007 (Murtaugh, 2009) and many nsp2 truncated viruses reported in Thailand (Kedkovid et al., 2010).

In addition, outbreaks of PRRS occurred in commercial swine herds in several areas around the world are reported. For example, the outbreak of type 1-related PRRSV isolates in Britain (Frossard et al., 2012), Belarus, Lithuania, Ukraine, Latvia and Russia (Balka et al., 2008; Balka et al., 2012) and also in Czech Republic (Jankova et al., 2011). On the other hand, the type 2-related isolates especially the Highly pathogenic-PRRSV (HP-PRRSV) were commonly reported in China (Wang et al., 2012), Lao People's Democratic Republic (Ni et al., 2012), Vietnam (Feng et al., 2008), Thailand (Nuntawan Na Ayudhya et al., 2011; Nilubol et al., 2013[b]) and also other countries within the nearby regions including Bhutan, Cambodia, Malaysia, Myanmar, Philippines, Singapore, South Korea and Russia (An et al., 2011). These are evident that the novel PRRSV both the virulent and the benign ones are still causing outbreaks and continuing to be endemic in most swine farms worldwide.

2.6 Genetic diversity of PRRSV

PRRSV is a virus with a very high genetic diversity, compared to other viruses. It was found that the virus genetics are very different (Thanawongnuwech et al., 2004[a]). It is expected that the genetic variation of the PRRSV is correlated to the pathogenesis of the virus (Meng et al., 1995). However, there are no evidence to describe the results of the different genes or proteins of PRRSV affecting to the virulence and its pathogenesis. Analogically, between the type 1 and type 2 PRRSV, the two viruses shared genetic similarity only about 60-63% (Allende et al., 1999). Genetic differences within the genotype might share the genetic similarity only about 61.3-62.5% (Bouvet et al., 2001). This indicated that PRRSV of both types are very different genetically. The genetic sequences of the North American strains share similarity about 87-95% having amino acid translated from ORF2 to ORF7 similarity about 91-99%, 86-98%, 89-99%, 83-99%, 98-100% and 95-100%, respectively (Meng et al., 1995; Gao et al., 2004; Thanawongnuwech et al., 2004[a]).

According to previous reports, PRRSV was assumed as one of the highest evolutionary divergence viruses. With its excessive nucleotide substitution rates, as high as $4.7\text{-}9.8\text{x}10^{-2}$ per site per year, PRRSV were determined as one of the highest evolution among RNA viruses. Several viral mechanisms including recombination, nucleotide insertions and deletions play an important role in the PRRSV diversity. Recombination activity due to RNA strand cleavage and re-ligation of subgenomic mRNA must be high, especially in the process of transcription or genomic replication of large amount subgenomic mRNA production. Mostly if two different PRRSV infected into the same cell, recombination would increase and the substitutions of the virus would be enhanced (Murtaugh et al., 2010). Concurrently, evidences of the PRRSV recombination were widely reported worldwide (van Vugt, 2001; Li B, 2009; Liu D, 2011; Franzo et al., 2012). Moreover, host-mutated mechanisms also affect to PRRSV evolution. As a key step in antibody diversification in host cell, activation-induced deaminase (AID) such as apolipoprotein B editing catalytic polypeptide (APOBEC) also raise the chance of host-induced mutation (La

Rue et al., 2008; La Rue et al., 2009). This cytidine deamination from C to U may force mutation in the viral genetic and drive an evolution of PRRSV as well as other immunological selection (Murtaugh et al., 2010).

Genetically, most studies focus on the structural protein especially GP5, N protein and E protein (Verheije et al., 2002; Rowland et al., 2003; Rowland and Yoo, 2003; Plagemann, 2004[a]; Plagemann, 2004[b]; Lee et al., 2006; Lee and Yoo, 2006). There is a study of the nonstructural protein such as NSP2 being used for phylogenetic analysis (Kedkovid et al., 2010). However, the study on the mechanisms of the correlation between genetic characteristics and pathogenesis of the virus cannot clearly explain because the virulence and pathogenesis of the virus are not clearly associated with the location of the mutation. Anyway, studies in some major nonstructural protein gene are important since some proteins coded by the crucial genes are important for the virus survival and severity, for examples, NSP2, ORF5 and ORF7. NSP2 coded by NSP2 gene functions as protease activity, necessary for replication and survival of the PRRSV in cells. Moreover, NSP2 is the most genetically variable region of PRRSV (Kedkovid et al., 2010). Similarly, ORF5 of PRRSV is one of the most variable genomic sequences used for genetic variation determination (Murtaugh et al., 2010).

Although many researches proved that there are many viral epitopes reside on the M, GP2a, GP3 and also GP4 can induce neutralizing antibodies as the GP5 (Kimman et al., 2009). However, the neutralizing antibodies induced by GP5 appears to be the most effective antibodies. Using peptide mapping analysis, major neutralization epitope were proved as locating at the position 36-52 in the middle of the GP5 ectodomain (Plagemann et al., 2004). This region is known to be hypervariable for genetic change. Biological activities as well as antibodies targeting and protein folding of this site depend on multiple N-linked glycosylation which is flanking to this epitope (Kimman et al., 2009). Due to this mechanism, both type 1 and type 2 PRRSV do contain the hyper variable region in GP5 gene located at

amino acid position (aa) 32-40. Two variable regions located at aa 57-70 and 121-130 as well as three conserved regions at aa 41-56, 71-120 and 131-200 (Kimman et al., 2009).

Due to the property of the highest diversity of PRRSV structural protein and its correlation to neutralizing immunogenicity, ORF5 nucleotide sequences were mostly used in the polygenetic analysis of PRRSV. Based on publicly available ORF5 sequences data, the most phylogenetic classification of both type 1 and type 2 worldwide were generated continuously. The largest phylogenetic analysis of type 1 PRRSV was performed in 2010 (Shi et al., 2010). According to this study, type 1 PRRSV was divided into 3 subtypes. Subtype I is the global type 1 PRRSV which was divided into 12 clades (A to L) with interclade diversity more than 10%. Subtype II consisted of type 1 PRRSV isolated from Russia, Lithuania and Belarus. Interestingly, sequences from the Eastern Europe located in subtype II were sampled later than the Lelystad virus identification containing divergent sequences that prior to those of the Lelystad virus sequences (Oleksiewicz et al., 2000; Forsburg et al., 2002; Stadejek et al., 2002; Mateu et al., 2003; Indik et al., 2005; Pesch et al., 2005; Stadejek et al., 2006; Balka et al., 2008; Stadejek et al., 2008). This indicated that type 1 PRRSV emerged in Eastern Europe prior to the first recognized in Western Europe in late 1980s and might be the area that the virus evolved in the past (Stadejek et al., 2006). Evidently, the hypothesis as the first PRRSV outbreak occurred before the first recognition events in late 1990s was introduced. Many retrospective studies proved this hypothesis since PRRSV positive serological tests were found in samples as old as 1979 in North America (Carman et al., 1995; Hill et al., 1990).

Similar to the phylogenetic study of the type 1 PRRSV, ORF5 sequences were use to describe the genetic diversity of the type 2 PRRSV (Shi et al., 2010). More than 8,500 ORF5 sequences of type 2 PRRSV involved in this study. The phylogenetic analysis resulted in the separation of 9 lineages of type 2 PRRSV around the world with the interlineage divergence more than 10%. Seven from nine lineages were represented as US or North American isolates with only two lineages, lineage 3 and 4, were dominated by Asian isolates including

the isolates from China, Japan, Hong Kong and Taiwan. This result indicates that North American is an important area for the type 2 PRRSV evolution at that time containing the widest genetic diversity of the type 2 viruses.

However, as increasing of swine industries in China, the evolution of type 2 PRRSV in China has become greater. This situation is correlated with multiple factors especially swine population. PRRSV circulation in larger swine herds in China could infect and re-infect many times, more than the circulation in smaller herds in other countries. Mutation and recombination could occur more in this situation. Since the outbreaks of the NSP2 deleted type 2 PRRSV (HP-PRRSV) in China in 2006, NSP2 nucleotide sequences were widely used to determine genetic variations and correlations to the HP-PRRSV prototypes (Kedkovid et al., 2011; Han et al., 2006). This non-structural protein gene is mostly used in characterization and determination of PRRSV distribution in China as well as in the neighboring countries including Vietnam, Laos PDR Cambodia, Burma and Thailand.

Nonetheless, type 1 and type 2 PRRSV were spread geographically and genetics of the viruses had evolved over the time, but due to global transportation at the present, both type 1 and type 2 PRRSV emerge synchronously in many areas around the world. The categorization of a new PRRSV isolate is not limited on the geographic site of the isolation. Categorization of a novel PRRSV isolate should base on genetic likeliness to the original prototypes as well as its geographic site (Brar et al., 2011) especially in the current situation that intergenotype diversity has revealed genetic divergences as high as 20% (Meng, 2000; Plagemann, 2003; Han et al., 2006).

2.7 Highly Pathogenic PRRSV (HP-PRRSV)

Since the first outbreak of the PRRSV in 1987, the virus has spread into several continents such as Europe and Asia in 1990 (Thanawongnuwech et al., 2004). Later, antigenic and genetic heterogeneities as quasispecies evolution of the PRRSV have been reported (Schommer et al., 2001; Goldberg et al., 2003). Unavoidably, the swine high fever (SHF) syndrome caused by HP-PRRSV in China emerged. After the Chinese HP-PRRSV

outbreaks, the pigs production in more than 10 provinces in China were affected causing more than 400,000 pigs died and 2,000,000 pigs infected in 2006 (Tong et al., 2007; Xiao et al., 2010) and continuing to be the major problem until now.

Following to the outbreaks of the disease, many isolates of the HP-PRRSV were fully characterized. According to the genomic characteristics of the HP-PRRSV, NSP2 nucleotide deletion was clearly demonstrated (Tong et al., 2007). Comparing between the HP-PRRSV and the native China PRRSV isolates, CH-1a, HB-1 and BJ-4, multiple genomic mutations in ORF5 and 30 amino acids deletion at the position 482 and 533 to 561 in NSP2 were clearly described (Tian et al., 2007; Tong et al., 2007; Xiao et al., 2010). Moreover, recent data mentioned that the virus might originate from the CH-1a PRRSV which was isolated in the southern China (An et al., 2010). In addition, recombination between the Chinese modified live virus (MLV) vaccine and a local strain PRRSV was evident (Li et al., 2007).

Actually, the major source of PRRSV infection in farms is mostly due to the introduction of infected replacement pigs (Pesente et al., 2006). Thus, one of the most effective intervention strategies to prevent transmission is routinely surveillance and monitoring for the virus from the farm origins. To combine with the epidemiological data, eradication procedure may be possible in some farms depending on many factors (Thanawongnuwech, 2010). For this reason, an appropriate Thai HP-PRRSV detection methods such as RT-PCR is needed for a rapid and accurate detection to control the outbreaks in farms and also for the whole nation.

2.8 The study of PRRSV in Thailand

After the first outbreak of PRRSV in Thailand in 1989 (Damrongwatanapokin et al., 1996), many PRRSV outbreaks have been reported in Thailand. The first research about genetic diversity of PRRSV in Thailand was done in 2004 (Thanawongnuwech et al., 2004). In this period, Thai PRRSV belonged to type 1 PRRSV more than the other. Type 1 PRRSV in Thailand appeared to be from the Northern Europe and one of the type 1 Thai isolate, 02CB12, shared more than 99% similarity nucleotide sequences with the modified live

vaccine virus (Porcilis®). On the other hand, the study discovered that type 2 Thai PRRSV isolates were clustered within the same group and more closely related to the PRRSV isolated from Canada. This can conclude that Thai PRRSV at that time might be introduced by pig importation and also by vaccines using in farms. According to the study in 2011, Thai PRRSV isolates between 2000 and 2008 were collected. The study demonstrated novel introductions of PRRSV strains in Thailand. Type 2 PRRSV isolates collected in Thailand in this period were originated from at least three independent introductions. This represented co-circulation of diverse PRRSV strains in some areas especially in 3 major Thai swine producing provinces including Chonburi, Ratchaburi and Nakhonpathom. The collected Thai isolates were divided into 3 clusters within lineage 1, lineage 5 and the orphan cluster relation to lineage 6-9 from the phylogenetic tree generated by the worldwide type 2 isolates according to the study by Shi and the others in 2010 (Tun et al., 2010).

In 2010, the first study of NSP2 genetic variation of Thai PRRSV was reported. The research discovered that 90% of Thai PRRSV isolates were NSP2-truncated virus that might have evolved from a virus previously introduced in the past, not from the one recently introduced. Interestingly, Thai PRRSV included in this study had nucleotide deletion in the NSP2 coding region that occurred in the course of the individual self-evolution of the PRRSV previously introduced in Thailand (Kedkovid et al., 2010). This result indicated that the viruses circulating in Thailand in that period were not related to the HP-PRRSV emerged in China at the same time. Later, in late 2010, the first report of the HP-PRRSV outbreak in Thailand was documented. The viruses collected from Phitsanulok and Nongkhai in that year contains 30 amino acids deletion similar to the HP-PRRSV prototypes reported in China since 2007 (Nuntawan Na Ayudhya et al., 2011). These 2 studies raised up the important of NSP2 gene characterization of Thai PRRRSV. Genetic characteristics of this particular gene are represent the origin and variation of PRRSV circulating in the areas compared to the viruses in the past and other areas around the world.

