Porcine reproductive and respiratory syndrome virus: genetic diversity, pathogenesis and modified-live vaccines

Mr. Adthakorn Madapong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Pathobiology
Department of Veterinary Pathology
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2020

ไวรัสพีอาร์อาร์เอส: ความหลากหลายทางพันธุกรรม พยาธิกำเนิดและวัคซีนป้องกันโรคชนิดเชื้อเป็น

นายอรรถกร มาดาป้อง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	Porcine reproductive and respiratory syndrome virus: genetic diversity, pathogenesis and modified-live vaccines
By	Mr. Adthakorn Madapong
Field of Study	Veterinary Pathobiology
Thesis Advisor	Assistant Professor Dr. DACHRIT NILUBOL, D.V.M., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF VETERINARY SCIENCE
(Professor Dr. ROONGROJE THANAWONGNUWECH, D.V.M., Ph.D.)

DISSERTATION COMMITTEE

..... Chairman
(Associate Professor Dr. MORAKOT KAEWTHAMASORN, D.V.M., Ph.D.)

..... Thesis Advisor
(Assistant Professor Dr. DACHRIT NILUBOL, D.V.M., Ph.D.)

..... Examiner
(Assistant Professor Dr. AUNYARATANA THONTIRAVONG, D.V.M., Ph.D.)

..... Examiner
(Associate Professor Dr. ANGKANA TANTITUVANONT, Ph.D.)

..... External Examiner
(Associate Professor Dr. KAMPON KAEOKET, D.V.M., Ph.D.)

อรรถกร มาตาป้อง : ไวรัสพีอาร์อาร์เอส: ความหลากหลายทางพันธุกรรม พยาธิกำเนิดและวัคซีนป้องกันโรคชนิดเชื้อเป็น. (Porcine reproductive and respiratory syndrome virus: genetic diversity, pathogenesis and modified-live vaccines) อ.ที่ปรึกษาหลัก : ผศ. น.สพ. ดร.เดชฤทธิ์ นิลอุบล

ไวรัสพีอาร์อาร์เอส (porcine reproductive and respiratory syndrome virus; PRRSV) ก่อให้เกิดโรคทางระบบสืบพันธุ์และระบบทางเดินหายใจในสุกร ที่เรียกว่าโรคพีอาร์อาร์เอส (PRRS) ปัจจุบันมีวัคซีนป้องกันโรคชนิดเชื้อเป็นหลายชนิดจำหน่าย การเลือกใช้วัคซีนเพื่อป้องกันโรคดังกล่าวจึงมีความสำคัญ วัตถุประสงค์ของการศึกษานี้เริ่มต้นจากการศึกษาความหลากหลายทางพันธุกรรมของไวรัสพีอาร์อาร์เอสในฝูงสุกรของประเทศไทยตั้งแต่ปีพ.ศ. 2544-2560 ร่วมกับการศึกษาพยาธิกำเนิดของการติดไวรัสพีอาร์อาร์เอสที่แยกได้ในประเทศไทยในสุกรทดลอง จากนั้นทำการศึกษาประสิทธิภาพและช่องทางการฉีดวัคซีนป้องกันโรคพีอาร์อาร์เอสชนิดเชื้อเป็นที่มีจำหน่ายต่อการติดไวรัสพีอาร์อาร์เอสของประเทศไทย ผลจากการศึกษาพบว่า ร้อยละ 75 ของฝูงสุกรของประเทศไทยมีการติดไวรัสทั้งสองสายพันธุ์ร่วมกัน (co-infection) โดยไวรัสสายพันธุ์ยุโรปของประเทศไทยทั้งหมดถูกจัดอยู่ในซับไทป์ 1 (subtype 1) โดยมีไวรัสในเคลด A (clade A) เป็นไวรัสเด่น (dominant strain) ของสายพันธุ์ยุโรป ส่วนไวรัสสายพันธุ์อเมริกาเหนือของประเทศไทยมีความหลากหลายทางพันธุกรรมที่มากกว่า โดยมีไวรัสในลินิเอจ 8 (lineage 8) ซับลินิเอจ 8.7/HP-PRRSV-2 (sublineage 8.7/HP-PRRSV-2) เป็นไวรัสเด่นของสายพันธุ์อเมริกาเหนือซึ่งมีความใกล้เคียงกับไวรัสพีอาร์อาร์เอส สายพันธุ์รุนแรงที่เคยก่อโรคระบาดในประเทศไทยเมื่อปีพ.ศ. 2553 สำหรับผลการศึกษาพยาธิกำเนิดพบว่าไวรัสพีอาร์อาร์เอสสายพันธุ์ยุโรป (AN06EU4204) และสายพันธุ์อเมริกาเหนือ (FDT10US23, HP-PRRSV-2) ที่แยกได้ในประเทศไทยสามารถก่อโรคในสุกรได้เหมือนกัน แต่การติดไวรัสทั้งสองสายพันธุ์ร่วมกัน (co-infection) จะมีปริมาณไวรัสและรอยโรคที่ปอดมากกว่าการติดไวรัสเพียงสายพันธุ์ใดสายพันธุ์หนึ่ง ซึ่งบ่งบอกถึงความเป็นไปได้ในการเกิดโรคระบาด จากนั้นเมื่อทำการทดสอบประสิทธิภาพและช่องทางการฉีดวัคซีนที่แตกต่างกันพบว่า วัคซีนป้องกันโรคพีอาร์อาร์เอสชนิดเชื้อเป็นที่มีจำหน่าย ไม่ว่าจะฉีดด้วยวิธีเข้ากล้ามเนื้อ (intramuscular; IM) หรือฉีดเข้าใต้ผิวหนัง (intradermal; ID) สามารถกระตุ้นการตอบสนองของภูมิคุ้มกันได้ไม่แตกต่างกัน โดยมีการตอบสนองของภูมิคุ้มกันชนิดสารน้ำอย่างรวดเร็วเมื่อวัดด้วยวิธีอีไลซา (ELISA) แต่การตอบสนองของภูมิคุ้มกันชนิดพึ่งเซลล์เกิดได้ช้าและมีความจำเพาะกับไอโซเลตของไวรัสที่ใช้ในการทดสอบ อย่างไรก็ตามวัคซีนที่ใช้ในการศึกษาทั้งหมดสามารถลดปริมาณไวรัสในกระแสเลือด (viremia) และรอยโรคที่ปอด (lung lesion) ต่อการติดไวรัสพีอาร์อาร์เอสของประเทศไทย อีกทั้งยังพบว่าการฉีดวัคซีนเข้าใต้ผิวหนัง (ID) มีปริมาณของเซลล์ที่สร้างอินเตอร์เฟอรอน-แกมมา (interferon-gamma secreting cells) ที่มากกว่าและเหนี่ยวนำการผลิตอินเตอร์ลิวคิน-10 (IL-10) ที่น้อยกว่า เมื่อเทียบกับการฉีดเข้าใต้กล้ามเนื้อ (IM) จากการศึกษานี้สรุปได้ว่าไวรัสพีอาร์อาร์เอสทั้งสองสายพันธุ์ของประเทศไทยมีการเปลี่ยนแปลงอยู่ตลอดเวลา ไม่เกี่ยวข้องซึ่งกันและกัน โดยมีความหลากหลายทางพันธุกรรมที่สูงขึ้น ขึ้นอยู่กับการนำเข้ามาของไวรัสใหม่ในฝูงสุกร และการติดไวรัสทั้งสองสายพันธุ์ร่วมกัน (co-infection) จะมีความรุนแรงของโรคที่มากกว่า ส่วนการใช้วัคซีนป้องกันโรคชนิดเชื้อเป็นสามารถให้ความคุ้มโรคเพียงบางส่วน (partial protection) ต่อการติดไวรัสพีอาร์อาร์เอส ซึ่งการฉีดวัคซีนเข้าใต้ผิวหนัง (ID) อาจเป็นหนึ่งทางเลือกในการฉีดวัคซีนป้องกันโรคพีอาร์อาร์เอสในอนาคต

สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5875522831 : MAJOR VETERINARY PATHOBIOLOGY

KEYWORD: Porcine reproductive and respiratory syndrome virus, Genetic diversity, Pathogenesis, Modified-live virus vaccine

Adthakorn Madapong : Porcine reproductive and respiratory syndrome virus: genetic diversity, pathogenesis and modified-live vaccines. Advisor: Asst. Prof. Dr. DACHRIT NILUBOL, D.V.M., Ph.D.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a causative agent of PRRS that reproductive failure in sows and respiratory problems in piglets is the hallmark of the disease. Recently, several PRRSV modified live vaccines (MLV) are available, and vaccine selection is a concern. Therefore, the objectives of this study were: 1) to investigate the genetic diversity of Thai PRRSV isolates during 2001-2017; 2) to evaluate the pathogenicity of Thai PRRSV isolates; 3) to determine the efficacy of PRRSV MLV against Thai field PRRSV infection and 4) to investigate the effectiveness of PRRSV MLV when administered via intramuscular (IM) and intradermal (ID) routes against Thai field PRRSV infection. Our results showed that all Thai PRRSV-1 isolates were in subtype 1, which clade A was a dominant strain of PRRSV-1. Meanwhile, Thai PRRSV-2 in lineage 8, sublineage 8.7/HP-PRRSV-2, was the dominant strain of Thai PRRSV-2. When compared the pathogenicity, we noticed that either Thai field PRRSV-1 or PRRSV-2 isolates induced similar clinical disease, and co-infection with both PRRSV species able to cause more severity than those of single infection. For the study of vaccine efficacy, all commercially available PRRSV MLV induce similar humoral- and cell-mediated immune responses with partial cross-protection against Thai field PRRSV infection. Besides, vaccination via IM and ID able to activate an immune response in pigs with partial cross-protection against PRRSV infection. ID vaccination induces more interferon-gamma secreting groups and provide lower interleukin-10 (IL-10) than the IM vaccination. In conclusion, the Thai field PRRSV evolved separately and developed their clusters with higher genetic diversity, and the severity of the co-infection of both PRRSV species is remark. Regardless of vaccine species, all commercial PRRSV MLV provides partial protection against heterologous PRRSV infection, and the ID route might be a choice for PRRSV MLV vaccination in the future.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

ACKNOWLEDGEMENTS

This research was financially supported by Thailand Science Research and Innovation (TSRI), Research and Researchers for Industries (RRi) Ph.D. Scholarship (CODE: PHD59I0040), and Chulalongkorn University Special Task Force for Activating Research Research (CU-STAR): swine viral evolution and vaccine research (SVEVR). I would like to express my sincere gratitude to the people who have supported and assisted me in the completion of this dissertation, especially to:

Assistant Professor Dr. Dachrit Nilubol, my principal advisor, and Associate Professor Dr. Angkana Tantituvanont, for the opportunity and support throughout this dissertation, for believing in me and encourage me to be a better researcher/scientist. I deeply appreciate your supports during my study.

Associate Professor Dr. Morakot Kaewthamasorn, Associate Professor Dr. Kampon Kaeoket, external examiner, and Assistant Professor Dr. Arunyaratana Thontiravong, committee, for giving me suggestions on my dissertation.

Dr. Gun Teemyasen (D.V.M.), and Miss Thitima Tripipat, for your techniques and advice. Members of DN1214, including Dr. Puwich Chaikhumwang, Mr. Parin Watcharavongtip, Mr. Christropher Jame Stott (D.V.M.), Miss Pathumporn Jermsutjarit, Miss Sunit Meebumroong (D.V.M), Miss Kanokon Sawattrakool (D.V.M.), Mr. Arsa Wichaikul, and Mr. Panumas Kongpanna. Thank you very much for making good memories and joyful working. I appreciate the time we spent together.

Mr. Kittikun Madapong, my brother. Mr. Hirun and Mrs. Rungfaa Madapong, my father and mother, for your precious support, and I know you did it all you could just to make sure my life was good. Without you, success in my life could not have come. I am so proud to be your son.

And finally: my beloved partner, Dr. Kepalee Saeng-chuto, for your support and encouragement. You are always beside me during the study. My life during research is much more fun and I appreciate your love and kindness.

Adthakorn Madapong

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	antigen presenting cells
BSA	bovine serum albumin
bp	base pair
°C	degree Celsius
CD	cluster of differentiation
CMI	cell-mediated immune response
CO_2	carbondioxide
DPC	days post-challenge
DPI	days post-infection
DPV	days post-vaccination
E	envelope
FITC	fluorescein isothiocyanate
GP	glycoprotein
HP-PRRSV-2	highly pathogenic-PRRSV-2
ID	intradermal
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IM	intramuscular
MAb	monoclonal antibody
MHC	major histocompatibility complex
mL	milliliter (s)
MLV	modified live vaccine
mM	millimolar
µl	microliter (s)
µM	micromolar
µm	micron
MOI	multiplicity of infection
M protein	membrane protein

NK	natural killer cell
N protein	nucleocapsid protein
Nsp	nonstructural protein
ORF	open reading frame
PAM	pulmonary alveolar macrophage
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
rpm	rounds per minute
RT-PCR	reverse transcriptase polymerase chain reaction
$TCID_{50}$/ml	tissue culture infective dose 50 per milliliter (s)
TNF	tumor necrosis factor
Treg	regulatory T lymphocyte (s)
×g	times gravity

CHAPTER 1
IMPORTANT AND RATIONALE

Porcine reproductive and respiratory syndrome (PRRS) is one of important diseases that cause economic losses in swine production worldwide. PRRS caused by PRRS virus (PRRSV) and have two major clinical forms of disease in pigs, including reproductive failure in pregnant sows (Karniychuk et al., 2012) and respiratory problems in all ages of pigs. PRRSV is enveloped single-stranded positive-sense RNA virus of the family *Arteriviridae*, genus *Porarterivirus* (Adams et al., 2016). PRRSV is classified into two distinct species, PRRSV-1 (formerly European or genotype I) and PRRSV-2 (formerly North American or genotype II). PRRSV-1 was restricted to Europe, while PRRSV-2 presented in the North American continents (Collins et al., 1992; Wensvoort et al., 1991). In 2006, highly pathogenic PRRSV (HP-PRRSV-2) was emerged and caused huge impact losses in swine production in China (Tian et al., 2007). Since then, HP-PRRSV-2 is distributed and endemic in several regions especially in the South East Asia countries including Korea, Vietnam, and Thailand (Kim et al., 2011; Nilubol et al., 2012).

In Thailand, PRRSV has been detected since 1989 which both species can be isolated from swine herds. In the previous study demonstrated that 66.42% of PRRSV isolated from Thai swine herds was PRRSV-1. Meanwhile, 33.58% of PRRSV isolates was PRRSV-2 (Thanawongnuwech et al., 2004). In the present, PRRSV in Thai swine herds showed higher genetically variations and increasing of PRRSV-2 because PRRSV-2 modified-live vaccines (MLV) had been progressively used in swine herds (Nilubol et al., 2012). In the previous study of genetic diversity of Thai field PRRSV isolates demonstrated that both PRRSV species have evolved separately. In addition, Thai PRRSV isolates, either PRRSV-1 or PRRSV-2, develop their own clusters without geographical influence. Interestingly, both PRRSV-1 and PRRSV-2 had major dominant cluster which always detectable regardless to the new PRRSV introduction. Moreover, the majority of Thai swine herds were concurrently infected with both PRRSV species without any specie being dominant (Nilubol et al., 2013).

To control PRRSV, various types of vaccines had been used. Especially, the used of attenuated or modified-lived vaccines, either PRRSV-1 or PRRSV-2, showed promise efficacious against PRRSV infection as demonstrated by the reduction of disease severities, clinical sings and lung lesion as well as viremic phase (Labarque et

al., 2003; Labarque et al., 2000). However, these vaccines shared various efficacies against the disease form partial to none against heterologous protection (Martinez-Lobo et al., 2013; Mengeling et al., 2003; Park et al., 2014). In addition, the problems of PRRSV MLV used are reported including return to virulent of vaccine virus (Botner et al., 1997), increasing of genetic mutation (Nilubol et al., 2014), immune suppressive effect (Bassaganya-Riera et al., 2004). It has been already reported that the commercial PRRSV MLV is more effective in controlling homologous rather than heterologous infection (Kimman et al., 2009; Murtaugh and Genzow, 2011) and the used of PRRSV MLV depends on circulating PRRSV in the fields. However, in the presence of both PRRSV-1 and PRRSV-2, what PRRSV MLV should be use. Especially, in the regions which high genetic diversities of PRRSV isolates had been reported.

Therefore, in the present study had the following four aims; 1) to investigate the genetic diversity of Thai field PRRSV isolates using phylogenetic analysis; 2) to evaluate the pathogenicity of Thai field PRRSV isolates in experimental animal; 3) to test the efficacy of PRRSV MLV against Thai field PRRSV infection in experimental animal , and 4) to evaluate the alternative route of PRRSV MLV vaccination in experimental animals.

CHAPTER 2

OBJECTIVES, HYPOTHESES AND CONCEPTUAL FRAMEWORK

2.1 Objectives of study

1. To evaluate the genetic diversity and strain domination of porcine reproductive and respiratory syndrome virus in Thai swine herds based on ORF5 gene
2. To study the pathogenicity of Thai field porcine reproductive and respiratory syndrome virus in experimental pigs
3. To evaluate the efficacy of commercial porcine reproductive and respiratory syndrome virus modified-live vaccines against Thai filed porcine reproductive and respiratory syndrome virus isolates in experimental pigs
4. To investigate the alternative route of porcine reproductive and respiratory syndrome virus modified-live vaccine administration

2.2 Hypotheses

1. Genetic variation and dominant strain of porcine reproductive and respiratory syndrome virus (PRRSV) will be observed in Thai swine herds
2. Co-infection of porcine reproductive and respiratory syndrome virus-1 (PRRSV-1) and porcine reproductive and respiratory syndrome virus-2 (PRRSV-2) shows more severity compared to that of single infection with either porcine reproductive and respiratory syndrome virus-1 (PRRSV-1) or porcine reproductive and respiratory syndrome virus-2 (PRRSV-2) alone
3. Porcine reproductive and respiratory syndrome virus modified-live vaccines that commercially available in Thailand will be provided protection against Thai filed PRRSV infection
4. Intradermal vaccination can improve efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccine

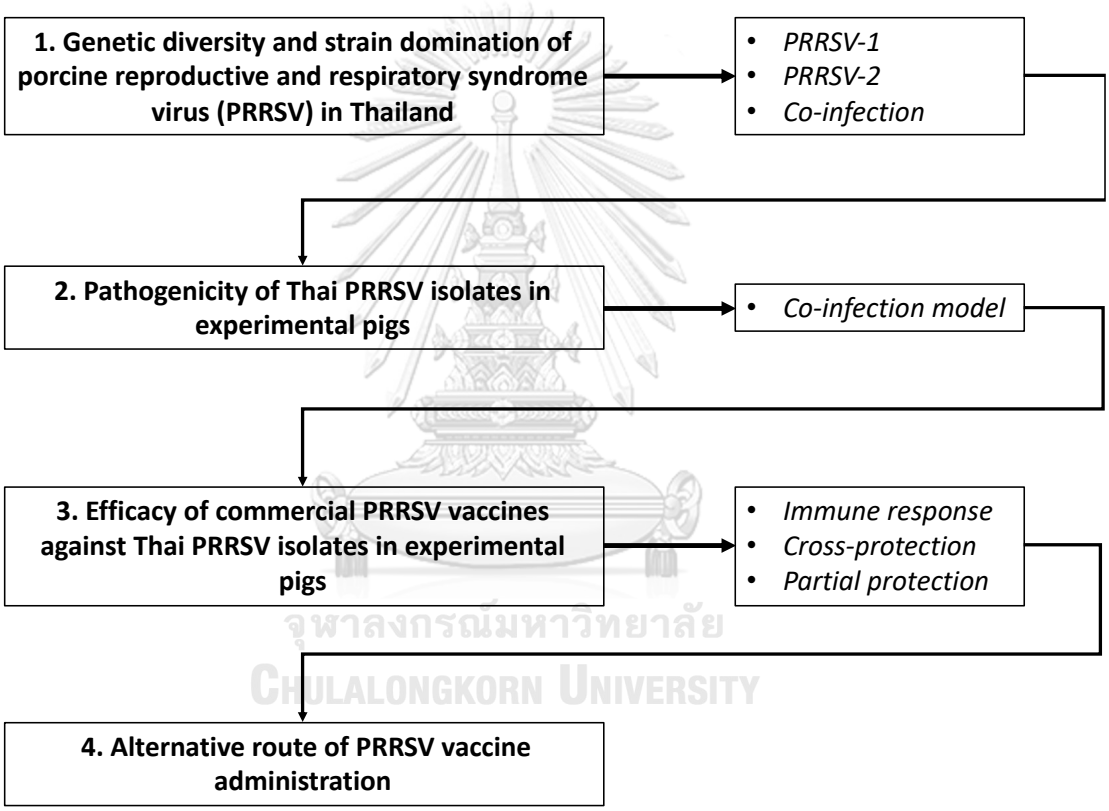
2.3 Keywords (Thai):

ไวรัสพีอาร์อาร์เอส ความหลากหลายทางพันธุกรรม พยาธิกำเนิด วัคซีนป้องกันโรคชนิดเชื้อเป็น

2.4 Keywords (English):

Porcine reproductive and respiratory syndrome virus, Genetic diversity, Pathogenesis, Modified-live virus vaccine

2.5 Conceptual framework



2.6 Place of study

1. Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.
2. Private commercial swine farms in Ratchaburi province, Thailand.

2.7 Advantage of study

1. Genetic diversity of Thai porcine reproductive and respiratory syndrome virus
2. Pathogenicity of Thai porcine reproductive and respiratory syndrome virus isolates
3. Efficacy of commercial porcine reproductive and respiratory syndrome virus modified-live vaccines against Thai field porcine reproductive and respiratory syndrome virus isolates
4. Alternative route of porcine reproductive and respiratory syndrome virus vaccine administration

CHAPTER 3
LITERATURE REVIEW

3.1 Porcine reproductive and respiratory syndrome virus (PRRSV)

Porcine reproductive and respiratory syndrome (PRRS) has been one of the most economically swine diseases worldwide. The etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped single-stranded positive-sense RNA genome (Rossow et al., 1995). PRRSV virion shows a roughly spherical- or oval-shaped particle of 50-69 nm in diameter with relatively smooth external surface PRRSV is member of the Order *Nidovialase*, family *Arteriviridae*, Genus *Porarterivirus* according to the International Committee of Taxonomy of Viruses (Adams et al., 2016). Presently, there are four distinct species including in these genus (Porarterivirus), PRRSV-1 and PRRSV-2 (with 30-45% variation in nucleotide sequences), along with other two virus does not affect pigs, including Lactate dehydrogenase-elevating virus (LDEV) and Rat Arterivirus 1 (RA1) (Lunney et al., 2016).

Initially, PRRS was referred to as mystery swine disease and mystery reproductive syndrome and was characterized as “Blue-Ear Pig” disease which is primarily transmitted via aerosol and affects mostly young boars and sows in the US swine farms (Rossow, 1998). Generally, the clinical symptoms of PRRS can be divided into two major forms including respiratory failure and reproductive disorder. The virus induces respiratory symptoms in nursery to finishing pigs, such as respiratory distress, viral pneumonia, and increased susceptibility to secondary infections associated with porcine respiratory disease complex (PRDC) (Beyer et al., 1998). PRRSV also induces reproductive disorder in breeding herds, which is characterized by late-term abortion, mummification, stillborn piglets, weakening newborn piglets, and affect semen quality (Christopher-Hennings et al., 1998; Rossow, 1998).

In China 2006, highly pathogenic PRRSV-2 (HP-PRRSV-2), with deletion of 30 amino acids in the non-structural protein 2 (nsp2) gene, emerged and reported to associated with porcine high fever disease, resulting in high mortality in both young and old pigs along with severe respiratory pathology (Tian et al., 2007). Then, HP-PRRSV-2 subsequently extended to Southeast Asia countries. In 2010, the first outbreak of HP-PRRSV-2 in Thailand was reported (Nilubol et al., 2012). HP-PRRSV-2 is characterized as a variant specie of PRRSV, which shares genetically background with

either PRRSV-1 or PRRSV-2 (Zhou et al., 2011), and shows different clinical disease outcomes from typical PRRSV. HP-PRRSV-2 causes fatal disease and associated with high morbidity and mortality rate in infected pigs (Gao et al., 2015; Li et al., 2015). Since then, HP-PRRSV-2 become and endemic strain in Thai swine herds and other neighboring countries (Nilubol et al., 2012; Nilubol et al., 2013).

3.2 PRRSV genome characterization

PRRSV genome is packed by nucleocapsid proteins surrounding by surface glycoproteins and membrane proteins. The genome size of PRRSV is approximately 15 kb with at least 11 known open reading frames (ORFs) as show in **Table 1**, with replicase genes located at the 5'-end followed by the genes encoding structural proteins toward the 3'-end (Pasternak et al., 2006). The majority of the genome, approximately 60%-70%, encodes non-structural proteins (nsps) involved in replication (ORF1a and ORF1b), whereas ORFs 2-7 encodes structural proteins (N, M, GP2-GP5, E) (Dokland, 2010) (**Figure 1**). Using ORF5 gene in molecular epidemiological studies, genetic variability has been described (Shi et al., 2010a; Stadejek et al., 2013). PRRSV replicase genes compose of two ORFs, ORF1a and ORF1b, which occupy the 5′-proximal three-quarters of the genomes. Both genes are expressed from the viral genome, with expression of ORF1b depending on a conserved ribosomal frameshifting mechanism. Subsequently, extensive proteolytic cleaving of the resulting pp1a and pp1ab polyproteins yields at least 14 functional nsps, most of which assemble into a membrane-associated replication and transcription complex (RTC) (Li et al., 2012). PRRSV also have a set of eight structural proteins, including a small non-glycosylated protein and a set of glycosylated ones: GP2a-b, GP3, GP4, GP5, and GP5a, M and N proteins (Meulenberg et al., 1995).

Figure 1 PRRSV genome characterization.

Table 1 Characteristics and functions of PRRSV genes and proteins [adapted from (Lunney et al., 2016)]

Genes	Proteins	Known or predicted properties/functions
ORF1a	Nsp1α	Protease PLPα*; zinc-finger protein; interferon (IFN) antagonist
	Nsp1β	Protease PLPβ; IFN antagonist
	Nsp2	Protease PLP2; deubiquitinating enzyme; IFN antagonist; transmembrane protein involved in membrane modification forming replication complex
ORF1a'-TF	Nsp2TF**	Contain PLP2 domain
	Nsp2N	Contain PLP2 domain
ORF1a	Nsp3	Transmembrane (TM) domain protein involved in membrane modification; forming replication complex
	Nsp4	Main protease SP*; apoptosis inducer; IFN-antagonist
	Nsp5	TM protein
	Nsp6	N/A
	Nsp7α	Recombinant nsp7 is highly antigenic
	Nsp7β	
	Nsp8	N-terminal domain of nsp9
ORF1b	Nsp9	RNA-dependent RNA polymerase
	NSP10	RNA NTPase/helicase, zinc-binding domain
	Nsp11	Uridylate-specific endoribonuclease (NendoU)

	Nsp12	
ORF2a	GP2a	Minor glycosylated structural protein;
ORF2b	E	Minor glycosylated structural protein; envelop
ORF3	GP3	Minor glycosylated structural protein
ORF4	GP4	Minor glycosylated structural protein
ORF5	GP5	Major glycosylated structural protein; most variable structure protein
ORF5a	ORF5a	Minor unglycosylated, hydrophobic structural protein
ORF6	M	Major glycosylated structural protein; highly conserved
ORF7	N	Unglycosylated and phosphorylated structural protein; highly antigenic; IFN antagonist

*PLP; papain-like cysteine protease; SP; serine protease

**NspTF is expressed though an alternative transframe (TF) open reading frame (ORF) underlying the nsp2-coding region by -2 ribosomal frameshifting (Fang et al., 2012), whereas the -1 ribosomal frameshift at the same position yields a truncated nsp2 variant, nsp2N (Li et al., 2014b).

3.3 PRRSV cell tropism and replication

PRRSV has very narrow cell tropism which is restricted for curtain subpopulation of swine monocyte/macrophage or myeloid lineages, notably pulmonary intravascular macrophages, subsets of macrophages in lymph nodes and spleen, and intravascular macrophages of the placenta and umbilical cord (Duan et al., 1997; Lawson et al., 1997). Despite its restricted cell tropism, PRRSV is able to replicate in several non-permissive cell lines by transfection of these cells with the viral genomic RNA. This finding suggests that the cell tropism is depending on specific entry of mediators in the target cells (Delputte et al., 2004; Meulenberg et al., 1998), mostly heparin sulphate and sialoadhesin (Delputte et al., 2002) as well as scavenger receptor CD163 (Van Gorp et al., 2010). PRRSV can be replicated in porcine alveolar macrophage (PAM) culture (Wensvoort et al., 1991) and swine testis (ST) cells (Plana et al., 1992). Although, among many different cell line tested, only the African green monkey kidney cell line, MA-104, and its derivatives such as MARC-145, CL-2621, and CRL11171, are fully permissive to PRRSV replication *in vitro* (Kim et al., 1993).

Viral replication starts by interaction of viral glycoproteins with different cellular receptors (Shi et al., 2015). CD163 and CD169 play an important role during PRRSV infections, uncoating of the viral particle, activation of clathrin-mediated endocytosis and release of viral genome into the cytoplasm (Yun and Lee, 2013). After cell entry, PRRSV caused a series of intracellular modifications to complete its replication cycle. At latter stages of viral replication, the mature virions accumulate in the intracellular membrane compartments and then released into the extracellular space through exocytosis pathway (Thanawongnuwech et al., 1997).

PRRSV infection can be divided into 3 major phases, including acute infection, persistence, and extinction. The acute infection shows PRRSV replicate in lung, mainly in the pulmonary alveolar macrophages (PAM) or intravascular macrophages (PIM), resulting in viremia by 6-12 hours post-infection (pi) which can be detected several weeks without antibody detection. Second phase is persistence which the viremia is not detected in blood. At this stage, PRRSV replicates mainly in lymphoid organs and potential transmit virus to naïve pigs (Allende et al., 2000). Afterward, virus replication continually decreases and become disappeared in the host. PRRSV replication dose not establish a steady-state equilibrium but continuously declines over time which lymphoid organs served as virus replication site before viral extinction (Allende et al., 2000).

3.4 Humoral immune response against PRRSV infection

PRRSV infection induces an antibody response within 7-9 days pi (dpi) without the presence of neutralizing antibodies (NAbs) which play an important role in PRRSV protection. The NAbs appear only later, typically more than 28 dpi (Loving et al., 2015). In contrast to NAbs, early detection of PRRSV-specific antibodies are non-neutralizing and do not correlated with PRRSV protection (Lopez et al., 2007; Lopez and Osorio, 2004). The earliest antibodies are directed against nucleocapsid (N) protein which appear around first week pi and remain constant in blood for several months (Horter et al., 2002).

The NAbs titers against PRRSV are relatively low and do not confer cross-protection against other PRRSV isolates. These NAbs are usually specific for the vaccine (homologous), with lower or no titers of heterologous viruses (Vu et al., 2011; Zhou et al., 2012). The NAbs are consistently detected by day 28 dpi or later for both PRRSV species and directed against GP5, that contains the major neutralizing

epitopes (Gonin et al., 1999; Nelson et al., 1993), leading to polygonal B-cell activation (Mulupuri et al., 2008). Due to slow response, irregular appearance of PRRSV-NAbs after PRRSV infection was unable to prevent the appearance of viremia. The potential mechanisms responsible for delayed NAbs include glycan shielding effects of N-linked glycosylation in GPs (Ansari et al., 2006), presences of immunodominant decoy epitope in GP5 upstream of the neutralizing epitopes (Ostrowski et al., 2002), antibody-dependent enhancement of viral entry (Cancel-Tirado et al., 2004), suppression of immune responses and prevent of normal B-cell repertoire development (Butler et al., 2014).

3.5 Cell-mediated immune response against PRRSV infection

Cell-mediated immunity (CMI) is crucial important in intracellular microbe infections. The CMI is mediated by T lymphocytes, through helper T cells (Th, $CD4^+$) and cytotoxic T cells (CTL, $CD8^+$) functions. Th cells recognize peptide fragment derived from protein antigens bound to the MHC class II molecules. After recognition, the naïve T cell may differentiate into different T cell subsets depend on the cytokines in the microenvironment (Abbas and Janeway, 2000; Shevach, 2006). The cytotoxic T cells recognize class I MHC-associated peptide. After recognition process, the cytotoxic T cells can kill infected cells expressing antigens (Abbas and Janeway, 2000).

Several studies indicate that PRRSV can suppress production of type I IFN, both IFN-α and IFN-β (Beura et al., 2010; Loving et al., 2015; Miller et al., 2004). The Type I IFN plays role in the inhibition of viral replication, increase natural killer cell (NK cells) function, and MHC class I molecules on virus-infected cells, and enhance Th cells development (Abbas and Janeway, 2000). In addition, PRRSV can suppress TNFα and IL-1 productions. In contrast, induction of IL-10 production had been reported (Lopez-Fuertes et al., 2000; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003).

Development of PRRSV-specific CMI response can be detected after 4 weeks pi, corresponded with the NAbs response (Bautista and Molitor, 1997). Cytokine response are mainly interferon (IFN)-γ and interleukin (IL)-12 (Lopez Fuertes et al., 1999). The IFN-γ activates macrophages, NK cells and T lymphocytes which involved in both innate and adaptive immune response (Abbas and Janeway, 2000). Using ELISPOT IFN-γ assay demonstrated that PRRSV-specific T cell response was detected

(Shevach, 2006)as early as 2 weeks pi without alternation of T cell in lymphoid tissues during or post PRRSV infections (Xiao et al., 2004). PRRSV-specific IFN-γ secreting cells firstly detected at 3 weeks post pi or vaccination and gradually increased and reached the highest numbers at 10 weeks post pi or vaccination. IFN-γ secreting cells were mainly double positive cells ($CD4^+CD8^+$), with a small portion of $CD4^-/CD8\alpha\beta^+$ cytotoxic T-cells (Meier et al., 2003). However, the increased IFN-γ secreting cells are very late compared to the other viral infections, such as pseudorabies (within 6 days pi) or classical swine fever (within 1 week pi) (Hoegen et al., 2004; Suradhat et al., 2001).

Cross-reactivity against divergent of PRRSV can show different intensity and be differently immune reactivity was evident upon stimulation with various virus isolates in terms of frequency and CD8 phenotype of PRRSV-specific IFN-γ-secreting cells. The modulation of cytokines in vaccinated pigs appeared to be more dependent on vaccination or infection condition than on stimulation by different isolates; change in IL-10 appear to be more relevant than those of TNFα at gene and protein levels (Costers et al., 2009; Ferrari et al., 2013; Xiao et al., 2004).

3.6 Genetic diversity and evolution of PRRSV

PRRSV is genetically heterogenous by its nature (Meng, 2000) and emerged almost simultaneously in the North America and Western Europe in the late 1980 and early 1990, respectively (Stevenson et al., 1993; Wensvoort et al., 1991). Presently, PRRSV is classified into two distinct species, PRRSV-1 (formerly called genotype I or EU genotype) and PRRSV-2 (formerly called genotype II or US genotype) (Adams et al., 2016). The two species of the virus resemble 60% of nucleotide homology to each other although their biological characteristics are very similar (Kim and Yoon, 2008).

The relative nucleotide sequence identity between PRRSV-1 isolates and PRRSV-2 isolates is approximately 97.8-99.7% in the ORF1, 81.1-98.3% in the ORF2, 81.0-98.0% in the ORF3, 85.2-98.3% in the ORF4, and 82.7-88.8% in the ORF5, respectively (Dortmans et al., 2019; Meng et al., 1995a; Meng et al., 1995b; Nelsen et al., 1999). The ORF6 and ORF7 genes are relatively conserved among both PRRSV-1 and PRRSV-2 isolates, but genetic variation was observed in these genes (Meng et al., 1995c). The ORF5 gene encodes glycoprotein 5 (GP5), major glycosylated structural protein, is highly variable

In Thailand, PRRSV has been detected since 1989 which both PRRSV species can be isolated from swine herds. In the previous study demonstrated that 66.42% of PRRSV isolated from Thai swine herds was PRRSV-1. Meanwhile, 33.58% was PRRSV-2 (Thanawongnuwech et al., 2004). In the present, PRRSV in Thai swine herds shows higher genetically variations and increasing of PRRSV-2 in Thai swine herds because of the increasingly used of PRRSV-2 modified-live vaccines (MLV) to control and prevent the disease (Nilubol et al., 2012). In the previous study of genetic diversity of Thai PRRSV isolates based on ORF5 gene, demonstrated that Thai PRRSV-1 isolates were divided into 3 clusters (Cluster I, II, and II) which showed nucleotide similarity ranged from 84.7% to 99.8% and amino acid similarity ranged from 84.2% to 95.5%, respectively. Meanwhile, Thai PRRSV-2 isolates were grouped into 4 clusters (Cluster I, II, III, and IV) and had nucleotide similarity ranged from 83.4% to 99.8% and amino acid similarity ranged from 80.8% to 99.5%, respectively (Nilubol et al., 2013).

In the previous study of genetic diversity of Thai field PRRSV isolates based on ORF5 gene demonstrated that both PRRSV species have evolved separately with a temporal influent on strain development. In addition, Thai PRRSV isolates, either PRRSV-1 or PRRSV-2, develop their own clusters without geographical influence. Interestingly, from the results of Thai PRRSV evolution study, both PRRSV-1 and PRRSV-2 had major dominant cluster which always detectable regardless to the new PRRSV introduction (Nilubol et al., 2013). However, in the presence of both PRRSV-1 and PRRSV-2, what PRRSV MLV should be use. Especially, in the regions which high genetic diversities of PRRSV isolates had been reported.

3.7 vaccine against PRRSV

Since the discovery of PRRSV, multiple vaccines against PRRSV are commercially available, especially modified-live vaccines, that have been launched against both PRRSV-1 and PRRSV-2; including Porcilis® PRRS (PRRSV-1 MLV, MSD Animal Health, The Netherlands), Amervac® PRRS and UNISTRAIN® PRRS (PRRSV-1 MLV, Hipra Laboratorios S.A., Spain), Prysvac-183 (PRRSV-1 MLV, Syva Laboratorios, Spain), Fostera™ PRRS (PRRSV-2 MLV, Zoetis, USA), Ingelvac® PRRS MLV and Ingelvac® PRRS ATP (PRRSV-2 MLV, Boehringer Ingelheim, Germany), and Prime Pac® PRRS (PRRSV-2 MLV, MSD Animal Health, The Netherlands). However, existing evidence suggests that all commercially available PRRSV MLV elicit only relatively weak

humoral and cell-mediated immune response (Diaz et al., 2006; Zuckermann et al., 2007). Based on challenge experiments to evaluate vaccine efficacy, it appears that PRRSV MLV do confer late but effectively protection, against genetically homologous PRRSV isolates, but provides only partial or no protection heterologous PRRSV infections (Murtaugh et al., 2002; Roca et al., 2012). Safety of PRRSV MLV is concern as shedding and persistence of vaccine viruses have been reported. Vaccinated pigs with PRRSV MLV can develop viremia up to 4 weeks post vaccination, and shedding to naïve animals (Martinez-Lobo et al., 2013). Consequently, reversion to virulence of vaccine viruses and recombination between vaccine virus and wild type PRRSV are serious concerns as showed in several studies (Botner et al., 1997; Madsen et al., 1998).

CHAPTER 4

Genetic diversity of porcine reproductive and respiratory syndrome virus in Thailand during 2001-2017

Manuscript in preparation

Genetic diversity of porcine reproductive and respiratory syndrome virus in Thailand during 2001-2017

Adthakorn Madapong, Kepalee Saeng-chuto, Chirstopher James Stott, Angkana Tantituvanont and Dachrit Nilubol

In this chapter, we investigated the genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) based on the ORF5 gene of serum samples collected from Thai swine herds during 2001-2017. Our findings showed that all Thai PRRSV-1 isolates are grouped in subtype 1 which clade A was a dominant strain of PRRSV-1. Meanwhile, Thai PRRSV-2 isolates are

4.1 Abstract

The objective of the present study was to investigate the genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) based on the ORF5 gene from serum samples collected from Thai swine herds during 2001-2017. The co-existence of PRRSV-1 and PRRSV-2 was observed in 75% of investigated herds. According to the international systematic classification, all Thai PRRSV-1 isolates belonged to Subtype 1 that were grouped into 3 clades; A, D and H, respectively. Meanwhile, Thai PRRSV-2 isolates were grouped into 3 lineages; lineages 1, 5 and 8, respectively, which lineage 8 can be divided into 2 sublineages; sublineage 8.7/Classical and 8.7/HP-PRRSV-2, respectively. PRRSV-1 and PRRSV-2 isolates in Clade A and sublineage 8.7/HP-PRRSV-2 were dominant strain in Thai swine herds, respectively. Both PRRSV species have evolved separately with a temporal influence on strain development and are separate from those of other countries.

Keywords: Porcine reproductive and respiratory syndrome virus; Genetic diversity; Phylogenetic tree; ORF5

4.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating diseases in swine production worldwide (Wensvoort et al., 1991). PRRS is caused by PRRS virus (PRRSV), an enveloped, positive single-stranded RNA virus, belongs to order *Nidoviralase*, family *Arterivirus*, genus *Porarterivirus* (Adams et al., 2016). PRRSV genome is approximately 15 kbp in length contains at least 11 known open reading frames (ORF); two large ORFs (ORF1a and b) and eight small ORFs (ORF2-7) (Cavanagh, 1997). ORF1a and ORF1b are encoded into replicase and non-structural proteins, while ORF2 to 7 encodes structural proteins (Conzelmann et al., 1993). Glycoprotein (GP5) is the most variable regions and associated with the neutralizing epitopes (Wissink et al., 2005), and has been used in phylogenetic analyses and studies on PRRSV genetic diversity (Meng, 2000)

Currently, PRRSV is divided into two distinct species, PRRSV-1 and PRRSV-2 (Adams et al., 2016) and circulating in swine production areas worldwide (Kimman et al., 2009) which had been evolving independently on each continent (Nelson et al., 1993). However, the co-existence of both PRRSV species has been increasingly evident in several countries, including Korea, China, and Thailand (Kim et al., 2008; Nilubol et al., 2012; Nilubol et al., 2013; Yoon et al., 2008). These concurrent infection of both PRRSV species leads to the necessity for further investigations to better understand the genetic diversity of the Thai PRRSV isolates and to improve the current control program.

Recently, systematic classification of PRRSV, either PRRSV-1 or PRRSV-2, was conducted based on comprehensive phylogenetic analyses (Shi et al., 2010b; Stadejek et al., 2008). However, the international systematic classification had less applied in several studies of PRRSV genetic diversity. Therefore, the objectives of this study were to investigate the genetic diversity of Thai field PRRSV isolates by phylogenetic analysis of the complete ORF5 sequences during 2001-2018. In addition, previously reported Thai PRRSV isolates and global PRRSV were included in this study.

4.3 Materials and Methods

4.3.1 Sample collection

Blood samples were collected from 5 major swine producing regions in Thailand during 2001-2018 including North, Northeast, Central, East and South. Herd selection in each region was based on the permission of the owner. One thousand, five hundred and forty-two samples from 102 swine herds were collected in this study. Of the 102 herds, 10, 12, and 10 herds are in the provinces in the North, Northeast, and South regions of Thailand, respectively. Seventy herds are in the Central and East regions that have the highest pig densities.

The provinces in the North region of Thailand were included Chiang Man, Chiang Rai, Uttaradit, Nakhon Sawan and Phichit, respectively. The provinces in the Northeast region of Thailand wer Ubon Ratchathani, Nakhon Ratchasima, Chaiyaphum, Burirum, Khon Kean and Nakhon Phanom, respectively. The provinces in the Central region of Thailand were Nakhon Pathom, Ratchaburi, Kanchanaburi, Saraburi, Lopburi, Phetchaburi, Suphanburi and Ang Thong, respectively. Two provinces are in the East region of Thailand, Chon Buri and Chachoengsao., and Nakhon Sri Thammarat, which in the South region of Thailand respectively.

4.3.2 PCR and sequence determination

Total RNA was extracted from sera using NucleoSpin® RNA Virus (Macherey-Nagel, Duren, Germany) in accordance with the manufacturer's instructions. The extracted RNA was converted to cDNA using M-MuLV Reverse Transcriptase (New England BioLabs Inc., MA, USA). ORF5 was amplified using previously reported primers (Nilubol et al., 2014), and PCR amplification was performed using *Taq* high-fidelity DNA polymerase (Invitrogen™, Carlsbad, CA, USA). The amplified PCR products were purified using a PCR purification kit (Macherey-Nagel, Duren, Germany) and cloned into plasmid vector for the subsequent transformation of *Escherichia coli* cells using commercial kit (pGEM-T® Easy Vector, Promega, WI, USA) according to manufacturer's instructions. Plasmid was extracted using NucleoSpin® Plasmid Extraction (Macherey-

Nagel, Duren, Germany) and sequencing was performed at Biobasic Inc. (Markham, Ontario, Canada) using an ABI Prism 3730XL DNA sequencer.

4.3.3 Sequence analysis

Three datasets of complete ORF5 genes were used to perform the genetic analysis. The first dataset included the complete ORF5 genes of the Thai PRRSV isolates that collected during 2001-2018. The second dataset was reference PRRSV isolates that included PRRSV-1 and PRRSV-2 prototypes (Lelestad virus and VR-2332), PRRSV-1 modified-live vaccines (Porcilis® PRRS, MSD Animal Health, The Netherlands; Prysvac-183, Laboratorios Syva, Spain; Amervac® PRRS, Laboratorios Hipra, Spain), and PRRSV-2 modified-live vaccines (Fostera™ PRRS, Zoetis, USA; Ingelvac® PRRS MLV and ATP, Boehringer Ingelheim, Germany; Prime Pac® PRRS, MSD Animal Health, The Netherlands) and sequences from China. The third dataset consisted of complete ORF5 gene that represented global PRRSV-1 and PRRSV-2 isolates that were available in GenBank.

The ORF5 sequences were aligned using the CLUSTALW method (Thompson et al., 1994). The phylogenetic trees of the PRRSV-1 and PRRSV-2 isolates were constructed separately to investigate the genetic relationship. Each phylogenetic tree was constructed based on non-redundant ORF5 sequences of PRRSV. Neighbor-joining trees with 1,000 bootstrap replicates were also constructed from the aligned nucleotide sequences using MEGA6 (Nilubol et al., 2013).

To analyze the genetic diversity of the PRRSV-1 and PRRSV-2, the percentages of identity at the nucleotide and amino acid levels between the isolates was calculated as previously described (Forsberg et al., 2002)

4.4 Results

4.4.1 PRRSV detection by PCR

PRRSV detection by PCR was showed in **Table 2**. Sera was positive for PRRSV in 1,432 out of 1,542 samples and was divided into 3 groups: PRRSV-1, PRRSV-2, and co-infection, respectively. Sera was positive for either PRRSV-1 or PRRSV-2 only in 62 out of 1,432 (4%), and 298 out of 1,432 (21%) samples, respectively. Meanwhile, the co-infection of both PRRSV-1 and PRRSV-2 in sera was in 1,072 out of 1,432 (75%) samples. The nucleotide sequences revealed that both PRRSV-1 and PRRSV-2 co-existed in 100 of 102 swine herds which mostly located in the central region of Thailand.

Table 2 PRRSV detection in Thailand during 2001-2018. From 1,542 samples, 1,432 sera were positive for PRRSV.

PRRSV detection	No. of positive samples	Percentages (%)
PRRSV-1	62	4
PRRSV-2	298	21
Co-infection	1,072	75

4.4.2 Phylogenetic analysis of Thai PRRSV isolates

The nucleotide and amino acid divergence of the Thai PRRSV-1 isolates ranged from 84.4 to 99.8% and 84.5 to 99.6%, respectively. All Thai PRRSV-1 isolates were grouped into Subtype 1 which were further divided into 3 clades; clade A, D and H (**Figure 2**). Four hundred and ninety-three PRRSV-1 isolates were grouped in clade A (82.44%) along with PRRSV-1 prototype (Lelystad virus) and PRRSV-1 MLV (Porcilis® PRRS, MSD Animal Health, The Netherlands). In subtype 1, clade D consisted of 14 PRRSV-1 isolates (2.34%). Meanwhile, subtype 1, clade H consisted of 91 isolates (15.22%) (**Figure 2**).

For the PRRSV-2, the nucleotide and amino acid sequence divergence ranged from 82.4 to 99.8% and 80.5 to 99.5%, respectively. Thai PRRSV-2 isolates were grouped into 4 major groups; lineage 1, lineage 5, sublineage 8.7/Classical and sublineage 8.7/HP-PRRSV-2 (**Figure 3**). Two hundred and ninety-six PRRSV-2 isolates

were grouped in lineage 1 (35.49%). In lineage 5 consisted of 22 Thai PRRSV-2 isolates (2.64%). Meanwhile Thai PRRSV-2 in lineage 8 were further divided into 2 sublineags: 8.7/Classical consisted of 143 isolates (17.15%), and 8.7/HP-PRRSV-2 consisted of 373 PRRSV-2 isolates (44.72%) (**Figure 3**), respectively.

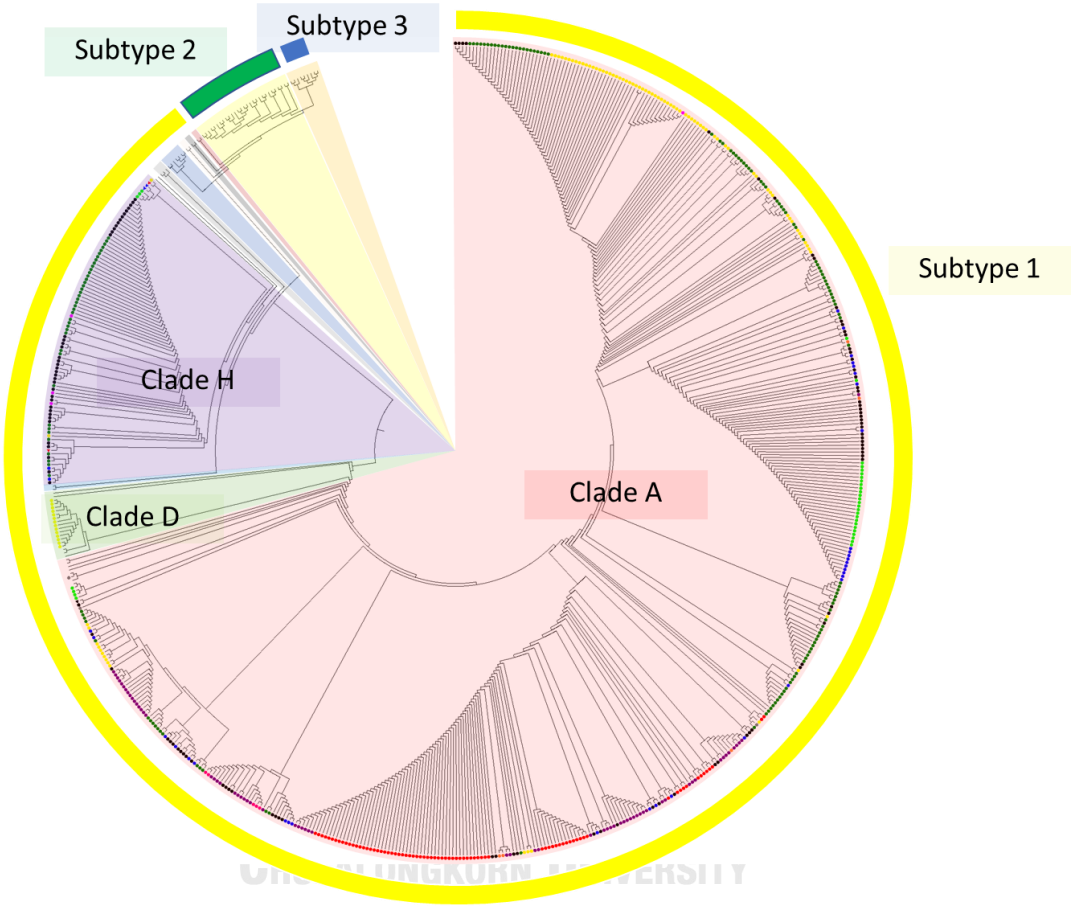


Figure 2 Phylogenetic analysis of Thai PRRSV-1 isolates based on the ORF5 gene during 2001-2018. Total 598 complete sequences of the ORF5 gene were analyzed. The color of the node markers indicates the year of sample collection.

