ประสิทธิภาพวัคซีนชนิดเชื้อเป็นสายพันธุ์ย่อย 8 ในการต้านเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงที่แยกได้จากประเทศไทย

นายพลกฤษณ์ เจริญชนิกานต์

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EFFICACY OF A MODIFIED LIVE-VIRUS (MLV) VACCINE OF LINEAGE 8 AGAINST A THAI HIGHLY PATHOGENIC PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (HP-PRRSV) CHALLENGE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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พลกฤษณ์ เจริญชนิกานต์ : ประสิทธิภาพวัคซีนชนิดเชื้อเป็นสายพันธุ์ย่อย 8 ในการต้านเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงที่แยกได้จากประเทศไทย (EFFICACY OF A MODIFIED LIVE-VIRUS (MLV) VACCINE OF LINEAGE 8 AGAINST A THAI HIGHLY PATHOGENIC PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (HP-PRRSV) CHALLENGE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. สว่าง เกษแดงสกลวุฒิ, 56 หน้า.

การศึกษาครั้งนี้ประเมินประสิทธิภาพวัคซีนชนิดเชื้อเป็นสายพันธุ์ย่อย 8 ในการต้านเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงที่แยกได้จากประเทศไทย โดยใช้สุกรปลอดเชื้อไวรัสพีอาร์อาร์เอสจำนวน 81 ตัว จากนั้นแบ่งการทดลองออกเป็น 2 การทดลองโดยมีช่วงห่างของการติดเชื้อหลังการทำวัคซีนเป็นข้อแตกต่างหลัก ซึ่งมีช่วงห่าง 42 วันหลังทำวัคซีนในการทดลองที่ 1 (n= 42) และช่วงห่าง 28 วันหลังทำวัคซีนในการทดลองที่ 2 (n= 39) แต่ละการทดลองจะมีวิธีการทดลองที่คล้ายกัน คือ สุกรจะถูกแบ่งออกเป็น 3 กลุ่มได้แก่ กลุ่มควบคุมผลลบ กลุ่มสุกรให้เชื้อแต่ไม่ฉีดวัคซีน กลุ่มสุกรให้เชื้อและฉีดวัคซีน ในสุกรกลุ่มฉีดวัคซีนจะฉีดวัคซีนเชื้อเป็นสายพันธุ์ย่อย 8 ที่อายุ 3 สัปดาห์ จากนั้นให้เชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงที่แยกได้จากประเทศไทย (10PL01) ผ่านทางจมูกทั้งในสุกรกลุ่มที่ฉีดและไม่ฉีดวัคซีน จากผลการทดลองพบว่ากลุ่มสุกรให้เชื้อและฉีดวัคซีนมีค่าเฉลี่ยของอุณหภูมิร่างกายเมื่อวัดผ่านทวารหนัก ความรุนแรงของอาการทางคลินิก คะแนนรอยโรคปอด ระดับไตเตอร์ของไวรัสในเลือดและเนื้อเยื่อปอดลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ไม่ฉีดวัคซีน ($p < 0.05$) นอกจากนี้พบว่ากลุ่มสุกรให้เชื้อและฉีดวัคซีนทั้งสองการทดลองมีอัตราการรอดชีวิตสูงกว่ากลุ่มที่ไม่ฉีดวัคซีน และพบว่ากลุ่มสุกรที่มีช่วงห่างของการติดเชื้อหลังการทำวัคซีน 42 วัน วัคซีนจะแสดงประสิทธิภาพดีกว่ากลุ่มสุกรที่มีช่วงห่างการติดเชื้อหลังการทำวัคซีน 28 วัน โดยสรุปวัคซีนเชื้อเป็นสายพันธุ์ย่อย 8 สามารถช่วยเพิ่มอัตราการรอดชีวิตต่อการติดเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงที่แยกได้จากประเทศไทยได้ทั้งในช่วงห่าง 42 และ 28 วันหลังทำวัคซีน

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5775309431 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: EFFICACY, HP-PRRSV, LINEAGE 8, PIGS, MLV VACCINE

PONLAKRIT CHAROENCHANIKRAN: EFFICACY OF A MODIFIED LIVE-VIRUS (MLV) VACCINE OF LINEAGE 8 AGAINST A THAI HIGHLY PATHOGENIC PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (HP-PRRSV) CHALLENGE. ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, CO-ADVISOR: ASST. PROF. DR. SAWANG KESDANGSAKONWUT, 56 pp.

To evaluate the efficacy of a new PRRS MLV of lineage 8 against a Thai HP-PRRSV (10PL01), eighty-one, PRRSV-free piglets were obtained from a PRRS-free herd and were divided into two experiments with the major difference of infection timing after vaccination, 42 days in experiment 1 (n= 42) and 28 days in experiment 2 (n= 39). Each experiment had similar protocol containing three groups including a negative control, unvaccinated challenged and vaccinated challenged groups. Pigs in vaccination groups were immunized with A PRRS MLV of lineage 8 at 3 weeks of age. Then, unvaccinated challenged and vaccinated challenged groups were intranasally inoculated with a Thai HP-PRRSV (10PL01). Vaccinated challenged pigs showed significantly lower levels of mean rectal temperatures, clinical severity, lung lesion scores, and viral titers in serum and lung tissue compared to the unvaccinated challenged pigs ($p < 0.05$). Vaccinated challenged pigs had higher survival rate than those of unvaccinated challenged pigs in both experiments. It should be noted that pigs challenged 42 days after vaccination showed a better performance than pigs challenged 28 days after vaccination. In conclusion, PRRS MLV of lineage 8 was able to improve the survival rate against the Thai HP-PRRSV infection in both 42 days and 28 days vaccination-to-infection protocols.

Department:	Veterinary Pathology	Student's Signature .....
Field of Study:	Veterinary Pathobiology	Advisor's Signature .....
Academic Year:	2015	Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

BSA	=	bovine serum albumin
CU-VDL	=	Chulalongkorn University-Veterinary Diagnostic Laboratory
^{o}C	=	Degree celcius (centrigrade)
DPC	=	days post challenge
DPI	=	days post-infection
DPV	=	days post vaccination
ELISA	=	enzyme-linked immunosorbent assay
EU	=	European
GP	=	glycoproteins
HP-PRRSV	=	highly pathogenic PRRSV
IFN-γ-SCs	=	interferon-γ-secreting cells
IHC	=	Immunohistochemistry
mAbs	=	monoclonal antibodies
MEM	=	Minimum essential medium
MLV	=	Modified live-Virus
NA	=	North American
NEG	=	negative control
nsp	=	non-structural proteins
PAMs	=	pulmonary alveolar macrophages
PBS	=	phosphate buffered saline
pi	=	post-infection
PRRS	=	Porcine reproductive and respiratory syndrome
PRRSV	=	PRRS virus
RT-PCR	=	reverse transcriptase polymerase chain reaction
UNV/CHA	=	unvaccinated challenged

VAC/CHA	=	vaccinated challenged
white blood cell	=	WBC

CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV) belonging to family *Arteriviridae*. PRRSV is an enveloped, single-stranded, and positive-sense RNA virus (de Groot et al., 2012) classified into two genotypes; the European (EU) or genotype 1 (Lelystad virus) (Terpstra et al., 1991) and the North American (NA) or genotype 2 (VR-2332) (Benfield et al., 1992). PRRSV genotype 2 can be separated into nine lineages. The major member of lineage 5 is VR2332, a genotype 2 PRRSV prototype that used as a seed virus for Ingelvac PRRS® MLV (Shi et al., 2010). PRRSV causes two main clinical signs, including reproductive failure in breeding pigs and respiratory diseases in nursery and growing-finishing pigs. Reproductive failure includes abortion, decreased numbers of weaned pigs, increased numbers of stillborn piglets, mummified fetuses, and weak piglets (Cavanagh, 1996; Zimmerman et al., 2006). PRRSV affects to swine producing areas and leads to economic losses in many countries. It is approximated 660 million dollars a year in the USA (Butler et al., 2014). In 2006, highly pathogenic PRRSV (HP-PRRSV) had been reported in China (Tian et al., 2007). HP-PRRSV is a new lineage of PRRSV containing discontinuous deletion of 30 amino acids in the non-structural proteins (nsp) 2 belonging to lineage 8 (Shi et al., 2010) and clustered in sublineage 8.7 (Tun et al., 2011; Thuy et al., 2013). HP-PRRSV induces high morbidity (50-100%) and high mortality rates (20-100%). The clinical signs of HP-PRRSV are high fever (40-42 ºC), severe respiratory distress, shivering, and erythematous rash (Tian et al., 2007). In Thailand, genotype 2 had been predominant and classified in lineage 1 and

sublineages 5.1, 5.2 and 8.7, which sublineage 8.7 was predominant, since 2010 (Jantafong and Lekcharoensuk, 2014; Jantafong et al., 2015). The first HP-PRRS outbreak had been reported in NongKhai province in 2010 (Nilubol et al., 2012). Then, Thai HP-PRRSV was isolated in Udonthani (10UT01) and Phitsanulok (10PL01) (Na Ayudhya et al., 2012). However, HP-PRRSV was firstly detected from serum samples in 2008 (Jantafong and Lekcharoensuk, 2014).

The control of PRRS is based on four aspects; 1) early diagnosis and monitoring, 2) biosecurity, 3) herd management, and 4) vaccination (Scortti et al., 2006[a, b]). Nowadays, vaccination is one of the main strategies to control the HP-PRRSV (Tian et al., 2009). Current vaccines are commercially available both modified live and killed PRRSV vaccines. Ingelvac PRRS® MLV is belonging to lineage 5 (Park et al., 2014). PRRSV MLV vaccine had been used to control a field outbreaks of PRRS (Gillespie and Carroll, 2003). PRRS MLV vaccine can reduce the severity of clinical signs, such as duration of viremia and viral shedding (Murtaugh et al., 2002), lung lesions, weight loss (Cano et al., 2007) and morbidity and mortality rates (Mengeling et al., 2003). In addition, homologous genotype of PRRSV MLV vaccines provided better protective efficacy than the killed PRRSV vaccines (Murtaugh et al., 2002; Labarque et al., 2003). PRRSV MLV vaccines provide complete and partial protections when challenged with homologous and heterologous strains, respectively (Li et al., 2014). However, current vaccines are ineffective due to the antigenic and genetic heterogeneity of PRRSV field isolates (Meng, 2000). Killed PRRSV vaccines were failed to elicit protective immunity even against homologous virus challenge (Renukaradhya et al., 2015[a]). PRRSV MLV vaccine offers solid protection against clinical disease induced by homologous infection, but none of the current vaccines is able to provide complete prevention of respiratory infection (Kimman et al., 2009). Current PRRSV-positive swine farms have been suffering mostly from early PRRSV infection. Therefore, early vaccination was used. However, the efficacy of the vaccines when

implementing with a short interval before facing with a natural PRRSV infection is still unknown.

Almost all HP-PRRSV isolates in Southeast Asian are clustered in the sublineage 8.7 and genetic similarity between them are quite high (Shi et al., 2010; Jantafong et al., 2015). Recently, a new MLV vaccine against HP-PRRSV using PRRSV strain P129 is commercially available. This vaccine strain derived from P129 was genetically classified into the same lineage as HP-PRRSV (Shi et al., 2010). Recent study showed when pigs were immunized with this new MLV vaccine at 3 weeks of age and challenged with the Vietnamese HP-PRRSV at 5 weeks post vaccination provided partial protection (Do et al., 2015). It could be hypothesized that the studied MLV vaccine could be an effective vaccine of choices inducing protection against the Thai HP-PRRSV. This new PRRS MLV vaccine of lineage 8 provided a partial protection and still provided better protection than the vaccine of lineage 5. However, the efficacy and levels of protection of the vaccine are of interest when challenged at 4 weeks and 6 weeks after vaccination.

