ศักยภาพในการนำเชื้อไวรัส พี อาร์ อาร์ เอส ของยุงสกุล *Culex* ที่พบในฟาร์มสุกร จังหวัดนครปฐม

นาย กฤษฎากรณ์ พริ้งเพราะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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POTENTIAL VECTORS OF PRRSV IN MOSQUITOES GENUS *Culex* FROM A PRRSV-POSITIVE SWINE FARM IN NAKHON PATHOM PROVINCE

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กฤษฎากรณ์ พริ้งเพราะ : ศักยภาพในการนำเชื้อไวรัส พี อาร์ อาร์ เอส ของยุงสกุล *Culex* ที่พบในฟาร์มสุกร จังหวัดนครปฐม อ.ที่ปรึกษา : รุ่งโรจน์ ธนาวงษ์นุเวช, ที่ปรึกษาร่วม : สุดจิตต์ จุ่งพิวัฒน์ จำนวน 54 หน้า. ISBN 974-17-6906-7

การศึกษานี้มีจุดมุ่งหมายในการสำรวจชนิดและความชุกของยุงแต่ละชนิดที่พบในฟาร์มสุกร จังหวัดนครปฐม และศึกษาศักยภาพในการนำเชื้อไวรัส พี อาร์ อาร์ เอส ของยุงชนิดที่พบได้มากที่สุดในฟาร์มสุกร จังหวัดนครปฐม ผลการสำรวจชนิดของยุงตั้งแต่เดือน มีนาคม ถึงเดือน สิงหาคม 2547 พบยุง *Culex tritaeniorhychus* มากที่สุด คิดเป็นค่าเฉลี่ยร้อยละ 85.68 ยุง *Culex gelidus* พบร้อยละ 12.13 ยุง *Anopheles spp.* ร้อยละ 2.06 และยุง *Mansonia uniformis* ร้อยละ 0.13 ตามลำดับ โดยเดือนที่พบปริมาณยุงมากที่สุด คือเดือน กรกฎาคม มิถุนายนและสิงหาคมตามลำดับ การทดสอบศักยภาพในการนำเชื้อไวรัส พี อาร์ อาร์ เอส ของยุง *C. tritaeniorhychus* ทำโดยการทดสอบระยะเวลาที่ตรวจพบเชื้อไวรัส พี อาร์ อาร์ เอส และการทดสอบความสามารถในการนำเชื้อไวรัสจากสุกรทดลองฉีดเชื้อ ไปยังสุกรปลอดเชื้อ ผลการศึกษาตรวจพบสารพันธุกรรมของเชื้อไวรัส พี อาร์ อาร์ เอส ในยุงนาน 48 ชั่วโมง ภายหลังการให้ยุงดูดเลือดสุกรทดลองที่ฉีดเชื้อปริมาณไวรัส $10^{2.75}$ $TCID_{50}$/ml ซึ่งเชื้อไวรัส พี อาร์ อาร์ เอส สามารถมีชีวิตในยุง *C. tritaeniorhychus* ได้นาน 2 ชั่วโมงภายหลังการกัดและดูดเลือดสุกรทดลองฉีดเชื้อ การทดสอบความสามารถในการนำเชื้อไวรัส พี อาร์ อาร์ เอส ของยุง *C. tritaeniorhychus* โดยให้ยุงทดลองกัดและดูดเลือดสุกรทดลองฉีดเชื้อไวรัส นำยุงมากัดและดูดเลือดสุกรที่ปลอดเชื้อ ควบคู่กับการบดยุงและฉีดเข้าไปในสุกรทดลองในกลุ่มเดียวกัน ในช่วงระยะเวลาต่าง ๆ ผลการศึกษาพบว่า สุกรทดลองตัวรับที่ได้รับการฉีดตัวอย่างยุงบด ในช่วงเวลา 30 นาทีหลังการกัดและดูดเลือดสุกรฉีดเชื้อ สามารถติดเชื้อไวรัส พี อาร์ อาร์ เอส ได้ ผลจากการศึกษานี้ สรุปได้ว่ายุง ที่พบได้มากที่สุดในฟาร์มสุกร จังหวัดนครปฐมคือยุง *C. tritaeniorhychus* และยุงชนิดนี้มีศักยภาพ ในการเป็นพาหะนำเชื้อไวรัส พี อาร์ อาร์ เอส แบบกลไก การป้องกันหรือควบคุมปริมาณยุงในฟาร์ม เป็นอีกแนวทางหนึ่งในการลดการแพร่ระบาดของเชื้อไวรัส พี อาร์ อาร์ เอส ในฟาร์มสุกร

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

สาขาวิชา.....พยาธิชีววิทยาทางสัตวแพทย์.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD : PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME / MOSQUITOES / VECTOR

Kidsadagon Pringproa : POTENTIAL VECTORS OF PRRSV IN MOSQUITOES, GENUS *Culex,* FROM A PRRSV-POSITIVE SWINE FARM IN NAKHON PATHOM PROVINCE. THESIS ADVISOR: Roongroje Thanawongnuwech, THESIS CO-ADVISOR: Sudchit Chungpivat, 54 PAGES ISBN 974-17-6906-7

The aims of this study were to survey the prevalence of mosquito species observed in a pig farm and to determine whether they could serve as a potential vectors in the role of PRRSV transmission among pigs. Mosquito survey was conducted during March to August, 2004. At least, 3 genus, 4 species, *Culex tritaeniorhynchus*, *Culex gelidus*, *Anopheles* spp. and *Mansonia uniformis* were identified in the farm. Of which *C. tritaeniorhynchus* was predominant (85.68%). Others were found at 12.13%, 2.06% and 0.13%, respectively. To determine whether *C. tritaeniorhynchus* could serve as a potential vector of PRRSV transmission, 2 experiments were conducted: the duration of PRRSV within mosquitoes and the assessment to transmit PRRSV from PRRSV-infected pig to naive pigs by mosquitoes. By RT-PCR detection from the *C. tritaeniorhynchus* samples, the PRRSV was detected up to 48 hours post feeding on a PRRSV-infected pig. The virus was also isolated from *C. tritaeniorhynchus* and the live PRRS virus could be isolated from mosquito samples up to 2 hours post feeding on an infected pig. To assess whether *C. tritaeniorhynchus* could transmit PRRSV from PRRSV-infected pig to the naive pigs, the direct mosquitoes to pig protocol and the swine bioassay were developed. The results showed that all naïve pigs used in the direct mosquitoes to pig protocol were negative, whereas, the swine bioassay using the mosquitoes which fed 30 minutes on the infected pig was positive for PRRSV detection. The results of this study indicated that *C. tritaeniorhynchus*, a predominant mosquito species found in a pig farm, was able to transmit PRRSV mechanically and is likely to serve as a potential role of PRRSV transmission in the pig farm. These results are useful for the prevention and control of PRRSV transmission in the pig farms and for a better understanding of PRRS epidemiology.

Department……Pathology……Student signature……

Field of study…….Veterinary Pathobiology….Advisor's signature……

Academic year……2004……Co-advisor's signature……

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ABBREVIATION

bp	base pairs
°C	degree Celsius (centrigate)
CU-VDL	Veterinary Diagnostic Laboratory, Chulalongkorn University
df	day post feeding
dpi	day post infection
DW	distilled water
ELISA	enzyme-linked immunosorbant assay
EU	European genotype
et al.	et alii, and others
g	gram (s)
h	hour (s)
H & E	hematoxylin & eosin
Ig	Immunoglobulin
IHC	immunohistochemistry
IPMA	Indirect immunoperoxidase monolayer assay
kb	kilobase pair
kd	kilodalton
Mab	Monoclonal antibody
min	minute (s)
ml	mililitre (s)
mM	milimole
mm^3	milimetre (s)
µl	microlitre
ORF	open reading frame
PAMs	pulmonary alveolar macrophage
pH	the negative logarithm of hydrogen ion concentration
PIMs	pulmonary intravascular macrophage
PRRS	porcine reproductive and respiratory syndrome

rpm	round per minutes
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
sec	second (s)
$TCID_{50}$/ml	tissue culture infectious dose
US	American genotype
V	Volt
VI	Virus isolation

CHARPTER I

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) caused by PRRS virus (PRRSV) is one of the most economically devastating diseases of the global pig industry today. The virus affects pigs of all ages causing poor conception rate, late - term abortion, stillborn and weak live-born pigs, post-weaning pneumonia and increase in mortality rate in nursery pigs. PRRSV has emerged in the late 1980s which resulted in reproductive failure and respiratory disease of infected pigs in the North America and Europe (Albina, 1997) and later in Asia. Since the absence of a specific etiology, it was previously named mystery swine disease. Subsequently, in the early 1990s, mystery swine disease had spread quickly throughout the major pig – producing areas of the United States as well as in Europe. The clinical outbreaks with signs similar to the mystery swine disease were reported. However, no link was founded between the outbreaks in the United States and those in Europe. Finally, the agent of mystery swine disease was identified in 1991 in Europe and was designated as Lelystad virus (Wensvoort et al., 1991; Rossow, 1998). Subsequently, the terminology 'porcine reproductive and respiratory syndrome virus (PRRSV)' was introduced by the Office International des Epizooties (O.I.E.) in early 1991 and PRRSV has been used since then (Denac et.al., 1997; Zimmerman, 2002; Plagemann, 2003). The viruses isolated either in Europe or in the United States, are known as the European genotype or the US genotype, respectively (Nelsen et al., 1999). The first retrospective report of PRRSV infection in Thailand revealed that Thai pigs had seroconversion to PRRSV since 1996 and the genomic organization of the first isolate was similar to the US genotype (Damrongwatanapokin et al., 1996; Damrongwatanapokin et al 1998).

PRRSV is a small, enveloped RNA virus and is classified as a member of the genus *Arterivirus*, family Arteriviridae in the order Nidovirales. Other viruses in the genus *Arterivirus* are Lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). All of these viruses are

enveloped and have an average diameter of 40-60 nm. The Arterivirus also process common biological properties, including primary replication in host macrophages and establishment of asymptomatic persistent infection in the host (Yoon, 2002).

Generally, PRRSV contains an electron-dense icosahedral nucleocapsid ranging between 25 to 30 nm in diameter. The genome of the virus is polyadenylated, single-stranded, nonsegmented, positive-sense RNA, and has approximately 15 kb in genomic size consisting with 8 open reading frams (ORFs). ORF1a and 1b, producing non-structural proteins, are located at the 5' end of genome and comprising approximately 80% of the viral genome. ORFs2-5, ORF6 and ORF7 are located at the 3' end of the genome and encode for envelop glycoprotein, matrix protein and nucleocapsid protein, respectively (Denac et al., 1997; Benfield et al., 1999; Yoon, 2002). The study on the pathogenesis of PRRSV infected pig demonstrated that the virus infects and replicates in the cells of mononuclear phagocytic systems (MPs) such as monocytes and macrophages, especially alveolar macrophages (Thanawongnuwech et.al., 2000b). The infection results in immunosuppressive effect, therefore leading to secondary infection with bacterial or other viruses. PRRSV-infected pigs, moreover, show the syndrome of reproductive signs and respiratory signs prolonging time to market in finisher pigs.