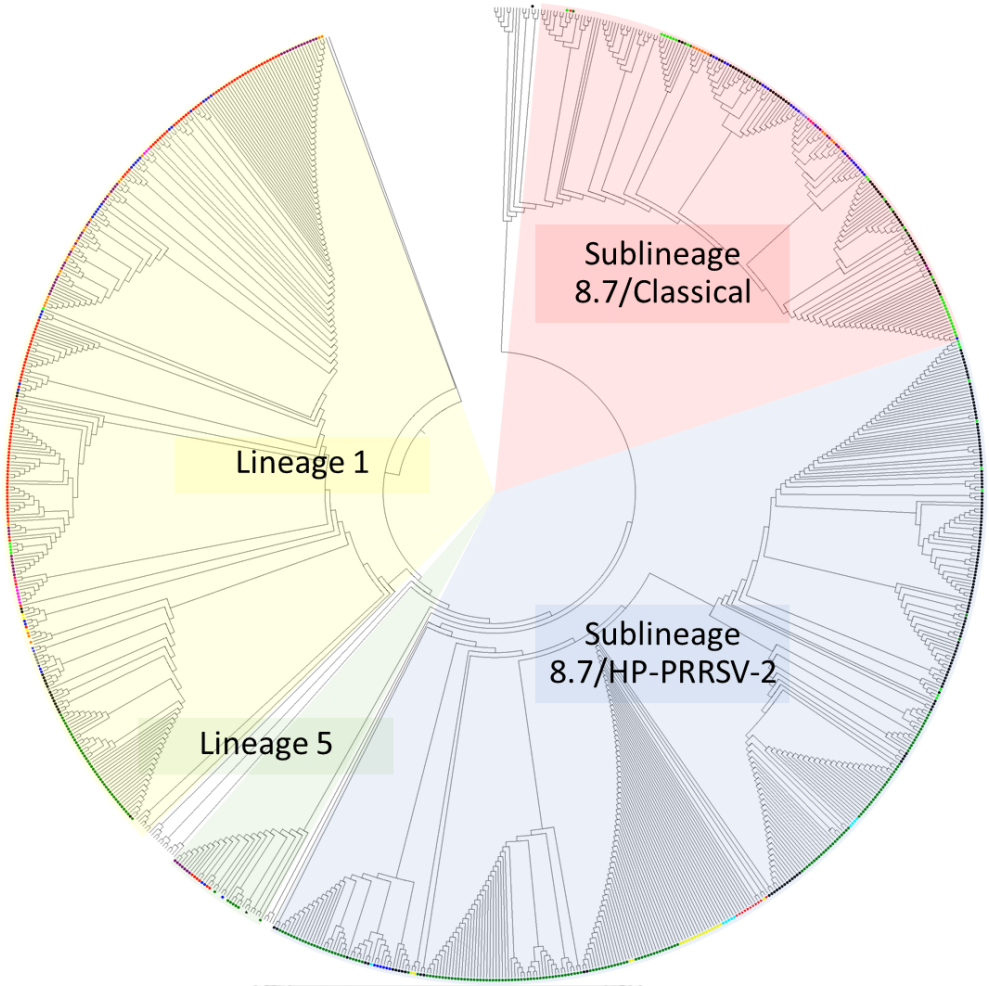


Figure 3 Phylogenetic analysis of Thai PRRSV-2 isolates based on the ORF5 gene during 2001-2018. Total 834 complete sequences of the ORF5 gene were analyzed. The color of the node markers indicates the year of sample collection.

4.5 Discussion and Conclusion

The genetic diversity based on the ORF5 gene of Thai PRRSV isolates during 2001-2017 was investigated in the present study. Our finding showed that both PRRSV species, PRRSV-1 and PRRSV-2, were detected and none of 102 swine herds were infected with only PRRSV-2. Most Thai swine herds were concurrently infected with both PRRSV species without any specie being dominant. Moreover, the detection of PRRSV-1 and PRRSV-2 in the present study were represented to be coinfected in the same pig from the herds that were co-infected with both PRRSV species. Based on the phylogenetic analysis of Thai PRRSV isolates in 2001-2018 demonstrated that Thai PRRSV isolates, both PRRSV-1 and PRRSV-2, develop their own clusters. The subtype 1, clad A was a dominant strain of Thai PRRSV-1. Meanwhile, the sublineage 8.7/HP-PRRSV-2 was a dominant strain of Thai PRRSV-2.

In Thailand, a retrospective serological study found that PRRSV had been circulating in Thai swine herds as early as 1989 and both PRRSV species showed co-circulate in Thai swine herds since 2001 (Thanawongnuwech et al., 2004). According to previous study, Thai PRRSV-1 isolates in clade A were closely related to each other and had more highly homologous to the Lelystad virus and PRRSV-1 MLV-like virus (Porcilis® PRRS) with nucleotide and amino acid sequence similarities of 97.8-98.5% and 96.5-99.0%, respectively (Nilubol et al., 2013; Stadejek et al., 2008). The Thai PRRSV-1 isolates in clade D were closely related to PRRSV-1 MLV-like virus (Amervac® PRRS) that were first detected in 2008 even though this vaccine has been available in 2004. The Thai PRRSV-1 isolates in clade H were closely related to Spanish PRRSV-like and Belgium PRRSV-like, which were detected in 2010-2013.

The Thai PRRSV-2 isolates are grouped into 3 lineages: 1, 5 and 8. The Thai PRRSV-2 isolates in lineage 1 were closely related to the Canadian isolates that might be introduced into Thai swine herds around 1990s (Tun et al., 2011). The Thai PRRSV-2 isolates in lineage 5 were closely related to PRRSV-2 MLV-like virus (Ingelvac® PRRS MLV). Meanwhile, the Thai PRRSV-2 isolates in the lineage 8 are divided into 2 huge groups: Classical and HP-PRRSV-2 (Shi et al., 2010b). The HP-PRRSV-2, in particular JXA-1 like viruses, was emerged in China in 2006 and subsequently spread to

neighboring countries including Vietnam, Cambodia and Laos (Tian et al., 2007). Then, the HP-PRRSV-2 was detected in swine herds in Thailand, Myanmar, Philippines and Singapore, and caused an outbreak in these countries (An et al., 2011; Feng et al., 2008; Nilubol et al., 2012). In Thailand, the first epidemic outbreak of HP-PRRSV-2 initiated in August 2010 and may have been introduced through the illegal transport of infected materials from bordering countries, especially form Vietnam to Thailand thorough Laos (Nilubol et al., 2012). Our results demonstrate that HP-PRRSV-2 are circulated and endemic in those regions of Thailand since its emergence. Almost all HP-PRRSV-2 isolates were in sublineage 8.7/HP-PRRSV-2 and closely related to the JXA1-like and 09HEN1-like viruses that are predominantly circulated in Southeast Asia (Nilubol et al., 2012; Shi et al., 2010b).

In conclusion, both PRRSV species have evolved continuously and developed clusters that are genetically separated form that of the other countries. The introduction of new isolates could be diverse the genetic variation of PRRSV, especially for the PRRSV-2. However, the mechanisms of genetic diversity and evolution analyses of both PRRSV species in Thailand are under investigation.

CHAPTER 5

Pathogenesis of Thai field porcine reproductive and respiratory syndrome virus

Manuscript in preparation

Pathogenesis of Thai field porcine reproductive and respiratory syndrome virus isolates in experimental pigs

Adthakorn Madapong, Kepalee Saeng-chuto, Puwich Chaikhumwang, Angkana Tantituvanont and Dachrit Nilubol

In this chapter, we investigated the pathogenicity of two different Thai field PRRSV isolates, including PRRSV-1 (subtype 1, clade A) and HP-PRRSV-2 (sublineage 8.7/HP-PRRSV-2), either alone or in combination conditions in experimental pigs. Our findings revealed different pathological outcomes between single infection with each PRRSV isolate and co-challenge with Thai field PRRSV isolates. Especially for the co-challenged pigs, that showed prominent clinical severity and lung lesions.

5.1 Abstract

The objective of the present study was to evaluate the pathogenesis of experimental infection with Thai porcine reproductive and respiratory syndrome virus (PRRSV) isolates, either alone or co-infection conditions in term of virus distribution, viremia, macroscopic- and microscopic lung lesions and PRRSV-antigens in lung tissues. Thirty-six, PRRSV-free, 3-week-old pigs were allocated into 4 groups with 9 pigs each: G1 (PRRSV-1), G2 (HP-PRRSV-2), G3 (Co-challenge) and G4 (Non-challenge). Pigs in the G1 and G2 were intranasally inoculated with Thai PRRSV-1 isolate (AN01EU4204) and PRRSV-2 (FDT10US23, HP-PRRSV-2). Pigs in G3 was intranasally co-inoculated with both Thai PRRSV-1 (AN01EU4204) and PRRSV-2 (FDT10US23, HP-PRRSV-2). Pigs in G4 served as control. Following challenge, pigs in the G3 showed severe clinical signs and had significantly ($p < 0.05$) higher viremia and lung lesions than those of the other challenged groups as well as PRRSV-antigens and virus distribution in tissues. Our results demonstrated a marked difference in pathogenicity of PRRSV isolates. The co-infection of both Thai PRRSV species induce more severity of the disease than those of single infection with either PRRSV-1 or PRRSV-2.

Keywords: Porcine reproductive and respiratory syndrome virus; Pathogenesis, Co-challenge, HP-PRRSV-2, Experimental pig

5.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a causative agent of PRRS disease, that affect swine production worldwide. The major clinical symptoms of the disease are reproductive failure in sows and mild- to severe respiratory diseases, leading to impaired growth in weaning pigs and secondary infection with other pathogens (Horter et al., 2001; Rossow et al., 1995). PRRSV is small enveloped, positive single-stranded RNA virus belonging to the family *Arteriviridae* in the order *Nidoviralase* (Snijder and Meulenberg, 1998). Two distinct species of PRRSV have been identified; PRRSV-1 and PRRSV-2 (Adams et al., 2016).

In Thailand, PRRSV had been detected in swine herds since 1989 which was PRRSV-2. Then, subsequent survey study of PRRSV diversity demonstrated that both PRRSV species was co-existed in Thai swine herds, and the PRRSV-1 isolates was more dominant than that of PRRSV-2 isolates (Thanawongnuwech et al., 2004). In addition, the previous study of genetic diversity of Thai PRRSV isolates in 2010-2011 showed that both PRRSV species have evolved separately and developed their own clusters without geographical influence on strain development within Thailand (Nilubol et al., 2013).

The severity and duration of PRRSV outbreak is variable, some swine farms may be devastated by high production losses, whereas other herds may have subclinical of the disease without losses (Rossow et al., 1999). Moreover, differences in severity of the disease depending on viral strain and immune status of pigs in the swine unit (Lunney et al., 2016). However, the data of pathogenesis of Thai field PRRSV isolates is not available. Therefore, the aims of the present study was to compared the pathogenicity of both Thai field PRRSV species, either alone or in co-infection in experimental pigs by defining the virus distribution, humoral immune response, lung lesions, and virus antigens in lung tissues.

5.3 Materials and Methods

5.3.1 PRRSV isolates

PRRSV isolates used in the present study refer to the AN06EU4204 and FDT10US23 which were Thai PRRSV-1 and PRRSV-2 (HP-PRRSV-2), respectively (Nilubol et al., 2013). The PRRSV isolates AN06EU4204 and FDT10US23 are in clade A, subtype 1 and sublineage 8.7/HP-PRRSV-2, based on international systematic classification according to previously described (Shi et al., 2010b; Stadejek et al., 2008). The ORF5 sequences of these PRRSV isolates are available in GenBank under accession number JQ04075 and JN255836, respectively. These PRRSV isolates were obtained from weaned pigs from two different herds experiencing PRRS outbreaks during 2010-2011 (Nilubol et al., 2012). Both swine herds are in the western region of Thailand. Based on the ORF5 gene, both Thai PRRSV isolates are phylogenetically clustered in endemic clades of which that could represent PRRSV isolates endemically infection in swine herds in this region. PRRSV isolates were propagated and plaque purified in MARC-145 cells as previously described methods (Geldhof et al., 2012; Nilubol et al., 2004). The virus titer was determined based on a procedure described previously (Nilubol et al., 2004). PRRSV genome was sequenced as previously described (Delrue et al., 2010; Nilubol et al., 2013).

5.3.2 Experimental design

All animal procedures in this study was conducted in accordance with the Guide for the Care and Used of Laboratory Animal of the National Research Council of Thailand according to protocols approved by The Chulalongkorn University Animal Care and Use Committee, protocol number 1731047.

Thirty-six, 3-week-old, PRRSV-free piglets were purchased form PRRSV-free herds and randomly allocated based on the stratification weighs onto 4 treatment groups of 9 pigs each (**Table 3**); G1 (PRRSV-1), G2 (HP-PRRSV-2), G3 (Co-challenge) and G4 (Non-challenge). At 0 days post-challenge (DPC), pigs in the G1 and G2 groups were intranasally inoculated with 4 ml of tissue culture inoculum of PRRSV-1 (AN06EU4204 isolate, third passage of porcine alveolar macrophage, $10^{5.2}$ $TCID_{50}$/ml)

and HP-PRRSV-2 (FDT10US23 isolate, fifth passage of MARC-145 cells, $10^{5.4}$ $TCID_{50}$/ml), 2 ml/nostril, respectively. Pigs in the G3 group was intranasally co-challenged with 4 ml of tissue culture inoculum of PRRSV-1 (AN06EU4204, $10^{5.2}$ $TCID_{50}$/ml) and HP-PRRSV-2 (FDT10US23, $10^{5.4}$ $TCID_{50}$/ml) isolates, at 2 ml of each isolate/nostril. Pigs in the G4 group was served as negative control. Each group was housed in separated room with separated air spaces and monitored daily for physical condition and clinical respiratory disease throughout the experiment.

Blood samples were collected at 0, 3, 5, 7, 10, 14, 21, and 28 DPC. Sera were separated and analyzed for PRRSV-specific antibody response using ELISA (IDEXX, USA) and serum neutralization (SN) assay. PRRSV RNA in sera was analyzed using real time quantitative reverse transcriptase PCR (RT-qPCR) as previously described (Madapong et al., 2017).

Rectal temperature was daily recorded throughout the experiment. The severity of clinical score were daily evaluated in accordance with previously described (Halbur et al., 1995a). In brief, a score of 0 = normal; 1 = mild dyspnea and/or tachypnea when stressed; 2 = mild dyspnea and/or tachypnea when at rest; 3 = moderate dyspnea and/or tachypnea when stressed; 4 = moderate dyspnea and/or tachypnea when at rest; 5 = severe dyspnea and/or tachypnea when stressed and at rest.

At 7 DPC, three pigs of each group were necropsied, and lung lesion will be evaluated. Sera and tissue samples will be collected to perform virus isolation in cell culture, detection of PRRSV using PCR, and immunohistochemistry (IHC).

5.3.3 Serological analysis

Sera were separated and analyzed for PRRSV-specific antibodies using ELISA (IDEXX, USA) in accordance with manufacturer's instructions. The PRRSV-specific antibody titers were reported as an S/P ratio, and the serum samples will be considered positive if the S/P ratio was greater than 0.4.

Serum neutralization (SN) assay was conducted using either PRRSV-1 (AN06EU4204) and PRRSV-2 (FDT10US23, HP-PRRSV-2) as previously described

(Madapong et al., 2017). The SN titers were reported as the highest dilution resulting in a 90% reduction in the number of fluorescent units per well.

5.3.4 PRRSV detection and quantification

Total RNA was extracted from sera and tissue samples using NucleoSpin® Virus RNA extraction kit (Macherey-Nagel, Germany) and converted into cDNA. Copy number of PRRSV was quantified using previously published TaqMan® probe-baseed real-time RT-PCR (Egli et al., 2001; Madapong et al., 2020). The ORF5 sequence of the PRRSV isolated from tissue samples was analyzed by PRRSV-specific primers as previously described (Nilubol et al., 2013).

5.3.5 Pathological examination and immunohistochemistry (IHC)

PRRSV-induced macroscopic- and microscopic lung lesion scores were evaluated as previously described (Halbur et al., 1995b). For macroscopic lung lesion, the lungs were given a score to estimate the percentage of the lung affected by pneumonia. Each lobe was assigned a number to reflex the approximate percentage of the volume of the entire lung and the percentage volume from each lobe added to obtain the entire lung score (range from 0 to 100% of affected lung). Sections were collected from all lung lobes as previously described (Halbur et al., 1995a). Lung tissues were fixed with 10% neutral buffered formalin for 7 days and routinely processed and embedded in paraffin in an automated tissue processor. Section were cut at 5 µm and stained with hematoxylin and eosin (H&E). For microscopic lung lesion analysis, the lung sections were examined in a blinded manner and given an estimated score of the severity of the interstitial pneumonia. Briefly, 0 = normal; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia, and 4 = severe diffuse interstitial pneumonia. The mean values of microscopic score of each group were calculated.

Immunohistochemistry was performed using monoclonal antibodies which recognized PRRSV-1 and PRRSV-2 antigens, respectively (Madapong et al., 2020). To obtain quantitative data, slides were analyzed with the NIH Image J 1.50i Program

(http://rsb.info.nih.gov/ij). In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm2) was determined as previously described (Halbur et al., 1996a; Park et al., 2014). The mean values were calculated.

5.3.6 Statistical analysis

The data from repeated measurements were analyzed using one-way analysis of variance (ANOVA) to determine the presence of significant differences between treatment groups for each day. *p*-value < 0.05 was considered as statistically significant difference.

Table 3 Experimental design

Treatment groups	No. of pigs	Details	PRRSV isolates
G1	9	Sigle PRRSV-1 challenge	AN06EU4204
G2	9	Single HP-PRRSV-2 challenge	FDT10US23
G3	9	Co-challenged with PRRSV-1 and HP-PRRSV-2	AN06EU4204 + FDT10US23
G4	9	No-challenge	-

5.4 Results

5.4.1 Clinical signs and rectal temperature

Pigs in the G4 group remained in normal conditions and rectal temperatures were in physiological range throughout the experiment. Pigs in the G3 group had moderately labored abdominal respiration between 3-7 DPC. While, pigs in the G2 showed mild transient dyspnea. In contrast, pigs in the G1 group did not showed PRRSV-related clinical signs such as increased respiration rate and coughing. The rectal temperature was increased in the at 3 DPC and 5 DPC in the G3 and G2 groups, respectively. Meanwhile, the rectal temperature of pigs in the G1 group remained in physiological range throughout the experiment.

5.4.2 PRRSV-specific antibody response

No PRRSV-specific antibody response detected in the G4 groups throughout the experiment. The PRRSV-specific antibody titers were first detected in all challenged groups at 3 DPC and reached above cut-off levels (S/P > 0.4) at 7 DPC. Then, the antibody titers were continually increased at 10 DPC and remained at plateau levels until the end of the experiment (**Figure 4**). The antibody titers of pigs in the G1 group had significantly ($p < 0.05$) lower than that of the other challenged groups at 7 and 10 DPC. There were no statistical differences in the antibody titers among challenged groups at 14-28 DPC. No PRRSV-specific neutralizing antibodies detected in any of pigs throughout the experiment.

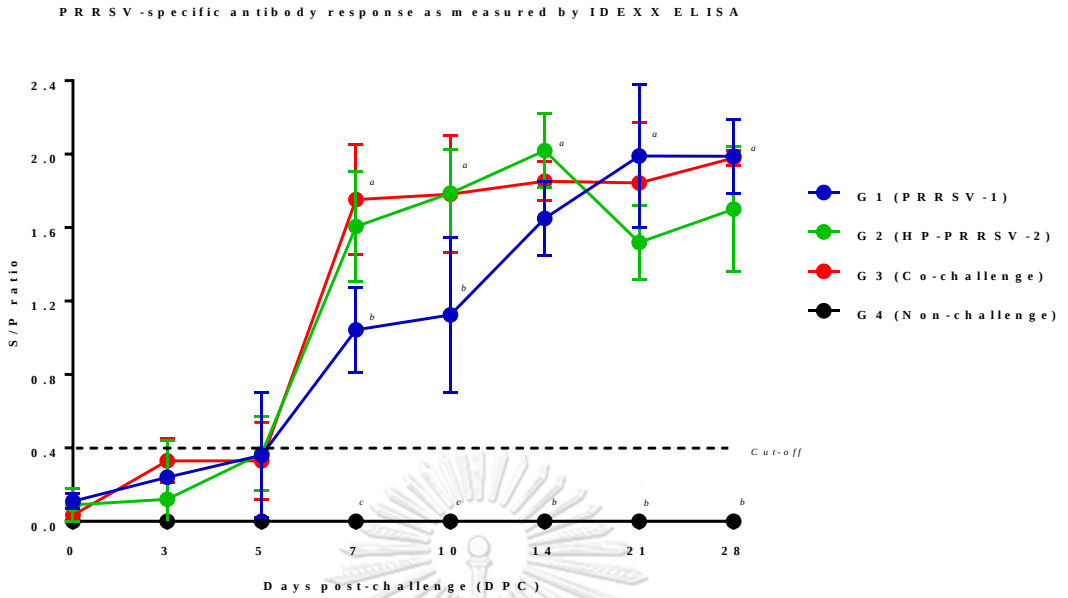


Figure 4 PRRSV-specific antibody response as measured by IDEXX ELISA following challenge.

5.4.3 Quantification of PRRSV in blood

The mean genomic copies of PRRSV RNA in blood was showed in **Figure 5.** Pigs in the G4 group had no PRRSV viremia until the end of experiment. PRRSV viremia was first detected in all challenged groups at 3 DPC, continually increased and reached peak at 7 DPC. Then, PRRSV RNA was slightly decreased and dropped to basal levels at 14-28 DPC, respectively. At 3 DPC, PRRSV RNA of all challenged groups had significantly ($p < 0.05$) higher levels than that of the G4 group. Pigs in the G3 group had significantly higher ($p < 0.05$) PRRSV RNA than that of the other challenged groups at 5-10 DPC. However, there was no difference in PRRSV RNA among the G1 and G2 groups at 5-10 DPC (**Figure 5**).

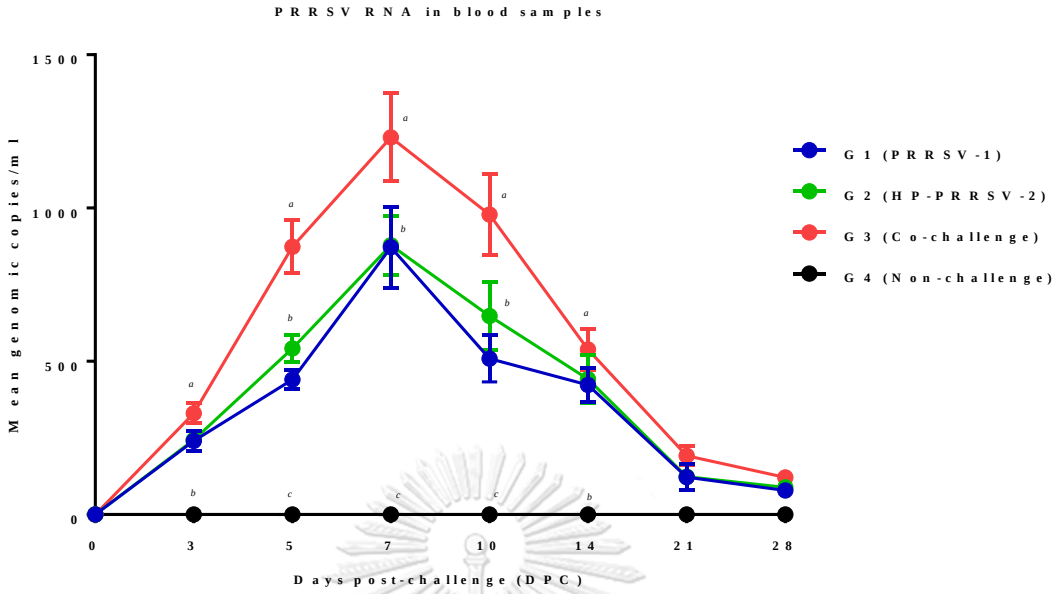


Figure 5 The mean genomic copies of PRRSV RNA in blood samples following challenge.

5.4.4 Macroscopic lung lesions

Macroscopic lesions were present predominantly in the middle, caudal lobes. The pneumonia was characterized by multifocal, mottled tan-colored areas, with irregular and indistinct borders. No macroscopic lung lesions were detected in any of pigs in the G4 groups. Pigs in the G3 group was the highest and had significantly ($p >$ 0.05) higher macroscopic lung lesion scores than those of the G1 and G2 groups at 7 DPC. Meanwhile, pigs inoculated with PRRSV-1 (G1) had relatively lower macroscopic lung lesion scores than those of the G2 groups but showed no statistical differences among groups (**Table 4**).

5.4.5 Microscopic lesions

Microscopic lesions were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes. Like macroscopic lesion score, the lungs of G4 group were normal. Among challenged groups, the co-challenged pigs of the G3 group had significantly ($p < 0.05$) higher microscopic lung

lesion scores than that of the other challenged groups. Microscopic lung lesion scores of the G1 group had relatively lower compared to that of the G2 group without statistical difference (**Table 4**).

5.4.6 Immunohistochemistry

The mean number of PRRSV-positive cells was significantly higher ($p < 0.05$) in the co-challenged group compared to that of the other groups. Meanwhile, the G1 group had relatively lower PRRSV-positive cells than in the G2 group. There was no difference in the PRRSV-positive cells between the G1 and G2 groups (**Table 4**).

Table 4 Macroscopic- and microscopic lung lesion scores, and PRRSV-positive cells in lung tissues. Values are displayed in mean±SEM. The different lowercase letters represent differences between treatment groups ($p < 0.05$)

Treatment groups	Macroscopic scores	Microscopic scores	PRRSV-antigen scores
G1 (PRRSV-1)	54.86 ± 1.28^{b}	1.76 ± 0.11^{b}	5.4 ± 0.4^{b}
G2 (HP-PRRSV-2)	57.84 ± 1.48^{b}	1.83 ± 0.14^{b}	6.3 ± 0.2^{b}
G3 (Co-challenge)	76.67 ± 7.69^{a}	2.37 ± 0.17^{a}	17.4 ± 1.2^{a}
G4 (Non-challenge)	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.0 ± 0.0^{c}

5.5 Discussion and Conclusion

The present study demonstrates differences in pathogenicity following infection with single Thai PRRSV-1 and PRRSV-2 (HP-PRRSV-2) isolates or in co-challenge with both PRRSV species in experimental pigs. The infection of Thai PRRSV-1, AN06EU4204, induce relatively lower respiratory clinical disease, viremia and lung lesions than that of the FDT10US23 isolate inoculation. In addition, the co-infection of both Thai PRRSV specie, AN06EU4204 and FDT10US23, induce more severity in terms of respiratory clinical sings, viral load in blood, increased of the lung lesion scores and viral antigen in tissues, than that of the infection with either PRRSV-1 or PRRSV-2 alone.

Intranasal inoculation with Thai field PRRSV isolates, either PRRSV-1 or PRRSV-2, rapidly resulted in viremia and virus distribution in several tissues. Viremia was initially detected at 3 DPC, peaked at 7 DPC and continually decreased at 10 to 28 DPC. These results agree with previous reports of viremia caused by PRRSV-1 and PRRSV-2 (HP-PRRSV-2) (Karniychuk et al., 2010; Zuckermann et al., 2007)

The more virulent PRRSV isolate replicates faster and able to induce more severe interstitial pneumonia than less virulent isolate regardless of its species (Halbur et al., 1995a; Halbur et al., 1996b). Therefore, microscopic pulmonary lesion scores and virus distribution in the lungs are the most important criteria for determining the virulence of PRRSV isolate. In the present study showed that pigs infected with Thai field HP-PRRSV-2, FDT10US23 isolate, had more higher lung lesions and PRRSV-antigens than did the pigs infected with PRRSV-1, AN06EU4204. These results suggest that Thai PRRSV isolates have different virulence based on the macro- and microscopic lung lesion scores and PRRSV-antigen in lung tissues, and the FDT10US23 isolate may be more virulent than the AN06EU4204 isolate.

PRRSV replicate extensively in pulmonary alveolar macrophage (Van Breedam et al., 2010; Van Gorp et al., 2010) and severe lung pathological lesions are main symptom of HP-PRRSV infection (Zhou et al., 2011). We found that the co-inoculated pigs showed a complete disappearance of lung structure, histiocytic interstitial pneumonia with increased of macrophages, and thickening of interlobular septal.

According to previous report suggested that the lack of immune response activated by HP-PRRSV infection are closely related with to acute lung injury (Han et al., 2014; Han et al., 2013).

PRRSV-specific antibodies were first detected at 3 DPC, reached above the cut-off level at 7 DPC and remained constant until the end of the experiments as measured by ELISA. Our results of antibody response are in agreement with previous studies that PRRSV-specific antibody response was initially detected at 7 DPC as measured by ELISA (Diaz et al., 2005; Yoon et al., 1992). However, the SN titers against PRRSV is usually detected at 28-35 DPC, the absence of SN titers in the present study was probably due to the short period of the experiment (Labarque et al., 2000).

Since the co-existence of both PRRSV species is endemic in several swine producing regions including Thailand. The results of the present study provide the pathogenicity of either single PRRSV infection or concurrent infection of both PRRSV species. The co-infection caused more severe clinical sings, increased viremia, and induction of lung lesions rather by single infection with either PRRSV-1 or PRRSV-2 (HP-PRRS) alone. The difference of these pathogenicity with different PRRSV isolates could help to explain the variability observed in the field outbreaks of PRRS.

CHAPTER 6

Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines

This work has been published in the topic of

Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines

Archives of Virology

January 2017, Volume 162, pp 139-146

(Appendix A)

Adthakorn Madapong, Gun Temeeyasen, Kepalee Saeng-chuto, Thitima Tripipat, Wichian Navasakuljinda, Alongkot Boonsoongnern, Angkana Tantituvanont, and Dachrit Nilubol

In this chapter, we investigated the induction of antibody response of pigs following vaccination with six different commercially available PRRSV MLV and evaluated the shedding pattern of vaccine viruses in sentinel pigs. Our finding provided criterions for PRRSV MLV selection based on antibody responses and safety concern based on the shedding and persistence of vaccine viruses.

6.1 Abstract

The antibody response and pattern of shedding of vaccine virus following vaccination with modified live genotype I and II porcine reproductive and respiratory syndrome virus (PRRSV) vaccines (MLVs) were investigated. Ninety PRRSV-free pigs were divided randomly seven groups including the NEG, EU1, EU2, US1, US2, US3 and US4 groups. The NEG group was unvaccinated. The EU1, EU2, US1, US2, US3 and US4 groups were vaccinated with the following MLVs: Amervac® PRRS, Porcilis® PRRS, Fostera™ PRRS, Ingelvac® PRRS MLV, Ingelvac® PRRS ATP, and PrimePac™ PRRS+, respectively. Sera were quantitatively assayed for viral RNA using qPCR. Antibody responses were measured using Idexx ELISA and serum neutralization (SN). Shedding of vaccine virus was investigated using sentinel pigs and by detection of viral RNA in tonsil scrapings. Antibody responses were detected by ELISA at 7-14 days post-vaccination (DPV) and persisted at high titers until 84 DPV in all MLV groups. The SN titers were delayed and isolate specific. SN titers were higher for the homologous virus than for heterologous viruses. Age-matched sentinel pigs introduced into the EU2, US2 and US3 groups at 60 DPV seroconverted. In contrast, sentinel pigs introduced at 84 DPV remained negative in all the MLV groups. Vaccine viral RNA was detected in tonsil scrapings from the EU2, US2 and US3 groups at 84-90 DPV. No viral RNA was detected beyond 70 DPV in the EU1, US1 and US4 groups. In conclusion, all MLV genotypes induced rapid antibody responses, which were measured using ELISA. The development of SN antibodies was delayed and isolate specific. However, the shedding pattern was variable and depend on the by virus isolate used to manufacture the vaccines.

Keywords: Porcine reproductive and respiratory syndrome virus, Modified live vaccine, Antibody response, Shedding, Sentinel pig

6.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) has caused severe economic damage to the swine industry worldwide since its emergence in the late 1980s (Done et al., 1996). This syndrome is characterized by reproductive disorders in sows, including abortion, reduced numbers of weaned pigs due to an increase in the number of stillborn pigs, mummified fetuses, and weakness, and respiratory disorders in pigs from nursery to finishing.

Because of the economic losses caused by PRRSV outbreaks, various types of PRRSV vaccines have been developed and implemented on pig farms with varying degree of success. Several vaccination trials have shown the replication of live immunogen in pigs to be a crucial requirement for generating robust protective immunity against PRRSV infection (Murtaugh and Genzow, 2011; Plana-Duran et al., 1997). Therefore, a modified-live vaccine (MLV) rather than a killed or subunit vaccine has been deemed to be the most efficacious type of vaccine against PRRSV infection to date and has been employed regularly in both experimental and field-scale trials since its first introduction in 1994.

Currently, various types of MLV vaccines, including genotypes I and II, are commercially available. In regions where a single infection with either genotype I or II has been reported, MLV targeting the circulating genotype should be used. However, in co-infected herds, which genotype of PRRSV MLV should be used to successfully control the disease is less obvious. Criteria for vaccine selection could be the induction of immune responses and the shedding patter of the vaccine virus. The induction of immune responses following MLV administration may vary according to the virus isolate used to produce the vaccine (Ferrari et al., 2013; Murtaugh and Genzow, 2011). Occasionally, vaccination with genotype II MLV can yield undesired outcomes, such as delayed immune responses, low potency of humoral or cell-mediated immune activation, the induction of regulatory IL-10 and/or T-cells (Treg), the suppression of pro-inflammatory cytokine production, and a reduced level of type I (α/β) and type II (γ) interferon. These undesired outcomes could potentially lead to reduced protective efficacy of a vaccine or, in the worst case, to increased

susceptibility to infection by other pathogens (LeRoith et al., 2011). In addition, the safety of MLV of all genotypes remains doubtful because the persistence of MLV, the development of viremia, transmission of a vaccine to non-vaccinated pigs, and clinical signs in vaccinated pigs have been documented following vaccination (Mengeling et al., 1999). Therefore, this study aimed to evaluate the induction of humoral immune responses and viral vaccine shedding following vaccination with either genotype I or II MLV. In the present study, the induction of antibody responses and shedding patterns of six different MLVs were compared.

6.3 Materials and Methods

6.3.1 Ethics statement

All animal procedures were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council of Thailand according to protocols approved by the Chulalongkorn University IACUC.

6.3.2 Experimental design

A cohort of 90 seven- to eight-week-old castrated male PRRSV-free pigs were randomly assigned based on a stratification by weight into the following seven treatment groups: NEG, EU1, EU2, US1, US2, US3 and US4. Groups of pigs were housed in separated rooms with separated air spaces (**Table 5**). The NEG group included 30 pigs that were left unvaccinated. Pigs in the EU1 and EU2 groups were vaccinated intramuscularly with Amervac® PRRS and Porcilis® PRRS, respectively, which are both PRRSV genotype I MLVs. The US1, US2, US3 and US4 groups were vaccinated intramuscularly with Fostera™ PRRS, Ingelvac® PRRS MLV, Ingelvac® PRRS ATP and PrimePac™ PRRS+, respectively, which are all PRRSV genotype II MLVs. The dosage and route of administration were in accordance with the respective manufacturer's directions.

Following vaccination, all groups were monitored for changes in physical condition and were scored for clinical respiratory disease. Blood samples were collected at 0, 3, 5, 7, 14, 21, 28, 35, 42, and 84 days post-vaccination (DPV). Sera were separated from blood samples and assayed for the presence of antibody using ELISA and a serum neutralization (SN) assay against homologous and heterologous isolates. The viral load in serum was measured using real-time quantitative PCR (qPCR). Tonsil scraping samples were collected at 60, 70, 84 and 90 DPV and assayed for the presence of viral RNA by RT-PCR. Individual pigs were restrained using a snare, and samples were then collected by scraping the palatine tonsil with an elongated spoon. Scrapings were mixed with 1 ml of DMEM supplemented with 50 µg of

gentamicin/ml and filtered through a 0.22 μm nitrocellulose membrane. Filtrates were then stored at -80ºC for later use.

Table 5 Experimental design

Treatment groups	No. of pigs	Vaccination	Vaccines	Manufacturer
NEG	30	No	-	-
EU1	10	Yes	Porcilis® PRRS	MSD Animal Health, The Netherlands
EU2	10	Yes	Amervac® PRRS	Hipra, Spain
US1	10	Yes	Fostera™ PRRS	Zoetis, USA
US2	10	Yes	Ingelvac® PRRS MLV	Boehringer Ingelheim, USA
US3	10	Yes	Ingelvac® PRRS ATP	Boehringer Ingelheim, USA
US4	10	Yes	PrimePac™ PRRS	MSD Animal Health, The Netherlands

6.3.3 Vaccines and viruses

Homologous and heterologous viruses were used to perform a serum neutralization (SN) assay. Homologous virus refers to a vaccine isolate. To retrieve homologous virus, each vaccine (except Fostera™ PRRS) was re-constituted in DMEM media. Then, the virus was propagated in MARC-145 cells using a previously described method (Park et al., 2014). Virus was harvested by a cycle of freezing and thawing. Supernatant containing the virus was stored at -80ºC before subsequent use. Because of the inability of the Fostera™ PRRS vaccine virus to be generated using MARC-145 cells, the homologous virus used to generate to Fostera™ PRRS was a virus that was isolated from pigs that were previously vaccinated with Fostera™ PRRS. Heterologous viruses refer to the SB_EU02 and ST_US02 isolates, which are Thai PRRSV genotype I and II field isolates. The SB_EU02 and ST_US02 were isolated from farms experiencing PRRS outbreaks.

6.3.4 Clinical evaluation

Rectal temperature was recorded daily for two consecutive weeks by the same personnel at the same time. The severity of clinical respiratory disease was evaluated daily for two consecutive weeks following vaccination, and on a weekly basis for 2 more weeks using a scoring system for each pig following stress induction as described previously (Halbur et al., 1995c). In brief, a score of 0 is normal. Pigs with scores of 1 and 2 display mild dyspnea and/or tachypnea when stressed and when at rest, respectively. Scores of 3 and 4 indicate moderate dyspnea and/or tachypnea when stressed and when at rest, respectively. Pigs with a score of 5 displayed severe dyspnea and/or tachypnea when stressed or at rest.

6.3.5 Quantification of PRRSV RNA in serum and RT-PCR in tonsil scraping samples

Total RNA was extracted from serum and tonsil scarping samples using NucleoSpin® RNA Virus (Macherey-Nagel, Germany) according to the instructions provided by the manufacture. The RNA quality was measured using a NanoDrop Spectrophotometer (Colibri spectrometer, Titertek Berthold, Germany). cDNA was synthesized from viral RNA immediately after the extraction process. The reaction contained 1X M-MuLV reverse transcriptase reaction buffer, 0.5 mM each dNTP, 2.5 µM random hexamers, 13.2 U of RNase inhibitor (RiboLock™, Fermentas, Vilnius, Lithuania), 6.6 U of M-MuLV reverse transcriptase (New England Biolabs, Ipswich, UK), 0.5 µg of viral RNA, and RNase-free water up to 25 µl. The reaction was carried out at 42ºC for 60 min, followed by the inactivation of reverse transcriptase at 95ºC for 10 min. All cDNA samples were kept at -20ºC until used for quantitative PCR (qPCR).

qPCR assays for determination of genomic copy number of PRRSV RNA in serum samples were conducted using an ABI PRISM 7500® Real-Time PCR platform (Applied Biosystem, USA). Primers specific for the ORF5 EU and US strains were used for qPCR (Nilubol et al., 2014). Each qPCR reaction contained 0.1 µg of cDNA, 0.2 µM ORF5EU or ORF5US primers (as appropriate for each sample), 1X EvaGreen real-time PCR master mix E4® (GeneOn, Germany), and deionized water to yield a 20-µl final

volume. pGEM®-T Easy Vector (Promega, WI, USA) containing an insert of 704 bp from ORF5EU or 780 bp from ORF5US were used to construct standard curves in these qPCR assays. Thermocycling conditions for qPCR started with an initial denaturation step at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 45 s, annealing at 55ºC for 45 s, and extension with fluorescence acquisition at 72ºC for 45 s. A standard curve was made for each pair of primers.

To detect the presence of virus in tonsil scrapings, RT-PCR and PCR amplification were performed using GoTaq® Green Master Mix (Promega, WI, USA). All reactions were performed as described above for qPCR. PCR amplicons were visualized by agarose gel electrophoresis.

6.3.6 Antibody detection

Sera were assayed for the presence of PRRSV-specific antibody by ELISA and serum neutralization (SN) assay. PRRSV Idexx ELISA (HeardCheck PRRS X3, Idexx Laboratories Inc., Wesbrook, Maines, USA) was used in accordance with the manufacturer's instructions. The presence or absence of antibody was determined by calculating the sample-to-positive control (S/P) ratio of the test. Serum samples were considered positive for PRRSV antibody if the S/P ratio was greater than 0.4.

SN assays were performed using the homologous isolate along with two different heterologous PRRSV isolates, SB_EU02 and ST_US02 as described previously (Nilubol et al., 2004). The SN antibody titers were reported as the highest serum dilution that resulted in a 90% reduction in the number of fluorescent focus units per well. Geometric mean titers were calculated.

6.3.7 Sentinel pigs

Viral shedding patterns were monitored by placing two groups of two age-matched sentinel pigs in contact with the principal pigs at 60 and 84 DPV for 7 days. Subsequently, sentinel pigs were removed and housed in a separate unit for an additional 2 weeks to monitor them for seroconversion, using ELISA.

6.3.8 Statistical analysis

Data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed for each day by ANOVA to determine whether there were significant differences between treatment groups. If the *p*-value in the ANOVA table was ≤ 0.05, differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $p \leq 0.05$ rejection level.

6.4 Results

6.4.1 Rectal temperature and clinical observations

The rectal temperatures of the pigs in the control group and all vaccinated groups were within normal physiological ranges throughout the experimental period. None of the pigs in any of the groups displayed any clinical respiratory disease throughout the study except for those in the EU2 and US3 groups. There were 5/10, 3/10 and 6/10 of pigs in the EU2, US2, and US3 groups, respectively, that showed respiratory signs at 20 DPV. At 21 DPV, all pigs in the EU2 group were injected once intramuscularly with tulathromycin. At 28 DPV, all pigs in the EU2, US1 and US2 groups were in-feed medicated with amoxycillin at 300 ppm for 7 consecutive days. There was one pig in the US3 group that died at 35 DPV, and the necropsy revealed paleness of skin and gastric ulceration. PCR results from organ sample, including lung, bronchial and mesenteric lymph nodes, were positive for PRRSV. At 35 DPV, two pigs from the EU2 and US3 groups were euthanized because of the severity of clinical disease.

6.4.2 Quantification of PRRSV RNA in serum

PRRSV RNA was not detectable in the NEG and EU1 groups throughout the study (**Figure 6**). In the US2 and US3 groups, the viral RNA copy numbers were highest at 3 DPV and then slowly decline until they were below the limit of detection at 21 and 28 DPV, respectively. In the EU2 group, the viral RNA copy number peaked at 3 DPV and decreased until it was below the limit of detection at 7 DPV. In the US1 group, the viral RNA copy number was detectable after 3 DPV and peaked at 7 DPV. Then, the viral RNA copy number level gradually declined until it could not be detected at 28 DPV.

The viral RNA copy number of the EU1 group was significantly lower compared with the other vaccinated groups at 3 DPV. At 7 DPV, the viral RNA copy number in the US1 group was not different from those of the other genotype II MLV groups but was significantly higher than those of the genotype I MLV groups.

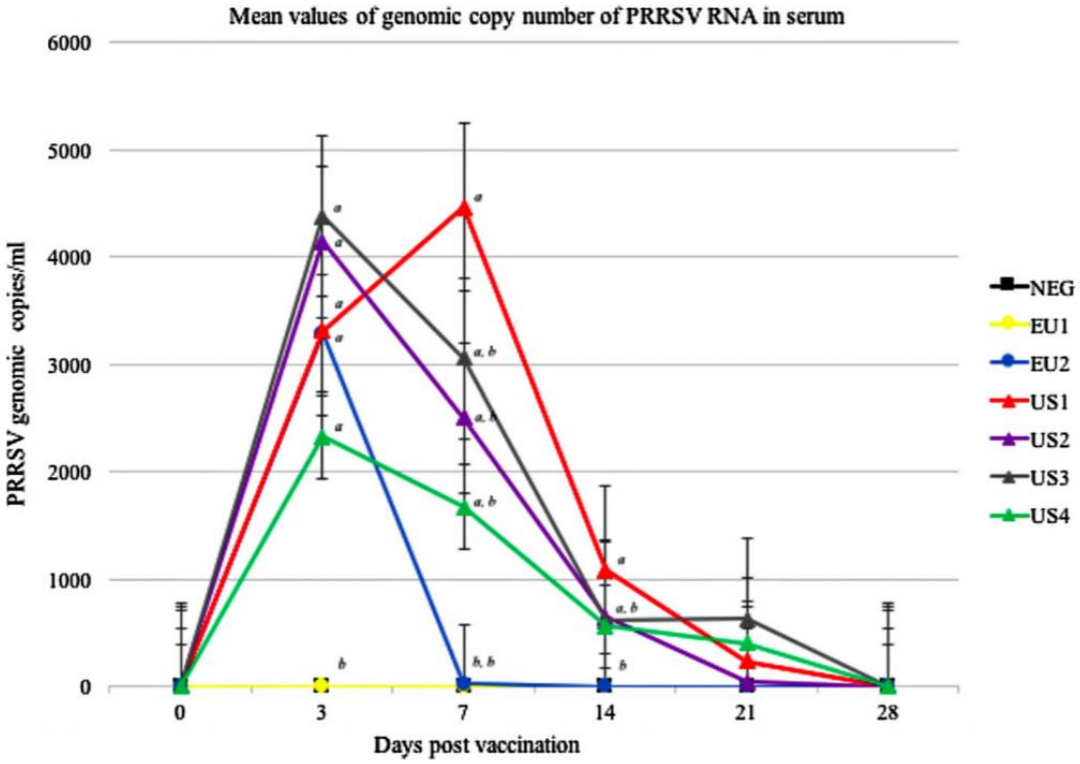


Figure 6 Mean values of the genomic copy number of PRRSV RNA in serum of the NEG (black square), EU1 (yellow circle), EU2 (blue circle), US1 (red triangle), US2 (purple triangle), US3 (gray triangle) and US4 (green triangle) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups.

6.4.3 RT-PCR in tonsil scrapings

Viral RNA was not detected in any pigs in the EU1 group. In contrast, viral RNA in the EU2 group was detected in 3 of 9, 2 of 9 and 1 of 9 pigs at 60, 70 and 90 DPV, respectively (**Table 6**). For genotype II MLVs, viral RNA was detected in 1 of 10 and 2 of 10 pigs in the US1 and US4 groups, respectively, at 60 DPV. In the US2 group, viral RNA was detected on all sampling days and was still detectable in 2 of 10, 3 of 10, 1 of 10 and 1 of 10 of pigs at 60, 70, 84 and 90 DPV, respectively. In the US3 group, viral RNA remained detectable in 1 of 8, 2 of 8 and 1 of 8 of pigs at 60, 70 and 84 DPV, respectively.

Table 6 Detection of viral RNA in tonsil scrapings from vaccinated pigs and sentinel pigs

Treatment groups	Vaccines	Tonsil scraping samples				Sentinel pigs	
		Days post-vaccination				Days post-vaccination	
		60	70	80	90	60	84
NEG	-	0/10[a]	0/10	0/10	0/10	Negative	Negative
EU1	Porcilis® PRRS	0/10	0/10	0/10	0/10	Negative	Negative
EU2	Amervac® PRRS	3/9	2/9	0/9	1/9	Positive	Negative
US1	Fostera™ PRRS	1/10	0/10	0/10	0/10	Negative	Negative
US2	Ingelvac® PRRS MLV	1/10	2/10	1/10	1/10	Positive	Negative
US3	Ingelvac® PRRS ATP	1/8	2/8	1/8	0/8	Positive	Negative
US4	PrimePac™ PRRS	1/10	2/10	0/10	0/10	Negative	Negative

[a]The number of positive pigs by PCR/total number of pigs in the groups

6.4.4 Antibody responses as measured by ELISA

Pigs in the NEG group remained serologically negative throughout the experiment (**Figure 7**). Our findings revealed a similar pattern in all vaccinated group. Antibody responses were first detected at 7 DPV at the earliest in some pigs of the vaccinated groups, but the average antibody level was below the cutoff level (S/P ratio at 0.4). At 14 DPV, the average antibody responses of the EU2, US1, US2 and US4 groups were significantly higher than those of the EU1 and US4 groups, in which the average antibody levels were below the cutoff level. At 21 DPV, the average antibody responses of all vaccinated groups were above the cutoff level and remained constant until the end of the experiment. There were no differences between any of the vaccinated groups from 21 to 84 DPV.

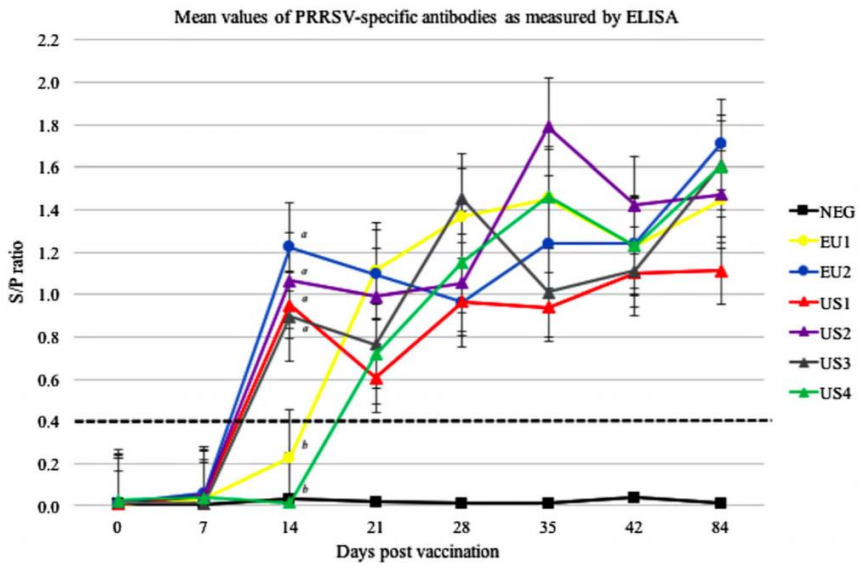


Figure 7 Mean values of PRRSV-specific antibodies as measured by ELISA of the NEG (black square), EU1 (yellow circle), EU2 (blue circle), US1 (red triangle), US2 (purple triangle), US3 (gray triangle) and US4 (green triangle) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups. A dashed line indicates the cutoff level (S/P ratio of 0.4).

6.4.5 Antibody responses as measured by serum neutralization (SN) assay

Pigs in the NEG group remained serologically negative throughout the experiment. In all vaccinated groups, the SN assay against homologous virus in all vaccinated groups showed a similar pattern of SN titers that could be detected as early as 28 DPV (**Figure 8A**). Titers reached a peak level at 35 or 42 DPV and then declined by 84 DPV. At 28 DPV, the EU2 group had significantly higher SN titers against the homologous virus compared with the other vaccinated groups. In contrast, the US4 group had significantly lower SN titers compared with the other vaccinated groups. At 35 DPV, the EU2 and US2 groups had significantly higher SN titers compared with the other vaccinated groups. At 42 DPV, the EU2, US1, US2 and US4 groups had significantly higher SN titers compared with the EU1 and US3 groups.

For the heterologous genotype I virus (SB_EU02), the kinetics of the SN response differed in a manner that was dependent on the MLV (**Figure 8B**). Compared with homologous virus, the SN titers were relatively low. The SN titers remained at a similar level from 28 to 42 DPV and then declined by 84 DPV. However, the SN titers in the US1 group were significantly higher than in the other vaccinated groups from 28 to 84 DPV and were significantly higher than those for the homologous virus.

In the case of heterologous genotype II virus (ST_US02), the kinetics of the SN response were like those evoked using heterologous genotype I virus (SB_EU02) (**Figure 8C**). SN titers were lower than with heterologous genotype I virus in all groups, except for the EU2 group. The SN titers of the EU2 group were significantly higher on 35 and 42 DPV compared with the other vaccinated groups.