Keywords

Efficacy, HP-PRRSV, Lineage 8, Pigs, MLV Vaccine

Objective of the study

To evaluate the efficacy of a new PRRS MLV of lineage 8 against a Thai HP-PRRSV (10PL01) when challenge pigs at 4 and 6 weeks post vaccination

CHAPTER II

LITERATURE REVIEW

2.1 Etiology

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRSV belonging to the member of *Arteriviridae* family and genus *Arterivirus*. PRRSV is a single-stranded, positive-sense RNA and enveloped virus (de Groot et al., 2012). PRRSV genome is approximately 15 kb in length consisting of nine open reading frames (ORFs) (Meulenberg et al., 1993). Non-structural proteins (nsp) are encoded by ORF1a and ORF1b. Structural proteins are encoded by ORFs 2–7 and ORFs 2-5 encode glycoproteins (GP) 2-5 (Fig. 1), that can glycosylation proteins in envelope (Wu et al., 2001). Major envelope and nucleocapsid proteins are encoded by ORF 5 and ORF 7, respectively. ORF 5 is mostly used for phylogenetic analysis due to genetically highly variable (Murtaugh et al., 1995) and its major roles in pathogenesis, such as entry to host cell and apoptosis (Sur et al., 1998). Moreover, it composes of a PRRSV neutralizing epitope (Wissink et al., 2003). PRRSV affects to swine industry worldwide and leads to economic losses in many countries. It is approximated 660 million dollars a year in the USA and these economic losses such as reproductive failure, increased pneumonia, reduced pig growth etc. (Holtkamp et al., 2012). In 1991, PRRSV was discovered in the Netherlands. PRRSV is divided into two genotypes, European (EU) or genotype 1 and North American (NA) or genotype 2. The prototype PRRSV of genotype 1 is Lelystad virus and genotype 2 is VR-2332. Although PRRSV genotype 1 and 2 induce similar clinical signs, but these two types have nucleotide similarity only 70% (Allende et al., 2000[a]). PRRSV causes two main clinical

signs: reproductive failure in breeding pigs and respiratory diseases in nursery and growing-finishing pigs (Cavanagh, 1996; Zimmerman et al., 2006).

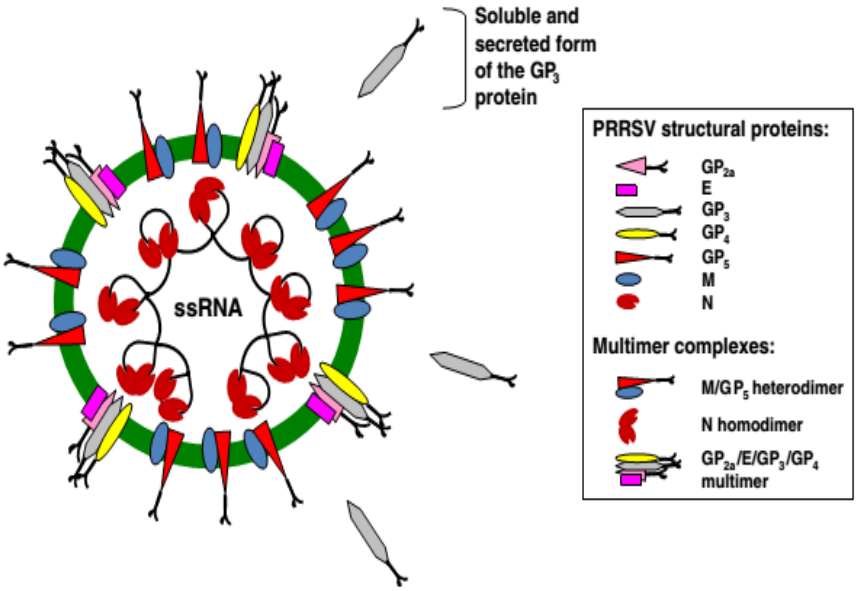


Figure 1. Schematic representation of the PRRSV particle (Gagnon, 2010).

2.2 Transmission

PRRSV transmission can be both direct and indirect routes. Direct route is the transmission among pig population including vertical and horizontal transmissions. Vertical transmission occurs in mid to late gestation. Horizontal transmission is caused by direct contact between infected and naïve pigs. PRRSV can be detected in most body fluids such as blood, feces, semen, saliva, milk and colostrum. Pigs with persistent PRRSV infection can be detected in breeding gilts up to 120 days post-infection (DPI) and up to 86 DPI for virus shedding. Indirect routes are transmitted by fomites, insects, transport vehicles and aerosols. Fomites particularly boots and coveralls are potential sources of virus. Moreover, needles can be a mode of PRRSV transmission between pigs. Potential route of mechanical and indirect transmissions

are transport vehicles and aerosol transmission. PRRSV can be spread in aerosols up to 3 km (Cho and Dee, 2006).

2.3 Pathogenesis

The infection of PRRSV is originated by virions attachment to the highly sulfated, negatively charged glycosaminoglycans on the surface of susceptible cells and subsequent to bind to the CD169 (Vanderheijden et al., 2003), that triggers receptor-mediated clathrin-dependent endocytosis (Nauwynck et al., 1999). PRRSV genome is released in the cell cytoplasm through a reaction mediated by CD163 (Calvert et al., 2007) and other cellular factors (Misinzo et al., 2008). The stages of PRRSV infection can be into three stages such as acute infection, persistent infection and extinction. In acute infection, the lung is specific site of viral infection. PRRSV mainly replicates in macrophages and dendritic cells of the lungs lead to viremia by 6–12 hours post-infection (pi) and viremia can be prolong for several weeks while presence of circulating antibodies (Wills et al., 1997; Allende et al., 2000[b]; Rowland et al., 2003). The lesion is represent by acute diffuse interstitial pneumonia and microscopic lung lesions are characterized by marked thickening of alveolar septa with infiltration of the lymphocytes and histiocytes and type 2 pneumocyte proliferation (Halbur et al., 1993; Rossow et al., 1995). In persistent infection, the virus mainly replicates in lymphoid organs such as tonsil and lymph nodes (Wills et al., 1997; Allende et al., 2000[b]; Rowland et al., 2003). Continuous virus replication in the regional lymph nodes can be efficient spread of virus to naïve pigs by oral-nasal secretions and semen (Christopher-Hennings et al., 2008). Then, the viral replication gradually decays until the virus turn into extinct in the host. In extinction stage, the virus is disappears but the viral replication can be maintained until 250 days after infection (Wills et al., 2003). Consequently, PRRSV replication is not set a steady-state

equilibrium but gradually decrease, with lymphoid organs as the site of the last evidence of virus replication before viral extinction (Allende et al., 2000[b]).

2.4 Clinical signs and antibody response

Variation of clinical signs in PRRSV infected pig is due to complex interaction between host and virus. The duration of acute viremia is around 28 days and pulmonary alveolar macrophages (PAMs) is the first target. Acute infection is shown by respiratory distress as a result of inflammatory cytokines releasing into the infected lung. PRRSV viremia occurs (Boddicker et al., 2012) after the primary virus replication in lymphoid tissue and lung. PRRSV can be isolated from lymph nodes up to 100 days after infection (Rowland et al., 2003). The mechanism of PRRSV persistent infection has relied on many factors such as; (1) antigenic and genetic drift, (2) complex structure of virion, (3) disestablish of interferon gene induction, (4) re-direction of the humoral response towards nonsurface proteins (Chand et al., 2012). PRRSV compromises lung immune response, such as reducing number and function of PAMs, induce immune cells apoptosis, imbalance cytokines between pro-inflammatory and anti-inflammatory cytokines, mucociliary transport system impair, reducing the bactericidal activity of macrophages. Therefore, infected pigs are highly susceptible to secondary infections (Gómez-Laguna et al., 2013).

After exposure PRRSV, anti-PRRSV antibody can be detected at 7–9 DPI. However, this early antibody response is no evidence to plays a role in the protection against PRRSV infection (Yoon et al., 1994; Labarque et al., 2000). Early antibody response cannot neutralize PRRSV in vitro (Yoon et al., 1994). PRRSV neutralizing antibodies appear only at periods equal or higher than 28 DPI (Meier et al., 2000). PRRSV-specific IgM can be detected at 7 DPI with peak titers between 14 and 21 DPI and decreasing to undetectable around 40 DPI. PRRSV-specific IgG peak

titers at 21 to 28 DPI (Loemba et al., 1996). Commercial PRRSV serologic test (IDEXX Labs, Portland Maine, US) is detected antibodies against the N protein. These antibodies appear at first week PI and prolong for several months, however do not correlate with protection (Lopez and Osorio, 2004).

2.5 Highly pathogenic PRRSV

In 2006, highly pathogenic porcine reproductive and respiratory syndrome virus was found in China. The Chinese HP-PRRSV is a new PRRSV lineage having discontinuous deletion of 30 amino acids in the non-structural proteins (nsp) 2 and belongs to lineage 8 (Shi et al., 2010) that clustered in sublineage 8.7 (Tun et al., 2011; Thuy et al., 2013). HP-PRRSV caused high morbidity (50-100%) and high mortality rates (20-100%). The clinical signs of HP-PRRSV were high fever (40-42 ºC), severe respiratory distress, shivering and erythematous rash (Tian et al., 2007). The pathological finding of infected pigs, such as pulmonary hemorrhage and edema, splenic infarct, urinary bladder filled with reddish brown urine, renal hemorrhage, putrescent of cardiac muscle, liver with yellow-white necrosis or hemorrhage, lymph node with petechial hemorrhage and arthritis with swollen joints and intestine ulceration (Tian et al., 2007).

2.6 Epidemiology of HP-PRRSV in Thailand

In Thailand, both PRRSV genotypes have been circulating in Thai swine farms but genotype 2 was more prevalent than genotype 1 (Jantafong and Lekcharoensuk, 2014). Genotype 2 has been predominant and classified in three lineages: 1, 5 (5.1 and 5.2) and 8 (8.7). Thai PRRSV are separated into two clusters: classical NA and HP-PRRSV and HP-PRRSV is predominant, especially after 2010 (Jantafong and Lekcharoensuk, 2014; Jantafong et al., 2015). The first HP-PRRS outbreak had been

reported in NongKhai province in 2010 (Nilubol et al., 2012). Then, Thai HP-PRRSV was isolated in Udonthani (10UT01) and Phitsanulok (10PL01) (Fig. 2) (Na Ayudhya et al., 2012). However, HP-PRRSV was firstly detected from serum samples in 2008 (Jantafong and Lekcharoensuk, 2014).

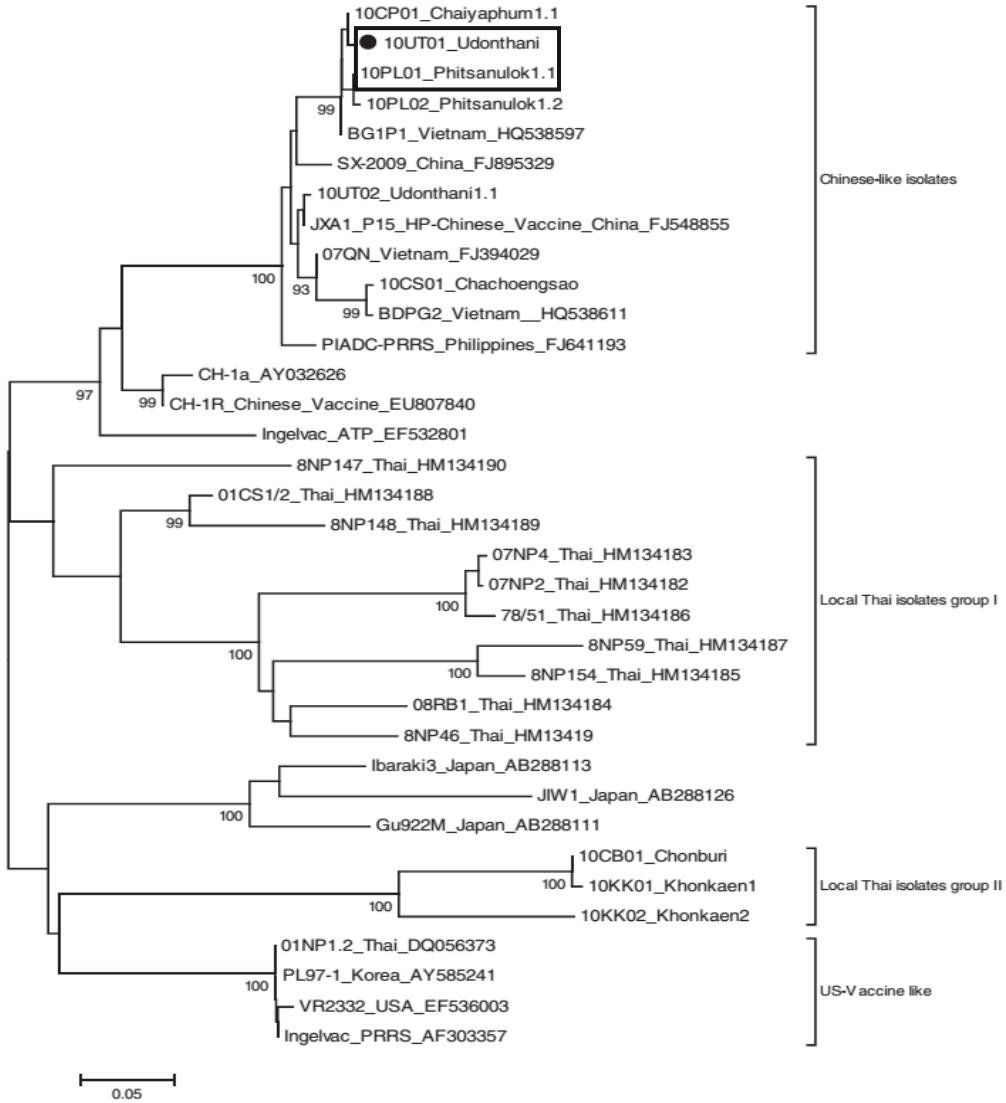


Figure 2. Thai HP-PRRSV was isolated in Udonthani (10UT01) and Phitsanulok (10PL01) (Na Ayudhya et al., 2012).