Pigs are susceptible to PRRSV by several routes of exposure including oral, intranasal, intramuscular, intraperitoneal and intravaginal routes. Consequently, the transmission of PRRSV among pig populations can occur by exposure to PRRSV-infected pigs, contaminate semen, contaminate needles, fomites, insects and mammary secretion and transplacental infection as well as airborne transmission (Rossow, 1998; Wagstrom et al., 2001). Previous reports found that some insects such as houseflies (*Musca domestica* Linnaeus) and mosquitoes (*Aedes vaxans*) could serve as mechanical vectors for PRRSV transmission (Otake et al., 2002; Otake et al., 2003a). In addition, several studies have shown that the mosquito transmission can serve as a vector of several pathogens under the filed and experimental conditions, including malaria, Japanese encephalitis, West Nile virus and swine fever virus (Stewatt et al., 1975; Hubalek et al., 1999; Johansen et al., 2002). Recent report has found the role of

mechanical vectors of PRRSV in the mosquito vector (*Aedes vaxans*), the predominant mosquito species seen in the United States. In addition, the infectious PRRSV could survive in the intestinal tract of mosquitoes for up to 6 h following the feeding on an infected pig. That finding also suggested that PRRSV did not replicate within mosquitoes to establish a sufficient concentration of the virus during the 14-days incubation period. Therefore, the study suggested that mosquitoes could not serve as the biological vectors for PRRSV transmission (Otake et al., 2003a).

Since Thailand has differences in the geographical region and in the mosquitoes species from the North America, the objectives of this study are to survey mosquito species seen in the pig farm in Nakhon Pathom, Thailand and to determine whether the mosquitoes genus *Culex* could serve as a potential role of PRRSV transmission among pigs. Since the results from the preliminary study showed that mosquito genus *Culex* was the predominant specie seen in the pig farm in Thailand.

CHARPTER II

REVIEW LITERATURES

2.1 History of PRRSV

Porcine reproductive and respiratory syndrome (PRRS), one of the most important pig diseases spreading in most swine raising countries, is caused by PRRS virus (PRRSV). This particular virus is not only found worldwide but also caused considerably economic losses in the swine industry (Benfield et al., 1999). The original source of the virus is unclear (Albina, 1997). However, it was reasonable to postulate that the virus might cross from other host species (Zimmerman, 2000). Plagemann (2003) suggested that the PRRSV might derive from lactate dehydrogenase-elevating virus (LDV) and might have wild boars as an intermediate host.

This unrecognized virus had caused serious outbreaks across the North America and European continents in the late 1980s. Clinical signs initially reported included severe reproductive losses, extensive post-weaning pneumonia, reduction of performance and increase in mortality of infected pigs. The attempts to diagnose were unsuccessful, and “mystery swine disease” was named at that time (Zimmerman, 2000).

Subsequently in 1991, the etiologic agent of mystery swine disease was identified and named Lylestad virus in The Netherlands (Wensvoort et al., 1991). Shortly, the virus was also isolated in the USA and Canada and porcine reproductive and respiratory syndrome was introduced in early 1991. PRRSV composed of 2 genotypes, US genotype and EU genotype, has distinct sub-population with distantly antigenic relation. Hence, it is difficult to establish the exact epidemiological relationship between the 2 genotypes (Suares et al., 1996; Albina, 1997; Nelsen et al., 1999).

2.2 Biological characteristics of PRRSV

Porcine reproductive and respiratory syndrome virus (PRRSV) is classified as a member of the genus *Arterivirus* of the family Arteriviridae in the order Nidovirales. PRRSV is enveloped, nonsegmented, single stranded, positive-sense RNA virus. Other viruses in the genus *Arterivirus* include Lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). All of those viruses are enveloped and have diameter between 40-60 nm. The Arteriviruses also process common biological properties, including primary replication in the host macrophages and establishment of asymptomatic persistent infection in their hosts (Yoon, 2002).

PRRSV is spherical in shape and ranges between 48-83 nm in diameter. The virus contains an electron-dense icosahedral nucleocapsid ranging from 25 to 30 nm in diameter. The study on genomic organization reveals that PRRSV is approximately 15 kb in size and consists of 8 open reading frames (ORFs). The product of each ORF has been identified. ORFs1a and b encode for RNA replicase, the only nonstructural proteins of PRRSV. ORFs2 to 7 are though to encode for structural proteins of the virus. The three major structural proteins which are envelop protein (E protein), matrix protein (M protein) and nucleoprotein (N protein) are produced from ORFs 5, 6 and 7, respectively (Denac et al., 1997; Wootton et al., 1998; Yoon, 2002). The study on molecular organization of the Lelystad virus suggested that ORFs 2, 3 and 4 encoded for viral envelop glycoprotein. The same study also suggested that those proteins were present at low level in viral particles or poorly immunogenic (Yoon, 2002). The schematic representation of PRRSV virion is shown in figure 1.

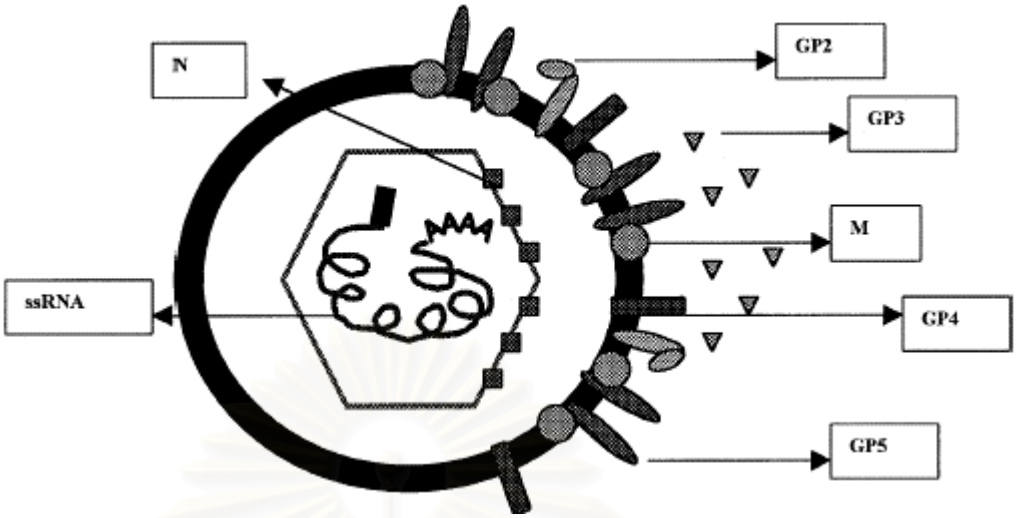


Figure 1. Schematic representation of PRRSV virion. The viral genome consists single-stranded, non-segmented, positive-sense RNA. The major viral proteins are N protein (nucleoprotein), M protein (matrix protein), GP 2-5 protein (glycoprotein 2-5). (Modified from Dea et al., 2000)

Comparative analysis of the predicted N protein amino acid sequence indicates that the N protein is well conserved among PRRSV isolates (Wootton et al, 1998). In addition, antibodies direct against N protein are most abundant in PRRSV- infected pigs and serological tests mostly rely on those antibodies (Murtaugh et al., 2002).

The study on viral replication has been demonstrated that monocyte / macrophage lineage cells, such as pulmonary intravascular macrophages (PIMs) and pulmonary alveolar macrophages (PAMs), are the primary cells supporting the replication of the virus both in vitro and in vivo (Rossow, 1998 ; Thanawongnuwech et al., 2000b; Yoon, 2002). Importantly, PRRSV infection is able to induce apoptosis of bystander macrophages and lymphocytes leading to immunosuppressive effect (Sirinarumitr et al., 1998). On the other hand, the replication is also demonstrated in vitro in establish cell lines such as African green monkey kidney cell line, MA-104 and its permissive clone, MARC-145.

Studies with immune responses to PRRSV have demonstrated that antibodies respond to PRRSV are high within the first week after infection but neutralizing

antibodies are not detected until 5-7 weeks post-infection. This phenomenon might be due to the up regulation of IL-10 cytokine during PRRSV infection (Thanawongnuwech et al., 2001; Feng et al., 2003; Suradhat et al., 2003).

No chemical inactivating agents except for chloroform have been tested in vitro against PRRSV. Treatment of virus with chloroform reduces virus infectivity by more than 99.99% (Yoon, 2000). With the presence of viral envelope, any lipid solvent or detergent should have an adverse effect on the virus infectivity. In addition, previous report suggested that quaternary ammonium compound had much stronger effects against envelop virus such as PRRSV than that of either iodine disinfectant or chlorine disinfectant (Shirai et al., 2000). However, other effects such as temperature or pH were not examined in the study, since these factors may important for virucidal effect of disinfection. Yoon (2000) suggested that the virus was stable for several months at -70 °C and at least 1 month for 4 °C. In contrast, at higher temperature the virus was rapidly inactivated. The report also suggested that at 37 °C the virus was complete inactivation within 48 hours and at 56 °C within 45 minutes. Another factor affecting the infectivity of PRRSV such as pH has also been reported. In culture medium at pH 7.5, the estimated half - life of PRRSV (EU genotype) is 140, 20, 3 and 0.1 hours at 4°, 21°, 37° and 56 °C, respectively.

2.3 Disease transmission

At present, PRRSV is endemic in nearly all pig-producing countries. A few countries such as Australia and Switzerland are believed to be free from PRRSV (Zimmerman, 2002). The rapid spread of PRRSV throughout the world has demonstrated that transmission is one of the most significant characteristics of this virus.

Pigs are susceptible to PRRSV infection by several routes of exposure, including oral, intranasal, intramuscular, intraperitoneal and intravaginal routes (Prieto et al., 1997; Brierk et al., 2001; Hennings et al., 2001; Zimmerman, 2002; Van der Linden et al., 2003). PRRSV is highly infectious. Yoon et al (1999) suggested that only 10 or fewer PRRSV particles infection by either intranasal or intramuscular route was sufficient to

cause infection. Moreover, the previous report indicated that insemination the gilts with semen containing PRRSV could result in transmission of the virus and caused early embryonic infection and death, respectively (Prieto et al., 1997). Infection of susceptible animals results in virus shedding in saliva, nasal secretion, urine, semen and mammary secretions (Albina, 1997; Wagstrom et al., 2001; Zimmerman, 2002). However, many researchers suggested that the major routes of PRRSV transmission could occur by close contact between pigs, by airborne transmission or by contaminated semen (Albina, 1997). In addition, those studies did not demonstrate the role of fomites as a potential route of transmission. Mortensen et al. (2002) suggested that aerosol transmission was a major mode of transmission of PRRSV in Danish' herds during 1996-1997, and PRRSV, US genotype could introduce into the herd by the following pathways: vaccination, purchasing of PRRSV- infected or MLV-vaccinated animals, using of infected-semen, airborne transmission, using of contaminated equipment and visitors or vectors.

Airborne transmission, also, has been demonstrated by Kristensen et al. (2004) that PRRSV-infected pigs are able to spread to other pig units over a short distance. However, airborne transmission of PRRSV is needed a high number of infected pigs to establish enough viral concentration and a long interval time of exposure.

Normally, PRRSV transmission by direct contact between infected and susceptible animals usually occurs by intensive animal management when purchasing of uninfected weaners or replacement breeding stock into a positive herd (Albina, 1997; Zimmerman, 2002). It should be noted that persistent infected sows are able to shed virus to naïve contact sows even in the absence of detectable viremia (Bierk et al., 2001).