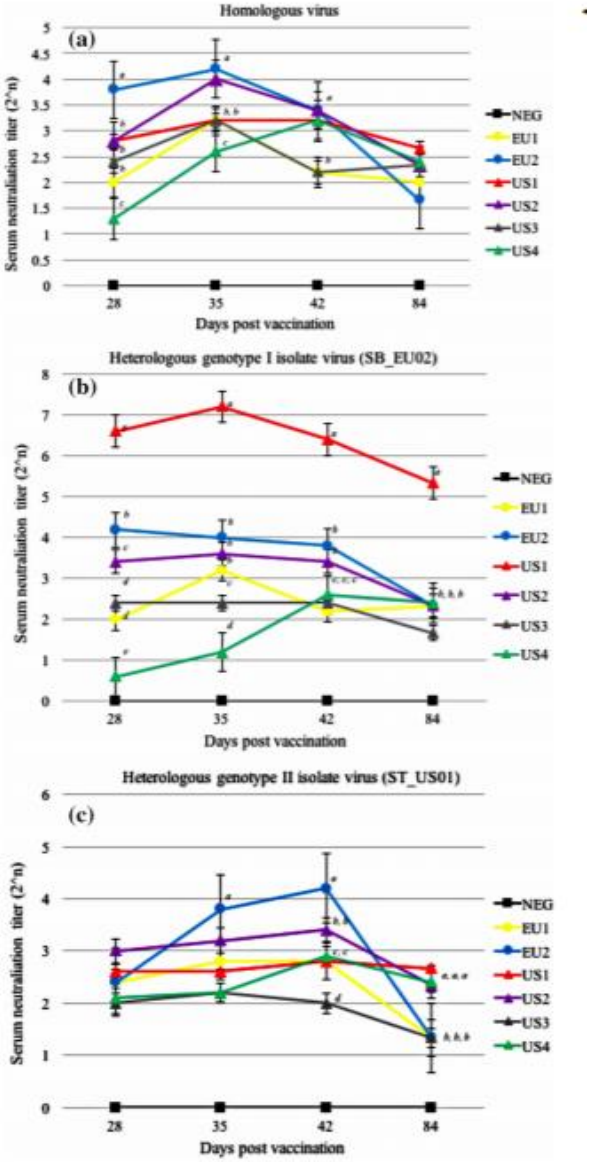


Figure 8 Antibody responses as measured by serum neutralizing (SN) assay using (**A**) homologous virus, (**B**) heterologous genotype I virus (SB_EU02), and (**C**) heterologous genotype II virus (ST_US02) of the NEG (black square), EU1 (yellow circle), EU2 (blue circle), US1 (red triangle), US2 (purple triangle), US3 (gray triangle) and US4 (green triangle) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups.

6.4.6 Sentinel pigs

Sentinel pigs introduced to the EU2, US2 and US3 groups at 60 DPV seroconverted, but those in the EU1, US1 and US4 groups did not. Sentinel pigs that were placed in contact with pigs of all groups on 84 DPV did not seroconvert over a 7- or 14-day period of observation.

6.5 Discussion and Conclusion

We compared the efficacy of six different PRRSV MLVs in the induction of antibody responses in PRRSV-free pigs. All six MLVs rapidly induced antibody responses as measured by ELISA. The antibody responses were detected as early as 7-14 DPV in all MLV-genotype-dependent manner. The antibody levels in all vaccinated groups were similar at 21 DPV. It was notable that there was a difference in early antibody detection between two genotype I MLVs. The EU1 group had a significantly lower S/P ratio than the EU2 group at 14 DPV, and the S/P ratio was below 0.4, the cutoff level. Surprisingly, the EU2 group produced an antibody response at a similar level when compared with the genotype II MLVs. In summary, there was no difference in the antibody responses as measured by ELISA for any of the MLV genotypes. The results of the present study suggest that the MLV genotype is not the key factor in the induction of immunity, but the specific virus isolate used for the vaccines might play an important role. Moreover, it is notable that the antibody detection in the present study was performed using Idexx ELISA, which can simultaneously detect specific antibodies against PRRSV genotype I and II infections. However, these findings may not be applicable when using other diagnostic kits.

Following MLV vaccination, antibody responses measured by the SN assay were delayed regardless of the MLV genotype and isolate in the vaccines. The responses were detected as early as 28 DPV. In addition, the response was isolate-specific. Homologous responses generated using a homologous virus induced a higher, although delayed response compared with the heterologous responses generated using either heterologous genotype I or II viruses. Heterologous responses were lower and shorter in duration. Our finding of differences in responses was not surprising. PRRSV isolates differed in their susceptibility to neutralization (Martinez-Lobo et al., 2011), and the mechanisms associated with this susceptibility remain poorly characterized, although the influence of N-linked glycosylation in decoy epitope regions could be one key factor (Nilubol et al., 2013, 2014; Plagemann et al., 2002). A previous study demonstrated that a heterologous response could be higher or lower (Ferrari et al., 2013), depending on the isolates that were used in the assay.

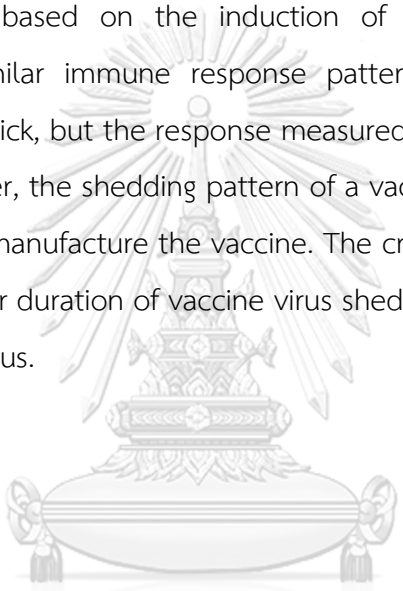
In addition to their ability to induce an immune response, the shedding patterns of the vaccine viruses were investigated using three different measurements, including the duration of viremia, the detection of viral RNA in tonsils, and infection of sentinel pigs. After vaccination, we detected a difference in the shedding patterns between MLVs. The two genotype I MLVs had a shorter viremic phase compared with genotype II MLVs. However, the magnitude of viral titers was not different. In addition, there was a difference in the shedding pattern of genotype I MLV, although one genotype I MLV had a shedding pattern that resembled that of a genotype II MLV. This finding could indicate the absence of viremia or that the quantity of virus in the serum was lower than the limit of detection of the real-time PCR assay. Within genotype II MLVs, all three MLVs caused viremia as early as 3 DPV, and it then declined thereafter. In contrast, the titers of one genotype II MLV continued to increase until 7 DPV and then declined. These findings suggested that the viremic phase of MLV was associated with the virus isolate used in the vaccine, not the virus genotype.

To further evaluate the viral shedding pattern, sentinel pigs were used. Sentinel pigs were housed along with principal pigs of the EU2, US2 and US3 groups in the same pen beginning on day 60, and they were found to undergo seroconversion. However, sentinel pigs introduced at 84 DPV remained uninfected, as indicated by their failure to seroconvert. The shedding patterns of vaccine viruses over this long period of time have not yet been investigated. Compared to wild-type PRRSV, vaccine viruses should be shed to sentinel pigs over a shorter time. Previous studies conducted by several investigators to characterize several wild-type field isolates of PRRSV and the duration of PRRSV shedding to sentinel pigs have suggested that virus shedding to sentinel pigs occurred on average 60 to 70 days after exposure (Wills et al., 2003). Although vaccine viruses were not transmitted to sentinel pigs at 84 DPV, the detection of viral-RNA-positive samples in the tonsil scraping samples might represent a risk factor for the shedding of vaccine viruses.

The PCR results from tonsil scrapings at 84 DPV indicated that vaccinated pigs still harbored viral RNA. However, whether the RNA-positive samples represented infectious viruses was not determined. The detection of viral RNA does not

necessarily indicate the isolation of infectious virus. Any viral genomic material needs to be tested further to determine whether the pigs may still be infectious and contagious. Using a swine bioassay, it was demonstrated that homogenates from tonsils collected from pigs infected with the PRRSV strain VR-2332 at 105 days post-exposure remained infectious (Horter et al., 2001). Viral RNA was detected in the tonsils, suggesting that viruses remained present in both groups of pigs but were not transmitted to contact sentinel pigs. Determining whether virus shedding can be reinitiated will require further study.

In conclusion, based on the induction of immune responses, all MLV genotypes yield a similar immune response pattern. Measurement of antibody response by ELISA is quick, but the response measured using SN assay is delayed and isolate specific. However, the shedding pattern of a vaccine virus is influenced by the isolate that is used to manufacture the vaccine. The criteria for MLV selection should be based on the shorter duration of vaccine virus shedding and the broader response against heterologous virus.



CHAPTER 7

Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2

This work has been published in the topic of

Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2

Scientific Reports

February 2020, Volume 10, 1649

(Appendix B)

Adthakorn Madapong, Kepalee Saeng-chuto, Alongkot Boonsoongnern, Angkana Tantituvanont and Dachrit Nilubol

In this chapter, we investigated the cell-mediated immune (CMI) response of six different commercially available PRRSV MLV and protective efficacy against PRRSV infection in the experimental condition. Our findings reveal the pattern of CMI response of PRRSV MLV following vaccination and showed reliable protective efficacy against heterologous PRRSV infections.

7.1 Abstract

Cell-mediated immunity (CMI), IL-10, and the protective efficacy of modified-live porcine reproductive and respiratory syndrome virus (PRRSV) vaccines (MLV) against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV) were investigated. Seventy, PRRSV-free, 3-week-old, pigs were allocated into 7 groups. Six groups were intramuscularly vaccinated with MLV, including Porcilis (PRRSV-1 MLV, MSD Animal Health, The Netherlands), Amervac (PRRSV-1 MLV, Laboratorios Hipra, Spain), Fostera (PRRSV-2 MLV, Zoetis, USA), Ingelvac PRRSV MLV and Ingelvac PRRS ATP (PRRSV-2 MLV, Boehringer Ingelheim, USA), and Prime Pac PRRS (PRRSV-2 MLV, MSD Animal Health, The Netherlands). Unvaccinated pigs were left as control. Lymphocyte proliferative response, IL-10 and IFN-γ production were determined. At 35 days post-vaccination (DPV), all pigs were inoculated intranasally with 2 ml of each PRRSV-1 ($10^{5.4}$ $TCID_{50}$/ml) and PRRSV-2 ($10^{5.2}$ $TCID_{50}$/ml, HP-PRRSV). Following challenge, sera were quantitatively assayed for PRRSV RNA. Pigs were necropsied at 7 days post-challenge. Viremia, macro- and microscopic lung lesion together with PRRSV antigen presence were evaluated in lung tissues. The results demonstrated that, regardless of vaccine genotype, CMI induced by all MLVs was relatively slow. Increased production of IL-10 in all vaccinated groups was observed at 7 and 14 DPV. Pigs in Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher levels of IL-10 compared to Porcilis, Fostera and Prime Pac groups at 7 and 14 DPV. Following challenge, regardless to vaccine genotype, vaccinated pigs had significantly lower lung lesion scores and PRRSV antigens than those in the control group. Both PRRSV-1 and PRRSV-2 RNA were significantly reduced. Prime Pac pigs had lowest PRRSV-1 and PRRSV-2 RNA in serum, and micro- and macroscopic lung lesion scores ($p < 0.05$) compared to other vaccinated groups. In conclusion, PRRSV MLVs, regardless of vaccine genotype, can reduce viremia and lung lesions following co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). The main difference between PRRSV MLV is the production of IL-10 following vaccination.

Keywords: Porcine reproductive and respiratory syndrome virus, Modified-live virus vaccine, Cell-mediated immunity, Co-challenge, Protective efficacy, HP-PRRSV

7.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a devastating disease in pigs characterized by reproductive and respiratory failures. PRRS virus (PRRSV), an enveloped, positive-sense single-stranded RNA virus belonging to the *Arteriviridae* family, order *Nidovirales,* is the causative agent (Cavanagh, 1997). Two antigenically distinct genotypes of PRRSV, PRRSV-1 and PRRSV-2, have been recognized. The genomes of both genotypes are 15 kb in length and consist of 10 open reading frames (ORFs). The genotypes of PRRSV-1 and PRRSV-2 are markedly different based on the full-length genomes, which share only approximately 60% similarity at the nucleotide level (Nelson et al., 1993).

PRRSV is recognized for its high genetic variation. Presently, PRRSV-1 and PRRSV-2 have continuously evolved into 3 subtypes and 9 lineages, respectively (Shi et al., 2010b; Stadejek et al., 2008). PRRSV-1 and PRRSV-2 have independently evolved in the European and North American (NA) continents. However, in Asia, the co-existence of both types has been increasingly evident in several countries, including Thailand, China, and Korea (Chen et al., 2017; Kim et al., 2011; Nilubol et al., 2013). Additionally, variants of PRRSV-2 endemically present in Asia are genetically related to HP-PRRSV lineage 8.7/HP-PRRSV (Do et al., 2016; Nilubol et al., 2012; Zhou et al., 2011).

Several PRRSV modified-live vaccines (MLV) against PRRSV-1 and PRRSV-2 have been commercially available and licensed in several countries worldwide depending on circulating virus genotypes. The use of PRRSV MLV depends on PRRSV genotype circulating in that region. However, questions have been raised as to what types of MLV should be used in the co-presence of PRRSV-1 and PRRSV-2. The criteria for vaccine selection should include the induction of the cell-mediated immunity (CMI) and the protection against PRRSV infection, especially against genotypes and isolates that are circulating in the affected region. Therefore, the present study was conducted to investigate CMI, IL-10, and protective efficacy of commercial PRRSV-1 and PRRSV-2 MLVs against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). Our results revealed that vaccination with PRRSV MLVs,

regardless of vaccine genotype, provide partial cross-protection against PRRSV infection. Additionally, this approach provided novel information regarding the vaccine selection for use in the presence of co-existence of both PRRSV genotypes.

7.3 Materials and Methods

7.3.1 Ethical statement for experimental procedures

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council of Thailand according to protocols reviewed and approved by the Chulalongkorn University Animal Care and Use Committee (protocol number 1731047).

Seventy, 21-day-old pigs were procured from a PRRS-free herd. Upon arrival, pigs were randomly allocated based on the stratification of weight into 7 treatment groups consisting of NonVac, Porcilis, Amervac, Fostera, Ingelvac MLV, Ingelvac ATP and Prime Pac (**Table 7**). Following a week of acclimatization, pigs were vaccinated with PRRS MLVs. NonVac was left unvaccinated. Porcilis and Amervac were vaccinated with Porcilis PRRS (PRRSV-1, MSD Animal Health, Boxmeer, the Netherlands) and Amervac PRRS (PRRSV-1, Laboratorios Hipra, Girona, Spain), respectively. Fostera, Ingelvac MLV, Ingelvac ATP and Prime Pac were vaccinated with Fostera PRRS (PRRSV-2, Zoetis, Troy Hills, USA), Ingelvac PRRS MLV (PRRSV-2, Boehringer Ingelheim, Rhein, Germany), Ingelvac PRRS ATP (PRRSV-2, Boehringer Ingelheim, Rhein, Germany) and Prime Pac PRRS (PRRSV-2, MSD Animal Health, Boxmeer, the Netherlands), respectively. Dosages and routes of administration were in accordance with manufacturers' instructions. Blood samples were collected at 0, 7, 14, 21, 28, 35 days post-vaccination (DPV). Peripheral blood mononuclear cells (PBMC) were isolated and assayed for lymphocyte proliferative response. IFN-γ and IL-10 were measured using flowcytometry, and ELISPOT or ELISA. At 35 DPV, all pigs were inoculated intranasally with PRRSV. Each pig received 2 ml (1 ml/nostril) of each PRRSV-1 (AN06EU4204) and PRRSV-2 (FDT10US23) at $10^{5.4}$ $TCID_{50}$/ml and $10^{5.2}$ $TCID_{50}$/ml, respectively. Sera were collected at 0, 3, 5, and 7 days post-challenge (DPC) and quantitatively assayed for PRRSV RNA using qPCR. All pigs were necropsied at 7 DPC. The severity of PRRSV-induced pneumonic lung lesion was scored (Halbur et al., 1995a). Lung tissues were collected for histopathological examination and immunohistochemistry (IHC).

Table 7 Experimental design. The pigs were allocated into seven treatment groups and vaccinated with six different PRRSV MLVs. The NonVac group was kept as unvaccinated control group.

Treatment groups	No. of pigs	Vaccination	Vaccines	Vaccine genotype	Dosage and route of administration	Manufacturers
NonVac	10	No	-	-	-	-
Porcilis	10	Yes	Porcilis PRRS	PRRSV-1	2 ml, intramuscular	MSD Animal Health, The Netherlands
Amervac	10	Yes	Amervac PRRS	PRRSV-1	2 ml, intramuscular	Laboratorios Hipra, Spain
Fostera	10	Yes	Fostera PRRS	PRRSV-2	2 ml, intramuscular	Zoetis, USA
Ingelvac MLV	10	Yes	Ingelvac PRRS MLV	PRRSV-2	2 ml, intramuscular	Boehringer Ingelheim, Germany
Ingelvac ATP	10	Yes	Ingelvac PRRS ATP	PRRSV-2	2 ml, intramuscular	Boehringer Ingelheim, Germany
Prime Pac	10	Yes	*Prime Pac* PRRS	PRRSV-2	1 ml, intramuscular	MSD Animal Health, The Netherlands

7.3.2 Virus isolates

Homologous and heterologous viruses were used as recall antigens in *in vitro* CMI and IL-10 assays. Homologous viruses refer to vaccine strains as previously described (Madapong et al., 2016). Heterologous viruses refer to AN06EU4204 and FDT10US23, which were Thai PRRSV-1 and PRRSV-2 (HP-PRRSV) isolates, respectively. AN06EU4204 and FDT10US23 are in Clade A, Subtype 1 and Lineage 8.7/HP-PRRSV, respectively, based on systematic classification previously described (Shi et al., 2010b; Stadejek et al., 2008). ORF5 gene sequences of AN06EU4204 and FDT10US23 are available in GenBank under accession numbers JQ040750 and JN255836, respectively. The nucleotide and amino acid similarities based on the ORF5 gene between these two isolates and PRRSV MLVs were summarized in **Table 8**.

Table 8 Nucleotide and amino acid similarities based on ORF5 gene between vaccine strains and Thai PRRSV isolates.

PRRSV (isolates)	**Classification***	**Nucleotide and amino acid similarities**						
		Level of similarity	Porcilis® PRRSV	Amervac® PRRSV	Fostera™ PRRS	Ingelvac® PRRS MLV	Ingelvac® PRRS ATP	Prime Pac® PRRS
PRRSV-1 (AN06EU4204)	Subtype I (Clade A)	Nucleotide	95.8%	92.7%	68.5%	68.3%	68.2%	67.9%
		Amino acid	92.0%	89.1%	60.9%	58.2%	55.5%	55.7%
PRRSV-2 (FDT10US23)	Lineage 8.7/HP-PRRSV	Nucleotide	68.8%	69.9%	94.0%	88.8%	90.2%	90.5%
		Amino acid	58.7%	59.8%	91.5%	87.5%	89.5%	91.8%

*International systematic classification was based on previously described, including PRRSV-1 (Stadejek et al., 2008) and PRRSV-2 (Shi et al., 2010b), respectively.

7.3.3 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using gradient density centrifugation (Lymphosep, Biowest, Riverside, MO, USA) as previously described (Ferrari et al., 2013). Isolated PBMC were resuspended in 1 ml complete media (RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 µg/ml gentamicin). The viability of PBMC were determined by Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) staining and more than 90% viability was used for lymphocyte proliferation assay, lymphocytes producing either IL-10 or IFN-γ, IFN-γ ELISPOT assay, and *in vitro* stimulation for IL-10 detection as described below.

7.3.4 Lymphocyte proliferation assay

The lymphocyte proliferation assay assesses cell proliferation using membrane-bound 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) and cell surface markers using flow cytometry.

Briefly, 1×10^7 cells/ml PBMC were incubated with CFSE at 37°C for 10 min. After washing, CFSE-stained PBMC at 1×10^6 cells were seeded into 96-well plate and co-cultured with MARC-145 cell lysate (mock suspension), PHA (10 µg/ml, Sigma-Aldrich, St. Louis, MO, USA), homologous and heterologous PRRSV at 0.01 multiplicity of infection (MOI). Following 5-day incubation, PBMC were stained with mouse anti-porcine CD4-FITC antibody (clone 74-12-14, SouthernBiotech, Birmingham, AL, USA) and mouse anti-porcine CD8-SPRD antibody (clone 76-2-11, SouthernBiotech, Birmingham, AL, USA). After washing, PBMC were suspended in 2% paraformaldehyde. The proliferation of T lymphocyte populations was measured using flow cytometry analysis (Beckman FC550, Beckman Coulter, Brea, CA, USA) with CXP software. The relative proliferative indices (PI) were calculated by using the percentage of proliferating cells in the virus stimulated well divided by the percentage of proliferating cells in the mock suspension well.

7.3.5 Lymphocytes producing either IL-10 or IFN-γ

The percentage of PRRSV-specific lymphocytes producing either IL-10 or IFN-γ after *in vitro* stimulation with homologous or heterologous PRRSV were evaluated using a method previously described (Ferrari et al., 2013). Briefly, 1×10^6 PBMC were seeded into a 96-well plate containing mock suspension, PMA (25 ng/ml) /ionomycin (1 µM) (Sigma-Aldrich, St. Louis, MO, USA), and homologous and heterologous PRRSV at 0.01 MOI, and incubated for 96 hours. Following incubation, protein transport inhibitor (BD GolgiStop, BD Biosciences, San Jose, CA, USA) was added 12 hours prior to cell harvesting and labeled PBMC were stained with mouse anti-porcine CD4-FITC antibody (clone 74-12-4, SouthernBiotech, Birmingham, AL, USA) and mouse anti-porcine CD8-SPRD antibody (clone 76-2-11, SouthernBiotech, Birmingham, AL, USA). Cells were subsequently fixed with fixation buffer (Leucoperm reagent A, Bio-Rad Laboratories, Hercules, CA, USA) for 15 min, washed and then separately incubated with either mouse anti-porcine IFN-γ-biotin antibody (clone P2C11, BD Pharmingen, San Jose, CA, USA) or mouse anti-porcine IL-10-biotin antibody (clone 945A 1A9 26C2, Invitrogen, Carlsbad, CA, USA) in Leucoperm reagent B (Bio-rad Laboratories, Hercules,

CA, USA). Subsequently, streptavidin-PE-Cy7 (Thermo Fisher Scientific, Waltham, MA, USA) were added and incubated for 30 min at 4°C. After washing, stained cells were suspended in 2% paraformaldehyde and analyzed by flow cytometer (Beckman FC550, Beckman Coulter, Brea, CA, USA) with CXP software. The results are based on lymphocyte gating on a forward scatter versus side scatter graph after acquiring at least 20,000 cell events.

7.3.6 Enzyme-linked immunospot (ELISPOT) assay

The numbers of PRRSV-specific interferon-γ-producing cells (IFN-γ-PC) were determined using ELISPOT kit (R&D Systems, Minneapolis, MN, USA). Briefly, 2×10^5 PBMC were stimulated with either homologous or heterologous PRRSV at 0.01 MOI or PHA (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 20 hours at 37°C in 5% CO_2. Spots were counted by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). PRRSV-specific IFN-γ-PC was expressed as spot forming colonies per million of PBMCs in each well.

7.3.7 Quantification of porcine interleukin-10

Porcine interleukin-10 (IL-10) concentration was quantified in the supernatant of stimulated PBMC (2×10^6 cells/well) cultured *in vitro* for 20 hours with homologous and heterologous PRRSV (0.01 of MOI) or PHA (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) using the porcine ELISA IL-10 kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction.

7.3.8 Quantification of PRRSV RNA

The PRRSV RNA in serum was evaluated by quantitative PCR (qPCR) after PRRSV challenge. The primers specific for the ORF5 gene of either PRRSV-1 or PRRSV-2 and detection conditions were described previously (Madapong et al., 2016). In brief, total RNA was extracted using NucleoSpin RNA Virus extraction kit (Macherey-Nagel, Duren, Germany) in accordance with manufacturer's instructions. The quality of RNA was measured using spectrophotometer (Colibri, Titertek-Berthold, Pforzheim,

Germany), and converted to cDNA. All cDNA was used for quantitative PCR (qPCR). PRRSV RNA was quantified using ABI PRISM 7500 Real time PCR platform (Applied Biosystem, CA, USA). Primers specific for the ORF5 gene of either PRRSV-1 or PRRSV 2 were used. Each qPCR reaction contained 0.1 µg of cDNA, 0.2 µM of each primer, 1x Eva Green real-time-PCR master mix E4 (GeneOn GmbH, Ludwigshafen, Germany), and deionized water to yield a 20 ul final volume. The thermal profile for qPCR was 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and fluorescence acquisition at 72°C for 45 s. pGEM-T Easy Vector (Promega, WI, USA) containing an inserted ORF5 gene of each PRRSV was used to construct plasmid standards. A standard curve was generated using serial diluted plasmid standards of 10^0-10^7 copies/µl. Copy number of the PRRSV RNA was calculated using standard curve method.

7.3.9 Pathological examination and immunohistochemistry

All pigs were necropsied at 7 DPC. PRRSV-induced pneumonic lung lesions were macroscopically and microscopically evaluated as previously described (Halbur et al., 1995a). For the macroscopic lung lesion score, each lung lobe was assigned a number to reflex the approximate percentage of the volume of the entire lung and the percentage volume form each lobe added to the entire lung score (ranged from 0 to 100% of the affected lung). For the microscopic lung lesion score, lung sections were blind. Histopathological changed were examined and an estimated score of the severity of the interstitial pneumonia was given as follows: 0 = no microscopic lesions; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; and 4 = severe interstitial pneumonia. The mean values of microscopic score of each group were calculated.

Immunohistochemistry was performed using monoclonal antibodies (MAbs) A35 and JP24, which recognized PRRSV-1 and PRRSV-2 antigens, respectively (kindly provided by Dr. Erwin van den Born, the Netherlands). Tissues were processed and placed on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA). Sections were deparaffinized, rehydrated using an alcohol gradient and air-dried. All

slides were treated with proteinase K (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 30 min. Endogenous alkaline phosphatase was quenched with 0.3% hydrogen peroxide for 5 min. All slides were then incubated with BSA for 30 min. The slides were separately incubated with monoclonal antibodies overnight at 4°C in a humidified chamber. After washing, PRRSV antigen was visualized by binding with secondary antibody conjugated with horseradish peroxidase conjugated (HRP)-labeled polymer followed by immersion in peroxidase (Agilent, Santa Clara, CA, USA). Slides were counterstained with Meyer's hematoxylin, dehydrated through graded concentrations of ethanol and xylene, and then mounted. Lung tissues from pigs in the unvaccinated unchallenged group served as negative controls. To obtain quantitative data, slides were analyzed with the NIH Image J 1.50i Program (http://rsb.info.nih.gov/ij). In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2) was determined as previously described (Halbur et al., 1996a; Park et al., 2014). The mean values were calculated.

7.3.10 Statistical analysis

The data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed by ANOVA to determine the presence of significant differences between treatment groups for each day. If the *p*-value for the ANOVA was < 0.05, the differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $p < 0.05$ significance level.

7.4 Results

7.4.1 Lymphocyte proliferation response using CFSE

Upon *in vitro* stimulation with either homologous or heterologous PRRSV, all vaccination groups, regardless of vaccine genotype, had relatively low lymphocyte proliferative indices following vaccination. A significantly increased response was not observed in any vaccination group, and the responses were not different among all of the vaccination groups (**Figure 9**).

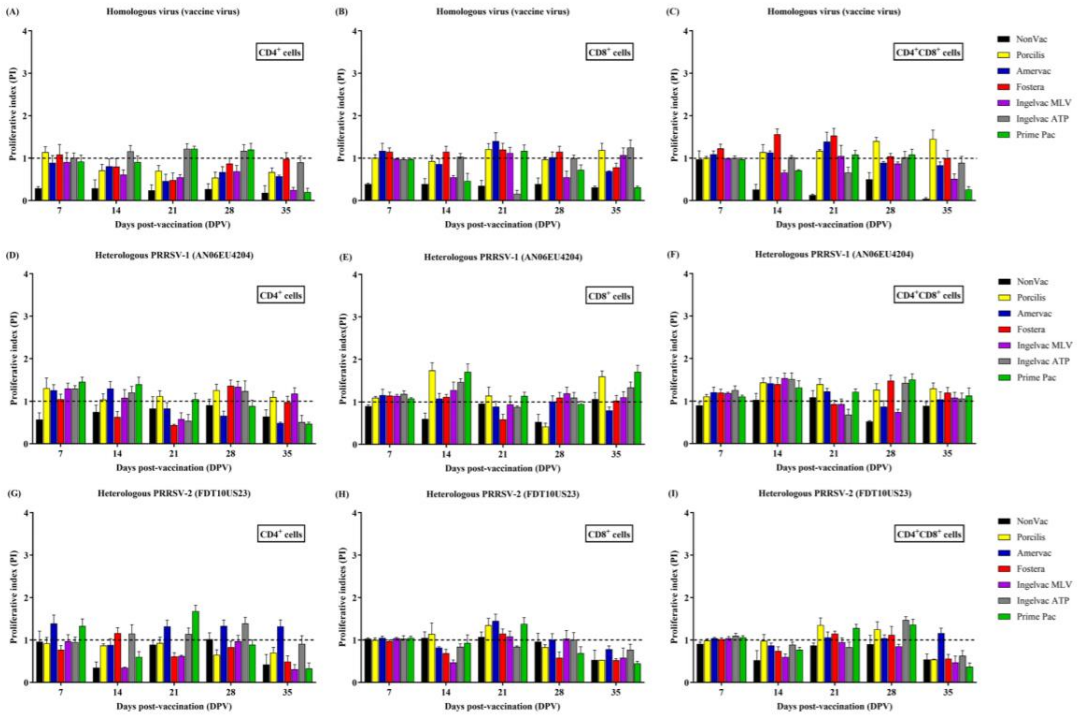


Figure 9 Lymphocyte proliferative index (PI) following vaccination. (**A-C**) homologous virus (vaccine strain), (**D-F**) heterologous PRRSV-1 (AN06EU4204), and (**G-I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations were identified by flow cytometry using CFSE and cell surfaces staining, including $CD4^+$ cells (**A, D** and **G**), $CD8^+$ cells (**B, E** and **H**), and $CD4^+CD8^+$ cells (**C, F** and **I**), respectively. Values are expressed as mean ± SEM. Dash lines indicate the cut-off level.

7.4.2 Lymphocyte populations producing IL-10

Following vaccination, lymphocyte populations producing IL-10 (L-IL-10) were detected in all vaccination groups at 7 and 14 DPV, regardless of vaccine genotype (**Figure 10**). The percentage of L-IL-10 declined to a nondetectable level from 21 to 35 DPV. L-IL-10 was mainly produced by $CD4^+$ cells. At 7 DPV, the Ingelvac MLV group had the highest amount of $CD4^+IL\text{-}10^+$ cells as compared to the PRRSV-1 or PRRSV-2 MLV vaccination and NonVac groups (**Figure 10A**). $CD4^+IL\text{-}10^+$ cells in the Amervac, Ingelvac MLV and Ingelvac ATP groups were significantly higher than those in the other vaccination groups at 14 DPV. The Porcilis, Fostera and Prime Pac groups had the lowest amount of $CD4^+IL\text{-}10^+$ cells as compared to other PRRSV-1 and PRRSV-2 MLV vaccination groups (p <0.05) at both 7 and 14 DPV.

Similar to $CD4^+IL\text{-}10^+$ cells, all vaccination groups had significantly more $CD8^+IL\text{-}10^+$ cells as compared to the NonVac group (**Figure 10B**). Although there was no difference in $CD8^+IL\text{-}10^+$ cells among vaccination groups at 7 DPV, the Amervac, Ingelvac MLV, and Ingelvac ATP groups had significantly more $CD8^+IL\text{-}10^+$ cells than did the Porcilis, Fostera and Prime Pac groups at 14 DPV. Additionally, the Amervac group had the highest amount of $CD8^+IL\text{-}10^+$ cells as compared to other vaccination groups as 14 DPV. All vaccination groups had relatively more $CD4^+CD8^+IL\text{-}10^+$ cells than did the NonVac group, and $CD4^+CD8^+IL\text{-}10^+$ cell numbers were not different between the vaccination groups (**Figure 10C**).

Similar to homologous virus stimulation, L-IL-10 was detected in all vaccination groups at 7 and 14 DPV after stimulation with PRRSV-1 (AN06EU4204) and was not detected from 21 to 35 DPV (**Figure 10D-F**). All vaccination groups had higher amounts of $CD4^+IL\text{-}10^+$ cells than did the NonVac group at 7 and 14 DPV (**Figure 10D**). The Amervac group had the highest amount of $CD4^+IL\text{-}10^+$ cells as compared to the PRRSV-1 and PRRSV-2 MLV vaccination groups at 7 DPV (p <0.05). Meanwhile, at 14 DPV, there were no differences in $CD4^+IL\text{-}10^+$ cells among all of the vaccination groups.

The Porcilis, Amervac, Fostera and Prime Pac groups had significantly more $CD8^+IL\text{-}10^+$ cells than the NonVac, Ingelvac MLV and Ingelvac ATP groups at 7 DPV (**Figure 10E**). However, at 14 DPV, all vaccination groups had significantly more

$CD8^+IL\text{-}10^+$ cells than the NonVac group. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly more $CD8^+IL\text{-}10^+$ cells than the Porcilis, Fostera and Prime Pac groups (p <0.05).

All vaccination groups had more $CD4^+CD8^+IL\text{-}10^+$ cells than the NonVac group at 7 and 14 DPV (**Figure 10F**). At 7 DPV, no significant differences were detected in the amount of $CD4^+CD8^+IL\text{-}10^+$ among all of the vaccination groups. In contrast, the Ingelvac MLV and Ingelvac ATP groups had more $CD4^+CD8^+IL\text{-}10^+$ cells than the other vaccination groups (p <0.05) at 14 DPV.

Following heterologous stimulation with PRRSV-2 (FDT10US23), L-IL-10 was detected at 7 and 14 DPV but not at 21 to 35 DPV (**Figure 10G-I**). At both 7 and 14 DPV, all vaccination groups had higher levels than the NonVac group. At 7 DPV, the Amervac and Fostera groups had significantly more $CD4^+IL\text{-}10^+$ cells than the Porcilis, Ingelvac MLV, Ingelvac ATP and Prime Pac groups (p <0.05). However, at 14 DPC, the amount of $CD4^+IL\text{-}10^+$ cells were the highest in the Ingelvac MLV and Ingelvac ATP groups as compared to the other vaccination groups (**Figure 10G**). In contrast, $CD8^+IL\text{-}10^+$ cells were only detected at 7 DPV (**Figure 10H**) in all vaccination groups, and the Porcilis group had more $CD8^+IL\text{-}10^+$ cells than the other groups. There were no differences in $CD4^+CD8^+IL\text{-}10^+$ cells among all of the vaccination groups after stimulation with PRRSV-2 (**Figure 10I**).

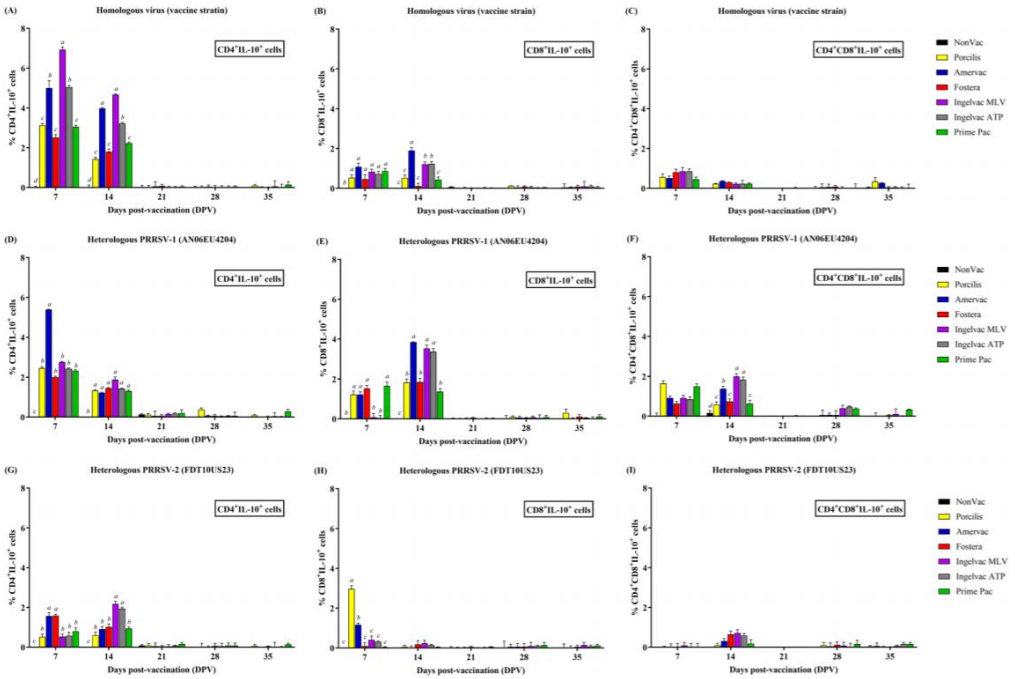


Figure 10 Lymphocyte populations producing IL-10 following vaccination. (**A-C**) homologous virus (vaccine strain), (**D-F**) heterologous PRRSV-1 (AN06EU4204), and (**G-I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations producing IL-10 were identified by flow cytometry using cell surfaces and intracellular IL-10 staining, including $CD4^+IL\text{-}10^+$ cells (**A, D** and **G**), $CD8^+IL\text{-}10^+$ cells (**B, E** and **H**), and $CD4^+CD8^+IL\text{-}10^+$ cells (**C, F** and **I**), respectively. Values are expressed as mean ± SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

7.4.3 Lymphocyte populations producing IFN-γ

Lymphocyte populations producing IFN-γ (L-IFN-γ) were detected after stimulation with either homologous or heterologous PRRSV as early as 21 DPV at levels less than 1% in all vaccination groups and showed no statistical differences between vaccination groups. Soon after detection, L-IFN-γ gradually increased until 35 DPV (**Figure 11**). The lymphocyte population response was toward both $CD4^+$ and $CD8^+$. Immediately after homologous stimulation, all vaccination groups had relatively more $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ cells than the NonVac group but showed no differences thereafter (**Figure 11A-C**). Similar to homologous stimulation, all vaccination groups had relatively more $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ cells after stimulation with heterologous PRRSV-1 (AN06EU4204) than the NonVac group but showed no difference among vaccination groups (**Figure 11D-F**). However, after heterologous PRRSV-2 (FDT10US23) stimulation, $CD8^+$IFN-γ^+ cells in all vaccination groups were detected only at 21 DPV and were significantly greater in number than in the NonVac group (**Figure 11H**).

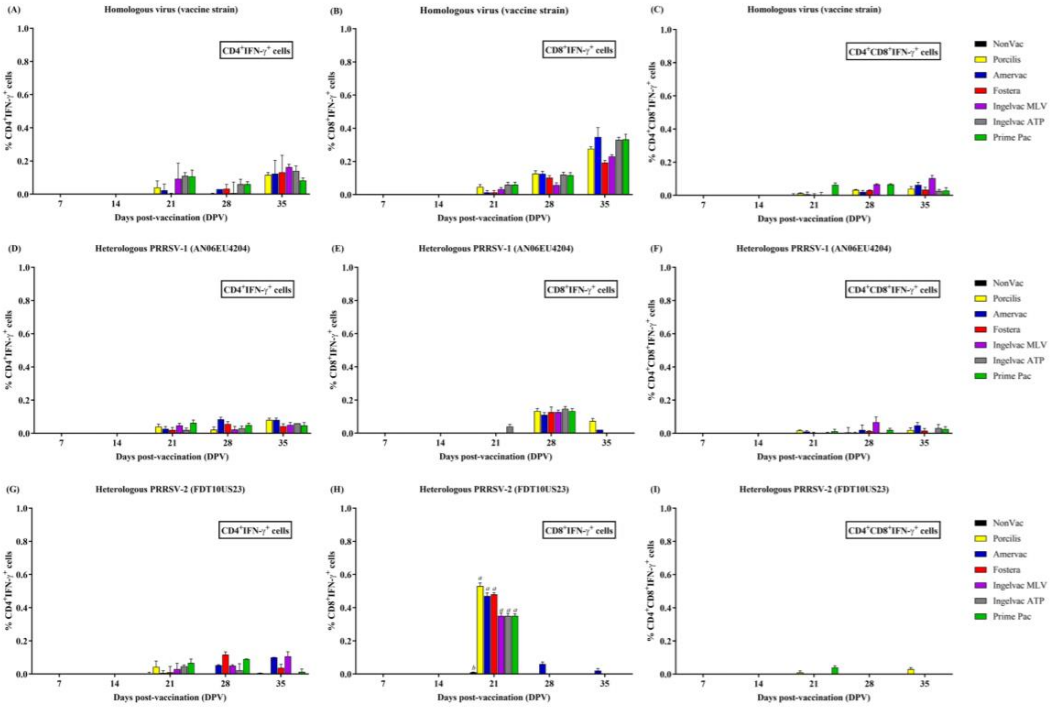


Figure 11 Lymphocyte populations producing IFN-γ following vaccination. (**A-C**) homologous virus (vaccine strain), (**D-F**) heterologous PRRSV-1 (AN06EU4204), and (**G-I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations producing IFN-γ were identified by flow cytometry using cell surfaces and intracellular IFN-γ staining, including $CD4^+IFN\text{-}\gamma^+$ cells (**A, D** and **G**), $CD8^+IFN\text{-}\gamma^+$ cells (**B, E** and **H**), and $CD4^+CD8^+IFN\text{-}\gamma^+$ cells (**C, F** and **I**), respectively. Values are expressed as mean ± SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

7.4.4 The number of PRRSV-specific IFN-γ-PC

Regardless of homologous or heterologous stimulation, IFN-γ-PC of all vaccination groups were first detected at 35 DPV (**Figure 12**). After homologous stimulation, all vaccination groups had significantly more IFN-γ-PC than the NonVac group at 35 DPV and 7 DPC. The Fostera group had significantly fewer IFN-γ-PC than the other vaccination groups at 35 DPV (**Figure 12A**). After heterologous PRRSV-1 (AN06EU4204) stimulation, all vaccination groups had significantly more IFN-γ-PC than NonVac group at 35 DPV and 7 DPC. The Fostera group had significantly fewer IFN-γ-PC than the other vaccination groups at 35 DPV (**Figure 12B**). After heterologous PRRSV-2 (FDT10US23) stimulation, IFN-γ-PC numbers were lower in all vaccination groups than after homologous stimulation with the exception of the Prime Pac group, which had significantly more IFN-γ-PC than the other groups at 35 DPV and 7 DPC. IFN-γ-PC were less abundant in Amervac the group than the other vaccination groups and were not different from those in the NonVac group at 35 DPV (**Figure 12C**).

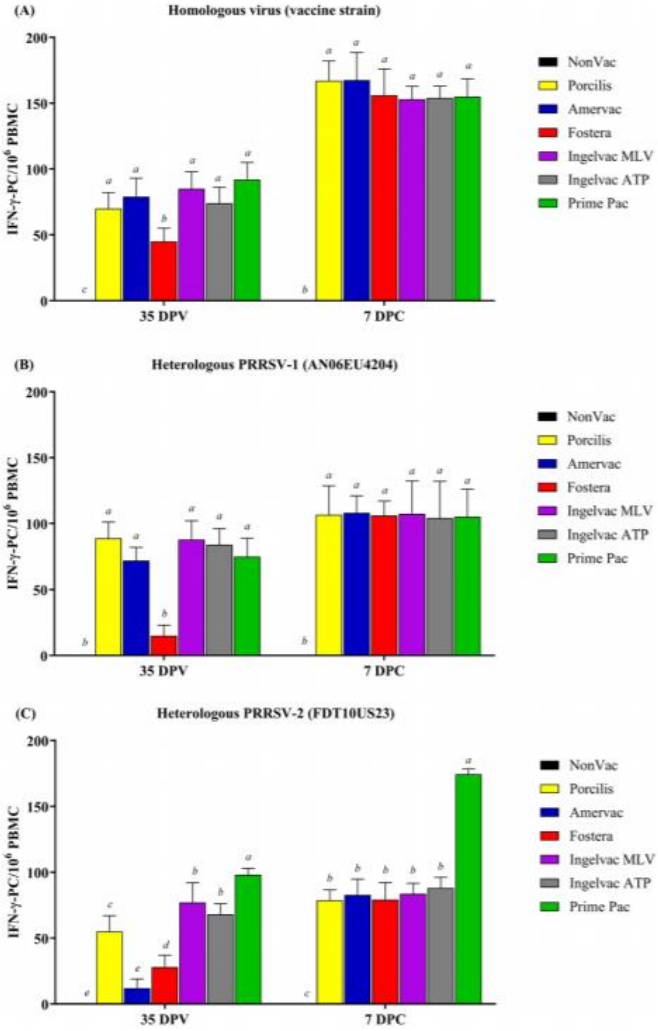


Figure 12 Evaluation of PRRSV-specific IFN-γ-PC following vaccination and at 7 days post-challenge (DPC) using in vitro stimulation. (**A**) homologous virus (vaccine strain), (**B**) heterologous PRRSV-1 (AN06EU4204), and (**C**) heterologous PRRSV-2 (FDT10US23), respectively. Values are expressed as mean ± SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

7.4.5 Porcine IL-10 production

After homologous stimulation, IL-10 levels in all vaccination groups increased and were significantly higher than that in the NonVac group at 7 DPV (**Figure 13A**). IL-10 levels of all vaccination groups peaked at 14 DPV and gradually decreased until they were similar to that of the NonVac group at 35 DPV. At 7 DPV, no differences were detected in IL-10 levels between the vaccination groups. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels at 14 DPV than the Porcilis, Fostera and Prime Pac groups. The IL-10 levels of the Ingelvac MLV and Ingelvac ATP groups remained significantly higher at 21 DPV compared to those of the other vaccination groups. No differences were detected in IL-10 among all of the vaccination groups at 28 or 35 DPV.

After heterologous PRRSV-1 (AN06EU4204) stimulation, IL-10 levels were significantly higher in all vaccination groups than that in the NonVac group (**Figure 13B**). The Amervac group had a significantly higher IL-10 level than did the other vaccination groups at 7 DPV. However, no differences were detected in IL-10 in all vaccination groups from 14 to 28 DPV, except for the Fostera group. The IL-10 level was significantly lower in the Fostera group on 28 DPV than those in the other vaccination groups.

After heterologous PRRSV-2 (FDT10US23) stimulation, all vaccination groups had significantly higher IL-10 levels than the NonVac group (**Figure 13C**). The Amervac and Fostera groups had significantly higher IL-10 levels at 7 DPV as compared to those of the other vaccination groups. The IL-10 levels in all vaccination groups, except for the Ingelvac MLV and Ingelvac ATP groups, continuously decreased from 7 to 35 DPC. The Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels at 14 and 21 DPV as compared to those of the other vaccination groups. At 28 DPV, IL-10 levels were significantly lower in the Fostera and Prime Pac groups than in the other vaccination groups but were still significantly higher than that the NonVac group. No statistical differences were observed in IL-10 levels between vaccination groups at 35 DPV.

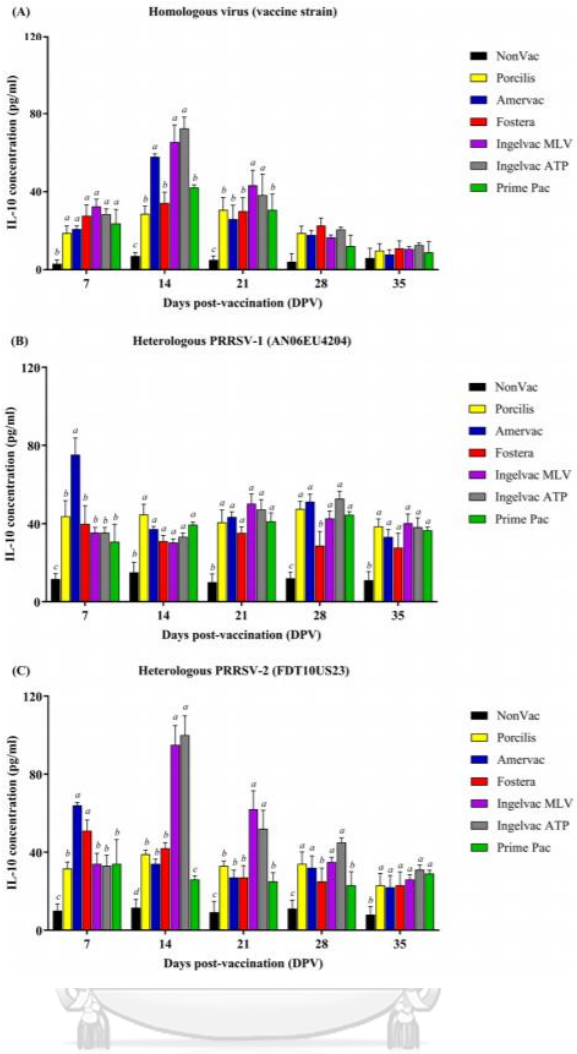


Figure 13 Quantification of porcine IL-10 in supernatant of stimulated PBMC following vaccination. (**A**) homologous virus (vaccine strain), (**B**) heterologous PRRSV-1 (AN06EU4204), and (**C**) heterologous PRRSV-2 (FDT10US23), respectively. Values are expressed as mean ± SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

7.4.6 PRRSV RNA in serum

Serum PRRSV RNA quantification after co-challenge was summarized in **Table 9**. Regardless of vaccine genotype, all vaccination groups had significantly (p <0.05) lower levels of both PRRSV-1 and PRRSV-2 RNA as compared to those of the NonVac group at 3, 5 and 7 DPC. Although, the PRRSV-2 MLV vaccination groups had significantly lower PRRSV-1 RNA levels compared to those of the PRRSV-1 MLV vaccination groups at 3 and 7 DPC, no differences were observed at 5 DPC between the PRRSV-2 MLV vaccination and the Amervac groups. At 5 DPC, serum PRRSV-1 RNA increased in all PRRSV-2 MLV vaccination groups as compared to those at 3 DPC. In contrast, PRRSV-1 RNA levels were reduced in all PRRSV-1 MLV vaccination groups at 5 and 7 DPC as compared to those at 3 DPC. The reduction in serum PRRSV RNA was not genotype-related but was associated with the isolates used in MLV. PRRSV-1 RNA was lower in the Porcilis group than in the Amervac group at 5 and 7 DPC. Meanwhile, no differences were detected in PRRSV-1 RNA levels between the PRRS-2 MLV vaccination groups at 5 DPC.

The PRRSV-2 RNA results are similar to those of PRRSV-1 RNA. All vaccination groups had significantly lower PRRSV-2 RNA as compared to that of the NonVac group, regardless of the vaccine genotype. In addition, PRRSV RNA levels were not different between vaccination groups at 3 DPC. At 5 and 7 DPC, PRRSV-2 RNA levels remained similar levels compared to those at 3 DPC in all vaccination groups except the Amervac and Prime Pac groups, which had significantly lower serum PRRSV-2 RNA at 5 and 7 DPC as compared to the other vaccination groups.

7.4.7 Pathological examination

For macroscopic lung lesion scores, the NonVac group had the highest PRRSV-induced pneumonic lung scores at 7 DPC (**Table 9**). In contrast, the lung lesion scores of all vaccination groups were significantly lower than that of the NonVac group regardless of genotype. The Porcilis group had the highest macroscopic lung lesion scores as compared to the other vaccination groups. The Prime Pac group had a significantly lower scores as compared to the other vaccination groups.

Microscopic lung lesions associated with PRRSV infection were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia. The microscopic lung lesion score results were concordant with the macroscopic lung lesion score results. All vaccination groups, except the Porcilis group, had significantly lower microscopic lung lesion scores compared to the NonVac group (**Table 9**).

7.4.8 Immunohistochemistry

Regardless of vaccine genotype, the mean number of PRRSV-positive cells was significantly (p <0.05) lower in all vaccination groups as compared to the NonVac group using either A35 or JP24 MAbs (**Table 9**). The mean number of PRRSV-positive cells stained with A35 MAb in the Porcilis group was significantly (p <0.05) higher than those in the other vaccination groups. No differences were detected in the mean number of PRRSV-positive cells between the Amervac group and the other PRRSV-2 MLV vaccination groups. In contrast, the Fostera and Prime Pac groups had significantly lower mean numbers of PRRSV-positive cells stained with JP24 MAb as compared to the Amervac, Ingelvac MLV and Ingelvac ATP groups.

Table 9 Results of PRRSV RNA in sera of non-vaccinated and vaccinated pigs following co-challenge with PRRSV-1 and PRRSV-2, lung lesion scores and immunohistochemistry at 7 days post-challenge (DPC).

		DPC*	Treatment groups						
			NonVac	Porcilis	Amervac	Fostera	Ingelvac MLV	Ingelvac ATP	Prime Pac
PRRSV RNA (1,000 copies/ml)	PRRSV-1	0	0.0±0.0[¥]	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		3	2.3±0.2^{a}	1.4±0.3^{b}	1.3±0.2^{b}	0.2±0.0^{d}	0.6±0.1^{c}	0.5±0.2^{c}	0.2±0.0^{d}
		5	2.7±0.3^{a}	0.4±0.1^{c}	0.8±0.2^{b}	0.9±0.1^{b}	0.8±0.2^{b}	0.9±0.3^{b}	1.1±0.2^{b}
		7	1.8±0.2^{a}	0.4±0.1^{c}	0.8±0.2^{b}	0.5±0.2^{c}	0.1±0.0^{d}	0.5±0.1^{c}	0.4±0.1^{c}
	PRRSV-2	0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		3	2.3±0.2^{a}	1.4±0.2^{b}	1.7±0.2^{b}	1.5±0.2^{b}	1.2±0.2^{b}	1.4±0.3^{b}	1.4±0.2^{b}
		5	2.7±0.2^{a}	1.5±0.3^{b}	0.8±0.3^{c}	1.3±0.3^{b}	1.8±0.3^{b}	1.3±0.3^{b}	0.8±0.2^{c}
		7	2.9±0.2^{a}	1.0±0.3^{b}	0.5±0.3^{c}	1.3±0.3^{b}	1.1±0.3^{b}	1.1±0.5^{b}	0.7±0.2^{c}
Macroscopic lung scores		7	72.7±8.8^{a}	59.0±4.4^{a}	45.0±5.7^{b}	55.3±5.5^{b}	54.7±1.7^{b}	54.6±6.4^{b}	42.7±4.6^{c}
Microscopic lung scores		7	1.40±0.08^{a}	1.24±0.06^{a}	0.92±0.08^{b}	0.82±0.08^{b}	0.83±0.08^{b}	0.82±0.08^{b}	0.87±0.08^{b}
PRRSV-antigen score by IHC[§]	A35	7	15.2±1.8^{a}	6.0±0.7^{b}	3.2±0.4^{c}	4.7±0.4^{c}	4.2±0.3^{c}	4.4±0.3^{c}	3.5±0.2^{c}
	JP24	7	8.2±1.4^{a}	4.9±0.4^{b}	3.9±0.5^{b}	2.3±0.3^{c}	4.0±0.4^{b}	4.1±0.5^{b}	2.6±0.2^{c}

*Days post-challenge (DPC). [§]Immunohistochemistry (IHC) using A35 and JP24, monoclonal antibodies specifically against PRRSV-1 and PRRSV-2 antigens, respectively. [¥]Values are displayed in mean ± SEM. The different lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day.