According to the PRRSV detection in the reproductive affected pigs since 2005 to 2010, the study demonstrated that 73.7% of the PRRSV positive samples in this period revealed to type 2 PRRSV while only 55.0% and 28.7% were type 1 PRRSV and mixed infection, respectively. This study suggested that type 2 PRRSV is the major causes of reproductive problems in swine farm in Thailand (Surapat et al., 2011). This conclusion was extended in the consequent study in 2012. This retrospective study was done with all kinds of samples submitted to the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). Frequently, analysis of this study indicated the prevalence of PRRSV was 32.6% including type 2, type 1 and mixed type PRRSV (54.5%, 31.0% and 14.5%, respectively). Interestingly, the prevalence in the cold season was 2.8%. This indicated that climate changes were also one of the factors affected to the prevalence of PRRSV outbreak in Thailand (Tummaruk et al., 2013).

On the other hand, genetic diversity of ORF5 gene of Thai PRRSV was reported. During 2010 to 2011, Thai PRRSV isolates were collected and sequenced to determine genetic diversity in ORF5 region. The research proved that Thai type 1 and type PRRSV isolates developed clusters separately from the viruses in other countries. By the way, this research found that PRRSV strains collected from different areas might contain the same genetic characteristics. This result proved that geographical factor was not affected to the virus mixing and strain development of PRRSV in Thailand. Thai type 1 PRRSV isolated in this study contains 84.7-99.8% nucleotide divergence and 84.2 to 99.5% amino acid divergence and divided into 3 clusters. On the other hand, with introductions of other PRRSV isolates into the country, type 2 PRRSV contains more genetic diversity as 83.4 to 99.8% and 80.8 to 99.5% for nucleotide and amino acid divergence, respectively. Thai type 2 PRRSV were divided into 4 clusters, similar to other isolates collected in other regions such as HP-PRRSV in China and also modified life virus vaccines (Nilubol et al., 2012).

According to the study of 5 pig farms in 2012, HP-PRRSV outbreaks in Thai swine farms were confirmed. Sequences of NSP2 gene from the collected PRRSV isolates

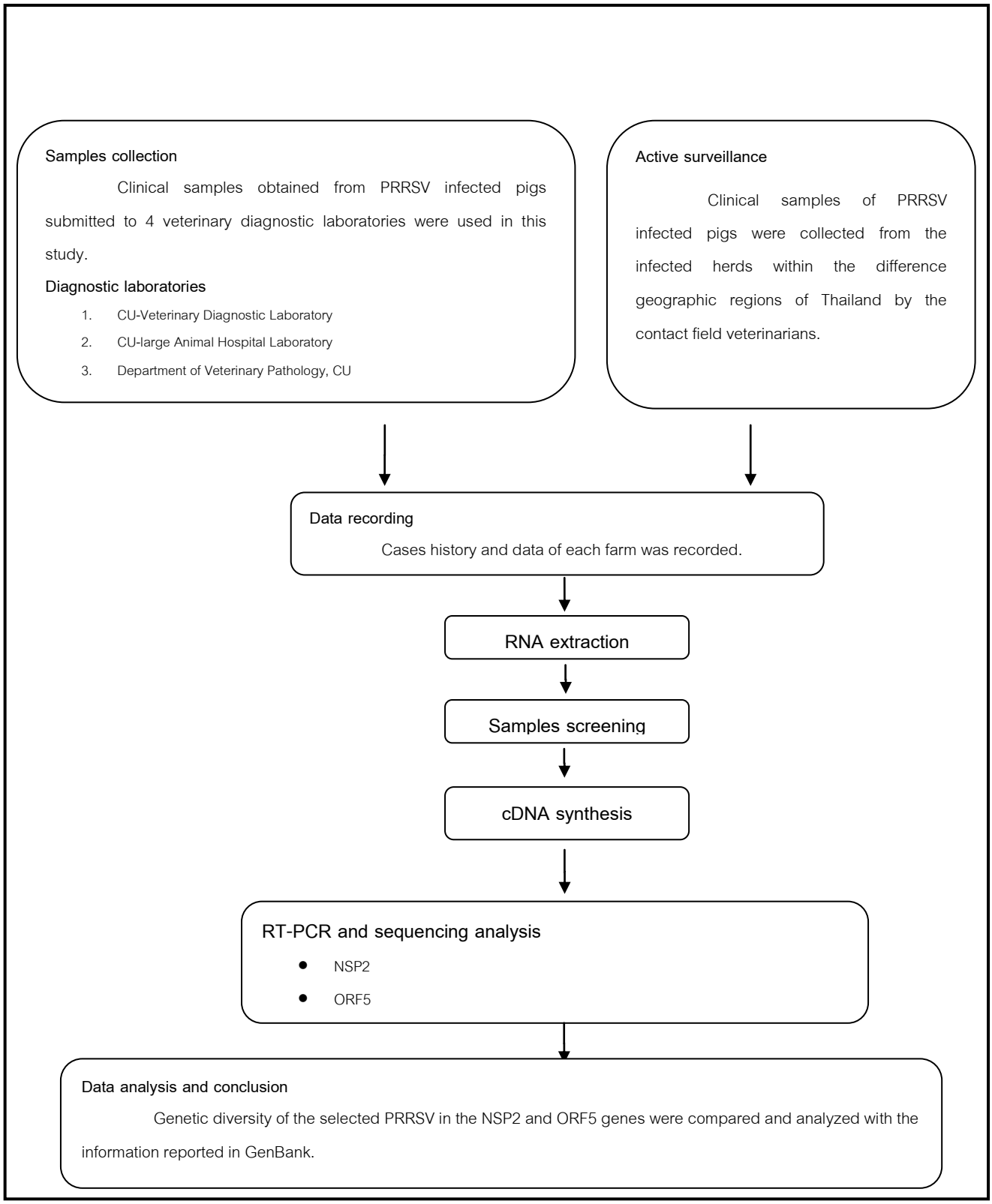
represented the unique characteristic of HP-PRRSV (30 amino acids deletion) and raised up a scenario as the cause of virus spreading in Thailand possibly introduced from Vietnam and Lao PDR via infected pigs and illegal transportation (Nilubol et al., 2013b). However, current genetic characteristics of Thai PRRSV causing infectious problems in Thai swine farms are still unclear, so further research is needed for the current PRRSV situation.

Chapter III

Materials and Methods

This study was divided into 3 parts. The first part was the sample collection from 4 collaborated veterinary diagnostic laboratories and from contact veterinarians working in the high risk areas. The second part was the viral nucleic acid extraction, identification and genetic characterization of selected Thai PRRSV isolates based on geographical areas. The selected isolates were obtained from different areas unless those isolates had caused problematic and shared different genetic characteristics on either NSP2 or ORF5. Finally, the third part was the nucleotide sequencing analysis of the studied Thai PRRSV and phylogenetic tree analysis. The overall process of this study is shown in Figure 1.

Figure1. Experimental plan



3.1 Sample collection

Serum, tissue and oral fluid samples from clinical cases of PRRSV infected pigs were submitted to four collaborated veterinary diagnostic laboratories including Chulalongkorn University-veterinary diagnostic laboratory (CU-VDL), Chulalongkorn University-large animal hospital laboratory, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University and Veterinary diagnostic laboratory, Faculty of Veterinary Medicine, Khon Kaen University (KKU) and the collaborative-contact veterinarians working in the high risk area.

Samples obtained from these 4 diagnostic laboratories were limited to the clinical cases which were submitted after February until October 2012. Primarily, each sample from the 4 laboratories was proven as type 2 PRRSV infected samples by standard RT-PCR method of each laboratory. On the one hand, samples submitted as an active surveillance from the contact veterinarians were testified as type 2 PRRSV positive samples by RT-PCR method as well. Data of each clinical case including farm information, location and severity of the outbreaks and other important information of the infected herd were recorded. The submitted samples were kept in -80°C until used.

3.2 Sample processing

Serum samples were stored in -80°C until RNA extraction and RT-PCR were performed. Tissue samples of each case were homogenized with beads and Minimum Essential Medium Eagle (MEM). The mixtures were centrifuged with 3500 rpm for 15-20 minutes. The supernatant were collected, filtered via a 0.45μm syringe-driven filter and stored in -80°C until used. Oral fluid samples were also collected and centrifuged with 3500 rpm for 3-5 minutes. The supernatant was collected and stored in -80°C until used. However, to save the laboratory expenses, 5 submitted samples from the same source were pooled into 1 testing sample as the standard procedure of a diagnostic laboratory.

3.3 Viral RNA extraction

Viral RNA were extracted from 150 µl of the processed samples by NucleoSpin® Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) following to the manufacturer recommended method. In brief, the samples were centrifuged and 200 µl of the supernatant were collected and mixed up with lysis buffer and carrier RNA. After mixing and incubating, binding solution was added to bind the RNA. Then, washing buffer was used to remove the residual elements. The RNA was eluted by 70°C nuclease free water for the final volume of 50 µl. Finally, the extracted RNA was stored in -80°C until used.

3.4 PRRSV samples screening

Samples submitted from 4 veterinary diagnostic laboratories were screened for the specific PRRSV nucleotide using the standard procedure of each laboratory. Samples from the contact veterinarians were primarily proven as the PRRSV positive samples by the RT-PCR method. Samples from the contact veterinarians were screened by specific PRRSV ORF7 multiplex RT-PCR as standard method of the Chulalongkorn University-veterinary diagnostic laboratory (CU-VDL). Only positive PRRSV samples with clearly clinical information were selected as the studied samples.

3.5 cDNA synthesis

For a long-lasting storage, the extracted RNA were synthesized to be cDNA using GoScript™ Reverse Transcription System (Promega, USA) as the method of manufacturer recommendation.

3.6 Reverse Transcriptase-Polymerase Chain Reaction, amplification of NSP2 gene and ORF5 sequences

Fragments including NSP2 and ORF5 genes of PRRSV were amplified by RT-PCR. Five micro-liters of extracted RNA in a 25µl reaction mixture contained 1µM/µl of forward and reverse primers published by Feng et al., 2008 (as presenting in Table 1), 10µl of 2x AccessQuick® Master Mix (Promega, USA), 1µl of AMV Reverse-Transcriptase (Promega, USA) and nuclease-free water to make up the final volume.

Table 1 Primer sequences of ORF5 and NSP2 genes

Primers name	Nucleotide sequences	Location	PCR product size (bp)	Reference
ORF5-F	5'-ATG TTG GGG AAG TGC TTG ACC-3'	1-600	600	Feng et al., 2008
ORF5-R	5'-GTA GAG ACG ACC CCA TTG TTC CGC-3'			
Nsp2-F	5'-AGGAAGGTCAGATCCGATTG-3'	1538-1894	370	Hao et al., 2011
Nsp2-R	5'-CGTCTGAGGACGCAGACA-3'			

3.7 Agarose gel electrophoresis and PCR product purification

The RT-PCR products were separated by 1.5% agarose gel electrophoresis. Agarose gels were immersed in Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide. Stained gels were visualized by UV transilluminator. The expected band of PCR product was cut and purified with NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany). For viral sequences, the cut gels were processed following to the manufacturer recommended protocol.

3.8 Viral sequences and phylogenetic analysis

Sequences of serum or infected tissues of pigs from different herds originating from different geographic regions of Thailand after 2010 were investigated. DNA sequencing was carried out by 1st BASE Company (Singapore) with primers used in the previous RT-PCR reaction. Sequences were edited and corrected by the BioEdit® computer programs (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The whole genome PRRSV sequences from Thai PRRSV isolates were compared with other PRRSV sequences reported in GenBank (http://www.ncbi.nlm.nih.gov/) by Bioedit® and MEGA5® software. Finally, the

phylogenetic trees were constructed by MEGA5® (Tamura et al., 2007) using the neighbor-joining method with 1000 bootstrapping replicates.

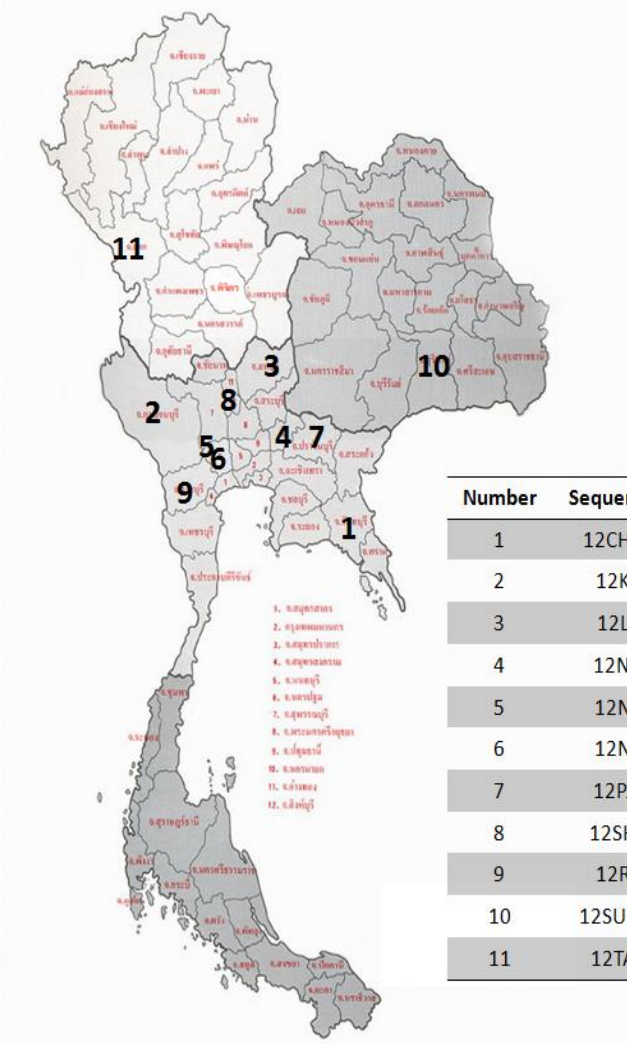
Chapter IV

Results

4.1 Samples collection and screening

During February until October 2012, 367 samples of 39 swine commercial farms were submitted to 4 collaborated veterinary diagnostic laboratories and contact veterinarians. Among them, only 33 farms were reported clearly about clinical signs, age affected and/or severity of the diseases in farms and were suspected as clinical PRRS cases while the remaining 6 farms were not affected or the information was not provided. Seventeen samples from 33 farms were proven as type 2 PRRSV positive samples by PRRSV specific RT-PCR but none of them were tested as type 1 PRRSV positive.