Shedding of virus by infected animals results in environmental contamination and might create the potential transmission via contaminated fomites. However, PRRSV is labile and quickly inactivated by either chloroform or higher temperature (Yoon, 2002). Interestingly, several studies reported the possibility of PRRSV-transmission by vectors. Otake et al. (2003a) demonstrated the possibility of PRRSV transmission by mosquitoes, *Aedes vaxans*, as a mechanical vector of PRRSV in pig farms. In addition, the

houseflies, *Musca domestica*, could also serve as a mechanical vector for PRRSV (Otake et al., 2003 b; Otake et al., 2003c).

On the other hand, spreading of PRRSV might depend on the environmental condition as demonstrated by Dee et al. (2003) that during warm weather the mechanical transmission of PRRSV infrequently occurs.

In Thailand, PRRSV-antibodies was found positive as early as in 1989, and the percentage of seropositive animals have been increased since then (Damrongwatanapokin et al., 1996). In addition, there is no geographical influence on the spreading of PRRSV in Thailand and both US and EU genotypes co-exist with the high prevalence of the EU genotype (Thanawongnuwech et al., 2004).

2.4 Mosquito vectors

Mosquitoes are the most medically important arthropod vectors of many diseases both in human beings and in animals. The transmission of many pathogens using mosquito vectors in human include malaria, lymphatic filariasis and many of viral diseases (Beerntsen et al., 2000), whereas, the diseases transmission via mosquito vector in animals are swine fever and equine infectious anemia (Tidwell et al., 1972; Stewatt et al., 1975; Weber et al., 1988). The transmission of swine fever virus via mosquitoes was reported mechanically by *Aedes aegypti* (Tidwell et al., 1972; Stewart et al., 1975).

In addition, many diseases in animals and human beings such as malaria, Japanese encephalitis and West Nile virus are known as mosquito – born diseases (Johansen et.al., 2002). Pandey et al. (1999) reported that Japanese encephalitis virus was isolated from a pool of mosquitoes, *Culex tritaeniorhynchus*, in Chiangmai, Thailand. The findings indicated the prevalence of Japanese encephalitis virus within mosquitoes, and it could be the source of infection among pig population since *Culex tritaeniorhynchus* is normally seen in the pig farm.

Interestingly, mosquitoes are known to serve as a mechanical vector for PRRSV (Otake, 2002a). The previous report revealed that mosquitoes, *Aedes vaxans*, a

predominant specie seen in the US pig farms could carry PRRSV up to 6 hrs after feeding on the PRRSV-infected pigs.

Naturally, mosquito vectors are divided into 2 groups according to the mechanisms of the pathogen transmission: biological and mechanical transmission. Mechanical transmission is the transmission that mosquitoes carry the pathogen from infected host to the uninfected host without maturation, multiplication or development of the pathogen within the mosquitoes, whereas biological transmission is the transmission in which the pathogen has undergone in one of the mechanisms mentioned above. In addition, the pathogen is able to survive within the mosquitoes for a longer period and different pathogens have differences in developmental sites (Weber et al., 1988; Beernsten et.al., 2000).

The role of mosquitoes as a mechanical vector occurs when mosquitoes feed on an infected animal. The virus contaminated blood then transmits to the susceptible animals by simply transferring via contaminated mouthparts of the vector (Webb et al., 1989). The potential for mechanical transmission of virus from an insect bite is influenced by various factors including the viral titer in the infected blood, the amount of blood introduced into an uninfected animals and the frequency of interrupted feeding (William et al., 1981; Webb et al., 1989).

In theory, arthropod transmission could occur biologically, which would require virus replication in arthropod tissue especially in the salivary gland (Beernsten et al., 2000). West Nile virus is one of mosquito born diseases that has mosquitoes as a biological vector (Hubalek et al., 1999). The mechanisms of disease occur when blood meal containing virus is ingested. West Nile virus then penetrates through the wall of mid gut, and replicates in this site before entering to the hemolymph-filled hemocoel. The virus then travels through the body and replicates again at the salivary gland until reaching the concentration for infection. The transmission occurs when the mosquitoes bite and release the virus from salivary gland to the recipient host.

Generally, there are three major routes of migration and developmental sites within the mosquito as depicted in figure 2. Mostly, there are 3 major pathogens

composing of viruses, malaria parasite and filarial worms requiring mosquitoes for the transmission.

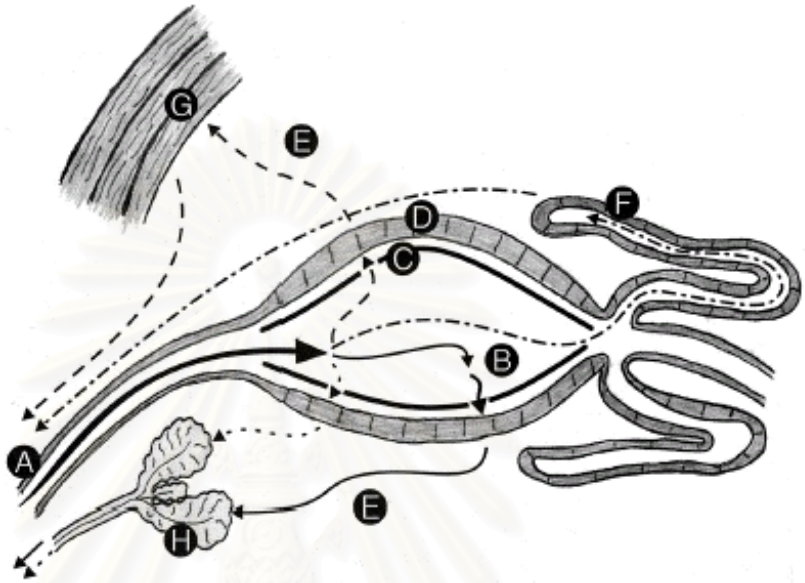


Figure 2. Migratory routes and developmental sites within mosquito for viruses, malaria parasite and filarial worms. The letter A to F and A to G are the developmental sites for filarial worms. While, developmental sites of viruses are defined by the letters A to H, and migratory routes are presented by lines (_____). Following ingestion in a blood meal (A), viruses enter the midgut (B) and then enter the midgut epithelial cells (D), replicate, exist the cells, and travel through the hemolymph-filled hemocoel (E) to the salivary glands (H), where the viruses again replicate and reside until injected into another vertebrate host. (Modified from Beerntsen et al., 2000)

A survey of mosquito species in the poultry farms in Thailand was done by Chungpivat et al. (1986). At least 7 species of mosquitoes including *Culex gelidus, Culex tritaeniorhynchus, Culex quinquefasciatus, Mansonia uniformis, Mansonia annulifera, Mansonia indiana* and *Anopheles barbirostis* were identified in the poultry farms. A survey of mosquito species in the pig farms was also reported in Malaysia in 1994 (Vythilingam et al., 1994). However, there is no report about mosquito species in

the pig farms in Thailand. As a result, the prevalence of each mosquito species is needed to be done in pig farms for future research works.

2.5 Clinical symptoms

Clinical symptoms of PRRS in pigs are characterized by the reproductive disorders in breeders and respiratory disorders in pigs of all ages. Reproductive failure associated with PRRSV infection includes poor conception rate, late-term abortions, increase in number of stillborn piglets, mummified fetuses and dead, while respiratory problems mostly associated with secondary bacterial infection (Segales et.al., 2002; Yoon et al., 2002).

Clinical symptoms of PRRS relate with the natural habitat of the virus. The virus, in general, infects cells in mononuclear phagocytic system (MPS) such as macrophages and monocytes, and uses these cells as a major replication site. When infected, those cells are subjected to die either by cell lysis or by apoptosis, leading to poor functions of the immune system. The infected –pigs are susceptible to secondary infections either with viral or with bacterial infection. The respiratory syndrome is developed and known as porcine respiratory disease complex (PRDC) (Thacker et al., 1999).

Secondary infections interacting with PRRSV infection are varied depending on the co-infected pathogens. Epidemiological studies in the US strongly suggested that swine influenza virus, the second most frequently identified virus in PRDC pigs, has become an endemic respiratory pathogen (Choi et al., 2003). In addition, respiratory bacterial infection such as *Mycoplasma hyopneumoniae, Sterptococcus suis, Haemophillus parasuis* and *Pasteurella multocida* are usually identified in the PRDC case (Thacker et al., 1999; Thanawongnuwech et al., 2000a; Choi et al., 2003). Since pulmonary intravascular macrophages (PIMs) play an important role in the clearance of blood-born pathogens, and PRRSV infection results in apoptosis or cell lysis of these cells, decreasing of phagocytic activity of PRRSV-infected PIMs may lead to susceptibility to bacterial infection (Thanawongnuwech et al., 2000a; Thanawongnuwech et al., 2000b). In addition, another previous report suggested that *Mycoplasma*

hyopneumoniae could potentiate the lesion of PRRSV-infected pigs (Thacker et al., 1999).

Moreover, the genotype of PRRSV is believed to be one of the major influenced factors in PRRSV infection. It has been demonstrated that PRRSV of the US genotype produced more severe lesions than that of the EU genotype in both macroscopic lesions and microscopic lesions (Halbur et al., 1995). In addition, the variability in clinical signs of PRRSV infection also influences on farm-management (Mortensen et al., 2002; Goldberg et al., 2004). As a result, the virulence of PRRSV infection may vary in each pig producing area, due to the genotype of PRRSV, type of secondary pathogens or the quality of farm - management.

2.6 Diagnosis

A tentative diagnosis of PRRSV infection is performed by obvious clinical signs in the reproductive and respiratory systems. However, because of the similarity of the clinical signs with other bacterial or viral diseases, and no pathognomonic gross or microscopic lesions in the PRRSV-infected pigs, several diagnostic tools are required for a definitive diagnosis. Tests normally performed in most laboratories include virus isolation, the detection of viral antigens or genomic materials, and/or antibody detection (Yoon et al., 2002).

The detection of infectious PRRSV by swine bioassay has been developed (Swenson et al., 1994), and it is considered to be the most sensitive test for PRRSV in live pigs (Benson et al., 2002). However, this technique is needed to confirm the presence of PRRSV infection by other techniques, and it is time consuming, laborious and expensive (Christopher-Hennings et al., 1995). In general, naïve pigs are susceptible to PRRSV of all ages, but the severity is depended on either the virulence of PRRSV strain or the age of the pigs (Thanawongnuwech et al., 1998; Yoon and Stevenson, 2002)

The detection of viral antigens or genetic materials can be done by using Immunohistochemistry and Fluorescense antibody test or In situ hybridization test

(Sirinarumitr et al., 1997; Chantamaneechote et al., 2000). The direct fluorescent antibody technique (FA) was developed to test on the frozen-tissue section; whereas, the Immunohistochemistry and In situ hybridization techniques were developed to test in the formalin-fixed tissues (Yoon and Stevenson, 2002). Although those techniques are rapid, inexpensive and specific, but the sensitivity is depended on a PRRSV-specific monoclonal antibody used in each test.

