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ORAL FLUID SAMPLES USED FOR PRRS DIAGNOSIS AND MANAGEMENT

Miss Yonlayong Woonwong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Pathobiology
Department of Veterinary Pathology
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ยลยง วุ้นวงษ์ : การใช้ตัวอย่างน้ำลายในการตรวจวินิจฉัยและการจัดการโรคพีอาร์อาร์เอส (ORAL FLUID SAMPLES USED FOR PRRS DIAGNOSIS AND MANAGEMENT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สพ.ญ. ดร.เยาวลักษณ์ ปัญญสิงห์, หน้า.

โรคพีอาร์อาร์เอส (PRRS) เป็นโรคที่ก่อให้เกิดความสูญเสียต่อธุรกิจสุกรทั่วโลก วิธีการจัดการและการวินิจฉัยโรคที่เหมาะสมจึงมีความสำคัญในการควบคุมเชื้อไวรัสพีอาร์อาร์เอส (PRRSV) อย่างไรก็ตาม การวินิจฉัยโรคจากตัวอย่างน้ำลายสุกรยังคงขาดองค์ความรู้ในเรื่องวิธีการใช้สำหรับฟาร์มในประเทศไทย โดยการศึกษาในครั้งนี้ ทำการศึกษาวิธีการเตรียมตัวอย่างน้ำลายสุกร ก่อนขั้นตอนการสกัดตัวอย่างเพื่อให้ได้ผลผลิตพีซีอาร์ที่มากขึ้น และศึกษาการใช้ตัวอย่างน้ำลายสุกรในการตรวจหาสถานะของเชื้อไวรัสพีอาร์อาร์เอสในช่วงการปรับสภาพสุกรสาวและในช่วงคลอดจนถึงอนุบาล จากการศึกษาพบว่า การเตรียมตัวอย่างน้ำลายด้วยวิธีการเพิ่มปริมาณสารตั้งต้น น่าจะเป็นวิธีพื้นฐานที่เหมาะสมสำหรับใช้กับชุดสกัดแบบคอลัมน์ ในการเพิ่มความสามารถในการตรวจหาเชื้อไวรัสพีอาร์อาร์เอส ในขณะที่วิธีการเตรียมตัวอย่างแบบอื่นอาจส่งผลเสียต่อคุณภาพสารพันธุกรรมในตัวอย่างได้ นอกจากนี้จากการศึกษาในภาคสนามพบว่า การวินิจฉัยโรคจากตัวอย่างน้ำลายเป็นวิธีที่สามารถปฏิบัติได้ง่าย ประหยัด คำนึงถึงสวัสดิภาพสัตว์ และให้ผลการตรวจที่น่าเชื่อถือ แต่ยังคงพบว่ามีข้อจำกัดในการใช้งาน ผลการศึกษาพบว่าการตรวจด้วยตัวอย่างน้ำลายสุกรมีความสัมพันธ์เชิงบวกอย่างมีนัยสำคัญทางสถิติกับตัวอย่างที่เป็นมาตรฐานคือตัวอย่างซีรัม ค่าเฉลี่ย S/P ratio จากตัวอย่างน้ำลายมีระดับสูงกว่าตัวอย่างซีรัมอย่างมีนัยสำคัญทางสถิติ แต่ยังคงพบว่ามีรูปแบบการตอบสนองที่เหมือนกัน ในระหว่างขั้นตอนการปรับสภาพสุกรสาว ตัวอย่างน้ำลายสุกรสามารถใช้ในการประเมินความสำเร็จของกระบวนการปรับสภาพได้ อย่างไรก็ตามการใช้ตัวอย่างน้ำลายอาจให้มีข้อจำกัดในการตรวจหาเชื้อไวรัสพีอาร์อาร์เอส ในระยะเริ่มต้นของการติดเชื้อ การศึกษาในช่วงคลอดถึงอนุบาลพบว่าการวินิจฉัยจากตัวอย่างน้ำลายสุกรให้ผลที่เหมาะสมในการประเมินสถานะการติดเชื้อไวรัสพีอาร์อาร์เอสโดยเฉพาะอย่างยิ่งในกรณีที่มีความชุกโรคในระดับปานกลางถึงสูง และพบว่าการเปลี่ยนแปลงของระดับแอนติบอดีต่อเชื้อไวรัสพีอาร์อาร์เอสที่ตรวจพบในตัวอย่างน้ำลายสอดคล้องกับการตรวจพบเชื้อไวรัสพีอาร์อาร์เอสในระดับสูง กล่าวโดยสรุปจากการศึกษาครั้งนี้พบว่า ตัวอย่างน้ำลายสุกรมีความเหมาะสมสำหรับใช้ในการประเมินการติดเชื้อไวรัสพีอาร์อาร์เอสในภาคสนาม นอกจากนี้การศึกษาเพื่อหาค่าพื้นฐานสำหรับการตรวจและแนวทางการใช้งานจริงสำหรับน้ำลายสุกรจึงมีความจำเป็น การศึกษานี้เพื่อที่จะให้ได้ข้อมูลที่เป็นประโยชน์สำหรับเกษตรกรในการนำไปใช้และเพื่อการศึกษาต่อไปในอนคต

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5575329831 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: ORAL FLUID, PRRSV, MONITORING, PRE- PREPARATION, ACCLIMATIZATION, MANAGEMENT, GILT, NURSERY PIGS

YONLAYONG WOONWONG: ORAL FLUID SAMPLES USED FOR PRRS DIAGNOSIS AND MANAGEMENT. ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, CO-ADVISOR: DR.YAOWALAK PANYASING, pp.

Porcine reproductive and respiratory syndrome (PRRS) is an economically significant swine disease having a negative impact to the swine industry worldwide. Appropriate management strategies and diagnostic tests are crucial to successfully control PRRS virus (PRRSV). However, the application for oral fluid-based diagnosis has not been evaluated in the Thai farms previously. In this study, pre-extraction methods of oral fluids were evaluated to improve the PCR product yielded. Moreover, the oral fluids utilization for PRRSV monitoring during the gilt acclimatization and farrowing to nursery period was investigated. The results demonstrated that increasing sample volume might be a suitable simple method for column-based extraction kit to improve PRRSV detection. Whereas, other modified pre-extractions could possibly impact nucleic acid quality in the samples. In addition, field evaluation demonstrated that oral fluid testing provided convenient, economical, satisfied and animal welfare friendly method of sample collection with some limitations. Significant positive correlation was found between oral fluid results and the results from serum samples. Mean S/P ratios of oral fluid samples showed significantly higher levels than those of from the serum samples with similar patterns. During acclimatization period, oral fluid testing could be used to monitor the success of acclimatization. However, it did not completely monitor all PRRSV infection in acute phase of infection. In the farrowing to nursery study, oral fluid testing provided satisfied performance to determine PRRS status especially when having moderate to high prevalence. The changes in anti-PRRSV antibodies status in oral fluids after horizontal exposure were concurrently with the presence of high viral loads. In conclusion, oral fluid testing is a suitable sample for PRRSV monitoring. Additionally, the baseline and application for oral fluid-based diagnosis are of essential to explore on its utilization. The finding data would beneficial to the farmers and the future utilization for successful PRRS prevention and control.

Department:	Veterinary Pathology	Student's Signature	....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	....
Academic Year:	2017	Co-Advisor's Signature	....