As the epidemiological aspect, PRRSV positive samples were collected from 17 farms located in 11 provinces in 4 regions of Thailand including North-eastern (Surin), Western (Tak, Kanchanaburi and Ratchaburi), Eastern (Prachinburi and Chantaburi) and Central (Lopburi, Singhburi, Nakhonnayok, Nakhonpathom and Suphanburi) (Figure 2). A total of 17 samples included 12SURIN01, 12KB01, 12LB01, 12RB01, 12RB03, 12RB04, 12NP01, 12NP02, 12NP03, 12NP04, 12NN01, 12SHB01, 12TAK01, 12PJB01, 12PJB02, 12SPB01 and 12CHTB01. All samples included serums, tissues and oral fluid samples were taken from the PRRS suspected pigs with clinical signs such as reproductive failure in sows and respiratory diseases in piglets. Details of all samples are shown in Table 2 and the details of the 17 PRRSV positive samples are shown in Table 3.



Number	Sequences ID	Location	Part of Thailand
1	12CHTB01	Chantaburi	Eastern
2	12KB01	Kanchanaburi	Western
3	12LB01	Lopburi	Central
4	12NN01	Nakhonnayok	Central
5	12NP01	Nakhonpathom	Central
6	12NP02	Nakhonpathom	Central
7	12PJB01	Prachinburi	Eastern
8	12SHB01	Singhaburi	Central
9	12RB01	Ratchaburi	Western
10	12SURIN01	Surin	North-western
11	12TAK01	Tak	Western

Figure 2: Location of the farms where 11 studied Thai PRRSV sequences were collected.

Table 2: Data of the submitted samples for PRRSV testing from 4 diagnostic laboratories and active surveillances during February to October 2012

No.	Sample ID	Location	Sample type	Clinical signs	Age affected	History			
						Morbidity (%)	Mortality (%)	Herd status	Vaccine
1	SURIN-1	Surin	serum	Reproductive diseases, PRDC	piglets	20-40	5	+	MLV type 2
2	KB-1	Kanchanaburi	serum	Respiratory signs, slow growth	Growing pigs	10-15	0-1	+	No
3	LB-1	Lopburi	serum	Respiratory signs, slow growth	Growing pigs	10-15	0-1	+	No
4	RB-1	Ratchaburi	serum	Reproductive failure, SMEDI	sows	10-20	10	+	MLV type 2
5	CHTB-1	Chantaburi	serum, tissue	Reproductive failure, abortion	sows	25	0	-	No
6	NP-1	Nakhonpathom	Serum, tissue	Respiratory diseases in weaning pigs	Weaning pigs	30	10	+	MLV type 2
7	NP-2	Nakhonpathom	serum	Reproductive failure, weak piglets	Sows, suckling pigs	10	5	+	MLV type 2
8	NP-1	Nakhonpathom	serum	Mild reproductive failure, respiratory disease in weaning pigs	Sows, weaning pigs	NA	NA	+	MLV type 2
9	SHB-1	Singhaburi	oral fluid, serum	Reproductive failure	sows	10	5-6	+	No
10	SHB-2	Singhaburi	serum	No	No	No	No	No	No
11	TAK-1	Tak	serum	Reproductive failure	sows	5-10	0	NA	NA
12	SB-1	Saraburi	oral fluid	Reproductive failure	sows	NA	NA	+	No
13	NN-1	Nakhonnayok	serum	Mild reproductive failure, respiratory disease in weaning pigs	Sows, weaning pigs	25	10	+	NA

Table 2: Data of the submitted samples for PRRSV testing from 4 diagnostic laboratories and active surveillances during February to October 2012 (cont.)

No.	Sample ID	Location	Sample type	Clinical signs	Age affected	History			
						Morbidity (%)	Mortality (%)	Herd status	Vaccine
14	SHB-3	Singhburi	serum	Reproductive failure	Sows	10%	0	+	NA
15	NP-4	Nakhonpathom	Tissue (from weak piglets), serum	reproductive failure, respiratory disease in weaning pigs	Sows, weaning pigs	10-20%	5	+	MLV type 2
16	KB-2	Kanchanaburi	serum	No	No	No	No	NA	No
17	NP-5 (139/55)	Nakhonpathom	tissue	NA	NA	NA	NA	NA	NA
18	NP-6 (143/55)	Nakhonpathom	tissue	NA	NA	NA	NA	NA	NA
19	NP-7	Nakhonpathom	serum	Respiratory diseases in suckling to weaning pigs	Suckling and weaning pigs	>15%	5	+	MLV type 2
20	NP-8	Nakhonpathom	serum	Respiratory diseases in suckling to weaning pigs	Suckling and weaning pigs	NA	NA	+	MLV type 2
21	CHS-1	Chachoengsao	serum	Respiratory diseases in suckling to weaning pigs	Suckling and weaning pigs	NA	NA	+	NA

Table 2: Data of the submitted samples for PRRSV testing from 4 diagnostic laboratories and active surveillances during February to October 2012 (cont.)

No.	Sample ID	Location	Sample type	Clinical signs	Age affected	History			
						Morbidity (%)	Mortality (%)	Herd status	Vaccine
22	SB-2	Saraburi	Tissue, serum	Reproductive failure, respiratory disease in piglets	Sows and piglets	NA	NA	+	MLV type 2
23	RY-1	Rayong	serum	NA	NA	NA	NA	NA	NA
24	RB-2	Ratchaburi	serum	Reproductive failure	Sows	5-6	0	+	Type 2 MLV
25	NP-9	Nakhonpathom	serum	Respiratory diseases in growing pigs	Growing pigs	NA	NA	+	MLV type 2
26	RB-3	Ratchaburi	serum	Respiratory diseases in weaning pigs	Weaning pigs	5	NA	+	MLV type 2
27	PJB-1	Prachinburi	serum	Reproductive failure and weak piglets	Sows and piglets	NA	NA	+	No
28	NN-2	Nakhonnayok	serum	Respiratory diseases in weaning pigs	Weaning pigs	<10	0	+	MLV type 2
29	SPB-1	Supanburi	serum	Respiratory diseases in nursery pigs	Weaning pigs	NA	NA	+	MLV type 2

Table 2: Data of the submitted samples for PRRSV testing from 4 diagnostic laboratories and active surveillances during February to October 2012 (cont.)

No.	Sample ID	Location	Sample type	Clinical signs	Age affected	History			
						Morbidity (%)	Mortality (%)	Herd status	Vaccine
30	ANGTONG-1	Angtong	serum	Respiratory diseases in weaning pigs	Weaning pigs	10	0	+	MLV type 2
31	ANGTONG-2	Angtong	serum	Reproductive failure, respiratory disease in piglets	Sows and piglets	NA	NA	+	MLV type 2
32	RB-4	Ratchaburi	serum	Reproductive failure	Sows	NA	NA	+	NA
33	NP-10	Nakhonpathom	serum	Reproductive failure	Sows	NA	NA	+	NA
34	CHB-1	Chonburi	serum	Reproductive failure and mild respiratory diseases in nursery pigs	Sows and nursery pigs	5-10	0	NA	NA
35	RB-5	Ratchaburi	serum	Respiratory diseases in nursery pigs	Weaning pigs	NA	NA	+	MLV type 2
36	RB-6	Ratchaburi	serum	Reproductive failure	Sows	5-10	0	+	NA

Table 2: Data of the submitted samples for PRRSV testing from 4 diagnostic laboratories and active surveillances during February to October 2012 (cont.)

No.	Sample ID	Location	Sample type	Clinical signs	Age affected	History			
						Morbidity (%)	Mortality (%)	Herd status	Vaccine
37	RB-7	Ratchaburi	serum	Reproductive failure, respiratory disease in piglets	Sows and piglets	NA	NA	NA	NA
38	CHB-2	Chonburi	serum	Reproductive failure, respiratory disease in piglets	Sows and piglets	NA	NA	NA	NA
39	PB-1	Petchaburi	serum	No	No	No	No	+	NA

Table 3: Data of the PRRSV positive samples (n=17) during February to October 2012

Sample ID	PRRSV positive samples	Provinces	Regions	Sequenced samples NSP2 and ORF5	Type of PRRSV
SURIN-1	12SURIN01	SURIN	NORTHEASTERN	✓	Type 2
TAK-1	12TAK01	TAK	WESTERN	✓	Type 2
KB-1	12KB01	KANCHANABURI	WESTERN	✓	Type 2
RB-1	12RB01	RATCHABURI	WESTERN	✓	Type 2
RB-2	12RB02	RATCHABURI	WESTERN	-	-
RB-3	12RB03	RATCHABURI	WESTERN	-	-
RB-4	12RB04	RATCHABURI	WESTERN	-	-
LB-1	12LB01	LOPBURI	CENTRAL	✓	Type 2
SHB-1	12SHB01	SINGBURI	CENTRAL	✓	Type 2
NN-1	12NN01	NAKHONNAYOK	CENTRAL	✓	Type 2
NP-1	12NP01	NAKHONPATHOM	CENTRAL	✓	Type 2
NP-2	12NP02	NAKHONPATHOM	CENTRAL	✓	Type 2
NP-3	12NP03	NAKHONPATHOM	CENTRAL	-	-
NP-4	12NP04	NAKHONPATHOM	CENTRAL	-	-
SPB-1	12SPB01	SUPHANBURI	CENTRAL	-	-
PJB-1	12PJB01	PRACHINBURI	EASTERN	✓	Type 2
CHTB-1	12CHTB01	CHANTHABURI	EASTERN	✓	Type 2

4.2 Viral nucleotide amplification

After tested as PRRSV positive, viral RNA samples extracted from 17 PRRSV positive samples were successfully converted to cDNA for further RT-PCR examination. The cDNA products were examined for both partial NSP2 and ORF5 gene of PRRSV by PCR. The PCR product sizes of both genes were predicted to be 370 and 550 bp, respectively (Figure 2 and Figure 3). A total of 17 samples included 12SURIN01, 12KB01, 12LB01, 12RB01, 12RB03, 12RB04, 12NP01, 12NP02, 12NP03, 12NP04, 12NN02, 12SHB01, 12TAK01, 12RB02, 12PJB01, 12PJB02, 12SPB01 and 12CHTB01 were positive to both NSP2 and ORF5 PCR. The PCR products of each sample were separated by agarose gel electrophoresis (Figure 3 and 4). Then, the gels were cut and purified for sequencing.

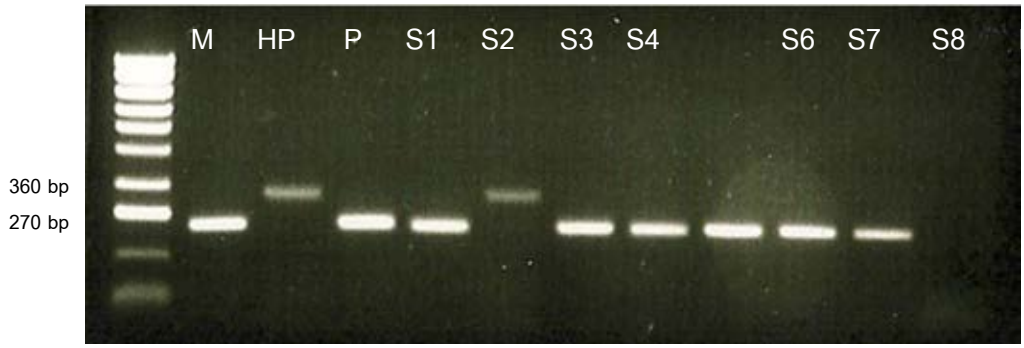


Figure 3: RT-PCR product of partial NSP2 gene; M: 100 bp DNA ladder, S1-S10: samples

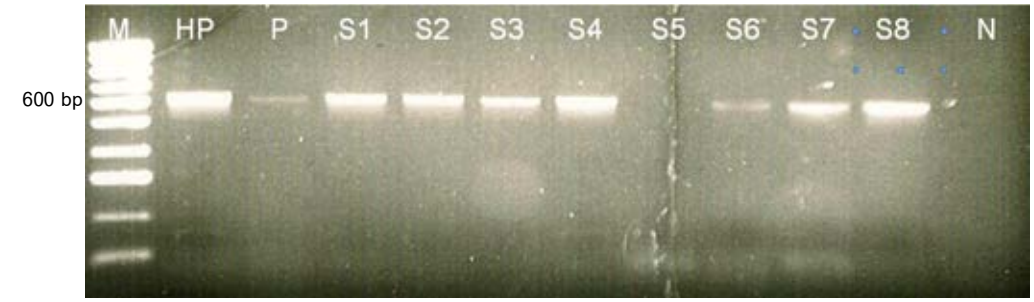


Figure 4: RT-PCR product of ORF5 gene; M: 100 bp DNA ladder, HP: HP-PRRSV positive control, P: type 2 PRRSV positive control (01NP1.2), S1-S8: samples and N: negative control

4.3 Sequences and phylogenetic analysis

4.3.1 Partial NSP2 sequences

According to the total 17 PRRSV positive samples, only 11 samples were sequenced successfully included 12SURIN01, 12TAK01, 12RB01, 12KB01, 12LB01, 12SHB01, 12NN01, 12NP01, 12NP02, 12PJB01 and 12CHTB01, respectively. The 7 samples left included 12RB02, 12RB03, 12RB04, 12NP03, 12NP04, 12SPB01 and 12PJB02 were not successfully sequenced for more than 3 times and were excluded from the study. The partial NSP2 sequences were located between nucleotide positions 2520-2588 of a total 7,512 nucleotides of the ORF1a. The sequences were blasted with the worldwide sequences data provided in NCBI database to affirm as the NSP2 gene sequences of PRRSV. Then, the sequences were aligned with reference isolate sequences and phylogenetic tree of partial NSP2 sequences was constructed.