Commonly, the virus can be isolated by culturing with pulmonary alveolar macrophages (PAMs) or African monkey kidney cell line, especially MA-104 clone identified as MARC-145, from many tissues of clinical specimens such as lymph nodes, tonsil and lung. Moreover, other cell lines such as CL2621 and CRL11171 have been reported to support virus replication in vitro (Bautista et al., 1993; Yoon and Stevenson, 2002). However, a previous report also suggested that PAMs were more sensitive for PRRSV isolation than any other continuous cell lines (Bautista et al., 1993). That report gave the possible reason that antibody may enhance PRRSV to replicate in PAMs.

The detection of serum antibodies using Indirected immunofluorescense antibody test (IFA), Enzyme linked immunosorbent assay (ELISA), Serum neutralization test (SN) or Immunoperoxidase monolayer assay (IPMA) have also been reported. However, the sensitivity and specificity are varied in each test. The IFA test was thought to have high specificity (99.5%) (Yoon and Stevenson, 2002). Similarly, the IPMA was also considered to be highly specific and sensitive. A previous report has shown that IPMA was more sensitive than that of ELISA (Nodelijk et al., 1996). However, depending on the low critical value of IPMA to consider the positive results comparable to the reliability and the easier in using of the commercial ELISA kit, the ELISA has commonly used in many laboratories (Nodelijk et al., 1996; Yoon and Stevenson, 2002).

Similarly, Reverse transcriptase - Polymerase chain reaction (RT-PCR) for viral genomic detection is wildly used in many laboratories (Larochelle et al. 1997; Spagnuolo et al., 1998; Witte et al., 2000; Benson et al., 2002; Yoon et al., 2002). In addition, it has been reported that PCR was suitable for PRRSV detection from boar semen since the cytotoxicity to various cell line of boar semen (Christopher-Hennings et al., 1995). Recently, the comparison of virus isolation, immunohistochemistry, serology and RT-PCR

was performed in fetal samples (Benson et al., 2002). The results of that report indicated that in autolyzed specimen, RT-PCR was sensitive than that of virus isolation, immunohistochemistry or fetal serology.

Moreover, nested multiplex reverse transcriptase - polymerase chain reaction (nm RT-PCR) has been developed in order to differentiate PRRSV genotype (Thanawongnuwech et al., 2002). The nm RT-PCR is able to detect at least 10 $TCID_{50}$/ml of PRRSV (Thanawongnuwech et al., 2004). The PCR-based assays are believed to be highly sensitive and highly specific. However, false positive should be considered when used contaminated samples.

2.6 Control and prevention

PRRS has caused major damages to the pig industries all over the world. The clinical signs of PRRSV-infected pigs are highly variable due to strain and genetic variation of the virus, known as quasispecies (Rowland et al., 1999). In addition, the clinical signs of infected pigs are also variable from genetic factors and farm-management (Goldberg et al., 2000). The bigger of the herd size the more susceptible of PRRSV transmission within herd could occur and this may lead to the difficulty on controlling PRRSV infection (Mortensen et al., 2002).

Many attempts have developed to control PRRS such as segregated early weaning or vaccination (Barfoed et al., 2004). Currently, several commercial vaccines have been developed and are available in some countries. However, the efficacy of those vaccines is still questionable. In order to mimic coinfection of PRRSV with other bacterial, several studies have been established. Previous study suggested that injection the pigs with ceftiofur hydrochloride (Excenel®) was the most effective in controlling mortality associated with PRRSV and *Streptococcus suis* coinfection. Interestingly, the PRRSV vaccinated group had higher numbers in mortality rate compared to the untreated positive control group (Halbur et al., 2000).

The key success for prevention and control of PRRSV is still unclear. However, understanding of the route of PRRSV-transmission is necessary. Currently, several

known routes of transmission include exposure with infected pigs, aerosol transmission, insemination with PRRSV-infected semen or transmission via fomites (Albina, 1997; Dee et al, 2003). It should be noted that the role of transmission by vectors is not clearly demonstrated.

In conclusion, since the successful model to control of PRRS in pig farms is no longer solution, the good farm management and the prevention of PRRSV-positive herd from secondary bacterial infection is the way that can minimized the losses from this agent.

2.7 Summary

Since emerging in 1980s, porcine reproductive and respiratory syndrome (PRRS) has become a major pig disease throughout the pig producing areas of the world. The impact of PRRS results in economic losses due to prolong to market of finishing pigs. The etiologic agent of this syndrome has been identified as a member of genus *Arterivirus*, order Nidovirales. PRRS is an enveloped, positive-sense, single stranded RNA virus (Yoon, 2002). Several reports have shown that the virus primarily infect the cells of mononuclear phagocytic system especially pulmonary alveolar macrophages (PAMs) (Thanawongnuwech et al., 2000b; Yoon, 2002). PRRSV infection induces apoptosis and cell lysis leading to decreasing of phagocytic activity and bactericidal activity of those cells (Thanawongnuwech et al., 2000b). Subsequently, PRRSV- infected pigs will be more susceptible to secondary bacterial infection.

The success of prevention and control of PRRSV infection is still under the current investigation. Several applications including vaccines, management and other treatments have shown that there is no absolute model for controlling this disease. In addition, the control of PRRSV transmission in pig farm is one of the most importance strategies to control the infection. This study intends to partly fulfill the need and expects to gain more information regarding the transmission of PRRSV via mosquito vectors in a pig farm in Thailand.

2.8 Objectives of the study

The objective of this study is to survey and identify the mosquito species found in the pig farm in Nakhon Pathom province, Thailand and to determine the potential vector of PRRSV by *Culex spp.* captured from a PRRSV-positive pig farm in Nakhon Pathom province, Thailand.

CHARPTER III

MATERIALS AND METHODS

In order to survey and identify the mosquito species found in the pig farm, and to determine whether PRRSV could be transmitted from PRRSV-infected pigs to naive pigs by mosquitoes, 3 experiments were established.

Experiment 3.1: Mosquitoes survey and mosquito colonization

3.1.1 Survey and identification of mosquito species

During March – August, 2004, mosquitoes were captured once a month from a PRRSV-positive pig farm in Nakhon Pathom province, Thailand, during 18.00-21.00 pm. The mosquitoes were collected by using mouth aspirator while feeding on the pig. After that the mosquitoes were kept in small plastic cages (3x5 cm.), 200 mosquitoes/cage (Figure 3), and then brought to the Insectary room, Veterinary Parasitology Unit, Chulalongkorn University. Species identification was done by using illustrated keys of the medically important mosquitoes of Thailand (Rattanarithikul et.al., 1994), and the number of mosquitoes in each species was counted and recorded. In addition, the total of 50 mosquitoes/species were randomly selected and tested for the presence of PRRSV right after capturing and tested again after 7 days.

3.1.2 Mosquitoes colonization

In order to establish the colony of the predominant mosquito species, *Culex tritaeniorhynchus*, all captured mosquitoes were reared in the insectary at the temperature of 25 – 39 °C with 80 – 90% relative humidity. The female mosquitoes were allowed to lay eggs on the decholinated water. Then, the eggs rafts were transfered to the plastic tray (15x30 cm.) containing decholinated water and rice straw. Eggs were allowed to develop into larvae and pupae, respectively. Subsequently, 200 pupas were collected with wild-mouthed dropper and transferred to a small plastic cup containing decholinated water and kept in mosquito cages (30x30x30 cm.). Emerging adult males

and females mosquitoes were fed and maintained with 10% sucrose solution. Mice were used as a source of blood meal for mosquitoes when eggs were needed for maintain of colony.

Figure 3. Plastic cages containing mosquitoes captured from a pig farm.

Experiment 3.2: Assessment of the presence of PRRSV within mosquitoes after feeding on PRRSV-infected pig.

3.2.1 Source of mosquitoes.

All mosquitoes used in this study were from an established colony of *Culex tritaeniorhynchus* reared at 25 – 39 °C with 80 – 90% relative humidity as described above. The mosquitoes were divided into 2 cages. All mosquitoes were starved for 6-8 hours to ensure feeding success.

3.2.2 Experimental pigs.

Two 3 week old piglets were purchased from a commercial, PRRSV free herd. Upon arrival, the pigs were tested for the presence of antibodies of Swine fever virus by neutralizing peroxidase link assay (NPLA), and Pseodorabies virus and PRRSV by Enzyme linked immunosorbent assay (ELISA). Nested multiplex reverse transcriptase - polymerase chain reaction (Nested multiplex RT-PCR) was also done using pooled sera to verify their PRRSV – negative status. Pigs were housed in a separated room at the

isolation facility, Faculty of Veterinary Science, Chulalongkorn University. This facility consisted of a series of rooms that were ventilated separately containing individual slurry pits to prevent the cross – contamination of the pathogens between groups. All pigs were received the Ceftiofur injection (Excenel® kindly provided by Pfizer animal health,Thailand) 3 days prior to initiated the studies. After acclimatization for 3 days, a pig in the infected group was inoculated intranasally with 4 ml of the US genotype PRRSV (O1NP1) at a concentration of 10^4 $TCID_{50}$/ml. The other group was served as a negative control group. Animals were cared following the standards of the Animal care committee, Faculty of Veterinary Science, Chulalongkorn University. In order to prevent cross contamination of PRRSV between groups, stricted biosecurity measures were implemented.

3.2.3 Experimental protocol.

At 7 Days post infection (DPI), the mosquito infection model was performed. The time period was selected based on the previous published data indicating peaked PRRS – viremia (Panprapa. 2004). To allow mosquitoes to feed on infected pigs, pigs in both PRRSV-infected group and negative control group were anesthesied with Pentobarbital sodium (20 mg/kg. body weight). The anesthesied pigs was placed upon the mosquitoes cages, and then mosquitoes were allowed to feed on each pig through the mesh roof of the cage for 30 minutes (Appendix B). Blood sampling from the pigs was also done for viral titration at the same time. A total of 300 engorged female mosquitoes in each group were collected and placed in a new mosquito cages. The full-fed mosquitoes were kept in an insectary room and were given 10% sucrose solution until used. PRRSV detection from fed mosquitoes was performed by using pooled 30 mosquitoes at 0, 2, 4, 6, 12, 24, 48, 72 h and 7 days after feeding on the pigs. At each time point, the mosquitoes were knocked with low temperature at appropriated time and pooled in sterile tubes. In order to prevent cross contamination, the exterior surface wash from mosquito pooled samples was performed. Pooled mosquito samples were washed with 1 ml of minimum essential medium (MEM) by centrifuging at 5000 rpm for 1 min, and dissected. The legs of mosquitoes were removed and placed in a new

microcentrifuge tube containing 1 ml of MEM. The mosquito legs were label according to each sampling time and tested for the presence of PRRSV by RT-PCR. The remaining pooled mosquitoes were crashed against the tube wall with sterile swab containing 8 ml of MEM and centrifuged at 4500 for 5 min, before collecting the supernatant. The supernatants derived from mosquito pooled samples were tested for the presence of PRRSV by both RT-PCR and viral isolation.

3.2.4 Diagnostic analysis

PRRSV antigens were detected by nmRT-PCR from the samples of exterior washing fluid, legs and homogeneous pooled samples in each time point (Thanawongnuwech et al., 2002), whereas, the viability of PRRSV was detected by virus isolation from homogeneous pooled samples.