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CONTENTS

Page

THAI ABSTRACT iv

ENGLISH ABSTRACT v

ACKNOWLEDGEMENTS vi

CONTENTS vii

LIST OF ABBREVIATIONS 1

CHAPTER 1 4

INTRODUCTION 4

1.1 Importance and Rationale 4

1.2 Objectives of the study 9

1.3 Literature Reviews 9

Porcine reproductive and respiratory syndrome: 9

PRRSV infection in a breeding herd: 10

PRRS control: 11

Gilt acclimatization program: 13

Parity segregation system: 14

PRRS Monitoring program: 15

Basic concepts of oral fluid: 17

Oral fluid immunity: 18

PCR inhibitions and NaOH treatment: 19

Oral fluid collection and processing: 19

Oral fluid collection: 19

Oral fluid processing: 20

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Oral fluid used in swine medicine: ... 21

CHAPTER 2 ... 22

MODIFIED PRE-EXTRACTION METHODS TO INCREASE PRRSV qRT-PCR DETECTION FROM SWINE ORAL FLUID SAMPLES ... 22

Abstract ... 23

2.1 Introduction ... 24

2.2 Material and Methods ... 25

2.3 Results and Discussion ... 26

2.4 Acknowledgements ... 28

Table ... 29

Table 2.1 ... 29

CHAPTER 3 ... 30

ORAL FLUID SAMPLES USED FOR PRRSV ACCLIMATIZATION PROGRAM AND SOW PERFORMANCE MONITORING IN ENDEMIC PRRS-POSITIVE FARMS ... 30

Abstract ... 31

3.1 Introduction ... 32

3.2 Material and Methods ... 35

Animals and Farms: ... 35

Sample collection: ... 37

Quantification of PRRSV RNA: ... 38

PRRSV antibody detection by ELISA: ... 39

PRRSV Monitoring: ... 39

Data analysis: ... 40

3.3 Results ... 40

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Gilt status: 40

qRT-PCR results: 41

PRRSV antibody responses in serum and oral fluid samples: 42

Sow performance: 42

3.4 Discussion 43

3.5 Acknowledgements 48

3.6 Funding information 48

3.7 Compliance with ethical standards 48

3.8 Conflict of interest 48

Table & Figures 49

Table 3.1 49

Figure 3.1 50

Figure 3.2 51

Figure 3.3 52

CHAPTER 4 53

DETECTION OF PRRSV CIRCULATION USING ORAL FLUID SAMPLES FOR NURSERY MANAGEMENT IN ENDEMICALLY PRRSV-INFECTED FARMS 53

Abstract 54

4.1 Introduction 55

4.2 Material and Methods 57

Ethics statement: 57

Trial farms: 57

Oral fluids and serum collections: 59

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Quantification of PRRSV RNA: ... 60

Detection of PRRSV-specific antibody: ... 60

Performance monitoring of sows and nursery pigs: ... 61

Data analysis: ... 61

4.3 Results ... 62

Detection of PRRSV RNA in oral fluid and serum samples: ... 62

Serology test: ... 63

Production parameters: ... 63

4.4 Discussion ... 64

4.5 Conclusion ... 68

4.6 Acknowledgements ... 69

Tables & Figures ... 70

Table 4.1 ... 70

Table 4.2 ... 71

Table 4.3 ... 72

Figure 4.1 ... 73

Figure 4.2 ... 75

Figure 4.3 ... 77

CHAPTER 5 ... 78

CONCLUSION ... 78

5.1 Research summary ... 78

5.2 Research limitation and further investigation ... 84

REFERENCES ... 86

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

APPENDIX 97

Appendix A: Oral fluid collection 98

Appendix B: Virus titration and Immunoperoxidase monolayer assay (IPMA) 99

VITA 100

LIST OF ABBREVIATIONS

Ab	antibody
ADG	average daily gain
ADV	Aujeszky’s disease virus
ANOVA	analysis of variance
APP	*Actinobacillus pleuropneumoniae*
BSA	bovine serum albumin
Cm	centimeter (s)
CSFV	classical swine fever virus
Ct	Cycle threshold
CU-VDL	Veterinary Diagnosis Laboratory, Chulalongkorn University
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
et al.	et alii, and others
EVAP	evaporative cooling system
FCG	feed conversion per gain
FCR	feed conversion ratio
FMDV	foot-and-mouth disease virus

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

g	gram (s)
IACUC	Institutional Animal Care and Use Committee
kg	kilogram (s)
M	mole (s)
mAb	monoclonal antibody
MEM	minimum essential media
ml	millilitre (s)
MLV	modified live virus
NaOH	sodium hydroxide
OF	oral fluids
ORF7	Open reading frame 7, nucleocapsid protein
P1	primiparous sow
P2+	multiparous sow
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV2	porcine circovirus type 2 virus
PEDV	porcine epidemic diarrhea virus
PPV	porcine parvovirus

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

PRDC	porcine respiratory disease complex
PRRS	porcine reproductive and respiratory syndrome
PRRSV	porcine reproductive and respiratory syndrome virus
qRT-PCR	real-time quantitative reverse transcription-PCR
RNA	ribonucleic acid
S/P ratio	sample-to-positive ratio
SIV	swine influenza virus
$TCID_{50}$	tissue culture infectious dose 50
USAMC-AFRIMS	U.S. Army Medical Component-Armed Forces Research Institute of Medical Sciences
WPE	week post exposure
WPF	week post farrowing
°C	degree Celsius (centigrade)
µl	microliter (s)

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CHAPTER 1

INTRODUCTION

1.1 Importance and Rationale

Nowadays, pork industry has become one of the most important food supplies worldwide. Swine production has been growing strong in Asia, and the trend has changed from small holders to intensive farming. The intensive farming contains over-crowed animals that induce stress and trauma. It commonly has more or less disease circulation depending on the management (Amadori and Zanotti, 2016). In the intensive farming, porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important disease affecting the swine production. In swine, PRRS can cause reproductive failure in breeders and respiratory problems in growers. Therefore, PRRS could be a very severe disease in naïve breeding herds and are usually followed by porcine respiratory disease complex (PRDC) in growing pigs. To achieve this problem, the appropriated disease control methods including diagnosis, monitoring, surveillance, vaccination, pre-medication, treatment and sanitation are of importance. Although the different PRRS control protocols have been established, ongoing problems associated with PRRS virus (PRRSV) still occur. Therefore, the impacts of PRRS monitoring strategies on the successful of PRRS controls should not be

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overlooked. Generally, swine diagnostic sample often use blood samples. However, blood collection procedure induces stress, trauma, infection and costly. Key determinant in choosing the diagnostic sample is of interest in terms of animal welfare and sampling cost based on the farmer concern. Because the ban of animal cruelty is becoming society concern having a high impact on animal production and the conventional sampling cost can affect on the overall financial of the farm. Interestingly, recognition on the benefits of oral fluids use for PRRS monitoring has recently become apparent due to its advantages over the use of serum samples. Many studies both in medical and veterinary fields reported that saliva and oral fluid samples were suitable for many assays, e.g., polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) (Kittawornrat et al., 2010; Decorte et al., 2015; Sattler et al., 2015). In addition, oral fluid sampling method is a non-invasive method and could reduce stress during sampling (Prickett et al., 2008b; Kittawornrat et al., 2010). Oral fluid is the liquid found in the oral cavity which is a mixture of saliva and oral mucosal transudate. It contains hormones, drugs, pathogens, non-specific defense proteins and immunity (Challacombe et al., 1978). Previous study showed that the levels of agent and antibody in oral fluid samples were similarly found as in blood samples (Kittawornrat et al., 2010; Goodell et al., 2013). For this reason, oral fluid samples become an alternative sample for swine disease diagnosis worldwide. The agents and antibodies have been detected in oral fluid including porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) (Prickett et al.,

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2008a), foot-and-mouth disease virus (FMDV) (Vosloo et al., 2015) and swine influenza virus (SIV) (Romagosa et al., 2012; Panyasing et al., 2013).

Although rope testing has become a valuable tool for diagnosis and surveillance, the study showed that various inhibitors found in oral fluid specimens can affect the test results. Inhibitor substances found in swine oral fluid, such as mucin, soil, feces and feed continue to increase the viscosity and adhesion with antigens and antibodies in oral fluid samples. Moreover, the detection limits of pen-based sample and improper post-collection processing may also influence the test results. For these reasons, the agent and antibody levels are usually varied in oral fluid samples. The preliminary study using dirty oral fluid samples showed that the sample matrix caused the problem with PCR analysis. So, the pre-treatment protocol for PCR testing should be developed for increasing the production yields. Previous studies from human diagnosis showed that pre-extraction method was developed for neutralization of PCR inhibitors from forensic samples by NaOH treatment (Michael et al., 1998). Therefore, NaOH treatment may be a valuable complementary tool for improving the PCR analysis in dirty oral fluid samples. To answer this question, the study on pre-extraction method using NaOH treatment for real-time PCR detection was performed.

The other point of interest is about the application of rope testing in the fields especially in the gilt acclimatization, suckling and nursery periods. Generally, controlling PRRSV circulation in the breeding herd require strict biosecurity program

and good farm management. Utilizations of various strategies in the sow herd including gilt acclimatization, parity segregation and vaccination have been suggested for an effective PRRSV control (Thai Swine Veterinary Association [TSVA], 2011). In general, gilts and primiparous sows are the major sources of virus introduction into breeding herd because their immunity against PRRSV is not as effective as the others. Gilt acclimatization is one of the most effective ways to reduce risks of PRRS outbreak in PRRS-positive herds. The major objective of acclimatization program is to prepare the replacement gilts for fully developed protective immunity without PRRSV shedding when moved to the breeding herd. Replacement gilts are immunized by exposing to PRRSV in combination of three different ways including contacting with PRRSV-positive donors, vaccination with modified live virus or exposure with live virus (Corzo et al., 2010; Thai Swine Veterinary Association [TSVA], 2011). Furthermore, primiparous sows and their progeny are more susceptible to PRRSV infection than others, and can lead to disease outbreak in the farrowing unit. After weaning, the virus can be transmitted from persistently infected weaned piglets to susceptible pigs. Therefore, the implementation of disease-specific control programs will become easier when all breeding gilts and their litters are separated in one location. P0-P1 segregation involves minimizing production losses from PRRSV infection in gilts/primiparous sows by separating their gestation and farrowing units from the other multiparous pigs (Moore et al., 2005). However, even the P0-P1 segregation system have shown satisfied successes, the risks of reinfection are still high in case of poor biosecurity program.