2.7 Current vaccines

Vaccination is one of the main strategies to control the HP-PRRSV (Tian et al., 2009). Current available vaccines are modified live and killed PRRSV vaccines. Nowadays, commercial type 2 PRRSV MLV such as Ingelvac PRRS® MLV classified in lineage 5 (Park et al., 2014) and Prime Pac® PRRS classified in lineage 7 (Thuy et al., 2013). Benefits of PRRS-MLV vaccination include reducing the severity of clinical signs, as well as the duration of viremia and viral shedding (Murtaugh et al., 2002), lung lesions, weight loss (Cano et al., 2007), morbidity and mortality rates (Mengeling et al., 2003) and improving animal production under field conditions (Renukaradhya et al., 2015[b]). Moreover, homologous genotype of PRRSV MLV vaccines provided better protective efficacy than those of the killed PRRSV vaccines (Murtaugh et al., 2002; Labarque et al., 2003). PRRSV MLV vaccines provide complete and partial protections when challenged with homologous and heterologous strains, respectively (Li et al., 2014). However, current vaccines are ineffective due to the antigenic and genetic heterogeneity of PRRSV field isolates (Meng, 2000). Killed PRRSV vaccines failed to elicit protective immunity even against homologous virus challenge (Renukaradhya et al., 2015[a]). PRRSV MLV vaccines offer solid protection against clinical disease induced by homologous infection, but none of the current vaccines is able to provide complete prevention of respiratory infection (Kimman et al., 2009).

2.8 The new PRRS MLV vaccine of lineage 8

A new MLV vaccine against HP-PRRSV originated from a virulent US PRRS isolate (P129) belonging to lineage 8 based on classification system, was introduced into the international market to control respiratory disease in growing pigs (Fig. 3) (Park et al., 2014). Recent study showed that the new commercial PRRS MLV vaccine of lineage 8 could reduce the level of viremia, nasal shedding and the severity of

PRRSV-induced lesions after challenged with heterologous strains under experimental conditions. Moreover, this particular MLV vaccine could induce PRRSV-specific interferon-γ-secreting cells (IFN-γ-SCs) leading to reduce PRRSV viremia (Park et al., 2014). In addition, the new commercial PRRS MLV vaccine of lineage 8 provides better protection than that of the vaccine of lineage 5 against the Vietnamese HP-PRRSV when immunized at 3 weeks of age with 5 weeks for vaccination-to-challenge period (Do et al., 2015). Furthermore, this particular MLV vaccine could reduce fever, levels of HP-PRRSV viremia and induce high numbers of HP-PRRSV-specific IFN-γ-SCs in vaccinated challenged pigs (Do et al., 2015). Recent study showed that the new commercial PRRS MLV vaccine of lineage 8 could reduce the level of viral load in blood, lung lesions and induce high numbers of type 1 and type 2 PRRSV-specific IFN-γ-SCs against heterologous dual challenge of PRRSV genotypes 1 and 2 compared with vaccination of pigs with type 1 PRRSV (Choi et al., 2016).

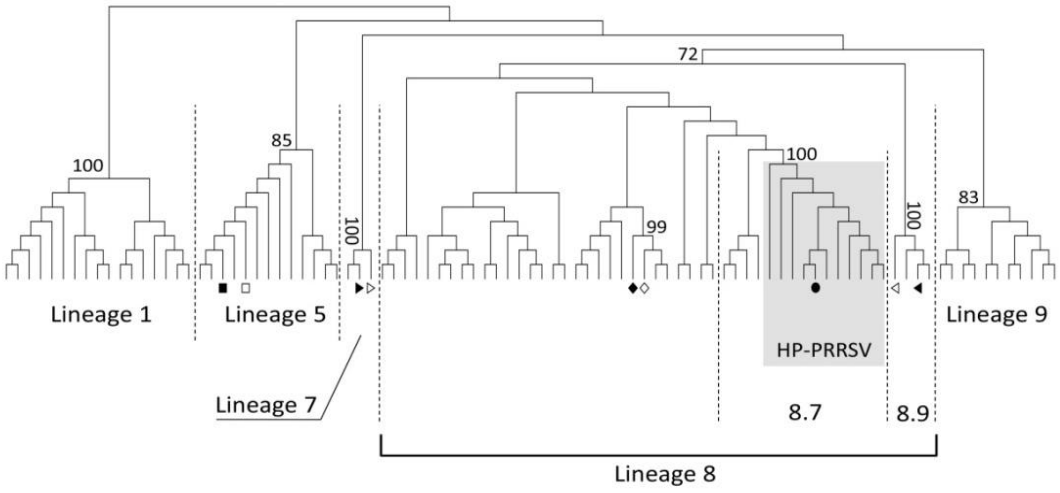


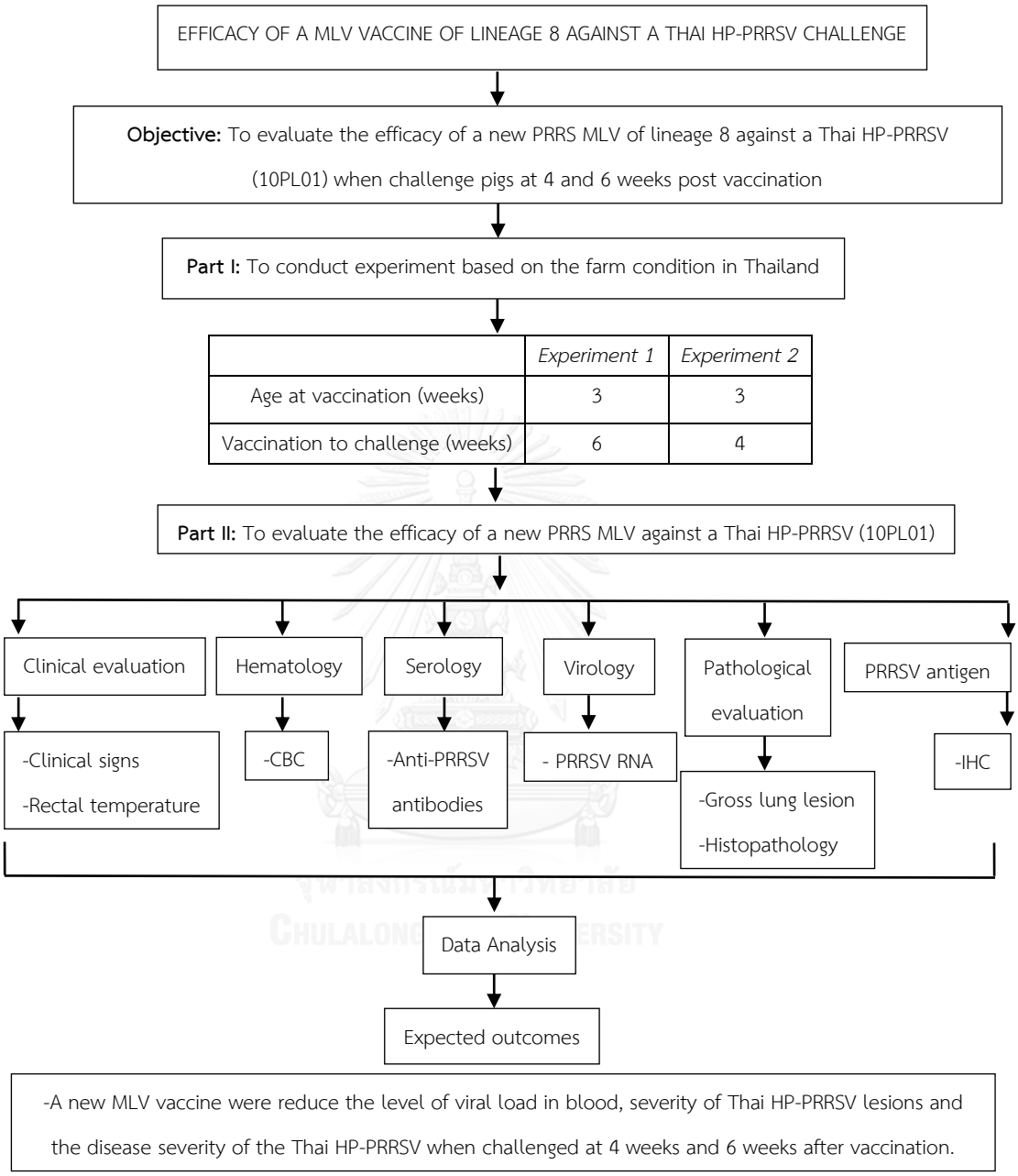
Figure 3. Dendrogram of type 2 PRRSV. The tree was constructed based on ORF5 sequences using Neighbor-joining method. The ORF5 sequences included in the tree were from 4 major type 2 PRRSV lineages, including lineage 1, 5, 8, and 9. Sequences from lineage 7 is also demonstrated. Bootstrap values of each lineage and some clusters are shown. Grey box represents a Chinese HP-PRRSV cluster, which is in the sublineage 8.7. ● represents 10PL01, a Thai isolate of HP-PRRSV. ■ and □ represent Ingelvac MLV vaccine strain and its parental strain, VR2332, respectively. ▶ and ▷ represent PrimePac vaccine strain and its parental strain, Neb-1, respectively. ◆ and ◇ represent the new PRRS MLV vaccine strain and its parental strain, P129, respectively. ◀ and ◁ represent Ingelvac ATP vaccine strain and its parental strain, JA142, respectively.

CHAPTER III

MATERIALS AND METHODS

The study was divided into two parts. The first part was conducted two experiments (*experiment 1* and *experiment 2*) based on the farm conditions in Thailand for elucidate the protection induced by a new PRRS MLV vaccine. In *experiment 1*, pigs were immunized at 3 weeks of age and then were challenged with the HP-PRRSV at 42 days post vaccination (DPV). In *experiment 2*, a shorter vaccination-to-challenge period was applied by using 3 weeks old pigs and challenged at 28 DPV. The second part was evaluated the efficacy of a PRRS MLV against a Thai HP-PRRSV (10PL01) such as clinical evaluation, hematology, serology, virology, pathological evaluation and PRRSV antigen detection. The overall experimental plan of this study is shown in Figure 4.

Figure 4. Experimental plan



3.1 Pigs

A total of 81 pigs (castrated male, crossbreeds), at the age of 3 weeks old were used. Pigs were originated from a PRRSV-free farm. The PRRSV-negative status of pigs were reconfirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) prior to introduce to the research facility. All pigs were kept in the experimental facilities at the Faculty of Veterinary Medicine, Kasetsart University, Kumpangsan Campus, Nakorn Pathom Province, Thailand, until terminated. The experimental facilities are considered as biosafety level 2. The pigs were acclimatized to the research facility for 3 days before starting the experiment. All pigs received injection using antibiotics before the experiments.

3.2 Vaccine and virus inoculums

A new PRRS MLV vaccine based on a virulent US PRRS isolates (P129) belonging to lineage 8 were used in piglet immunization. The pigs were immunized by intramuscular injection with 2 ml of the vaccine, according to manufacturer's protocol. The Thai HP-PRRSV (10PL01) was isolated from Phitsanulok province (Na Ayudhya et al., 2012) and used as virus inoculums provided by Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). The inoculum virus was propagated using MARC145 and titrated at 10^4 $TCID_{50}$ in 5 ml of Minimum essential medium (MEM) medium. Virus inoculation was done via intranasal route. The titer of inoculum virus was determined before and after challenge according to standard virus titration methods using Marc-145 cells (10^4 $TCID_{50}$ in 5 ml of MEM medium).