Nested Multiplex Reverse transcriptase - Polymerase chain reaction (nmRT-PCR)

The nmRT-PCR was done for detection of PRRSV and differentiation of the genotypes. PCR mix (Promega® One step RT- PCR kit, USA) was 25 µl in volume containing Access Quick Master mix 12.5 µl, AMV reverse transcriptase enzyme 0.5 µl, Sense primer 0.5 µl, antisense primer 0.5 µl, RNase free water 8 µl and DNA template 3 µl. In this study, the outer primers for ORF1b of PRRSV were 5'-: AGG TCC TCG AAC TTG AGC TG-3' (sense) and 5'-CCT CCT GTA TGA ACT TGC-3' (antisense) (Thanawongnuwech et al., 2002). Then, the PCR mix was placed in a thermoregulator Thermohybaid (Hybaid, UK) and the PCR condition was modified using the following programs : reverse transcription at 50°C for 30 min, initial PCR reaction at 95 °C for 30 min, denaturation at 94 °C for 20 sec, primer annealing at 50 °C for 30 sec and primer extension at 72 °C for 30 sec for 35 cycle, final extension at 72 °C for 15 min and holding at 4 °C (Thanawongnuwech et al. 2002). The PCR product was then subjected to test by the multiplex PCR. The master mix for Taq DNA polymerase (Fermentus, Canada) was also prepared at 25 µl in volume containing 10X buffer PCR 5 µl, 10 mM dNTP mix 1 µl, 25 mM of $MgCL_2$ 1.5 µl, RNAse free water 13 µl, Taq polymerase (5U/ul) 0.5 µl, 0.5 µl of primer sense and 0.5 µl of primer antisense of US genotype, 0.5 µl of primer sense

and 0.5 µl of primer antisense of the EU genotype and DNA template 2 µl. In this study, the primers used for the US genotype were 5'- GTA TGA ACT TGC AGG ATG-3' (sense) and 5'- GGA GCA GTG ACT AAG AGA-3' (antisense), whereas the primers used for EU genotype were 5'- GCC GAC AAT ACC ATG TGC TG-3' (sense) and 5'- GTA ACT GAA CAC CAT ATG CTG-3' (antisense). The PCR mix was placed in a thermoregulator Thermohybaid (Hybaid, UK) and the PCR condition was modified with the following programs : initial PCR activation at 94 °C for 3 min, denaturation at 94 °C for 20 sec, primer annealing at 48 °C for 30 sec and primer extension at 72 °C for 30 sec for 35 cycle, final extension at 72 °C for 15 min and holding at 4 °C (Thanawongnuwech et al., 2002). The PCR products were detected using electrophoresis at 100 V, 500 A for 65 min in 2% agarose gel (Gene pure, Spain). The gel electrophoresis was stained with ethidium bromide (Promega, USA) for 15 min and then placed in distilled water. The PCR products of ORF1b were analyzed by the UV illuminator and interpreted as a US genotype (107 bp) or an EU genotype (186 bp).

Virus isolation and Immunoperoxidase monolayer assay (IMPA)

Briefly, MARC-145 cells, were trypsinizes and separated into individual cell in minimum essential media (MEM) (Hyclone, USA) using 5 % fetal calf serum (Biowest, France), filled in a 96 well plate (Corning incorporated, USA), 200 µl / well, and incubated in 5% CO_2 at 37 °C for 48 hr or until monolayer was seen. Sample was inoculated in each well 100 µl each and was left for 1 hr at 37 °C at 5% CO_2 before filling with 2% fetal calf serum in MEM. One hundred µl of the supernatant from the 2nd passage was inoculated onto the cell line and incubated for 1 hour at 37 °C. Then supernatant was discarded and MEM with 2 % fetal calf serum was placed onto the cell, 200 µl / well, and then incubated in 5% CO2 at 37 °C for 48 hr. At least 3 passages were done in order to confirm the presence of PRRSV in the sample.

The plate, then was fixed with 4% formalin 50 µl / well at room temperature for 30 min and washed 3 times with 0.5% PBST*. The N protein of PRRSV was detected using the monoclonal antibody ISU15 (Kindly provided by Dr. K.B.Platt, Iowa State University, USA). The ISU15 was diluted with 1%BSA (Sigma, Germany) in 0.5%PBST

(1:300) , and 50 µl of these dilution was filled in each well and incubated at room temperature for 60 min. Then, 50 µl of the conjugated anti-mouse immunoglobulin G (Dako, Denmark) (1:300) was added into each well and incubated at room temperature for 60 min. Then, the plate was washed 3 times with 0.5% PBST followed by filling of DAB substrate, 100 µl / well in room temperature for 30 – 60 min and washed with tab water. The positive cells were seen with dark brown granule in the cytoplasm under inverted light microscope.

Experiment 3.3: Experiment of PRRSV transmission from PRRSV-infected pigs to naive pigs by *Culex tritaeniorhynchus* .

3.3.1 Mosquitoes

The colony of *Culex tritaeniorhynchus* was colonized and raring as described previously at the Insectary room, Veterinary Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University. Initially, a total of 50 mosquitoes were randomly selected and tested for the presence of PRRSV by RT-PCR. The adult were staved for 6-8 hours before to ensure the feeding success.

3.3.2 Experimental pigs

Ten 3 week old piglets were purchased from the same source as the experiment 2, and were tested similarly to ensure of being negative for PRRSV. The pigs were divided into 5 groups, 2 pigs each. Group A was a PRRSV-infected group (Donor pigs), Group B to E were the recipient pigs. In each group, pigs were labeled according to the experimental designs as pig 1 for mosquito-contact protocol and pig 2 for swine bioassay, and then housed in a separated room in the isolation facility, Faculty of Veterinary Science, Chulalongkorn University. This facility consists of series of rooms that were ventilated separately and contained individual slurry pits to prevent the cross – contamination of the pathogens between groups. The animals were cared following the standards of the Animal care committee of Faculty of Veterinary Science, Chulalongkorn University entire the study. After quarantine for 3 days and tested for the PRRSV-

negative status, the pig in group A was inoculated intranasally with 4 ml of PRRSV, the US genotype (01NP1) at a concentration of 10^4 $TCID_{50}$/ml.

3.3.3. Attempts to transmit PRRSV from PRRSV-infected group (Donor pig) to the Recipient pigs using *Culex tritaeniorhynchus*.

At day 7 post infection, the peaked PRRS – viremia was expected according to the previous study (Panprapa, 2004). The PRRSV-infected pigs (group A) were anesthesized with Pentobarbital sodium, blood sampling was performed and the mosquitoes were allowed to feed on the infected pigs. During feeding on the infected pigs, the mosquitoes were interrupted and the total of 150 mosquitoes were collected and placed in a new small plastic cage. A total of 4 small plastic cages of mosquitoes were placed in a humidity incubation room. The attempts of PRRSV transmission by mosquitoes were performed in pigs in group B to E at appropriated time as mentioned below.

Approximately 30 minutes post feeding on infected pigs, a total of 100 mosquitoes was allowed to feed on the pig number 1 (group B) similar to the donor pigs. A total of 50 remaining mosquitoes are tested for the presence of infectious PRRSV using swine bioassay by intramuscular injection of the grounded filtered mosquitoes into the remaining pig (Pig 2) as described by Stewart et al. (1975). Similarly, the other 3 small plastic cages containing partially fed mosquitoes, were allowed to feed on the recipient pigs in group C, D and E at the 6, 24 hrs. and 7 days post feeding, respectively.

3.3.4 PRRSV detection.

PRRSV detection was performed on sera of the recipient pigs at 3, 7, 14 and 21 days post-contact with mosquitoes using either RT-PCR or ELISA. On day 14 post infection all pigs were euthanasized with overdose of Pentobarbital sodium and necropsied. Samples from bronchial alveolar larvage, lungs and lymph node were collected for viral isolation and RT-PCR in each pig. The same organs from each pig

were also collected and fixed with 10% buffer formalin for histopathology and immunohistochemistry detection.

Immunohistochemistry

Briefly, sections on poly-L-lysine coated slide were deparafinized and rehydrated using step xylene and ethanol, DW and PBS, respectively. Tissues were digested with trypsin at 37°C for 30 min and washed with PBS 3 times. The endogenous peroxidase enzyme was removed by using 3% H_2O_2 in methanol for 30 min at room temperature, then with DW 5 min and with PBS 3 times. Nonspecific background staining was blocked with 10%BSA (Sigma, USA) in DW at 37°C for 30 min and then washed with PBS 3 times. PRRSV antigens reacted with monoclonal mouse anti-PRRSV antibody (SDOW17, USA, kindly provided by Dr. E. Thacker, Ames, Iowa) using 1:1000 at 4°C overnight and then washed with PBS 3 times. The IgG was attached with peroxidase antiperoxidase (Nichirel, Japan) at 37°C for 1 hour and then washed with PBS 3 times. After that Diminobenzidine tetra chloride (DAB) solution preparing before reacted with Ag-Ab complex and yielded the brown granular staining in the cytoplasm of PRRSV-infected cell considered as a positive cell. The reaction was stopped by rinsing with DW. The slides were counter stained with mayer hematoxylin for 30 second and then dehydrated and graded in ethanol and xylene, respectively.

CHARPTER IV

RESULTS

Experiment 1: Mosquito surveys from the pig farm

The results of mosquito species identification from a pig farm in Nakhon Pathom Province, Thailand during March, 2004 to August, 2004 are shown in table 1. The total number of mosquitoes collected by approximately 6 peoples in each month was identified as *Culex tritaeniorhynchus*, *Culex gelidus*, *Anopheles spp*. and *Mansonia uniformis*. The mosquito population, in general, was highest during the study (July and June) (Table 1). The percentage average of density in each species was 85.68%, 12.13%, 2.06% and 0.13%, respectively. The techniques of mosquito collection in the pig farm are shown in appendix B. The predominant mosquito species in this farm was *Culex tritaeniorhynchus* during the study. This specie was used as a model in the following studies.

Table 1. Mosquito density and species identification from the pig farm in Nakhon Pathom Province, Thailand.

	Number of mosquitoes	Species identification			
2004		*Ct*	*Cg*	*Anopheles.spp*	*M.uniformis*
March	4,200	78.92 %	19.73 %	1.28 %	0.07 %
April	5,400	93.67 %	0.78 %	5.50 %	0.05 %
May	5,400	92.68 %	4.72 %	2.59 %	0.01 %
June	8,000	69.44 %	29.76 %	0.69 %	0.11 %
July	11,600	88.53 %	10.00 %	1.06 %	0.41 %
August	7,600	90.83 %	7.80 %	1.24 %	0.13 %
Total	42,200	85.68 %	12.13 %	2.06 %	0.13 %

Ct = Culex tritaeniorhynchus *Cg = Culex gelidus*

Experiment 2: Assessment of the duration of PRRSV within mosquitoes after feeding on PRRSV-infected pig.