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Thus, understanding of PRRS status is essential in order to control PRRSV spreading within the herd.

Interestingly, rope testing has been reported as an alternative diagnostic sample for disease surveillance and monitoring in swine herds (Kittawornrat et al., 2014; Trang et al., 2014). Previous studies reported that pen-based oral fluid samples had a higher percentage of PCR positive finding and provided a longer detection period when compared with pooled blood sample in the same pen (Kittawornrat et al., 2010; Goodell et al., 2013; Decorte et al., 2015). Positive results of RT-PCR analysis using oral fluid samples respected to PRRSV shedding and circulating in infected herd (Biernacka et al., 2016). However, rope testing still has its limitation when used in the field practice because the specific protocol for disease surveillance using oral fluid samples is still unclear. Implementation of oral fluid-based PRRS monitoring should be explored for the benefit information of the oral fluid assay since the swine production has various critical stages of production, especially during gilt acclimatization, suckling and nursery period. Therefore, another objective of this study was to evaluate the results of oral fluid used in PRRSV status monitoring during the gilt acclimatization and suckling to nursery period. Using oral fluid sample as an alternative sample was evaluated together with a traditional serum sample and production parameters to yield the best result analysis. The comparison was done in two PRRS endemic farms with different management systems with and without parity segregation system. Finally, it is hoped

that this investigation results would provide beneficial information for disease management and surveillance in the swine productions.

1.2 Objectives of the study

1. To study the efficacy of modified pre-extraction methods for improving the PCR analysis in oral fluid samples.
2. To determine whether oral fluid samples could be used for monitoring gilt acclimatization program for PRRSV infection.
3. To determine and describe the PRRSV transmission during the suckling and nursery period using oral fluid samples.
4. To determine the effect of parity distribution with disease status using oral fluid samples.

1.3 Literature Reviews

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Porcine reproductive and respiratory syndrome:

After its initial recognition in the early 1990s, in the United State (Collins et al., 1992; Yoon et al., 1992) and in the Netherlands (Terpstra et al., 1991; Wensvoort et al., 1991), PRRSV subsequently was identified in many countries throughout the world. During the last three decades, many swine industries suffered from economic losses in all production stages due to PRRS disease. PRRS is characterized by two major clinical appearances, i.e., reproductive failure in breeders and respiratory problems in growers to finishers. The total annual losses in the US swine industry from PRRS were $666

million in 2010 which increased from the annual cost in 2005 about $104 million (Holtkamp et al., 2013). Massive losses in the breeder were due to reduced farrowing rates and number of weaned pigs, whereas, in the growing pigs, losses resulted in high mortality rate and decreased growth rate.

PRRSV is classified in the genus *Arterivirus* family *Arteriviridae*. PRRSV is an enveloped single-stranded positive sense RNA virus. Infected pigs can shed virus via saliva, urine, semen, nasal secretion, feces and mammary secretion. PRRSV can transmit via both vertical and horizontal transmission. Infected sows can transmit the virus to their offspring by transplacental route and/or by direct contact during lactation period. For horizontal transmission, unstable pigs (shedder) can transmit virus to susceptible pigs via direct or indirect contact. The parenteral exposure by using the same needle repeatedly is the major route of direct contract (Dee et al., 2002; Pileri and Mateu, 2016).

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PRRSV infection in a breeding herd:

The most common routes of PRRSV entered negative herds are commonly via the introduction of infected animals, semen or contaminated objects, or through spread from neighboring farms (Pileri and Mateu, 2016). Once the virus introduced into the farm, PRRSV tends to circulate within the herd indefinitely. PRRSV was reported to create persistent infection and become endemic. Gilts and primiparous sows are the major sources of virus introduction to the breeding herd. They are more susceptible

to PRRSV infection and become persistently infected due to comingling with different immune status gilts (Dee, 1997). They could subsequently shed the virus to other sows and piglets resulting in an outbreak in the farrowing unit. Thereafter, congenital infection in the newborn may become persistently infected carrier and can shed PRRSV in all productive stages (Karniychuk and Nauwynck, 2013). Previous studies showed that infection rate of PRRSV infection were different among sow parity, litters, groups, pens and farm conditions (Houben et al., 1995; Ramirez et al., 2012). A large population may quickly become infected under conditions in which susceptible animals are commingled with infected animals especially at weaning (Dee et al., 1996).

PRRS control:

Several strategies for controlling PRRSV in conventional swine farm are described, however the success rate is highly variable among farms. It should be noted that genetic variation and disease characteristics of PRRSV are highly variable. Thus, identifying the source of virus and whereabout the virus recirculation should be considered. Importantly, the objective of PRRSV management must be clearly determined first whether to eradicate the virus or to control the clinical disease and stay stabilized with the virus before starting any program. PRRSV eradication method has been demonstrated as an effective method for the successful elimination of resident PRRSV circulation within a herd. However, whole herd depopulation-repopulation may have the potential to perform, in particular, in an individual farm

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which located in low pig density area or in lower PRRS exposure area (Corzo et al., 2010). Unfortunately, several eradication activities have failed, possibly due to the introduction of the new strains through unidentified routes from poor biosecurity management or the worker movement (DeBuse, 2007). Then, the key to success of depopulation-repopulation method needs to have a high level of quarantine, monitoring and biosecurity program.

The current issues focus on PRRS control options by raising herd immunity and preventing upsurges of viral challenge. Immunization using vaccination is the easiest and safest controlled method to stabilize a herd. Currently, there are two types of commercial PRRS vaccines: a modified-live virus (MLV) vaccine and a killed virus (KV) vaccine. PRRSV vaccination can be used with four different purposes including stopping disease outbreaks, acclimatizing gilts, boosting PRRSV-immune sows and immunizing piglets. Generally, PRRS KV vaccine is safe to use, but its capacity to induce a protective immunity still incomplete (Charerntantanakul, 2012). In contrast, many studies have shown beneficial effects of commercial PRRS MLV in controlling outbreaks, reducing shedding, and preventing economic losses (Martelli et al., 2009; Zhao et al., 2012). Although the effectiveness of MLV vaccine use is also limited, combination between vaccination and other control programs could be a useful implementation in the PRRSV stabilized farms.

Gilt acclimatization program:

Introducing replacement gilts into a breeding herd may provoke health problem and disease outbreak as the following: 1) introduction of a new strain from a supply farm; 2) incoming gilts are susceptible to the recipient herd pathogens. Therefore, gilt acclimatization strategy is the most effective way to diminish these problems. The purpose of the acclimatization program is to prepare the replacement gilts for fully protective immunity without PRRSV shedding when moving to the sow herd (Dee, 1997). A previous report showed that the successful gilt acclimatization program is one of the major methods to reduce risks of PRRS outbreak in PRRS-positive herd by improving farrowing rate and litter size, decrease wean-to-service intervals and herd status (Vashisht et al., 2008). The acclimatization program can be divided into 4 phases (isolation, acclimation, cool-down and introduction to the sow herd) (Pig Improvement Company [PIC], 2015). To prevent the introduction of new pathogens into the recipient herd, incoming gilts were quarantined and monitored at least 4 weeks after replacement. The acclimatization barn should be stayed in semi-isolation area of the farm. In acclimation phase, gilts will be immunized by slowly expose to the organisms and pathogens existing in the recipient herd including: 1) contacting with PRRSV-positive donors; 2) vaccination with a modified live vaccine or; 3) exposure with field-derived strains (Corzo et al., 2010; Thai Swine Veterinary Association [TSVA], 2011). Culled sows are often used as donor exposures but may be poor sources of PRRSV for acclimatization. Weaned pigs and their secretions (e.g. serum and feces) may be

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preferable for inducing immunity against local PRRSV, but are also at higher risk of other infections. The aim of vaccination is to improve the consistency of gilts immunity. By vaccination, the vaccinated gilts are exposed to the virus at the same amount and it is safe for other specific infectious agents. Moreover, to induce complete protective immunity, MLV vaccine exposure and proper timing of vaccine-induced immunity must be concerned especially in naïve gilts (Scortti et al., 2008). After done with PRRSV exposure, gilts should be quarantined in the cool-down phase at least 4 weeks before entering into the sow herds. The cool-down period should spend enough time for eliminating PRRSV from the pig's body and for developing fully immunity against PRRSV (Lambert et al., 2012).