NSP2 gene of important type 2 PRRSV included type 2 PRRSV prototypes, type 2 PRRSV vaccine isolates, current and the past isolates of Thai PRRSV, HP-PRRSV isolates reported in many countries and the vaccine isolates of HP-PRRSV were combined in the phylogenetic tree of the NSP2 gene. The reference isolates were collected from NCBI database shown in Table 4.

According to the alignment, 10 sequenced samples included 12SURIN01, 12TAK01, 12RB01, 12KB01, 12SHB01, 12NN01, 12NP01, 12NP02, 12PJB01 and 12CHTB01 having 30 amino acids deletion at the position 869 and 921 to 949 of NSP2 as presented in the HP-PRRSV prototypes and Thai HP-PRRSV isolates (Figure 5). Moreover, according to the phylogenetic tree, the 10 studied sequences were closely related to the HP-PRRSV group. Only 12LB01 having full amino acid sequences without deduced characteristic and the sequence was closely related to the endemic Thai PRRSV isolates

reported in 2008 such as 8NP147 and Ingelvac ATP PRRSV vaccine isolate as shown in Figure 4.

Table 4: The reference PRRSV isolates used for partial NSP2 gene comparisons

References isolates	Location	Year	GenBank accession number
BG1P1	Vietnam	2010	HQ538597.1
HN12P5	Vietnam	2010	HQ538598.1
JLPJ1	China	2010	HM232822.1
JXA1	China	2007	EF112445.1
WUH4	China	2011	JQ326271.1
PIADC-PRRS	Philippines	2008	FJ641193.1
HEB1	China	2007	EF112447.1
GXNN12	China	2007	JX046237.1
07QN	Vietnam	2007	FJ394029
07BJ	China	2007	FJ393459.1
JXwn06	China	2009	EF641008.1
GXHCH26-2007	China	2007	JX046226.1
BDPG2	Vietnam	2010	HQ538611.1
Ingelvac ATP	USA	2006	EF532801.1
01CS1/2	Thailand	2010	HM134188.1
8NP148	Thailand	2008	HM134189.1
8NP59	Thailand	2008	HM134187.1
8NP154	Thailand	2008	HM134185.1
08RB1	Thailand	2008	HM134184.1
8NP46	Thailand	2008	HM134191.1
07NP4	Thailand	2007	HM134183.1

78/51	Thailand	2007	HM134186.1
07NP2	Thailand	2007	HM134182.1
8NP147	Thailand	2008	HM134190.1
JIW1	Japan	2000	AB288126.1
Ibaraki3	Japan	1993	AB288113.1
Gu922M	Japan	1992	AB288111.1
HN1	China	2003	AY457635.1
01NP1.2	Thailand	2001	EF153486.1
PL97-1	South Korea	1997	AY585241.1
VR2332	USA	1992	EF536003
Ingelvac PRRS	USA	2001	AF303357.1
Lelystad	The Netherland	2001	M96262
10PL01	Thailand	2010	NA
10PL02	Thailand	2010	NA
10CP01	Thailand	2010	NA
10CP02	Thailand	2010	NA
10CP03	Thailand	2010	NA
10CP04	Thailand	2010	NA
10UT01	Thailand	2010	NA
10UT02	Thailand	2010	NA
10UT03	Thailand	2010	NA
10CS01	Thailand	2010	NA
10CB01	Thailand	2010	NA
10KK01	Thailand	2010	NA
10KK02	Thailand	2010	NA

```
                845        855        865        875        885        895
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332         PVPAPRRKVG SDCGSPVSLG GDVSNSWEDL AVSSPFDLPT PPEPATPSSE LVIVSSPQCI
12CHTB01       -----?RKVR SDCGSPVLMG DNVPNGSE-? TVGGPLNFPT PSELMTPMSE PVLVPASQFV
12KB01         ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12NN01         -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12NP01         ------?KVR SDCGGPVLIG DNVPSGSE-R TVGGPLNFPT PSKLMTPMSE PALVPASQFV
12NP02         -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12PJB01        -----?RKVR SDCGSPVLMG DNVPNDSE-? TVGGPLNFPT PSELMTPMSG PVLMPASQFV
12RB01         ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12SHB01        ------?KVR SDCGSPVLMG NNVPNGSE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA
12SURIN01      ------RKVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMAPMSE PALVPASQFV
12TAK01        ------?KVR SDCGSPVLMG DNVPNGCE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA
12LB01         ------RKIR SDCGSSILLG DNVPNSWEDL TVGGPLDLPA PPEPVTPPRE LAPMPAPQHI

                905        915        925        935        945        955
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332         FRPATPLSEP APIPAPRGTV SRPVTPLSEP IPVPAPRRKF QQVKRLSSAA AIPPYQNEPL
12CHTB01       PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTTLTHQDEPL
12KB01         PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTTLTHQDEPL
12NN01         PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTPTHQDEPL
12NP01         PTLITPLIGS APVPAPRRTV ---------- ---------- ---------- TTLTHQDEPL
12NP02         PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTPTHQDEPL
12PJB01        PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TALTHQDEPL
12RB01         PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTLTHQDEPL
12SHB01        PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------- TTLTHQDEPL
12SURIN01      PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------- TTLTHQDEPL
12TAK01        PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------- TTLTHQDEPL
12LB01         FRPVTPLSEP APVPAPRRTV FRPMTSLSEP ILVSAPRHKF QQVEKANLAT TTLTHQDEPL

                965        975        985        995        1005       1015
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332         DLSASSQTEH EASPPAPPQS GGVPGVEGHE AEETLSEISD MSGNIKPASV SSSSSLSSVR
12CHTB01       DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12KB01         DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12NN01         DLSASS---- ---------- ---------- ---------- ---------- ----------
12NP01         DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12NP02         DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12PJB01        DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12RB01         DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12SHB01        DLSASS---- ---------- ---------- ---------- ---------- ----------
12SURIN01      DLSASSQ?-- ---------- ---------- ---------- ---------- ----------
12TAK01        DLSASSQ--- ---------- ---------- ---------- ---------- ----------
12LB01         DLSASSQT-- ---------- ---------- ---------- ---------- ----------
```

Figure 5: Partial NSP2 gene nucleotide sequences alignment of the current Thai PRRSV isolates. One and 29 deduced amino acids were labeled in boxes. These 30 amino acids deletion were genetic characteristics of HP-PRRSV representatives.

The phylogenetic tree was divided into 3 groups including HP-PRRSV related, type 2 PRRSV vaccine-like and local Thai type 2 PRRSV. Most of the current Thai PRRSV sequences were grouped into the HP-PRRSV related group except 12LB01 was categorized into the local Thai type 2 PRRSV group (Figure 6).

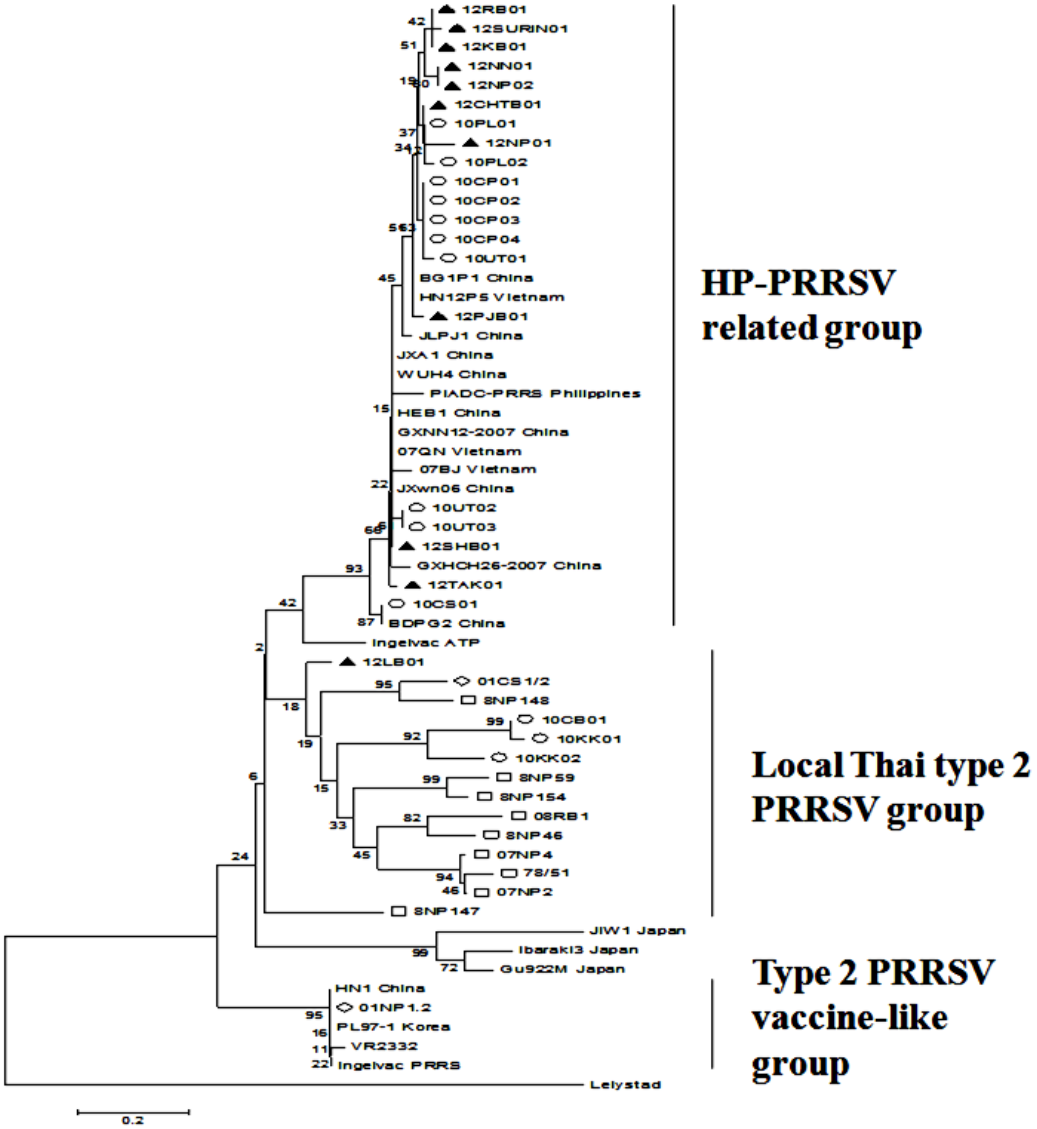


Figure 6: Phylogenetic tree based on partial NSP2 gene sequences. The markers were labeled in different shape depending on timing of the sequences including triangle for current Thai PRRSV isolates obtained in this study, circle for Thai PRRSV isolates collected

in 2010, square for Thai type 2 PRRSV isolates reported in 2007-2008 and diamond for Thai type 2 PRRSV isolates reported in 2001.

4.3.2 ORF5 sequences

Eleven samples of the ORF5 gene of current Thai PRRSV were sequenced successfully. The sequences were located between nucleotide positions 57-483 of total 600 nucleotides of ORF5. The phylogenetic tree was constructed, 11 sequences of current studied Thai type 2 PRRSV were combined with type 2 PRRSV prototypes, type 2 PRRSV vaccine isolates, current and previous isolates of Thai PRRSV. Moreover, HP-PRRSV isolates and the vaccine isolates of HP-PRRSV were included in the phylogenetic tree as well. The reference isolates for ORF5 phylogenetic tree construction were collected from NCBI database as shown in Table 5.

Table 5: Reference PRRSV isolates used for ORF5 gene comparisons

References isolates	Location	Year	GenBank accession number
O7HEN	China	2007	FJ393457.1
07NM	China	2007	FJ393456
Jiangxi-3	China	2007	EU200961
HEB1	China	2007	EF112447.1
Hen-JY	China	2006	AB359236.1
HUB1	China	2006	EF075945.1
Jsyx	China	2006	EU939312.1
JXA1	China	2007	EF112445
HUB2	China	2007	EF112446.1
BJ	China	2007	EU825723.1
HuN	China	2007	EF517962.1
XH-GD	China	2007	EU624117.1

07QN	Vietnam	2007	FJ394029
HLM-09	China	2009	HQ843179.1
UDT0810US 5/28-160	Thailand	2010	JN255819
HB-2(sh)/2002	China	2002	AY262352
P129	USA	2002	AF494042.1
Prime Pac	USA	1999	AF066384
SP	Singapore	2000	AF184212.1
Ingelvac ATP	USA	2006	EF532801.1
CH-1a	China	2001	AY032626
07NP4	Thailand	2007	FJ908077
08NP147	Thailand	2008	FJ90078
08RB51	Thailand	2008	FJ90080
SCP1210-U.S.-7-79-1	Thailand	2010	JN255837
02PB1	Thailand	2002	AY297116
08NP148	Thailand	2008	FJ908079
01NP1	Thailand	2001	AY297112
02KK1	Thailand	2002	AY297115
02SB3	Thailand	2002	AY297118
01UD6	Thailand	2001	AY297113
02CB13	Thailand	2002	AY297114
00CS1	Thailand	2000	AY297111
08RB154	Thailand	2008	FY908081
08RB160	Thailand	2008	FJ90802
LMY	South Korea	2006	DQ473474
SD23983	USA	2012	JX258843.1
ISU-P	USA	2008	EF532816.1

HN1	China	2003	AY457635.1
VR2332	USA	1992	EF536003
PL97-1	South Korea	1997	AY585241.1
CC-1	China	2006	EF153486.1
01NP1.2	Thailand	2001	DQ056373
BJ-4	China	2001	AF331831
RespPRRS MLV	USA	2005	AF066183
Lelystad	The Netherland	2000	M96262
FDT10US-2-1	Thailand	2010	JN255834
UDT1210US-25-1	Thailand	2012	JN255833
JXA1-P160	China	2009	KC422731.1
NB/04	China	2004	FJ536165
HB1-(sh)/2002	China	2002	AY150312
SHB	China	2005	EU864232.1
UD1210US/61-E03	Thailand	2010	JN255827

Similar to the phylogenetic tree of partial NSP2 gene, ORF5 gene phylogenetic tree was divided into 3 groups including HP-PRRSV related group, type 2 PRRSV vaccine-like group and local Thai type 2 PRRSV group. Likewise, most of the current Thai PRRSV sequences were categorized into the HP-PRRSV related group except 12LB01 which was grouped into the local Thai type 2 PRRSV group (Figure 7).