On day 7 post infection, the presence of PRRSV viremia was detected in the infected pigs and had a viral titer at $10^{2.70}$ $TCID_{50}$/ml. About 30 mosquitoes (*Culex tritaeniorhynchus*) pooled sample were tested for PRRSV by RT-PCR and found negative before used. Then, the mosquitoes (*Culex tritaeniorhynchus*) were allowed to feed on an infected-pig for 30 minutes. After feeding, 300 engorged mosquitoes were collected and placed in a new small plastic cages, fed with 10% sucrose solution and placed at the Insectary room, Veterinary Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University. In order to test for the presence of PRRSV, a total of 30 mosquitoes were collected and tested by either RT-PCR or Virus isolation in each time point at 0, 2, 4, 6, 12, 24, 48, 72 hr. 7 and 14 days post feeding. The results are summarized in table 2. Pooled mosquito legs samples and pooled washing fluid from exterior surface of the mosquito samples were also tested for PRRSV by RT-PCR and found negative. The mosquito pooled samples from the samples at 0 hr to 48 hr post feeding were tested positive for PRRSV by RT-PCR, whereas virus isolation was able to detect the infectious PRRSV from mosquito pooled samples only at 0 hr and 2 hr post feeding. The positive samples for RT-PCR had PCR products at 107 bp as shown in figure 4, while the samples positive for virus isolation using MARC-145 cell line was shown in figure 5.

Table 2. The presence of PRRSV in mosquitoes after feeding on PRRSV-infected pigs

	RT-PCR			VI
Time post feeding on infected-pig	Whole body	Legs	Wash	
0 hour	+ ve	- ve	- ve	+ ve
2 hours	+ ve	- ve	- ve	+ ve
4 hours	+ ve	- ve	- ve	- ve
6 hours	+ ve	- ve	- ve	- ve
12 hours	+ ve	- ve	- ve	- ve
24 hours	+ ve	- ve	- ve	- ve
48 hours	+ ve	- ve	- ve	- ve
72 hours	- ve	- ve	- ve	- ve
7 days	- ve	- ve	- ve	- ve
14 days	- ve	- ve	- ve	- ve

RT-PCR = nested multiplex reverse transcriptase polymerase chain reaction

Wash = washing fluid from exterior surface of mosquitoes

Legs = leg of mosquitoes (pooled sample)

VI = virus isolation from homogeneous mosquito pooled sample

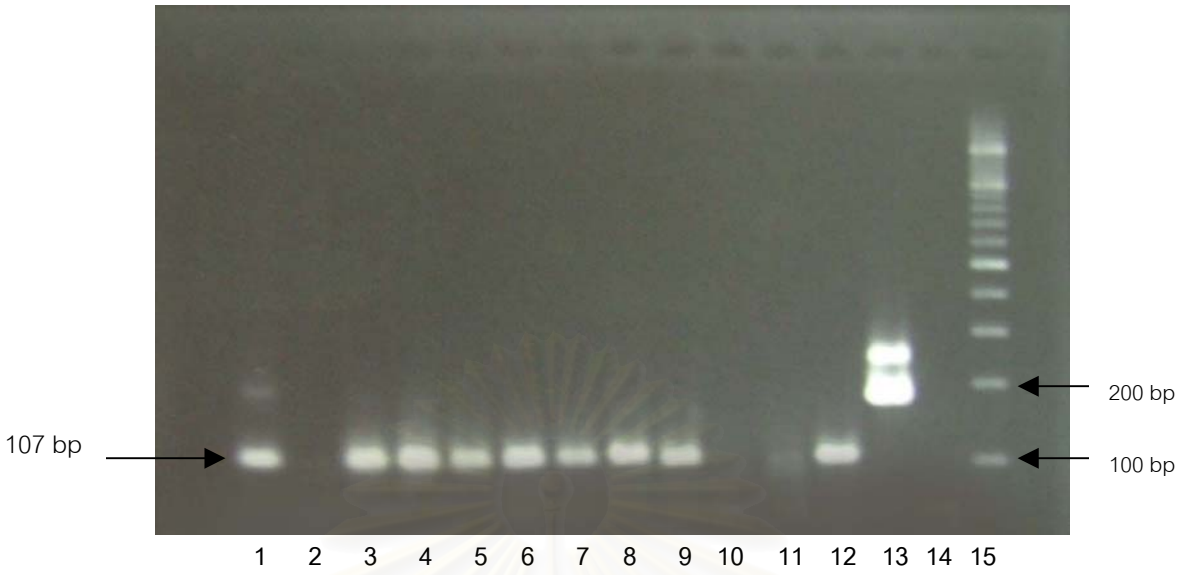


Figure 4: Nested multiplex PCR for PRRSV. Lanes: 1, PRRSV-infected pig serum; 2, negative control of *C. tritaeniorhynchus*. Lanes 3-11 is the pooled sample of *C. tritaeniorhynchus* at 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 7 days. Lane: 12, US PRRSV positive; 13, EU PRRSV; 14, negative control; L, DNA ladder.

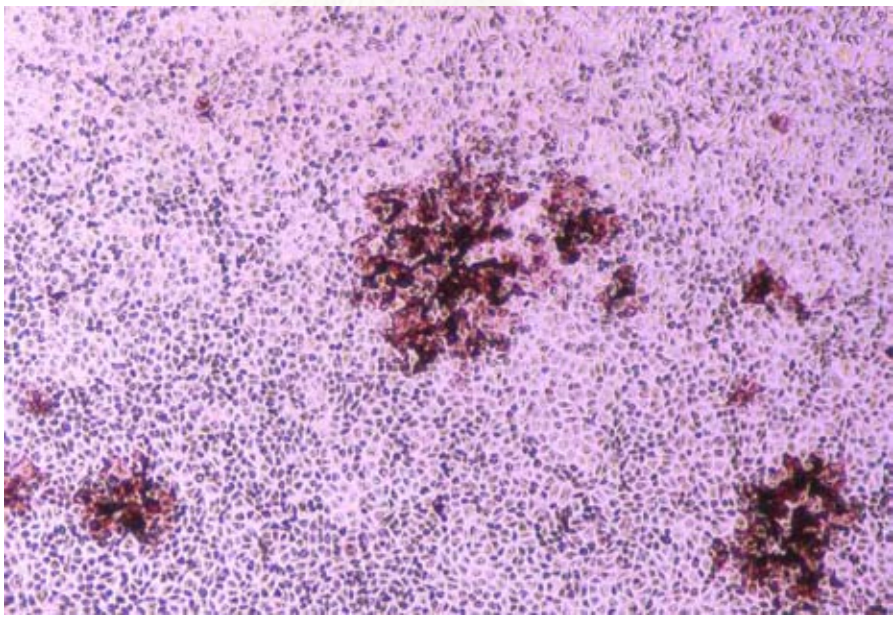


Figure 5: Immunoperoxidase monolayer assay revealing the presence of PRRSV by red granules staining in the infected cells. (20x)

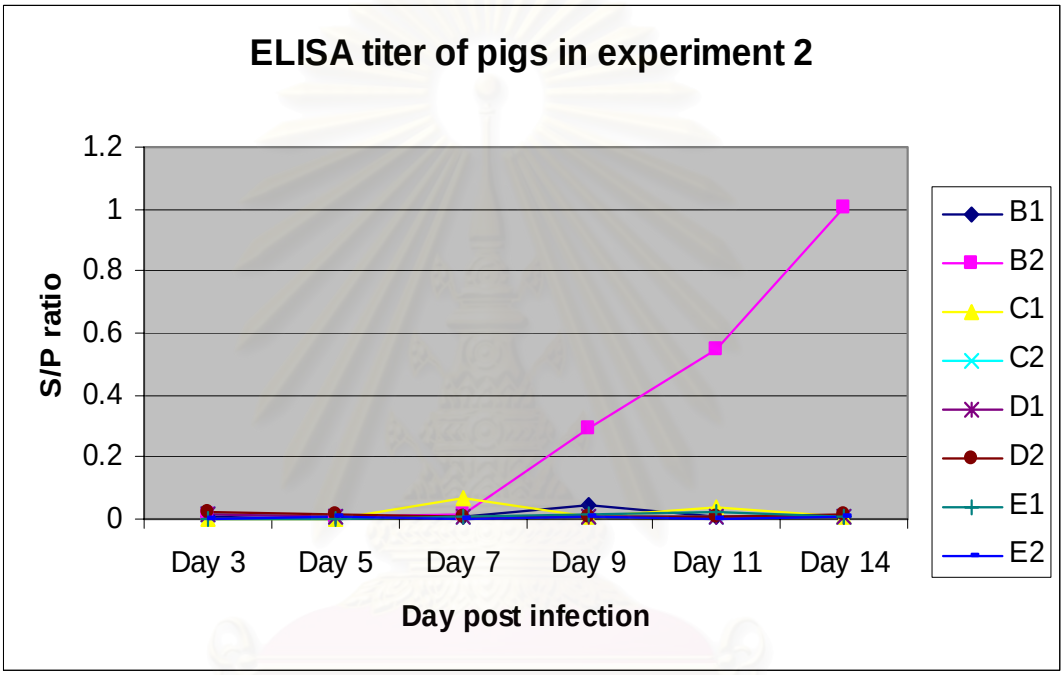
Experiment 3: PRRSV transmission from donor pigs to recipient pigs by *Culex tritaeniorhynchus* .

In order to confirm the presence of PRRSV-negative status of mosquitoes (*Culex tritaeniorhynchus*), a total of 30 female mosquitoes were collected and tested by RT-PCR. The donor pig had PRRSV-viremia at the time of mosquitoes contact protocol taken place (day 7 post infection) with the virus titer of $10^{2.23}$ $TCID_{50}$/ml. All recipient pigs were tested negative for PRRSV by both ELISA and RT-PCR. After feeding on the donor pigs, the mosquitoes fed on the recipient pigs were homogenized and injected to the swine bioassay in pigs group B (30 min), group C (6 hrs), group D (7 days) and group E (14 days) one pig each, respectively. Briefly, a total of 100 mosquitoes which partially fed on an infected-pig were transferred to a new small plastic cage and allowed to feed on the pig number 1 in each group at the appropriated time above. Another set of 50 mosquitoes were used in the swine bioassay to pig number 2 in each group.

On day 14 post contact with infected mosquitoes, all recipient pigs were euthanasized and necropsied. Macroscopic findings of PRRSV-infected pigs (group A) showed multifocal tan-mottled consolidation involving 8% of the lungs (Figure 7), associated with tracheobronchial lymph node enlargement. PRRSV antigen in the infected lung was confirmed using immunohistochemistry (IHC). Secondary bacterial infection was found in the pig of group E, while the other pig had no-remarkable lesion. Only the recipient pig from group B (pig number 2 or B2 pig) was positive for PRRSV swine bioassay, detected by ELISA, RT-PCR and virus isolation, whereas the other swine bioassay pigs had no evidence of the presence of PRRSV infection when detected by both ELISA and RT-PCR (Figure 6). The ELISA result of B2 pig revealed positive results for PRRSV since day 10 post injection by homogenated mosquitoes.

The result of virus isolation was positive only from the serum sample of B2 pig, whereas samples from bronchial alveolar larvage, lymph nodes and lungs were negative in all pigs. In addition, immunohistochemistry results from the lung were negative in all pigs.

Figure 6: Enzyme linked immunosorbent assay (ELISA) in the recipient pigs.



S/P ratio $\geq$ 0.04 considered positive

B1,C1,D1 and E1 pigs were mosquito-contact protocol pigs.

B2,C2,D2 and E2 pigs were swine bioassay pigs.

Table 4: PRRSV results in the recipient pigs at necropsy.