3.3 Experimental design

To elucidate the protection induced by a new PRRSV MLV vaccine in young pigs, two experiments (*experiment 1* and *experiment 2*) were conducted based on

the farm condition in Thailand for example an early or late PRRSV infection. In *experiment 1*, pigs were immunized at 3 weeks of age and then were challenged with the HP-PRRSV at 42 days post vaccination (DPV) for efficacy evaluation of a new PRRS MLV against a Thai HP-PRRSV (10PL01). In *experiment 2*, a shorter vaccination-to-challenge period were applied by using 3 weeks old pigs and challenged at 28 DPV. Both animal experiment protocols were approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC number 13310019, 1431086).

Experiment 1: Forty two 3 week-old pigs were randomly allocated into three groups, including negative control (NEG) group (n=10), unvaccinated challenged (UNV/CHA) group (n= 16) and vaccinated challenged (VAC/CHA) group (n= 16). Each group was housed in a separate room. At 3 weeks of age, pigs in the vaccination group were immunized by a studied PRRSV MLV vaccine. At 42 DPV, pigs in the unvaccinated challenged and vaccinated challenged group were commingled and then challenged with the Thai-HP-PRRSV (10PL01) via intranasal route. The negative control pigs were mock inoculated with virus free-MEM as the challenge groups. Half of the pigs in each group were randomly selected to necropsy at 10 and 17 days post challenge (DPC).

Experiment 2: The protocol is similar to *experiment 1* with major differences such as the vaccination-to-challenge period. Thirty-nine pigs of the same age were randomly allocated to three groups; negative control (NEG) group (n= 9), unvaccinated challenged (UNV/CHA) group (n= 15) and vaccinated challenged (VAC/CHA) group (n= 15). At 3 weeks of age, the vaccinated group was immunized. At 28 DPV, the challenge control group and the vaccination group were challenged with the same Thai HP-PRRSV at the dose. All pigs were euthanized and necropsied at 14 DPC (Table 1).

Table 1. Experimental design

Experiment	Group	Days post vaccination				
		0	28	42	52	59
Experiment 1	UNV/CHA (n= 16)	-	-	HP-PRRSV	Necropsy (10 DPC)	Necropsy (17 DPC)
	VAC/CHA (n= 16)	Vaccine[1]	-	HP-PRRSV		
	NEG (n=10)	-	-	-		
Experiment 2	UNV/CHA (n= 15)	-	HP-PRRSV	Necropsy (14 DPC)		
	VAC/CHA (n= 15)	Vaccine[1]	HP-PRRSV			
	NEG (n= 9)	-	-			

[1] Vaccination

3.4 Sample collection

In both *experiment 1* and *2*, whole blood and serum were collected weekly in the same pigs. In *experiment 1*, whole blood and serum were collected at (-3), 3, 7, 14, 21, 28, 35, and 42 DPV and 4, 7, 14, and 17 DPC. In *experiment 2*, the sample collection were done at (-4), 3, 7, 14, 21 and 28 DPV and 3, 7, and 14 DPC.

3.5 Hematology and serology

Whole blood samples were evaluated for complete blood count using automated hematology analyzer. Leukopenia was defined as a total white blood cells (WBC) count less than 9,000 cells/µl. Sera were detected for PRRSV antibody using IDEXX PRRS X3 ELISA test kit (IDEXX laboratories, Inc., Westbrook, ME, USA). ELISA were done as the manufacturer's instructions. S/P ratio equal to or greater than 0.4 is considered as positive.

3.6 Clinical evaluations

Clinical signs and rectal temperature (℃) were monitored daily during post vaccination and post challenge periods. The monitoring was done at 0–7 DPV for both experiments. After the challenge, clinical signs and rectal temperature were monitored at 0–10 DPC. Fever was defined as a rectal temperature higher than 40 ℃. Clinical signs were monitored, including general condition, depression, respiratory distress, coughing, sneezing and appetite. General condition, depression and respiratory distress were scored according to the degree of severity; 0 (normal), 1 (mild), 2 (moderate), and 3 (severe). General condition were scored from 0 (normal appearance) to 3 (severe abnormal condition). Depression was scored from 0 (normal) to 3 (severe depression or death). Respiratory distress was scored from 0 (normal) to 3 (severe dyspnea and abdominal breathing). The appearance of

coughing and sneezing were scored; 0 (absence) and 1 (presence). The presence of anorexia were scored; 0 (absence) and 1 (loss of appetite). Body weight was recorded at 0 DPC and prior to euthanization.

3.7 Detection of PRRSV RNA

PRRSV RNA was extracted from serum and lung tissues for quantitative viral genomic cDNA copy numbers. Briefly, NucleoSpin® RNA virus kit (MACHEREY-NAGEL GmbH &Co. KG, Germany) were used for viral RNA extraction. Viral RNA were quantified using previously published TaqMan® probe-based real-time RT-PCR (Egli et al., 2001) with minor modification. Primers and probes were as follows; reverse primer USalignEU-R (5' AAATGIGGCTTCTCIGGITTTT 3′) and forward primer USalignEU-F (5′ TCAICTGTGCCAGITGCTGG 3′) and US-PRRSV-specific probe FAM_US_rev (5′ *FAM*-TCCCGGTCCCTTGCCTCTGGA-*TAMRA* 3′). The target gene was highly conserved ORF 7 region of virus. RT-PCR mixture (25 µl) was based on SuperScript™ III One-Step RT-PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1× Reaction Mix, 0.4 mM of each primer and probe, 0.5 µl of SuperScript® III RT/Platinum® Taq Mix, and 0.5 µl of viral RNA. RT-PCR was carried out in MyGo Pro real-time PCR machine (IT-IS International, Ltd, UK). After RT at 50 °C for 30 minutes and denaturation at 92 °C for 2 minutes, 35 PCR cycles (95 °C for 15 seconds, 50 °C for 30 seconds and 60 °C for 30 seconds). The product size was 105 base pair.

3.8 Pathological evaluation

All pigs were euthanized using an intravenous injection of pentobarbital sodium (Nembutal®, CEVA Santé Animale, France) and necropsied at the end of experiment or when moribund. Upon necropsied, lung was collected and stored as both fresh frozen (at -80°C) and formalin-fixed tissues. Percentage of lung

consolidation of each lobe were determined as previously described (Halbur et al., 1995). For histopathology study, formalin-fixed, paraffin-embedded tissues of the lung was used. Microscopic examination was used to confirm the presence of interstitial pneumonia. Severity of interstitial pneumonia were scored as previously described (Halbur et al., 1995); 0 = no microscopic lesions, 1 = mild interstitial pneumonia, 2 = moderate multifocal interstitial pneumonia, 3 = moderate diffuse interstitial pneumonia; 4 = severe interstitial pneumonia.

3.9 Immunohistochemistry (IHC)

IHC was used to confirm the presence of PRRSV antigen in the lesion of lung and the tracheobronchial lymph node. IHC staining for PRRSV antigen was performed as previously described using SDOW17 monoclonal antibodies (mAbs) (Rural Technologies, Inc., Brookings, SD, USA) specific for PRRSV nucleocapsid as a primary antibody (Laohasittikul et al., 2004). Briefly, Sections were subjected for antigen retrieval treatment by 0.1 % trypsin and block non-specific endogenous peroxidase by 3% H_20_2 in absolute methanol. Sections were blocked by 10% bovine serum albumin (BSA) and incubated with a PRRSV monoclonal antibody (SDOW17 diluted 1: 1000 in sterile 1x phosphate buffer saline (PBS)) for 12-14 hours at 4°C. Sections were incubated with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) for 45 min at room temperature and incubated with Dako REAL™ DAB+ Chromogen (diluted 1: 50 in Dako REAL™ Substrate Buffer) for 25 seconds at room temperature. Sections were counter stained with hematoxylin.

3.10 Statistical analysis

Comparison of each parameter was done between VAC and UNV groups in each experiment. Survival rate at the end of both studies was compared and analyzed by Fisher's exact test. Continuous variables (rectal temperature, virus titer in serum and lung tissue, total white blood cells count and, lung lesion score) were analyzed by the independent *t*-test. Some continuous variables were analyzed by Fisher's exact test as frequencies of positive animals (e.g. viral titer in serum: frequency of PRRSV viremic animals, CBC: frequency of leukopenic animals). Ordinal scale variables (clinical score) were calculated as 1) frequency of positive animals and 2) cumulative proportion within the positive animals and analyzed by Fisher's exact test. Average value and frequency value were reported as "mean (± SD)" and "percentage (number of positive animals/total number of animals)", unless otherwise stated. Statistical significance was set at $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Survival rate

In *experiment 1*, all pigs (16/16) in VAC group survived until the end of the experiment comparing with 87.5% (14/16) of UNV group (Fig. 5). However, they were not significantly different. In *experiment 2*, VAC group showed significantly higher survival rate than UNV group at 80% (12/15) and 20% (3/15), respectively. A peak mortality rate of UNV group was occurring at 8 DPC (40%; 6/15). VAC pigs were significantly lower than UNV pigs at 14 (Fig. 5). In both *experiment 1* and *2*, NEG group survived until the end of the experiment (data not shown).

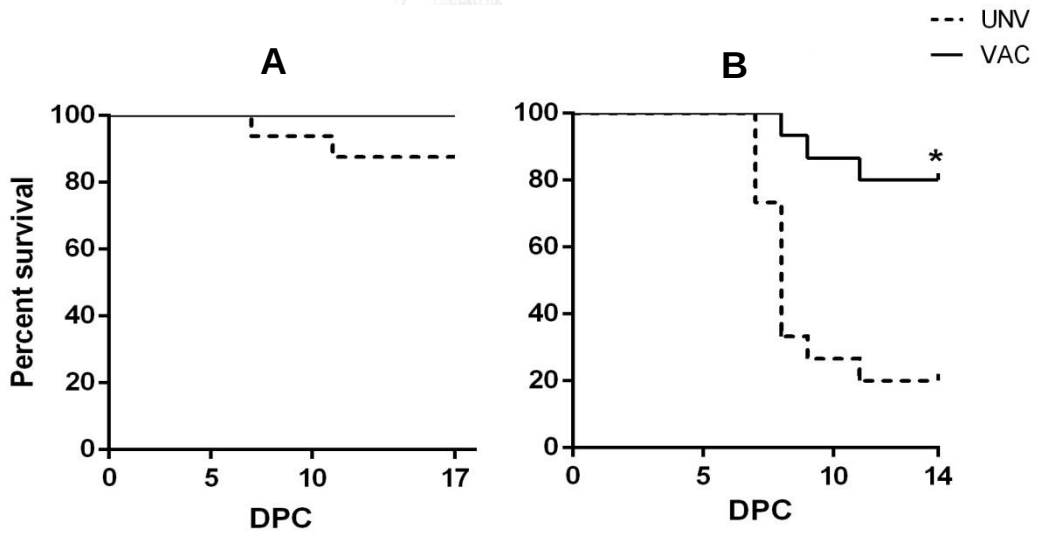


Figure 5. Survival rate of pigs in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

4.2 Clinical evaluation

Experiment 1: VAC pigs showed lower levels of rectal temperature, depression, and respiratory distress than those of the UNV pigs. Rectal temperature of VAC pigs was significantly lower than UNV pigs at 1 DPC and during 7 - 10 DPC (Fig. 6a). At 10 DPC, mean rectal temperature of VAC group [39.3 ± 0.51°C] and NEG group [39.3 ± 0.12°C] were not significantly different (data not shown). The Frequency of pigs showing signs of fever was significantly lower than that of UNV group at 7 - 10 DPC (data not shown). The frequency of pigs showing signs of depression in VAC group was significantly lower than that of UNV group at 4 and 6 – 10 DPC (Fig. 7a). Cumulative frequency of moderate and severe forms of depression in moribund pigs was observed in UNV group during 5 – 9 DPC [peaked at 7 DPC: 25% (4/16)]. However, pigs in VAC group showed only mild form of depression but not significantly different (Fig. 8a). For respiratory distress, pigs in VAC group showed significantly lower frequency at 1, 2, and 10 DPC (Fig. 9a). During 4 – 10 DPC, cumulative frequency of moderate and severe degree of respiratory distress in UNV group was significantly higher than that of VAC group at 4, 5 and 7-10 DPC (Fig. 10a). Clinical signs were not observed in NEG group throughout the experiment (data not shown).