7.5 Discussion and Conclusion

The present study was conducted to investigate CMI, IL-10 levels and protective efficacy of PRRSV-1 and PRRSV-2 MLVs against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). Following PRRSV MLV vaccination, regardless of MLV genotype, the induction of CMI against PRRSV as measured by lymphocyte proliferative response and IFN-γ-PC against homologous stimulation was relatively delayed and low in magnitude. The response was observed beginning from 28-35 DPV. Additionally, the magnitude of the response was not different between vaccination groups. Although there was no difference in CMI, IL-10 was different between vaccination groups. Regardless of MLV genotype, increased IL-10 production was observed in all vaccination groups after vaccination. IL-10 levels were significantly higher in all vaccination groups at 7 DPV than in the unvaccinated control. The magnitude of the increase in IL-10 level is not genotype-related but rather is influenced by the virus isolate used to manufacture the vaccine. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels than the Porcilis, Fostera and Prime Pac groups. The Prime Pac group had the lowest IL-10 level. Following challenge, regardless of MLV genotype, all vaccinated pigs were partially protected against co-challenge with PRRSV-1 and PRRSV-2, as demonstrated by significantly reduced viremia against both genotypes, lung lesion scores and PRRSV antigens in lung tissues at 7 DPC as compared to the unvaccinated group, and the Prime Pac group demonstrated significantly greater reductions than the other vaccination groups. The results of reduced viremia and lung lesions suggest that protective efficacy against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV) is not genotype-related but rather is influenced by the virus isolate used to manufacture the vaccine. The results of the study suggest that all PRRSV MLVs are relatively similar in their protective efficacy against concurrent heterologous PRRSV-1 and PRRSV-2 (HP-PRRSV) challenge. The use of either genotype of PRRSV MLV to control PRRS in herds co-infected with both PRRSV genotypes would provide some level of protection against heterologous PRRSV infection. Other control strategies, including

strict biosecurity, to prevent external PRRSV introduction will enhance a successful PRRSV control program.

Although CMI against either PRRSV MLV or field infection has been intensively studied (Diaz et al., 2005; Ferrari et al., 2013; Kim et al., 2015; Park et al., 2014; van Woensel et al., 1998b; Zuckermann et al., 2007), no study has performed a comparative study between both MLV genotypes. The CMI results against the homologous virus in the present study demonstrated that all PRRSV MLVs induce relatively slow CMI responses as measured by lymphocyte proliferative response and the number of IFN-γ-PC, regardless of vaccine genotype. Based on the lymphocyte proliferative response, it was demonstrated that none of the PRRSV MLVs induced a detectable response until 35 DPV. The results of the CMI response analysis reported herein assessing CFSE-labeled lymphocyte proliferation are in agreement with those of previous reports showing that a PRRSV-specific CMI response appears late, approximately 4-6 weeks post-vaccination as determined by lymphocyte blastogenesis and other assays (Bassaganya-Riera et al., 2004; Ferrari et al., 2013; Martelli et al., 2009; Meier et al., 2003). In contrast to the lymphocyte proliferative response, the CMI response, as measured by the enumeration of IFN-γ-PC, demonstrated that all MLV isolates induced a delay in the detectable level of response. After *in vitro* stimulation with homologous vaccine viruses, IFN-γ-PC were detected in pigs vaccinated with either PRRSV-1 or PRRSV-2 MLVs at 35 DPV and showed significantly higher numbers in the vaccination groups than in the NonVac group, albeit the numbers were relatively low. The number of IFN-γ-PC, however, increased rapidly by 7 DPC. The results of the delayed CMI response induced by MLV are in accordance with those of previous studies in which vaccination with either PRRSV-1 or PRRSV-2 MLV elicited a relatively slow CMI response (Diaz et al., 2006; Ferrari et al., 2013; Zuckermann et al., 2007). The findings of the present study suggest that all commercial PRRSV MLVs induce a relative slow CMI response, regardless of vaccine genotype. Such responses are directed toward homologous stimulation.

It is noteworthy that the effective CMI response was directed toward the homologous response. The use of heterologous stimulation, either by PRRSV-1 or PRRSV-2, showed contrasting results to homologous stimulation. The heterologous response was somewhat unpredictable and unrelated to the genetic similarity between the vaccine and the challenge viruses. A previous study reported similar findings in that homologous stimulation upregulates IFN-γ-PC following vaccination, while heterologous virus stimulation showed varied IFN-γ-PC upregulation (Ferrari et al., 2013). Heterologous stimulation with one virus was able to upregulate IFN-γ-PC as high as homologous stimulation, while another virus was not able to do so despite high genetic similarity. In the present study, the frequencies of IFN-γ-PC in PBMC varied after stimulation with heterologous recall viruses. Stimulation with either heterologous PRRSV-1 or PRRSV-2 induced low amounts of IFN-γ-PC in the Amervac and Fostera groups (**Figure 12B, C**). In contrast, some vaccination groups, in particular the Prime Pac group, showed increased amounts of IFN-γ-PC after stimulation with heterologous PRRSV-2 (**Figure 12C**). Our results are in accordance with those of previous studies suggesting that viral recognition is also directed against antigens of genetically divergent virus isolates regardless of the vaccine strain (Ferrari et al., 2013). In addition, a cellular immune response such as IFN-γ-PC depends on the virus isolate used for *in vitro* stimulation, and different PRRSV isolates can interact differently to stimulate immune cells (Correas et al., 2017; Diaz et al., 2006).

Following vaccination, all vaccination groups had significantly higher IL-10 levels compared to the unvaccinated group (**Figure 13A-C**). The IL-10 level decreased at 14 DPV and was not different between the MLV-vaccinated and unvaccinated groups at 21 DPV. It is noteworthy that while the IL-10 levels of most of the vaccination groups displayed a gradual declining trend after 7 DPV, the Amervac, Ingelvac MLV and Ingelvac ATP groups had increased levels of IL-10 until 14 DPV before showing a decline. Our result demonstrated that the patterns of IL-10 levels following PRRSV MLV vaccination were different regardless of genotype of MLV but were rather influenced by the PRRSV isolate used to manufacture the vaccine. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10

levels than the Porcilis, Fostera and Prime Pac groups. These varying IL-10 levels among the vaccination groups may be due to the different virus isolates used in vaccine production or *in vitro* stimulation (Darwich et al., 2010; Diaz et al., 2006; Silva-Campa et al., 2010; Silva-Campa et al., 2009; Subramaniam et al., 2011). The differences in IL-10 levels among the PRRSV MLV vaccination groups are not surprising. Previous reports have demonstrated that PRRSV isolates vary in the degree of IL-10 production both *in vivo* and *in vitro* (Chung and Chae, 2003; Diaz et al., 2005). IL-10 induction by PRRSV might depend on the virus isolate used in the experiment (Darwich et al., 2010; Diaz et al., 2006; Silva-Campa et al., 2010; Silva-Campa et al., 2009; Subramaniam et al., 2011). Our findings support the conclusion that PRRSV MLVs, regardless of vaccine genotype, are able to induce IL-10 upregulation, thus resembling a natural PRRSV infection (Suradhat and Thanawongnuwech, 2003). The level of IL-10 production depends on the virus isolate used in the vaccine (Diaz et al., 2006). This finding can be used as one of several criteria to select a vaccine to use for PRRSV control. A higher level of IL-10 can potentially induce more adverse effects following vaccination with PRRSV MLVs. A previous report demonstrated that following vaccination with Ingelvac MLV and Amervac, pigs had higher lung lesion scores compared to other vaccination groups (Martinez-Lobo et al., 2013). This could be because IL-10 induction is higher in these groups than in other PRRSV MLV vaccination groups.

It is noteworthy that, regarding to the CMI response in the present study, we only investigated the dynamic change of immune cells against different PRRSV MLV vaccines using the lymphocyte proliferative assay. Our findings illustrated variations observed in the proliferative indices between PRRSV MLV vaccines. Although the CMI response as measured by the lymphocyte proliferative assay between vaccinated groups were difference, the degree of clinical protection after PRRSV infection was similar. The results suggested that CMI might not fit as immunological correlation for PRRSV protection. In agreement with our findings, previous studies found that the protection against PRRSV infection does not correlate with CMI response (Li et al., 2014a; Xiao et al., 2004). In addition, the dynamic change of immune cells seems not to correlate with other cytokines including IL-10. IL-10 is expressed by many cells of

the adaptive immune system, including Th1, Th2 and Th17 cell subsets, Treg, $CD8^+$ T cells and B cells (Maloy and Powrie, 2001; Moore et al., 2001; Roncarolo et al., 2006; Trinchieri, 2007). It is also produced by cells of innate immune system including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils and neutrophils (Moore et al., 2001). The uncorrelated results could be due to IL-10 produced from these cells. Unfortunately, the defined immune cell subpopulations involved in the different CMI response between PRRSV MLV vaccines in the present study are not fully characterize due to the limitation of cell-specific antibodies. Additional studies to measure subpopulations of immune cells secreting cytokines against PRRSV MLV vaccines are needed for further investigation.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine (Opriessnig et al., 2002). The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with field viruses (Labarque et al., 2003; van Woensel et al., 1998a). In the present study, vaccination with either PRRSV-1 or PRRSV-2 MLVs reduced the level of PRRSV viremia, lung lesions, both macroscopically and microscopically, and PRRSV antigen in the lung tissues of vaccinated pigs following co-challenge with heterologous PRRSV-1 and PRRSV-2 compared to the non-vaccinated control. Based on pneumonic lung lesions, all PRRSV MLVs provide some level of protection against co-infection with PRRSV-1 and PRRSV-2, regardless of vaccine genotype. The lung lesion scores and PRRSV antigens in lung tissues were significantly reduced in the vaccination groups compared to the unvaccinated group after challenge with heterologous viruses (Diaz et al., 2005; Kim et al., 2015; Park et al., 2014; van Woensel et al., 1998b). Our results are in agreement with those of a previous single challenge study that demonstrated partial cross-protection by PRRSV MLV (Jeong et al., 2016; Kim et al., 2015; Kristensen et al., 2018; Martelli et al., 2009; Park et al., 2015; Park et al., 2014; Roca et al., 2012). On the other hand, our cross-protection results are in contrast with those of another previous dual-challenge study in which vaccination with PRRSV-1 MLV reduced only PRRSV-1 viremia and not PRRSV-2 viremia (Choi et al., 2016). Pigs vaccinated with PRRSV-1 MLV showed no reductions in PRRSV-2 antigens in lung tissues. The

discrepancy between these findings could be due to the virus isolate used in the two studies. It is possible that the differences are attributable to our challenge strain of PRRSV-2 having higher levels of virulence. To postulate, additional studies are needed.

In conclusion, based on the overall results of the present study, all commercially available PRRSV MLVs are capable of inducing relatively low and delayed CMI response. Differences in IL-10 responses post vaccination were noted between the different vaccines. Vaccination with PRRS MLVs will reduce viremia and lung lesions after heterologous PRRSV challenge regardless of vaccine genotype.

CHAPTER 8

Immune response and protective efficacy of intramuscular and intradermal vaccination with porcine reproductive and respiratory syndrome virus 1(PRRSV-1) modified live vaccine against highly pathogenic PRRSV-2 (HP-PRRSV-2) challenge, either alone or in combination with PRRSV-1

This work has been published in the topic of

Immune response and protective efficacy of intramuscular and intradermal vaccination with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine against highly pathogenic PRRSV-2 (HP-PRRSV-2) challenge, either alone or in combination with PRRSV-1

Veterinary Microbiology

May 2020, Volume 244, 108655

(Appendix C)

Adthakorn Madapong, Kepalee Saeng-chuto, Puwich Chaikhumwang, Angkana Tantituvanont, Kriangsak Saardrak, Rafael Pedrazuela Sanz, Joel Martinez Alvarez and Dachrit Nilubol

In this chapter, we investigated and compared the immune response and protective efficacy of PRRSV MLV between intramuscular and intradermal vaccination in experimental pigs. Our findings showed alternative route of PRRSV MLV vaccination that could be improve the efficacy of PRRSV MLV against PRRSV infection.

8.1 Abstract

The study was conducted to evaluate the immune response of pigs vaccinated intramuscularly (IM) or intradermally (ID) with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine (MLV). The protective efficacy was evaluated upon challenge with highly pathogenic (HP)-PRRSV-2, either alone or in combination with PRRSV-1. Forty-two, castrated male, PRRSV-free pigs were randomly allocated into 7 groups of 6 pig each. IM/HPPRRSV2, IM/CoChallenge, ID/HPPRRSV2 and ID/CoChallenge groups were vaccinated IM or ID with PRRSV-1 MLV (UNISTRAIN® PRRS, Laboratorios Hipra S.A., Amer, Spain) in accordance to the manufacturer's directions. NV/HPPRRSV2 and NoVac/CoChallenge groups were nonvaccinated/challenged controls. NoVac/NoChallenge group was left as the control. Antibody response, IFN-γ -secreting cells (IFN-γ-SC) and IL-10 production were evaluated following vaccination. At 35 days post vaccination (DPV), all challenged groups were intranasally inoculated with HP-PRRSV-2, either alone or in combination with PRRSV-1. PRRSV viremia and lung lesion scores were evaluated following challenge. The results demonstrated that ID vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC than that of IM vaccinated pigs. Following challenge with HP-PRRSV-2 either alone or with PRRSV-1, PRRSV viremia and lung lesions, both macroscopically and microscopically, were significantly reduced in vaccinated pigs than that of nonvaccinated pigs, regardless to the route of vaccine administration. ID vaccinated pigs had significantly lower levels of PRRSV viremia and lung lesion scores than that of IM vaccinated pigs. The results of the study suggested that the administration of PRRSV-1 MLV, either IM or ID, provided partial protection against HP-PRRSV-2, either alone or when cochallenged with PRRSV-1, as demonstrated by the reduction in lung lesions and viremia. The ID route might represent an alternative to improve vaccine efficacy, as it resulted in lower IL-10 levels and higher IFN-γ-SC levels.

Keywords: Porcine reproductive and respiratory syndrome virus, Modified live virus vaccine, Intramuscular, Intradermal, Immune response, Protective efficacy, Challenge

8.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease in pigs characterized by respiratory distress in finishing pigs and reproductive disorders in breeding sows. PRRS is caused by PRRS virus (PRRSV), an enveloped, positive-sense, single-stranded RNA virus belonging to the order *Nidovirales* and family *Arteriviridae* (Cavanagh, 1997). At present, two genetically distinct PRRSV species, PRRSV-1 and PRRSV-2, have been recognized (Kuhn et al., 2016). Two PRRSV species are similar in the genome organization but their genomes are markedly different, with genetic similarities of only 60% and 56% at the nucleotide and amino acid levels, respectively (Forsberg et al., 2002). The classification into two distinct species is based on the continents where the viruses were first discovered. PRRSV-1 was first discovered in the European continent and currently has further evolved into 4 subtypes (Stadejek et al., 2008). Meanwhile, PRRSV-2 was first discovered in the North American continent and further evolved into 9 distinct lineages (Shi et al., 2010b).

PRRSV that predominantly exists in Asian countries are different from those in the European and North American continents. At present, the co-existence of both PRRSV-1 and PRRSV-2 has been increasingly reported in several Asian countries, including China, Korea, and Vietnam (Chen et al., 2011; Kim et al., 2011). In Thailand, both PRRSV-1 and PRRSV-2, have been reported (Nilubol et al., 2013). The co-existence of both species was evident at both individual pig and herd levels. Based on international systematic classification according to previously described methods (Shi et al., 2010b; Stadejek et al., 2008), PRRSV-1 in Thailand includes mainly isolates in clade A, subtype 1. PRRSV-2 includes lineages 1, 5 and 8. PRRSV-2 in the lineage 8 is mainly clustered in sublineage 8.7 in which both 8.7/HP-PRRSV-2 and 8.7/Classical North America (NA) have been reported (Shi et al., 2010b). This sublineage 8.7/HP-PRRSV-2, a highly pathogenic isolate that causing high mortality, has been a predominant virus in the region (Nilubol et al., 2013). The co-existing of both PRRSV species could generates more severe clinical diseases than does a single infection with either species in which could subsequently complicate a successful PRRSV control program in the region. The more severe clinical diseases were experimentally

demonstrated as pigs co-infected with both species had significantly higher levels of pneumonic lung lesion at 7 days post challenge (DPC) than those challenged with either PRRSV species (Choi et al., 2015).

With the availability of modified live vaccine (MLV) for both PRRSV species, it is difficult to justify which species of PRRSV MLV should be used to successfully control PRRS in the region where the coinfection does exist. Recently, a live attenuated vaccine based on PRRSV-1 (UNISTRAIN® PRRS) is commercially available in both intramuscular (IM) and intradermal (ID) administration. This vaccine, administered IM, already demonstrated partial protection against heterologous PRRSV-1 (Bonckaert et al., 2016) or PRRSV-2 (Roca et al., 2012), However, it has not been evaluated against the coinfection of PRRSV-1 and PRRSV-2. In addition, there has not been any reports on the induction of immune response, IL-10 production and protective efficacy of the intradermal administration.

IL-10 is a cytokine that functions in immunoregulation, including the downregulation of the expression of Th1 cytokines and the enhancement of B cell proliferation and antibody production (Saraiva and O'Garra, 2010). In PRRSV infection, IL-10 levels are reportedly associated with severity of clinical disease (van Reeth and Nauwynck, 2000) and can delay the immune response. Therefore, the objectives of the present study were to investigate the immune response of pigs vaccinated IM or ID with PRRSV-1 MLV (UNISTRAIN® PRRS). The protective efficacy was evaluated against the challenge with HP-PRRSV-2 (sublineage 8.7/HP-PRRSV-2), either alone or in combination with PRRSV-1 (clade A, subtype 1).

8.3 Materials and Methods

8.3.1 Experimental design

All animal procedures were conducted in accordance with the recommendations in the Guild for the Care and Use of Laboratory Animal of the National Research Council of Thailand according to protocols reviewed and approved by the Chulalongkorn University Animal Care and Use Committee (protocol number 1731047).

Forty-two, 3-week-old, castrated male pigs were procured from a PRRSV-free herd. Upon arrival, sera were collected individually and assayed for the presence of viral RNA and PRRSV specific antibody using PCR and ELISA to confirm their PRRSV negative status. Pigs were randomly allocated into the following 7 groups of 6 pigs each based on weight stratification. As shown in **Table 10**: IM/HPPRRSV2, ID/HPPRRSV2, NoVac/HPPRRSV2, IM/CoChallenge, ID/CoChallenge, NoVac/CoChallenge and NoVac/NoChallenge. The IM/HPPRRSV2 and IM/CoChallenge groups were vaccinated once via IM route with a 2 ml dose of UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain). The ID/HPPRRSV2 and ID/CoChallenge groups were vaccinated once via ID route with a 0.2 ml dose of UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain) using Hipradermic® needle-free vaccinator. The NoVac/HPPRRSV2, NoVac/CoChallenge and NoVac/NoChallenge groups were left non-vaccination.

At 35 days post vaccination (DPV), the IM/HPPRRSV2, ID/HPPRRSV2 and NoVac/HPPRRSV2 groups were intranasally challenged with 4 ml of tissue culture inoculum of HP-PRRSV-2 (FDT10US23 isolate, fifth passage of MARC-145 cells, $10^{5.2}$ $TCID_{50}$/ml), 2 ml/nostril. The IM/CoChallenge, ID/CoChallenge and NoVac/CoChallenge groups were intranasally cochallenged with 4 ml of tissue culture inoculum of PRRSV-1 and HP-PRRSV-2 isolates, at 2 ml of each isolate/nostril. The inoculum composed of 2 ml of tissue culture supernatant of each PRRSV-1 (AN06EU4204 isolate, third passage of porcine alveolar macrophages, $10^{5.4}$ $TCID_{50}$/ml) and HP-PRRSV-2 (FDT10US23 isolate, fifth passage of MARC-145 cells, $10^{5.2}$ $TCID_{50}$/ml).

The NoVac/NoChallenge group was kept as nonvaccinated/nonchallenged control. Pigs in each group were kept in separated room with separated air spaces and monitored daily for physical condition and clinical respiratory disease throughout the experiment.

Blood samples were collected at 0, 7, 14, 21, 28, 35 DPV and 3, 7, 14, and 35 days post challenge (DPC). Nasal swabs were collected at 0, 3, 7, 14 and 35 DPC using individually packaged sterile swabs which were immersed in 1 ml of RNAlater™ solution (Thermo-Fisher Scientific, MA, USA) and kept at -80°C for further processed (**Figure 14A**). Sera were separated and measured for antibody response using commercial ELISA kit and serum neutralization (SN) assay. PRRSV RNA was quantitatively assayed in sera and nasal swab samples using real-time quantitative RT-PCR (RT-qPCR). Peripheral blood mononuclear cells (PBMC) were isolated and used for *in vitro* stimulation to measure IL-10 production using ELISA kit and IFN-γ-secreting cells (IFN-γ-SC) using ELISPOT assay. Three pigs from each group were necropsied at 7 and 35 DPC. PRRSV-induced pneumonic lung lesions were scored using previously described (Halbur et al., 1995a)

Table 10 Experimental design. Seven treatment groups included 4 vaccinated- and 3 non-vaccinated groups. Routes of vaccine administration included either intramuscular (IM) or intradermal (ID). At 35 DPV, pigs in challenged groups were intranasally inoculated with HP-PRRSV-2, either alone or combination with PRRSV-1. Pigs in Nonvaccinated (NoVac)/nonchallenged (NoChallenge) group served as the control.

Treatment groups	Pigs no.	Vaccination	Vaccine	Dosage and route of administration	PRRSV challenge	
					PRRSV-1 (AN06EU4204)	HP-PRRSV-2 (FDT10US23)
IM/HPPRRSV2	6	Yes	UNISTRATAIN® PRRS (Laboratorios Hipra, S.A., Amer, Spain)	2 ml, intramuscular (IM)	No	Yes
ID/HPPRRSV2	6	Yes	UNISTRATAIN® PRRS (Laboratorios Hipra, S.A., Amer, Spain)	0.2 ml, intradermal (ID)	No	Yes
NoVac/HPPRRSV2	6	No	-	-	No	Yes
IM/CoChallenge	6	Yes	UNISTRATAIN® PRRS (Laboratorios Hipra, S.A., Amer, Spain)	2 ml, intramuscular (IM)	Yes	Yes
ID/CoChallenge	6	Yes	UNISTRATAIN® PRRS (Laboratorios Hipra, S.A., Amer, Spain)	0.2 ml, intradermal (ID)	Yes	Yes
NoVac/CoChallenge	6	No	-	-	Yes	Yes
NoVac/NoChallenge	6	No	-	-	No	No

8.3.2 PRRSV vaccine and viruses

PRRSV vaccine used was UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain), available in 2 different preparations, IM and ID vaccination. Dosage and administration routes were in accordance with the manufacturer's instructions. In brief, a 2 ml dose was used for IM vaccination (batch no. 3Z79-4). Meanwhile, a 0.2 ml dose was used for ID vaccination (batch no. 6D16). ID vaccination was performed using Hipradermic® needle-free device (Laboratorios Hipra S.A., Amer, Spain).

For *in vitro* stimulation assay, homologous and heterologous viruses were used as recall antigens. Homologous virus refers to a vaccine strain as previously described (Madapong et al., 2017). Heterologous viruses refer to the AN06EU4204 and FDT10US23 PRRSV isolates which contained Thai PRRSV-1 and PRRSV-2 (HP-PRRSV-2),

respectively. The PRRSV isolates AN06EU4204 and FDT10US23 are in clade A, subtype 1 and sublineage 8.7/HP-PRRSV-2, based on international systematic classification according to previously described methods (Shi et al., 2010b; Stadejek et al., 2008). The ORF5 genome sequences of the AN06EU4204 and FDT10US23 isolates are available in GenBank under accession numbers JQ040750 and JN255836, respectively. These two PRRSV isolates were isolated from weaned pigs from two different herds experiencing PRRS outbreaks during 2010-2011 (Nilubol et al., 2012). Both swine herds are located in the western region of Thailand. Based on the ORF5 gene, both Thai PRRSV isolates are phylogenetically clustered in endemic clades of which could represent PRRSV isolates endemically infected in swine herds in this region. In addition, the two viruses were genetically distinct from the PRRSV MLV used in the present study. The nucleotide and amino acid similarities based on the ORF5 gene between Thai PRRSV isolates and vaccine virus are summarized in **Table 11**.

Table 11 Nucleotide and amino acid similarities based on the ORF5 gene between vaccine virus and PRRSV-1 and HP-PRRSV-2 isolates used to challenge in this study. International systematic classification was based on previously described, including PRRSV-1 (Stadejek et al., 2008) and HP-PRRSV-2 (Shi et al., 2010b).

PRRSV isolates	Classification	Similarity level (%) between vaccine virus and PRRSV isolates	
		Nucleotide	Amino acid
PRRSV-1 (AN06EU4204)	Subtype 1 (Clade A)	92.7	89.1
HP-PRRSV-2 (FDT10US23)	Sublineage 8.7/HP-PRRSV-2	69.9	59.8

8.3.3 Clinical evaluation

Clinical signs and rectal temperature were monitored daily post vaccination and post challenge periods for two consecutive weeks by the same personnel at the same time. The severity of clinical respiratory disease was evaluated using a scoring system for each pig following stress induction as previously described (Halbur et al., 1995a): 0 = normal, 1 = mild dyspnea and/or tachypnea when stressed, 2 = mild

dyspnea and/or tachypnea when at rest, 3 = moderate dyspnea and/or tachypnea when stressed, 4 = moderate dyspnea and/or tachypnea when at rest, 5 = severe dyspnea and/or tachypnea when stressed, and 6 = severe dyspnea and/or tachypnea when at rest.

8.3.4 Antibody detection

Serum samples were tested for the presence of PRRSV-specific antibody by commercial ELISA kit: IDEXX PRRS X3 Ab test (IDEXX laboratories Inc., MA, USA) and serum neutralization (SN) assay. ELISA assay was performed according to the manufacturer's recommendations. Serum samples were considered positive for PRRSV antibody if the S/P ratio was greater than 0.4.

Serum neutralization (SN) assay was performed using homologous (vaccine virus), heterologous PRRSV-1 (AN06EU4204 isolates) and heterologous HP-PRRSV-2 (FDT10US23 isolate), as previously described (Nilubol et al., 2004). The presence of virus specific cytopathic effect (CPE) in each well was recorded after incubating for 7 days. The SN titers were determined as the reciprocal of the highest dilution in which no evidence of the virus growth was detected. Geometric mean titers were calculated.

8.3.5 Isolation of porcine PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of heparinized blood samples by gradient density centrifugation (Lymphosep™, Biowest, MO, USA) according to previously described protocol (Ferrari et al., 2013). The isolated PBMC were counted by inverted microscope and concentration was accessed in cRPMI-1640 (RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 µg/ml of gentamycin). The viability of PBMC were determined by Trypan blue (Sigma-Aldrich, MO, USA) staining and more than 90% viability was used for *in vitro* stimulation for IL-10 production and enzyme-linked immunospot (ELISPOT) assay as describe below.

8.3.6 Porcine interleukin-10

Following vaccination, porcine interleukin-10 (IL-10) concentration in supernatant of stimulated PBMC was quantified using porcine ELISA interleukin-10 (IL-10) commercial kit (Quantikine® ELISA porcine IL-10, R&D System, MN, USA) according to manufacturer's instructions. In brief, 2 x 10^6 PBMC in cRPMI-1640 were seeded into 96-well plates and cultured *in vitro* for 24 hours with homologous virus at 0.01 multiplicity of infection (MOI) or phytohemagglutinin (10 µg/ml, Sigma-Aldrich, MO, USA).

8.3.7 PRRSV-specific interferon-γ-secreting cells

The number of PRRSV-specific interferon-γ-secreting cells (IFN-γ-SC) were determined in PBMC using commercial ELISPOT IFN-γ kit (ELISpot porcine IFN-γ, R&D System, MN, USA), processed according to manufacturer's instructions and previously described (Park et al., 2014) with minor modification. Briefly, 2 x 10^5 PBMC in cRPMI-1640 medium were seeded into 96-well plates and stimulated with either homologous or heterologous PRRSV isolates at 0.01 MOI for 24 hours at 37°C in 5% CO_2, humidified atmosphere. The linear response was tested between 0.01 and 0.1 multiplicity of infection (MOI). Phytohemagglutinin (10 µg/ml, Sigma-Aldrich, MO, USA) and cRPMI-1640 medium was used as positive and negative control, respectively. The spots were counted by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany), and the background values were subtracted from the respective count of the stimulated cells and the immune response was expressed as number of IFN-γ-SC per 1 x 10^6 PBMC.

8.3.8 Quantification of PRRSV RNA

PRRSV RNA was extracted form serum and nasal swabs samples using NucleoSpin® Virus (Macherey-Nagel, Duren, Germany) according to manufacturer's instruction. The RNA quality was measured using a NanoDrop spectrophotometer (Colibri spectrometer, Titertek Berthold, Pforzheim, Germany). Copy number of viral RNA was then 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 USalingEU-R, 5′ AAATGIGGCTTCTCIGGITTTT 3′; forward primer USalingEU-F, 5′ TCAICTGTGCCAGITGCTGG 3′; EU-PRRSV specific probe FAM_EU (5′ *CAL 560* CCCAGCGCCAGCAACCTAGGG *BHQ1* 3′; and US-PRRSV specific probe FAM_US (5′ *FAM* TCCCGGTCCCTTGCCTCTGGA *BHQ1* 3′). RT-qPCR mixture (20 µl) was based on QuantiNova™ Probe RT-PCR kit (Qiagen®, Hilden, Germany), 1X Probe RT-PCR Master Mix, 1X QN Probe RT-Mix, 0.8 µM of each primer, 0.2µM of each probe, 1 µl of cDNA (0.5 µg), and RNase-free water up to 20 µl. The reaction was carried out in QuantStudio™ 3 Real-time PCR machine (Thermo-Fisher Scientific, Waltham, MA, USA).

8.3.9 Pathological examination

Pigs were necropsied at 7 and 35 DPC. Macro- and microscopic lung lesions were scored according to a previously described methods (Halbur et al., 1995a). For macroscopic lung lesion, the lungs were given a score to estimate the percentage of the lung affected by pneumonia. Each lobe was assigned a number to reflex the approximate percentage of the volume of the entire lung and the percentage volume from each lobe added to obtain the entire lung score (range from 0 to 100% of affected lung). Sections were collected from all lung lobes as previously described (Halbur et al., 1995a). Lung tissues were fixed with 10% neutral buffered formalin for 7 days and routinely processed and embedded in paraffin in an automated tissue processor. Sections were cut at 5 µm and stained with hematoxylin and eosin (H&E). For microscopic lung lesion analysis, the lung sections were examined in a blinded manner and given an estimated score of the severity of the interstitial pneumonia. Briefly, 0 = normal; 1 = mild interstitial pneumonia, 2 = moderate multifocal interstitial pneumonia, 3 = moderate diffuse interstitial pneumonia, and 4 = severe diffuse interstitial pneumonia. The mean values of microscopic score of each group were calculated.

8.3.10 Statistical analyses

Data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed for each day by ANOVA to determine whether there were significant differences between treatment groups for each day. If the *p*-value in the ANOVA table was < 0.05, differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $P < 0.05$ rejection level.

8.4 Results

8.4.1 Reduced clinical disease following challenge in vaccinated pigs

All vaccinated pigs displayed no clinical abnormalities following vaccination and rectal temperatures were within the normal physiological range (data not shown). Following challenge, pigs in all groups displayed the clinical respiratory disease associated with PRRSV, including fever and dyspnea. Nonvaccinated pigs displayed more severe clinical diseases than those of the vaccinated pigs.

8.4.2 IM and ID vaccination induced similar antibody response as measured by ELISA

Regardless of the route of administration, an increased PRRSV-specific antibody response was first detected at 7 DPV in all vaccinated groups, but the level was less than the cut-off level (S/P ratio < 0.4) (**Figure 14B**). Significantly increased antibody titers, above the cut-off level, were observed in all vaccinated groups from 14 to 35 DPV. The levels were significantly higher in the vaccinated groups than in the nonvaccinated groups. Following challenge, no increased antibody responses were observed in any vaccinated groups.

8.4.3 IM and ID vaccination induced similar antibody response as measured by SN assay

Regardless of the route of vaccination and PRRSV isolates of recall antigen used, the SN titers of all vaccinated groups were first detected at 21 DPV (**Figure 14C-E**) and significantly higher ($P < 0.05$) than those of the nonvaccinated groups throughout the experiment.

In homologous virus stimulation (**Figure 14C**), the SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV. Pigs in the ID/HPPRRSV2 and ID/CoChallenge groups had significantly ($P < 0.05$) higher SN titers than those in the IM/HPPRRSV2 and IM/CoChallenge groups at 21 and 28 DPV. However, the SN titers were not different between vaccinated groups at 35 DPV.

Following challenge, the SN titers of all vaccinated groups gradually increased. At 7 DPC, the IM/CoChallenge and ID/CoChallenge groups had significantly higher SN titers than the IM/HPPRRSV2 and ID/CoChallenge groups, but no difference was observed between vaccinated groups at 14 DPV. However, at 35 DPC, the SN titers of the IM/HPPRRSV2 and ID/CoChallenge groups were significantly higher than those of the ID/HPPRRSV2 and IM/CoChallenge groups.

In heterologous PRRSV-1 (AN06EU4204) stimulation (**Figure 14D**). the SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV. The IM/HPPRRSV2 group had significantly lower SN titers than the other groups at 28 and 35 DPV. Following challenge, the SN titers of all vaccinated groups remained constant from 7 to 14 DPC but slightly increased at 35 DPC. At 7 DPC, the IM/CoChallenge and ID/CoChallenge groups had significantly higher SN titers than the IM/HPPRRSV2 and IM/CoChallenge groups, but there was no difference in the SN titers among the vaccinated groups at 14 DPC. However, at 35 DPC, the IM/HPPRRSV2 and ID/CoChallenge groups had significantly higher SN titers than the ID/HPPRRSV2 and IM/CoChallenge groups.

In heterologous HP-PRRSV-2 (FDT10US23) stimulation the SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV (**Figure 14E**). Following challenge, the SN titers of all vaccinated groups were decreased from 7 to 14 DPC. Increased SN titers were increased in all vaccinated groups at 35 DPC. The SN titers of the IM/CoChallenge and ID/CoChallenge groups were significantly higher than those of the IM/HPPRRSV2 and ID/HPPRRSV2 groups at 35 DPC.

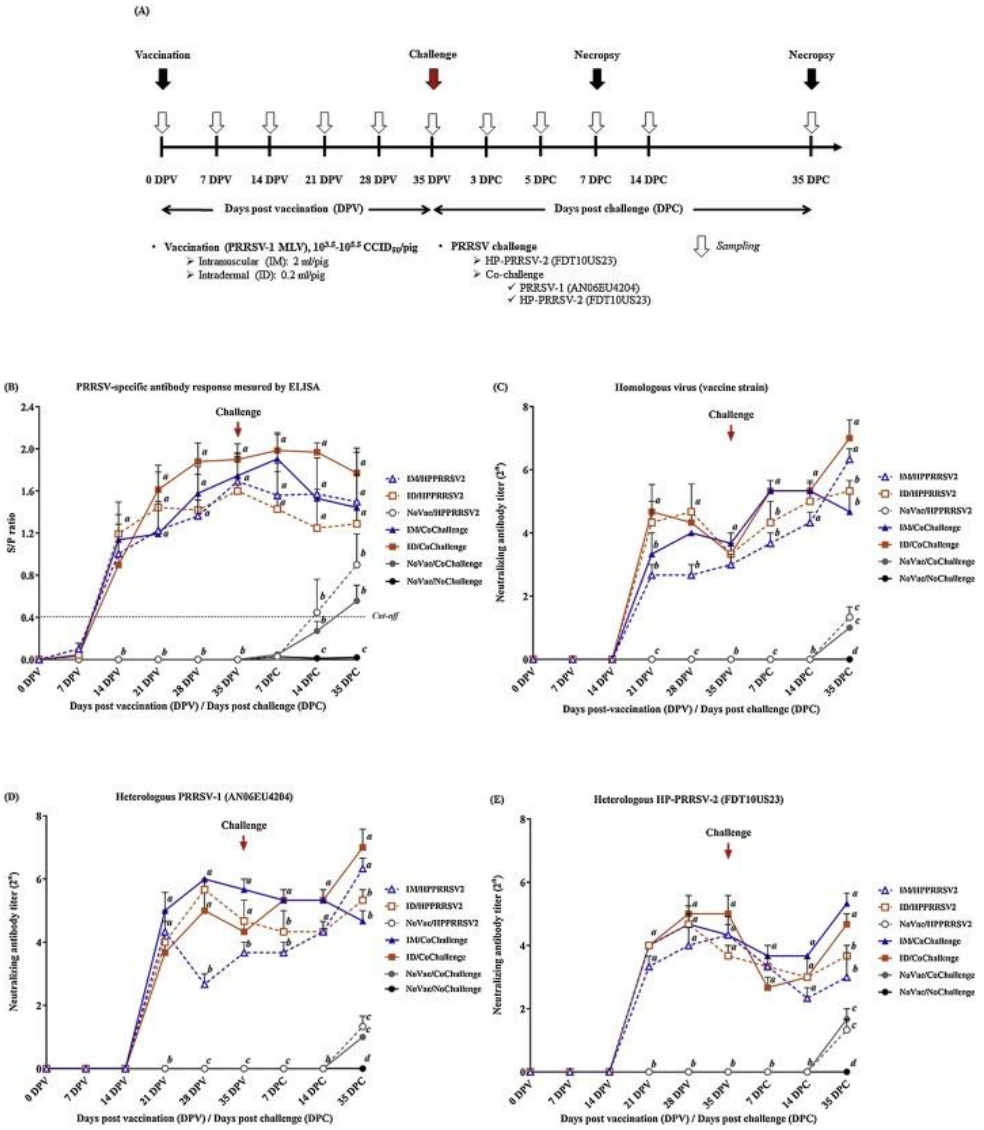


Figure 14 (**A**) Experimental design showing PRRSV vaccine administration details and sampling time points. PRRSV-1: (UNISTRAIN® PRRS); MLV: modified-live vaccine; $CCID_{50}$: 50% of the cell culture infectious dose. Level of PRRSV-specific antibodies measured by (**B**) ELISA and serum neutralization (SN) assay after stimulation with (**C**) homologous virus (vaccine strain), (**D**) heterologous PRRSV-1 (AN06EU4204) and (**E**) heterologous HP-PRRSV-2 (FDT10US23). Values are expressed as the mean ± SEM. Sample-to-positive (S/P) ratios equal to or greater than 0.4 (dashed line) are considered positive. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (a-d) indicate significant differences between treatment groups ($P < 0.05$) for each day.

8.4.4 ID vaccination induced lower IL-10 production than IM vaccination

The induction of IL-10 production was different between vaccinated groups in which delayed response was observed in ID vaccinated pigs (**Figure 15A**). IL-10 was observed at 7 DPV in IM vaccinated pigs and increased to the highest level at 21 DPV. In contrast, IL-10 was observed at 21 DPV in ID vaccinated pigs. All IM vaccinated groups had significantly higher IL-10 levels than that of ID vaccinated and control groups at 7 and 14 DPV. There was no difference in IL-10 levels between ID vaccinated and nonvaccinated groups at 7 and 14 DPV. At 7 and 14 DPV, increased IL-10 levels were observed only in the IM/HPPRRSV2 and IM/CoChallenge groups, which had significantly ($P < 0.05$) higher IL-10 levels (range from 1.78 ± 0.4 to 10.42 ± 1.2 pg/ml) than the ID/HPPRRSV2 and ID/CoChallenge groups. Then, the IL-10 levels continuously increased and reached the maximum level (range from 10.34 ± 1.7 to 11.45 ± 1.3 pg/ml) at 21 DPV without a significant difference among vaccinated groups. Subsequently, the IL-10 levels of all vaccinated groups continually declined at 28 and 35 DPV. At 28 and 35 DPV, the IL-10 of the IM/HPPRRSV2 and IM/CoChallenge groups were statistically ($P < 0.05$) higher levels (range from 4.35 ± 1.0 to 9.74 ± 1.4 pg/ml) than those of the ID/HPPRRSV2 and ID/CoChallenge groups (range from 0 to 6.23 ± 1.2 pg/ml), respectively.

8.4.5 ID vaccination induced higher IFN-γ-SC than IM vaccination

Following homologous virus stimulation, IFN-γ-SC were first detected in the ID vaccinated groups at 28 DPV and continuously increased, reaching the highest levels at 35 DPV. In contrast, IFN-γ-SC were first detected in the IM vaccinated groups at 35 DPV (**Figure 15B**). The ID vaccinated groups had significantly more IFN-γ-SC than the IM vaccinated groups at 28 and 35 DPV. Following challenge, the IFN-γ-SC in the IM/CoChallenge (155 ± 12 and 170 ± 16 cells/10^6 PMBC) and ID/CoChallenge (120 ± 12 and 155 ± 15 cells/10^6 PBMC) groups continually increased and had significantly higher frequencies than those in the IM/HPPRRSV2 and ID/HPPRRSV2 groups at 7 and 14 DPC, respectively. The IFN-γ-SC of all vaccinated groups were decreased at 35 DPC.

After stimulation with heterologous PRRSV-1 (AN6EU4204), no IFN-γ-SC were detected in any vaccinated groups following vaccination (**Figure 15C**). Following challenge, significantly more IFN-γ-SC were observed in all vaccinated groups than in nonvaccinated groups. At 7 DPC, the most IFN-γ-SC (316.67 ± 25.49 cells/10^6 PBMC) were observed in the ID/CoChallenge group ($P < 0.05$). Meanwhile, the IFN-γ-SC in the vaccinated groups were not different at 14 and 35 DPC, except for the IM/CoChallenge group, which had significantly fewer IFN-γ-SC (26.67 ± 6.15 cells/10^6 PBMC) than the other vaccinated groups.

Similar to heterologous PRRSV-1 stimulation, after stimulation with heterologous HP-PRRSV-2 (FDT10US23), no IFN-γ-SC were detected in any vaccinated groups following vaccination (**Figure 15D**). Following challenge, significantly more IFN-γ-SC were observed in all vaccinated groups than in the nonvaccinated groups. At 7 DPC, the ID/CoChallenge group had the significantly more IFN-γ-SC of 416.67 ± 34 cells/10^6 PBMC than the other vaccinated groups. At 14 DPC, significantly more IFN-γ-SC were observed in the IM/CoChallenge and ID/CoChallenge groups than in the IM/HPPRRSV2 and ID/HPPRRSV2 groups. However, there was no difference in IFN-γ-SC among the vaccinated groups at 35 DPC.

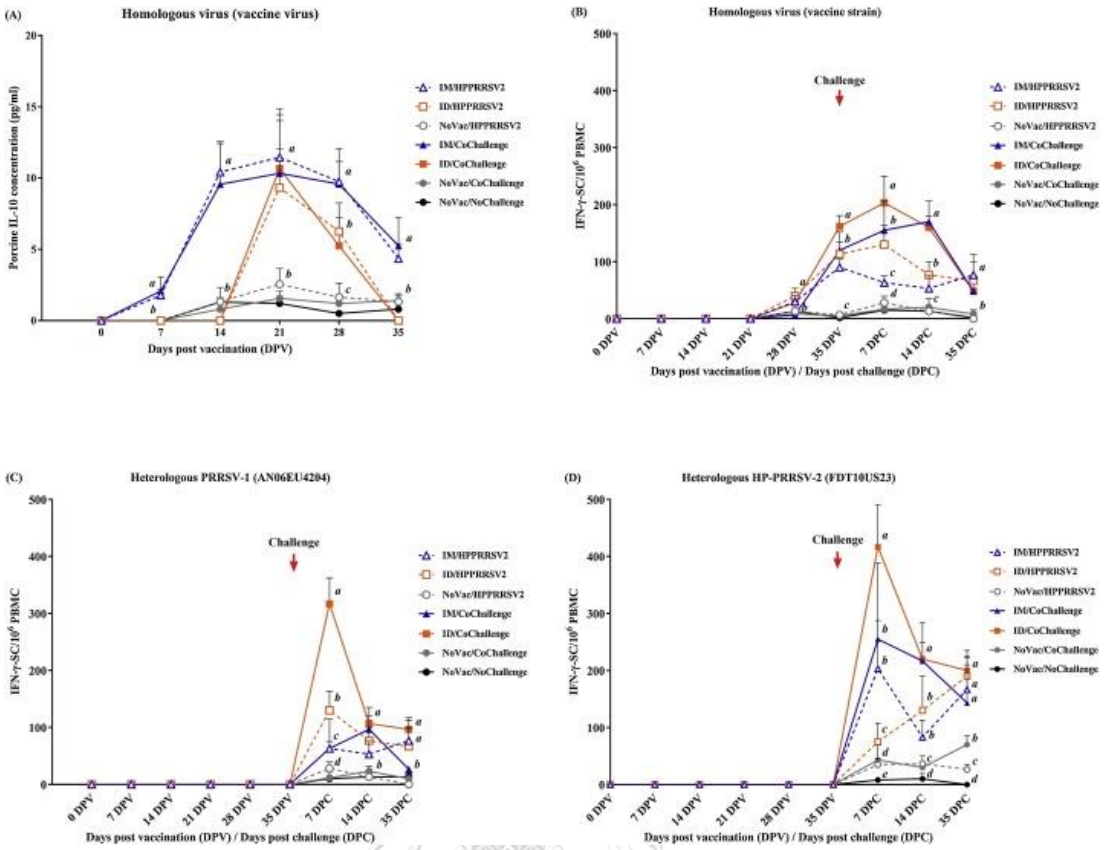


Figure 15 Analysis of in vitro stimulation. (**A**) Quantification of porcine IL-10 in the supernatant of stimulated PBMC with homologous virus (vaccine strain) following vaccination. (**B-D**) Evaluation of PRRSV-specific IFN-γ-secreting cells (SC) after stimulation with (**B**) homologous virus (vaccine virus), (**C**) heterologous PRRSV-1(AN06EU4204) and (**D**) heterologous HP-PRRSV-2 (FDT10US23). Values are expressed as the mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (a-e) indicate significant differences between treatment groups ($P < 0.05$) for each day.

8.4.6 IM and ID vaccination reduced PRRSV viremia and nasal shedding following challenge

Following PRRSV-1 MLV vaccination, there was no significant difference in the amount of PRRSV-1 RNA in the sera of the IM and ID vaccinated groups. PRRSV-1 RNA was first detected in all vaccinated groups at 14 DPV with lower levels (< 100 copies) and remained constant until 35 DPV (**Figure 16A**). Following challenge, PRRSV-1 RNA was detected in the blood of the IM/CoChallenge, ID/CoChallenge and NoVac/CoChallenge groups only and rapidly increased, reaching peaks at 7 DPC. Then, the amount of PRRSV-1 RNA was continually decreased to basal levels from 14 to 35 DPC. At 3 DPC, there was no difference in PRRSV-1 RNA among the groups. The IM/CoChallenge and ID/CoChallenge groups had significantly less ($P < 0.05$) PRRSV-1 RNA than the NoVac/CoChallenge group at 7 and 14 DPC. However, there was no difference in PRRSV-1 RNA among the groups at 35 DPC (**Figure 16A**).

Following PRRSV-1 MLV vaccination, no HP-PRRSV-2 RNA was detected in the blood of any of the groups. HP-PRRSV-2 RNA was first detected at 3 DPC and continually increased and reached peaks at 7 DPC. Then, HP-PRRSV-2 RNA continued to decrease at 14 DPC and remained at basal at 35 DPC (**Figure 16B**). The pigs in the NoVac/CoChallenge group had the highest HP-PRRSV-2 RNA levels of 1,038 ± 122 and 493 ± 112 copies/ml at 7 and 14 DPC, respectively. At 7 DPC, the IM/HPPRRSV2 and ID/HPPRRSV2 groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the other groups. At 14 DPC, all vaccinated groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the nonvaccinated/challenged groups. There was no difference in HP-PRRSV-2 RNA among the groups at 35 DPC (**Figure 16B**).

The IM/CoChallenge and ID/CoChallenge groups had significantly lower ($P < 0.05$) PRRSV-1 RNA in the nasal swabs than the NoVac/CoChallenge group from 3 to 14 DPC, but no differences were observed at 35 DPC. There were no differences in PRRSV-1 RNA between the IM/CoChallenge and ID/CoChallenge groups at 3, 7 and 14 DPC (**Figure 16C**). The genomic copies of HP-PRRSV-2 RNA in nasal swabs were relatively higher than those of PRRSV-1 RNA but had similar patterns to PRRSV-1 RNA (**Figure 16D**). The HP-PRRSV-2 RNA in nasal swabs continually increased in all groups at 3 DPC and reached the highest levels at 7 DPC. Afterward, the HP-PRRSV-2 RNA

quickly decreased at 14 DPC and remained at basal levels at 35 DPC. The ID/HPPRRSV2 group had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA of 50 ± 5 and 201.6 ± 12 copies/ml than the other groups at 3 and 7 DPC, respectively. At 14 DPC, all vaccinated groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the NoVac/HPPRRSV2 and NoVac/CoChallenge groups. There was no difference in HP-PRRSV-2 RNA in nasal swabs among the groups at 35 DPC. PRRSV RNA, both PRRSV-1 and HP-PRRSV-2, was not detected in the blood and nasal swabs from the NoVac/NoChallenge group throughout the experiment.

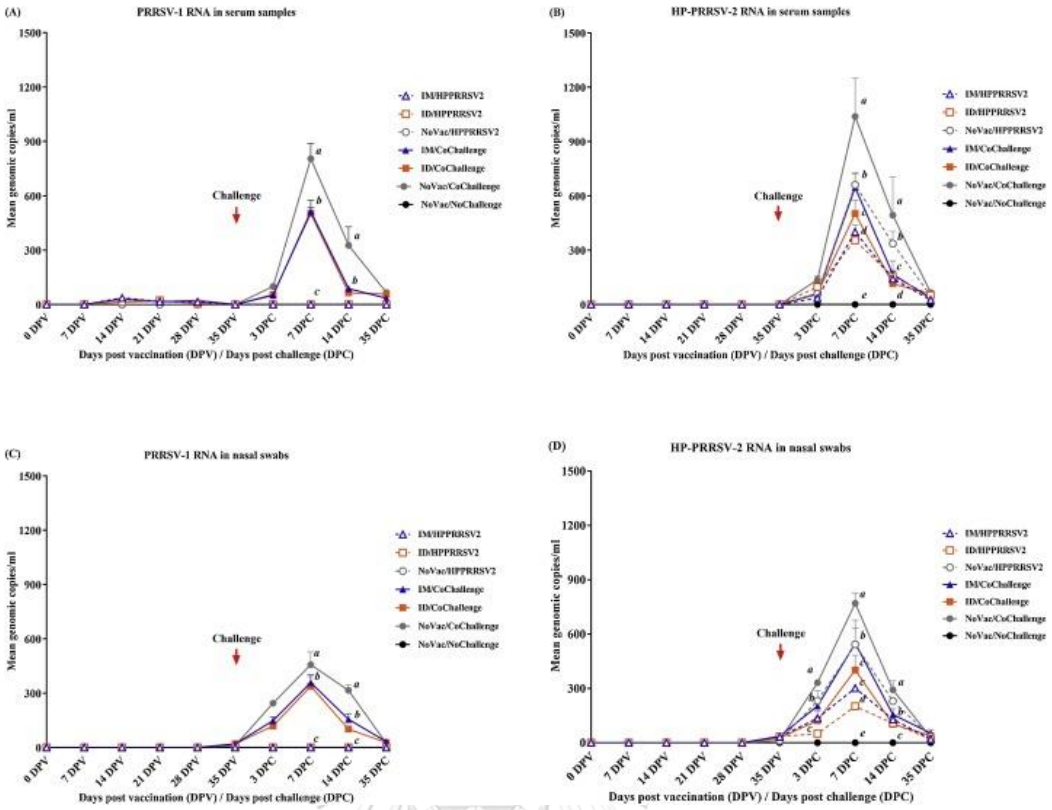


Figure 16 Mean genomic copy number of PRRSV-1 and HP-PRRSV-2 RNA in the (**A-B**) serum and (**C-D**) nasal swabs of all treatment groups. Values are expressed as the mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (a-e) indicate significant differences between treatment groups ($P < 0.05$) for each day.