Parity segregation system:

In general, gilts and primiparous sows are more sensitive to the infection than multiparous sows since their immunity to herd-specific pathogens are not fully effective. Therefore, disease like infectious infertility or mastitis-metritis-agalactia (MMA) are more susceptible to gilts after farrowing. The piglets might receive inadequate maternal immunity and become ill (e.g. diarrhea, crushing, stunting). One of the control methods is a P0-P1 segregation system which manage the persistently-infected primiparous sows and their progeny in another area. The P0-P1 segregation system is the effective method to reduce chance of PRRSV shedding from infected primiparous sows to the others. The system will separate gilts (P0) and primiparous sows (P1) from

higher parity sows (P2+). To stabilize the disease in nursery, piglets of P0 and P1 sows are housed separately until the end of finishing period. For the success of P0-P1 segregation system, farm management requires high labors, costs and a good biosecurity program (Moore et al., 2005).

PRRS Monitoring program:

Monitoring program is an important tool for a success of PRRS control. Of the effective disease monitoring program, information such as performance parameters, clinical appearance, pathogenesis, and laboratory diagnosis are necessary to determine PRRSV infection status. PRRSV is often characterized by reproductive failure in breeder and pneumonia in young pigs which mostly related with the increases of abortions, pre-weaning mortality rate (PWM), mummified and stillbirth (Brouwer et al., 1994). Therefore, the change of production parameters can be used as indicators of disease outbreak. A previous case reported that the higher abortion and post-weaning mortality rates were correlated with early PRRS outbreak (Silva et al., 2015). In growing unit, increased mortality rate, and decreased feed efficiency and average daily gain are the important parameters related with PRRSV infection. Moreover, in the situation that clinical signs and abnormal parameters are detected, diagnostic tests are required to find the cause of the problem and to establish the appropriate control program.

Currently, several laboratory techniques can be used to identify the antigens and antibodies in diagnostic samples including serum, tissues, saliva, feces, semen etc.

RT-PCR analysis is an acceptable assay to identify PRRSV for monitoring the prevalence of PRRSV. ELISA is a common serological assay used to determine PRRS serological status. This assay is rapid, sensitive, specific, and is standardized for demonstration of seroconversion. Antibody response can be detected within 7–10 days and persists up to 4-12 months after infection. ELISA S/P ratios can be used to identify animals into categories of susceptible, infectious, and resistant immune status (Holtkamp et al., 2011). However, the serology of PRRSV is not a valid approach for differentiation the previously infected or vaccinated herds. Therefore, the interpretation of ELISA results should be analyzed from the multiple points of sample collection.

Nodelijk et al. (1996) showed that 86-95% seroprevalence was detected in the sow herd during an acute PRRS outbreak. At the later stage, non-infected sows would get infected showing the seroconversion. These sub-population sows might be a susceptible group for PRRSV reinfection repeatedly within the herd. Transmission between breeders often causes the infection cycle in breeding herd leading to persistently infected piglets causing the virus transmission from nursery or finishing units (Evans et al., 2008). PRRSV can remain persistently infected and circulate within a herd for several years depending on the management to stabilize the sow herd. PRRSV was continuingly detected in the sentinel pigs in the farrow-to-finish farm after the outbreak several months in the un-stabilized sow herd (Bilodeau et al., 1994).

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Generally, blood samples are commonly used for PRRSV detection. Blood collection procedure induces stress for both animals and practitioners and quite costly. Recently, oral fluid sampling has been applied as a diagnostic sample for the swine industry. This sampling method is cost effective, convenient and efficient for both pathogen and antibody detection at individual and pen level (Cuong et al., 2014; Kittawornrat et al., 2014).

Basic concepts of oral fluid:

Oral fluid, a water-like substance, is a mixture of saliva and oral mucosal transudate in the oral cavity. Saliva is produced by salivary glands for lubricating function, bolus formation and digestion (Llena, 2006). Pig has three paired of major parotid, mandibular, and sublingual glands and scattered submucosal minor glands. The main function of salivary glands is to produce the saliva containing digestive enzyme, mucin, mucus, water etc. Moreover, the saliva has non-specific defense proteins such as anti-microbial peptide, lysozyme, peroxidase and others responsible for innate immunity (Kutta et al., 2008). Furthermore, the salivary glands are considered as one of the functional unit of mucosal immune system because the salivary glands have mucosa-associated lymphoid tissue (MALT) structure, a special compartment of mucosal immunity. Moreover, saliva also has non-specific defense proteins, mucosal immunity and agents, whereas, oral mucosal transudate, which crosses from the

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capillaries of oral mucosa and gingiva, contains both systemic immunity and oral agents.

Oral fluid immunity:

Oral fluid immunity is divided into 2 groups (mucosal and systemic immunity). The mucosal immunity produces from MALT in salivary glands which called salivary duct-associated lymphoid tissue (DALT) (Nair and E Schroeder, 1986). MALT is located along the surface of the mucosal tissue. This lymphoid tissue is an inductive site and an effector site of the mucosal immune response. Generally, the mucosal immune system acts as common mucosal immune system demonstrating that the immune responses are stimulated from any inductive sites. It can deliver to other effector sites (Mestecky et al., 1978). The DALT is important effector site of mucosal immune responding to the pathogens in gut-associated lymphoid tissue (GALT) and nose-associated lymphoid tissue (NALT) (Cesta, 2006). The main antibody presented in mucosal area is secretory immunoglobulin A (sIgA). Interestingly, the sIgA is important molecules to control the oral microorganism (Marcotte and Lavoie, 1998). Furthermore, the sIgA inhibits the adhesion of the pathogen to epithelium or teeth by opsonizing for phagocytosis, inducing complement fixation and neutralizing pathogen (Walker, 2004). Moreover, the systemic immune response including serum-derived antibodies (IgG, IgM, IgA) had been demonstrated in oral fluid (Challacombe et al., 1978). It should be noted that the systemic immune response in saliva may cross the

vessel by passive transduction of serum component from oral mucosa and gingival capillaries into saliva (Chiappin et al., 2007). Accordingly, saliva and salivary glands are the important parts of oral immunity including mucosal immune response and systemic immune response to control the pathogens in the oral cavity.

PCR inhibitions and NaOH treatment:

The presentation of the PCR inhibitors in oral fluid specimen may cause a difficulty with PCR analysis. The inhibitory substances both from environment and oral fluid components especially mucin often bind or inactivate the agents, and can reduce the efficacy of an enzymatic reaction (Chittick et al., 2011). Therefore, the inactivation or block the activity of PCR inhibitors in the samples are important to yield the best PCR results. In the medical field, NaOH was used for the neutralization of PCR inhibitions to purify and recover the DNA. Interestingly, NaOH treatment could inactivate the inhibitors by denaturation and NaOH was washed out before the PCR analysis. However, the data showed that NaOH treatment might not be good when having low yield DNA samples (Bourke et al., 1999; Vongpaisarnsin et al., 2011).

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Oral fluid collection and processing:

Oral fluid collection: The swine oral fluid samples usually collect from a group of pigs (pen-based oral fluid sampling) having 20-25 pigs in a pen. A previous study showed that type of sampling materials did affect the results which cotton rope is the best absorbent when compared with hemp and nylon (Olsen et al., 2013a). In general,

oral fluid collection at pen level (20-25 pigs) requires only one rope per pen. However, in a larger pen, the require numbers of the rope usually are 2-4 ropes per pen to increase the test sensitivity (Gonggrijp et al., 2014). Firstly, the cotton ropes (1/2" diameter for piglet-nursery pigs, 5/8" diameter for grow-finish pigs) are hanged at the shoulder height to the pigs in each pen waiting for the pigs to chew on the ropes about 20-30 minutes. After that, the ropes are inserted into a plastic bag by cutting off the wet part of the ropes and then, extracting the oral fluid samples from the ropes by hand or mechanical compression. Finally, oral fluid samples are collected and stored at 4°C for submitting to laboratory, or at -20°C or −80°C for a longer storage. In addition, pigs should be trained before sampling. The study showed that the training program in individually-housed boar was effective for oral sampling (Kittawornrat et al., 2010).