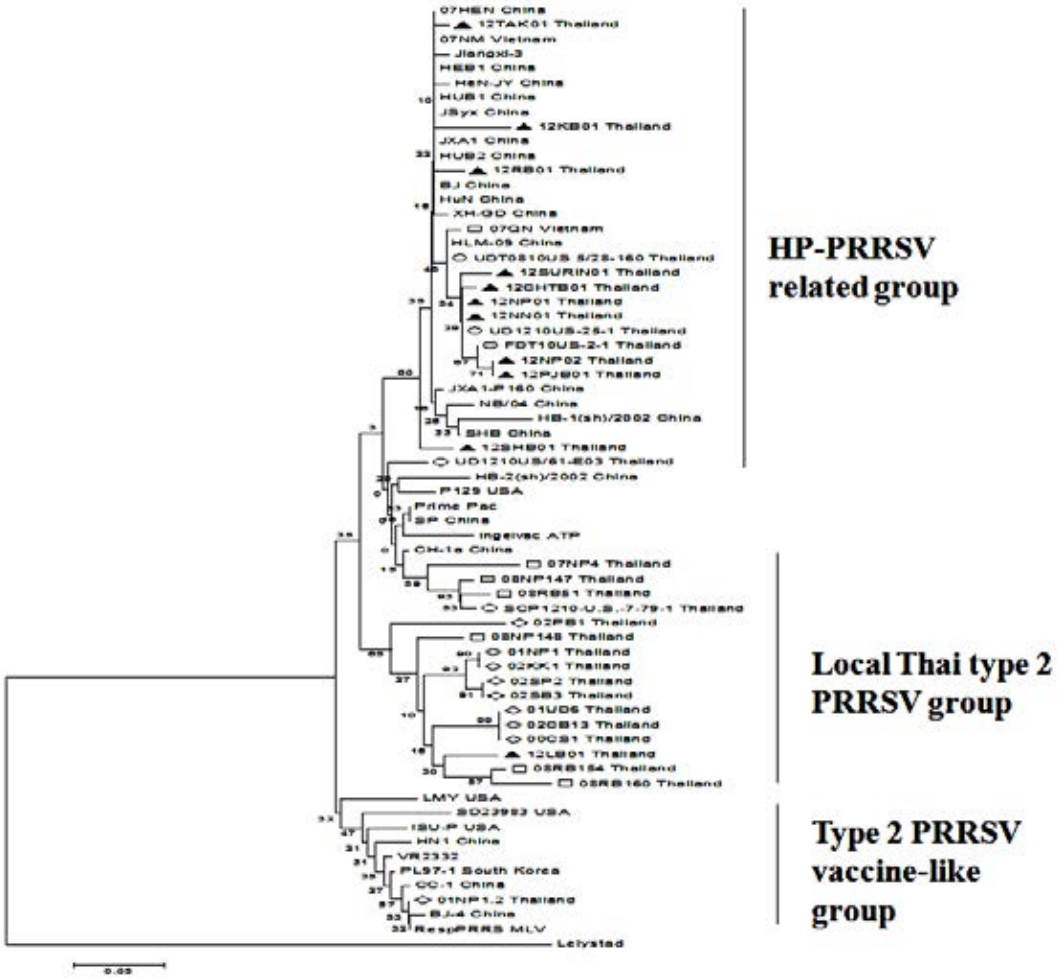


Figure 7: Phylogenetic tree based on ORF5 sequences. The markers were labeled in different shapes depending on timing of the sequences including triangle for the current Thai PRRSV isolates obtained in this study, circle for Thai PRRSV isolates collected between 2010 and 2011, square for Thai type 2 PRRSV isolates collected between 2007 and 2008 and diamond for Thai type 2 PRRSV isolates reported between 2000 and 2002.

4.4 Amino acid sequences homology analysis

4.4.1 partial NSP2 gene amino acid sequences homology

Similar to the phylogenetic tree of partial NSP2 gene, most of the partial NSP2 sequences of the current Thai PRRSV were categorized into the HP-PRRSV related group but 1 sequence sample, 12LB01, was closed to the local Thai PRRSV isolates. Consequently, the amino acid identity values among the current Thai PRRSV sequences were varied between 47.4 and 100 percent. Markedly, most of the current Thai PRRSV isolates such as 12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12RB01, 12SHB01, 12SURIN01 and 12TAK01 were related to the previous Thai HP-PRRSV isolates such as 10PL01 as well as the other HP-PRRSV in other countries such as HEB1 (China) and 07QN (Vietnam). On the other hand, 12LB01 was closely related to the local Thai PRRSV isolates which were collected in 2008 such as 8NP154 and shared similarity to the other type 2 PRRSV including VR2332 and the type 2 PRRSV vaccine isolates such as Ingelvac ATP and Ingelvac PRRS while the others were shared similarity to the HP-PRRSV vaccine isolate, JXA1. The partial NSP2 gene amino acid sequences homology are summarized in table 6.

Perspectively, amino acid identity values of the partial NSP2 gene between the current studied Thai PRRSV sequences and the other PRRSV isolates were varied due to the current Thai PRRSV were composed of both local Thai type 2 PRRSV and HP-PRRSV related virus. Since 10PL01 is the representative of Thai PRRSV isolates which emerged in 2010, the identity values of the current Thai PRRSV sequences and the representative of Thai PRRSV collected in 2010 (10PL01) were varied between 49.1-98.8%. On the contrary, 8NP154 is the representative of the local Thai type 2 PRRSV collected in 2008, the identity values between the current Thai PRRSV sequences and 8NP154 were between 39.4-64.4%. Similarly, the identity values of the current Thai PRRSV sequences and 01NP1.2, the Thai type 2 PRRSV prototype, varied about 36.4-64.4%. Comparing to type 1 and type 2 PRRSV prototypes, Lelystad and VR2332, identity values among the current studied Thai PRRSV

and the prototypes are 6.7-13.4% and 35.5-63.5%, respectively. However, the current Thai PRRSV sequences shared more similarity to the HEB1 and 07QN sequences since HEB1 is the Chinese HP-PRRSV prototype and 07QN is Vietnamese HP-PRRSV representative. The identity values varied between 49.1-94.3% and 50.8-96.5% for HEB1 and 07QN, respectively. Moreover, the current Thai PRRSV sequences showed varied identity values between commercial type 2 PRRSV isolates, Ingelvac ATP and Ingelvac PRRS, 50.8-64.4 and 36.4-64.4%, respectively. Interestingly, for JXA1, a chinese HP-PRRSV vaccine, the identity values were higher than the type 2 PRRSV vaccine since the similarity values between the current Thai PRRSV sequences and JXA1 varied between 50-86.3%. The percentage identity of partial NSP2 gene sequences as compared between the current Thai PRRSV sequences and reference isolates are shown in Table 6 and amino acid sequences alignment comparisons between the PRRSV are shown in Figure 9.

Table 6: Sequences identical analysis (%) of partial NSP2 amino acid sequences among the current Thai PRRSV isolates and with the represent PRRSV isolates of the previous Thai isolates (01NP1.2, 10PL01 and 8NP154), type 1 PRRSV prototype (Lelystad virus), type 2 PRRSV prototype (VR2332), type 2 PRRSV vaccine isolates (Ingelvac ATP, Ingelvac PRRS and JXA-1) and HP-PRRSV (HEB1 and 07QN).

Isolates	10PL01	8NP154	01NP1.2	Lelystad	VR2332	Ingelvac_ATP	Ingelvac_PRRS	JXA1	HEB1	07QN
12CHTB01	0.988	0.406	0.398	0.084	0.389	0.525	0.398	0.931	0.92	0.92
12KB01	0.977	0.423	0.381	0.092	0.372	0.525	0.381	0.92	0.909	0.909
12LB01	0.491	0.635	0.644	0.134	0.635	0.771	0.644	0.5	0.491	0.508
12NN01	0.943	0.406	0.389	0.084	0.381	0.508	0.389	0.909	0.897	0.92
12NP01	0.92	0.406	0.364	0.067	0.355	0.508	0.364	0.863	0.852	0.852
12NP02	0.943	0.406	0.389	0.084	0.381	0.508	0.389	0.909	0.897	0.92
12PJB01	0.931	0.406	0.381	0.084	0.372	0.516	0.381	0.897	0.886	0.909
12RB01	0.977	0.423	0.381	0.092	0.372	0.525	0.381	0.92	0.909	0.909
12SHB01	0.897	0.398	0.398	0.084	0.389	0.533	0.398	0.954	0.943	0.965
12SURIN01	0.965	0.415	0.372	0.092	0.364	0.516	0.372	0.909	0.897	0.897
12TAK01	0.897	0.398	0.398	0.084	0.389	0.542	0.398	0.954	0.943	0.965

4.4.2 GP5 amino acid sequences homology

Similar to partial NSP2 gene sequences, amino acid identity values of GP5 among 11 sequences of the current Thai PRRSV were 57-100% as compared to each other. Coincidently, 12LB01 shared the lowest similarity among the current Thai PRRSV in agreement with the result of ORF5 phylogenetic tree. The 12LB01 is the only one current Thai PRRSV isolate related to the local Thai type 2 PRRSV. The identity values of 12LB01 with the other 10 current Thai PRRSV sequences collected in 2012 were between 57.0-86.6%. The current Thai type 2 GP5 amino acid sequences shows high similarity to the previous representative HP-PRRSV collected in 2010 (UDT0810US 5/28-16). The identity values were about 64.5-97.8%. However, compared with the local Thai PRRSV collected in 2008 and 2001, the current Thai PRRSV amino acid sequences showed similarity between 58.0-91.5% and 57.5-88.7%, respectively, since most of the current Thai PRRSV sequences were categorized in the HP-PRRSV related group and more closely related to the isolates collected in 2010 than the isolates collected in 2008 and 2001. In addition, similar to partial NSP2 sequences, the current Thai PRRSV ORF5 sequences showed limited similarity to the Lelystad virus. The identity matrix between the current Thai PRRSV and the Lelystad virus were only 41.2-48.8%. On the other hand, type 2 PRRSV prototype, VR2332, shared more similarity to the current Thai PRRSV sequences since the identity values were 58.5-88.7%. Moreover, the HP-PRRSV prototype, HEB1, and the Vietnamese HP-PRRSV representative, 07QN, showed closer relation to the current Thai PRRSV (62.0-96.0% and 64.5-97.8% similarity, respectively). By the way, the most important aspect of the GP5 amino acid similarity is to compare with the PRRSV vaccine isolates. Limitedly, GP5 amino acid sequences of the current Thai PRRSV shared similarity to the commercial type 2 PRRSV vaccine isolates, Ingelvac ATP, RespPRRS MLV and Prime Pac, between 60.5-88.0%, 57.5-88.7% and 61.0-92.2%, respectively. By the way, the highest similarity between the current Thai PRRSV sequences and the type 2 PRRSV vaccine isolates was with JXA1 (65.6-97.8%). The percentage identity between GP5 amino acid sequences and the reference

PRRSV isolates are shown in Table 7 and amino acid sequences alignment comparisons between PRRSV isolates are shown in Figure 10.

The amino acids comparisons among the current Thai PRRSV and the reference isolate, VR2332, found amino acid substitution in many regions (Figure 7). In signal peptide domain, the obtained sequences began at the amino acid position 19 of GP5, 6 amino acids mutation were found. In ectodomain, 15 positions of amino acids were transformed. In this region, 2 asparagine (N) were changed in the position 44 and 58 which may affect to the glycosylation sites of the GP5. Notably, new asparagines were found separately in 3 positions in this region. In transmembrane region, 1, 2 and 7 amino acids transformation were found in transmembrane protein 1, transmembrane protein 2 and transmembrane protein 3, respectively. Finally, endodomain of the GP5 were sequenced from amino acid position 129 to 161. According to the incomplete sequences, 7 amino acid mutation points were found in this region. The amino acid mutation points and the functional domain of the GP5 were presented in the sequences as shown in Figure 8.