8.4.7 IM and ID vaccination reduced macroscopic and microscopic lung lesions following challenge

The macroscopic lung lesions induced by PRRSV were characterized by multifocal, tan-molted areas with irregular and indistinct borders (**Figure 17**). Pigs in the NoVac/HPPRRSV2 and NoVac/CoChallenge groups had significantly higher lung scores of 71.3 ± 3.2 and 84.0 ± 3.5 than those in the vaccinated challenged groups at 7 DPC. Significantly fewer macroscopic lung lesions were observed in the ID/HPPRRSV2 and ID/CoChallenge groups than in the IM/HPPRRSV2 and IM/CoChallenge groups, respectively (**Table 12**). In addition, the ID/HPPRRSV2 group had significantly lower macroscopic lung lesion scores of 27.3 ± 2.4 than the other groups. There was no difference in macroscopic lung lesion scores among the groups at 35 DPC.

The microscopic lung lesions associated with PRRSV infection were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia (**Figure 18**). Microscopic lung lesion scores were concordant with the macroscopic lung lesion scores. All vaccinated groups, regardless to the route of administration, had significantly lower microscopic lung lesion scores than the nonvaccinated/challenged groups (**Table 12**). Pigs in the ID/HPPRRSV2 and ID/CoChallenge groups had significantly lower microscopic lung lesion scores of 1.33 ± 0.14 and 1.51 ± 0.11 than those in the IM/HPPRRSV2 and NoVac/HPPRRSV2 groups of 2.38 ± 0.11 and 2.37 ± 0.07 at 7 DPC. There were no microscopic lung lesions in the groups at 35 DPC (Supplementary Information).

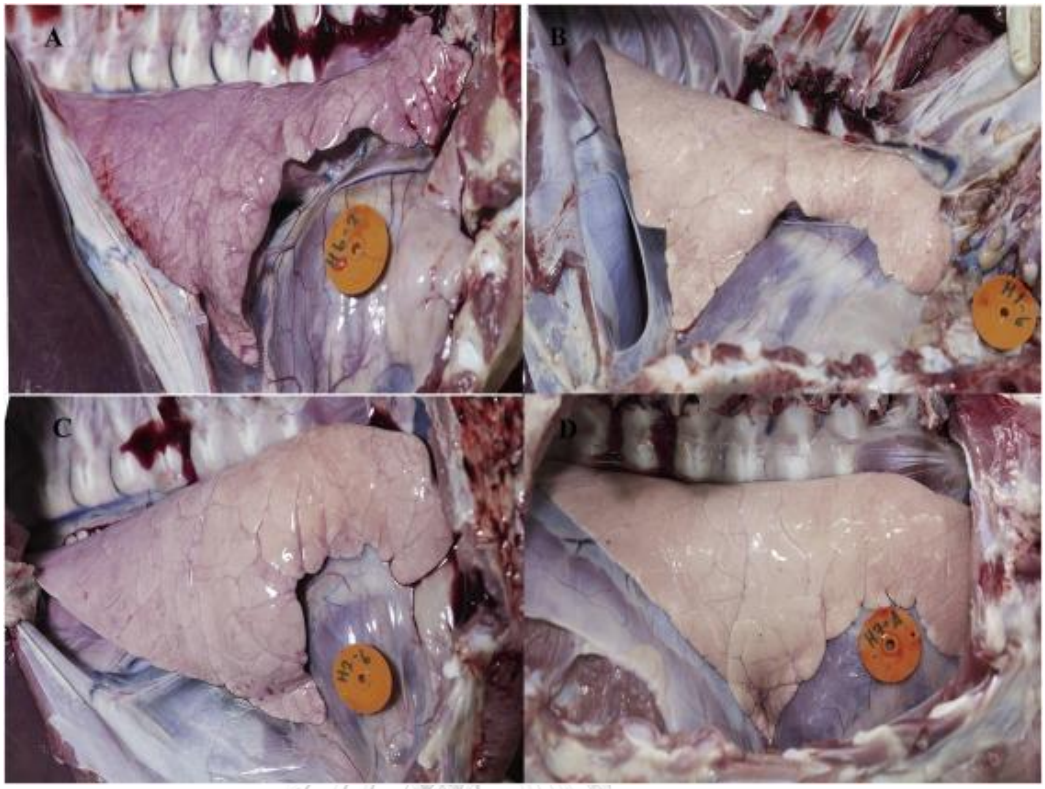


Figure 17 Macroscopic lung lesions following challenge at 7 DPC of the (**A**) nonvaccinated/challenged, (**B**) IM vaccinated pigs, (**C**) ID vaccinated pigs and (**D**) nonvaccinated/nonchallenge pigs.

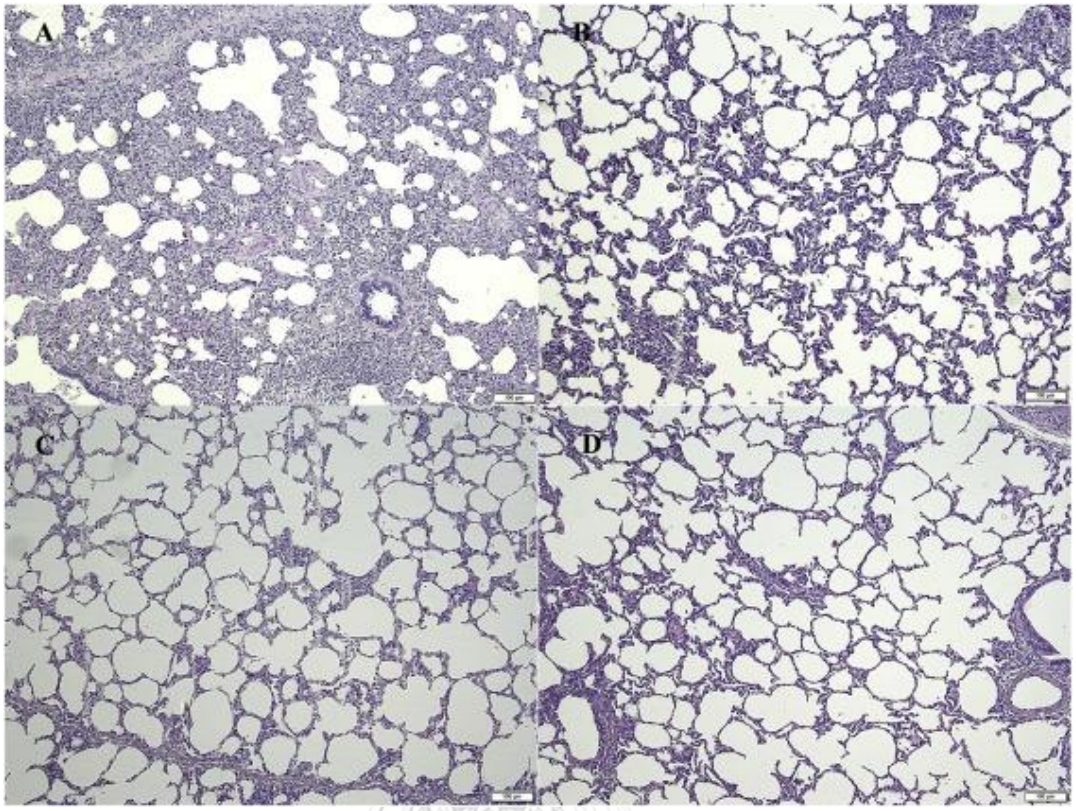


Figure 18 Microscopic lung lesions following challenge of the (**A**) nonvaccinated/challenged, (**B**) IM vaccinated pigs, (**C**) ID vaccinated pigs and (**D**) nonvaccinated/nonchallenge pigs. H&E staining. Bar =100 µm.

Table 12 Macro- and microscopic lung lesion scores following challenge. Values expressed as mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (a-e) indicate significant differences between treatment groups ($P < 0.05$) for each day post-challenge (DPC).

Treatment groups	Macroscopic scores		Microscopic scores	
	7 DPC	35 DPC	7 DPC	35 DPC
IM/HPPRRSV2	58.0 ± 2.0^{C}	0 ± 0	1.62 ± 0.11^{b}	0 ± 0
ID/HPPRRSV2	27.3 ± 2.4^{e}	0 ± 0	1.33 ± 0.14^{c}	0 ± 0
NoVac/HPPRRSV2	71.3 ± 3.2^{b}	2.0 ± 1.0	2.38 ± 0.11^{a}	0 ± 0
IM/CoChallenge	62.3 ± 2.4^{c}	2.0 ± 0.3	1.88 ± 0.06^{b}	0 ± 0
ID/CoChallenge	41.0 ± 7.0^{d}	1.0 ± 0.3	1.51 ± 0.10^{c}	0 ± 0
NoVac/CoChallenge	84.0 ± 3.5^{a}	2.0 ± 1.0	2.37 ± 0.07^{a}	0 ± 0
NoVac/NoChallenge	0 ± 0^{e}	0 ± 0	0 ± 0^{d}	0 ± 0

8.5 Discussion and Conclusion

The present study was conducted to investigate the immune response and IL-10 production of pigs vaccinated IM or ID with PRRSV-1 MLV. The protective efficacy was evaluated upon challenge with HP-PRRSV-2 either alone or in combination with PRRSV-1. It was demonstrated that pigs vaccinated, either IM or ID, induce a similar patterns of antibody response as measured by ELISA and SN assays. The discrepancy is observed in cell mediated immune (CMI) response in which ID vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC levels than that of IM-vaccinated pigs. Following challenge with HP-PRRSV-2, either alone or cochallenge with PRRSV-1, PRRSV viremia and lung lesions, both macroscopically and microscopically, were significantly reduced in vaccinated pigs than that of nonvaccinated pigs, regardless to the route of vaccine administration. It is notably that ID vaccinated pigs had significantly lower levels of viremia and lung lesion scores than that of IM vaccinated pigs.

Recently, new routes of vaccine administration including an ID administration through needle-free devices have intensively been studies to improve the efficacy of vaccines. Needle-free device have been used as advantageous methods to cross the epidermal barrier and efficiently deliver antigens into the dermal layer (Giudice and Campbell, 2006), requiring a smaller volume of fluid than the more conventional IM route (Giudice and Campbell, 2006). The most important advantages of the ID administration by a needle-less device are that it is less invasive, painless, safe, quick and easy. Furthermore, the ID administration could induce a stronger CMI response compared to that of the IM administration. The superior efficacy of the ID vaccination, regarding to the induction of the immune response, was demonstrated in a previous report in which the ID vaccination delivered by a needle-free device can prime a stronger specific immune response, both humoral and CMI, against Aujeszky's disease compared to that of induced by the IM vaccination (Ferrari et al., 2011).

Regarding to CMI response, delivery through the intradermal route could induce T cell polarization through the Th1 pathway, favoring the induction of IFN-γ.

This phenomenon was evident in the present study. ID vaccinated pigs had a significantly higher level of PRRSV-specific IFN-γ-SC than that of IM vaccinated pigs. The observed results are in accordance with previous reports in which ID vaccinated pigs induce relatively more IFN-γ-SC than IM-vaccinated pigs (Ferrari et al., 2013; Martelli et al., 2009). One factor likely contributing to this finding is the presence of skin-resident immune cells able to sufficiently capture antigens directly from the skin. The skin is rich in professional antigen presenting cells (APC), including epidermal Langerhans cells (LC) and dermal dendritic cells, which are known to migrate to draining lymph nodes and trigger immune responses (Combadiere and Liard, 2011).

Another possibility of higher IFN-γ-SC in ID vaccinated pigs than IM vaccinated pigs could be due to the lower IL-10 levels. Our results demonstrated that the IL-10 production was delayed, and the level was significantly lower in ID vaccinated pigs than in IM vaccinated pigs in the early phase following vaccination. The results are in agreement with a previous report in which vaccinated pigs, both IM and ID, can induce IL-10 production, but ID vaccinated pigs induced relatively lower IL-10 levels compared to that of IM vaccinated pigs (Ferrari et al., 2013). However, the delivery of antigen through the intradermal route could target dendritic cells. IL-10 is a cytokine of the Th2 response. The delivery through this route could induce T cell polarization through the Th1 pathway, favoring other cytokines that act against Th2 (Tesfaye et al., 2019). However, the mechanisms of IL-10 induction following vaccination by the IM and ID routes are not understood.

IL-10 is a cytokine with multiple effects on immunoregulation and inflammation. It functions in T cell polarization by downregulating the expression of Th1 cytokines, including IL-12 and IFN-γ, MHC class II antigens, and costimulatory molecules on macrophages. IL-10 also enhances B cell survival, proliferation, and antibody production. Additionally, IL-10 has a central role in limiting pathogen-induced immunopathology and is associated with the induction of tolerance and regulatory T lymphocytes (Treg) (LeRoith et al., 2011). The exploitation of IL-10 appear to be a common mechanism of immunosuppression by intracellular pathogens that specifically target macrophages for infection. Considering the

restricted tissue tropism of PRRSV, it is conceivable that PRRSV used IL-10 to suppressing the host immune response. In PRRSV infection, either by natural infection of MLV vaccination, increased IL-10 production was observed in both *in vitro* and *in vivo* (Flores-Mendoza et al., 2008). These undesired outcomes potentially resulted in the slow induction of effective immunity, the failure of other vaccines and increased susceptibility to secondary infection by the other pathogens, causing porcine respiratory disease complex. In PRRSV infection, IL-10 is also related to the severity of clinical diseases (van Reeth and Nauwynck, 2000).

With respect to the humoral immune response, the results of the present study demonstrated that the induction of the humoral immune response against PRRSV was not different between IM- and ID-vaccinated pigs. The results demonstrated no difference in the induction of the humoral immune response as measured by ELISA between IM- and ID-vaccinated pigs and are in agreement with previous studies (Ferrari et al., 2013).

The results of the study demonstrated that pigs vaccinated ID or IM with PRRSV-1 MLV (UNISTRAIN® PRRS) conferred partial heterologous protection against HP-PRRSV-2, either alone or in combination with PRRSV-1. The findings reported herein are in agreement with previous reports (Bonckaert et al., 2016; Roca et al., 2012). Based on a single challenge with either virulent PRRSV-1 (Lena) (Bonckaert et al., 2016) or HP-PRRSV-2 (Roca et al., 2012), PRRSV-1 MLV (UNISTRAIN® PRRS), administered through the IM route, can confer a partial protection as evidenced by reduced viremia. The mechanism of partial cross protection of the PRRSV-1 MLV against heterologous PRRSV-2 is not known but might be due to the induction of cross neutralizing reactivity against heterologous HP-PRRSV-2. This was observed by increased SN titers against HP-PRRSV-2 in the vaccinated pigs in the present study. In addition, different type of PRRSV-1 MLV could potentially have various activities against PRRSV-2. A previous report comparing the efficacy of 2 different PRRSV-1 MLV vaccines demonstrated that one PRRSV-1 MLV had low protection against HP-PRRSV-2 (Madapong et al., 2020). However, further investigations are needed to be performed.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine (Opriessnig et al., 2002). The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with virulent viruses (Labarque et al., 2003). PRRSV viremia plays a central role to its pathogenesis. High PRRSV in serum associated with the development of interstitial pneumonic lung lesions (Han et al., 2013). Therefore, vaccine mediated reduction of PRRSV viremia is critical for controlling the infection pigs. Our results are in accordance with previous studies on the efficacy of PRRSV MLV vaccination showing that all vaccine species provide partial protection against challenge with heterologous PRRSV strains with a wide range of protection (Bonckaert et al., 2016; Ferrari et al., 2013; Martelli et al., 2009; Roca et al., 2012). Notably, ID vaccinated pigs had significantly lower macroscopic and microscopic lung lesion scores than IM-vaccinated pigs. This finding could be because the ID route induce a strong cell-mediated immune response as evidenced by the number of IFN-γ-SC.

In conclusion, the results of the study suggested that PRRSV-1 MLV administered by either IM or ID can provide partial heterologous protection against challenge with HP-PRRSV-2, either alone or in conjunction with PRRSV-1, as demonstrated by reduced lung lesions and viremia. The ID route might represent an alternative to improve vaccine efficacy, as it induced lower IL-10 levels and more IFN-γ-SC.

CHAPTER 9
GENERAL CONCLUSION

Based on the phylogenetic analysis of Thai PRRSV isolates in 2001-2017 demonstrated that Thai PRRSV isolates, both PRRSV-1 and PRRSV-2, develop their own clusters. The subtype 1, clad A was a dominant strain of Thai PRRSV-1. Meanwhile, the sublineage 8.7/HP-PRRSV-2 was a dominant strain of Thai PRRSV-2. In Thailand, a retrospective serological study found that PRRSV had been circulating in Thai swine herds as early as 1989 and both PRRSV species showed co-circulate in Thai swine herds since 2001 (Thanawongnuwech et al., 2004). According to previous study, Thai PRRSV-1 isolates in clade A were closely related to each other and had more highly homologous to the Lelystad virus and PRRSV-1 MLV-like virus (Porcilis® PRRS) with nucleotide and amino acid sequence similarities of 97.8-98.5% and 96.5-99.0%, respectively (Nilubol et al., 2013; Stadejek et al., 2008). The Thai PRRSV-1 isolates in clade D were closely related to PRRSV-1 MLV-like virus (Amervac® PRRS) that were first detected in 2008 even though this vaccine has been available in 2004. The Thai PRRSV-1 isolates in clade H were closely related to Spanish PRRSV-like and Belgium PRRSV-like, which were detected in 2010-2013.

The Thai PRRSV-2 isolates are grouped into 3 lineages: 1, 5 and 8. The Thai PRRSV-2 isolates in lineage 1 were closely related to the Canadian isolates that might be introduced into Thai swine herds around 1990s (Tun et al., 2011). The Thai PRRSV-2 isolates in lineage 5 were closely related to PRRSV-2 MLV-like virus (Ingelvac® PRRS MLV). Meanwhile, the Thai PRRSV-2 isolates in the lineage 8 are divided into 2 huge groups: Classical and HP-PRRSV-2 (Shi et al., 2010b). The HP-PRRSV-2, in particular JXA-1 like viruses, was emerged in China in 2006 and subsequently spread to neighboring countries including Vietnam, Cambodia and Laos (Tian et al., 2007). Then, the HP-PRRSV-2 was detected in swine herds in Thailand, Myanmar, Philippines and Singapore, and caused an outbreak in these countries (An et al., 2011; Feng et al., 2008; Nilubol et al., 2012). In Thailand, the first epidemic outbreak of HP-PRRSV-2 initiated in August 2010 and may have been introduced through the illegal transport of infected materials from bordering countries, especially form Vietnam to Thailand

thorough Laos (Nilubol et al., 2012). Our results demonstrate that HP-PRRSV-2 are circulated and endemic in those regions of Thailand since its emergence. Almost all HP-PRRSV-2 isolates were in sublineage 8.7/HP-PRRSV-2 and closely related to the JXA1-like and 09HEN1-like viruses that are predominantly circulated in Southeast Asia (Nilubol et al., 2012; Shi et al., 2010b). In conclusion, both PRRSV species have evolved continuously and developed clusters that are genetically separated form that of the other countries. The introduction of new isolates could be diverse the genetic variation of PRRSV, especially for the PRRSV-2. However, the mechanisms of genetic diversity and evolution analyses of both PRRSV species in Thailand are under investigation.

The present study demonstrates differences in pathogenicity following infection with single Thai PRRSV-1 and PRRSV-2 (HP-PRRSV-2) isolates or in co-challenge with both PRRSV species in experimental pigs. The infection of Thai PRRSV-1, AN06EU4204, induce relatively lower respiratory clinical disease, viremia and lung lesions than that of the FDT10US23 isolate inoculation. In addition, the co-infection of both Thai PRRSV specie, AN06EU4204 and FDT10US23, induce more severity in terms of respiratory clinical sings, viral load in blood, increased of the lung lesion scores and viral antigen in tissues, than that of the infection with either PRRSV-1 or PRRSV-2 alone. The more virulent PRRSV isolate replicates faster and able to induce more severe interstitial pneumonia than less virulent isolate regardless of its species (Halbur et al., 1995a; Halbur et al., 1996b). Therefore, microscopic pulmonary lesion scores and virus distribution in the lungs are the most important criteria for determining the virulence of PRRSV isolate. In the present study showed that pigs infected with Thai field HP-PRRSV-2, FDT10US23 isolate, had more higher lung lesions and PRRSV-antigens than did the pigs infected with PRRSV-1, AN06EU4204. These results suggest that Thai PRRSV isolates have different virulence based on the macro- and microscopic lung lesion scores and PRRSV-antigen in lung tissues, and the FDT10US23 isolate may be more virulent than the AN06EU4204 isolate.

Since the co-existence of both PRRSV species is endemic in several swine producing regions including Thailand. The results of the present study provide the pathogenicity of either single PRRSV infection or concurrent infection of both PRRSV

species. The co-infection caused more severe clinical sings, increased viremia, and induction of lung lesions rather by single infection with either PRRSV-1 or PRRSV-2 (HP-PRRS) alone. The difference of these pathogenicity with different PRRSV isolates could help to explain the variability observed in the field outbreaks of PRRS.

In chapter 6, we compared the efficacy of six different PRRSV MLV in the induction of antibody responses in PRRSV-free pigs. All six PRRSV MLV rapidly induced antibody responses as measured by ELISA. The antibody responses were detected as early as 7-14 DPV in all PRRSV MLV-specie-dependent manner. The antibody levels in all vaccinated groups were similar at 21 DPV. In summary, there was no difference in the antibody responses as measured by ELISA for any of the PRRSV MLV. The results of the present study suggest that the specie of PRRSV MLV is not the key factor in the induction of immunity, but the specific virus isolate used for the vaccines might play an important role. Moreover, it is notable that the antibody detection in the present study was performed using IDEXX ELISA, which can simultaneously detect specific antibodies against PRRSV-1 and PRRSV-2 infections. However, these findings may not be applicable when using other diagnostic kits.

Our finding of differences in SN responses was not surprising. PRRSV isolates differed in their susceptibility to neutralization (Martinez-Lobo et al., 2011), and the mechanisms associated with this susceptibility remain poorly characterized, although the influence of N-linked glycosylation in decoy epitope regions could be one key factor (Nilubol et al., 2013, 2014; Plagemann et al., 2002). A previous study demonstrated that a heterologous response could be higher or lower (Ferrari et al., 2013), depending on the isolates that were used in the assay.

In addition to their ability to induce an immune response, the shedding patterns of the vaccine viruses were investigated using three different measurements, including the duration of viremia, the detection of viral RNA in tonsils, and infection of sentinel pigs. After vaccination, we detected a difference in the shedding patterns between PRRSV MLV. These findings suggested that the viremic phase of PRRSV MLV vaccination was associated with the virus isolate used in the vaccine, not the specie of PRRSV. In conclusion, based on the induction of humoral immune responses, all PRRSV MLV yield a similar response pattern. Measurement of antibody response by

ELISA is quick, but the response measured using SN assay is delayed and isolate specific. However, the shedding pattern of a vaccine virus is influenced by the isolate that is used to manufacture the vaccine. The criteria for PRRSV MLV selection should be based on the shorter duration of vaccine virus shedding and the broader response against heterologous virus.

In chapter 7, we conducted to investigate CMI, IL-10 levels and protective efficacy of PRRSV-1 and PRRSV-2 MLV against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). Following PRRSV MLV vaccination, regardless of MLV specie, the induction of CMI against PRRSV as measured by lymphocyte proliferative response and IFN-γ-PC against homologous stimulation was relatively delayed and low in magnitude. Additionally, the magnitude of the response was not different between vaccination groups. Although there was no difference in CMI, IL-10 was different between vaccination groups. Regardless of PRRSV MLV specie, increased IL-10 production was observed in all vaccination groups after vaccination. The magnitude of the increase in IL-10 level is not specie-related but rather is influenced by the virus isolate used to manufacture the vaccine. The results of reduced viremia and lung lesions suggest that protective efficacy against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV) is not specie-related but rather is influenced by the virus isolate used to manufacture the vaccine. we suggest that all PRRSV MLV are relatively similar in their protective efficacy against concurrent heterologous PRRSV-1 and PRRSV-2 (HP-PRRSV) challenge. The use of either PRRSV-1 or PRRSV-2 MLV to control PRRS in herds co-infected with both PRRSV species would provide some level of protection against heterologous PRRSV infection. Other control strategies will enhance a successful PRRSV control program.

Although CMI against either PRRSV MLV or field infection has been intensively studied (Diaz et al., 2005; Ferrari et al., 2013; Kim et al., 2015; Park et al., 2014; van Woensel et al., 1998b; Zuckermann et al., 2007), no study has performed a comparative study between them. Our findings of the present study suggest that all commercial PRRSV MLV induce a relative slow CMI response, regardless of vaccine specie. Such responses are directed toward homologous stimulation. Our results

agree with those of previous studies suggesting that viral recognition is also directed against antigens of genetically divergent virus isolates regardless of the vaccine specie (Ferrari et al., 2013). In addition, a cellular immune response such as IFN-γ-PC depends on the virus isolate used for *in vitro* stimulation, and different PRRSV isolates can interact differently to stimulate immune cells (Correas et al., 2017; Diaz et al., 2006). Here, we demonstrated that the patterns of IL-10 levels following PRRSV MLV vaccination were different regardless of PRRSV MLV specie but were rather influenced by the PRRSV isolate used to manufacture the vaccine (Diaz et al., 2006). These varying IL-10 levels may be due to the different virus isolates used in vaccine production or *in vitro* stimulation (Darwich et al., 2010; Diaz et al., 2006; Silva-Campa et al., 2010; Silva-Campa et al., 2009; Subramaniam et al., 2011) and support the conclusion that all PRRSV MLV can induce IL-10 upregulation, thus resembling a natural PRRSV infection (Suradhat and Thanawongnuwech, 2003). Our findings can be used as one of several criteria to select a vaccine to use for PRRSV control. A higher level of IL-10 can potentially induce more adverse effects following vaccination with PRRSV MLV. It is noteworthy that, regarding to the CMI response in the present study, we only investigated the dynamic change of immune cells against different PRRSV MLV vaccines using the lymphocyte proliferative assay. Our findings illustrated variations observed in the proliferative indices between PRRSV MLV vaccines. Although the CMI response as measured by the lymphocyte proliferative assay between vaccinated groups were difference, the degree of clinical protection after PRRSV infection was similar. The results suggested that CMI might not fit as immunological correlation for PRRSV protection. In agreement with our findings, previous studies found that the protection against PRRSV infection does not correlate with CMI response (Li et al., 2014a; Xiao et al., 2004). Unfortunately, the defined immune cell subpopulations involved in the different CMI response between PRRSV MLV vaccines in the present study are not fully characterize due to the limitation of cell-specific antibodies. Additional studies to measure subpopulations of immune cells secreting cytokines against PRRSV MLV vaccines are needed for further investigation.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine (Opriessnig et al., 2002). The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with field viruses (Labarque et al., 2003; van Woensel et al., 1998a). Our results are in agreement with those of a previous single challenge study that demonstrated partial cross-protection by PRRSV MLV (Jeong et al., 2016; Kim et al., 2015; Kristensen et al., 2018; Martelli et al., 2009; Park et al., 2015; Park et al., 2014; Roca et al., 2012). In conclusion, all commercially available PRRSV MLV are capable of inducing relatively low and delayed CMI response. Differences in IL-10 responses post vaccination were noted between the different vaccines. Vaccination with PRRS MLV will reduce viremia and lung lesions after heterologous PRRSV challenge regardless of vaccine specie.

In chapter 8, we conducted to investigate the immune response and IL-10 production of pigs vaccinated IM or ID with PRRSV-1 MLV. The protective efficacy was evaluated upon challenge with HP-PRRSV-2 either alone or in combination with PRRSV-1. It was demonstrated that pigs vaccinated, either IM or ID, induce similar patterns of antibody response as measured by IDEXX ELISA and SN assays. The discrepancy is observed in cell mediated immune (CMI) response in which ID vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC levels than that of IM-vaccinated pigs. Following challenge with HP-PRRSV-2, either alone or co-challenge with PRRSV-1, PRRSV viremia and lung lesions, both macroscopically and microscopically, were significantly reduced in vaccinated pigs than that of nonvaccinated pigs, regardless to the route of vaccine administration. It is notably that ID vaccinated pigs had significantly lower levels of viremia and lung lesion scores than that of IM vaccinated pigs.

Recently, the ID administration through needle-free devices have intensively been studies to improve the efficacy of vaccines. Needle-free device have been used as advantageous methods to cross the epidermal barrier and efficiently deliver antigens into the dermal layer (Giudice and Campbell, 2006), requiring a smaller volume of fluid than the more conventional IM route (Giudice and Campbell, 2006).

The most important advantages of the ID vaccination are that it is less invasive, painless, safe, quick and easy. Furthermore, this administration could induce a stronger CMI response. The advantages of the ID vaccination, regarding to the induction of the immune response, was demonstrated in a previous report in which the ID vaccination delivered by a needle-free device can prime a stronger specific immune response, both humoral and CMI, against Aujeszky's disease compared to that of induced by the IM vaccination (Ferrari et al., 2011).

Regarding to CMI response, delivery through the intradermal route could induce T cell polarization through the Th1 pathway, favoring the induction of IFN-γ. This phenomenon was evident in the present study. ID vaccinated pigs had a significantly higher level of PRRSV-specific IFN-γ-SC than that of IM vaccinated pigs. The observed results are in accordance with previous reports in which ID vaccinated pigs induce relatively more IFN-γ-SC than IM-vaccinated pigs (Ferrari et al., 2013; Martelli et al., 2009). One factor likely contributing to this finding is the presence of skin-resident immune cells able to sufficiently capture antigens directly from the skin, which are known to migrate to draining lymph nodes and activate immune responses (Combadiere and Liard, 2011).

Another possibility of higher IFN-γ-SC in ID vaccinated pigs than IM vaccinated pigs could be due to the lower IL-10 levels. The delivery through this route could induce T cell polarization through the Th1 pathway, favoring other cytokines that act against Th2 (Tesfaye et al., 2019). However, the mechanisms of IL-10 induction following vaccination by the IM and ID routes are not understood. With respect to the humoral immune response, the results of the present study demonstrated that the induction of the humoral immune response against PRRSV was not different between IM- and ID-vaccinated pigs and are in agreement with previous studies (Ferrari et al., 2013).

The results of the study demonstrated that pigs vaccinated ID or IM with PRRSV-1 MLV (UNISTRAIN® PRRS) conferred partial heterologous protection against HP-PRRSV-2, either alone or in combination with PRRSV-1. The findings reported herein are in agreement with previous studies (Bonckaert et al., 2016; Roca et al.,

2012). Based on a single challenge with either virulent PRRSV-1 (Lena) (Bonckaert et al., 2016) or HP-PRRSV-2 (Roca et al., 2012), PRRSV-1 MLV (UNISTRAIN® PRRS), administered through the IM route, can confer a partial protection as evidenced by reduced viremia. The mechanism of partial cross protection of the PRRSV-1 MLV against heterologous PRRSV-2 is not known but might be due to the induction of cross neutralizing reactivity against heterologous HP-PRRSV-2. This was observed by increased SN titers against HP-PRRSV-2 in the vaccinated pigs in the present study. In addition, different type of PRRSV-1 MLV could potentially have various activities against PRRSV-2. A previous report comparing the efficacy of 2 different PRRSV-1 MLV vaccines demonstrated that one PRRSV-1 MLV had low protection against HP-PRRSV-2 (Madapong et al., 2020). However, further investigations are needed to be performed.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine (Opriessnig et al., 2002). The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with virulent viruses (Labarque et al., 2003). PRRSV viremia plays a central role to its pathogenesis. High PRRSV in blood associated with the development of lung lesions (Han et al., 2013). Therefore, vaccine mediated reduction of PRRSV viremia is critical for controlling the infection in pigs. Our results agree with previous studies on the efficacy of PRRSV MLV vaccination showing that all vaccine species provide partial protection against challenge with heterologous PRRSV strains with a wide range of protection (Bonckaert et al., 2016; Ferrari et al., 2013; Martelli et al., 2009; Roca et al., 2012). Notably, ID vaccinated pigs had significantly lower lung lesions than IM-vaccinated pigs. This finding could be because the ID route induce a strong cell-mediated immune response as evidenced by the number of IFN-γ-SC.

In conclusion of chapter 8, we suggested that PRRSV-1 MLV administered by either IM or ID provide partial heterologous protection against challenge with HP-PRRSV-2, either alone or in conjunction with PRRSV-1, as demonstrated by reduced

lung lesions and viremia. The ID route might represent an alternative to improve vaccine efficacy, as it induced lower IL-10 levels and more IFN-γ-SC.

REFERENCES

Abbas, A.K., Janeway, C.A., Jr., 2000. Immunology: improving on nature in the twenty-first century. Cell 100, 129-138.

Adams, M.J., Lefkowitz, E.J., King, A.M., Harrach, B., Harrison, R.L., Knowles, N.J., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Mushegian, A.R., Nibert, M., Sabanadzovic, S., Sanfacon, H., Siddell, S.G., Simmonds, P., Varsani, A., Zerbini, F.M., Gorbalenya, A.E., Davison, A.J., 2016. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). Arch Virol 161, 2921-2949.

Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A., 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J Virol 74, 10834-10837.

An, T.Q., Tian, Z.J., Leng, C.L., Peng, J.M., Tong, G.Z., 2011. Highly pathogenic porcine reproductive and respiratory syndrome virus, Asia. Emerg Infect Dis 17, 1782-1784.

Ansari, I.H., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J Virol 80, 3994-4004.

Bassaganya-Riera, J., Thacker, B.J., Yu, S., Strait, E., Wannemuehler, M.J., Thacker, E.L., 2004. Impact of immunizations with porcine reproductive and respiratory syndrome virus on lymphoproliferative recall responses of CD8+ T cells. Viral Immunol 17, 25-37.

Bautista, E.M., Molitor, T.W., 1997. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunol 10, 83-94.

Beura, L.K., Sarkar, S.N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A.K., Osorio, F.A., 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J Virol 84, 1574-1584.

Beyer, J., Fichtner, D., Schirrmeier, H., Granzow, H., Polster, U., Weiland, E., Berndt, A., Wege, H., 1998. Arterivirus PRRSV. Experimental studies on the pathogenesis of respiratory disease. Adv Exp Med Biol 440, 593-599.

Bonckaert, C., van der Meulen, K., Rodriguez-Ballara, I., Pedrazuela Sanz, R., Martinez, M.F., Nauwynck, H.J., 2016. Modified-live PRRSV subtype 1 vaccine UNISTRAIN(R) PRRS provides a partial clinical and virological protection upon challenge with East European subtype 3 PRRSV strain Lena. Porcine Health Manag 2, 12.

Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S., Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet Rec 141, 497-499.

Butler, J.E., Lager, K.M., Golde, W., Faaberg, K.S., Sinkora, M., Loving, C., Zhang, Y.I., 2014. Porcine reproductive and respiratory syndrome (PRRS): an immune dysregulatory pandemic. Immunol Res 59, 81-108.

Cancel-Tirado, S.M., Evans, R.B., Yoon, K.J., 2004. Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. Vet Immunol Immunopathol 102, 249-262.

Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142, 629-633.

Chen, N., Cao, Z., Yu, X., Deng, X., Zhao, T., Wang, L., Liu, Q., Li, X., Tian, K., 2011. Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China. J Gen Virol 92, 880-892.

Chen, N., Liu, Q., Qiao, M., Deng, X., Chen, X., Sun, M., 2017. Whole genome characterization of a novel porcine reproductive and respiratory syndrome virus 1 isolate: Genetic evidence for recombination between Amervac vaccine and circulating strains in mainland China. Infect Genet Evol 54, 308-313.

Choi, K., Lee, J., Park, C., Jeong, J., Chae, C., 2015. Comparison of the pathogenesis of single or dual infections with type 1 and type 2 porcine reproductive and respiratory syndrome virus. J Comp Pathol 152, 317-324.

Choi, K., Park, C., Jeong, J., Kang, I., Park, S.J., Chae, C., 2016. Comparison of commercial type 1 and type 2 PRRSV vaccines against heterologous dual challenge. Vet Rec 178, 291.

Christopher-Hennings, J., Nelson, E.A., Nelson, J.K., Rossow, K.D., Shivers, J.L., Yaeger, M.J., Chase, C.C., Garduno, R.A., Collins, J.E., Benfield, D.A., 1998. Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. Vet Pathol 35, 260-267.

Chung, H.K., Chae, C., 2003. Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). J Comp Pathol 129, 205-212.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., et al., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4, 117-126.

Combadiere, B., Liard, C., 2011. Transcutaneous and intradermal vaccination. Hum Vaccin 7, 811-827.

Conzelmann, K.K., Visser, N., Van Woensel, P., Thiel, H.J., 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193, 329-339.

Correas, I., Osorio, F.A., Steffen, D., Pattnaik, A.K., Vu, H.L.X., 2017. Cross reactivity of immune responses to porcine reproductive and respiratory syndrome virus infection. Vaccine 35, 782-788.

Costers, S., Lefebvre, D.J., Goddeeris, B., Delputte, P.L., Nauwynck, H.J., 2009. Functional impairment of PRRSV-specific peripheral CD3+CD8high cells. Vet Res 40, 46.

Darwich, L., Diaz, I., Mateu, E., 2010. Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. Virus Res 154, 123-132.

Delputte, P.L., Meerts, P., Costers, S., Nauwynck, H.J., 2004. Effect of virus-specific antibodies on attachment, internalization and infection of porcine

reproductive and respiratory syndrome virus in primary macrophages. Vet Immunol Immunopathol 102, 179-188.

Delputte, P.L., Vanderheijden, N., Nauwynck, H.J., Pensaert, M.B., 2002. Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. J Virol 76, 4312-4320.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P.L., Nauwynck, H.J., 2010. Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. BMC Biotechnol 10, 48.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J., Mateu, E., 2005. Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. J Gen Virol 86, 1943-1951.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J., Mateu, E., 2006. Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology 351, 249-259.

Do, H.Q., Trinh, D.T., Nguyen, T.L., Vu, T.T., Than, D.D., Van Lo, T., Yeom, M., Song, D., Choe, S., An, D.J., Le, V.P., 2016. Molecular evolution of type 2 porcine reproductive and respiratory syndrome viruses circulating in Vietnam from 2007 to 2015. BMC Vet Res 12, 256.

Dokland, T., 2010. The structural biology of PRRSV. Virus Res 154, 86-97.

Done, S.H., Paton, D.J., White, M.E., 1996. Porcine reproductive and respiratory syndrome (PRRS): a review, with emphasis on pathological, virological and diagnostic aspects. Br Vet J 152, 153-174.

Dortmans, J., Buter, G.J., Dijkman, R., Houben, M., Duinhof, T.F., 2019. Molecular characterization of type 1 porcine reproductive and respiratory syndrome viruses (PRRSV) isolated in the Netherlands from 2014 to 2016. PLoS One 14, e0218481.

Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Effects of origin and state of differentiation and activation of monocytes/macrophages on their

susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). Arch Virol 142, 2483-2497.

Egli, C., Thur, B., Liu, L., Hofmann, M.A., 2001. Quantitative TaqMan RT-PCR for the detection and differentiation of European and North American strains of porcine reproductive and respiratory syndrome virus. J Virol Methods 98, 63-75.

Fang, Y., Treffers, E.E., Li, Y., Tas, A., Sun, Z., van der Meer, Y., de Ru, A.H., van Veelen, P.A., Atkins, J.F., Snijder, E.J., Firth, A.E., 2012. Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. Proc Natl Acad Sci U S A 109, E2920-2928.

Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T.H., Nguyen, V.C., Liu, D., Bui, Q.A., To, L.T., Wang, C., Tian, K., Gao, G.F., 2008. Porcine respiratory and reproductive syndrome virus variants, Vietnam and China, 2007. Emerg Infect Dis 14, 1774-1776.

Ferrari, L., Borghetti, P., Gozio, S., De Angelis, E., Ballotta, L., Smeets, J., Blanchaert, A., Martelli, P., 2011. Evaluation of the immune response induced by intradermal vaccination by using a needle-less system in comparison with the intramuscular route in conventional pigs. Res Vet Sci 90, 64-71.

Ferrari, L., Martelli, P., Saleri, R., De Angelis, E., Cavalli, V., Bresaola, M., Benetti, M., Borghetti, P., 2013. Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. Vet Immunol Immunopathol 151, 193-206.

Flores-Mendoza, L., Silva-Campa, E., Resendiz, M., Osorio, F.A., Hernandez, J., 2008. Porcine reproductive and respiratory syndrome virus infects mature porcine dendritic cells and up-regulates interleukin-10 production. Clin Vaccine Immunol 15, 720-725.

Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Botner, A., 2002. The genetic diversity of European type PRRSV is similar to

that of the North American type but is geographically skewed within Europe. Virology 299, 38-47.

Gao, L., Wang, L., Huang, C., Yang, L., Guo, X.K., Yu, Z., Liu, Y., Yang, P., Feng, W.H., 2015. HP-PRRSV is attenuated by de-optimization of codon pair bias in its RNA-dependent RNA polymerase nsp9 gene. Virology 485, 135-144.

Geldhof, M.F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U.U., Nauwynck, H.J., 2012. Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Vet Res 8, 182.

Giudice, E.L., Campbell, J.D., 2006. Needle-free vaccine delivery. Adv Drug Deliv Rev 58, 68-89.

Gonin, P., Pirzadeh, B., Gagnon, C.A., Dea, S., 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J Vet Diagn Invest 11, 20-26.

Halbur, P., Paul, P., Frey, M., Landgraf, J., Eernisse, K., Meng, X.-J., Lum, M., Andrews, J., Rathje, J., 1995a. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Veterinary Pathology Online 32, 648-660.

Halbur, P.G., Miller, L.D., Paul, P.S., Meng, X.J., Huffman, E.L., Andrews, J.J., 1995b. Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet Pathol 32, 200-204.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Andrews, J.J., Lum, M.A., Rathje, J.A., 1996a. Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 33, 159-170.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995c. Comparison of the pathogenicity of two US

porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 32, 648-660.

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1996b. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J Vet Diagn Invest 8, 11-20.

Han, D., Hu, Y., Li, L., Tian, H., Chen, Z., Wang, L., Ma, H., Yang, H., Teng, K., 2014. Highly pathogenic porcine reproductive and respiratory syndrome virus infection results in acute lung injury of the infected pigs. Vet Microbiol 169, 135-146.

Han, K., Seo, H.W., Oh, Y., Kang, I., Park, C., Chae, C., 2013. Comparison of the virulence of European and North American genotypes of porcine reproductive and respiratory syndrome virus in experimentally infected pigs. Vet J 195, 313-318.

Hoegen, B., Saalmuller, A., Rottgen, M., Rziha, H.J., Geldermann, H., Reiner, G., Pfaff, E., Buttner, M., 2004. Interferon-gamma response of PBMC indicates productive pseudorabies virus (PRV) infection in swine. Vet Immunol Immunopathol 102, 389-397.

Horter, D., Chang, C.C., Pogranichnyy, R., Zimmerman, J., Yoon, K.J., 2001. Persistence of porcine reproductive and respiratory syndrome in pigs. Adv Exp Med Biol 494, 91-94.

Horter, D.C., Pogranichniy, R.M., Chang, C.C., Evans, R.B., Yoon, K.J., Zimmerman, J.J., 2002. Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet Microbiol 86, 213-228.

Jeong, J., Choi, K., Kang, I., Park, C., Chae, C., 2016. Evaluation of a 20year old porcine reproductive and respiratory syndrome (PRRS) modified live vaccine (Ingelvac((R)) PRRS MLV) against two recent type 2 PRRS virus isolates in South Korea. Vet Microbiol 192, 102-109.

Karniychuk, U.U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T.A., Nauwynck, H.J., 2010. Pathogenesis and antigenic characterization of a new

East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet Res 6, 30.

Karniychuk, U.U., Van Breedam, W., Van Roy, N., Rogel-Gaillard, C., Nauwynck, H.J., 2012. Demonstration of microchimerism in pregnant sows and effects of congenital PRRSV infection. Vet Res 43, 19.

Kim, H.K., Park, S.J., Rho, S.M., Han, J.Y., Nguyen, V.G., Park, B.K., 2011. One year's study of dynamic and evolution of types I and II PRRSV in a swine farm. Vet Microbiol 150, 230-238.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch Virol 133, 477-483.

Kim, T., Park, C., Choi, K., Jeong, J., Kang, I., Park, S.J., Chae, C., 2015. Comparison of Two Commercial Type 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Modified Live Vaccines against Heterologous Type 1 and Type 2 PRRSV Challenge in Growing Pigs. Clin Vaccine Immunol 22, 631-640.

Kim, W.I., Kim, J.J., Cha, S.H., Yoon, K.J., 2008. Different biological characteristics of wild-type porcine reproductive and respiratory syndrome viruses and vaccine viruses and identification of the corresponding genetic determinants. J Clin Microbiol 46, 1758-1768.

Kim, W.I., Yoon, K.J., 2008. Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. Virus Genes 37, 380-391.

Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M., Stockhofe-Zurwieden, N., 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27, 3704-3718.

Kristensen, C.S., Kvisgaard, L.K., Pawlowski, M., Holmgaard Carlsen, S., Hjulsager, C.K., Heegaard, P.M.H., Botner, A., Stadejek, T., Haugegaard, S., Larsen, L.E., 2018. Efficacy and safety of simultaneous vaccination with two modified live virus vaccines against porcine reproductive and respiratory syndrome virus types 1 and 2 in pigs. Vaccine 36, 227-236.

Kuhn, J.H., Lauck, M., Bailey, A.L., Shchetinin, A.M., Vishnevskaya, T.V., Bao, Y.M., Ng, T.F.F., LeBreton, M., Schneider, B.S., Gillis, A., Tamoufe, U., Diffo, J.L., Takuo, J.M., Kondov, N.O., Coffey, L.L., Wolfe, N.D., Delwart, E., Clawson, A.N., Postnikova, E., Bollinger, L., Lackemeyer, M.G., Radoshitzky, S.R., Palacios, G., Wada, J., Shevtsova, Z.V., Jahrling, P.B., Lapin, B.A., Deriabin, P.G., Dunowska, M., Alkhovsky, S.V., Rogers, J., Friedrich, T.C., O'Connor, D.H., Goldberg, T.L., 2016. Reorganization and expansion of the nidoviral family Arteriviridae. Archives of Virology 161, 755-768.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2003. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet Microbiol 95, 187-197.

Labarque, G.G., Nauwynck, H.J., Van Reeth, K., Pensaert, M.B., 2000. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J Gen Virol 81, 1327-1334.

Lawson, S.R., Rossow, K.D., Collins, J.E., Benfield, D.A., Rowland, R.R., 1997. Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. Virus Res 51, 105-113.

LeRoith, T., Hammond, S., Todd, S.M., Ni, Y., Cecere, T., Pelzer, K.D., 2011. A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with Mycoplasma hyopneumoniae. Vet Immunol Immunopathol 140, 312-316.

Li, J., Chen, Z., Zhao, J., Fang, L., Fang, R., Xiao, J., Chen, X., Zhou, A., Zhang, Y., Ren, L., Hu, X., Zhao, Y., Zhang, S., Li, N., 2015. Difference in microRNA expression and editing profile of lung tissues from different pig breeds related to immune responses to HP-PRRSV. Sci Rep 5, 9549.

Li, X., Galliher-Beckley, A., Pappan, L., Trible, B., Kerrigan, M., Beck, A., Hesse, R., Blecha, F., Nietfeld, J.C., Rowland, R.R., Shi, J., 2014a. Comparison of host immune responses to homologous and heterologous type II porcine

reproductive and respiratory syndrome virus (PRRSV) challenge in vaccinated and unvaccinated pigs. Biomed Res Int 2014, 416727.

Li, Y., Tas, A., Snijder, E.J., Fang, Y., 2012. Identification of porcine reproductive and respiratory syndrome virus ORF1a-encoded non-structural proteins in virus-infected cells. J Gen Virol 93, 829-839.

Li, Y., Treffers, E.E., Napthine, S., Tas, A., Zhu, L., Sun, Z., Bell, S., Mark, B.L., van Veelen, P.A., van Hemert, M.J., Firth, A.E., Brierley, I., Snijder, E.J., Fang, Y., 2014b. Transactivation of programmed ribosomal frameshifting by a viral protein. Proc Natl Acad Sci U S A 111, E2172-2181.

Lopez-Fuertes, L., Campos, E., Domenech, N., Ezquerra, A., Castro, J.M., Dominguez, J., Alonso, F., 2000. Porcine reproductive and respiratory syndrome (PRRS) virus down-modulates TNF-alpha production in infected macrophages. Virus Res 69, 41-46.

Lopez Fuertes, L., Domenech, N., Alvarez, B., Ezquerra, A., Dominguez, J., Castro, J.M., Alonso, F., 1999. Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res 64, 33-42.

Lopez, O.J., Oliveira, M.F., Garcia, E.A., Kwon, B.J., Doster, A., Osorio, F.A., 2007. Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clin Vaccine Immunol 14, 269-275.

Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Vet Immunol Immunopathol 102, 155-163.

Loving, C.L., Osorio, F.A., Murtaugh, M.P., Zuckermann, F.A., 2015. Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol 167, 1-14.

Lunney, J.K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., Renukaradhya, G.J., 2016. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci 4, 129-154.

Madapong, A., Saeng-Chuto, K., Boonsoongnern, A., Tantituvanont, A., Nilubol, D., 2020. Cell-mediated immune response and protective efficacy of porcine

reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2. Sci Rep 10, 1649.

Madapong, A., Temeeyasen, G., Saeng-Chuto, K., Tripipat, T., Navasakuljinda, W., Boonsoongnern, A., Tantituvanont, A., Nilubol, D., 2016. Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines. Arch Virol.

Madapong, A., Temeeyasen, G., Saeng-Chuto, K., Tripipat, T., Navasakuljinda, W., Boonsoongnern, A., Tantituvanont, A., Nilubol, D., 2017. Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines. Arch Virol 162, 139-146.

Madsen, K.G., Hansen, C.M., Madsen, E.S., Strandbygaard, B., Botner, A., Sorensen, K.J., 1998. Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Arch Virol 143, 1683-1700.

Maloy, K.J., Powrie, F., 2001. Regulatory T cells in the control of immune pathology. Nat Immunol 2, 816-822.

Martelli, P., Gozio, S., Ferrari, L., Rosina, S., De Angelis, E., Quintavalla, C., Bottarelli, E., Borghetti, P., 2009. Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. Vaccine 27, 3788-3799.

Martinez-Lobo, F.J., de Lome, L.C., Diez-Fuertes, F., Segales, J., Garcia-Artiga, C., Simarro, I., Castro, J.M., Prieto, C., 2013. Safety of Porcine Reproductive and Respiratory Syndrome Modified Live Virus (MLV) vaccine strains in a young pig infection model. Vet Res 44, 115.

Martinez-Lobo, F.J., Diez-Fuertes, F., Simarro, I., Castro, J.M., Prieto, C., 2011. Porcine Reproductive and Respiratory Syndrome Virus isolates differ in their susceptibility to neutralization. Vaccine 29, 6928-6940.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-gamma response of swine

to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18-31.

Meng, F., Hoversten, M.T., Thompson, R.C., Taylor, L., Watson, S.J., Akil, H., 1995a. A chimeric study of the molecular basis of affinity and selectivity of the kappa and the delta opioid receptors. Potential role of extracellular domains. J Biol Chem 270, 12730-12736.

Meng, J., Zhao, S., Zhao, T., Doyle, M.P., 1995b. Molecular characterisation of Escherichia coli O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. J Med Microbiol 42, 258-263.

Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet Microbiol 74, 309-329.

Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1995c. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch Virol 140, 745-755.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Koehler, K.J., 2003. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet Microbiol 93, 13-24.

Mengeling, W.L., Vorwald, A.C., Lager, K.M., Clouser, D.F., Wesley, R.D., 1999. Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. Am J Vet Res 60, 334-340.

Meulenberg, J.J., Bos-de Ruijter, J.N., van de Graaf, R., Wensvoort, G., Moormann, R.J., 1998. Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. J Virol 72, 380-387.

Meulenberg, J.J., Petersen-den Besten, A., De Kluyver, E.P., Moormann, R.J., Schaaper, W.M., Wensvoort, G., 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology 206, 155-163.

Miller, L.C., Laegreid, W.W., Bono, J.L., Chitko-McKown, C.G., Fox, J.M., 2004. Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. Arch Virol 149, 2453-2463.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19, 683-765.

Mulupuri, P., Zimmerman, J.J., Hermann, J., Johnson, C.R., Cano, J.P., Yu, W., Dee, S.A., Murtaugh, M.P., 2008. Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. J Virol 82, 358-370.