Group	PCR			VI			IHC
	serum	larvage	organs	serum	larvage	organs	
3o min. pf							
B1	- ve	- ve	- ve	- ve	- ve	- ve	- ve
B2	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve
6 hours. pf							
C1	- ve	- ve	- ve	- ve	- ve	- ve	- ve
C2	- ve	- ve	- ve	- ve	- ve	- ve	- ve
24 hours. pf							
D1	- ve	- ve	- ve	- ve	- ve	- ve	- ve
D2	- ve	- ve	- ve	- ve	- ve	- ve	- ve
7 days. pf							
E1	- ve	- ve	- ve	- ve	- ve	- ve	- ve
E2	- ve	- ve	- ve	- ve	- ve	- ve	- ve

pf = time post feeding, RT-PCR = nested multiplex reverse transcriptase polymerase chain reaction
VI = virus isolation IHC = immunohistochemistry, ELISA = enzyme linked immunosorbent assay
larvage = bronchial alveolar larvage, Organs = lymph nodes and lungs

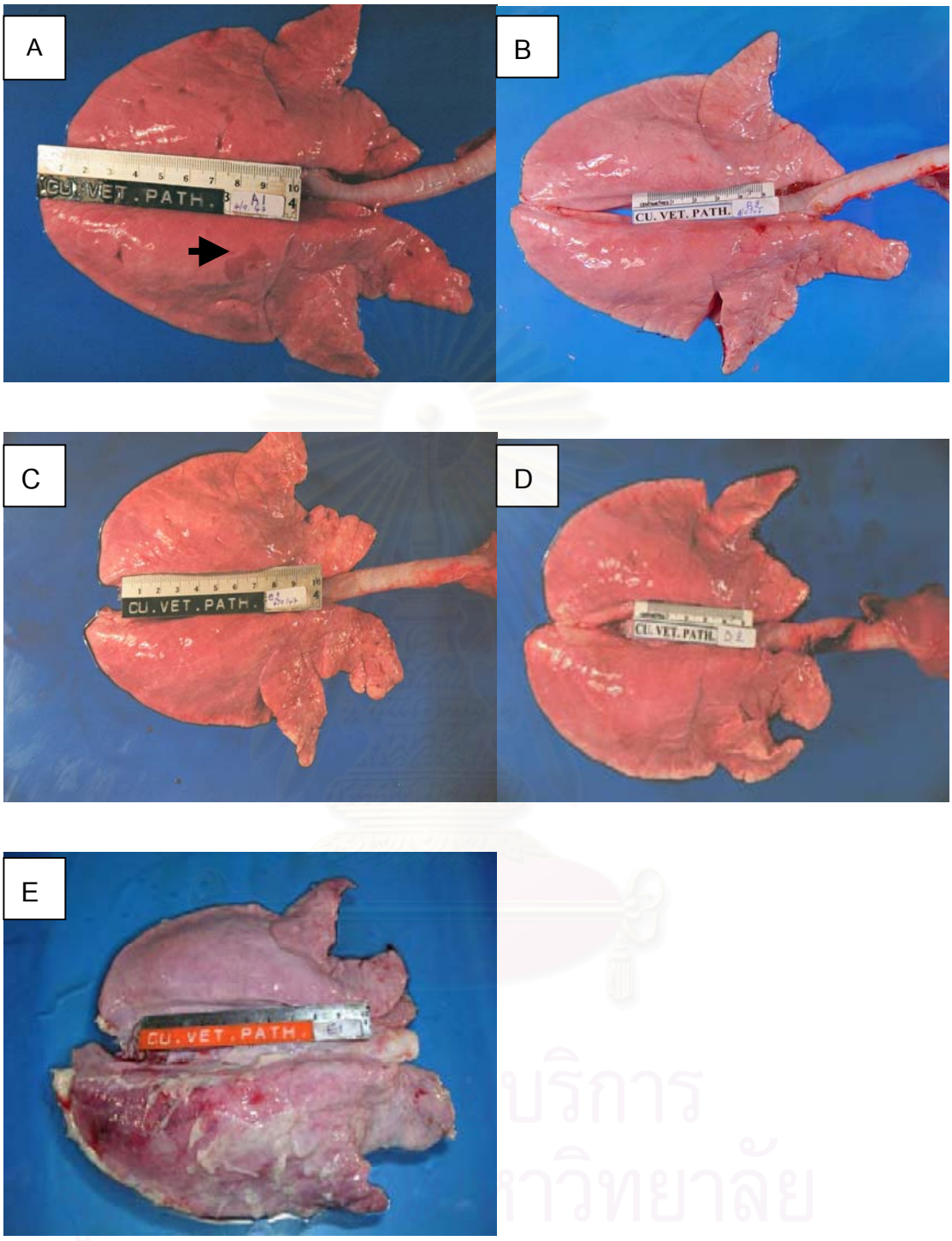


Figure 7: Lung, pig; 14 day post inoculation with 01NP1 PRRSV (A) and 14 days post contact with infected mosquitoes (B-E). Mild multifocal tan-mottled consolidation of lung in group A (A, arrows), whereas others showed no-remarkable lesion (B-D). The pig in group E had secondary bacterial infection with no evidence of PRRSV-induced lesion.

CHARPTER V

DISCUSSION

The results of this study strongly suggested that *Culex tritaeniorhynchus*, a predominant mosquito species seen in a pig farm, could serve as a potential mechanical vector for PRRSV in a pig farm.

In experiment 1, the survey of mosquito species found in a pig farm showed that *Culex tritaeniorhynchus* was the predominant specie seen in the pig farm, *Culex gelidus, Anopheles* spp. and *Mansonia uniformis* were regularly seen in the pig farm in Nakhon Pathom Province but in less number. This study was performed monthly during March – August 2004. Importantly, the results of this study correlate with the previous report in Malaysia that *Culex tritaeniorhynchus* was the predominant mosquito specie founded in the pig farms following by *Culex gelidus*, *Anopheles* spp. and *Mansonia spp.*, respectively (Vythilingam et al., 1994). As a result, there was a suitable breeding place for *Culex tritaeniorhynchus* around the selected farm. Although, it has been reported that the prevalence of *Culex* spp. was high following the rainfall pattern and varied in seasonal changed (Vythilingam et al., 1994), the presence of numerous breeding places such as paddy-field, tanks, ponds, etc may serve as the alternative breeding places for *Culex* spp. (Vythilingam et al., 1992; Geevarghese et al., 1994). Consequently, these may result in the highest prevalence of *Culex tritaeniorhynchus* in this farm. The abundance of vector species and its seasonal variation might have a bearing on disease transmission in pig farm; since, Macdonald et al (1967) showed that mosquitoes, *Culex tritaeniorhynchus,* have a preference on pigs more readily than man. Moreover, the result of our preliminary study indicated that PRRSV could detect within mosquitoes right after capturing from this farm by using RT-PCR (data not show). As a results, we hypothezied whether mosquitoes, *Culex tritaeniorhynchus*, could serve as a potential vector for PRRSV transmission in the pig farm.

In experiment 2, we confirmed our previous report that PRRSV could detected from the pooled of mosquito samples (*Culex tritaeniorhynchus*) for up to 48 hours post

feeding on a PRRSV-infected pig by RT-PCR technique. No evidence of virus multiplication was detected over a 14 - days period after feeding on a PRRSV-infected pig. In general, the positive for RT-PCR indicated the presence of genetic materials of PRRSV, but it does not necessary indicating the presence of the infectious PRRSV (Benson et al., 2002; Yoon and Stevenson, 2002). Therefore, we confirmed the presence of infectious PRRSV by virus isolation. Interestingly, the result of our study showed that the infectious virus can survive within the mosquitoes (*Culex tritaeniorhynchus*) for up to 2 hours after feeding on a PRRSV-infected pig. However, the virus isolation from other mosquito pooled samples at 4, 6, 12, 24, 48, 72 hrs, 7 or 14 days post feeding were negative. Since the extrinsic incubation periods of mosquito-born virus were approximately 5 to 14 days post feeding on the infected animals (Stewart et al., 1975; Beerntsen et al., 2000), it should be noted that PRRS virus replication had not occurred within the mosquitoes (*Culex tritaeniorhynchus*).

In contrast to the previous report that PRRSV found to persist within the gut of mosquitoes (*A. vaxans*) up to 6 hours after feeding on a PRRSV-infected pig (Otake et al, 2003a). The disagreement of the present study and that findings could be explained. One possible explanation is the specie of mosquitoes used in the study. The difference of mosquito species leads to the difference of carrying pathogens (Beerntsen et al., 2000). Moreover, the results of the previous study did not show the PRRSV titer of the PRRSV-infected pig. It should be noted that, the difference of PRRSV viremia in each experiment might lead to the difference in survival time of PRRSV within mosquitoes. In addition, several previous reports indicated that PRRSV titer of the PRRSV-infected-pig may vary widely depending either the age of the pig (Thanawongnuwech et al., 1998) or the strain of PRRS virus (Johnson et al., 2004).

The presence of infectious PRRSV for a longer time period within mosquitoes could determine that the mosquitoes might serve as the biological vectors for PRRSV. Since the character of the biological vector of mosquito-born virus is determined by the extrinsic incubation period, which is the period between the initial feeding and the time at which the mosquitoes are capable for infection. The extrinsic incubation period is variable depending on the genetic of virus, initial dose of virus, mosquito species and

environmental temperature (Meller, 2000). Moreover, the replication of the virus within the mosquitoes until reached a sufficient concentration for transmission, and the presence of the virus in hemocoel are the major characteristics of a biological vector (Beerntsen et al., 2000). Therefore, the presence of PRRSV at the hemolymph-filled hemocoel such as the legs of mosquitoes could determine the biological characteristic for the virus. However, this study demonstrated that there was no evidence of PRRSV within the legs of mosquitoes tested by RT-PCR; as well as, the inability to detect PRRSV several days after feeding on the PRRSV-infected pig. Therefore, our study suggested that *Culex tritaeniorhynchus* could not serve as a biological vector for PRRSV transmission.

During the experiment, it was essential to minimize the risk of cross contamination of PRRSV by plastic containers or contaminated mosquitoes, since it has been reported that PRRSV could be detected from the contaminated container during warm condition (Dee et al., 2003). In this study, the risk was minimized by using a new plastic container at each step, and the contaminated mosquitoes were tested by testing the washing fluid from the exterior surface of the mosquitoes. No PRRSV contamination was observed in this study. In conclusion, our study suggested that the infectious PRRSV could not survive within mosquitoes for longer than 2 hours, and confirmed that mosquitoes could not serve as a biological vector for PRRSV transmission.

In experiment 3, we demonstrated whether the mosquitoes are able to transmit the infectious PRRSV from the donor pigs to the naive pigs at each time point. In order to perform this experiment, swine bioassay was done along with the mosquito to pig-contact protocol. The results of this study showed that only the pig in group B (30 minutes post feeding) was positive for PRRSV by the swine bioassay. Swine bioassay is considered to be the most sensitive test for PRRSV in live pigs (Benson et al., 2002), and only 10 or fewer infectious PRRSV particles either by intranasal or by intramuscular route is sufficient to cause infection (Yoon et al., 1999). The PRRSV positive B2 pig indicated that the virus could survive within the mosquitoes up to 30 minutes post feeding. In contrast to the swine bioassay, the pigs used in the mosquito to pig-contact protocol did not have PRRSV tested by either ELISA or RT-PCR at all times. It is indicated that the

mosquitoes, *Culex tritaeniorhynchus,* used in this study are unlikely to transmit PRRSV mechanically in the experiment or even in the field condition. However, some explanations are needed to be considered. Firstly, it is possible that the PRRSV titers of the infected donor pigs did not exceed the thresholds of infection. Secondly, the limited number of mosquitoes available to complete the mechanical transmission trials must be considered. Since the success of the mechanical transmission depends on the virus concentration in the mouthpart of the mosquitoes (Webb et al., 1989), the higher number of mosquitoes used in the experiment the more susceptible of recipient pigs to the infection is expected.