Oral fluid processing: Field oral fluid samples are usually very dirty because the samples are mixed with many materials such as soil, feces and feed. Therefore, the post- collection processing has been developed for clearance those inhibitor substances. However, the study showed that the unprocessed oral fluid samples had higher levels of anti-PRRSV antibody than that of the processed samples (Olsen et al., 2013a). Furthermore, sample storage temperature also had an effect on the PCR analysis and ELISA S/P ratios demonstrating at −20, 4 and 10°C. Likewise, the stability of the samples was at least 12 days when stored at 4°C (Prickett et al., 2008).

Oral fluid used in swine medicine:

There are many research studies about oral fluid samples in swine. In 1976, Corthier (1976) reported that the levels of CSFV Thiverval strain antibodies were detected in pharyngeal secretions. (Weesendorp et al., 2008) reported that during CSFV outbreaks (highly, moderately virulent strains) the virus shedding in oropharangeal fluid was up to 1300 times to 5000 times of feces and urine. After that major swine pathogens have been studies for the present in oral fluid samples such as PRRSV, PCV2 and swine influenza virus (SIV). The data indicated that SIV RNA was detected in oral fluids for a longer period than found in nasal swabs (Goodell et al., 2013; Decorte et al., 2015). Similarly, PRRSV data analysis on both a pen-based and individual-based samples reported that oral fluids samples presented a longer duration and highly sensitive for PCR-detectable PRRSV than found in serum (Prickett et al., 2008b; Kittawornrat et al., 2010). Moreover, several studies suggested that using oral fluid samples could gain a better benefit in general PRRS surveillance (Cuong et al., 2014; De Regge and Cay, 2016).

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CHAPTER 2

MODIFIED PRE-EXTRACTION METHODS TO INCREASE PRRSV qRT-PCR DETECTION FROM SWINE ORAL FLUID SAMPLES

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(Submitted)

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Abstract

Oral fluid sample pre-preparation before extraction believe to yield better PCR results based on the sample quality. The objective of this study was to evaluate the effect of centrifugation, NaOH treatment and sample input volume on PRRSV detection from oral fluid samples. Negative control oral fluids, PRRSV-spiked oral fluids and oral fluids from PRRSV-inoculated pigs were obtained. Processed and unprocessed oral fluid samples were tested using different sample-processing methods. All samples were extracted using a Nucleospin® column based extraction kit and were later examined by qRT-PCR. The results showed that high-speed, rapid spin centrifugation might have an impact on antigen yielded. The results also indicated that the modified pre-extraction using NaOH treatment could possibly be used to reduce PCR inhibitors and was able to recover target nucleic acid. However, extensive NaOH treatment might degrade the target nucleic acid in the samples. It should be noted that the method using 200 µl sample input volume yielded the best PRRSV detection. However, using larger volume (250 µl) was not effective. The results suggested that all three pre-preparation methods could affect the PCR analysis and further study should be conducted to optimize the better pre-preparation for PCR processing assays.

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Keywords: NaOH, oral fluids, pre-preparation, PRRS, qRT-PCR, swine

2.1 Introduction

Currently, oral fluid samples have become samples of choices for swine diagnosis (Prickett et al., 2008b; Olsen et al., 2013b). However, the collected oral fluid samples may have contaminated materials containing inhibitor substances such as mucin, soil, feces and feed contaminants. These inhibitor substances could impact on the quality of antigens and antibodies, and cause the problem on polymerase chain reaction (PCR) analysis. Consequently, pre-preparation of the oral fluid samples before extraction should be conducted to yield the best results.

Therefore, several post-collection protocols have been developed for inhibitor substance clearance. Previous studies showed that the processed oral fluid samples with a long duration, high-speed centrifugation yielded lower levels of porcine reproductive and respiratory syndrome virus (PRRSV)-specific antibody and viral antigens (Rotolo et al., 2012; Olsen et al., 2013a). Furthermore, sample input volume for magnetic bead extraction also had a positive effect on the PCR analysis (Chittick et al., 2011). Interestingly, a previous study of human diagnosis on synovial fluid samples showed that pre-extraction method using NaOH treatment for neutralization of PCR inhibitors yielded better DNA recovering and purification (Bourke et al., 1999; Vongpaisarnsin et al., 2011). Therefore, it would be of interest on using NaOH treatment for improving the PCR analysis in contaminated oral fluid samples. The objective of this study was to evaluate the results of different pre-preparation methods of oral

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fluid samples before extraction for a modified quantitative real time PCR (qRT-PCR) assay.

2.2 Material and Methods

To evaluate the effect of different pre-preparation methods before extraction on the detection of PRRSV in contaminated oral fluid samples, total of 20 oral fluid samples, including negative control oral fluids (CSFV, PRRSV and SIV free confirmed by ELISA; n = 5), PRRSV-spiked oral fluids (n = 5) and the previous collected oral fluids from experimental PRRSV-inoculated pigs (n = 10) (Sirisereewan et al., 2017), were selected in this study. One-half of each negative oral fluid sample was spiked with PRRSV (01NP1) at low concentration 10^2 $TCID_{50}$/ml of oral fluids, and another half was kept as negative control oral fluids. The stock virus used in this study was provided by the Chulalongkorn University-Veterinary Diagnosis Laboratory (CU-VDL). Sample collection protocols and animal use was approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC number 1431086, 1531020).

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Following the collection, effect of a short duration, high-speed centrifugation was determined to clean the contaminated materials in oral fluid samples. The oral fluid samples were divided into 2 portions: 1) non-centrifuged and 2) centrifuged at 8,000 x g for 5 mins. Another part of the non-centrifuged oral fluid samples was treated

with a modified NaOH treatment at different concentration (0.2, 0.4, 0.6 M) by adding 50 µl of NaOH into 200 µl of oral fluid samples (Vongpaisarnsin et al., 2011).

Then, all oral fluid samples were processed for RNA extraction using a Nucleospin® commercial kit. Three different input sample volumes were evaluated using non-centrifuged oral fluid samples. Protocol 1 was conducted as recommended by the manufacturers to use 150 µl of sample volume, and protocols 2 and 3 were 200 µl, 250 µl, respectively. Finally, all samples were examined for the presence of PRRSV by qRT-PCR. Primers and probes were designed to bind specifically for ORF7 (Egli et al., 2001). The qRT-PCR reaction was performed as previously described (Sirisereewan et al., 2017).

The results of rapid centrifugation, NaOH treatment and sample input volume were evaluated on detectable PRRSV RNA data using analysis of variance (ANOVA) following by Tukey's multiple comparison tests.

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2.3 Results and Discussion

The objective of the current study was to evaluate different sample processing methods for PRRSV detection from swine oral fluid samples. Although the number of samples involved in this study was relatively small, the results showed marked differences among protocols (Tables 1). Overall, oral fluid samples from the negative control pens detected negative for all processing methods. In addition, uncentrifuged dirty oral fluid samples could have a negative effect on the column based extraction

method, presumably by reducing the concentration of target yield due to being trapped in the column based extraction kit.

The results indicated that the proportions of PRRSV-positive samples were reduced after centrifuged in all sample groups. The findings demonstrated that high-speed, rapid spin centrifugation might have the negative impact on the target antigen yielded. The viral RNA from the oral fluid samples might be hampered by the mucous component in the saliva or other organic matters and would be eliminated together with the pellet.

Interestingly, the proportion of PRRSV-positive and PRRSV concentration was increased when treated with 0.2 M NaOH compared with the unprocessed samples. However, 0.6 M NaOH treatment in PRRSV-spiked oral fluid samples yielded significantly lower viral titers than those of the unprocessed samples and other NaOH concentration treatments. It should be suggested that the modified pre-extraction method using 0.2 M NaOH treatment might be an alternative method for reducing PCR inhibitors and not harmful to target nucleic acid. However, extensive NaOH treatment could possibly degrade the nucleic acid in the samples (Bourke et al., 1999).

The results also indicated that the proportion of PRRSV-positive and PRRSV concentration were highest when increasing input sample volume at 200 µl, but declined when using 250 µl volume. The possible explanation for this result is that the initial larger sample volume could elute the target RNA or a large sample volume

might interfere the capacity of column based extraction kit and endogenous inhibitors might also be increased (Chittick et al., 2011). Accordingly, in order to improve the sensitivity of PRRSV detection by qRT-PCR, increased PCR enzyme concentration is suggested when using large-volume extraction (Wilson, 1997). The present study demonstrated that all three studied factors yielded variable results. Noticeably, sample processing using 200 µl sample input volume would be a simple method for this column based extraction kit demonstrating the best PRRSV detection but not significantly statistical differences. Future study should focus on the other optimizing methods for a better PCR analysis when using oral fluid samples.