Murtaugh, M.P., Genzow, M., 2011. Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine 29, 8192-8204.

Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol 15, 533-547.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol 73, 270-280.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J Clin Microbiol 31, 3184-3189.

Nilubol, D., Platt, K.B., Halbur, P.G., Torremorell, M., Harris, D.L., 2004. The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. Vet Microbiol 102, 11-18.

Nilubol, D., Tripipat, T., Hoonsuwan, T., Kortheerakul, K., 2012. Porcine reproductive and respiratory syndrome virus, Thailand, 2010-2011. Emerg Infect Dis 18, 2039-2043.

Nilubol, D., Tripipat, T., Hoonsuwan, T., Tipsombatboon, P., Piriyapongsa, J., 2013. Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes I and II in Thailand. Arch Virol 158, 943-953.

Nilubol, D., Tripipat, T., Hoonsuwan, T., Tipsombatboon, P., Piriyapongsa, J., 2014. Dynamics and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 following modified live PRRSV vaccination in a PRRSV-infected herd. Arch Virol 159, 17-27.

Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J Virol 76, 11837-11844.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J Virol 76, 4241-4250.

Park, C., Choi, K., Jeong, J., Chae, C., 2015. Cross-protection of a new type 2 porcine reproductive and respiratory syndrome virus (PRRSV) modified live vaccine (Fostera PRRS) against heterologous type 1 PRRSV challenge in growing pigs. Vet Microbiol 177, 87-94.

Park, C., Seo, H.W., Han, K., Kang, I., Chae, C., 2014. Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge. Vet Microbiol 172, 432-442.

Pasternak, A.O., Spaan, W.J., Snijder, E.J., 2006. Nidovirus transcription: how to make sense...? J Gen Virol 87, 1403-1421.

Plagemann, P.G., Rowland, R.R., Faaberg, K.S., 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch Virol 147, 2327-2347.

Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X., Mane, H., 1997. Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. Vet Microbiol 55, 361-370.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., San Gabriel, A., Pujols, J., Badiola, J.L., Ramos, J.A., et al., 1992. Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease. Vet Microbiol 33, 203-211.

Roca, M., Gimeno, M., Bruguera, S., Segales, J., Diaz, I., Galindo-Cardiel, I.J., Martinez, E., Darwich, L., Fang, Y., Maldonado, J., March, R., Mateu, E., 2012. Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. Vet J 193, 92-96.

Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., Levings, M.K., 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 212, 28-50.

Rossow, K.D., 1998. Porcine reproductive and respiratory syndrome. Vet Pathol 35, 1-20.

Rossow, K.D., Collins, J.E., Goyal, S.M., Nelson, E.A., Christopher-Hennings, J., Benfield, D.A., 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet Pathol 32, 361-373.

Rossow, K.D., Shivers, J.L., Yeske, P.E., Polson, D.D., Rowland, R.R., Lawson, S.R., Murtaugh, M.P., Nelson, E.A., Collins, J.E., 1999. Porcine reproductive and respiratory syndrome virus infection in neonatal pigs characterised by marked neurovirulence. Vet Rec 144, 444-448.

Saraiva, M., O'Garra, A., 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol 10, 170-181.

Shevach, E.M., 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity 25, 195-201.

Shi, C., Liu, Y., Ding, Y., Zhang, Y., Zhang, J., 2015. PRRSV receptors and their roles in virus infection. Arch Microbiol 197, 503-512.

Shi, M., Lam, T.T., Hon, C.C., Hui, R.K., Faaberg, K.S., Wennblom, T., Murtaugh, M.P., Stadejek, T., Leung, F.C., 2010a. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res 154, 7-17.

Shi, M., Lam, T.T., Hon, C.C., Murtaugh, M.P., Davies, P.R., Hui, R.K., Li, J., Wong, L.T., Yip, C.W., Jiang, J.W., Leung, F.C., 2010b. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J Virol 84, 8700-8711.

Silva-Campa, E., Cordoba, L., Fraile, L., Flores-Mendoza, L., Montoya, M., Hernandez, J., 2010. European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology 396, 264-271.

Silva-Campa, E., Flores-Mendoza, L., Resendiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W., Hernandez, J., 2009. Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. Virology 387, 373-379.

Snijder, E.J., Meulenberg, J.J.M., 1998. The molecular biology of arteriviruses. Journal of General Virology 79, 961-979.

Stadejek, T., Oleksiewicz, M.B., Scherbakov, A.V., Timina, A.M., Krabbe, J.S., Chabros, K., Potapchuk, D., 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch Virol 153, 1479-1488.

Stadejek, T., Stankevicius, A., Murtaugh, M.P., Oleksiewicz, M.B., 2013. Molecular evolution of PRRSV in Europe: current state of play. Vet Microbiol 165, 21-28.

Stevenson, G.W., Van Alstine, W.G., Kanitz, C.L., Keffaber, K.K., 1993. Endemic porcine reproductive and respiratory syndrome virus infection of nursery pigs in two swine herds without current reproductive failure. J Vet Diagn Invest 5, 432-434.

Subramaniam, S., Sur, J.H., Kwon, B., Pattnaik, A.K., Osorio, F.A., 2011. A virulent strain of porcine reproductive and respiratory syndrome virus does not up-regulate interleukin-10 levels in vitro or in vivo. Virus Res 155, 415-422.

Suradhat, S., Intrakamhaeng, M., Damrongwatanapokin, S., 2001. The correlation of virus-specific interferon-gamma production and protection against classical swine fever virus infection. Vet Immunol Immunopathol 83, 177-189.

Suradhat, S., Thanawongnuwech, R., 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J Gen Virol 84, 2755-2760.

Suradhat, S., Thanawongnuwech, R., Poovorawan, Y., 2003. Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. J Gen Virol 84, 453-459.

Tesfaye, D.Y., Gudjonsson, A., Bogen, B., Fossum, E., 2019. Targeting Conventional Dendritic Cells to Fine-Tune Antibody Responses. Front Immunol 10, 1529.

Thanawongnuwech, R., Amonsin, A., Tatsanakit, A., Damrongwatanapokin, S., 2004. Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. Vet Microbiol 101, 9-21.

Thanawongnuwech, R., Thacker, E.L., Halbur, P.G., 1997. Effect of porcine reproductive and respiratory syndrome virus (PRRSV) (isolate ATCC VR-2385) infection on bactericidal activity of porcine pulmonary intravascular macrophages (PIMs): in vitro comparisons with pulmonary alveolar macrophages (PAMs). Vet Immunol Immunopathol 59, 323-335.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research 22, 4673-4680.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One 2, e526.

Trinchieri, G., 2007. Interleukin-10 production by effector T cells: Th1 cells show self control. J Exp Med 204, 239-243.

Tun, H.M., Shi, M., Wong, C.L., Ayudhya, S.N., Amonsin, A., Thanawonguwech, R., Leung, F.C., 2011. Genetic diversity and multiple introductions of porcine reproductive and respiratory syndrome viruses in Thailand. Virol J 8, 164.

Van Breedam, W., Van Gorp, H., Zhang, J.Q., Crocker, P.R., Delputte, P.L., Nauwynck, H.J., 2010. The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. PLoS Pathog 6, e1000730.

Van Gorp, H., Van Breedam, W., Van Doorsselaere, J., Delputte, P.L., Nauwynck, H.J., 2010. Identification of the CD163 protein domains involved in infection of the porcine reproductive and respiratory syndrome virus. J Virol 84, 3101-3105.

van Reeth, K., Nauwynck, H., 2000. Proinflammatory cytokines and viral respiratory disease in pigs. Vet Res 31, 187-213.

van Woensel, P.A., Liefkens, K., Demaret, S., 1998a. Effect on viraemia of an American and a European serotype PRRSV vaccine after challenge with European wild-type strains of the virus. Vet Rec 142, 510-512.

van Woensel, P.A., Liefkens, K., Demaret, S., 1998b. European serotype PRRSV vaccine protects against European serotype challenge whereas an American serotype vaccine does not. Adv Exp Med Biol 440, 713-718.

Vu, H.L., Kwon, B., Yoon, K.J., Laegreid, W.W., Pattnaik, A.K., Osorio, F.A., 2011. Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. J Virol 85, 5555-5564.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., et al., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q 13, 121-130.

Wills, R.W., Doster, A.R., Galeota, J.A., Sur, J.H., Osorio, F.A., 2003. Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J Clin Microbiol 41, 58-62.

Wissink, E.H., Kroese, M.V., van Wijk, H.A., Rijsewijk, F.A., Meulenberg, J.J., Rottier, P.J., 2005. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J Virol 79, 12495-12506.

Xiao, Z., Batista, L., Dee, S., Halbur, P., Murtaugh, M.P., 2004. The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and

respiratory syndrome virus infection in pigs is independent of virus load. J Virol 78, 5923-5933.

Yoon, I.J., Joo, H.S., Christianson, W.T., Kim, H.S., Collins, J.E., Morrison, R.B., Dial, G.D., 1992. An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. J Vet Diagn Invest 4, 144-147.

Yoon, S.H., Song, J.Y., Lee, C.H., Choi, E.J., Cho, I.S., Kim, B., 2008. Genetic characterization of the Korean porcine reproductive and respiratory syndrome viruses based on the nucleocapsid protein gene (ORF7) sequences. Arch Virol 153, 627-635.

Yun, S.I., Lee, Y.M., 2013. Overview: Replication of porcine reproductive and respiratory syndrome virus. J Microbiol 51, 711-723.

Zhou, L., Ni, Y.Y., Pineyro, P., Sanford, B.J., Cossaboom, C.M., Dryman, B.A., Huang, Y.W., Cao, D.J., Meng, X.J., 2012. DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology 434, 96-109.

Zhou, Z., Ni, J., Cao, Z., Han, X., Xia, Y., Zi, Z., Ning, K., Liu, Q., Cai, L., Qiu, P., Deng, X., Hu, D., Zhang, Q., Fan, Y., Wu, J., Wang, L., Zhang, M., Yu, X., Zhai, X., Tian, K., 2011. The epidemic status and genetic diversity of 14 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) isolates from China in 2009. Vet Microbiol 150, 257-269.

Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M., Osorio, F., 2007. Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet Microbiol 123, 69-85.

APPENDIX

Appendix A

Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines

Adthakorn Madapong[1], Gun Teemeyasen[1], Kepalee Saeng-chuto[1], Thitima Tripipat[1], Wichian Navasakuljinda[2], Alongkot Boonsoongnern[3], Angkana Tantituvanont[4], Dachrit Nilubol[1,*]

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]Zoetis (Thailand) Limited, Bangkok, Thailand
[3]Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine Kamphaeng Sean Campus, Kasetsart University, Nakhon Pathom, Thailand
[4]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

*Corresponding author:
Assistant Professor Dr. Dachrit Nilubol
E-mail address: dachrit@gmail.com, ndachrit@chula.ac.th
Department of Veterinary Microbiology, Faculty of Veterinary Science
Chulalongkorn University, Bangkok 10330, Thailand

Keywords: Porcine reproductive and respiratory syndrome virus, modified live vaccine, antibody response, shedding, sentinel pig

Arch Virol (2017) 162:139–146
DOI 10.1007/s00705-016-3084-4

ORIGINAL ARTICLE

Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines

Adthakorn Madapong[1] · Gun Temeeyasen[1] · Kepalee Saeng-chuto[1] · Thitima Tripipat[1] · Wichian Navasakuljinda[2] · Alongkot Boonsoongnern[3] · Angkana Tantituvanont[4] · Dachrit Nilubol[1]

Received: 2 August 2016 / Accepted: 20 September 2016 / Published online: 30 September 2016

Abstract The antibody response and pattern of shedding of vaccine virus following vaccination with modified live genotype I or II porcine reproductive and respiratory syndrome virus (PRRSV) vaccines (MLVs) were investigated. Ninety PRRSV-free pigs were divided randomly seven, groups including the NEG, EU1, EU2, US1, US2, US3 and US4 groups. The NEG group was unvaccinated. The EU1, EU2, US1, US2, US3 and US4 groups were vaccinated with the following MLVs: AMERVAC® PRRS, Porcillis® PRRS, Fostera™ PRRS, Ingelvac® PRRS MLV, Ingelvac® PRRS ATP, and PrimePac™ PRRS+ , respectively. Sera were quantitatively assayed for viral RNA using qPCR. Antibody responses were measured using Idexx ELISA and serum neutralization (SN). Shedding of vaccine virus was investigated using sentinel pigs and by detection of viral RNA in tonsil scrapings. Antibody responses were detected by ELISA at 7-14 days post-vaccination (DPV) and persisted at high titers until 84 DPV in all MLV groups. The SN titers were delayed and isolate-specific. SN titers were higher for the homologous virus than for heterologous viruses. Age-matched sentinel pigs introduced into the EU2, US2 and US3 groups at 60 DPV seroconverted. In contrast, sentinel pigs introduced at 84 DPV remained negative in all of the MLV groups. Vaccine viral RNA was detected in tonsil scrapings from the EU2, US2 and US3 groups at 84-90 DPV. No viral RNA was detected beyond 70 DPV in the EU1, US1 and US4 groups. In conclusion, all MLV genotypes induced rapid antibody responses, which were measured using ELISA. The development of SN antibodies was delayed and isolate-specific. However, the shedding pattern was variable and depended on the by virus isolate used to manufacture the vaccine.

✉ Dachrit Nilubol
dachrit@gmail.com

[1] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

[2] Zoetis (Thailand) Limited, Bangkok, Thailand

[3] Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine Kamphaeng Saen Campus, Kasetsart University, Nakhon Pathom, Thailand

[4] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

Introduction

Porcine reproductive and respiratory syndrome (PRRS) has caused severe economic damage to the swine industry worldwide since its emergence in the late 1980s [1]. This syndrome is characterized by reproductive disorders in sows, including abortion, reduced numbers of weaned pigs due to an increase in the number of stillborn pigs, mummified fetuses, and weakness, and respiratory disorders in pigs from nursery to finishing.

Because of the economic losses caused by PRRSV outbreaks, various types of PRRSV vaccines have been developed and implemented on pig farms with varying degrees of success. Several vaccination trials have shown the replication of live immunogen in pigs to be a crucial requirement for generating robust protective immunity against PRRSV infection [2, 3]. Therefore, a modified-live vaccine (MLV) rather than a killed or subunit vaccine has been deemed to be the most efficacious type of vaccine against PRRSV infection to date and has been employed regularly in both experimental and field-scale trials since its first introduction in 1994.

Currently, various types of MLV vaccines, including genotypes I and II, are commercially available. In regions where a single infection with either genotype I or II has been reported, MLV targeting the circulating genotype should be used. However, in co-infected herds, which genotype of PRRSV MLV should be used to successfully control the disease is less obvious. Criteria for vaccine selection could be the induction of immune responses and the shedding pattern of the vaccine virus. The induction of immune responses following MLV administration may vary according to the virus isolate used to produce the vaccine [2, 4]. Occasionally, vaccination with genotype II MLV can yield undesired outcomes, such as delayed immune responses, low potency of humoral or cell-mediated immune activation, the induction of regulatory IL-10 and/or T-cells (T_{regs}), the suppression of pro-inflammatory cytokine production, and a reduced level of type I (α/β) and type II (γ) interferon. These undesired outcomes could potentially lead to reduced protective efficiency of a vaccine or, in the worst case, to increased susceptibility to infection by other pathogens [5]. In addition, the safety of MLV of all genotypes remains doubtful because the persistence of MLV, the development of viremia, transmission of a vaccine to non-vaccinated pigs, and clinical signs in vaccinated pigs have been documented following vaccination [6]. Therefore, this study aimed to evaluate the induction of humoral immune responses and viral vaccine shedding following vaccination with either genotype I or II MLV. In the present study, the induction of antibody responses and shedding patterns of six different MLVs were compared.

Materials and methods

Ethics statement

All animal procedures were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council of Thailand according to protocols approved by the Chulalongkorn University IACUC.

Experimental design

A cohort of 90 seven- to eight-week-old castrated male PRRSV-free pigs were randomly assigned based on a stratification by weight into the following seven treatment groups: NEG, EU1, EU2, US1, US2, US3 and US4. Groups of pigs were housed in separate rooms with separate air spaces (Table 1). The NEG group included 30 pigs that were left unvaccinated. Pigs in the EU1 and EU2 groups were vaccinated intramuscularly with AMERVAC® PRRS and Porcillis® PRRS, respectively, which are both PRRSV genotype I MLVs. The US1, US2, US3 and US4 groups were vaccinated intramuscularly with Fostera™ PRRS, Ingelvac® PRRS MLV, Ingelvac® PRRS ATP and PrimePac™ PRRS+ , respectively, which are all PRRSV genotype II MLVs. The dosage and route of administration were in accordance with the respective manufacturer's directions.

Following vaccination, all groups were monitored for changes in physical condition and were scored for clinical respiratory disease. Blood samples were collected at 0, 3, 5, 7, 14, 21, 28, 35, 42 and 84 days post-vaccination (DPV). Sera were separated from blood samples and assayed for the presence of antibody using ELISA and a serum neutralization (SN) assay against both homologous and heterologous isolates. The viral load in serum was measured using real-time quantitative PCR (qPCR). Tonsil scraping samples were collected at 60, 70, 84 and 90 DPV and assayed for the presence of viral RNA by RT-PCR. Individual pigs were restrained using a snare, and samples were then collected by scraping the palatine tonsil with an elongated spoon. Scraping were mixed with 1 ml of DMEM supplemented with 50 µg of gentamicin per ml and filtered through a 0.22-µm nitrocellulose membrane. Filtrates were then stored at -80 °C for later use.

Vaccines and viruses

Homologous and heterologous viruses were used to perform a serum neutralizing (SN) assay. Homologous virus refers to a vaccine isolate. To retrieve homologous virus, each vaccine (except Fostera™ PRRS) was re-constituted in DMEM media. Then, the virus was propagated in MARC-145 cells using a previously described method [7]. Virus was harvested by a cycle of freezing and thawing. Supernatant containing the virus was stored at -80 °C before subsequent use. Because of the inability of the Fostera™ PRRS vaccine virus to be generated using MARC-145 cells, the homologous virus used to generate to Fostera™ PRRS was a virus that was isolated from pigs that were previously vaccinated with Fostera™ PRRS. Heterologous viruses refer to the SB_EU02 and ST_US02 isolates, which are Thai PRRSV genotype I and II field isolates. SB_EU02 and ST_US02 were isolated from farms experiencing PRRS outbreaks.

Clinical evaluation

Rectal temperature was recorded daily for two consecutive weeks by the same personnel at the same time. The severity of clinical respiratory disease was evaluated daily for two consecutive weeks following vaccination, and on a weekly basis for 2 more weeks using a scoring system

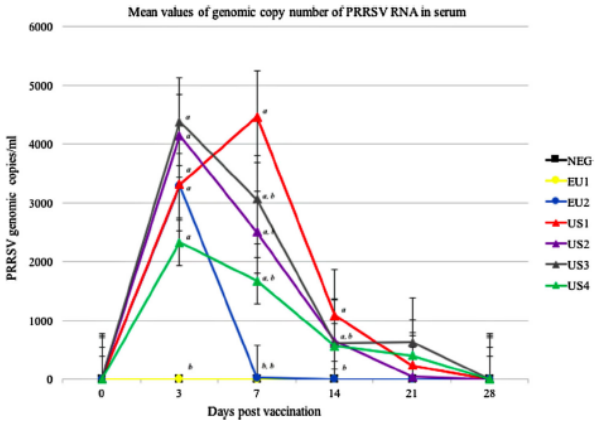


Fig. 1 Mean values of the genomic copy number of PRRSV RNA in serum of the NEG (—■—), EU1 (—●—), EU2 (—●—), US1(—▲—), US2 (—▲—), US3 (—▲—) and US4 (—▲—) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups

Statistical analysis

Data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed for each day by ANOVA to determine whether there were significant differences between treatment groups. If the p-value in the ANOVA table was ≤ 0.05, differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $p \leq 0.05$ rejection level.

Results

Rectal temperature and clinical observations

The rectal temperatures of the pigs in the control group and all vaccinated groups were within normal physiological ranges throughout the experimental period. None of the pigs in any of the groups displayed any clinical respiratory disease throughout the study except for those in the EU2 and US3 group. There were 5/10, 3/10 and 6/10 of pigs in the EU2, US2 and US3 groups, respectively, that showed respiratory signs at 20 DPV. At 21 DPV, all pigs in the EU2 group were injected once intramuscularly with tulathromycin. At 28 DPV, all pigs in the EU2, US1 and US2 groups were in-feed medicated with amoxycillin at 300 ppm for 7 consecutive days. There was one pig in the US3 group that died at 35 DPV, and the necropsy revealed paleness of skin and gastric ulceration. PCR results from organ samples, including lung, bronchial and mesenteric lymph nodes, were positive for PRRSV. At 35 DPV, two pigs from the EU2 and US3 groups were euthanized because of the severity of clinical disease.

Quantification of PRRSV RNA in serum

PRRSV RNA was not detectable in the NEG and EU1 groups throughout the study (Fig. 1). In the US2 and US3 groups, the viral RNA copy numbers were highest at 3 DPV and then slowly declined until they were below the limit of detection at 21 and 28 DPV, respectively. In the EU2 group, the viral RNA copy number peaked at 3 DPV and decreased until it was below the limit of detection at 7 DPV. In the US1 group, the viral RNA copy number was detectable after 3 DPV and peaked at 7 DPV. Then, the viral RNA copy number level gradually declined until it could not be detected at 28 DPV.

The viral RNA copy number of the EU1 group was significantly lower compared with the other vaccinated groups at 3 DPV. At 7 DPV, the viral RNA copy number in the US1 group was not different from those of the other genotype II MLV groups but was significantly higher than those of the genotype I MLV groups.

RT-PCR in tonsil scrapings

Viral RNA was not detected in any pigs in the EU1 group. In contrast, viral RNA in the EU2 groups was detected in 3 of 9, 2 of 9 and 1 of 9 pigs at 60, 70 and 90 DPV, respectively (Table 2). For genotype II MLVs, viral RNA was detected in 1 of 10 and 2 of 10 pigs in the US1 and US4 group, respectively, at 60 DPV. In the US2 group, viral RNA was detected on all sampling days and was still

Table 2 Detection of viral RNA in tonsil scrapings from vaccinated pigs and sentinel pigs

Treatment group	Vaccine	Tonsil scraping samples				Sentinel pigs	
		Days post-vaccination				Days post-vaccination	
		60	70	84	90	60	84
NEG	–	0/10[a]	0/10	0/10	0/10	Negative	Negative
EU1	Porcillis® PRRS	0/10	0/10	0/10	0/10	Negative	Negative
EU2	Amervac® PRRS	3/9	2/9	0/9	1/9	Positive	Negative
US1	Fostera™ PRRS	1/10	0/10	0/10	0/10	Negative	Negative
US2	Ingelvac® PRRS MLV	1/10	2/10	1/10	1/10	Positive	Negative
US3	Ingelvac® PRRS ATP	1/8	2/8	1/8	0/8	Positive	Negative
US4	*PrimePac*™ PRRS+	1/10	2/10	0/10	0/10	Negative	Negative

[a] The number of positive pigs by PCR/total number of pigs in the groups

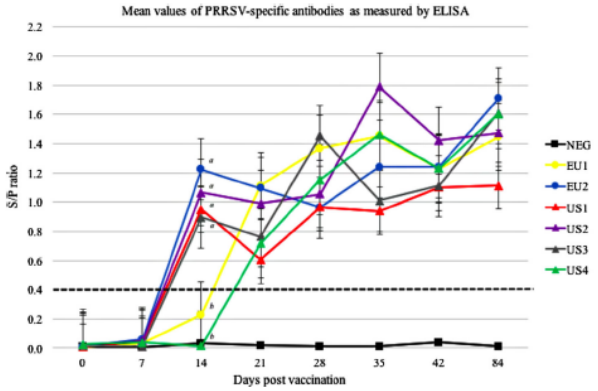


Fig. 2 Mean values of PRRSV-specific antibodies as measured by ELISA of the NEG (■), EU1 (●), EU2 (●), US1 (▲), US2 (▲), US3 (▲) and US4 (▲) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate statistical significant difference ($p < 0.05$) between groups. A dashed line indicates the cutoff level (S/P ratio of 0.4)

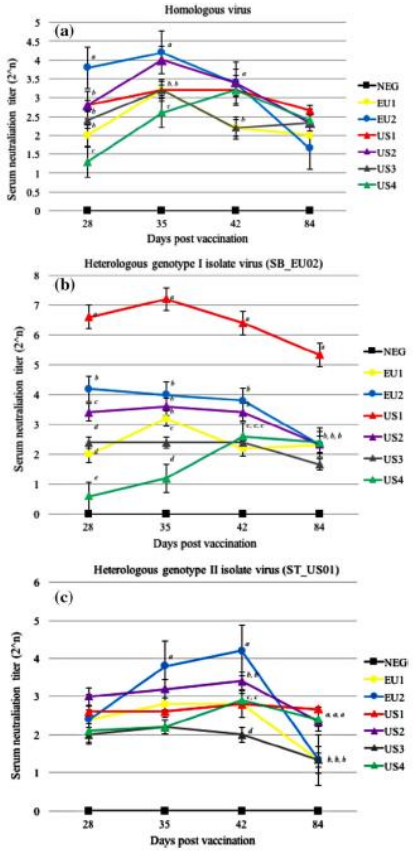
detectable in 2 of 10, 3 of 10, 1 of 10 and 1 of 10 of pigs at 60, 70, 84 and 90 DPV, respectively. In the US3 group, viral RNA remained detectable in 1 of 8, 2 of 8 and 1 of 8 of pigs at 60, 70 and 84 DPV, respectively.

Antibody responses as measured by ELISA

Pigs in the NEG group remained serologically negative throughout the experiment (Fig. 2). Our findings revealed a similar pattern in all vaccinated groups. Antibody responses were first detected at 7 DPV at the earliest in some pigs of the vaccinated groups, but the average antibody level was below the cutoff level (S/P ratio at 0.4). At 14 DPV, the average antibody responses of the EU2, US1, US2 and US3 groups were significantly higher than those of the EU1 and US4 groups, in which the average antibody levels were below the cutoff level. At 21 DPV, the average antibody responses of all vaccinated groups were above the cutoff level and remained constant until the end of the experiment. There were no differences between any of the vaccinated groups from 21 to 84 DPV.

Antibody responses as measured by serum neutralization (SN) assay

Pigs in the NEG group remained serologically negative throughout the experiment. In all vaccinated groups, the SN assay against homologous virus in all vaccinated groups (Fig. 3A) showed a similar pattern of SN titers that could be detected as early as 28 DPV. Titers reached a peak level at 35 or 42 DPV and then declined by 84 DPV. At 28 DPV, the EU2 group had significantly higher SN titers against the homologous virus compared with the other vaccinated groups. In contrast, the US4 group had significantly lower SN titers compared with the other vaccinated groups. At 35 DPV, the EU2 and US2 groups had significantly higher SN titers compared with the other vaccinated groups. At 42 DPV, the EU2, US1, US2 and US4 groups



◀**Fig. 3** **A** Antibody responses as measured by serum neutralizing (SN) assay using homologous virus as a recall antigen in the NEG (■), EU1 (—), EU2 (—), US1 (—), US2 (—), US3 (—) and US4 (—) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistical significant difference ($p < 0.05$) between groups. **B.** Antibody responses as measured by serum neutralization (SN) assay using heterologous genotype I virus (SB_EU02) as a recall antigen of the NEG (■), EU1 (—), EU2 (—), US1 (—), US2 (—), US3 (—) and US4 (—) groups, respectively. Variation is expressed as standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups. **C.** Antibody responses as measured by serum neutralizing (SN) assay using heterologous genotype II virus (ST_US01) as a recall antigen in the NEG (■), EU1 (—), EU2 (—), US1 (—), US2 (—), US3 (—) and US4 (—) groups. Variation is expressed as the standard deviation. Different letters in superscript indicate a statistically significant difference ($p < 0.05$) between groups

had significantly higher SN titers compared with the EU1 and US3 groups.

For the heterologous genotype I virus (SB_EU02), the kinetics of the SN response differed in a manner that was dependent on the MLV (Fig. 3B). Compared with homologous virus, the SN titers were relatively low. The SN titers remained at a similar level from 28 to 42 DPV and then declined by 84 DPV. However, the SN titers in the US1 group were significantly higher than in the other vaccinated groups from 28 to 84 DPV and were significantly higher than those for the homologous virus.

In the case of heterologous genotype II virus (ST_US01), the kinetics of the SN response were similar to those evoked using homologous genotype I virus (SB_EU02) (Fig. 3C). SN titers were lower than with homologous genotype I virus in all groups, except for the EU2 group. The SN titers of the EU2 group were significantly higher on 35 and 42 DPV compared with the other vaccinated groups.

Sentinel pigs

Sentinel pigs introduced to the EU2, US2 and US3 groups at 60 DPV seroconverted, but those in the EU1, US1 and US4 groups did not. Sentinel pigs that were placed in contact with pigs of all groups on 84 DPV did not seroconvert over a 7- or 14-day period of observation.

Discussion

We compared the efficacy of six different PRRSV MLVs in the induction of antibody responses in PRRSV-free pigs. All six MLVs rapidly induced antibody responses as measured by ELISA. The antibody responses were detected as early as 7-14 DPV in an MLV-genotype-dependent manner. The antibody levels in all vaccinated groups were similar at 21 DPV. It was notable that there was a difference in early antibody detection between two genotype I MLVs. The EU1 group had a significantly lower S/P ratio than the EU2 group at 14 DPV, and the S/P ratio was below 0.4, the cutoff level. Surprisingly, the EU2 group produced an antibody response at a similar level when compared with the genotype II MLVs. In summary, there was no difference in the antibody responses as measured by ELISA for any of the MLV genotypes. The results of the present study suggest that the MLV genotype is not the key factor

in the induction of immunity, but the specific virus isolate used for the vaccines might play an important role. Moreover, it is notable that the antibody detection in the present study was performed using Idexx ELISA, which can simultaneously detect specific antibodies against PRRSV genotype I and II infections. However, these findings may not be applicable when using other diagnostic kits.

Following MLV vaccination, antibody responses measured by the SN assay were delayed regardless of the MLV genotype and isolate in the vaccines. The responses were detected as early as 28 DPV. In addition, the response was isolate-specific. Homologous responses generated using a homologous virus induced a higher, although delayed response compared with the heterologous responses generated using either heterologous genotype I or II viruses. Heterologous responses were lower and shorter in duration. Our finding of differences in responses was not surprising. PRRSV isolates differed in their susceptibility to neutralization [11], and the mechanisms associated with this susceptibility remain poorly characterized, although the influence of N-linked glycosylation in decoy epitope regions could be one key factor [9, 12, 13]. A previous study demonstrated that a heterologous response could be higher or lower [4], depending on the isolates that were used in the assay.

In addition to their ability to induce an immune response, the shedding patterns of the vaccine viruses were investigated using three different measurements, including the duration of viremia, the detection of viral RNA in tonsils, and infection of sentinel pigs. After vaccination, we detected a difference in the shedding patterns between MLVs. The two genotype I MLVs had a shorter viremic phase compared with genotype II MLVs. However, the magnitude of viral titers was not different. In addition, there was a difference in the shedding pattern of genotype I MLV, although one genotype I MLV had a shedding pattern that resembled that of a genotype II MLV. Viremia could not be detected in one genotype I MLV. This finding could indicate the absence of viremia or that the quantity of virus in the serum was lower than the limit of detection of the real-time PCR assay. Within genotype II MLVs, all three MLVs caused viremia as early as 3 DPV, and it then declined thereafter. In contrast, the titers of one genotype II MLV continued to increase until 7 DPV and then declined. These findings suggested that the viremic phase of MLV was associated with the virus isolate used in the vaccine, not the virus genotype.

To further evaluate the viral shedding pattern, sentinel pigs were used. Sentinel pigs were housed along with principal pigs of the EU2, US2 and US3 groups in the same pen beginning on day 60, and they were found to undergo seroconversion. However, sentinel pigs introduced at 84 DPV remained uninfected, as indicated by their failure to seroconvert. The shedding patterns of vaccine viruses over this long period of time have not yet been investigated. Compared to wild-type PRRSV, vaccine viruses should be shed to sentinel pigs over a shorter time. Previous studies conducted by several investigators to characterize several wild-type field isolates of PRRSV and the duration of PRRSV shedding to sentinel pigs have suggested that virus shedding to sentinel pigs occurred on average 60 to 70 days after exposure [14]. Although vaccine viruses were not transmitted to sentinel pigs at 84 DPV, the detection of viral-RNA-positive samples in the tonsil scraping samples might represent a risk factor for the shedding of vaccine viruses.

The PCR results from tonsil scrapings at 84 DPV indicated that vaccinated pigs still harbored viral RNA. However, whether the RNA-positive samples represented infectious viruses was not determined. The detection of viral RNA does not necessarily indicate the isolation of infectious virus. Any viral genomic material needs to be tested further to determine whether the pigs may still be infectious and contagious. Using a swine bioassay, it was demonstrated that homogenates from tonsils collected from pigs infected with the PRRSV strain VR-2332 at 105 days post-exposure remained infectious [15]. Viral RNA was detected in the tonsils, suggesting that viruses remained present in both groups of pigs but were not transmitted to contact sentinel pigs. Determining whether virus shedding can be reinitiated will require further study.

In conclusion, based on the induction of immune responses, all MLV genotypes yield a similar immune-response pattern. Measurement of the antibody response by ELISA is quick, but the response measured using an SN assay is delayed and isolate-specific. However, the shedding pattern of a vaccine virus is influenced by the isolate that is used to manufacture the vaccine. The criteria for MLV selection should be based on the shorter duration of vaccine virus shedding and the broader response against heterologous virus.

Acknowledgements The authors are grateful to the Thailand Research Fund and Government budget year 2015-16 for funding this research and partial funding provided by Special Task Force for Activating Research (STAR), Swine Viral Evolution and Vaccine Research (SVEVR), Chulalongkorn University.

Compliance with ethical standards

The study was funded by the Thailand Research Fund (Grant Number PHD59I0040) and Government budget year 2015.

Conflict of interest The authors declare that they have no conflicts of interest related to this work.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

1. Done SH, Paton DJ, White MEC (1996) Porcine reproductive and respiratory syndrome (PRRS): a review, with emphasis on pathological, virological and diagnostic aspects. Brit Vet J 152:153–174
2. Murtaugh MP, Genzow M (2011) Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine 29:8192–8204
3. Plana-Duran J, Bastons M, Urniza A, Vayreda M, Vila X, Mane H (1997) Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. Vet Microbiol 55:361–370
4. Ferrari L, Martelli P, Saleri R, De Angelis E, Cavalli V, Bresaola M, Benetti M, Borghetti P (2013) Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. Vet Immunol Immunopathol 151:193–206
5. LeRoith T, Hammond S, Todd SM, Ni Y, Cecere T, Pelzer KD (2011) A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with *Mycoplasma hyopneumoniae*. Vet Immunol Immunopathol 140:312–316
6. Mengeling WL, Vorwald AC, Lager KM, Clouser DF, Wesley RD (1999) Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. Am J Vet Res 60:334–340
7. Park C, Seo HW, Han K, Kang I, Chae C (2014) Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge. Vet Microbiol 172:432–442
8. Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA (1995) Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 32:648–660
9. Nilubol D, Tripipat T, Hoonsuwan T, Tipsombatboon P, Piriyapongsa J (2014) Dynamics and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 following modified live PRRSV vaccination in a PRRSV-infected herd. Arch Virol 159:17–27
10. Nilubol D, Platt KB, Halbur PG, Torremorell M, Harris DL (2004) The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. Vet Microbiol 102:11–18
11. Martinez-Lobo FJ, Diez-Fuertes F, Simarro I, Castro JM, Prieto C (2011) Porcine reproductive and respiratory syndrome virus isolates differ in their susceptibility to neutralization. Vaccine 29:6928–6940
12. Nilubol D, Tripipat T, Hoonsuwan T, Tipsombatboon P, Piriyapongsa J (2013) Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes I and II in Thailand. Arch Virol 158:943–953
13. Plagemann PG, Rowland RR, Faaberg KS (2002) The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch Virol 147:2327–2347
14. Wills RW, Doster AR, Galeota JA, Sur J-H, Osorio FA (2003) Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J Clin Microbiol 41:58–62
15. Horter D, Chang CC, Pogranichnyy R, Zimmerman J, Yoon KJ (2001) Persistence of porcine reproductive and respiratory syndrome in pigs. Adv Exp Med Biol 494:91–94

Appendix B

Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2

Adthakorn Madapong[1], Kepalee Saeng-chuto[1], Alongkot Boonsoongnern[2], Angkana Tantituvanont[3], Dachrit Nilubol[1,*]

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine Kamphaeng Sean Campus, Kasetsart University, Nakhon Pathom, Thailand
[3]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

*Corresponding author:
Assistant Professor Dr. Dachrit Nilubol
E-mail address: dachrit@gmail.com, ndachrit@chula.ac.th
Department of Veterinary Microbiology, Faculty of Veterinary Science
Chulalongkorn University, Bangkok 10330, Thailand

Keywords: Porcine reproductive and respiratory syndrome virus, modified-live virus vaccine, cell-mediated immunity, co-challenge, protective efficacy, HP-PRRSV

www.nature.com/scientificreports

SCIENTIFIC REPORTS
natureresearch

OPEN

Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2

Adthakorn Madapong[1], Kepalee Saeng-chuto[1], Alongkot Boonsoongnern[2], Angkana Tantituvanont[3] & Dachrit Nilubol[1*]

Cell-mediated immunity (CMI), IL-10, and the protective efficacy of modified-live porcine reproductive and respiratory syndrome virus (PRRSV) vaccines (MLV) against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV) were investigated. Seventy, PRRSV-free, 3-week old, pigs were allocated into 7 groups. Six groups were intramuscularly vaccinated with MLV, including Porcilis (PRRSV-1 MLV, MSD Animal Health, The Netherlands), Amervac (PRRSV-1 MLV, Laboratorios Hipra, Spain), Fostera (PRRSV-2 MLV, Zoetis, USA), Ingelvac PRRS MLV and Ingelvac PRRS ATP (PRRSV-2, Boehringer Ingelheim, USA), and Prime Pac PRRS (PRRSV-2 MLV, MSD Animal Health, The Netherlands). Unvaccinated pigs were left as control. Lymphocyte proliferative response, IL-10 and IFN-γ production were determined. At 35 days post-vaccination (DPV), all pigs were inoculated intranasally with 2 ml of each PRRSV-1 ($10^{5.4}$ $TCID_{50}$/ml) and PRRSV-2 ($10^{5.2}$ $TCID_{50}$/ml, HP-PRRSV). Following challenge, sera were quantitatively assayed for PRRSV RNA. Pigs were necropsied at 7 days post-challenge. Viremia, macro- and microscopic lung lesion together with PRRSV antigen presence were evaluated in lung tissues. The results demonstrated that, regardless of vaccine genotype, CMI induced by all MLVs was relatively slow. Increased production of IL-10 in all vaccinated groups was observed at 7 and 14 DPV. Pigs in Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher levels of IL-10 compared to Porcilis, Fostera and Prime Pac groups at 7 and 14 DPV. Following challenge, regardless to vaccine genotype, vaccinated pigs had significantly lower lung lesion scores and PRRSV antigens than those in the control group. Both PRRSV-1 and PRRSV-2 RNA were significantly reduced. Prime Pac pigs had lowest PRRSV-1 and PRRSV-2 RNA in serum, and micro- and macroscopic lung lesion scores ($p < 0.05$) compared to other vaccinated groups. In conclusion, PRRSV MLVs, regardless of vaccine genotype, can reduce viremia and lung lesions following co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). The main difference between PRRSV MLV is the production of IL-10 following vaccination.

Porcine reproductive and respiratory syndrome (PRRS) is a devastating disease in pigs characterized by reproductive and respiratory failures. PRRS virus (PRRSV), an enveloped, positive-sense single-stranded RNA virus belonging to the *Arteriviridae* family, order *Nidovirales*, is the causative agent[1]. Two antigenically distinct genotypes of PRRSV, PRRSV-1 and PRRSV-2, have been recognized. The genomes of both genotypes are 15 kb in

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. [2]Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine Kamphaeng Saen Campus, Kasetsart University, Nakon Pathom, Thailand. [3]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. *email: dachrit@gmail.com

Treatment groups	No. of pigs	Vaccination	Vaccines	Vaccine genotype	Dosage and route of administration	Manufacturers
NonVac	10	No	—	—	—	—
Porcilis	10	Yes	Porcilis PRRS	PRRSV-1	2 ml, intramuscular	MSD Animal Health, The Netherlands
Amervac	10	Yes	Amervac PRRS	PRRSV-1	2 ml, intramuscular	Laboratorios Hipra, Spain
Fostera	10	Yes	Fostera PRRS	PRRSV-2	2 ml, intramuscular	Zoetis, USA
Ingelvac MLV	10	Yes	Ingelvac PRRS MLV	PRRSV-2	2 ml, intramuscular	Boehringer Ingelheim, Germany
Ingelvac ATP	10	Yes	Ingelvac PRRS ATP	PRRSV-2	2 ml, intramuscular	Boehringer Ingelheim, Germany
Prime Pac	10	Yes	Prime Pac PRRS	PRRSV-2	1 ml, intramuscular	MSD Animal Health, The Netherlands

Table 1. Experimental design. The pigs were allocated into seven treatment groups and vaccinated with six different PRRSV MLVs. The NonVac group was kept as unvaccinated control group.

length and consist of 10 open reading frames (ORFs). The genotypes of PRRSV-1 and PRRSV-2 are markedly different based on the full-length genomes, which share only approximately 60% similarity at the nucleotide level[2].

PRRSV is recognized for its high genetic variation. Presently, PRRSV-1 and PRRSV-2 have continuously evolved into 3 subtypes and 9 lineages, respectively[3,4]. PRRSV-1 and PRRSV-2 have independently evolved in the European and North American (NA) continents. However, in Asia, the co-existence of both types has been increasingly evident in several countries, including Thailand, China, and Korea[5–7]. Additionally, variants of PRRSV-2 endemically present in Asia are genetically related to HP-PRRSV lineage 8.7/HP-PRRSV[8–10].

Several PRRSV modified-live vaccines (MLV) against PRRSV-1 and PRRSV-2 have been commercially available and licensed in several countries worldwide depending on circulating virus genotypes. The use of PRRSV MLV depends on PRRSV genotype circulating in that region. However, questions have been raised as to what types of MLV should be used in the co-presence of PRRSV-1 and PRRSV-2. The criteria for vaccine selection should include the induction of the cell-mediated immunity (CMI) and the protection against PRRSV infection, especially against genotypes and isolates that are circulating in the affected region. Therefore, the present study was conducted to investigate CMI, IL-10, and protective efficacy of commercial PRRSV-1 and PRRSV-2 MLVs against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). Our results revealed that vaccination with PRRSV MLVs, regardless of vaccine genotype, provide partial cross-protection against PRRSV infection. Additionally, this approach provided novel information regarding the vaccine selection for use in the presence of co-existence of both PRRSV genotypes.

Materials and Methods

Ethical statement for experimental procedures. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council of Thailand according to protocols reviewed and approved by the Chulalongkorn University Animal Care and Use Committee (protocol number 1731047).

Seventy, 21-day-old pigs were procured from a PRRS-free herd. Upon arrival, pigs were randomly allocated based on the stratification of weight into 7 treatment groups consisting of NonVac, Porcilis, Amervac, Fostera, Ingelvac MLV, Ingelvac ATP and Prime Pac (Table 1). Following a week of acclimatization, pigs were vaccinated with PRRS MLVs. NonVac was left unvaccinated. Porcilis and Amervac were vaccinated with Porcilis PRRS (PRRSV-1, MSD Animal Health, Boxmeer, the Netherlands) and Amervac PRRS (PRRSV-1, Laboratorios Hipra, Girona, Spain), respectively. Fostera, Ingelvac MLV, Ingelvac ATP and Prime Pac were vaccinated with Fostera PRRS (PRRSV-2, Zoetis, Troy Hills, USA), Ingelvac PRRS MLV (PRRSV-2, Boehringer Ingelheim, Rhein, Germany), Ingelvac PRRS ATP (PRRSV-2, Boehringer Ingelheim, Rhein, Germany) and Prime Pac PRRS (PRRSV-2, MSD Animal Health, Boxmeer, the Netherlands), respectively. Dosages and routes of administration were in accordance with manufacturers' instructions. Blood samples were collected at 0, 7, 14, 21, 28, 35 days post-vaccination (DPV). Peripheral blood mononuclear cells (PBMC) were isolated and assayed for lymphocyte proliferative response. IFN-γ and IL-10 were measured using flowcytometry, and ELISPOT or ELISA. At 35 DPV, all pigs were inoculated intranasally with PRRSV. Each pig received 2 ml (1 ml/nostril) of each PRRSV-1 (AN06EU4204) and PRRSV-2 (FDT10US23) at $10^{5.4}$ $TCID_{50}$/ml and $10^{5.2}$ $TCID_{50}$/ml, respectively. Sera were collected at 0, 3, 5 and 7 days post-challenge (DPC) and quantitatively assayed for PRRSV RNA using qPCR. All pigs were necropsied at 7 DPC. The severity of PRRSV-induced pneumonic lung lesion was scored[11]. Lung tissues were collected for histopathological examination and immunohistochemistry (IHC).

Virus isolates. Homologous and heterologous viruses were used as recall antigens in *in vitro* CMI and IL-10 assays. Homologous viruses refer to vaccine strains as previously described[12]. Heterologous viruses refer to AN06EU4204 and FDT10US23, which were Thai PRRSV-1 and PRRSV-2 (HP-PRRSV) isolates, respectively. AN06EU4204 and FDT10US23 are in Clade A, Subtype 1 and Lineage 8.7/HP-PRRSV, respectively, based on systematic classification previously described[3,4]. ORF5 gene sequences of AN06EU4204 and FDT10US23 are available in GenBank under accession numbers JQ040750 and JN255836, respectively. The nucleotide and amino acid similarities based on the ORF5 gene between these two isolates and PRRSV MLVs were summarized in Table 2.

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using gradient density centrifugation (Lymphosep, Biowest, Riverside, MO, USA) as previously described[13]. Isolated PBMC were resuspended in 1 ml complete media (RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 μg/ml gentamicin). The viability of PBMC

PRRSV (isolates)	Classification*	Nucleotide and amino acid similarities						
		Level of similarity	Porcilis® PRRSV	Amervac® PRRSV	Fostera™ PRRS	Ingelvac® PRRS MLV	Ingelvac® PRRS ATP	Prime Pac® PRRS
PRRSV-1 (AN06EU4204)	Subtype I (Clade A)	Nucleotide	95.8%	92.7%	68.5%	68.3%	68.2%	67.9%
		Amino acid	92.0%	89.1%	60.9%	58.2%	55.5%	55.7%
PRRSV-2 (FDT10US23)	Lineage 8.7/HP-PRRSV	Nucleotide	68.8%	69.9%	94.0%	88.8%	90.2%	90.5%
		Amino acid	58.7%	59.8%	91.5%	87.5%	89.5%	91.8%

Table 2. Nucleotide and amino acid similarities based on ORF5 gene between vaccine strains and Thai PRRSV isolates. *International systematic classification was based on previously described, including PRRSV-1[3] and PRRSV-2[4], respectively.

were determined by Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) staining and more than 90% viability was used for lymphocyte proliferation assay, lymphocytes producing either IL-10 or IFN-γ, IFN-γ ELISPOT assay, and *in vitro* stimulation for IL-10 detection as described below.

Lymphocyte proliferation assay. The lymphocyte proliferation assay assesses cell proliferation using membrane-bound 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) and cell surface markers using flow cytometry. Briefly, 1×10^7 cells/ml PBMC were incubated with CFSE at 37 °C for 10 min. After washing, CFSE-stained PBMC at 1×10^6 cells were seeded into 96-well plate and co-cultured with MARC-145 cell lysate (mock suspension), PHA (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA), homologous and heterologous PRRSV at 0.01 multiplicity of infection (MOI). Following 5-day incubation, PBMC were stained with mouse anti-porcine CD4-FITC antibody (clone 74-12-14, SouthernBiotech, Birmingham, AL, USA) and mouse anti-porcine CD8-SPRD antibody (clone 76-2-11, SouthernBiotech, Birmingham, AL, USA). After washing, PBMC were suspended in 2% paraformaldehyde. The proliferation of T lymphocyte populations was measured using flow cytometry analysis (Beckman FC550, Beckman Coulter, Brea, CA, USA) with CXP software. The relative proliferative indices (PI) were calculated by using the percentage of proliferating cells in the virus stimulated well divided by the percentage of proliferating cells in the mock suspension well.

Lymphocytes producing either IL-10 or IFN-γ. The percentage of PRRSV-specific lymphocytes producing either IL-10 or IFN-γ after *in vitro* stimulation with homologous or heterologous PRRSV were evaluated using a method previously described[13]. Briefly, 1×10^6 PBMC were seeded into a 96-well plate containing mock suspension, PMA (25 ng/ml)/ionomycin (1 μM) (Sigma-Aldrich, St. Louis, MO, USA), and homologous and heterologous PRRSV at 0.01 MOI, and incubated for 96 hours. Following incubation, protein transport inhibitor (BD GolgiStop, BD Biosciences, San Jose, CA, USA) was added 12 hours prior to cell harvesting and labeled PBMC were stained with mouse anti-porcine CD4-FITC antibody (clone 74-12-4, SouthernBiotech, Birmingham, AL, USA) and mouse anti-porcine CD8-SPRD antibody (clone 76-2-11, SouthernBiotech, Birmingham, AL, USA). Cells were subsequently fixed with fixation buffer (Leucoperm reagent A, Bio-Rad Laboratories, Hercules, CA, USA) for 15 min, washed and then separately incubated with either mouse anti-porcine IFN-γ-biotin antibody (clone P2C11, BD Pharmingen, San Jose, CA, USA) or mouse anti-porcine IL-10-biotin antibody (clone 945 A 1A9 26C2, Invitrogen, Carlsbad, CA, USA) in Leucoperm reagent B (Bio-rad Laboratories, Hercules, CA, USA). Subsequently, streptavidin-PE-Cy7 (Thermo Fisher Scientific, Waltham, MA, USA) were added and incubated for 30 min at 4 °C. After washing, stained cells were suspended in 2% paraformaldehyde and analyzed by flow cytometer (Beckman FC550, Beckman Coulter, Brea, CA, USA) with CXP software. The results are based on lymphocyte gating on a forward scatter versus side scatter graph after acquiring at least 20,000 cell events.

Enzyme-linked immunospot (ELISPOT) assay. The numbers of PRRSV-specific interferon-γ-producing cells (IFN-γ-PC) were determined using ELISPOT kit (R&D Systems, Minneapolis, MN, USA). Briefly, 2×10^5 PBMC were stimulated with either homologous or heterologous PRRSV at 0.01 MOI or PHA (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 20 hours at 37 °C in 5% CO_2. Spots were counted by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). PRRSV-specific IFN-γ-PC was expressed as spot forming colonies per million of PBMCs in each well.

Quantification of porcine interleukin-10. Porcine interleukin-10 (IL-10) concentration was quantified in the supernatant of stimulated PBMC (2×10^6 cells/well) cultured *in vitro* for 20 hours with homologous and heterologous PRRSV (0.01 of MOI) or PHA (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) using the porcine ELISA IL-10 kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction.

Quantification of PRRSV RNA. The PRRSV RNA in serum was evaluated by quantitative PCR (qPCR) after PRRSV challenge. The primers specific for the ORF5 gene of either PRRSV-1 or PRRSV-2 and detection conditions were described previously[12]. In brief, total RNA was extracted using NucleoSpin RNA Virus extraction kit (Macherey-Nagel, Duren, Germany) in accordance with manufacturer's instructions. The quality of RNA was measured using spectrophotometer (Colibri, Titertek-Berthold, Pforzheim, Germany), and converted to cDNA. All cDNA was used for quantitative PCR (qPCR). PRRSV RNA was quantified using ABI PRISM 7500 Real time PCR platform (Applied Biosystem, CA, USA). Primers specific for the ORF5 gene of either PRRSV-1 or PRRSV 2 were used. Each qPCR reaction contained 0.1 μg of cDNA, 0.2 μM of each primers, 1x Eva Green real-time-PCR

master mix E4 (GeneOn GmbH, Ludwigshafen, Germany), and deionized water to yield a 20 ul final volume. The thermal profile for qPCR was 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and fluorescence acquisition at 72 °C for 45 s. pGEM-T Easy Vector (Promega, WI, USA) containing an inserted ORF5 gene of each PRRSV was used to construct plasmid standards. A standard curve was generated using serial diluted plasmid standards of 10^0–10^7 copies/μl. Copy number of the PRRSV RNA was calculated using standard curve method.

Pathological examination and immunohistochemistry. All pigs were necropsied at 7 DPC. PRRSV-induced pneumonic lung lesions were macroscopically and microscopically evaluated as previously described[11]. For the macroscopic lung lesion score, each lung lobe was assigned a number to reflex the approximate percentage of the volume of the entire lung and the percentage volume form each lobe added to the entire lung score (ranged from 0 to 100% of the affected lung). For the microscopic lung lesion score, lung sections were blind. Histopathological changed were examined and an estimated score of the severity of the interstitial pneumonia was given as follows: 0 = no microscopic lesions; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; and 4 = severe interstitial pneumonia. The mean values of microscopic score of each group were calculated.