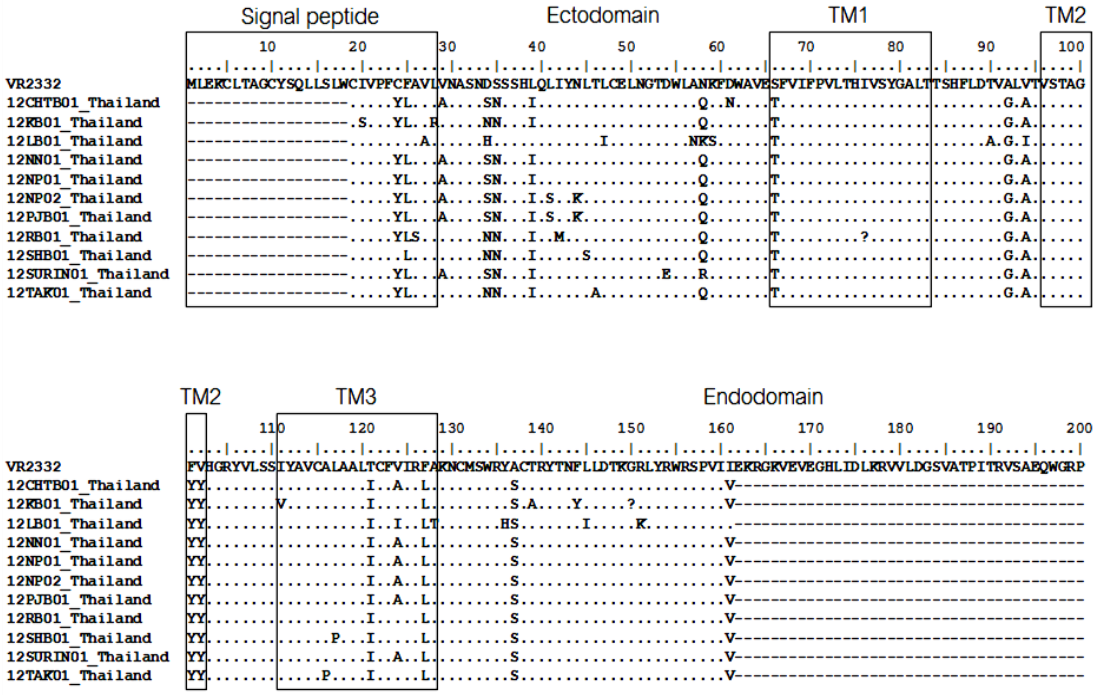


Figure 8: Amino acid substitution in GP5 sequences. The functional domains are reported similar to the previous study (Zhou et al., 2011) and indicated in boxes.

Table 7: Sequences identical analysis (%) of nucleotide and amino acids identity of the ORF5 gene among the current Thai PRRSV isolates and with the represent PRRSV isolates of the previous Thai isolates (01NP1.2, 08RB154 and UDT0810US 5/28-16), type 1 PRRSV prototype (Lelystad virus), type 2 PRRSV prototype (VR2332), type 2 PRRSV vaccine isolates (Ingelvac ATP, RespPRRS, Prime Pac and JXA-1) and HP-PRRSV (HEB1 and 07QN).

Isolates	UDT0810US_5/28-160	08RB154	01NP1.2	Lelystad	VR2332	Ingelvac_ATP	RespPRRS_MLV	Prime_Pac	JXA1	HEB1	07QN
12CHTB01	0.978	0.859	0.873	0.417	0.873	0.615	0.873	0.908	0.964	0.685	0.964
12KB01	0.645	0.58	0.575	0.488	0.585	0.855	0.575	0.61	0.655	0.935	0.645
12LB01	0.866	0.915	0.83	0.412	0.838	0.605	0.83	0.866	0.873	0.62	0.859
12NN01	0.992	0.873	0.887	0.417	0.887	0.625	0.887	0.922	0.978	0.695	0.978
12NP01	0.992	0.873	0.887	0.417	0.887	0.625	0.887	0.922	0.978	0.695	0.978
12NP02	0.978	0.859	0.873	0.412	0.873	0.615	0.873	0.908	0.964	0.685	0.964
12PJB01	0.978	0.859	0.873	0.412	0.873	0.615	0.873	0.908	0.964	0.685	0.964
12RB01	0.665	0.59	0.6	0.483	0.61	0.855	0.6	0.63	0.675	0.95	0.665
12SHB01	0.668	0.608	0.613	0.483	0.623	0.88	0.613	0.643	0.678	0.955	0.668
12SURIN01	0.978	0.88	0.88	0.412	0.88	0.62	0.88	0.915	0.964	0.685	0.964
12TAK01	0.676	0.601	0.606	0.485	0.616	0.865	0.606	0.636	0.686	0.96	0.676

```
                845        855        865        875        885        895
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     PVPAPRRKVG SDCGSPVSLG GDVSNSWEDL AVSSPFDLPT PPEPATPSSE LVIVSSPQCI
12CHTB01   -----?RKVR SDCGSPVLMG DNVPNGSE-? TVGGPLNFPT PSELMTPMSE PVLVPASQFV
12KB01     ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12LB01     ------RKIR SDCGSSILLG DNVPNSWEDL TVGGPLDLPA PPEPVTPPRE LAPMPAPQHI
12NN01     -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12NP01     ------?KVR SDCGGPVLIG DNVPSGSE-R TVGGPLNFPT PSKLMTPMSE PALVPASQFV
12NP02     -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12PJB01    -----?RKVR SDCGSPVLMG DNVPNDSE-? TVGGPLNFPT PSELMTPMSG PVLMPASQFV
12RB01     ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12SHB01    ------?KVR SDCGSPVLMG NNVPNGSE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA
12SURIN01  ------RKVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMAPMSE PALVPASQFV
12TAK01    ------?KVR SDCGSPVLMG DNVPNGCE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA

                905        915        925        935        945        955
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     FRPATPLSEP APIPAPRGTV SRPVTPLSEP IPVPAPRRKF QQVKRLSSAA AIPPYQNEPL
12CHTB01   PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12KB01     PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12LB01     FRPVTPLSEP APVPAPRRTV FRPMTSLSEP ILVSAPRHKF QQVEKANLAT TTLTHQDEPL
12NN01     PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTPTHQDEPL
12NP01     PTLITPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12NP02     PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTPTHQDEPL
12PJB01    PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TALTHQDEPL
12RB01     PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12SHB01    PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12SURIN01  PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12TAK01    PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL

                965        975        985        995       1005
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
1015
VR2332     DLSASSQTEH EASPPAPPQS GGVPGVEGHE AEETLSEISD MSGNIKPASV SSSSSLSSVR
12CHTB01   DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12KB01     DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12LB01     DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12NN01     DLSASS---- ---------- ---------- ---------- ---------- ----------
12NP01     DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12NP02     DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12PJB01    DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12RB01     DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12SHB01    DLSASS---- ---------- ---------- ---------- ---------- ----------
12SURIN01  DLSASSQ?-- ---------- ---------- ---------- ---------- ----------
12TAK01    DLSASSQ--- ---------- ---------- ---------- ---------- ----------
```

Figure 9: The amino acid sequences alignment of the current Thai PRRSV based on partial NSP2 gene.

```
                5         15         25         35         45         55
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     MLEKCLTAGC YSQLLSLWCI VPFCFAVLVN ASNDSSSHLQ LIYNLTLCEL NGTDWLANKF
12CHTB01_T ---------- --------?I PFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12KB01_Tha ---------- --------CS VPFYLA?RVN A??NNSSHIQ LIYNL?LCEL NGTDWLAQKF
12LB01_Tha ---------- --------CI VPFWFAALVN ASNH??SHLQ LIYNLTICEL NGTDWLNKSF
12NN01_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12NP01_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12NP02_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ SIYKLTLCEL NGTDWLAQKF
12PJB01_Th ---------- --------CI VPFYLAVLAN ASNSNSSHIQ SIYKLTLCEL NGTDWLAQKF
12RB01_Tha ---------- --------CI VPFYLSVLVN ASNNNSSHIQ LM?NLTLCEL NGTDWLAQKF
12SHB01_Th ---------- --------CI VPFC?AVLVN ASNNNSSHIQ LIYNS?LCEL NGTDWLAQKF
12SURIN01_ ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTEWLARKF
12TAK01_Th ---------- --------CI VPFYLAVLVN ASNNNSSHIQ LIYNLALCEL NGTDWLAQKF

                65         75         85         95        105        115
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     DWAVESFVIF PVLTHIVSYG ALTTSHFLDT VALVTVSTAG FVHGRYVLSS IYAVCALAAL
12CHTB01_T NWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12KB01_Tha DWAVETFVIF PVLTHIVSYG ALT?SHFLDT VGLATVSTAG YYHGRYVLSS VYAVCALAAL
12LB01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDA VGLITVSTAG YYHGRYVLSS IYAVCALAAL
12NN01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12NP01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12NP02_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12PJB01_Th DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12RB01_Tha DWAV??FVIF PVLTH?VSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCAL?AL
12SHB01_Th DWAVETFVIF PVLTHIVSYG AL?TSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCAP?AL
12SURIN01_ DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12TAK01_Th DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCPLAAL

               125        135        145        155        165        175
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     TCFVIRFAKN CMSWRYACTR YTNFLLDTKG RLYRWRSPVI IEKRGKVEVE GHLIDLKRVV
12CHTB01_T ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12KB01_Tha ICFVIRLAKN CMSWRYSCAR YTNYLLDTK? RLYRWRSPVI V--------- ----------
12LB01_Tha ICFIIRLTKN CMSWRHSCTR YTNFILDTKG KLYRWRSPVI ?--------- ----------
12NN01_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12NP01_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12NP02_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12PJB01_Th ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12RB01_Tha ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI V--------- ----------
12SHB01_Th ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12SURIN01_ ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?--------- ----------
12TAK01_Th ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRS??I V--------- ----------
```

Figure 10: The amino acid sequences alignment of the current Thai PRRSV based on ORF5 gene.

Chapter V

Discussion

5.1 Samples collection and classification

Porcine reproductive and respiratory syndrome virus is one of the major problematic swine diseases. Porcine respiratory disease complex (PRDC) and reproductive failure induced by PRRSV infection have a negative influence to swine health. The virus was first identified in Thailand since 1996 (Damrongwatanapokin et al., 1996). Both type 1 and type 2 PRRSV have been presented in Thailand since swine breeders and its related products such as semen were frequently imported from the European and North American countries (Thanawongnuwech et al., 2004). Consequently, due to NSP2 and ORF5 phylogenetic analysis, PRRSV in Thailand was proven as one separate group differed from the original isolates (Kedkovid et al., 2011; Tun et al., 2011). However, after the outbreak of the HP-PRRSV in Thailand since 2010, Thai PRRSV isolates were closely related to the HP-PRRSV isolates collected in original countries such as China and Vietnam (Nuntawan Na Ayudhya et al., 2010; Nilubol et al., 2012). Therefore, genetic characteristics of the current Thai PRRSV is of interest since the genetic characterization of the virus is variable due to the novel PRRSV strains were introduced into the country.

To gain insight into the genetic characteristics as well as molecular epidemiology of the current Thai PRRSV, partial NSP2 and ORF5 genes of selected 11 PRRSV samples collected in 4 regions of Thailand since February until October 2012 were analyzed. All studied samples were obtained from the clinically affected swine farms to demonstrate the major problematic isolates not the ineffective ones. According to the submitted samples, the PRRSV specific multiplex RT-PCR was used for PRRSV genotype diagnoses. The result indicated that up to 44% of the swine farms with clinical problems were affected by PRRSV mostly related to type 2 PRRSV. Suffered pigs were infected by type 2 PRRSV even PRRSV modified life virus vaccines were used in many studied farms. This scenario is common in

the intensive swine herds which were infected by other PRRSV strains different to the immunized one. Without the presence of type 1 PRRSV in the clinical cases collected in this study, the type 1 virus used to be the major PRRSV strains up to 66.42% in Thailand during the period of 2000 to 2002 (Thanawongnuwech et al., 2004) and became a group of local Thai type 1 PRRSV showing genetic similarity to each other (Tun et al., 2011). Moreover, according to the study during 2010 to 2011, Thai type 1 PRRSV were identified in many PRRSV endemic swine farms without showing clinical diseases (Nilubol et al., 2012). The previous report conformed to this current study in 2012 that Thai type 1 PRRSV might exist in Thai swine herds but caused subclinical problems especially in the situation that many sow herds were completely immunized by PRRSV vaccines. Different to type 2 PRRSV, type 1 PRRSV exhibits significantly low virulence. Problems due to type 1 PRRSV infection might subclinically appear when HP-PRRSV outbreaks. Since the novel type 2 PRRSV, HP-PRRSV, outbreaks in Thailand in 2010, most of the problems due to PRRSV infection in Thailand were caused by type 2 PRRSV infection. This condition resulting in all PRRS clinical cases collected in this study were identified as type 2 PRRSV in agreement with the phylogenetic analysis.

5.2 Sequences and phylogenetic analysis

Seventeen viral cDNA samples were successfully amplified for both partial NSP2 and ORF5 genes but only 11 samples were successfully sequenced. The eleven sequences were used for phylogenetic tree construction and analysed for amino acids identity as well as amino acids substitution.

The studied sequences of the current Thai PRRSV were collected from 11 farms located in 11 provinces in 4 regions of Thailand (Figure 10). The farm location of each studied Thai PRRSV sequence was analyzed comparing to the genetic diversity. The result based on partial NSP2 sequence of 2 pairs including 12NN01 from Nakhonnayok/12NP01 from Nakhonpathom and 12NP02 from Nakhonpathom/12PJB01 from Prachinburi were

highly homologous, 86.3 and 92.0% similarity, respectively, whereas the ORF5 sequences of both pairs were completely homologous (100% similarity). On the other hand, 2 pairs of partial NSP2 sequence comparison, 12KB01 from Kanchanaburi/12RB01 from Ratchaburi and 12NP02 from Nakhonpathom/12NN01 from Nakhonnayok showed 100% similarity while the ORF5 sequences showed 89.0 and 98.5% similarity, respectively. The results indicated that percent similarity compared by only one sequenced gene was not accurated for comparing the similarity of the virus. More than one gene or whole genome sequence is an ideal for sequence identity analysis of PRRSV since the genetic diversity of the virus is very high.