2.4 Acknowledgements

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Table

Table 2.1 Averaged PRRSV genomic copies/µl (log10) and positive sample percentages (%) of oral fluid samples yielded from various sample-processing method (centrifugation, NaOH treatment and sample input volume) comparing with the standard protocol

Methods	Negative control (n = 5)	PRRSV-spiked (n = 5)	PRRSV-inoculated (n = 10)
Control[1]	1.09±0.25[2] (0)	4.78±0.63[a] (60)	5.04±0.41 (70)
Centrifuged	0.74±0.40 (0)	4.33±0.53 (40)	4.10±0.47 (40)
NaOH treatment			
- 0.2M	1.20±0.48 (0)	5.59±0.54[a] (60)	4.78±0.55 (80)
- 0.4M	0.99±0.34 (0)	4.82±0.70[a] (60)	4.92±0.38 (60)
- 0.6M	0.57±0.30 (0)	2.20±0.71[b] (20)	3.57±0.43 (30)
Starting volume			
- 200 µl	1.53±0.17 (0)	5.96±0.51 (80)	5.62±0.34 (90)
- 250 µl	0.77±0.40 (0)	5.02±0.59 (60)	4.54±0.37 (70)

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[1] Unprocessed samples were conducted as recommended by the manufacturers (150 µl).

[2] Statistical analyses were performed between each processing method and the control method comparing among the same sample types (different superscripts demonstrate statistically significant, $p<0.05$).

CHAPTER 3

ORAL FLUID SAMPLES USED FOR PRRSV ACCLIMATIZATION PROGRAM AND SOW PERFORMANCE MONITORING IN ENDEMIC PRRS-POSITIVE FARMS

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Abstract

An effective gilt acclimatization program is one of the most important management strategies for controlling porcine reproductive and respiratory syndrome virus (PRRSV) infection. Recently, oral fluid samples have been used as alternative diagnostic samples for various swine diseases. This study utilized oral fluids for PRRSV monitoring during the gilt acclimatization period in PRRSV endemic farms. The study was performed in 2 selected commercial breeding herds (Farm A and Farm B). PRRSV RNA and PRRSV-specific antibodies were monitored using oral fluid and serum samples. Sow performance parameters related to PRRSV infection were recorded and assessed. After PRRSV exposure during acclimatization, viral RNA was demonstrated in oral fluids from 1 to 10 weeks post-exposure (WPE). PRRSV RNA was detected in serum at 1 and 4 WPE in Farm A and at 1, 4, 8, and 12 WPE in Farm B. Prolonged viremia of gilts from Farm B was possibly due to re-infection (within the herd) and later, reproductive problems were found in the breeding herd. The correlation of PRRSV RNA concentration in oral fluids and serum was evident. The S/P ratio values of PRRSV antibodies in oral fluid samples were higher and had similar patterns of antibody responses to the serum samples. The results suggest that the use of oral fluid samples for PRRSV monitoring during gilt acclimatization in endemic farms is effective, convenient, practical, and economical and would be most beneficial when used with other parameters.

Keywords: acclimatization, gilts, monitoring, oral fluid, PRRSV, reproductive performance

3.1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases in the swine industry worldwide. PRRS virus (PRRSV) causes reproductive failure in breeding swine and respiratory diseases in nursery to finishing pigs. Many swine farms have become PRRSV endemic in the breeding herds and suffer from production losses during all production stages (Holtkamp et al., 2011).

Improper gilt management causes PRRSV persistence in the sow herd (Dee, 1997). It is known that PRRSV-negative replacement gilts are susceptible to PRRSV infection. Soon after commingling and becoming infected, replacement gilts may persistently shed the virus, causing prolonged virus circulation within the breeding herd, and resulting in PRRSV-induced reproductive failure (Brouwer et al., 1994; Baysinger, 1997). Furthermore, these PRRS unstable sows may transmit the virus to their piglets, either vertically or horizontally, resulting in production losses due to porcine respiratory disease complex (PRDC) (Dee and Philips, 1998; Rajic, 2001).

To control PRRSV circulation in the breeding herd, a strict biosecurity program, good farm management, and an effective gilt acclimatization program must be employed. Previous reports showed that a successful gilt acclimatization program is one of the major methods to reduce the risk of PRRS outbreak in PRRS-positive herds, demonstrated by improved farrowing rate and litter size, decreased wean-to-service

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intervals, and improved disease status (Vashisht et al., 2008). The major objective of the acclimatization program is to produce replacement gilts with fully-developed protective immunity without PRRSV shedding when moved to the sow herd. The acclimatization program is commonly divided into 4 phases: isolation, acclimation, cool-down, and introduction to the sow herd. During the isolation phase, replacement gilts are moved to the isolation barn and must be quarantined at least 4 weeks to prevent new diseases entering the sow herd (Pig Improvement Company [PIC], 2015). In the acclimation phase, gilts must be immunized by exposing to PRRSV by one or the combination of three different ways including contact with PRRSV-positive donors, vaccination, or exposure to live viruses (Corzo et al., 2010; Thai Swine Veterinary Association [TSVA], 2011). Following the cool-down phase and prior to introducing the acclimated gilts into the sow herds, PRRSV must be eliminated by the immune system so that there is no virus shedding when animals are moved to the breeding herd (Lambert et al., 2012).

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Therefore, an appropriate monitoring program is necessary for evaluating the disease status and acclimatization program. Many diagnostic methods have been developed for detection of PRRSV shedding and exposure status. However, blood sampling, which is generally used as a diagnostic sample of choice, is not always an ideal sample as it can induce stress to animals, causes tissue trauma, and may infect animals with contaminated needles.

Currently, oral fluid is the sample of choice for the current diagnosis of many swine diseases worldwide (Ramirez et al., 2012; White et al., 2014; Pepin et al., 2015). Previous studies reported that pen-based oral fluid samples provided a higher chance of real- time quantitative reverse transcription- PCR (qRT- PCR) positive findings compared to pooled blood samples in the same pen (Goodell et al., 2013; Olsen et al., 2013b). Positive results of RT-PCR analyses using oral fluid samples showed PRRSV shedding and circulating in an infected herd (Biernacka et al., 2016). However, this method has some limitations in the field because the specific protocol for disease control, especially PRRSV infection, using oral fluid samples is still unclear. The context of oral fluid-based monitoring, especially during PRRS acclimatization program, has not been previously evaluated.

Therefore, the objective of this study is to determine the effectiveness of oral fluid testing in the field as an alternative sample for monitoring and evaluating PRRSV status during the gilt acclimatization program and sow performance after gilt introduction into the sow herd. The investigation results provided beneficial information for disease management and surveillance using oral fluid samples in the swine production. Additionally, this study determined whether oral fluid samples could be used for gilt acclimatization and PRRSV status monitoring in the sow herd.

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3.2 Material and Methods

Animals and Farms:

Two commercial one-site system farms (Farm A: 1,300 sows and Farm B: 5,000 sows) participated in this study. Both selected farms were PRRSV-positive based on PRRSV-specific antibody responses as measured by ELISA. The management protocols used in the studied farms were determined by the attending veterinarians and the farmers. According to the routine gilt management for PRRS control strategy of both farms, all incoming Landrace *x* large white crossbred gilts from both herds were exposed to live PRRSV by two methods: first immunization with a modified live virus (MLV) PRRSV vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim) and second via acclimatization with culled sows for at least two weeks. The PRRSV exposure protocol of both farms started at 20 weeks of age (0 weeks post-exposure, WPE).

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The acclimatization barn of Farm A is an open barn with natural ventilation located in a semi-isolated area. The barn consists of 12 pens in the front side and 100 individual stalls in the back side of the barn. All replacement gilts were internally replaced, moved from the finishing unit to the gilt acclimatization unit at 15-17 weeks of age (average weight 71.10 kg), and randomly housed 10 gilts in each pen. At 20 weeks-old, the replacement gilts were intramuscularly vaccinated with the MLV PRRSV vaccine and then, boosted again at 6 weeks post-vaccination. Culled sows were housed with the replacement gilts at 0 WPE and stayed for 2-4 weeks. Culled sows

were placed one per pen and changed every week. All vaccinated gilts were moved to individual stalls in the same barn at 8 WPE for cool-down period. Thereafter, the gilts were vaccinated with a herd-specific PRRSV killed virus vaccine at 9 WPE. The acclimatization program was finished at 12 WPE.