Immunohistochemistry was performed using monoclonal antibodies (MAbs) A35 and JP24, which recognized PRRSV-1 and PRRSV-2 antigens, respectively (kindly provided by Dr. Erwin van den Born, the Netherlands). Tissues were processed and placed on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA). Sections were deparaffinized, rehydrated using an alcohol gradient and air-dried. All slides were treated with proteinase K (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 30 min. Endogenous alkaline phosphatase was quenched with 0.3% hydrogen peroxide for 5 min. All slides were then incubated with BSA for 30 min. The slides were separately incubated with monoclonal antibodies overnight at 4 °C in a humidified chamber. After washing, PRRSV antigen was visualized by binding with secondary antibody conjugated with horseradish peroxidase conjugated (HRP)-labeled polymer followed by immersion in peroxidase (Agilent, Santa Clara, CA, USA). Slides were counterstained with Meyer's hematoxylin, dehydrated through graded concentrations of ethanol and xylene, and then mounted. Lung tissues from pigs in the unvaccinated unchallenged group served as negative controls. To obtain quantitative data, slides were analyzed with the NIH Image J 1.50i Program (http://rsb.info.nih.gov/ij). In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm^2) was determined as previously described[14,15]. The mean values were calculated.

Statistical analysis. The data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed by ANOVA to determine the presence of significant differences between treatment groups for each day. If the p-value for the ANOVA was <0.05, the differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $p < 0.05$ significance level.

Results

Lymphocyte proliferation response using CFSE. Upon *in vitro* stimulation with either homologous or heterologous PRRSV, all vaccination groups, regardless of vaccine genotype, had relatively low lymphocyte proliferative indices following vaccination. A significantly increased response was not observed in any vaccination group, and the responses were not different among all of the vaccination groups (Fig. 1).

Lymphocyte populations producing IL-10. Following vaccination, lymphocyte populations producing IL-10 (L-IL-10) were detected in all vaccination groups at 7 and 14 DPV, regardless of vaccine genotype (Fig. 2). The percentage of L-IL-10 declined to a nondetectable level from 21 to 35 DPV. L-IL-10 was mainly produced by $CD4^+$ cells. At 7 DPV, the Ingelvac MLV group had the highest amount of $CD4^+IL\text{-}10^+$ cells as compared to the PRRSV-1 or PRRSV-2 MLV vaccination and NonVac groups (Fig. 2A). $CD4^+IL\text{-}10^+$ cells in the Amervac, Ingelvac MLV and Ingelvac ATP groups were significantly higher than those in the other vaccination groups at 14 DPV. The Porcilis, Fostera and Prime Pac groups had the lowest amount of $CD4^+IL\text{-}10^+$ cells as compared to other PRRSV-1 and PRRSV-2 MLV vaccination groups ($p < 0.05$) at both 7 and 14 DPV.

Similar to $CD4^+IL\text{-}10^+$ cells, all vaccination groups had significantly more $CD8^+IL\text{-}10^+$ cells as compared to the NonVac group (Fig. 2B). Although there was no difference in $CD8^+IL\text{-}10^+$ cells among vaccination groups at 7 DPV, the Amervac, Ingelvac MLV, and Ingelvac ATP groups had significantly more $CD8^+IL\text{-}10^+$ cells than did the Porcilis, Fostera and Prime Pac groups at 14 DPV. Additionally, the Amervac group had the highest amount of $CD8^+IL\text{-}10^+$ cells as compared to other vaccination groups as 14 DPV. All vaccination groups had relatively more $CD4^+CD8^+IL\text{-}10^+$ cells than did the NonVac group, and $CD4^+CD8^+IL\text{-}10^+$ cell numbers were not different between the vaccination groups (Fig. 2C).

Similar to homologous virus stimulation, L-IL-10 was detected in all vaccination groups at 7 and 14 DPV after stimulation with PRRSV-1 (AN06EU4204) and was not detected from 21 to 35 DPV (Fig. 2D–F). All vaccination groups had higher amounts of $CD4^+IL\text{-}10^+$ cells than did the NonVac group at 7 and 14 DPV (Fig. 2D). The Amervac group had the highest amount of $CD4^+IL\text{-}10^+$ cells as compared to the PRRSV-1 and PRRSV-2 MLV vaccination groups at 7 DPV ($p < 0.05$). Meanwhile, at 14 DPV, there were no differences in $CD4^+IL\text{-}10^+$ cells among all of the vaccination groups.

The Porcilis, Amervac, Fostera and Prime Pac groups had significantly more $CD8^+IL\text{-}10^+$ cells than the NonVac, Ingelvac MLV and Ingelvac ATP groups at 7 DPV (Fig. 2E). However, at 14 DPV, all vaccination groups had significantly more $CD8^+IL\text{-}10^+$ cells than the NonVac group. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly more $CD8^+IL\text{-}10^+$ cells than the Porcilis, Fostera and Prime Pac groups ($p < 0.05$).

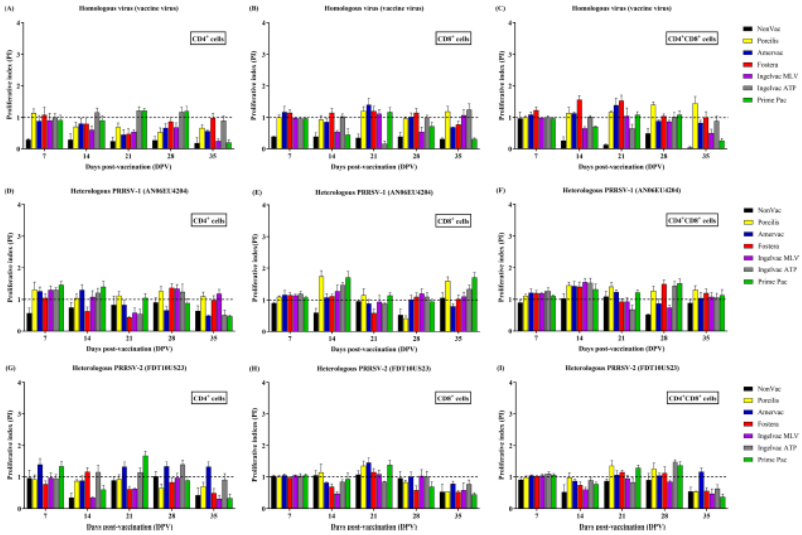


Figure 1. Lymphocyte proliferative index (PI) following vaccination. (**A**–**C**) Homologous virus (vaccine strain), (**D**–**F**) heterologous PRRSV-1 (AN06EU4204), and (**G**–**I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations were identified by flow cytometry using CFSE and cell surfaces staining, including $CD4^+$ cells (**A**,**D**,**G**), $CD8^+$ cells (**B**,**E**,**H**), and $CD4^+CD8^+$ cells (**C**,**F**,**I**), respectively. Values are expressed as mean±SEM. Dash lines indicate the cut-off level.

All vaccination groups had more $CD4^+CD8^+IL\text{-}10^+$ cells than the NonVac group at 7 and 14 DPV (Fig. 2F). At 7 DPV, no significant differences were detected in the amount of $CD4^+CD8^+IL\text{-}10^+$ among all of the vaccination groups. In contrast, the Ingelvac MLV and Ingelvac ATP groups had more $CD4^+CD8^+IL\text{-}10^+$ cells than the other vaccination groups ($p < 0.05$) at 14 DPV.

Following heterologous stimulation with PRRSV-2 (FDT10US23), L-IL-10 was detected at 7 and 14 DPV but not at 21 to 35 DPV (Fig. 2G–I). At both 7 and 14 DPV, all vaccination groups had higher levels than the NonVac group. At 7 DPV, the Amervac and Fostera groups had significantly more $CD4^+IL\text{-}10^+$ cells than the Porcilis, Ingelvac MLV, Ingelvac ATP and Prime Pac groups ($p < 0.05$). However, at 14 DPC, the amount of $CD4^+IL\text{-}10^+$ cells was the highest in the Ingelvac MLV and Ingelvac ATP groups as compared to the other vaccination groups (Fig. 2G). In contrast, $CD8^+IL\text{-}10^+$ cells were only detected at 7 DPV (Fig. 2H) in all vaccination groups, and the Porcilis group had more $CD8^+IL\text{-}10^+$ cells than the other groups. There were no differences in $CD4^+CD8^+IL\text{-}10^+$ cells among all of the vaccination groups after stimulation with PRRSV-2 (Fig. 2I).

Lymphocyte Populations Producing IFN-γ. Lymphocyte populations producing IFN-γ (L-IFN-γ) were detected after stimulation with either homologous or heterologous PRRSV as early as 21 DPV at levels less than 1% in all vaccination groups and showed no statistical differences between vaccination groups. Soon after detection, L-IFN-γ gradually increased until 35 DPV (Fig. 3). The lymphocyte population response was toward both $CD4^+$ and $CD8^+$. Immediately after homologous stimulation, all vaccination groups had relatively more $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells than the NonVac group but showed no differences thereafter (Fig. 3A–C). Similar to homologous stimulation, all vaccination groups had relatively more $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells after stimulation with heterologous PRRSV-1 (AN06EU4204) than the NonVac group but showed no difference among vaccination groups (Fig. 3D–F). However, after heterologous PRRSV-2 (FDT10US23) stimulation, $CD8^+IFN\text{-}\gamma^+$ cells in all vaccination groups were detected only at 21 DPV and were significantly greater in number than in the NonVac group (Fig. 3H).

The number of PRRSV-specific IFN-γ-PC. Regardless of homologous or heterologous stimulation, IFN-γ-PC of all vaccination groups were first detected at 35 DPV (Fig. 4). After homologous stimulation, all vaccination groups had significantly more IFN-γ-PC than the NonVac group at 35 DPV and 7 DPC. The Fostera group had significantly fewer IFN-γ-PC than the other vaccination groups at 35 DPV (Fig. 4A). After heterologous PRRSV-1 (AN06EU4204) stimulation, all vaccination groups had significantly more IFN-γ-PC than NonVac group at 35 DPV and 7 DPC. The Fostera group had significantly fewer IFN-γ-PC than the other vaccination

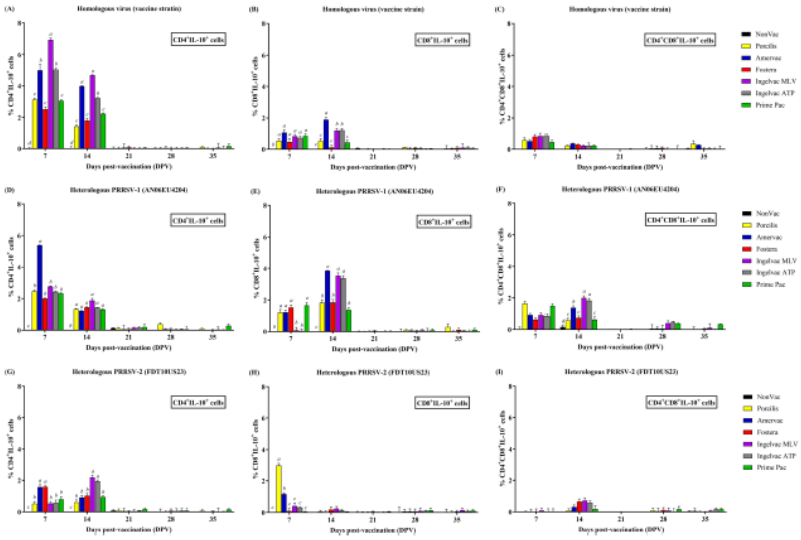


Figure 2. Lymphocyte populations producing IL-10 following vaccination. (**A**–**C**) Homologous virus (vaccine strain), (**D**–**F**) heterologous PRRSV-1 (AN06EU4204), and (**G**–**I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations producing IL-10 were identified by flow cytometry using cell surfaces and intracellular IL-10 staining, including $CD4^+IL\text{-}10^+$ cells (**A**,**D**,**G**), $CD8^+IL\text{-}10^+$ cells (**B**,**E**,**H**), and $CD4^+CD8^+IL\text{-}10^+$ cells (**C**,**F**,**I**), respectively. Values are expressed as mean±SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

groups at 35 DPV (Fig. 4B). After heterologous PRRSV-2 (FDT10US23) stimulation, IFN-γ-PC numbers were lower in all vaccination groups than after homologous stimulation with the exception of the Prime Pac group, which had significantly more IFN-γ-PC than the other groups at 35 DPV and 7 DPC. IFN-γ-PC were less abundant in Amervac the group than the other vaccination groups and were not different from those in the NonVac group at 35 DPV (Fig. 4C).

Porcine IL-10 production. After homologous stimulation, IL-10 levels in all vaccination groups increased and were significantly higher than that in the NonVac group at 7 DPV (Fig. 5A). IL-10 levels of all vaccination groups peaked at 14 DPV and gradually decreased until they were similar to that of the NonVac group at 35 DPV. At 7 DPV, no differences were detected in IL-10 levels between the vaccination groups. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels at 14 DPV than the Porcilis, Fostera and Prime Pac groups. The IL-10 levels of the Ingelvac MLV and Ingelvac ATP groups remained significantly higher at 21 DPV compared to those of the other vaccination groups. No differences were detected in IL-10 among all of the vaccination groups at 28 or 35 DPV.

After heterologous PRRSV-1 (AN06EU4204) stimulation, IL-10 levels were significantly higher in all vaccination groups than that in the NonVac group (Fig. 5B). The Amervac group had a significantly higher IL-10 level than did the other vaccination groups at 7 DPV. However, no differences were detected in IL-10 in all vaccination groups from 14 to 28 DPV, except for the Fostera group. The IL-10 level was significantly lower in the Fostera group on 28 DPV than those in the other vaccination groups.

After heterologous PRRSV-2 (FDT10US23) stimulation, all vaccination groups had significantly higher IL-10 levels than the NonVac group (Fig. 5C). The Amervac and Fostera groups had significantly higher IL-10 levels at 7 DPV as compared to those of the other vaccination groups. The IL-10 levels in all vaccination groups, except for the Ingelvac MLV and Ingelvac ATP groups, continuously decreased from 7 to 35 DPC. The Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels at 14 and 21 DPV as compared to those of the other vaccination groups. At 28 DPV, IL-10 levels were significantly lower in the Fostera and Prime Pac groups than in the other vaccination groups but were still significantly higher than that the NonVac group. No statistical differences were observed in IL-10 levels between vaccination groups at 35 DPV.

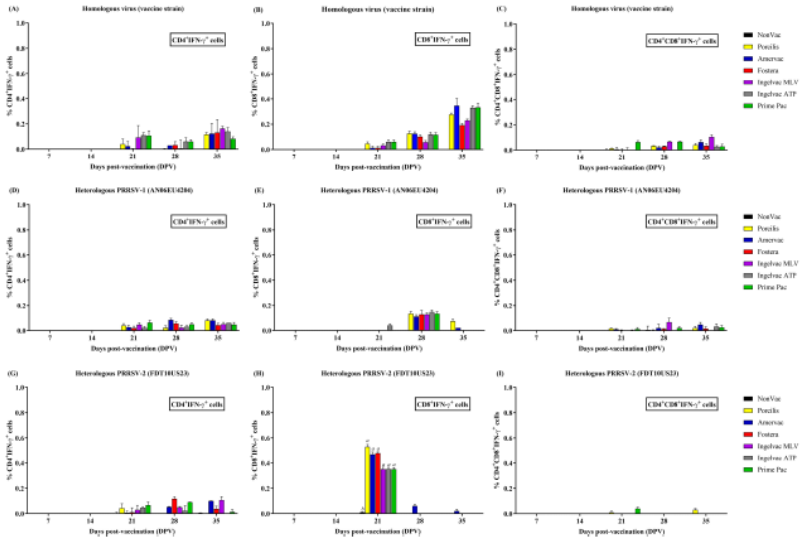


Figure 3. Lymphocyte populations producing IFN-γ following vaccination. (**A**–**C**) homologous virus (vaccine strain), (**D**–**F**) heterologous PRRSV-1 (AN06EU4204), and (**G**–**I**) heterologous PRRSV-2 (FDT10US23), respectively. The lymphocyte populations producing IFN-γ were identified by flow cytometry using cell surfaces and intracellular IFN-γ staining, including $CD4^+IFN\text{-}\gamma^+$ cells (**A**,**D**,**G**), $CD8^+IFN\text{-}\gamma^+$ cells (**B**,**E**,**H**), and $CD4^+CD8^+IFN\text{-}\gamma^+$ cells (**C**,**F**,**I**), respectively. Values are expressed as mean±SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

PRRSV RNA in serum. Serum PRRSV RNA quantification after co-challenge was summarized in Table 3. Regardless of vaccine genotype, all vaccination groups had significantly ($p < 0.05$) lower levels of both PRRSV-1 and PRRSV-2 RNA as compared to those of the NonVac group at 3, 5 and 7 DPC. Although, the PRRSV-2 MLV vaccination groups had significantly lower PRRSV-1 RNA levels compared to those of the PRRSV-1 MLV vaccination groups at 3 and 7 DPC, no differences were observed at 5 DPC between the PRRSV-2 MLV vaccination and the Amervac groups. At 5 DPC, serum PRRSV-1 RNA increased in all PRRSV-2 MLV vaccination groups as compared to those at 3 DPC. In contrast, PRRSV-1 RNA levels were reduced in all PRRSV-1 MLV vaccination groups at 5 and 7 DPC as compared to those at 3 DPC. The reduction in serum PRRSV RNA was not genotype-related but was associated with the isolates used in MLV. PRRSV-1 RNA was lower in the Porcilis group than in the Amervac group at 5 and 7 DPC. Meanwhile, no differences were detected in PRRSV-1 RNA levels between the PRRS-2 MLV vaccination groups at 5 DPC.

The PRRSV-2 RNA results are similar to those of PRRSV-1 RNA. All vaccination groups had significantly lower PRRSV-2 RNA as compared to that of the NonVac group, regardless of the vaccine genotype. In addition, PRRSV RNA levels were not different between vaccination groups at 3 DPC. At 5 and 7 DPC, PRRSV-2 RNA levels remained similar levels compared to those at 3 DPC in all vaccination groups except the Amervac and Prime Pac groups, which had significantly lower serum PRRSV-2 RNA at 5 and 7 DPC as compared to the other vaccination groups.

Pathological examination. For macroscopic lung lesion scores, the NonVac group had the highest PRRSV-induced pneumonic lung scores at 7 DPC (Table 3). In contrast, the lung lesion scores of all vaccination groups were significantly lower than that of the NonVac group regardless of genotype. The Porcilis group had the highest macroscopic lung lesion scores as compared to the other vaccination groups. The Prime Pac group had a significantly lower scores as compared to the other vaccination groups (Supplemetary information).

Microscopic lung lesions associated with PRRSV infection were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia. The microscopic lung lesion score results were concordant with the macroscopic lung lesion score results. All vaccination groups, except the Porcilis group, had significantly lower microscopic lung lesion scores compared to the NonVac group (Table 3).

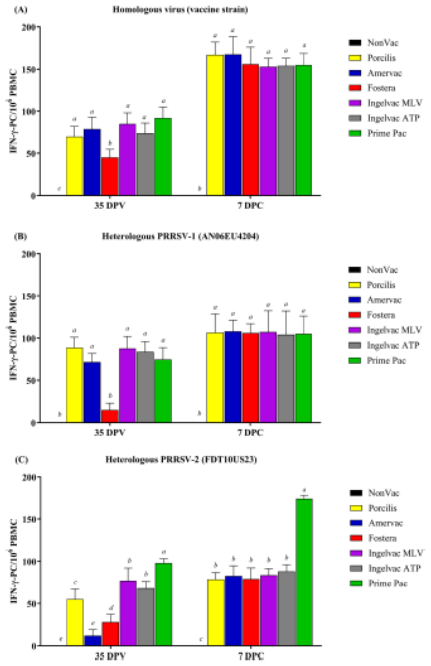


Figure 4. Evaluation of PRRSV-specific IFN-γ-PC following vaccination and at 7 days post-challenge (DPC) using *in vitro* stimulation. (**A**) Homologous virus (vaccine strain), (**B**) heterologous PRRSV-1 (AN06EU4204), and (**C**) heterologous PRRSV-2 (FDT10US23), respectively. Values are expressed as mean±SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

Immunohistochemistry. Regardless of vaccine genotype, the mean number of PRRSV-positive cells was significantly ($p < 0.05$) lower in all vaccination groups as compared to the NonVac group using either A35 or JP24 MAbs (Table 3). The mean number of PRRSV-positive cells stained with A35 MAb in the Porcilis group was significantly ($p < 0.05$) higher than those in the other vaccination groups. No differences were detected in the mean number of PRRSV-positive cells between the Amervac group and the other PRRSV-2 MLV vaccination groups. In contrast, the Fostera and Prime Pac groups had significantly lower mean numbers of PRRSV-positive cells stained with JP24 MAb as compared to the Amervac, Ingelvac MLV and Ingelvac ATP groups (Supplementary information).

Discussion

The present study was conducted to investigate CMI, IL-10 levels and protective efficacy of PRRSV-1 and PRRSV-2 MLVs against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV). Following PRRSV MLV vaccination, regardless of MLV genotype, the induction of CMI against PRRSV as measured by lymphocyte proliferative response and IFN-γ-PC against homologous stimulation was relatively delayed and low in magnitude. The response was observed beginning from 28–35 DPV. Additionally, the magnitude of the response was not different between vaccination groups. Although there was no difference in CMI, IL-10 was different between vaccination groups. Regardless of MLV genotype, increased IL-10 production was observed in all vaccination groups after vaccination. IL-10 levels were significantly higher in all vaccination groups at 7 DPV than in the unvaccinated control. The magnitude of the increase in IL-10 level is not genotype-related but rather is influenced by the virus isolate used to manufacture the vaccine. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels than the Porcilis, Fostera and Prime Pac groups. The Prime Pac group had the lowest IL-10 level. Following challenge, regardless of MLV genotype, all vaccinated pigs were partially protected against co-challenge with PRRSV-1 and PRRSV-2, as demonstrated by significantly reduced viremia against

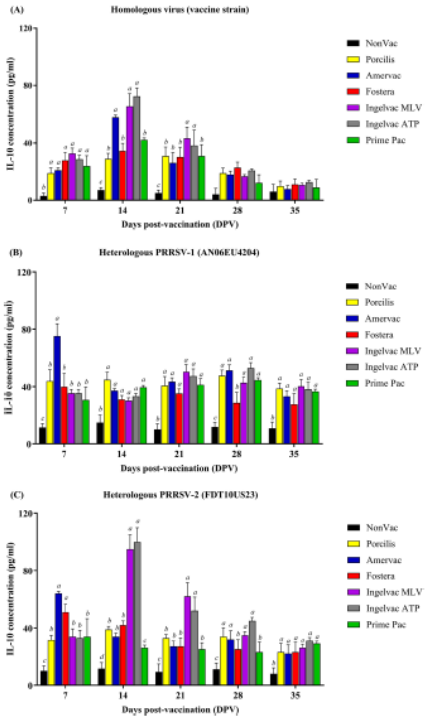


Figure 5. Quantification of porcine IL-10 in supernatant of stimulated PBMC following vaccination. (**A**) Homologous virus (vaccine strain), (**B**) heterologous PRRSV-1 (AN06EU4204), and (**C**) heterologous PRRSV-2 (FDT10US23), respectively. Values are expressed as mean ± SEM. Results were compared using two-way ANOVA multiple comparison test. Lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day post vaccination.

both genotypes, lung lesion scores and PRRSV antigens in lung tissues at 7 DPC as compared to the unvaccinated group, and the Prime Pac group demonstrated significantly greater reductions than the other vaccination groups. The results of reduced viremia and lung lesions suggest that protective efficacy against co-challenge with PRRSV-1 and PRRSV-2 (HP-PRRSV) is not genotype-related but rather is influenced by the virus isolate used to manufacture the vaccine. The results of the study suggest that all PRRSV MLVs are relatively similar in their protective efficacy against concurrent heterologous PRRSV-1 and PRRSV-2 (HP-PRRSV) challenge. The use of either genotype of PRRSV MLV to control PRRS in herds co-infected with both PRRSV genotypes would provide some level of protection against heterologous PRRSV infection. Other control strategies, including strict biosecurity, to prevent external PRRSV introduction will enhance a successful PRRSV control program.

Although CMI against either PRRSV MLV or field infection has been intensively studied[13,15–19], no study has performed a comparative study between both MLV genotypes. The CMI results against the homologous virus in the present study demonstrated that all PRRSV MLVs induce relatively slow CMI responses as measured by lymphocyte proliferative response and the number of IFN-γ-PC, regardless of vaccine genotype. Based on the lymphocyte proliferative response, it was demonstrated that none of the PRRSV MLVs induced a detectable response until 35 DPV. The results of the CMI response analysis reported herein assessing CFSE-labeled lymphocyte proliferation are in agreement with those of previous reports showing that a PRRSV-specific CMI response appears late, approximately 4–6 weeks post-vaccination as determined by lymphocyte blastogenesis and other assays[13,20–22]. In contrast to the lymphocyte proliferative response, the CMI response, as measured by the enumeration of IFN-γ-PC, demonstrated that all MLV isolates induced a delay in the detectable

		DPC*	Treatment groups						
			NonVac	Porcilis	Amervac	Fostera	Ingelvac MLV	Ingelvac ATP	Prime Pac
PRRSV RNA (1,000 copies/ml)	PRRSV-1	0	0.0 ± 0.0[a]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		3	2.3 ± 0.2[a]	1.4 ± 0.3[b]	1.3 ± 0.2[b]	0.2 ± 0.0[d]	0.6 ± 0.1[c]	0.5 ± 0.2[c]	0.2 ± 0.0[d]
		5	2.7 ± 0.3[a]	0.4 ± 0.1[c]	0.8 ± 0.2[b]	0.9 ± 0.1[b]	0.8 ± 0.2[b]	0.9 ± 0.3[b]	1.1 ± 0.2[b]
		7	1.8 ± 0.2[a]	0.4 ± 0.1[c]	0.8 ± 0.2[b]	0.5 ± 0.2[c]	0.1 ± 0.0[d]	0.5 ± 0.1[c]	0.4 ± 0.1[c]
	PRRSV-2	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		3	2.3 ± 0.2[a]	1.4 ± 0.2[b]	1.7 ± 0.2[b]	1.5 ± 0.2[b]	1.2 ± 0.2[b]	1.4 ± 0.3[b]	1.4 ± 0.2[b]
		5	2.7 ± 0.2[a]	1.5 ± 0.3[b]	0.8 ± 0.3[c]	1.3 ± 0.3[b]	1.8 ± 0.3[b]	1.3 ± 0.3[b]	0.8 ± 0.2[c]
		7	2.9 ± 0.2[a]	1.0 ± 0.3[b]	0.5 ± 0.3[c]	1.3 ± 0.3[b]	1.1 ± 0.3[b]	1.1 ± 0.5[b]	0.7 ± 0.2[c]
Macroscopic lung scores		7	72.7 ± 8.8[a]	59.0 ± 4.4[a]	45.0 ± 5.7[b]	55.3 ± 5.5[b]	54.7 ± 1.7[b]	54.6 ± 6.4[b]	42.7 ± 4.6[c]
Microscopic lung scores		7	1.40 ± 0.08[a]	1.24 ± 0.06[a]	0.92 ± 0.08[b]	0.82 ± 0.08[b]	0.83 ± 0.08[b]	0.82 ± 0.08[b]	0.87 ± 0.08[b]
PRRSV-antigen score by IHC[§]	A35	7	15.2 ± 1.8[a]	6.0 ± 0.7[b]	3.2 ± 0.4[c]	4.7 ± 0.4[c]	4.2 ± 0.3[c]	4.4 ± 0.3[c]	3.5 ± 0.2[c]
	JP24	7	8.2 ± 1.4[a]	4.9 ± 0.4[b]	3.9 ± 0.5[b]	2.3 ± 0.3[c]	4.0 ± 0.4[b]	4.1 ± 0.5[b]	2.6 ± 0.2[c]

Table 3. Results of PRRSV RNA in sera of non-vaccinated and vaccinated pigs following co-challenge with PRRSV-1 and PRRSV-2, lung lesion scores and immunohistochemistry at 7 days post-challenge (DPC). *Days post-challenge (DPC). §Immunohistochemistry (IHC) using A35 and JP24, monoclonal antibodies specifically against PRRSV-1 and PRRSV-2 antigens, respectively. ¥Values are displayed in mean ± SEM. The different lowercase letters represent significant differences between treatment groups ($p < 0.05$) at each day.

level of response. After *in vitro* stimulation with homologous vaccine viruses, IFN-γ-PC were detected in pigs vaccinated with either PRRSV-1 or PRRSV-2 MLVs at 35 DPV and showed significantly higher numbers in the vaccination groups than in the NonVac group, albeit the numbers were relatively low. The number of IFN-γ-PC, however, increased rapidly by 7 DPC. The results of the delayed CMI response induced by MLV are in accordance with those of previous studies in which vaccination with either PRRSV-1 or PRRSV-2 MLV elicited a relatively slow CMI response[13,19,23]. The findings of the present study suggest that all commercial PRRSV MLVs induce a relative slow CMI response, regardless of vaccine genotype. Such responses are directed toward homologous stimulation.

It is noteworthy that the effective CMI response was directed toward the homologous response. The use of heterologous stimulation, either by PRRSV-1 or PRRSV-2, showed contrasting results to homologous stimulation. The heterologous response was somewhat unpredictable and unrelated to the genetic similarity between the vaccine and the challenge viruses. A previous study reported similar findings in that homologous stimulation upregulates IFN-γ-PC following vaccination, while heterologous virus stimulation showed varied IFN-γ-PC upregulation[13]. Heterologous stimulation with one virus was able to upregulate IFN-γ-PC as high as homologous stimulation, while another virus was not able to do so despite high genetic similarity. In the present study, the frequencies of IFN-γ-PC in PBMC varied after stimulation with heterologous recall viruses. Stimulation with either heterologous PRRSV-1 or PRRSV-2 induced low amounts of IFN-γ-PC in the Amervac and Fostera groups (Fig. 4B,C). In contrast, some vaccination groups, in particular the Prime Pac group, showed increased amounts of IFN-γ-PC after stimulation with heterologous PRRSV-2 (Fig. 4C). Our results are in accordance with those of previous studies suggesting that viral recognition is also directed against antigens of genetically divergent virus isolates regardless of the vaccine strain[13]. In addition, a cellular immune response such as IFN-γ-PC depends on the virus isolate used for *in vitro* stimulation, and different PRRSV isolates can interact differently to stimulate immune cells[23,24].

Following vaccination, all vaccination groups had significantly higher IL-10 levels compared to the unvaccinated group (Fig. 5A–C). The IL-10 level decreased at 14 DPV and was not different between the MLV-vaccinated and unvaccinated groups at 21 DPV. It is noteworthy that while the IL-10 levels of most of the vaccination groups displayed a gradual declining trend after 7 DPV, the Amervac, Ingelvac MLV and Ingelvac ATP groups had increased levels of IL-10 until 14 DPV before showing a decline. Our result demonstrated that the patterns of IL-10 levels following PRRSV MLV vaccination were different regardless of genotype of MLV but were rather influenced by the PRRSV isolate used to manufacture the vaccine. The Amervac, Ingelvac MLV and Ingelvac ATP groups had significantly higher IL-10 levels than the Porcilis, Fostera and Prime Pac groups. These varying IL-10 levels among the vaccination groups may be due to the different virus isolates used in vaccine production or *in vitro* stimulation[23,25–28]. The differences in IL-10 levels among the PRRSV MLV vaccination groups are not surprising. Previous reports have demonstrated that PRRSV isolates vary in the degree of IL-10 production both *in vivo* and *in vitro*[17,29]. IL-10 induction by PRRSV might depend on the virus isolate used in the experiment[23,25–28]. Our findings support the conclusion that PRRSV MLVs, regardless of vaccine genotype, are able to induce IL-10 upregulation, thus resembling a natural PRRSV infection[20]. The level of IL-10 production depends on the virus isolate used in the vaccine[23]. This finding can be used as one of several criteria to select a vaccine to use for PRRSV control. A higher level of IL-10 can potentially induce more adverse effects following vaccination with PRRSV MLVs. A previous report demonstrated that following vaccination with Ingelvac MLV and Amervac, pigs had higher lung lesion scores compared to other vaccination groups[31]. This could be because IL-10 induction is higher in these groups than in other PRRSV MLV vaccination groups.

It is noteworthy that, regarding to the CMI response in the present study, we only investigated the dynamic change of immune cells against different PRRSV MLV vaccines using the lymphocyte proliferative assay. Our

findings illustrated variations observed in the proliferative indices between PRRSV MLV vaccines. Although the CMI response as measured by the lymphocyte proliferative assay between vaccinated groups were difference, the degree of clinical protection after PRRSV infection was similar. The results suggested that CMI might not fit as immunological correlation for PRRSV protection. In agreement with our findings, previous studies found that the protection against PRRSV infection does not correlate with CMI response[32,33]. In addition, the dynamic change of immune cells seems not to correlate with other cytokines including IL-10. IL-10 is expressed by many cells of the adaptive immune system, including Th1, Th2 and Th17 cell subsets, Treg, $CD8^+$ T cells and B cells[34–37]. It is also produced by cells of innate immune system including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils and neutrophils[35]. The uncorrelated results could be due to IL-10 produced from these cells. Unfortunately, the defined immune cell subpopulations involved in the different CMI response between PRRSV MLV vaccines in the present study are not fully characterize due to the limitation of cell-specific antibodies. Additional studies to measure subpopulations of immune cells secreting cytokines against PRRSV MLV vaccines are needed for further investigation.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine[38]. The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with field viruses[39,40]. In the present study, vaccination with either PRRSV-1 or PRRSV-2 MLVs reduced the level of PRRSV viremia, lung lesions, both macroscopically and microscopically, and PRRSV antigen in the lung tissues of vaccinated pigs following co-challenge with heterologous PRRSV-1 and PRRSV-2 compared to the non-vaccinated control. Based on pneumonic lung lesions, all PRRSV MLVs provide some level of protection against co-infection with PRRSV-1 and PRRSV-2, regardless of vaccine genotype. The lung lesion scores and PRRSV antigens in lung tissues were significantly reduced in the vaccination groups compared to the unvaccinated group after challenge with heterologous viruses[15–18]. Our results are in agreement with those of a previous single challenge study that demonstrated partial cross-protection by PRRSV MLV[15,16,22,41–44]. On the other hand, our cross-protection results are in contrast with those of another previous dual-challenge study in which vaccination with PRRSV-1 MLV reduced only PRRSV-1 viremia and not PRRSV-2 viremia[45]. Pigs vaccinated with PRRSV-1 MLV showed no reductions in PRRSV-2 antigens in lung tissues. The discrepancy between these findings could be due to the virus isolate used in the two studies. It is possible that the differences are attributable to our challenge strain of PRRSV-2 having higher levels of virulence. To postulate, additional studies are needed.

Conclusion

Based on the overall results of the present study, all commercially available PRRSV MLVs are capable of inducing relatively low and delayed CMI response. Differences in IL-10 responses post vaccination were noted between the different vaccines. Vaccination with PRRS MLVs will reduce viremia and lung lesions after heterologous PRRSV challenge regardless of vaccine genotype.

Received: 1 May 2019; Accepted: 19 January 2020;
Published online: 03 February 2020

References

1. Cavanagh, D. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Arch. Virol.* **142**, 629–633 (1997).
2. Nelson, E. A. *et al.* Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. *J. Clin. Microbiol.* **31**, 3184–3189 (1993).
3. Stadejek, T. *et al.* Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. *Arch. Virol.* **153**, 1479–1488, https://doi.org/10.1007/s00705-008-0146-2 (2008).
4. Shi, M. *et al.* Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. *J. Virol.* **84**, 8700–8711, https://doi.org/10.1128/JVI.02551-09 (2010).
5. Nilubol, D., Tripipat, T., Hoonsuwan, T., Tipsombatboon, P. & Piriyapongsa, J. Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes I and II in Thailand. *Arch. Virol.* **158**, 943–953, https://doi.org/10.1007/s00705-012-1573-7 (2013).
6. Kim, H. K. *et al.* One year's study of dynamic and evolution of types I and II PRRSV in a swine farm. *Vet. Microbiol.* **150**, 230–238, https://doi.org/10.1016/j.vetmic.2011.01.025 (2011).
7. Chen, N. *et al.* Whole genome characterization of a novel porcine reproductive and respiratory syndrome virus 1 isolate: Genetic evidence for recombination between Amervac vaccine and circulating strains in mainland China. *Infect. Genet. Evol.* **54**, 308–313, https://doi.org/10.1016/j.meegid.2017.07.024 (2017).
8. Nilubol, D., Tripipat, T., Hoonsuwan, T. & Kortheerakul, K. Porcine reproductive and respiratory syndrome virus, Thailand, 2010–2011. *Emerg. Infect. Dis.* **18**, 2039–2043, https://doi.org/10.3201/eid1812.111105 (2012).
9. Zhou, Z. *et al.* The epidemic status and genetic diversity of 14 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) isolates from China in 2009. *Vet. Microbiol.* **150**, 257–269, https://doi.org/10.1016/j.vetmic.2011.02.013 (2011).
10. Do, H. Q. *et al.* Molecular evolution of type 2 porcine reproductive and respiratory syndrome viruses circulating in Vietnam from 2007 to 2015. *BMC Vet. Res.* **12**, 256, https://doi.org/10.1186/s12917-016-0885-3 (2016).
11. Halbur, P. *et al.* Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Veterinary Pathol. Online* **32**, 648–660 (1995).
12. Madapong, A. *et al.* Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines. *Arch Virol*, https://doi.org/10.1007/s00705-016-3084-4 (2016).
13. Ferrari, L. *et al.* Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after *in vitro* homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. *Vet. Immunol. Immunopathol.* **151**, 193–206, https://doi.org/10.1016/j.vetimm.2012.11.006 (2013).
14. Halbur, P. G. *et al.* Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet. Pathol.* **33**, 159–170, https://doi.org/10.1177/030098589603300205 (1996).

15. Park, C., Seo, H. W., Han, K., Kang, I. & Chae, C. Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge. *Vet. Microbiol.* **172**, 432–442, https://doi.org/10.1016/j.vetmic.2014.05.030 (2014).
16. Kim, T. *et al.* Comparison of Two Commercial Type 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Modified Live Vaccines against Heterologous Type 1 and Type 2 PRRSV Challenge in Growing Pigs. *Clin. Vaccine Immunol.* **22**, 631–640, https://doi.org/10.1128/CVI.00001-15 (2015).
17. Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* **86**, 1943–1951, https://doi.org/10.1099/vir.0.80959-0 (2005).
18. van Woensel, P. A., Liefkens, K. & Demaret, S. European serotype PRRSV vaccine protects against European serotype challenge whereas an American serotype vaccine does not. *Adv. Exp. Med. Biol.* **440**, 713–718 (1998).
19. Zuckermann, F. A. *et al.* Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet. Microbiol.* **123**, 69–85, https://doi.org/10.1016/j.vetmic.2007.02.009 (2007).
20. Meier, W. A. *et al.* Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology* **309**, 18–31 (2003).
21. Bassaganya-Riera, J. *et al.* Impact of immunizations with porcine reproductive and respiratory syndrome virus on lymphoproliferative recall responses of CD8+ T cells. *Viral Immunol.* **17**, 25–37, https://doi.org/10.1089/088282404322875430 (2004).
22. Martelli, P. *et al.* Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. *Vaccine* **27**, 3788–3799, https://doi.org/10.1016/j.vaccine.2009.03.028 (2009).
23. Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology* **351**, 249–259, https://doi.org/10.1016/j.virol.2006.03.046 (2006).
24. Correas, I., Osorio, F. A., Steffen, D., Pattnaik, A. K. & Vu, H. L. X. Cross reactivity of immune responses to porcine reproductive and respiratory syndrome virus infection. *Vaccine* **35**, 782–788, https://doi.org/10.1016/j.vaccine.2016.12.040 (2017).
25. Darwich, L., Diaz, I. & Mateu, E. Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. *Virus Res.* **154**, 123–132, https://doi.org/10.1016/j.virusres.2010.07.017 (2010).
26. Silva-Campa, E. *et al.* Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. *Virology* **387**, 373–379, https://doi.org/10.1016/j.virol.2009.02.033 (2009).
27. Silva-Campa, E. *et al.* European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. *Virology* **396**, 264–271, https://doi.org/10.1016/j.virol.2009.10.024 (2010).
28. Subramaniam, S., Sur, J. H., Kwon, B., Pattnaik, A. K. & Osorio, F. A. A virulent strain of porcine reproductive and respiratory syndrome virus does not up-regulate interleukin-10 levels *in vitro* or *in vivo*. *Virus Res.* **155**, 415–422, https://doi.org/10.1016/j.virusres.2010.11.010 (2011).
29. Chung, H. K. & Chae, C. Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). *J. Comp. Pathol.* **129**, 205–212 (2003).
30. Suradhat, S. & Thanawongnuwech, R. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* **84**, 2755–2760, https://doi.org/10.1099/vir.0.19230-0 (2003).
31. Martinez-Lobo, F. J. *et al.* Safety of Porcine Reproductive and Respiratory Syndrome Modified Live Virus (MLV) vaccine strains in a young pig infection model. *Vet. Res.* **44**, 115, https://doi.org/10.1186/1297-9716-44-115 (2013).
32. Xiao, Z., Batista, L., Dee, S., Halbur, P. & Murtaugh, M. P. The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J. Virol.* **78**, 5923–5933, https://doi.org/10.1128/JVI.78.11.5923-5933.2004 (2004).
33. Li, X. *et al.* Comparison of host immune responses to homologous and heterologous type II porcine reproductive and respiratory syndrome virus (PRRSV) challenge in vaccinated and unvaccinated pigs. *Biomed. Res. Int.* **2014**, 416727, https://doi.org/10.1155/2014/416727 (2014).
34. Roncarolo, M. G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* **212**, 28–50, https://doi.org/10.1111/j.0105-2896.2006.00420.x (2006).
35. Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**, 683–765, https://doi.org/10.1146/annurev.immunol.19.1.683 (2001).
36. Trinchieri, G. Interleukin-10 production by effector T cells: Th1 cells show self control. *J. Exp. Med.* **204**, 239–243, https://doi.org/10.1084/jem.20070104 (2007).
37. Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* **2**, 816–822, https://doi.org/10.1038/ni0901-816 (2001).
38. Opriessnig, T. *et al.* Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. *J. Virol.* **76**, 11837–11844 (2002).
39. van Woensel, P. A., Liefkens, K. & Demaret, S. Effect on viraemia of an American and a European serotype PRRSV vaccine after challenge with European wild-type strains of the virus. *Vet. Rec.* **142**, 510–512 (1998).
40. Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H. & Pensaert, M. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. *Vet. Microbiol.* **95**, 187–197 (2003).
41. Park, C., Choi, K., Jeong, J. & Chae, C. Cross-protection of a new type 2 porcine reproductive and respiratory syndrome virus (PRRSV) modified live vaccine (Fostera PRRS) against heterologous type 1 PRRSV challenge in growing pigs. *Vet. Microbiol.* **177**, 87–94, https://doi.org/10.1016/j.vetmic.2015.02.020 (2015).
42. Jeong, J., Choi, K., Kang, I., Park, C. & Chae, C. Evaluation of a 20year old porcine reproductive and respiratory syndrome (PRRS) modified live vaccine (Ingelvac((R)) PRRS MLV) against two recent type 2 PRRS virus isolates in South Korea. *Vet. Microbiol.* **192**, 102–109, https://doi.org/10.1016/j.vetmic.2016.07.006 (2016).
43. Kristensen, C. S. *et al.* Efficacy and safety of simultaneous vaccination with two modified live virus vaccines against porcine reproductive and respiratory syndrome virus types 1 and 2 in pigs. *Vaccine* **36**, 227–236, https://doi.org/10.1016/j.vaccine.2017.11.059 (2018).
44. Roca, M. *et al.* Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. *Vet. J.* **193**, 92–96, https://doi.org/10.1016/j.tvjl.2011.11.019 (2012).
45. Choi, K. *et al.* Comparison of commercial type 1 and type 2 PRRSV vaccines against heterologous dual challenge. *Vet. Rec.* **178**, 291, https://doi.org/10.1136/vr.103529 (2016).

Acknowledgements

This work was supported by the government budget year 2017–2018 (Grant Number 5080001), Research and Researchers for Industries (RRI, Grant Number PHD59I0040), Agricultural Research Development Agency (ARDA, Public organization), and Chulalongkorn University Special Task Force for Activating Research (CU-STAR) Swine Virus Evolution and Vaccine Research (SVEVR).

Author contributions

A.M., K.S.c. and D.N. designed experiment. A.M. and K.S.c. processed the samples and performed laboratory analyses. A.B. and D.N. supervised the *ex vivo* experiment. A.B. and D.N. performed pathological examinations and analyses. D.N. supervised the statistical analysis. A.M., A.T. and D.N. drafted the manuscript and provided through discussion and critical manuscript reading.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-58626-y.

Correspondence and requests for materials should be addressed to D.N.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Appendix C

Immune response and protective efficacy of intramuscular and intradermal vaccination with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine against highly pathogenic PRRSV-2 (HP-PRRSV-2) challenge, either alone or in combination of PRRSV-1

Adthakorn Madapong[a], Kepalee Saeng-chuto[a], Puwich Chaikhumwang[b], Angkana Tantituvanont[b], Kriangsak Saardrak[C], Rafael Pedrazuela Sanz[d], Joel Miranda Alvarez[d], Dachrit Nilubol[a,*]

[a]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

[b]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

[c]Department of Animal Science at Kamphaeng Sean, Faculty of Agriculture at Kamphaeng Seang, Kasetsart University Kamphaeng Sean Campus, Nakhon Pathom 73140, Thailand

[d]Laboratorios Hipra S.A., Amer 17170, Spain

*Corresponding author:
Assistant Professor Dr. Dachrit Nilubol
E-mail address: dachrit@gmail.com, ndachrit@chula.ac.th
Department of Veterinary Microbiology, Faculty of Veterinary Science
Chulalongkorn University, Bangkok 10330, Thailand

Keywords: Porcine reproductive and respiratory syndrome virus, modified live vaccine, intramuscular, intradermal, immune response, protective efficacy, challenge

Veterinary Microbiology 244 (2020) 108655

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Immune response and protective efficacy of intramuscular and intradermal vaccination with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine against highly pathogenic PRRSV-2 (HP-PRRSV-2) challenge, either alone or in combination with of PRRSV-1

Adthakorn Madapong[a], Kepalee Saeng-chuto[a], Puwich Chaikhumwang[b], Angkana Tantituvanont[b], Kriangsak Saardrak[c], Rafael Pedrazuela Sanz[d], Joel Miranda Alvarez[d], Dachrit Nilubol[a,*]

[a] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[c] Department of Animal Science at Kamphaeng Saen, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[d] Laboratorios Hipra S.A., Amer 17170, Spain

ARTICLE INFO

Keywords:
Porcine reproductive and respiratory syndrome virus
Modified live virus vaccine
Intramuscular
Intradermal
Immune response
Protective efficacy
Challenge

ABSTRACT

The study was conducted to evaluate the immune response of pigs vaccinated intramuscularly (IM) or intradermally (ID) with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine (MLV). The protective efficacy was evaluated upon challenge with highly pathogenic (HP)-PRRSV-2, either alone or in combination with PRRSV-1. Forty-two, castrated male, PRRSV-free pigs were randomly allocated into 7 groups of 6 pig each. IM/HPPRRSV2, IM/CoChallenge, ID/HPPRRSV2 and ID/CoChallenge groups were vaccinated IM or ID with PRRSV-1 MLV (UNISTRAIN® PRRS, Laboratorios Hipra S.A., Amer, Spain) in accordance to the manufacturer's directions. NV/HPPRRSV2 and NoVac/CoChallenge groups were nonvaccinated/challenged controls. NoVac/NoChallenge group was left as the control. Antibody response, IFN-γ-secreting cells (IFN-γ-SC) and IL-10 production were evaluated following vaccination. At 35 days post vaccination (DPV), all challenged groups were intranasally inoculated with HP-PRRSV-2, either alone or in combination with PRRSV-1. PRRSV viremia and lung lesion scores were evaluated following challenge. The results demonstrated that ID vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC than that of IM vaccinated pigs. Following challenge with HP-PRRSV-2 either alone or with PRRSV-1, PRRSV viremia and lung lesions, both macroscopically and microscopically, were significantly reduced in vaccinated pigs than that of nonvaccinated pigs, regardless to the route of vaccine administration. ID vaccinated pigs had significantly lower levels of PRRSV viremia and lung lesion scores than that of IM vaccinated pigs. The results of the study suggested that the administration of PRRSV-1 MLV, either IM or ID, provided partial protection against HP-PRRSV-2, either alone or when cochallenged with PRRSV-1, as demonstrated by the reduction in lung lesions and viremia. The ID route might represent an alternative to improve vaccine efficacy, as it resulted in lower IL-10 levels and higher IFN-γ-SC levels.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease in pigs characterized by respiratory distress in finishing pigs and reproductive disorders in breeding sows. PRRS is caused by PRRS virus (PRRSV), an enveloped, positive-sense, single-stranded RNA virus belonging to the order *Nidovirales* and family *Arteriviridae* (Cavanagh, 1997). At present, two genetically distinct PRRSV species, PRRSV-1 and PRRSV-2, have been recognized (Kuhn et al., 2016). Two PRRSV species are similar in the genome organization but their genomes are markedly different, with genetic similarities of only 60 % and 56 % at the nucleotide and amino acid levels, respectively (Forsberg et al., 2002). The

*Corresponding author at: Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Henry-Dunant Road, Pathumwan, Bangkok 10330, Thailand.
E-mail address: dachrit.N@chula.ac.th (D. Nilubol).

https://doi.org/10.1016/j.vetmic.2020.108655
Received 21 October 2019; Received in revised form 5 March 2020; Accepted 18 March 2020

Table 1
Experimental design. Seven treatment groups included 4 vaccinated- and 3 non-vaccinated groups. Routes of vaccine administration included either intramuscular (IM) or intradermal (ID). At 35 DPV, pigs in challenged groups were intranasally inoculated with HP-PRRSV-2, either alone or combination with PRRSV-1. Pigs in Nonvaccinated (NoVac)/nonchallenged (NoChallenge) group served as the control.

Treatment groups	Pigs no.	Vaccination	Vaccine	Dosage and route of administration	PRRSV challenge	
					PRRSV-1(AN06EU4204)	HP-PRRSV-2(FDT01U823)
IM/HPPRRSV2	6	Yes	UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain)	2 ml, intramuscular (IM)	No	Yes
ID/HPPRRSV2	6	Yes	UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain)	0.2 ml, intradermal (ID)	No	Yes
NoVac/HPPRRSV2	6	No	–	–	No	Yes
IM/CoChallenge	6	Yes	UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain)	2 ml, intramuscular (IM)	Yes	Yes
ID/CoChallenge	6	Yes	UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain)	0.2 ml, intradermal (ID)	Yes	Yes
NoVac/CoChallenge	6	No	–	–	Yes	Yes
NoVac/NoChallenge	6	No	–	–	No	No

classification into two distinct species is based on the continents where the viruses were first discovered. PRRSV-1 was first discovered in the European continent and currently has further evolved into 4 subtypes (Stadejek et al., 2008). Meanwhile, PRRSV-2 was first discovered in the North American continent and further evolved into 9 distinct lineages (Shi et al., 2010).

PRRSV that predominantly exists in Asian countries are different from those in the European and North American continents. At present, the co-existence of both PRRSV-1 and PRRSV-2 has been increasingly reported in several Asian countries, including China, Korea, and Vietnam (Chen et al., 2011; Kim et al., 2011). In Thailand, both PRRSV-1 and PRRSV-2, have been reported (Nilubol et al., 2013). The co-existence of both species was evident at both individual pig and herd levels. Based on international systematic classification according to previously described methods (Shi et al., 2010; Stadejek et al., 2008), PRRSV-1 in Thailand includes mainly isolates in clade A, subtype 1. PRRSV-2 includes lineages 1, 5 and 8. PRRSV-2 in the lineage 8 is mainly clustered in sublineage 8.7 in which both 8.7/HP-PRRSV-2 and 8.7/Classical North America (NA) have been reported (Shi et al., 2010). This sublineage 8.7/HP-PRRSV-2, a highly pathogenic isolate that causing high mortality, has been a predominant virus in the region (Nilubol et al., 2013). The co-existing of both PRRSV species could generates more severe clinical diseases than does a single infection with either species in which could subsequently complicate a successful PRRSV control program in the region. The more severe clinical diseases were experimentally demonstrated as pigs co-infected with both species had significantly higher levels of pneumonic lung lesion at 7 days post challenge (DPC) than those challenged with either PRRSV species (Choi et al., 2015).