Experiment 2: Pigs in VAC groups showed less severity of depression while rectal temperature and respiratory distress were not different from pigs in UNV group. After HP-PRRSV inoculation, both UNV and VAC groups had increased rectal temperature (Fig. 6b). Frequency of pigs with signs of depression in VAC and UNV groups were not significantly different during 0 – 7 DPC (Fig. 7b). Cumulative frequency of moderate and severe degree of depression in moribund pigs in VAC groups was significantly lower at 4 and 7 DPC (Fig. 8b). Respiratory distress was rarely observed during 0 – 7 DPC in both groups (Fig. 9b). Both frequency of respiratory distressed pigs and frequency of moderate or severe degree of the respiratory

distress in VAC groups were increased during 8 – 9 DPC (Fig. 9b and 10b). Clinical signs were not observed in the NEG group throughout the experiment (data not shown).

In both *experiment 1* and *2*, Pigs in UNV groups showed more numerically abnormal general appearance (moderately to severely abnormal condition) than VAC groups. Few animals in both groups were recorded as coughing and sneezing. Clinical signs were not observed in the NEG group throughout the experiment (data not shown).

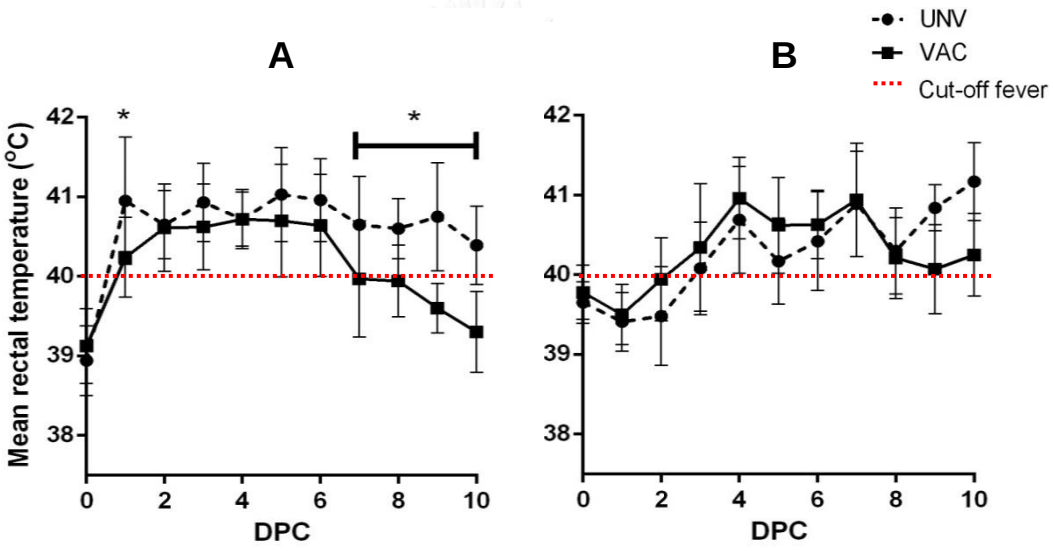


Figure 6. Mean rectal temperature of pigs in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

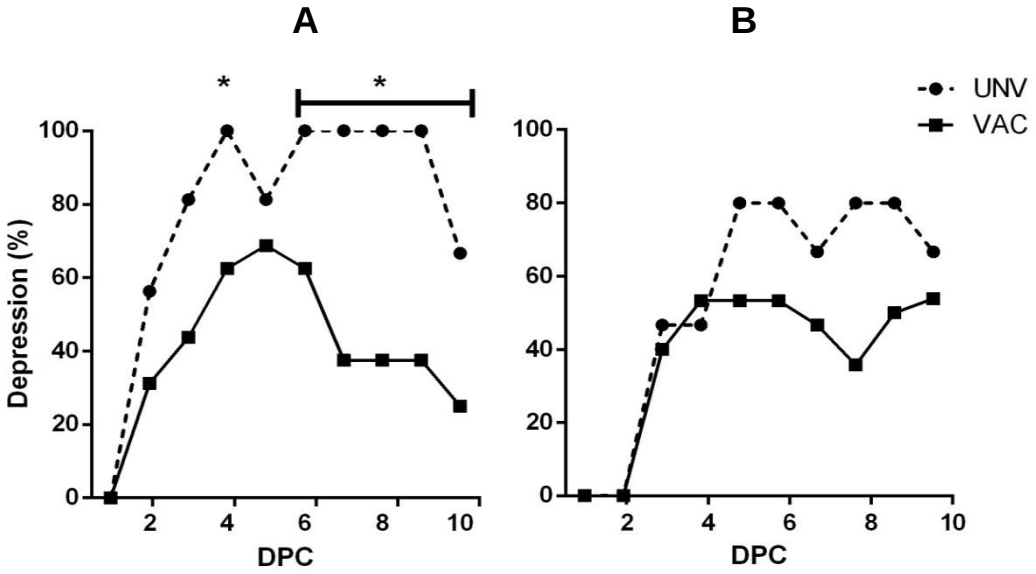


Figure 7. Frequency of pigs showing signs of depression in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

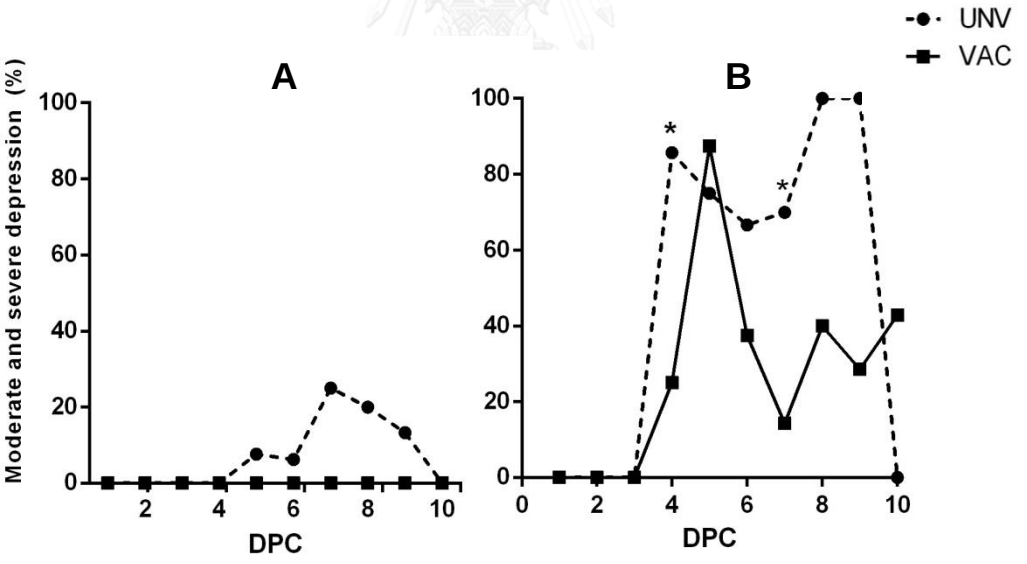


Figure 8. Cumulative frequency of moderate and severe forms of depression in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

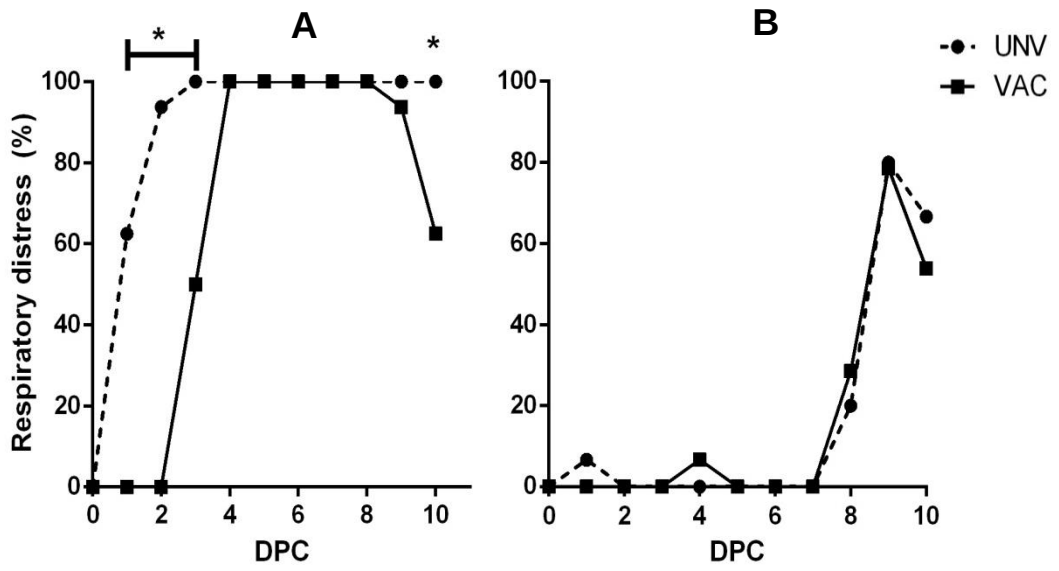


Figure 9. Frequency of pigs showing signs of respiratory distress in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

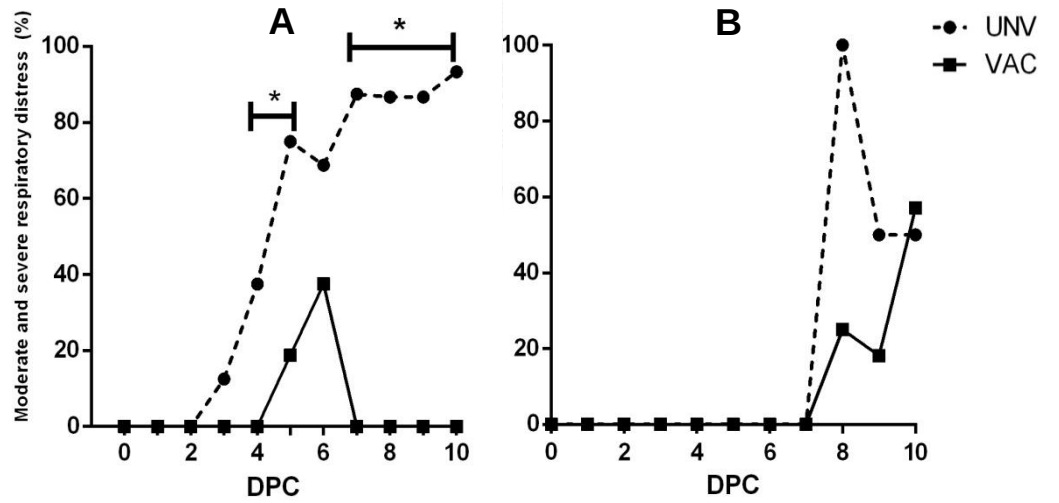


Figure 10. Cumulative frequency of moderate and severe degree of respiratory distress *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

4.3 Serology

In both experiments, anti-PRRSV antibody was detected in all pigs of VAC group after vaccination as early as 14 DPV, and all pigs of UNV group after HP-PRRSV

inoculation (Fig. 11) as early as 7 DPC. Anti-PRRSV antibody was detected in 100% of pigs in VAC group at 35 DPV in *experiment 1*, and 14 DPV in *experiment 2*. After virus inoculation, anti-PRRSV antibody was detected in 100% of pigs in UNV group at 14 DPC in *experiment 1*, and 7 DPC in *experiment 2*. Pigs in the NEG groups remained negative throughout the experiments (data not shown).

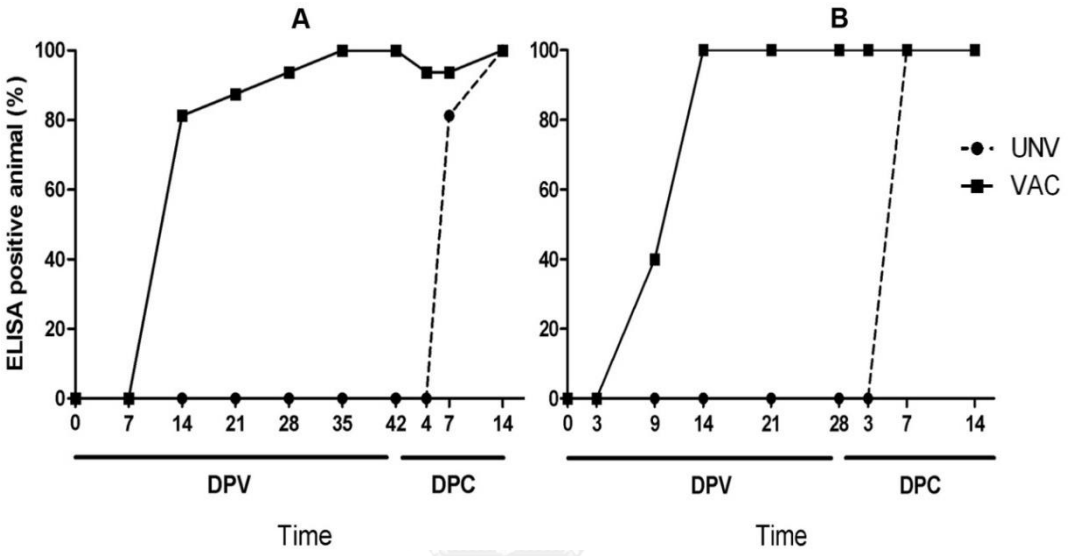


Figure 11. Percentage of positive pigs for anti-PRRSV antibody in *experiment 1* (a) and *experiment 2* (b).