The acclimatization barn of Farm B is an evaporative cooling system barn located in a semi-isolated area. The barn consists of 20 pens on both sides of a walkway. Similar to Farm A, all replacement gilts were internally replaced at 15-17 weeks of age (average weight 69.75 kg) and housed 20 pigs per pen. Gilts were intramuscularly vaccinated with the MLV PRRSV vaccine at 20 weeks of age. At 4 weeks after the first vaccination, all gilts were vaccinated with a herd-specific killed PRRSV virus vaccine (4 WPE) as recommended by the attending veterinarian. Thereafter, the gilts were revaccinated twice with the same MLV PRRSV vaccine at 6 and 9 WPE. Culled sows were later housed with the replacement gilts from 10 to 12 WPE.

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Other vaccination programs were implemented on both farms including foot-and-mouth disease virus (FMDV), classical swine fever virus (CSFV), Aujeszky's disease virus (ADV), porcine circovirus type 2 virus (PCV2), porcine parvovirus (PPV), *Erysipelothrix rhusiopathiae*, leptospirosis, and *Actinobacillus pleuropneumoniae*. Additionally, the replacement gilts in Farm B received feedback prepared by minced intestines of porcine epidemic diarrhea virus (PEDV) infected piglets (at 22 and 31

weeks of age) before introduction to the breeding herd in order to prevent PEDV outbreaks in the sow herd.

Sample collection:

Serum samples were collected from gilts in randomly selected pens of Farm A (5 gilts per pen) and Farm B (10 gilts per pen) at -3, 1, 4, 8, 12 and 15 WPE (3 days before insemination) to monitor PRRSV status of both farms. Blood samples were collected from the jugular vein using a single-use blood collection system (Monovette® 9ml Z, Sarstedt AG & Co, Germany). Serum samples were aliquoted into microcentrifuge tubes and stored at -80°C until tested.

The oral fluid sampling has previously proven to be an easy and friendly method. Therefore, oral fluid sample collections were more frequently conducted in this study whenever available in order to gain additional data. Pen-based oral fluids were collected at the beginning of acclimatization (Farm A: -3 to 8 WPE; Farm B: -3 to 12 WPE). Thereafter, the animals were moved to individual stalls and individual oral fluids were collected through 15 WPE. Samples from 6 randomly selected pens were collected at -3, 1, 2, 3, 4, 5, 6, 7, and 8 WPE, and individual samples from 15 selected pigs were collected at 9, 10, 11, 12, and 15 WPE in Farm A. In Farm B, samples from 9 selected pens were collected at -3, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 WPE and individual samples from 30 selected pigs were collected at 15 WPE. Those

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replacement gilts selected for blood sampling were also selected for individual oral fluid collection.

Briefly, oral fluid samples were collected by hanging a 100% cotton, 2.0 cm diameter rope over each pen or stall. For pen-based sampling, two cotton ropes were hung in each pen at the shoulder height of pigs and the pigs were allowed to chew on the ropes for 30 minutes. For individual oral fluid sampling, each individual cotton rope was carefully collected to prevent cross-contamination from the other gilts. Thereafter, wet part of each rope was inserted into a plastic bag and squeezed. The collected oral fluid samples were aliquoted and stored at -80°C until tested.

Quantification of PRRSV RNA:

All swine oral fluid and serum samples were processed for RNA extraction using NucleoSpin® RNA Virus kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). Serum samples were processed according to the manufacturer's instruction. A modified qRT-PCR reaction (25 μl) was performed using 200 μl of oral fluid samples on Corbett Roter-Gene™ 6000 (Qiagen – Germany) and using a commercial mastermix SuperScript™ III Platinum® One-step Quantitative RT-PCR system (Invitrogen, Carlsbad, CA, USA). All samples were tested for the presence of PRRSV nucleic acids using TaqMan® probe-based qRT-PCR as described elsewhere by Egli et al. (2001). Primers and probes were designed specifically to detect the ORF7 gene.

PRRSV antibody detection by ELISA:

All serum samples were tested for the presence of PRRSV antibodies by a commercial ELISA (IDEXX PRRS X3 ELISA test kit, IDEXX Laboratories, Inc., USA). All oral fluid samples were assayed by PRRSV Antibody Test Kit for Oral Fluids (IDEXX PRRS OF, IDEXX Laboratories, Inc., The Netherlands). The assay was performed as described in the manufacturer's instruction. An S/P ratio value greater than 0.4 was considered to be a PRRSV Ab-positive sample.

PRRSV Monitoring:

Data, including gilt information, vaccination, and clinical signs, were recorded by caretakers from the beginning of acclimatization through introduction into the sow herd. Health status and PRRSV-related information including returns to service, abortion, pseudopregnancy, culling, farrowing rate, the litter characteristics (the number piglets born alive, stillborn, mummified, average weaning weight, average lactation length, number of pigs weaned per litter, and pre-weaning mortality), wean-to-first-service interval, the percentage of sows in heat within 7 days after weaning, and culled sows were recorded from the beginning of the introduction through weaning by caretakers using a computerized recording system (PigLive® software, Kasetsart University, Thailand) as described by Udomprasert et al. (1993).

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Data analysis:

Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) on the results from the serum (Farm A, n=90; Farm B, n=180) and oral fluid samples (Farm A, n=129; Farm B, n=147). Viral titers and S/P ratio values of oral fluid and serum samples were presented as copy number means. For each farm, comparison between the use of serum samples and oral fluids were performed at each time point. Data were analyzed using the independent *t*-test to compare each variable of interest (qRT-PCR and ELISA) between sample types. Frequency of positive animals (qRT-PCR and ELISA) in both serum and oral fluid samples were analyzed by Fisher's exact test. The association of virus titers detected in oral fluid and serum samples was assessed using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

3.3 Results

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Gilt status:

Both Farms A and B demonstrated PRRSV genotype 2 circulating in the herds. Replacement rates of Farm A and Farm B were 44.83% and 52%, respectively. At the beginning, all gilts were qRT-PCR negative for PRRSV RNA detection in both serum and oral fluid samples. Gilts in both farms exhibited high anti-PRRSV antibody titers as shown by ELISA (Farm A: serum = 1.50±0.13, oral fluid = 0.89±0.14; Farm B: serum = 2.03±0.52, oral fluid = 2.38±0.09). During the acclimatization program, gilts in Farm B

showed clinical signs including depression, anorexia, and acute death (9 of 196 replacement gilts) between 6-8 WPE, and laboratory results confirmed *A. pleuropneumoniae* (APP) infection, whereas all gilts in Farm A looked clinically normal.

qRT-PCR results:

Farm A: Following the acclimatization program, PRRSV qRT-PCR positive results were detected in the serum samples at 1 and 4 WPE and disappeared at 8 WPE, whereas, the oral fluid samples exhibited longer detection, until 9 WPE (Figure 3.1a). However, there were no differences in the proportion of qRT-PCR positive results between serum and oral fluid samples during the monitoring period and the concentration of PRRSV titers detected by qRT-PCR were at low levels. An analysis of $\log_{10}$ genomic copies per µL showed significantly higher levels in the oral fluid samples compared to the levels in the serum samples at 8 WPE ($p = 0.015$).

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Farm B: PRRSV qRT-PCR positive results were detected in serum samples at 1, 4, 8 and 12 WPE, whereas, the oral fluid samples were found positive until 10 WPE. However, the levels of qRT-PCR positive results between serum and oral fluid samples were not significantly different. Both sample types had similar pattern of viral RNA loads declining after reaching the peak at 1 WPE (Figure 3.1b). Interestingly, serum samples exhibited higher levels of PRRSV genomic copy numbers compared to the levels found in the oral fluid samples at 12 WPE ($p = 0.002$), suggesting acute viremia.

The relationship of PRRSV RNA loads between 87 pairs of oral fluid and serum samples were analyzed over time using Pearson correlation. Analysis showed a Pearson's correlation coefficient of 0.55; P < 0.0001 (Figure 3.2).

PRRSV antibody responses in serum and oral fluid samples:

Farm A: After exposure, PRRSV antibody titers were detected in 100% of oral fluid samples, whereas, PRRSV ELISA results were positive in 80%, 73.34%, 86.67% and 86.67% of the serum samples at 4, 8, 12 and 15 WPE, respectively. PRRSV antibody titers of both sample types increased at 9 WPE until 12 WPE and declined at 15 WPE (Figure 3.3a). Mean S/P ratio values of oral fluid samples showed significantly higher levels than those of serum values at 8 (p = 0.0002), 12 (p < 0.0001) and 15 WPE (p < 0.0001).