According to the results in experiment 2, the viability of PRRSV was demonstrated within the mosquitoes for up to 2 hours after feeding on an infected pig when tested by virus isolation. However, the results of the experiment 3 showed that PRRSV remained infective just 30 minutes after feeding on the infected pig. One possible explanation is the various time period using in experiment 3. In the experiment 3, we had the time period in each group as the following 30 minutes (Group B), 6 hours (Group C), 12 hours (Group D) and 7 days (Group E). During 30 minutes to 6 hrs, PRRSV might not survive due to many factors such as lower titer in the viremic pig and differences in mosquito species or temperature (Yoon and Stevenson, 2002). Therefore, only the pig in group B swine bioassay (30 minutes) was positive for PRRSV infection.

The virus isolation of B2 pig indicated that PRRS virus could be isolated from serum, whereas the results of virus isolation from bronchial alveolar larvage, lungs and lymph nodes remained negative. Firstly, in young pigs, viremia persists for a prolonged period, and PRRSV is more stable in serum than in the tissues (Yoon and Stevenson, 2002). As a result, the PRRSV isolation from the serum is more sensitive than that of from the tissue samples. Secondly, PRRSV persistence may depend on age of the pigs and the stage of infection. Although previous study has shown that more antigen of PRRSV was detected higher in the lungs, lymph nodes and tonsils (Halbur et al., 1996), the fact that PRRSV distribution depending on the stage of infection could be an explanation for that matter.

Our study also found that immunohistochemistry staining for PRRSV antigens from naive pigs were negative, whereas RT-PCR and virus isolation were positive in B2 pig. It is suggested that immunohistochemistry is less sensitive than that of RT-PCR or virus isolation. These findings agree with the previous reported that RT-PCR and virus isolation are more sensitive than that of immunohistochemistry (Benson et al., 2002).

In summary, our study indicated that *Culex tritaeniorhynchus*, a predominant mosquito species found in a pig farm in Nakhon Pathom Province, was unable to transmit PRRSV biologically. Although, the inability of *Culex tritaeniorhynchus* to transmit PRRSV mechanically from infected donor pig to susceptible pigs indicated that mosquitoes were unlikely to transmit PRRSV mechanically in the field condition. However, the positive for PRRSV by swine bioassay of B2 pig indicated that mechanical transmission could occur and *Culex tritaeniorhynchus* could serve as a potential vector for PRRSV transmission in a pig farm.

Finally, this study, along with the previous reports that suggest mosquitoes could serve as a potential vector of PRRSV in pig farm (Otake et al., 2002). Although further studies in other mosquito species are needed; however, the results could be useful for preventing PRRSV transmission by mosquitoes in the PRRSV-negative pig farms.

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APPENDICES

APPENDIX A

The reagents for experiments

1. Viral RNA extraction

QIAamp® Viral RNA Mini Kit. (QIAquick ion-exchange column chromatography)

- Buffer AVL ; containing carrier RNA
- AW1 and AW2 ; two different wash buffer, has significant improved the purity of eluted RNA
- AVE ; RNAse-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNAse.

2. Total RNA extraction

SV RNA Red blood cell lysis solution

5 mM $MgCl_2$

10 mM NaCl

10 mM Tris-HCL (ph 7.0)

3. Reverse transcriptase polymerase chain reaction

QIAGEN® (One Step RT-PCR Kit, USA)

- QIAGEN One step RT-PCR Enzyme Mix ; Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase and HotStar Tag DNA polymerase. Omniscript and Sensiscript Reverse Transcriptase are recombinant heterodimeric enzymes expressed in *E.coli* HotStar Tag DNA polymerase is a modified form of a recombinant 94-kDa DNA polymerase (deoxynucleoside-triphosphate : DNA deoxynucleotidyltransferase, EC 2.7.7.7), originally isolated from *Thermus aquqticus*, expressed in *E.coli*.
- 5xQ-solution ; 12.5 mM $MgCl_2$
- 5x QIAGEN One step RT-PCR Buffer; KCL and $(NH_4)_2so_4$

2% agarose gel

- agarose gel	2 g
- TBE (Tris-borate EDTA)	100 ml

4. PCR product purification

Wizard® SV Gel and PCR Clean-Up System. (Promega, USA)

- Membrane Binding Solution ; guanidine isothiocyanate
- Membrane Wash Solution ;

5. Viral isolation

Trypsin versence

- Stock Trypsin (2.5% trypsin)	5 ml.
- Stock versience	2.5 ml.
- PBS	92.5 ml.

6. Indirect immunoperoxidase monolayer assay

0.5% PBST

- 20x PBS	80 ml.
- DW	1,920 ml.
- Tween20	10 ml.

4% formalin in 0.5%PBST

- 40% formalin	0.4 ml.
- 0.5%PBST	9.6 ml.

1% BSA in 0.5% PBST

- BSA	0.15 g.
- 0.5%PBST	15 ml.

The SDOW-17 (1:300)

- SDOW-17	20 ul.
- 1% BSA in 0.5% PBST	6 ml.

The mouse IgG conjugate (1:300)

- The mouse IgG conjugate	20 ul
- 1%BSA in 0.5% PBST	6 ml.

Substrate for IPMA

- AEC solution	0.5 ml.

- acetate buffer 9.5 ml.
- 30% H_2O_2 25 ul.

AEC solution

- 3-amino acid-9-ethylcarbazole 80 mg.
- Dimethyl formamide 20 ml.

Acetate buffer

- 0.1 M Glacial acetic acid 21 ml.
- 0.1 M Sodium acetate 79 ml.

7. Vial titration

Trypsin versience

- Stock trypsin (2.5% trypsin) 5 ml.
- Stock versience (1% EDTA) 2.5 ml.
- PBS 92.5 ml.

8. Immunohistochemistry

DAB solution

- 0.05% 3 – 3 diaminobenzidine tetrahydrochloride
- 0.05 M Tris HCL pH 7.6
- 0.03% H_2O_2

APPENDIX B

Mosquito collection techniques

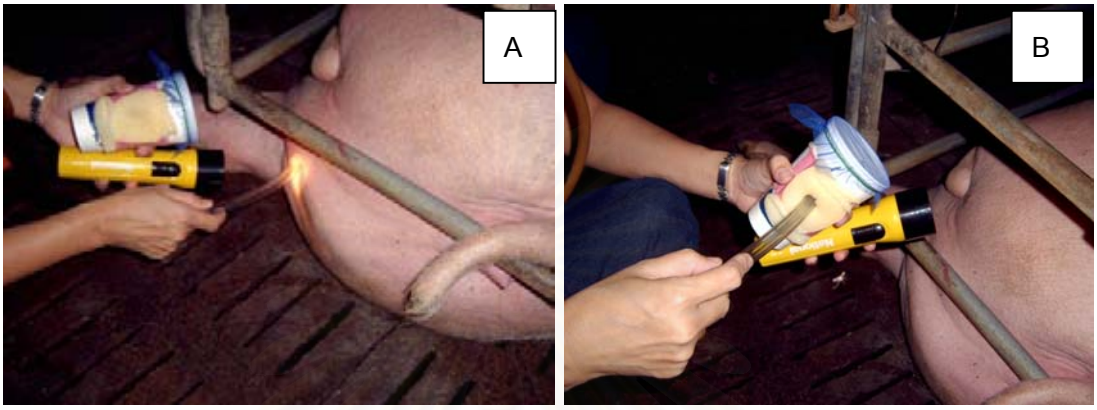

Figure A and B: Mosquitoes collection from the pig farm by using mouth aspirator

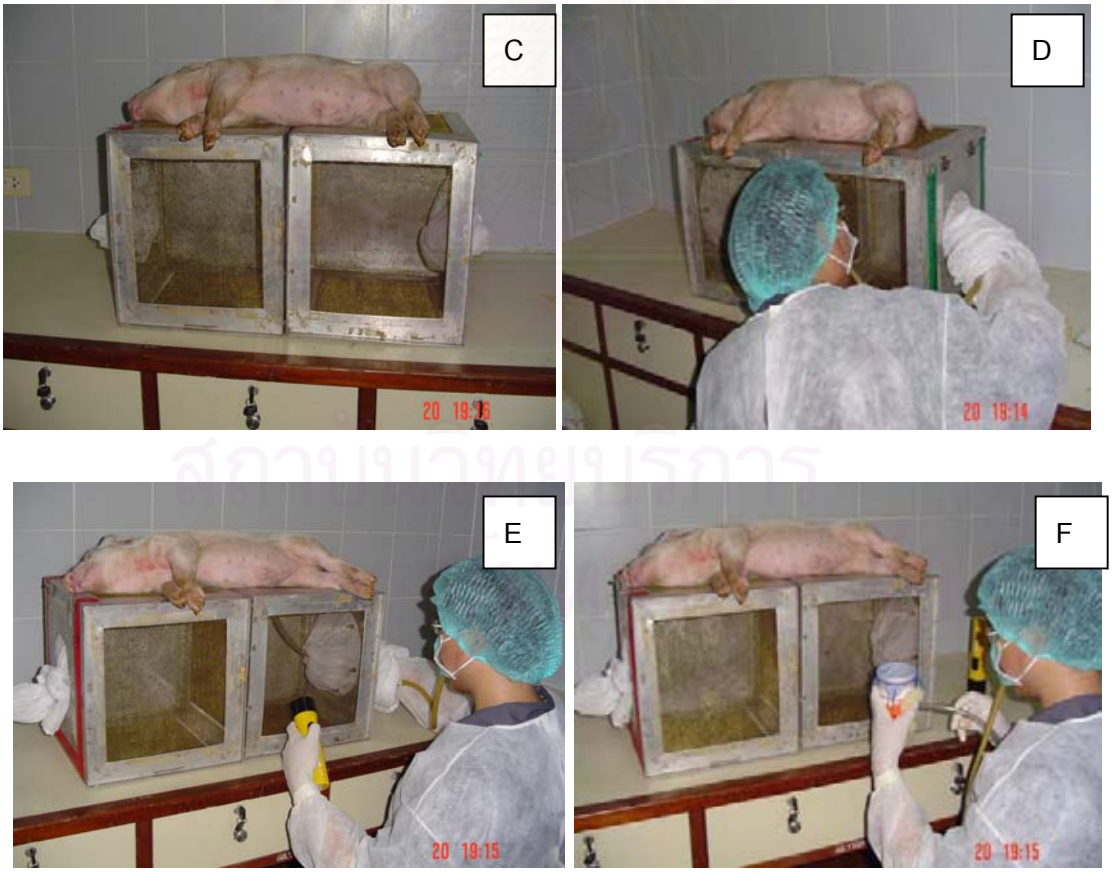

Figure C to F: The procedure for mosquitoes collection from the PRRSV-infected pig.

VITAE

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