5.2.1 partial NSP2 sequences analysis

The 368 bp of partial NSP2 gene sequences were amplified from the studied 11 samples. The sequences are located between nucleotide positions 2,550 to 2,888 of ORF1a compared to type 2 PRRSV prototype (VR2332). Approximate 123 amino acids which located in positions 840 to 963 of the polyprotein ORF1a. The amino acids sequences were covered the 30 amino acids deletion which were the major characteristics of HP-PRRSV isolates at the position 863 and 915-943 of ORF1a polyprotein or the position 481 and 533-561 of NSP2. This region of NSP2 gene is the most important part of this NSP2 study as well as the other studies of a novel PRRSV isolate because this region is the only one genetic characteristic of the Chinese HP-PRRSV. Moreover, not only be the major characteristic of the Chinese HP-PRRSV isolates, this region in NSP2 gene representing high variable region and would be useful for genetic variation analysis as well.

For PRRSV characterization, the obtained 11 sequences were aligned with NSP2 sequences of the reference PRRSV isolates including the prototype of type 2 PRRSV, VR2332, and the representative of HP-PRRSV, HEB1. The alignment revealed that 10 sequences including 12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12SHB01, 12RB01, 12SURIN01 and 12TAK01 contained 30 deduced amino acids in the same position as the HEB1. The results indicated that these 10 partial NSP2 sequences were a member of

the HP-PRRSV related group. On the other hand, 12LB01 contained no deduced amino acids in this particular region and was characterized as an endemic Thai type 2 PRRSV.

Based on phylogenetic tree analysis, the current Thai PRRSV sequences were separated into 2 different groups of type 2 PRRSV, HP-PRRSV related group and local Thai type 2 PRRSV group. The phylogenetic tree showed the 10 sequences (12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12SHB01, 12RB01, 12SURIN01 and 12TAK01) were closely related to the HP-PRRSV isolates collected in other previous HP-PRRSV outbreak countries including China, Vietnam and the Philippines as well. Moreover, these 10 sequences were also closely related to the Thai HP-PRRSV sequences obtained from the previous study in 2010 (Nuntawan Na Ayudhya et al., 2011). This indicated that the studied Thai PRRSV might derive from the HP-PRRSV isolates introduced in 2010 or the viruses might be newly introduced from other neighbouring countries such as Laos, Cambodia, Myanmar and Vietnam. The current Thai HP-PRRSV spread to different parts of Thailand due to multiple introductions after the first outbreak in 2010 since these 10 studied sequences showed some nucleotide differences and were located in different position in the phylogenetic tree. On the contrary, 12LB01 was closely related to the local Thai type 2 PRRSV isolates obtained in 2010. Moreover, 12LB01 was grouped into the same group as the other local Thai type 2 PRRSV isolates collected in 2007-2008 as well as 01NP1 which was demonstrated in 2001.

Partial NSP2 gene phylogenetic tree completely showed the scenario of the Thai type 2 PRRSV in Thailand in the past and the present. Type 2 PRRSV in Thailand might originate from the vaccine virus as well as its progeny due to the primary Thai type 2 PRRSV isolates, 01NP1.2 and 01NP1, were grouped in the type 2 PRRSV vaccine-like and local Thai type 2 PRRSV group, respectively. According to the study in 2007-2008, more NSP2 gene of Thai PRRSV were sequenced. The type 2 Thai PRRSV isolates collected between 2007 and 2008 showed some deduced amino acids in NSP2 gene (Kedkovid et al., 2011). However, the sequences were grouped into the local Thai type 2 PRRSV, closely related to

the previous isolate, 01NP1. Interestingly, after the introduction of HP-PRRSV into Thailand in 2010, the newly introduced PRRSV had made higher variation of the nucleotides in NSP2 gene of Thai type 2 PRRSV. Type 2 PRRSV isolates collected in 2010 consisted of both local Thai type 2 PRRSV and HP-PRRSV related group. The HP-PRRSV collected in Thailand at that time revealed novel introductions of HP-PRRSV in Thailand as well as existence of local Thai type 2 PRRSV. Currently, after 2 years of the first HP-PRRSV outbreak in Thailand, Thai type 2 PRRSV sequences still belonged to both 2 groups as presented in 2010. This indicated that Thai type 2 PRRSV might evolve into 2 major groups, local type 2 and the HP-PRRSV related group in the next few years since both isolates still dominating and persisting in Thai swine herds.

5.2.2 ORF5 sequences analysis

Glycoprotein 5 or GP5 is the primary envelope protein of PRRSV encoded by ORF5 gene, a 603 nucleotides gene locating in the position 13,618 to 14,220 in VR2332, the GP5 contains 200 amino acids in length. The N-terminal ectodomain on GP5 functions as a major neutralization epitope and the study of genetic diversity of ORF5 of PRRSV may plays an important role in the vaccine efficacy prediction as well as development of a novel subunit vaccine. In addition, since the GP5 is involved in receptor recognition of PRRSV, ORF5 is one of the most variable structural protein genes of PRRSV due to the influence of immune evasion strategy. Therefore, ORF5 is the most important gene for any genetic variation analysis of PRRSV.

Eleven ORF5 sequences of the studied Thai PRRSV and other PRRSV isolates including the previous Thai PRRSV sequences, HP-PRRSV isolates, PRRSV prototypes and reference isolates were used for the phylogenetic tree construction. The ORF5 phylogenetic tree was divided into 3 groups; HP-PRRSV related, local Thai type 2 PRRSV and type 2 PRRSV vaccine-like group. Concordantly, as the NSP2 based phylogenetic tree, 10 HP-PRRSV sequences (12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12SHB01, 12RB01, 12SURIN01 and 12TAK01) obtained in this study were located into the HP-PRRSV

related group and only 12LB01 was grouped into the local Thai type 2 PRRSV group. Ten HP-PRRSV sequences were closely related to the Thai HP-PRRSV which were collected in 2010 (UDT0810US 5/28-160, UDT1210US-25-1, FDT10US-2-1 and UD1210US/61-E03) similar to the other HP-PRRSV representative such as HEN1, JXA1 and 07QN. On the other side, 12LB01 was closely related to the 2010 Thai type 2 PRRSV isolates, SCP1210-U.S.-7-79-1, as well as the other Thai isolates which were collected between 2000-2002 and 2007-2008. Similar to partial NSP2 gene phylogenetic tree, the current Thai HP-PRRSV might derive from the HP-PRRSV introduced from neighbouring countries. Ten Thai HP-PRRSV sequences occurred by novel introductions of HP-PRRSV into the country. Interestingly, 12LB01 evolved from one of the local Thai type 2 PRRSV.

Similarly to NSP2, the ORF5 phylogenetic tree also represented the current scenario of the Thai type 2 PRRSV in the country. One of the earliest Thai type 2 isolate, 01NP1.2, showed closely genetic relationship with VR2332 and the other prototype and representatives of type 2 PRRSV vaccine-like isolates from many countries such as VR2332 and RespPRRS MLV from USA, CC-1 and BJ-4 collected in China and PL-97 from South Korea. Moreover, the primary Thai type 2 isolates collected in 200-202 including 00CS1, 01NP, 01UD6, 02CB13, 02KK1, 02PB1, 02SB3 and 02SP2, each isolates were categorized into the same group different to the other type 2 PRRSV isolates known as the local Thai type 2 PRRSV group. According to Thai type 2 isolated between 2007 and 2008, the type 2 PRRSV in Thailand evolved from the virus in the past and were grouped into the same group since the local Thai type 2 PRRSV, completely separated from the other isolates from other countries. However, after the HP-PRRSV outbreak in Thailand in 2010, the viruses isolated in Thailand were divided into 2 groups as the major emerging HP-PRRSV related group and the minor re-emerging local Thai type 2 PRRSV group. The present study in 2012 demonstrated that Thai type 2 PRRSV were still comprised of 2 groups.

5.3 Current Thai PRRSV distribution and correlation

Since the first PRRSV demonstrated in Thailand in 1989, the virus spread and continued to be one of the major important diseases in most pig farms. Many farms were affected as endemic PRRS and some farms were affected as newly emerged PRRS outbreaks. Type 2 PRRSV circulated and persisted in the country for many years. The major introduction of PRRSV into Thailand might be an importation of carrier breeders without clinical signs or by semen importation. Similarly, according to the previous report, PRRSV distribution between farms often occurs by infected pig introduction especially replacement gilts from a PRRSV positive to the negative farm (56%). Moreover, contamination in trucks and PRRSV positive semen also play an important role in PRRSV spreading for up to 20 and 3%, respectively. In high pig density areas, PRRSV distribution may be caused by vectors such as insects which were reported as PRRSV from positive farm distributed to the others within the area of 500 m distance (Le Potier et al., 1997). Specific PRRSV sequencing can be applied to the disease investigation in some cases with geographical proximity. Unfortunately, by advancement of modern transportation accompanied with limitation of data recording of some farms in Thailand, the geographical proximity of Thai PRRSV and its transmission route throughout the country were not completely elucidated.

In addition, according to the studies between 2007 and 2008 (Tun et al., 2011), the genetic analysis of both NSP2 and ORF5 genes of Thai PRRSV collected in this period revealed that PRRSV in Thailand were rather homology grouping depending on the origin of the isolates introduced since there was limited pig transportation among Thailand and neighbouring countries at that time. Moreover, since 1996, PRRSV positive pigs were not able to import into the country due to the legislation of the Department of livestock development of Thailand. Consequently, without new exotic PRRSV strain introduction, genetic diversity of local Thai PRRSV was unique and not variable compared to the others at that time. In 2010, a previous study suggested that the HP-PRRSV was firstly introduced into Thailand via transboundary transmission. Illegal transportation of infected pigs or related

products might play an important role in the viral introduction due to the early case was occurred in the Northern part of Thailand at the border (Nuntawan Na Ayudhya et al., 2012). Similarly, other research assumed that the first HP-PRRSV introduction transboundary introduced by an illegal transportation of dead pigs from Lao PDR to the illegal slaughter house in Thailand since one of the early HP-PRRS cases was reported in Nongkhai which is the border province located near Vientiane, the capital city of Lao PDR (Nilubol et al., 2012). After the first outbreak of HP-PRRSV in Thailand, the virus spread into many pig farms especially in the major pig production provinces such as Nakhonpathom and Ratchaburi. The HP-PRRS cases were reported in many swine herds throughout the country. Both sows and growing pigs were affected with the HP-PRRSV as well as suffered from the complicated infectious diseases for many years since the first outbreak was reported.

The samples collected in this study were the representatives of the PRRSV clinical cases which were affecting pigs in the studied period. Due to the virus were spread and distributed into almost all areas in the country, severity of the diseases were relatively lessen as naturally adaptation of the virus. Thus, after 2 years since the first introduction, the severity of the disease was relatively lower than the first period of the outbreaks. According to the data of the 10 HP-PRRSV yielded from the affected farms, morbidity and mortality rates varied between 5-30 and 0-10 percent, respectively. No HP-PRRSV affected farms in this study showed illness rate up to 50-100% and mortality rate up to 60% as the previous reported in 2012 (Nilubol et al., 2012). Interestingly, in the case of local Thai type 2 PRRSV (12LB01) affected farm, morbidity and mortality rate were as high as 100 and 30 percent, respectively. This information supported the previous research claimed that severity of PRRS was not associated to the 30 amino acids deletion in NSP2 gene due to the fact that severity of the disease is complicated by many factors not limited to the virus including immune status of the pigs, ages, environment as well as management of each farm.

Further information of the 11 affected farms was compiled. According to the intensive data, important correlation between the PRRSV from 2 farms in the same location

was revealed. There were boars trading between two farms which 12NP01 and 12NP02 were collected. These farms were located in Nakhonpathom and the 12NP01 collected farm was always buying boars from the other. This might be an important route of PRRSV distribution between these farms. Unfortunately, the data of each boar in the 12NP01 collected farm at that time was not provided. According to the amino acid identity analysis, amino acid sequences of both farms shared high similarity in the partial NSP2 and GP5 sequences, 86.3 and 98.5%, respectively.

According to the data of the farm which 12LB01 was collected, the farm was a growing-to-finishing site functioning as one finishing farm of an integrated livestock company. At the affected time, there was severely outbreak of PRRSV in other finishing farm adjacent to the studied farm. Unfortunately, the early outbreak farm belonged to another company without cooperation with the outsource laboratory and the virus sample from that farm was uncollectable.

The information of the other studied 10 farms was not linked PRRSV among them. The farms having 12CHTB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12RB01, 12SHB01 and 12TAK01 were completely 2-site farms with more than 2,000 sows whereas the farm having 12SURIN01 was a smaller one. Replacement gilts of each farm were internally supported. The farms having 12LB01 and 12KB01 were finishing farm. Pigs in these 2 farms were supplied from type 2 PRRSV vaccinated sow herds.

5.4 Genetic relationship between the current Thai PRRSV and PRRSV vaccine strain

In 2012, Ingelvac PRRS was the only one PRRSV modified live vaccines of Type 2 genotype legally distributed in Thailand. The parent strain of the virus vaccine is VR2332. Many farms in this study used this type 2 MLV vaccine. Ingelvac PRRS is one of the vaccines combined in vaccination program of the farms having 12NP01 and 12RB01 as well as the sow herds supplied growing pigs for the farms having 12LB01 and 12KB01.