4.4 Hematology

The Thai HP-PRRSV infection induced leukopenia in UNV group of both experiments. In *experiment 1*, both groups had decreased mean total white blood cells count after challenge but not reached to the cut-off leukopenia and VAC group had significantly higher mean total white blood cells count than that of UNV group at 2, 4, 7, 10 and 14 DPC (Fig. 12a). The frequency of pigs showing leukopenia in UNV group was significantly higher than that of VAC group at 2 and 4 DPC (Fig. 13a). In *experiment 2*, pigs from both groups had decreased mean total white blood cells count after challenge but not reached to the cut-off leukopenia and VAC group had significantly higher mean total white blood cells count than that of UNV group at 7

and 14 DPC (Fig. 12b) and one pig from UNV group showing leukopenia at 3 DPC (Fig. 13b). However, there was not significantly different. Leukopenic animals were not detected in pigs in the NEG group throughout the experiment (data not shown).

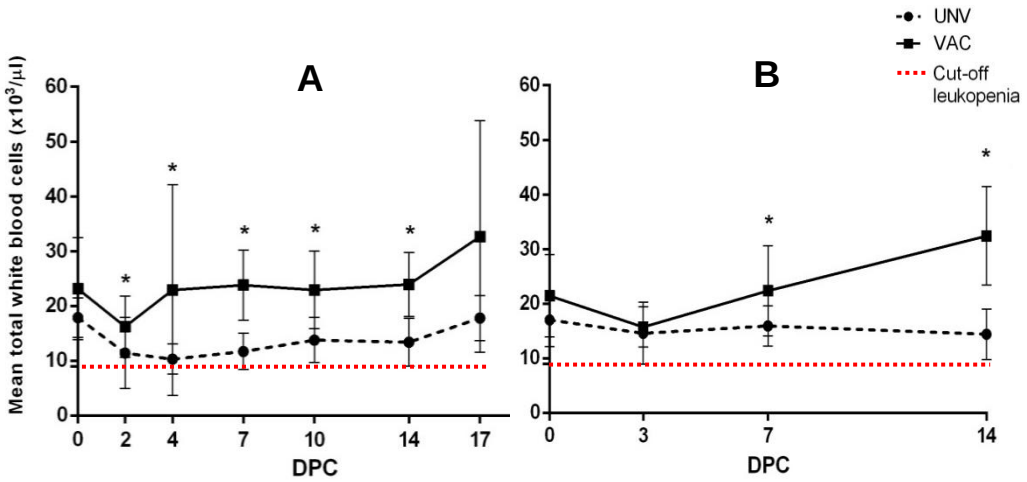


Figure 12. Mean total white blood cells count in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

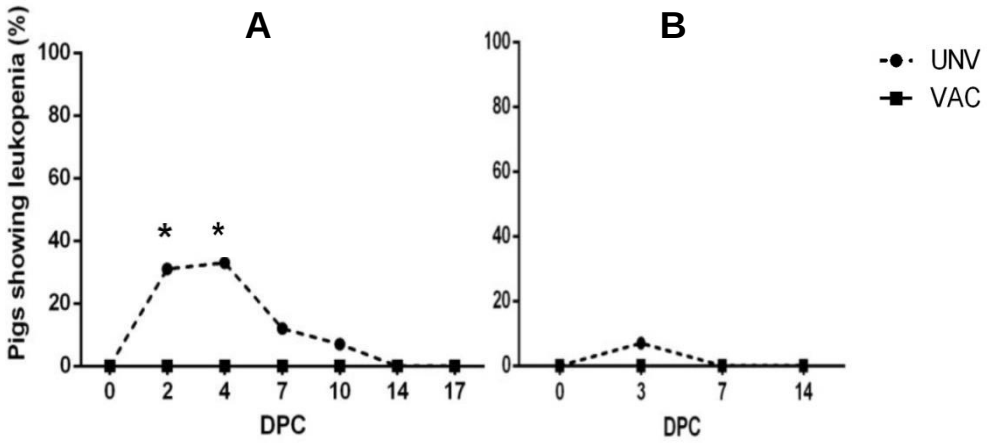


Figure 13. Frequency of pigs showing leukopenia in *experiment 1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

4.5 Pathological evaluation

4.5.1 Gross lung lesion

Experiment 1: Mean lung lesion scores demonstrated that pigs in VAC group showed significantly lower lung lesion scores than those of pigs in UNV group at both 10 and 17 DPC (Fig. 14a). Prior to the first necropsy examination date (at 10 DPC), one pig from UNV group died at 7 DPC with 47% lung lesion score. At 10 DPC, mean lung score of VAC group (9.13 ± 12.72 %, n = 8) was significantly lower than that of pigs in UNV group (44.43 ± 20.30%, n = 7). Prior to the second necropsy examination date (at 17 DPC), one pig from UNV group died at 11 DPC with 55% lung lesion score. At 17 DPC, mean lung score of pigs in UNV group was 29.43 ±16.40% (n = 8) which was significantly higher than that of pigs in VAC group (6.00 ± 6.39 %, n = 7). Lung lesion was not observed in the NEG group throughout the experiment (data not shown).

Experiment 2: During 7 – 11 DPC, prior to the necropsy examination date (at 14 DPC), 12 of 15 pigs from UNV group and 3 of 15 pigs from VAC group died. At this period, mean lung scores of pigs in UNV and VAC groups were not significantly different (Fig. 14b). At 14 DPC, mean lung scores of VAC group (40.73 ±18.18%, n = 12) was significantly lower than those of pigs in UNV group (64.58 ± 3.76%, n = 3) (Fig 14b and Fig 15). Lung lesion was not observed in the NEG group throughout the experiment (data not shown).

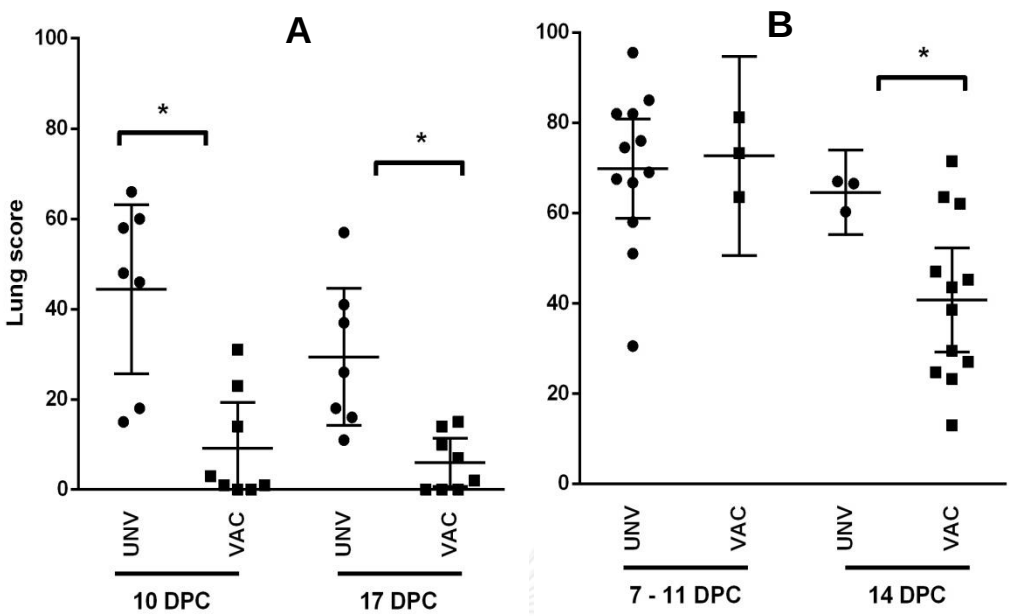


Figure 14. Mean lung lesion scores in *experiment 1* (a) and *experiment 2* (b).

Significant difference is indicated at *P* value < 0.05*.

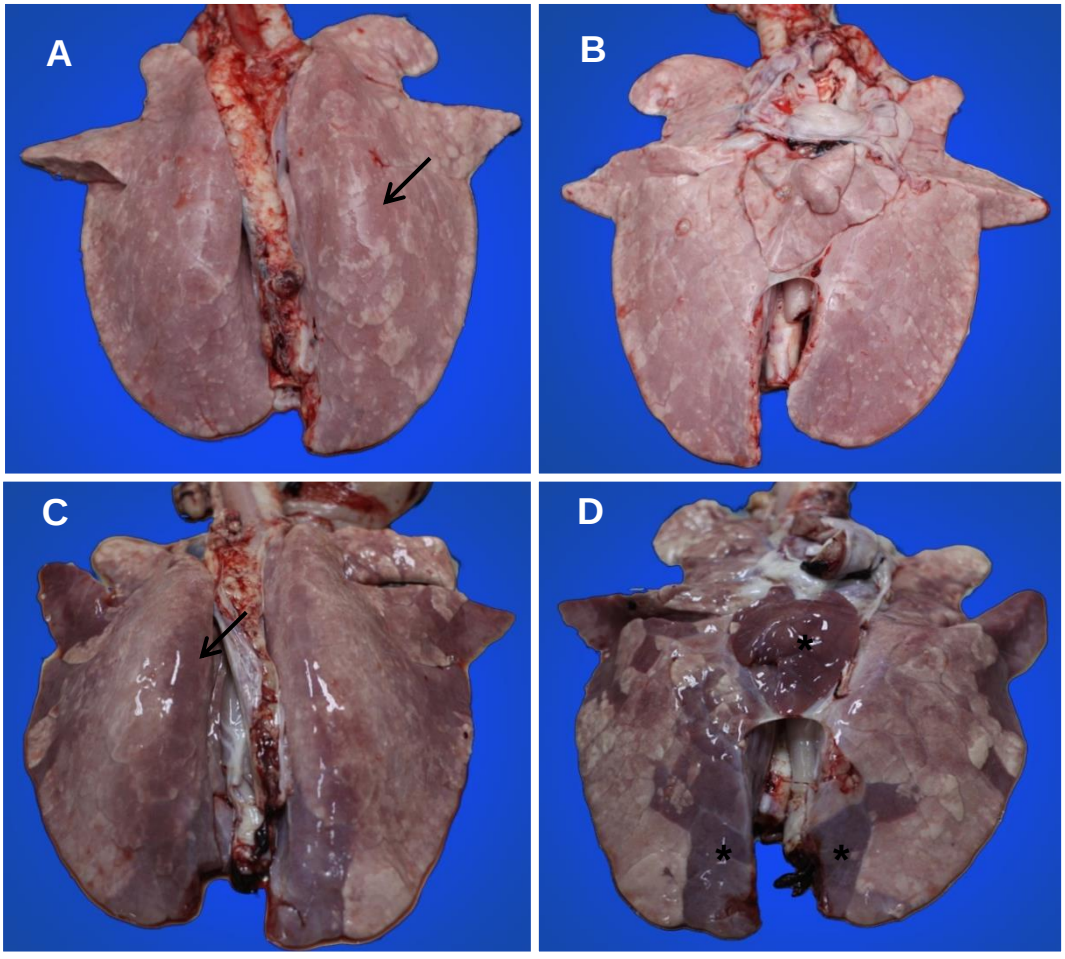


Figure 15. Gross lung lesion of VAC (a and b) and UNV group (c and d) in *experiment 2*. PRRSV-induced pneumonia is characterized by fail to collapse, multifocal, tan-mottled consolidated areas, with irregular and indistinct borders (arrow). Lungs from UNV group exhibiting well-demarcated and extensive dark-red consolidated areas (asterisk).