With the availability of modified live vaccine (MLV) for both PRRSV species, it is difficult to justify which species of PRRSV MLV should be used to successfully control PRRS in the region where the coinfection does exist. Recently, a live attenuated vaccine based on PRRSV-1 (UNISTRAIN® PRRS) is commercially available in both intramuscular (IM) and intradermal (ID) administration. This vaccine, administered IM, already demonstrated partial protection against heterologous PRRSV-1 (Bonckaert et al., 2016) or PRRSV-2 (Roca et al., 2012), However, it has not been evaluated against the coinfection of PRRSV-1 and PRRSV-2. In addition, there has not been any reports on the induction of immune response, IL-10 production and protective efficacy of the intradermal administration.

IL-10 is a cytokine that functions in immunoregulation, including the downregulation of the expression of Th1 cytokines and the enhancement of B cell proliferation and antibody production (Saraiva and O'Garra, 2010). In PRRSV infection, IL-10 levels are reportedly associated with severity of clinical disease (van Reeth and Nauwynck, 2000) and can delay the immune response. Therefore, the objectives of the present study were to investigate the immune response of pigs vaccinated IM or ID with PRRSV-1 MLV (UNISTRAIN® PRRS). The protective efficacy was evaluated against the challenge with HP-PRRSV-2 (sublineage 8.7/HP-PRRSV-2), either alone or in combination with PRRSV-1 (clade A, subtype 1).

2. Materials and methods

2.1. Experimental design

All animal procedures were conducted in accordance with the recommendations in the Guild for the Care and Use of Laboratory Animal of the National Research Council of Thailand according to protocols reviewed and approved by the Chulalongkorn University Animal Care and Use Committee (protocol number 1,731,047).

Forty-two, 3-week-old, castrated male pigs were procured from a PRRSV-free herd. Upon arrival, sera were collected individually and assayed for the presence of viral RNA and PRRSV specific antibody using PCR and ELISA to confirm their PRRSV negative status. Pigs were

randomly allocated into the following 7 groups of 6 pigs each based on weight stratification. As shown in Table 1: IM/HPPRRSV2, ID/HPPRRSV2, NoVac/HPPRRSV2, IM/CoChallenge, ID/CoChallenge, NoVac/CoChallenge and NoVac/NoChallenge. The IM/HPPRRSV2 and IM/CoChallenge groups were vaccinated once *via* IM route with a 2 mL dose of UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain). The ID/HPPRRSV2 and ID/CoChallenge groups were vaccinated once *via* ID route with a 0.2 mL dose of UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain) using Hipradermic® needle-free vaccinator. The NoVac/HPPRRSV2, NoVac/CoChallenge and NoVac/NoChallenge groups were left non-vaccination.

At 35 days post vaccination (DPV), the IM/HPPRRSV2, ID/HPPRRSV2 and NoVac/HPPRRSV2 groups were intranasally challenged with 4 mL of tissue culture inoculum of HP-PRRSV-2 (FDT10US23 isolate, fifth passage of MARC-145 cells, $10^{5.2}$ $TCID_{50}$/mL), 2 mL/nostril. The IM/CoChallenge, ID/CoChallenge and NoVac/CoChallenge groups were intranasally cochallenged with 4 mL of tissue culture inoculum of PRRSV-1 and HP-PRRSV-2 isolates, at 2 mL of each isolate/nostril. The inoculum composed of 2 mL of tissue culture supernatant of each PRRSV-1 (AN06EU4204 isolate, third passage of porcine alveolar macrophages, $10^{5.4}$ $TCID_{50}$/mL) and HP-PRRSV-2 (FDT10US23 isolate, fifth passage of MARC-145 cells, $10^{5.2}$ $TCID_{50}$/mL). The NoVac/NoChallenge group was kept as nonvaccinated/nonchallenged control. Pigs in each group were kept in separated room with separated air spaces and monitored daily for physical condition and clinical respiratory disease throughout the experiment.

Blood samples were collected at 0, 7, 14, 21, 28, 35 DPV and 3, 7, 14, and 35 days post challenge (DPC). Nasal swabs were collected at 0, 3, 7, 14 and 35 DPC using individually packaged sterile swabs which were immersed in 1 mL of RNAlater™ solution (Thermo-Fisher Scientific, MA, USA) and kept at −80 °C for further processed (Fig. 1A). Sera were separated and measured for antibody response using commercial ELISA kit and serum neutralization (SN) assay. PRRSV RNA was quantitatively assayed in sera and nasal swab samples using real-time quantitative RT-PCR (RT-qPCR). Peripheral blood mononuclear cells (PBMC) were isolated and used for *in vitro* stimulation to measure IL-10 production using ELISA kit and IFN-γ-secreting cells (IFN-γ-SC) using ELISPOT assay. Three pigs from each group were necropsied at 7 and 35 DPC. PRRSV-induced pneumonic lung lesions were scored using previously described (Halbur et al., 1995).

2.2. PRRSV vaccine and viruses

PRRSV vaccine used was UNISTRAIN® PRRS (Laboratorios Hipra S.A., Amer, Spain), available in 2 different preparations, IM and ID vaccination. Dosage and administration routes were in accordance with the manufacturer's instructions. In brief, a 2 mL dose was used for IM vaccination (batch no. 3Z79−4). Meanwhile, a 0.2 mL dose was used for ID vaccination (batch no. 6D16). ID vaccination was performed using Hipradermic® needle-free device (Laboratorios Hipra S.A., Amer, Spain).

For *in vitro* stimulation assay, homologous and heterologous viruses were used as recall antigens. Homologous virus refers to a vaccine strain as previously described (Madapong et al., 2017). Heterologous viruses refer to the AN06EU4204 and FDT10US23 PRRSV isolates which contained Thai PRRSV-1 and PRRSV-2 (HP-PRRSV-2), respectively. The PRRSV isolates AN06EU4204 and FDT10US23 are in clade A, subtype 1 and sublineage 8.7/HP-PRRSV-2, based on international systematic classification according to previously described methods (Shi et al., 2010; Stadejek et al., 2008). The ORF5 genome sequences of the AN06EU4204 and FDT10US23 isolates are available in GenBank under accession numbers JQ040750 and JN255836, respectively. These two PRRSV isolates were isolated from weaned pigs from two different herds experiencing PRRS outbreaks during 2010–2011 (Nilubol et al., 2012). Both swine herds are located in the western region of Thailand. Based on the ORF5 gene, both Thai PRRSV isolates are phylogenetically clustered in endemic clades of which could represent PRRSV isolates endemically infected in swine herds in this region. In addition, the two viruses were genetically distinct from the PRRSV MLV used in the present study. The nucleotide and amino acid similarities based on the ORF5 gene between Thai PRRSV isolates and vaccine virus are summarized in Table 2.

2.3. Clinical evaluation

Clinical signs and rectal temperature were monitored daily post vaccination and post challenge periods for two consecutive weeks by the same personnel at the same time. The severity of clinical respiratory disease was evaluated using a scoring system for each pig following stress induction as previously described (Halbur et al., 1995): 0 = normal, 1 = mild dyspnea and/or tachypnea when stressed, 2 = mild dyspnea and/or tachypnea when at rest, 3 = moderate dyspnea and/or tachypnea when stressed, 4 = moderate dyspnea and/or tachypnea when at rest, 5 = severe dyspnea and/or tachypnea when stressed, and 6 = severe dyspnea and/or tachypnea when at rest.

2.4. Antibody detection

Serum samples were tested for the presence of PRRSV-specific antibody by commercial ELISA kit: IDEXX PRRS X3 Ab test (IDEXX laboratories Inc., MA, USA) and serum neutralization (SN) assay. ELISA assay was performed according to the manufacturer's recommendations. Serum samples were considered positive for PRRSV antibody if the S/P ratio was greater than 0.4.

Serum neutralization (SN) assay was performed using homologous (vaccine virus), heterologous PRRSV-1 (AN06EU4204 isolates) and heterologous HP-PRRSV-2 (FDT10US23 isolate), as previously described (Nilubol et al., 2004). The presence of virus specific cytopathic effect (CPE) in each well was recorded after incubating for 7 days. The SN titers were determined as the reciprocal of the highest dilution in which no evidence of the virus growth was detected. Geometric mean titers were calculated.

2.5. Isolation of porcine PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from 10 mL of heparinized blood samples by gradient density centrifugation (Lymphosep™, Biowest, MO, USA) according to previously described protocol (Ferrari et al., 2013). The isolated PBMC were counted by inverted microscope and concentration was accessed in cRPMI-1640 (RPMI-1640 media supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 50 μg/mL of gentamycin). The viability of PBMC were determined by Trypan blue (Sigma-Aldrich, MO, USA) staining and more than 90 % viability was used for *in vitro* stimulation for IL-10 production and enzyme-linked immunospot (ELISPOT) assay as describe below.

2.6. Porcine interleukin-10

Following vaccination, porcine interleukin-10 (IL-10) concentration in supernatant of stimulated PBMC was quantified using porcine ELISA interleukin-10 (IL-10) commercial kit (Quantikine® ELISA porcine IL-10, R&D System, MN, USA) according to manufacturer's instructions. In brief, 2×10^6 PBMC in cRPMI-1640 were seeded into 96-well plates and cultured *in vitro* for 24 h with homologous virus at 0.01 multiplicity of infection (MOI) or phytohemagglutinin (10 μg/mL, Sigma-Aldrich, MO, USA).

2.7. PRRSV-specific interferon-γ-secreting cells

The number of PRRSV-specific interferon-γ-secreting cells (IFN-γ-SC) were determined in PBMC using commercial ELISPOT IFN-γ kit

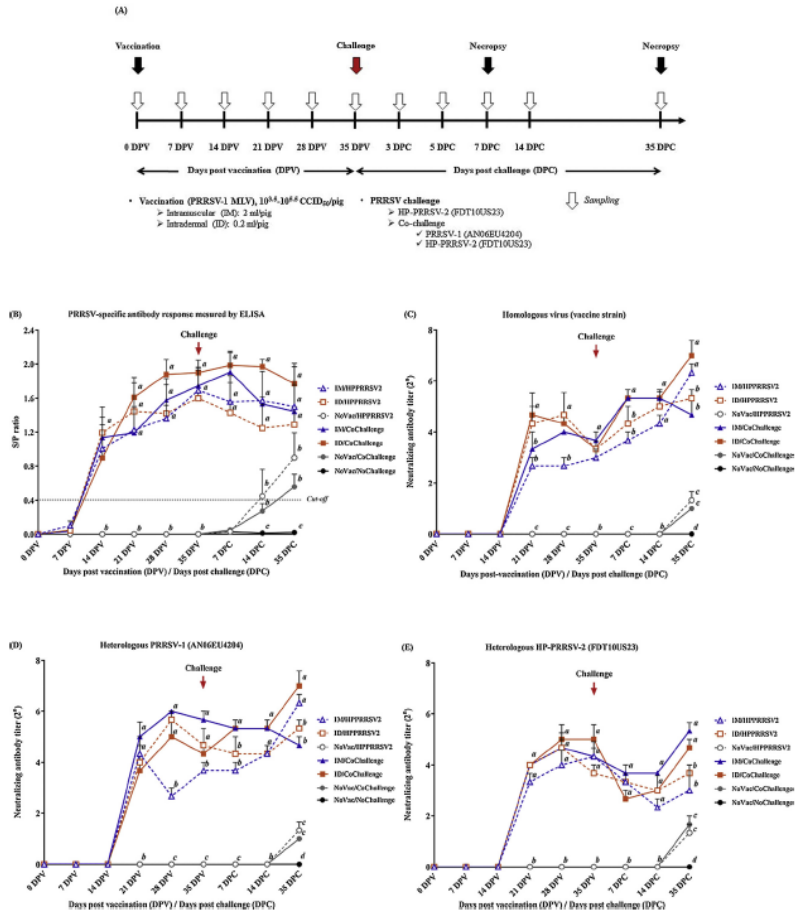


Fig. 1. (**A**) Experimental design showing PRRSV vaccine administration details and sampling time points. PRRSV-1: (UNISTRAIN® PRRS); MLV: modified-live vaccine; $CCID_{50}$: 50 % of the cell culture infectious dose. Level of PRRSV-specific antibodies measured by (**B**) ELISA and serum neutralization (*SN*) assay after stimulation with (**C**) homologous virus (vaccine strain), (**D**) heterologous PRRSV-1 (AN06EU4204) and (*E*) heterologous HP-PRRSV-2 (FDT10US23). Values are expressed as the mean ± SEM. Sample-to-positive (S/P) ratios equal to or greater than 0.4 (dashed line) are considered positive. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (*a-d*) indicate significant differences between treatment groups ($P < 0.05$) for each day.

(ELISpot porcine IFN-γ, R&D System, MN, USA), processed according to manufacturer's instructions and previously described (Park et al., 2014) with minor modification. Briefly, 2×10^5 PBMC in cRPMI-1640 medium were seeded into 96-well plates and stimulated with either homologous or heterologous PRRSV isolates at 0.01 MOI for 24 h at 37 °C in 5% CO_2, humidified atmosphere. The linear response was tested between 0.01 and 0.1 multiplicity of infection (MOI). Phytohemagglutinin (10 μg/mL, Sigma-Aldrich, MO, USA) and cRPMI-1640 medium was used as positive and negative control, respectively. The spots were counted by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany), and the background values were subtracted from the respective count of the stimulated cells and the immune response was expressed as number of IFN-γ-SC per 1×10^6 PBMC.

2.8. Quantification of PRRSV RNA

PRRSV RNA was extracted form serum and nasal swabs samples

Table 2
Nucleotide and amino acid similarities based on the ORF5 gene between vaccine virus and PRRSV-1 and HP-PRRSV-2 isolates used to challenge in this study. International systematic classification was based on previously described, including PRRSV-1 (Stadejek et al., 2008) and HP-PRRSV-2 (Shi et al., 2010).

PRRSV isolates	Classification	Similarity level (%) between vaccine virus and PRRSV isolates	
		Nucleotide	Amino acid
PRRSV-1(AN06EU4204)	Clade A, subtype 1	92.7	89.1
HP-PRRSV-2(FDT10US23)	Sublineage 8.7/ HP-PRRSV-2	69.9	59.8

Shi, M., Lam, T.T., Hon, C.C., Murtaugh, M.P., Davies, P.R., Hui, R.K., Li, J., Wong, L.T., Yip, C.W., Jiang, J.W., Leung, F.C., 2010. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J. Virol. 84.8700–8711.
Stadejek, T., Oleksiewicz, M.B., Scherbakov, A.V., Timina, A.M., Krabbe, J.S., Chabros, K., Potapchuk, D., 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch. Virol. 153.1479–1488.

using NucleoSpin® Virus (Macherey-Nagel, Duren, Germany) according to manufacturer's instruction. The RNA quality was measured using a NanoDrop spectrophotometer (Colibri spectrometer, Titertek Berthold, Pforzheim, Germany). Copy number of viral RNA was then 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 USalingEU-R, 5′ AAATGIGGCTTCTCIGGITTTT 3′; forward primer USalingEU-F, 5′ TCAICTGTGCCAGITGCTGG 3′; EU-PRRSV specific probe FAM_EU (5′ *CAL 560* CCCAGCGCCAGCAACCTA GGG *BHQ1* 3′; and US-PRRSV specific probe FAM_US (5′ *FAM* TCCCG GTCCCTTGCCTCTGGA *BHQ1* 3′). RT-qPCR mixture (20 µl) was based on QuantiNova™ Probe RT-PCR kit (Qiagen®, Hilden, Germany), 1X Probe RT-PCR Master Mix, 1X QN Probe RT-Mix, 0.8 µM of each primer, 0.2 µM of each probe, 1 µl of cDNA (0.5 µg), and RNase-free water up to 20 µl. The reaction was carried out in QuantStudio™ 3 Real-time PCR machine (Thermo-Fisher Scientific, Waltham, MA, USA).

2.9. Pathological examination

Pigs were necropsied at 7 and 35 DPC. Macro- and microscopic lung lesions were scored according to a previously described methods (Halbur et al., 1995). For macroscopic lung lesion, the lungs were given a score to estimate the percentage of the lung affected by pneumonia. Each lobe was assigned a number to reflex the approximate percentage of the volume of the entire lung and the percentage volume from each lobe added to obtain the entire lung score (range from 0 to 100 % of affected lung). Sections were collected from all lung lobes as previously described (Halbur et al., 1995). Lung tissues were fixed with 10 % neutral buffered formalin for 7 days and routinely processed and embedded in paraffin in an automated tissue processor. Sections were cut at 5 µm and stained with hematoxylin and eosin (H&E). For microscopic lung lesion analysis, the lung sections were examined in a blinded manner and given an estimated score of the severity of the interstitial pneumonia. Briefly, 0 = normal; 1 = mild interstitial pneumonia, 2 = moderate multifocal interstitial pneumonia, 3 = moderate diffuse interstitial pneumonia, and 4 = severe diffuse interstitial pneumonia. The mean values of microscopic score of each group were calculated.

2.10. Statistical analyses

Data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed for each day by ANOVA to determine whether there were significant differences between treatment groups for each day. If the p-value in the ANOVA table was < 0.05, differences between treatment groups were evaluated by pairwise comparisons using least significant differences at the $P < 0.05$ rejection level.

3. Results

3.1. Reduced clinical disease following challenge in vaccinated pigs

All vaccinated pigs displayed no clinical abnormalities following vaccination and rectal temperatures were within the normal physiological range (data not shown). Following challenge, pigs in all groups displayed the clinical respiratory disease associated with PRRSV, including fever and dyspnea. Nonvaccinated pigs displayed more severe clinical diseases than those of the vaccinated pigs.

3.2. IM and ID vaccination induced similar antibody response as measured by ELISA

Regardless of the route of administration, an increased PRRSV-specific antibody response was first detected at 7 DPV in all vaccinated groups, but the level was less than the cut-off level (S/P ratio < 0.4) (Fig. 1B). Significantly increased antibody titers, above the cut-off level, were observed in all vaccinated groups from 14 to 35 DPV. The levels were significantly higher in the vaccinated groups than in the non-vaccinated groups. Following challenge, no increased antibody responses were observed in any vaccinated groups.

3.3. IM and ID vaccination induced similar antibody response as measured by SN assay

Regardless of the route of vaccination and PRRSV isolates of recall antigen used, the SN titers of all vaccinated groups were first detected at 21 DPV (Fig. 1**C-E**) and significantly higher ($P < 0.05$) than those of the nonvaccinated groups throughout the experiment.

In homologous virus stimulation (Fig. 1C), the SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV. Pigs in the ID/HPPRRSV2 and ID/CoChallenge groups had significantly ($P < 0.05$) higher SN titers than those in the IM/HPPRRSV2 and IM/CoChallenge groups at 21 and 28 DPV. However, the SN titers were not different between vaccinated groups at 35 DPV. Following challenge, the SN titers of all vaccinated groups gradually increased. At 7 DPC, the IM/CoChallenge and ID/CoChallenge groups had significantly higher SN titers than the IM/HPPRRSV2 and ID/CoChallenge groups, but no difference was observed between vaccinated groups at 14 DPV. However, at 35 DPC, the SN titers of the IM/HPPRRSV2 and ID/CoChallenge groups were significantly higher than those of the ID/HPPRRSV2 and IM/CoChallenge groups.

In heterologous PRRSV-1 (AN06EU4204) stimulation (Fig. 1D). The SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV. The IM/HPPRRSV2 group had significantly lower SN titers than the other groups at 28 and 35 DPV. Following challenge, the SN titers of all vaccinated groups remained constant from 7 to 14 DPC but slightly increased at 35 DPC. At 7 DPC, the IM/CoChallenge and ID/CoChallenge groups had significantly higher SN titers than the IM/HPPRRSV2 and IM/CoChallenge groups, but there was no difference in the SN titers among the vaccinated groups at 14 DPC. However, at 35 DPC, the IM/HPPRRSV2 and ID/CoChallenge groups had significantly higher SN titers than the ID/HPPRRSV2 and IM/CoChallenge groups.

In heterologous HP-PRRSV-2 (FDT10US23) stimulation the SN titers of all vaccinated groups were first detected at 21 DPV and increased from 28 to 35 DPV (Fig. 1E). Following challenge, the SN titers of all vaccinated groups were decreased from 7 to 14 DPC. Increased SN titers were increased in all vaccinated groups at 35 DPC. The SN titers of the

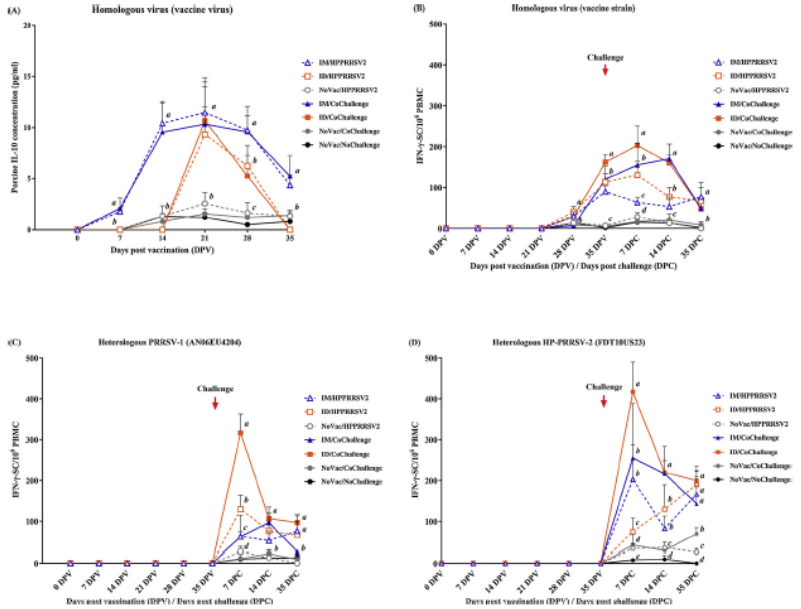


Fig. 2. Analysis of *in vitro* stimulation. (**A**) Quantification of porcine IL-10 in the supernatant of stimulated PBMC with homologous virus (vaccine strain) following vaccination. (**B-D**) Evaluation of PRRSV-specific IFN-©-secreting cells (SC) after stimulation with (**B**) homologous virus (vaccine virus), (**C**) heterologous PRRSV-1 (AN06EU4204) and (**D**) heterologous HP-PRRSV-2 (FDT10US23). Values are expressed as the mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (*a-e*) indicate significant differences between treatment groups ($P < 0.05$) for each day.

IM/CoChallenge and ID/CoChallenge groups were significantly higher than those of the IM/HPPRRSV2 and ID/HPPRRSV2 groups at 35 DPC.

3.4. ID vaccination induced lower IL-10 production than IM vaccination

The induction of IL-10 production was different between vaccinated groups in which delayed response was observed in ID vaccinated pigs (Fig. 2A). IL-10 was observed at 7 DPV in IM vaccinated pigs and increased to the highest level at 21 DPV. In contrast, IL-10 was observed at 21 DPV in ID vaccinated pigs. All IM vaccinated groups had significantly higher IL-10 levels than that of ID vaccinated and control groups at 7 and 14 DPV. There was no difference in IL-10 levels between ID vaccinated and nonvaccinated groups at 7 and 14 DPV. At 7 and 14 DPV, increased IL-10 levels were observed only in the IM/HPPRRSV2 and IM/CoChallenge groups, which had significantly ($P < 0.05$) higher IL-10 levels (range from 1.78 ± 0.4–10.42 ± 1.2 pg/mL) than the ID/HPPRRSV2 and ID/CoChallenge groups. Then, the IL-10 levels continuously increased and reached the maximum level (range from 10.34 ± 1.7–11.45 ± 1.3 pg/mL) at 21 DPV without a significant difference among vaccinated groups. Subsequently, the IL-10 levels of all vaccinated groups continually declined at 28 and 35 DPV. At 28 and 35 DPV, the IL-10 of the IM/HPPRRSV2 and IM/CoChallenge groups were statistically ($P < 0.05$) higher levels (range from 4.35 ± 1.0–9.74 ± 1.4 pg/mL) than those of the ID/HPPRRSV2 and ID/CoChallenge groups (range from 0 to 6.23 ± 1.2 pg/mL), respectively.

3.5. ID vaccination induced higher IFN-γ-SC than IM vaccination

Following homologous virus stimulation, IFN-γ-SC were first detected in the ID vaccinated groups at 28 DPV and continuously increased, reaching the highest levels at 35 DPV. In contrast, IFN-γ-SC were first detected in the IM vaccinated groups at 35 DPV (Fig. 2B). The ID vaccinated groups had significantly more IFN-γ-SC than the IM vaccinated groups at 28 and 35 DPV. Following challenge, the IFN-γ-SC in the IM/CoChallenge (155 ± 12 and 170 ± 16 cells/10^6 PMBC) and ID/CoChallenge (120 ± 12 and 155 ± 15 cells/10^6 PBMC) groups continually increased and had significantly higher frequencies than those in the IM/HPPRRSV2 and ID/HPPRRSV2 groups at 7 and 14 DPC, respectively. The IFN-γ-SC of all vaccinated groups were decreased at 35 DPC.

After stimulation with heterologous PRRSV-1 (AN6EU4204), no IFN-γ-SC were detected in any vaccinated groups following vaccination (Fig. 2C). Following challenge, significantly more IFN-γ-SC were observed in all vaccinated groups than in nonvaccinated groups. At 7 DPC, the most IFN-γ-SC (316.67 ± 25.49 cells/10^6 PBMC) were observed in the ID/CoChallenge group ($P < 0.05$). Meanwhile, the IFN-γ-SC in the vaccinated groups were not different at 14 and 35 DPC, except for the IM/CoChallenge group, which had significantly fewer IFN-γ-SC (26.67 ± 6.15 cells/10^6 PBMC) than the other vaccinated groups.

Similar to heterologous PRRSV-1 stimulation, after stimulation with heterologous HP-PRRSV-2 (FDT10US23), no IFN-γ-SC were detected in any vaccinated groups following vaccination (Fig. 2D). Following challenge, significantly more IFN-γ-SC were observed in all vaccinated

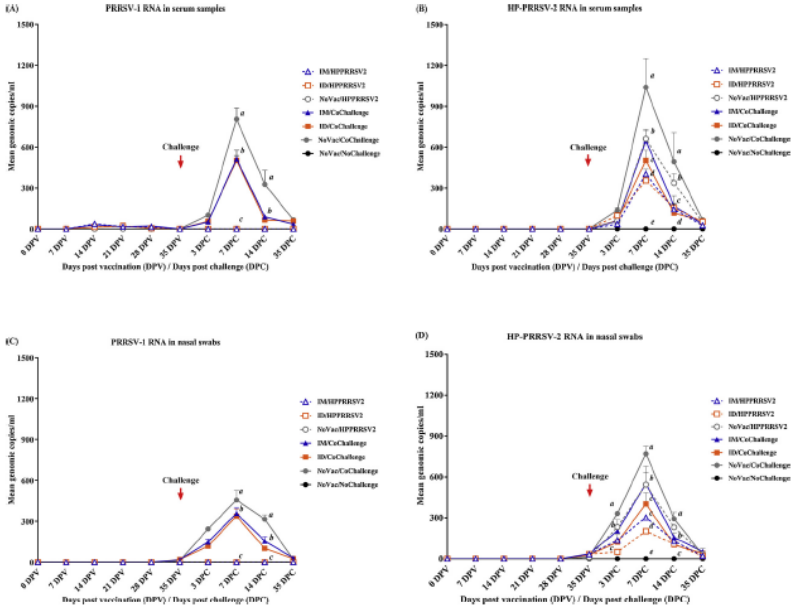


Fig. 3. Mean genomic copy number of PRRSV-1 and HP-PRRSV-2 RNA in the (**A-B**) serum and (**C-D**) nasal swabs of all treatment groups. Values are expressed as the mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (*a-e*) indicate significant differences between treatment groups ($P < 0.05$) for each day.

groups than in the nonvaccinated groups. At 7 DPC, the ID/CoChallenge group had the significantly more IFN-γ-SC of 416.67 ± 34 cells/10^6 PBMC than the other vaccinated groups. At 14 DPC, significantly more IFN-γ-SC were observed in the IM/CoChallenge and ID/CoChallenge groups than in the IM/HPPRRSV2 and ID/HPPRRSV2 groups. However, there was no difference in IFN-γ-SC among the vaccinated groups at 35 DPC.

3.6. IM and ID vaccination reduced PRRSV viremia and nasal shedding following challenge

Following PRRSV-1 MLV vaccination, there was no significant difference in the amount of PRRSV-1 RNA in the sera of the IM and ID vaccinated groups. PRRSV-1 RNA was first detected in all vaccinated groups at 14 DPV with lower levels (< 100 copies) and remained constant until 35 DPV (Fig. 3A). Following challenge, PRRSV-1 RNA was detected in the blood of the IM/CoChallenge, ID/CoChallenge and NoVac/CoChallenge groups only and rapidly increased, reaching peaks at 7 DPC. Then, the amount of PRRSV-1 RNA was continually decreased to basal levels from 14 to 35 DPC. At 3 DPC, there was no difference in PRRSV-1 RNA among the groups. The IM/CoChallenge and ID/CoChallenge groups had significantly less ($P < 0.05$) PRRSV-1 RNA than the NoVac/CoChallenge group at 7 and 14 DPC. However, there was no difference in PRRSV-1 RNA among the groups at 35 DPC (Fig. 3A).

Following PRRSV-1 MLV vaccination, no HP-PRRSV-2 RNA was detected in the blood of any of the groups. HP-PRRSV-2 RNA was first detected at 3 DPC and continually increased and reached peaks at 7 DPC. Then, HP-PRRSV-2 RNA continued to decrease at 14 DPC and remained at basal at 35 DPC (Fig. 3B). The pigs in the NoVac/CoChallenge group had the highest HP-PRRSV-2 RNA levels of 1038 ± 122 and 493 ± 112 copies/mL at 7 and 14 DPC, respectively. At 7 DPC, the IM/HPPRRSV2 and ID/HPPRRSV2 groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the other groups. At 14 DPC, all vaccinated groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the nonvaccinated/challenged groups. There was no difference in HP-PRRSV-2 RNA among the groups at 35 DPC (Fig. 3B).

The IM/CoChallenge and ID/CoChallenge groups had significantly lower ($P < 0.05$) PRRSV-1 RNA in the nasal swabs than the NoVac/CoChallenge group from 3 to 14 DPC, but no differences were observed at 35 DPC. There were no differences in PRRSV-1 RNA between the IM/CoChallenge and ID/CoChallenge groups at 3, 7 and 14 DPC (Fig. 3C). The genomic copies of HP-PRRSV-2 RNA in nasal swabs were relatively higher than those of PRRSV-1 RNA but had similar patterns to PRRSV-1 RNA (Fig. 3D). The HP-PRRSV-2 RNA in nasal swabs continually increased in all groups at 3 DPC and reached the highest levels at 7 DPC. Afterward, the HP-PRRSV-2 RNA quickly decreased at 14 DPC and remained at basal levels at 35 DPC. The ID/HPPRRSV2 group had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA of 50 ± 5 and 201.6 ± 12 copies/mL than the other groups at 3 and 7 DPC, respectively. At 14 DPC, all vaccinated groups had significantly lower ($P < 0.05$) HP-PRRSV-2 RNA than the NoVac/HPPRRSV2 and NoVac/CoChallenge groups. There was no difference in HP-PRRSV-2 RNA in nasal swabs among the groups at 35 DPC. PRRSV RNA, both PRRSV-1

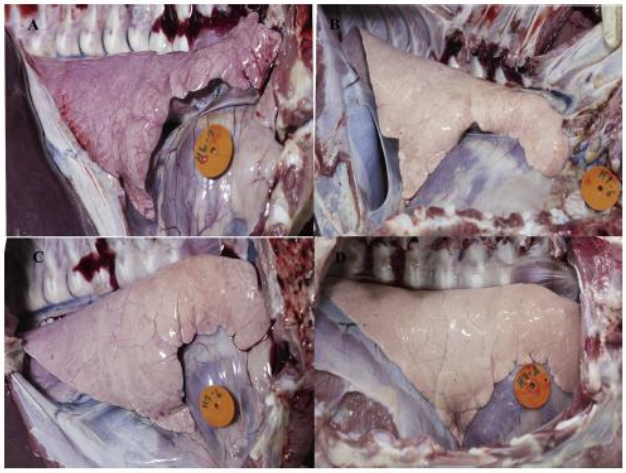


Fig. 4. Macroscopic lung lesions following challenge at 7 DPC of the (A) nonvaccinated/challenged, (B) IM vaccinated pigs, (C) ID vaccinated pigs and (D) nonvaccinated/nonchallenge pigs.

and HP-PRRSV-2, was not detected in the blood and nasal swabs from the NoVac/NoChallenge group throughout the experiment.

3.7. IM and ID vaccination reduced macroscopic and microscopic lung lesions following challenge

The macroscopic lung lesions induced by PRRSV were characterized by multifocal, tan-molted areas with irregular and indistinct borders (Fig. 4). Pigs in the NoVac/HPPRRSV2 and NoVac/CoChallenge groups had significantly higher lung scores of 71.3 ± 3.2 and 84.0 ± 3.5 than those in the vaccinated challenged groups at 7 DPC. Significantly fewer macroscopic lung lesions were observed in the ID/HPPRRSV2 and ID/CoChallenge groups than in the IM/HPPRRSV2 and IM/CoChallenge groups, respectively (Table 3). In addition, the ID/HPPRRSV2 group had significantly lower macroscopic lung lesion scores of 27.3 ± 2.4 than the other groups. There was no difference in macroscopic lung lesion scores among the groups at 35 DPC.

The microscopic lung lesions associated with PRRSV infection were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia (Fig. 5). Microscopic lung lesion scores were concordant with the macroscopic lung lesion scores. All vaccinated groups, regardless to the route of administration, had significantly lower microscopic lung lesion scores than the nonvaccinated/challenged groups (Table 3). Pigs in the ID/HPPRRSV2 and ID/CoChallenge groups had significantly lower microscopic lung lesion scores of 1.33 ± 0.14 and 1.51 ± 0.11 than those in the IM/HPPRRSV2 and NoVac/HPPRRSV2 groups of 2.38 ± 0.11 and 2.37 ± 0.07 at 7 DPC. There were no microscopic lung lesions in the groups at 35 DPC (Supplementary Information).

Table 3
Macro- and microscopic lung lesion scores following challenge. Values express as the mean ± SEM. The results were compared using two-way ANOVA for multiple comparisons. Different lowercase letters (*a-e*) indicate significant differences between treatment groups ($P < 0.05$) for each day post-challenge (DPC).

Treatment groups	Macroscopic scores		Microscopic scores	
	7 DPC	35 DPC	7 DPC	35 DPC
IM/HPPRRSV2	58.0 ± 2.0^{c}	0 ± 0	1.62 ± 0.11^{b}	0 ± 0
ID/HPPRRSV2	27.3 ± 2.4^{e}	0 ± 0	1.33 ± 0.14^{c}	0 ± 0
NoVac/HPPRRSV2	71.3 ± 3.2^{b}	2.0 ± 1.0	2.38 ± 0.11^{a}	0 ± 0
IM/CoChallenge	62.3 ± 2.4^{c}	2.0 ± 0.3	1.88 ± 0.06^{b}	0 ± 0
ID/CoChallenge	41.0 ± 7.0^{d}	1.0 ± 0.3	1.51 ± 0.10^{c}	0 ± 0
NoVac/CoChallenge	84.0 ± 3.5^{a}	2.0 ± 1.0	2.37 ± 0.07^{a}	0 ± 0
NoVac/NoChallenge	0 ± 0^{e}	0 ± 0	0 ± 0^{e}	0 ± 0

4. Discussion

The present study was conducted to investigate the immune response and IL-10 production of pigs vaccinated IM or ID with PRRSV-1 MLV. The protective efficacy was evaluated upon challenge with HP-PRRSV-2 either alone or in combination with PRRSV-1. It was demonstrated that pigs vaccinated, either IM or ID, induce a similar patterns of antibody response as measured by ELISA and SN assays. The discrepancy is observed in cell mediated immune (CMI) response in which ID vaccinated pigs had significantly lower IL-10 levels and higher IFN-γ-SC levels than that of IM-vaccinated pigs. Following challenge with HP-PRRSV-2, either alone or cochallenge with PRRSV-1, PRRSV viremia and lung lesions, both macroscopically and microscopically, were significantly reduced in vaccinated pigs than that of nonvaccinated pigs, regardless to the route of vaccine administration. It is notably that ID vaccinated pigs had significantly lower levels of viremia and lung lesion scores than that of IM vaccinated pigs.

Recently, new routes of vaccine administration including an ID administration through needle-free devices have intensively been studies to improve the efficacy of vaccines. Needle-free device have been used as advantageous methods to cross the epidermal barrier and efficiently deliver antigens into the dermal layer (Giudice and Campbell, 2006), requiring a smaller volume of fluid than the more conventional IM route (Giudice and Campbell, 2006). The most important

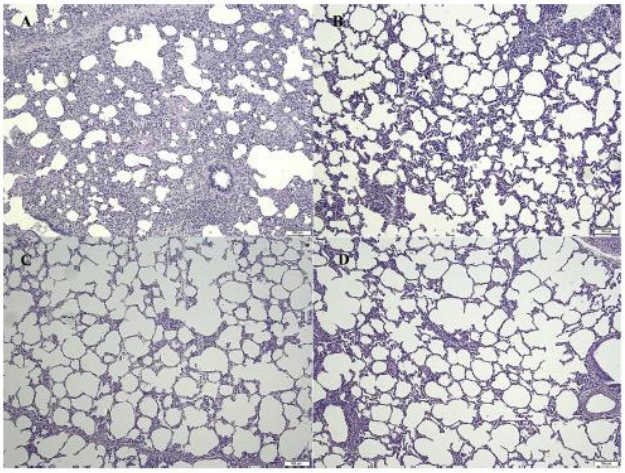


Fig. 5. Microscopic lung lesions following challenge of the (**A**) nonvaccinated/challenged, (**B**) IM vaccinated pigs, (**C**) ID vaccinated pigs and (**D**) nonvaccinated/nonchallenge pigs. H&E staining. Bar = 100 μm.

advantages of the ID administration by a needle-less device are that it is less invasive, painless, safe, quick and easy. Furthermore, the ID administration could induce a stronger CMI response compared to that of the IM administration. The superior efficacy of the ID vaccination, regarding to the induction of the immune response, was demonstrated in a previous report in which the ID vaccination delivered by a needle-free device can prime a stronger specific immune response, both humoral and CMI, against Aujeszky's disease compared to that of induced by the IM vaccination (Ferrari et al., 2011).

Regarding to CMI response, delivery through the intradermal route could induce T cell polarization through the Th1 pathway, favoring the induction of IFN-γ. This phenomenon was evident in the present study. ID vaccinated pigs had a significantly higher level of PRRSV-specific IFN-γ-SC than that of IM vaccinated pigs. The observed results are in accordance with previous reports in which ID vaccinated pigs induce relatively more IFN-γ-SC than IM-vaccinated pigs (Ferrari et al., 2013; Martelli et al., 2009). One factor likely contributing to this finding is the presence of skin-resident immune cells able to sufficiently capture antigens directly from the skin. The skin is rich in professional antigen presenting cells (APC), including epidermal Langerhans cells (LC) and dermal dendritic cells, which are known to migrate to draining lymph nodes and trigger immune responses (Combadiere and Liard, 2011).

Another possibility of higher IFN-γ-SC in ID vaccinated pigs than IM vaccinated pigs could be due to the lower IL-10 levels. Our results demonstrated that the IL-10 production was delayed, and the level was significantly lower in ID vaccinated pigs than in IM vaccinated pigs in the early phase following vaccination. The results are in agreement with a previous report in which vaccinated pigs, both IM and ID, can induce IL-10 production, but ID vaccinated pigs induced relatively lower IL-10 levels compared to that of IM vaccinated pigs (Ferrari et al., 2013). However, the delivery of antigen through the intradermal route could target dendritic cells. IL-10 is a cytokine of the Th2 response. The delivery through this route could induce T cell polarization through the Th1 pathway, favoring other cytokines that act against Th2 (Tesfaye et al., 2019). However, the mechanisms of IL-10 induction following vaccination by the IM and ID routes are not understood.

IL-10 is a cytokine with multiple effects on immunoregulation and inflammation. It functions in T cell polarization by downregulating the expression of Th1 cytokines, including IL-12 and IFN-γ, MHC class II antigens, and costimulatory molecules on macrophages. IL-10 also enhances B cell survival, proliferation, and antibody production. Additionally, IL-10 has a central role in limiting pathogen-induced immunopathology and is associated with the induction of tolerance and regulatory T lymphocytes (Treg) (LeRoith et al., 2011). The exploitation of IL-10 appear to be a common mechanism of immunosuppression by intracellular pathogens that specifically target macrophages for infection. Considering the restricted tissue tropism of PRRSV, it is conceivable that PRRSV used IL-10 to suppressing the host immune response. In PRRSV infection, either by natural infection of MLV vaccination, increased IL-10 production was observed in both *in vitro* and *in vivo* (Flores-Mendoza et al., 2008). These undesired outcomes potentially resulted in the slow induction of effective immunity, the failure of other vaccines and increased susceptibility to secondary infection by the other pathogens, causing porcine respiratory disease complex. In PRRSV infection, IL-10 is also related to the severity of clinical diseases (van Reeth and Nauwynck, 2000).

With respect to the humoral immune response, the results of the present study demonstrated that the induction of the humoral immune response against PRRSV was not different between IM- and ID-vaccinated pigs. The results demonstrated no difference in the induction of the humoral immune response as measured by ELISA between IM- and ID-vaccinated pigs and are in agreement with previous studies (Ferrari et al., 2013).

The results of the study demonstrated that pigs vaccinated ID or IM with PRRSV-1 MLV (UNISTRAIN® PRRS) conferred partial heterologous protection against HP-PRRSV-2, either alone or in combination with PRRSV-1. The findings reported herein are in agreement with previous reports (Bonckaert et al., 2016; Roca et al., 2012). Based on a single challenge with either virulent PRRSV-1 (Lena) (Bonckaert et al., 2016) or HP-PRRSV-2 (Roca et al., 2012), PRRSV-1 MLV (UNISTRAIN® PRRS), administered through the IM route, can confer a partial protection as evidenced by reduced viremia. The mechanism of partial cross

protection of the PRRSV-1 MLV against heterologous PRRSV-2 is not known but might be due to the induction of cross neutralizing reactivity against heterologous HP-PRRSV-2. This was observed by increased SN titers against HP-PRRSV-2 in the vaccinated pigs in the present study. In addition, different type of PRRSV-1 MLV could potentially have various activities against PRRSV-2. A previous report comparing the efficacy of 2 different PRRSV-1 MLV vaccines demonstrated that one PRRSV-1 MLV had low protection against HP-PRRSV-2 (Madapong et al., 2020). However, further investigations are needed to be performed.

Genetic similarity between the vaccine and field virus is not a good indicator of the protective efficacy provided by a PRRSV MLV vaccine (Opriessnig et al., 2002). The protective efficacy of a PRRSV MLV is usually determined by the reduction in viremia and lung lesions following challenge with virulent viruses (Labarque et al., 2003). PRRSV viremia plays a central role to its pathogenesis. High PRRSV in serum associated with the development of interstitial pneumonic lung lesions (Han et al., 2013). Therefore, vaccine mediated reduction of PRRSV viremia is critical for controlling the infection pigs. Our results are in accordance with previous studies on the efficacy of PRRSV MLV vaccination showing that all vaccine species provide partial protection against challenge with heterologous PRRSV strains with a wide range of protection (Bonckaert et al., 2016; Ferrari et al., 2013; Martelli et al., 2009; Roca et al., 2012). Notably, ID vaccinated pigs had significantly lower macroscopic and microscopic lung lesion scores than IM-vaccinated pigs. This finding could be because the ID route induce a strong cell-mediated immune response as evidenced by the number of IFN-γ-SC.

5. Conclusion

In conclusion, the results of the study suggested that PRRSV-1 MLV administered by either IM or ID can provide partial heterologous protection against challenge with HP-PRRSV-2, either alone or in conjunction with PRRSV-1, as demonstrated by reduced lung lesions and viremia. The ID route might represent an alternative to improve vaccine efficacy, as it induced lower IL-10 levels and more IFN-γ-SC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by Research and Researchers for Industries (RRI, grant number PHD59I0040), and Agricultural Research Development Agency (Public Organization), (ARDA, grant number CRP5705019990). Furthermore, the partial funding was supported by Chulalongkorn University Special Task Force for Activation Research (CU-STAR); swine virus evolution and vaccine research (SVEVR).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108655.

References

Bonckaert, C., van der Meulen, K., Rodríguez-Ballara, I., Pedrazuela Sanz, R., Martínez, M.F., Nauwynck, H.J., 2016. Modified-live PRRSV subtype 1 vaccine UNISTRAIN(R) PRRS provides a partial clinical and virological protection upon challenge with East European subtype 3 PRRSV strain Lena. Porcine Health Manag. 2, 12.

Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629–633.

Chen, N., Cao, Z., Yu, X., Deng, X., Zhao, T., Wang, L., Liu, Q., Li, X., Tian, K., 2011. Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China. J. Gen. Virol. 92, 880–892.

Choi, K., Lee, J., Park, C., Jeong, J., Chae, C., 2015. Comparison of the pathogenesis of single or dual infections with type 1 and type 2 porcine reproductive and respiratory syndrome virus. J. Comp. Pathol. 152, 317–324.

Combadiere, B., Liard, C., 2011. Transcutaneous and intradermal vaccination. Hum. Vaccin. 7, 811–827.

Egli, C., Thur, B., Liu, L., Hofmann, M.A., 2001. Quantitative TaqMan RT-PCR for the detection and differentiation of European and North American strains of porcine reproductive and respiratory syndrome virus. J. Virol. Methods 98, 63–75.

Ferrari, L., Borghetti, P., Gozio, S., De Angelis, E., Ballotta, L., Smeets, J., Blanchaert, A., Martelli, P., 2011. Evaluation of the immune response induced by intradermal vaccination by using a needle-less system in comparison with the intramuscular route in conventional pigs. Res. Vet. Sci. 90, 64–71.

Ferrari, L., Martelli, P., Saleri, R., De Angelis, E., Cavalli, V., Bresaola, M., Benetti, M., Borghetti, P., 2013. Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. Vet. Immunol. Immunopathol. 151, 193–206.

Flores-Mendoza, L., Silva-Campa, E., Resendiz, M., Osorio, F.A., Hernandez, J., 2008. Porcine reproductive and respiratory syndrome virus infects mature porcine dendritic cells and up-regulates interleukin-10 production. Clin. Vaccine Immunol. 15, 720–725.

Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Botner, A., 2002. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38–47.

Giudice, E.L., Campbell, J.D., 2006. Needle-free vaccine delivery. Adv. Drug Deliv. Rev. 58, 68–89.

Halbur, P., Paul, P., Frey, M., Landgraf, J., Eernisse, K., Meng, X.-J., Lum, M., Andrews, J., Rathje, J., 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. 32, 648–660.

Han, K., Seo, H.W., Oh, Y., Kang, I., Park, C., Chae, C., 2013. Comparison of the virulence of European and North American genotypes of porcine reproductive and respiratory syndrome virus in experimentally infected pigs. Vet. J. 195, 313–318.

Kim, H.K., Park, S.J., Rho, S.M., Han, J.Y., Nguyen, V.G., Park, B.K., 2011. One year's study of dynamic and evolution of types I and II PRRSV in a swine farm. Vet. Microbiol. 150, 230–238.

Kuhn, J.H., Lauck, M., Bailey, A.L., Shchetinin, A.M., Vishnevskaya, T.V., Bao, Y.M., Ng, T.F.F., LeBreton, M., Schneider, B.S., Gillis, A., Tamoufe, U., Diffo, J.L., Takuo, J.M., Kondov, N.O., Coffey, L.L., Wolfe, N.D., Delwart, E., Clawson, A.N., Postnikova, E., Bollinger, L., Lackemeyer, M.G., Radoshitzky, S.R., Palacios, G., Wada, J., Shevtsova, Z.V., Jahrling, P.B., Lapin, B.A., Deriabin, P.G., Dunowska, M., Alkhovsky, S.V., Rogers, J., Friedrich, T.C., O'Connor, D.H., Goldberg, T.L., 2016. Reorganization and expansion of the nidoviral family Arteriviridae. Arch. Virol. 161, 755–768.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2003. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet. Microbiol. 95, 187–197.

LeRoith, T., Hammond, S., Todd, S.M., Ni, Y., Cecere, T., Pelzer, K.D., 2011. A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with Mycoplasma hyopneumoniae. Vet. Immunol. Immunopathol. 140, 312–316.

Madapong, A., Temeeyasen, G., Saeng-Chuto, K., Tripipat, T., Navasakuljinda, W., Boonsoongnern, A., Tantituvanont, A., Nilubol, D., 2017. Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines. Arch. Virol. 162, 139–146.

Madapong, A., Saeng-Chuto, K., Boonsoongnern, A., Tantituvanont, A., Nilubol, D., 2020. Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2. Sci. Rep. 10, 1649.

Martelli, P., Gozio, S., Ferrari, L., Rosina, S., De Angelis, E., Quintavalla, C., Bottarelli, E., Borghetti, P., 2009. Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: clinical protection and cell-mediated immunity. Vaccine 27, 3788–3799.

Nilubol, D., Platt, K.B., Halbur, P.G., Torremorell, M., Harris, D.L., 2004. The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. Vet. Microbiol. 102, 11–18.

Nilubol, D., Tripipat, T., Hoonsuwan, T., Kortheerakul, K., 2012. Porcine reproductive and respiratory syndrome virus, Thailand, 2010-2011. Emerg. Infect. Dis. 18, 2039–2043.

Nilubol, D., Tripipat, T., Hoonsuwan, T., Tipsombatboon, P., Piriyapongsa, J., 2013. Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes I and II in Thailand. Arch. Virol. 158, 943–953.

Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J. Virol. 76, 11837–11844.

Park, C., Seo, H.W., Han, K., Kang, I., Chae, C., 2014. Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge. Vet. Microbiol. 172, 432–442.

Roca, M., Gimeno, M., Bruguera, S., Segales, J., Diaz, I., Galindo-Cardiel, I.J., Martinez, E., Darwich, L., Fang, Y., Maldonado, J., March, R., Mateu, E., 2012. Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. Vet. J. 193, 92–96.
Saraiva, M., O'Garra, A., 2010. The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10, 170–181.
Shi, M., Lam, T.T., Hon, C.C., Murtaugh, M.P., Davies, P.R., Hui, R.K., Li, J., Wong, L.T., Yip, C.W., Jiang, J.W., Leung, F.C., 2010. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J. Virol. 84, 8700–8711.
Stadejek, T., Oleksiewicz, M.B., Scherbakov, A.V., Timina, A.M., Krabbe, J.S., Chabros, K., Potapchuk, D., 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch. Virol. 153, 1479–1488.
Tesfaye, D.Y., Gudjonsson, A., Bogen, B., Fossum, E., 2019. Targeting conventional dendritic cells to fine-tune antibody responses. Front. Immunol. 10, 1529.
van Reeth, K., Nauwynck, H., 2000. Proinflammatory cytokines and viral respiratory disease in pigs. Vet. Res. 31, 187–213.

VITA

NAME	Adthakorn Madapong
DATE OF BIRTH	06 April 1989
PLACE OF BIRTH	Phetchaboon, Thailand
INSTITUTIONS ATTENDED	2007-2010 Bachelor of Science degree (Veterinary Technology), Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand
HOME ADDRESS	138/85 Ramintra 117, Min Buri, Bangkok, 10510, Thailand
PUBLICATION	1. Madapong, A., Saeng-chuto, K., Lorsirikool, A., Teemeyasen, G., Srijangwas, A., Tripipat, T., Wegner, M. and Nilubol, D. 2016. Complete genome sequence of porcine deltacoronavirus isolated in Thailand in 2015. Genome Announcement. 4(3): e00408-16 2. Madapong, A., Teemeeyasen, G., Saeng-chuto, K., Tripipat, T., Navasakuljinda, W., Boonsoongnern, A., Tuvanont, A. and Nilubol, D. 2017. Humoral immune responses and viral shedding following vaccination with modified live porcine reproductive and respiratory syndrome virus vaccines. Archives of Virology. 162: 139-146, https://doi.org/10.1007/s00705-016-3084-4 3. Madapong, A., Saeng-chuto, K., Boonsoongnern, A., Tantituvanont, A., Nilubol, D. 2020. Cell-mediated immune response and protective efficacy of porcine reproductive and respiratory syndrome virus modified-live vaccines against co-challenge with PRRSV-1 and PRRSV-2. Scientific Reports. 10: 1649, https://doi.org/10.1038/s41598-020-58626-y

4. Madapong, A., Saeng-chuto, K., Chaikhumwang, P., Tantituvanont, A., Saardrak, K., Pedrazuela S.R., Miranda A.J., Nilubol, D. 2020. Immune response and protective efficacy of intramuscular and intradermal vaccination with porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) modified live vaccine against highly pathogenic PRRSV-2 (HP-PRRSV-2) challenge, either alone or in combination with of PRRSV-1. Veterinary Microbiology. 244: 108655, https://doi.org/10.1016/j.vetmic.2020.108655