Interestingly, Ingelvac ATP, the other type 2 MLV vaccine not authorized for use in Thailand, was illegally used in the farm which 12NP02 was collected. The Ingelvac ATP was a new MLV vaccine from the same manufacturer as the Ingelvac PRRS but the parent strain of the seed was JA142 (Key et al., 2003). On the other hand, some studied farms such as the farms which 12CHTB01, 12PJB01, 12SHB01 and 12TAK01 were collected were not use any PRRSV vaccine and managed as PRRSV negative herds before the outbreaks. However, the vaccination data of the farms having12NN01 and 12SURIN01 were not provided.

The MLV vaccine was recommended for use in healthy gilts and sows in susceptible PRRSV positive herds. The manufacturer claimed that the vaccine can be used in any period of pig production except boars. Generally, Ingelvac PRRS MLV was applied in gilt vaccination program in acclimatization program or used for reducing PRRSV associated diseases in the affected herd. In Thailand, Ingelvac PRRS was utilized in sow herds and were revaccinated every 3-4 months. However, as the data provided in this study, the PRRSV vaccine application incompletely prevent the PRRS clinical signs in some herds. PRRSV specific immunity induced by vaccination can only reduce the suffering of the disease but cannot prevent infection of the PRRSV with different genetic background.

According to the GP5 amino acid sequence identity matrix, the 11 studied Thai PRRSV including 12CHTB01, 12KB01, 12LB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12RB01, 12SHB01, 12SURIN01 and 12TAK01 shared limited similarity with the parent strain of the vaccine,VR2332 (87.3, 58.5, 83.8, 88.7, 88.7, 87.3, 87.3, 61.0, 62.3, 88.0 and 61.6%, respectively). Moreover, the amino acid comparisons between GP5 amino acid sequences of the studied Thai PRRSV and the VR2332 revealed many amino acid substitutions in signal peptide domain, ectodomain, endodomain and transmembrane domain 1-3. The most important is the mutation in glycosylation site of the ectodomain, the attachment site of the PRRSV specific neutralizing antibody. Asparagine transformation of ectodomain was found in the position 44 (N$\rightarrow$K) of 12NP02 and 12PJB02 and in the position 58 of all sequences (N$\rightarrow$Q of 12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12RB01, 12SHB01

and 12TAK01, N→K of 12LB01 and N→R of 12SURIN01). On the other hand, many insertions of N amino acids in the ORF5 sequences were found as well. Asparagine conversions were found in the position 34(D→N) of 12KB01, 12RB01, 12SHB01and 12TAK01 and the position 35(S→N) of all Thai HP-PRRSV sequences. One asparagine was changed in the position 57(A→N) of the 12LB01 as well as in the position 61(D→N) of 12CHTB01. These mutations in the potential glycosylation sites might affect to the neutralizing antibody induced by the infection. Pig vaccinated with Ingelvac PRRS may induce PRRS specific neutralizing antibody not matching to the ectodomain of the current Thai PRRSV. However, effectiveness of vaccine was unable to be predicted completely since the vaccine efficacy is influenced by many factors not only genetic of the viruses.

After 2012, not only the Ingelvac PRRS vaccine is authorized in Thailand, several PRRSV MLV vaccines such as Amervac (Laboratorios Hipra, Spain) and Porcilis PRRS (MSD Animal Health, USA) have been legally used in Thailand. However, the later 2 novel vaccines belong to type 1 PRRSV and the applications in Thailand were limited at this time. More information is needed for farmers and also veterinarians to create the proper vaccination programs. Study of genetic characteristic of Thai PRRSV may be repeated in the future since genetic variation of the virus in 2 years between 2010 and 2012 shared high different, up to 85%. Moreover, the current studied Thai PRRSV sequences and the information provided by this study may be used for further research of the genetic characteristics of the Thai PRRSV in the future.

Conclusion

Since the HP-PRRSV outbreaks in Thailand in 2010, most of the problems due to PRRSV infection in Thailand were caused by a novel type 2 PRRSV infection. Infected pigs were suffered from the type 2 virus even PRRSV modified life vaccines were used in many farms. On the other hand, Thai type 1 PRRSV might exist in Thai swine herds but caused subclinical problems especially in the situation that many sow herds were completely immunized by PRRSV vaccines. According to partial NSP2 and ORF5 sequences obtained from clinically affected cases in this study, the partial NSP2 alignment revealed that 10 sequences including 12CHTB01, 12KB01, 12NN01, 12NP01, 12NP02, 12PJB01, 12SHB01, 12RB01, 12SURIN01 and 12TAK01 contained 30 deduced amino acids at the same position as the HP-PRRSV prototypes while 12LB01 contained no deduced amino acid and shared high identity to the type 2 PRRSV collected in Thailand in the past (2010). Similarly, based on the phylogenetic trees of partial NSP2 and ORF5 genes, the current Thai PRRSV were categorized into 2 groups of type 2 PRRSV, HP-PRRSV related group and local Thai type 2 PRRSV group. Ten sequences with 30 amino acids deletion were located in the HP-PRRSV related group and the 12LB01 was grouped into the local Thai type 2 PRRSV group. The current Thai HP-PRRSV sequences were closely related to the virus isolated in the other country including China, Vietnam and Philippines and shared similarity to the HP-PRRSV isolated in Thailand in 2010. This indicates that the current Thai PRRSV might derive from the HP-PRRSV isolates introduced in 2010 or the viruses might be the newly introduced from other neighbouring countries such as Cambodia, Laos, Myanmar and Vietnam. The HP-PRRSV sequences obtained from this study may be introduced by novel introductions from the neighbouring countries since genetic diversity among 10 sequences was shown and these sequences located in different positions in the phylogenetic trees. On contrary, 12LB01 was located into the same group as the other local Thai type 2 PRRSV collected in the past and showed high virulence as well as HP-PRRSV isolates. This indicates that not

only HP-PRRSV but all type 2 PRRSV groups can cause severe PRRS in the swine farms in Thailand due to many factors were involved in the severity of the disease.

Since both HP-PRRSV and local Thai type 2 PRRSV showed its virulence in the herd with poor management or any PRRSV negative herd, disease prevention should involve strictly biosecurity procedure as well as virus screening in replacement gilts. Moreover, quarantine period for animals, humans and objects should be deployed to prevent new PRRSV introduction. On the other hand, in the susceptible PRRSV positive herds, the MLV vaccine might be used in gilts and sows but the PRRSV vaccine application showed incompletely prevent the PRRS clinical signs in some herds. PRRSV specific immunity induced by vaccination can only reduce the suffering of the disease and may conceal some signs of an infected animal due to subclinical disease so the vaccination should be used with high awareness.

This study revealed complete genetic characteristics of the current Thai PRRSV which caused clinical PRRS in Thailand after the 2010 HP-PRRSV outbreaks. Further study should focus on the pathogenicity and immunogenicity of the virus to improve knowledge of the disease and its effects in Thailand. This information can be integrated to conclude and effective procedure for prevention and control of the disease in Thailand in the future.

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APPENDIX

Appendix A: Figure of partial NSP2 sequences alignment of current Thai PRRSV

```
                845        855        865        875        885        895
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332      PVPAPRRKVG SDCGSPVSLG GDVSNSWEDL AVSSPFDLPT PPEPATPSSE LVIVSSPQCI
12CHTB01    -----?RKVR SDCGSPVLMG DNVPNGSE-? TVGGPLNFPT PSELMTPMSE PVLVPASQFV
12KB01      ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12LB01      ------RKIR SDCGSSILLG DNVPNSWEDL TVGGPLDLPA PPEPVTPPRE LAPMPAPQHI
12NN01      -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12NP01      ------?KVR SDCGGPVLIG DNVPSGSE-R TVGGPLNFPT PSKLMTPMSE PALVPASQFV
12NP02      -------?VR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE SVLMPASQFV
12PJB01     -----?RKVR SDCGSPVLMG DNVPNDSE-? TVGGPLNFPT PSELMTPMSG PVLMPASQFV
12RB01      ------?KVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMTPMSE PALVPASQFV
12SHB01     ------?KVR SDCGSPVLMG NNVPNGSE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA
12SURIN01   ------RKVR SDCGSPVLMG DNVPNGSE-E TVGGPLNFPA PSELMAPMSE PALVPASQFV
12TAK01     ------?KVR SDCGSPVLMG DNVPNGCE-K TVGGPLNFPT PSEPMTPMSE PVLMPASRRA

                905        915        925        935        945        955
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332      FRPATPLSEP APIPAPRGTV SRPVTPLSEP IPVPAPRRKF QQVKRLSSAA AIPPYQNEPL
12CHTB01    PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12KB01      PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12LB01      FRPVTPLSEP APVPAPRRTV FRPMTSLSEP ILVSAPRHKF QQVEKANLAT TTLTHQDEPL
12NN01      PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTPTHQDEPL
12NP01      PTLITPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12NP02      PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTPTHQDEPL
12PJB01     PKLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TALTHQDEPL
12RB01      PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12SHB01     PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12SURIN01   PTLMTPLIGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL
12TAK01     PKLMTPLSGS APVPAPRRTV ---------- ---------- ---------T TTLTHQDEPL

                965        975        985        995        1005
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
1015
VR2332      DLSASSQTEH EASPPAPPQS GGVPGVEGHE AEETLSEISD MSGNIKPASV SSSSSLSSVR
12CHTB01    DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12KB01      DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12LB01      DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12NN01      DLSASS---- ---------- ---------- ---------- ---------- ----------
12NP01      DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12NP02      DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12PJB01     DLSASSQT-- ---------- ---------- ---------- ---------- ----------
12RB01      DLSASSQT?- ---------- ---------- ---------- ---------- ----------
12SHB01     DLSASS---- ---------- ---------- ---------- ---------- ----------
12SURIN01   DLSASSQ?-- ---------- ---------- ---------- ---------- ----------
12TAK01     DLSASSQ--- ---------- ---------- ---------- ---------- ----------
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Appendix B: Figure of ORF5 sequences alignment of current Thai PRRSV

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                5         15         25         35         45         55
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     MLEKCLTAGC YSQLLSLWCI VPFCFAVLVN ASNDSSSHLQ LIYNLTLCEL NGTDWLANKF
12CHTB01_T ---------- --------?I PFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12KB01_Tha ---------- --------CS VPFYLA?RVN A??NNSSHIQ LIYNL?LCEL NGTDWLAQKF
12LB01_Tha ---------- --------CI VPFWFAALVN ASNH??SHLQ LIYNLTICEL NGTDWLNKSF
12NN01_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12NP01_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTDWLAQKF
12NP02_Tha ---------- --------CI VPFYLAVLAN ASNSNSSHIQ SIYKLTLCEL NGTDWLAQKF
12PJB01_Th ---------- --------CI VPFYLAVLAN ASNSNSSHIQ SIYKLTLCEL NGTDWLAQKF
12RB01_Tha ---------- --------CI VPFYLSVLVN ASNNNSSHIQ LM?NLTLCEL NGTDWLAQKF
12SHB01_Th ---------- --------CI VPFC?AVLVN ASNNNSSHIQ LIYNS?LCEL NGTDWLAQKF
12SURIN01_ ---------- --------CI VPFYLAVLAN ASNSNSSHIQ LIYNLTLCEL NGTEWLARKF
12TAK01_Th ---------- --------CI VPFYLAVLVN ASNNNSSHIQ LIYNLALCEL NGTDWLAQKF

                   65         75         85         95        105        115
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     DWAVESFVIF PVLTHIVSYG ALTTSHFLDT VALVTVSTAG FVHGRYVLSS IYAVCALAAL
12CHTB01_T NWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12KB01_Tha DWAVETFVIF PVLTHIVSYG ALT?SHFLDT VGLATVSTAG YYHGRYVLSS VYAVCALAAL
12LB01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDA VGLITVSTAG YYHGRYVLSS IYAVCALAAL
12NN01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12NP01_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12NP02_Tha DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12PJB01_Th DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12RB01_Tha DWAV??FVIF PVLTH?VSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCAL?AL
12SHB01_Th DWAVETFVIF PVLTHIVSYG AL?TSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCAP?AL
12SURIN01_ DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCALAAL
12TAK01_Th DWAVETFVIF PVLTHIVSYG ALTTSHFLDT VGLATVSTAG YYHGRYVLSS IYAVCPLAAL

                  125        135        145        155        165        175
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VR2332     TCFVIRFAKN CMSWRYACTR YTNFLLDTKG RLYRWRSPVI IEKRGKVEVE GHLIDLKRVV
12CHTB01_T ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12KB01_Tha ICFVIRLAKN CMSWRYSCAR YTNYLLDTK? RLYRWRSPVI V---------- ----------
12LB01_Tha ICFIIRLTKN CMSWRHSCTR YTNFILDTKG KLYRWRSPVI ?---------- ----------
12NN01_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12NP01_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12NP02_Tha ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12PJB01_Th ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12RB01_Tha ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI V---------- ----------
12SHB01_Th ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12SURIN01_ ICFAIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRSPVI ?---------- ----------
12TAK01_Th ICFVIRLAKN CMSWRYSCTR YTNFLLDTKG RLYRWRS??I V---------- ----------
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BIOGRAPHY

Mister Korakrit Poonsuk was born on January, 5, 1987 in Bangkok, Thailand. He earned his Bachelor degree on Doctor of Veterinary Medicine from the Faculty of Veterinary Science, Chulalongkorn University in 2010. Soon after graduated, he was enrolled in the Master program in Veterinary Pathobiology program and has been working as a veterinarian technical service in K.M.P. Biotech Co., Ltd.