4.5.2 Histopathology

Upon lung microscopic examination, Mild to moderate degree of lymphohistiocytic interstitial pneumonia (characterized by thickening of alveolar walls by the infiltration of the lymphocytes and histiocytes) (Fig. 16a) and mild peribronchiolar lymphoid hyperplasia (characterized by accumulation of the lymphoid cells around bronchioli), were observed between VAC and UNV groups of both experiments. Microscopic lesion was not observed in the NEG group throughout the experiment (data not shown).

4.6 IHC

PRRSV antigen was detected in both lung and lymph node of both groups. PRRSV antigen was detected in pulmonary alveolar macrophages (Fig. 16b) and follicular macrophages in the lymph node (Fig. 16c). PRRSV antigen was characterized by dark-brown granular in the cytoplasm of the macrophage-liked cells of both lung and lymph node.

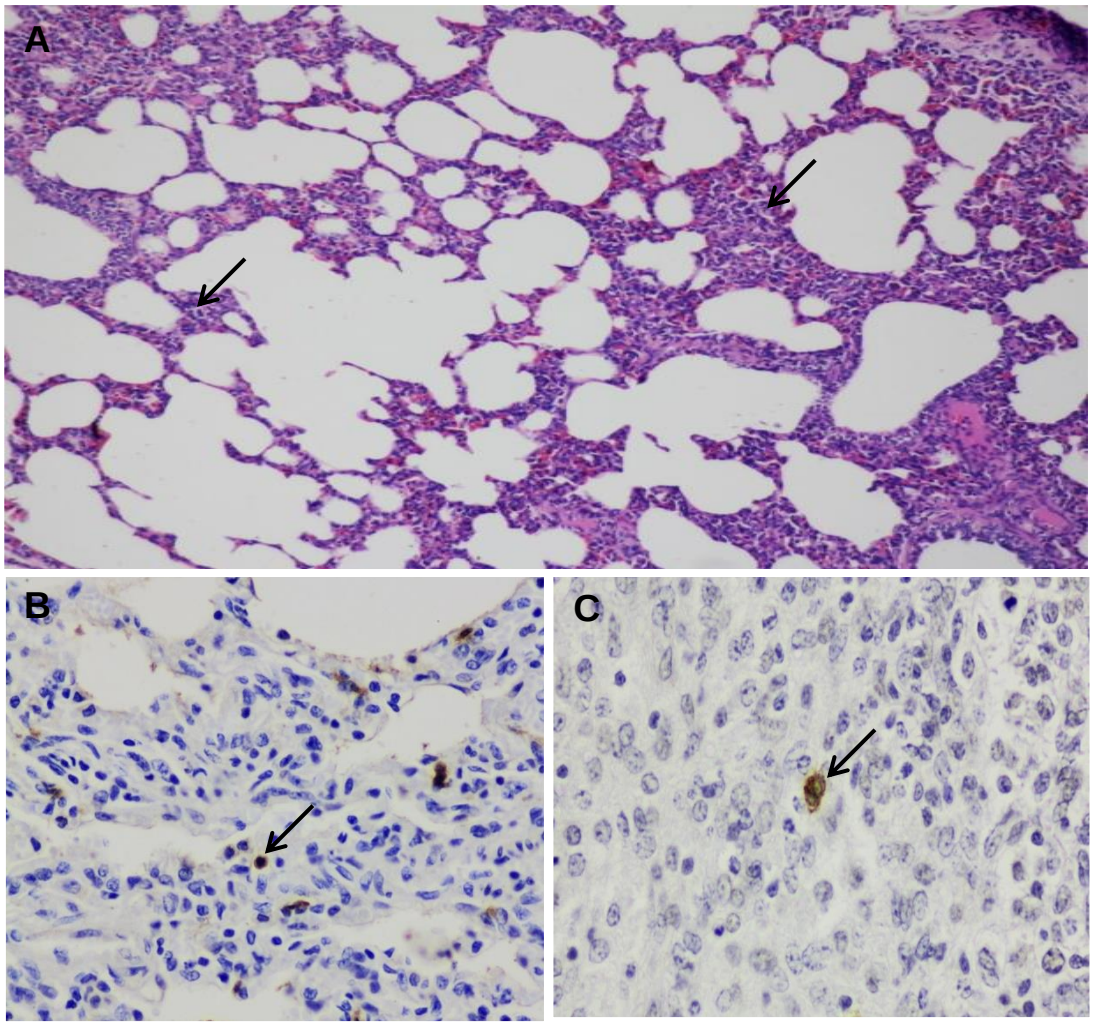


Figure 16. Microscopic findings and immunohistochemistry for the detection of PRRSV antigen in lungs and lymph node. Mild to moderate degree of lymphohistiocytic interstitial pneumonia (a) characterized by thickening of alveolar walls by the infiltration of the lymphocytes and histiocytes (arrow) (10×, H&E stain). PRRSV antigen (arrow) was detected in pulmonary alveolar macrophages (b) and follicular macrophages in the lymph nodes (c). Positive cells typically exhibited dark-brown granular PRRSV antigen in the cytoplasm (40×, SDOW17, Peroxidase/DAB+, Rabbit/Mouse, hematoxylin counter stain).

4.7 Virology

4.7.1 Viremia

In *experiment 1*, pigs in VAC group showed significantly lower frequency of PRRSV viremic pigs than that of UNV group at 10 and 14 DPC (Fig. 17a). Pigs in VAC group showed significantly lower copy number or viral load of PRRSV RNA, comparing with pigs in UNV group, at 2, 4, 7, 10, and 14 DPC (Fig.17c). No PRRSV genomic RNA was detected in pigs in NEG group (data not shown). In *experiment 2*, frequency of viremic pigs in both groups was not significantly different (Fig. 17b). However, pigs in VAC group showed significantly lower level of PRRSV genomic RNA than that of pigs in UNV group at 7 DPC (Fig. 17d). No PRRSV genomic RNA was detected in pigs in the NEG group (data not shown). Moreover, viremia from vaccine occurred at 14-21 DPV and disappeared before challenge (data not shown).

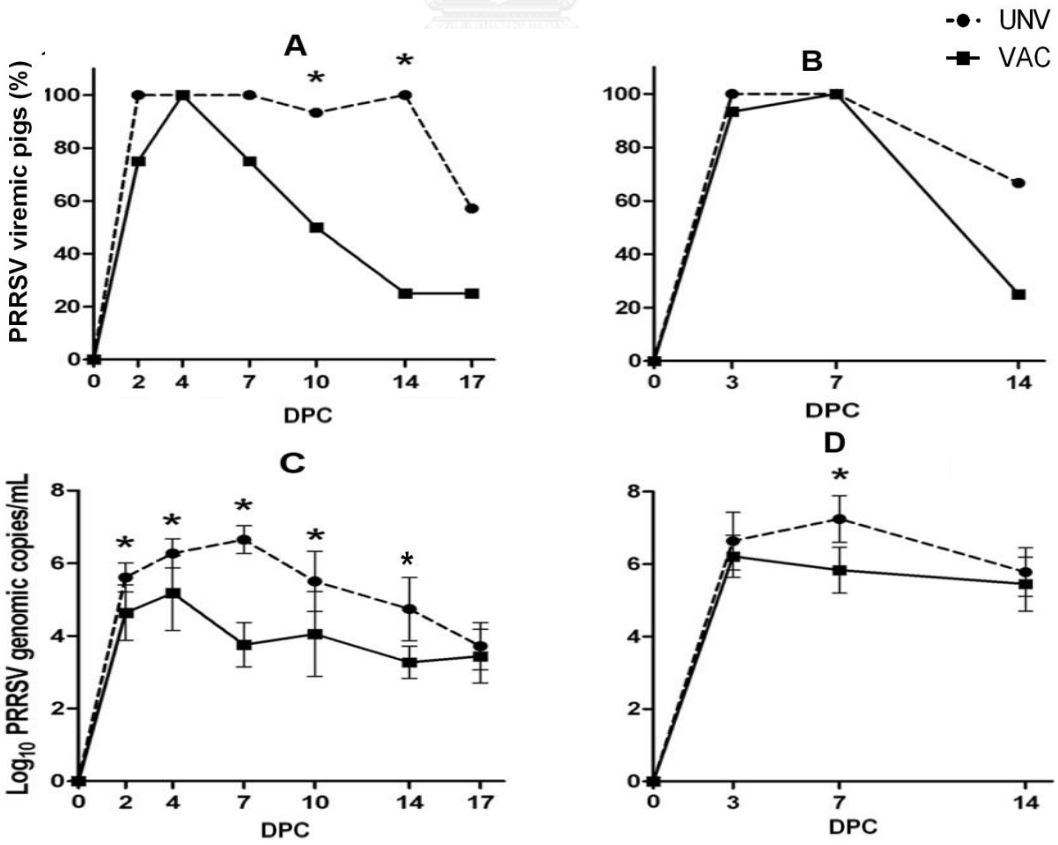


Figure 17. Frequency of PRRSV viremic pigs and mean values of the PRRSV genomic copy number from serum in *experiment 1* (a and c) and in *experiment 2* (b and d). Significant difference is indicated at *P* value < 0.05*.

4.7.2 Viral titer in the lung

In *experiment 1*, the quantity of PRRSV RNA in lung homogenate of pigs in VAC group was ($10^{2.5}$ copies/ml, n = 8) significantly lower than that of the level in UNV group ($10^{5.6}$ copies/ml, n = 7) (Fig. 18a) at 10 DPC. At 17 DPC, the level in VAC group was ($10^{1.3}$ copies/ml, n = 8) significantly lower than that of the level in pigs in UNV group ($10^{4.8}$ copies/ml, n = 7) (Fig. 18a). In experiment 2, the level in the VAC group was significantly lower than that of the level in UNV group at 14 DPC, no PRRSV RNA was detected in all pigs of VAC group (12/12) while 66.67% (2/3) were detected in the remaining pigs of UNV group ($10^{3.7}$ copies/ml, n = 3) (Fig. 18b). No PRRSV genomic RNA was detected in pigs in the NEG group (data not shown).

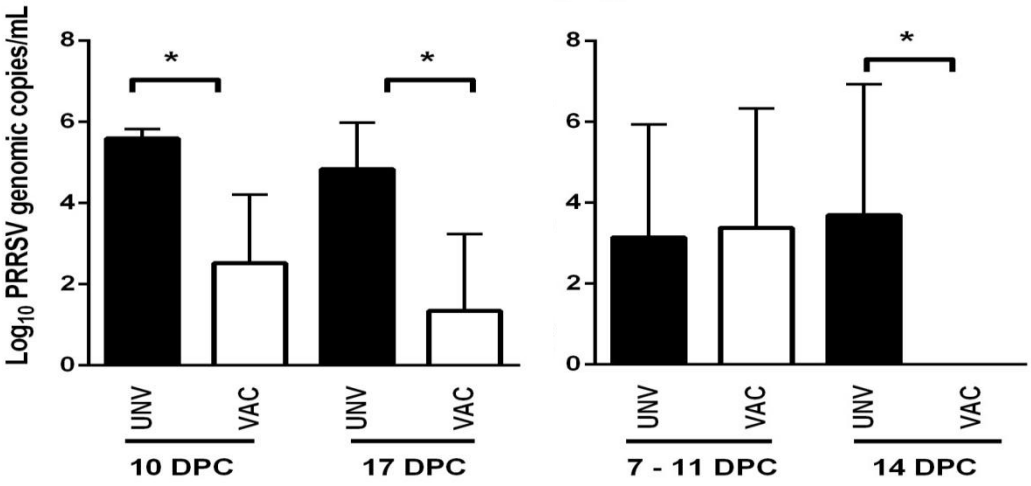


Figure 18. Mean values of PRRSV genomic copy number from lung homogenate in *experiment1* (a) and *experiment 2* (b). Significant difference is indicated at *P* value < 0.05*.

CHAPTER V

DISCUSSION AND CONCLUSION

Currently, HP-PRRSV is widespread and become a dominant strain in the Thai swine population (Jantafong et al., 2015). Evaluation of the efficacy and implementation of commercially available vaccines against HP-PRRSV infection is of importance. Previous study showed that a PRRS MLV of lineage 8 could provide varied degrees of protection against the Vietnamese HP-PRRSV infection (Do et al., 2015).

In the present study, efficacy of a new PRRS MLV of lineage 8 vaccine against the Thai HP-PRRSV infection was examined in 3-week-old piglets using 2 experiment protocols; a long (42 days) vaccination-to-infection period (*experiment 1*), and a short vaccination-to-infection period (28 days) (*experiment 2*) in order to deal with the different PRRSV-infected farm scenario. The results showed that the vaccine in both experiments could reduce the disease severity of Thai HP-PRRSV infection similar to the previous study when challenged at 35 DPV (Do et al. 2015).

Interestingly, the longer vaccination-to-infection period (42 DPV) study showed less severity of the disease outcomes in infected pigs coincided with lower virus titers in both serum and lung. HP-PRRSV infection showed greater severity in the short vaccination-to-infection period (28 DPV) study particularly in the unvaccinated piglets. This could be partly due to the higher sensitivity to HP-PRRSV infection of younger pigs, as previously reported of a typical PRRSV infection (Klinge et al., 2009) or the vaccine-induced immunity was not yet sufficient when challenged. Moreover, the difference between young and old pigs in viremic levels and virus excretion might be due to the combination between an altered macrophage response and

difference in cell subsets present in lymphoid organs and lungs (Van der Linden et al., 2003).

Higher sensitivity to the HP-PRRSV infection of younger pigs have been documented (Klinge et al., 2009) and younger pigs were demonstrated having higher levels of viremia due to the susceptibility of the macrophages (Thanawongnuwech et al., 1998). In addition, the cellular condition in the lymphoid organs such as T-cell distribution (presence of $CD4^{+}CD8^{+}$ in lungs and tonsil of old pigs) did affect the outcome of PRRSV infection (Zuckermann and Husmann, 1996). It should be noted that, the studied pigs were inoculated at 9 and 7 weeks of age, respectively, depending on each experimental protocol.

Antibody response of pigs in VAC group in *experiment 2* reached 100% detection earlier possibly due to differences in experimental setting or the different response of pigs in each experiment. Normally, PRRSV-specific antibodies are detected around 2 weeks and peaked around 4 weeks after vaccination. It should be noted that antibodies tested by a commercial ELISA could not reflect the protective responses. PRRSV-specific virus neutralizing antibodies appeared around 4 weeks after vaccination (Darwich et al., 2010) and associated with PRRSV vaccination-induced protection against homologous and heterologous challenge (Li et al., 2014). Unfortunately, PRRSV-specific virus neutralizing antibodies were not evaluated in this study.

The PRRS MLV vaccine could reduce viremia in the Thai HP-PRRSV infected pigs in both experiments. These finding are similar to the previous study when challenged at 35 DPV (Do et al., 2015). Prevention of PRRSV viremia by vaccination depends on the induction of cell-mediated immunity, especially the host IFN- γ response. It should be noted that IFN- γ inhibits PRRSV replication as previously described (Rowland et al., 2001). PRRSV-specific IFN-γ-SCs play key roles in the protective cell-mediated immunity against PRRSV infection (Meier et al., 2003) and

the important factors in viral clearance as previously described (Park et al., 2014). In addition, this particular MLV vaccine could induce high numbers of HP-PRRSV-specific IFN-γ-SCs in vaccinated challenged pigs as described previously (Do et al., 2015). It could be one of the main factors to explain viremic reduction in this study.

The PRRS MLV vaccine used in this study could improve the survival rate and clinical signs in the Thai HP-PRRSV infected pigs. In *experiment 2*, High mortality rate found in UNV group was possibly due to the susceptibility to PRRSV infection in younger pigs demonstrating by having higher viremic levels (Van der Linden et al., 2003), possibly related to the better efficacious viral replication in macrophages of younger pigs (Thanawongnuwech et al., 1998). Moreover, the differences of severity in both experiments might be due to the differences of pig ages when challenged relating to the presence of the viral load in blood (Diaz et al., 2005). In addition, the severity of respiratory signs was well correlated with the amount of viral load in blood (Johnson et al., 2004). However, viremia and the severity of clinical signs between VAC and UNV group were not significant in *Experiment 2*. It might be due to few survival pigs in UNV group after 8 DPC. Severity of clinical signs was related to the antibody response in both experiments since the vaccinated pigs showed less severity when compared with the unvaccinated pigs. Moreover, unvaccinated pigs in *experiment 2* reached 100% antibody detection earlier possibly due to higher sensitivity to the HP-PRRSV infection of younger pigs (Klinge et al., 2009).

The vaccine could be used against the severity of leukopenia. In this study, only pigs in UNV group of both experiments had transient leukopenia relating to the severity of clinical signs. In this study, transient leukopenia was related with the onset of fever and the results were similar to the previous report which commonly found at l-3 days after the onset of fever and persisted for 4-l0 days (Rossow et al., 1994; Lohse et al., 2004) and transient leukopenia in peripheral blood is resolved by 8–10

DPC (Nielsen and Bøtner, 1997). However, these finding were not significant in *Experiment 2* due to a few survival pigs in UNV group after 8 DPC.

The vaccine could reduce viral load in the lung, possibly due to viremic reduction in vaccinated pigs in both experiments. A good correlation between the extent of the viremia and the virus titer in the lung tissues have been documented (Duan et al., 1997). In experiment 2, no PRRSV RNA was detected in all pigs of VAC group possibly due to sampling technique. However, viremic reduction and mean lung scores of VAC group were provided sufficient information for the vaccine efficacy.

The vaccine could reduce lung lesions in vaccinated pigs, possibly due to reduce viral load in blood and lung of vaccinated pigs in both experiments. The pathogenic outcome of the PRRSV infected pigs were directly related to viral load in acute infection as reflected in viral titers in blood as previously described (Johnson et al., 2004). In this study, only pigs in UNV group of both experiments had higher lung lesion score as well as leukopenia. The vaccine could be used against the severity of leukopenia and gross lung lesions induced by HP-PRRSV. However, microscopic lung lesions and lesions found in tracheobronchial lymph node were not different from the unvaccinated pigs, possibly due to the challenge strain of PRRSV is highly virulent, resulting in extensive lung lesions occurring as early as 7 DPC and resolving by 21 DPC based on a previous study (Han et al., 2013). In addition, lymphoid necrosis was found at 7 DPC and lymph node hypertrophy and hyperplasia were later found at 28 DPC have been documented (Rossow et al., 1994).

The mechanisms of the partial protection including reducing severity of clinical signs and viral load in blood and lung tissues might be due to the induction of IFN-γ-SCs (Do et al., 2015). PRRSV-specific IFN-γ-SCs are the main factors in viral clearance (Meier et al., 2003; Park et al., 2014). Unfortunately, IL-10 and other cytokine profiles were not evaluated in this study. However, based on our pilot data

in *experiment 1*, the percentage of $CD^{3+}IL10^{+}$ cell population was firstly detected in pigs from the vaccinated groups at 42 DPV. Nonetheless, this population was detected in lower levels when compared to pigs in the unvaccinated groups after 7 DPC. The percentage of CD^{3+}IFN-γ^{+} cell population was detected at higher levels since 7 DPV and gradually decreased until 42 DPV. In contrast, this population was gradually increased and reached the highest levels at 7 DPV (data not shown).

The studied results also provided sufficient information on the timing of PRRS MLV of lineage 8 vaccination against the Thai HP-PRRSV infection. Previously, it was demonstrated that pigs vaccinated with a PRRS MLV of lineage 8 showed high level of IL-10 until 5 weeks post vaccination (Park et al., 2014). Interestingly, when challenge with HP-PRRSV as early as 4 weeks after vaccination, the vaccine could still provide satisfied protection against the infection. In addition, the parental strain of PRRS MLV is in the lineage 8 which genetically classified into the same lineage as the Chinese HP-PRRSV (Shi et al., 2010) and the studied vaccine could provide higher levels of cross protection against the Thai HP-PRRSV (sublineage 8.7) as described previously (Do et al., 2015). In this study, even different vaccination-to-infection periods when pigs infected by the Thai HP-PRRSV were implimented, vaccination still provides better results. More studies should be done if other currently available vaccines are effective enough when used against genetic heterogeneity of PRRSV field isolates (Meng, 2000).

Conclusion

A PRRS MLV of lineage 8 is able to provide protection against the Thai HP-PRRSV infection in both long and short vaccination-to-infection protocols. However, the better efficacy was depending on the longer timing of challenge. Vaccinated pigs showed significantly lower rectal temperatures, lower severity of clinical signs, lower lung lesion scores and lower viral titers (in both serum and lung).

Further studies are required to evaluate the effect of IL-10 and other cytokines, particularly the numbers of HP-PRRSV-specific IFN-γ-SCs against a Thai HP-PRRSV (10PL01) in vaccinated challenged pigs.

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APPENDIX

APPENDIX A

Detected for PRRSV antibody using IDEXX PRRS X3 ELISA test kit

1. The sample was diluted 1:40 with sample diluent (e.g. by diluting 5 µl of sample with 195 µl of sample diluent).
2. Dispense 100 µl of undiluted negative control in the first two wells (A1, B1). Then it was add 100 ml undiluted positive control in the next two wells (C1, D1).
3. Dispense 100 µl of diluted sample into two wells of the assay plate and incubate for 30 minutes at 18-25°C.
4. The plate was washed 3-5 times with 300 ml of washing solution.
5. Add 100 ml each of swine anti IgG conjugated with peroxidase labeled (HRPO) to each well and incubate for 30 minutes at 18-25 $^{\circ}$C.
6. The plate was wash 3-5 times with 300 ml of washing solution.
7. Add 100 ml of TMB substrate solution into each well and incubate 15 minutes at 18-25 $^{\circ}$C.
8. Add 100 ml of stop solution to each well to stop the reaction.
9. Measure and record the wavelength of A (650) samples and controls.
10. Calculations and interpretation of results.

Immunohistochemistry

1. Sections were deparaffinized in xylene followed by dehydration and rehydration in ethanols.
2. Sections were washed 1 times with distilled water for 5 minutes followed by washed 1 time with 1X PBS for 5 minutes.
3. Sections were antigen retrieval treatment by 0.1 % trypsin and incubate for 30 minutes at 37°C.
4. Sections were washed 3 times with 1X PBS for 5 minutes.

5. Sections were block non-specific endogenous peroxidase by 3% H_20_2 in absolute methanol for 20 minutes at room temperature.
6. Sections were washed 1 time with distilled water for 5 minutes followed by washed 2 times with 1X PBS for 5 minutes.
7. Sections were block by 10% BSA for 60 minutes at 37 °C.
8. Sections were washed 3 times with 1X PBS for 5 minutes.
9. Sections were incubated with a PRRSV monoclonal antibody (SDOW17 diluted 1: 1000 in sterile 1x PBS) for 12-14 hours at 4 °C.
10. Sections were washed 3 times with 1X PBS for 5 minutes.
11. Sections were incubated with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) for 45 min at room temperature.
12. Sections were washed 3 times with 1X PBS for 5 minutes.
13. Sections were incubated with Dako REAL™ DAB+ Chromogen (diluted 1: 50 in Dako REAL™ Substrate Buffer) for 25 seconds at room temperature.
14. Sections were washed 1 time with distilled water for stop the reaction.
15. Sections were washed 1 time with tap water for 5 minutes.
16. Sections were counter stain with hematoxylin for 30 seconds.
17. Sections were washed 1 time with tap water and dehydrate and mount.
18. Sections were dehydrate and mount.

Detection of PRRSV RNA from serum and lung tissues

1. NucleoSpin® RNA virus kit were used for viral RNA extraction.
2. Each organ (1 gram) was homogenised in 5 ml PBS, clarified by centrifugation, and stored at −70 °C before RNA extraction.
3. Copy number of viral RNA was then quantified using TaqMan® probe-based real-time RT-PCR.

4. Primers and probe were as follows; reverse primer USalignEU-R (5' AAATGIGGCTTCTCIGGITTTT 3′) and forward primer USalignEU-F (5′ TCAICTGTGCCAGITGCTGG 3′) and US-PRRSV-specific probe FAM_US_rev (5′ *FAM*-TCCCGGTCCCTTGCCTCTGGA-*TAMRA* 3′).
5. RT-PCR mixture (25 µl) was based on SuperScript™ III One-Step RT-PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1x Reaction Mix, 0.4 mM of each primer and probe, 0.5 µl of SuperScript® III RT/Platinum®Taq Mix, and 0.5 µl of viral RNA.
6. RT-PCR was carried out in MyGo Pro real-time PCR machine.

APPENDIX B

Inter-isolate genetic distance

Isolate		% Difference for isolate				
		1	2	3	4	5
1	10PL01					
2	PRRS MLV of lineage 8	8				
3	Prime Pac® PRRS	10	7			
4	Ingelvac PRRS® ATP	11	6	10		
5	Ingelvac PRRS® MLV	12	9	8	10	

VITA

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