Farm B: After exposure, all serum and oral fluid samples were positive for PRRSV antibody titers until 12 WPE and in oral fluids at 15 WPE. The exception being only 96.67% of the serum samples at 15 WPE were positive (Figure 3.3b). Oral fluid samples had higher S/P ratio values compared to the values of serum samples during the monitoring period and found significantly higher levels at 12 WPE (p = 0.002).

Sow performance:

At the end of acclimatization program, 103 of 111 replacement gilts from Farm A (92.79%) and 182 of 196 replacement gilts from Farm B (92.86%) were introduced into the breeding area. Culled gilts in these two farms mostly had lameness and major

reproductive problems unrelated to PRRSV exposure. The computerized performance parameters are summarized in Table 3.1. After the introduction to the breeding herd, all sow performance indices were in normal ranges in Farm A, whereas the percentage of gilts return to service after mating was above average (12.24% ± 1.89) in Farm B. Most sows were culled due to the illnesses relating to increased average wean-to-first-service intervals and decreased sows in heat by 7 days after weaning. The results indicated that increased pre-weaning mortality (16.31%) resulted in decreased weaned pigs per litters (10.67 ± 0.54 piglets) in Farm B. Furthermore, weaned sows from Farm B were more frequently culled (9%) after weaning. Laboratory results confirmed the disease outbreaks co-infected with PRRSV found in animals during the farrowing period in Farm B (data not shown).

3.4 Discussion

Based on the objective of this study, oral fluid testing was used for monitoring the PRRSV acclimatization program and sow performance on two different farms. The results indicated that oral fluid samples could be used for PRRSV monitoring during the acclimatization program. It should be noted that oral fluid-based diagnostic samples are economical, reliable, easy to work with, and noninvasive to the animals.

Prior to the acclimatization, all replacement gilts from both farms already had PRRSV antibodies as measured by the commercial ELISA in both sample types, but were found to be negative for PRRSV RNA based on the qRT-PCR. It should be noted

that those replacement gilts were internally replaced from PRRSV-endemic herds before moving to the acclimatization area. PRRSV ELISA antibodies normally persist for 4-10 months while the viremia could be found less than a month post-infection (Collins et al., 1996).

Pen-based oral fluid samples detected PRRSV RNA by qRT-PCR at the first week after PRRSV exposure protocols with virus shedding found continuously on both farms until 9-10 WPE. As expected, qRT-PCR detected the virus in serum from Farm A only at 1 and 4 WPE. Similar to the previous study by Trang et al. (2014). pen-based oral fluid samples could be used for PRRSV detection in gilts after the introduction to endemically-infected PRRSV sow herds for 8 weeks in spite of negative individual serum samples. However, in this study viral nucleic acid was detected at low levels in both sample types.

Interestingly, in Farm B, all oral fluid samples were negative for PRRSV RNA at the end of acclimatization period (12 WPE), whereas, two of thirty serum samples were positive. The numbers of PRRSV positive gilts and the concentration of PRRSV per pen might be insufficient for qRT-PCR detection by oral fluid samples. De Regge and Cay (2016) indicated that PRRSV qRT-PCR levels in oral fluid correlated well with the percentage of PRRSV positive serum per pen. Additionally, pen-based oral fluid samples would be PCR positive when greater than 30% of individual pigs are serum positive. Furthermore, some Farm B gilts might have been in the acute phase of PRRSV

infection at the late term of acclimatization due to reinfection and might not yet shed virus in the saliva. Possible routes of exposure producing this acute infection include multiple MLV vaccinations or reinfection with the herd's endemic PRRSV strain from the late exposure to the culled sows (Balka et al., 2016), or from the PRRSV-contaminated gut feedback utilized for PED control in Farm B. Similar to previous studies (Gerber et al., 2013; Pepin et al., 2015), acute PRRSV-infected pigs would be expected to exhibit higher levels of PRRSV RNA in serum compared to oral fluid samples.

Previous studies have already demonstrated a positive correlation between the ELISA results of individual serum and pen-based oral fluid samples (Decorte et al., 2015; Kuiek et al., 2015; De Regge and Cay, 2016). The present study is in agreement with those previous studies in that the detection of PRRSV antibody response in oral fluid samples by ELISA had similar pattern, but higher S/P ratio values, when compared with the antibody response in serum. The higher S/P ratio values in oral fluid samples have been reported in association with the IDEXX PRRS Oral Fluids Ab Test (IDEXX, 2013).

Increasing PRRSV antibody levels detected by ELISA at the late acclimatization period in Farm A might be a booster effect of an inactivated PRRSV vaccine. In contrast, the stability of PRRSV antibody levels found in Farm B might be a consequence of multiple PRRSV vaccinations with a homologous strain (McCaw, 2002). In this study,

acclimatized gilts had viremia and shed the virus in the saliva in spite of having high S/P ratios. Previous studies showed that PRRSV-ELISA antibody response did not correlate well with the protective immune status (Ouyang et al., 2013). Additionally, S/P ratio values are not reliable for seropositive animals having viremia or shedding the virus (Mengeling and Lager, 2000).

In this field-based study, the effectiveness of gilt acclimatization program was also investigated after moving to the sow herd. Several reproductive parameters in breeding herd were in acceptable ranges in Farm A. However, sows from Farm B showed higher percentage of returns to estrus associated with irregular intervals. It should be noted that gestating sows might have problems with poor management due to higher percentages of gilt replacement (52%), stress, or disease after service (Elbers et al., 1994). Moreover, litter parameters were also affected with changes in pre-weaning mortality and culled sows after weaning. Most sows were culled due to illnesses resulting in an increased average wean-to-first-service interval and decreased sows in heat by 7 days after weaning. It could be speculated that as a result of poor gilt management, a disease outbreak, particularly PRRS, might have occurred in the farrowing units and affected the production performances (Alexopoulos et al., 2005). It appears that having PRRSV positive results in the serum before the introduction to the sow herd in Farm B might result in sow problems in the breeding herd.

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This study indicated that viremia found at the end of acclimatization resulted in an ineffective cool-down period (Lambert et al., 2012). It should be noted that PRRSV co-infection with porcine epidemic diarrhea virus during the feedback protocol (Olanratmanee et al., 2010) and APP infection did exacerbate PRRSV clinical signs and those co-infections might have had a negative impact on PRRSV cool-down period as shown in Farm B. In addition, Farm A gilts also had a high percentage of gilt replacement (44.83%) and gilts were moved to individual stalls during the cool-down period. The use of individual stalls might also increase the efficiency of pathogen elimination compared to group-housing gilts during cool-down period. Therefore, the duration and method of cool-down period are of importance for complete clearance of viremia in infected gilts before the introduction to the sow herd.

In conclusion, the present study demonstrated that oral fluid samples offered distinct advantages over the serum samples in case of PRRSV-persistent infection. However, the results obtained from the serum samples provided better detection of acute infection in this field-based study. Therefore, oral fluid samples could be used for monitoring the effectiveness of PRRSV acclimatization in the gilt pool, especially when evaluated together with other sow performance parameters. Application of oral fluid samples used in other production periods such as in the farrowing, nursery and finishing phases is of interest and should be studied for future swine management strategies.

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3.5 Acknowledgements

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3.7 Compliance with ethical standards

The study protocol was approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC #1531020) with the farm owners' consent.

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3.8 Conflict of interest

The authors declare that they have no conflict of interest.

Table & Figures

Table 3.1 Descriptive analysis of the sow performance parameters after introduction to breeding herds (means ± SEM).

	Farm A	**Farm B**
Returns to service after mating (%)	3.92 ± 1.03	12.24 ± 1.89
Abortion (%)	0	0
Culling after mating (%)	2.36 ± 1.04	3.80 ± 0.80
Farrowing rate (%)	93.72 ± 0.80	82.46 ± 1.78
Average total born/litter (heads)	13.17 ± 0.29	14.39 ± 0.25
Born alive litter size (heads)	12.51 ± 0.26	12.76 ± 0.34
Stillborn (%)	3.36 ± 0.44	7.47 ± 0.88
Mummies (%)	1.66 ± 0.33	3.89 ± 0.74
Pigs weaned/litter (heads)	11.38 ± 0.19	10.67 ± 0.54
Average weaning weight (kg)	6.68 ± 0.14	7.06 ± 0.23
Pre-weaning mortality (%)	9.06 ± 1.60	16.31 ± 1.63
Average lactation length (days)	25.76 ± 0.12	29.59 ± 0.26
Average wean-to-first-service interval (days)	4.32 ± 0.28	8.26 ± 0.71
Sows in heat by 7 days after weaning (%)	91.90 ± 0.10	84.67 ± 0.88
Sows culled after weaning (%)	0	9 ± 2.08

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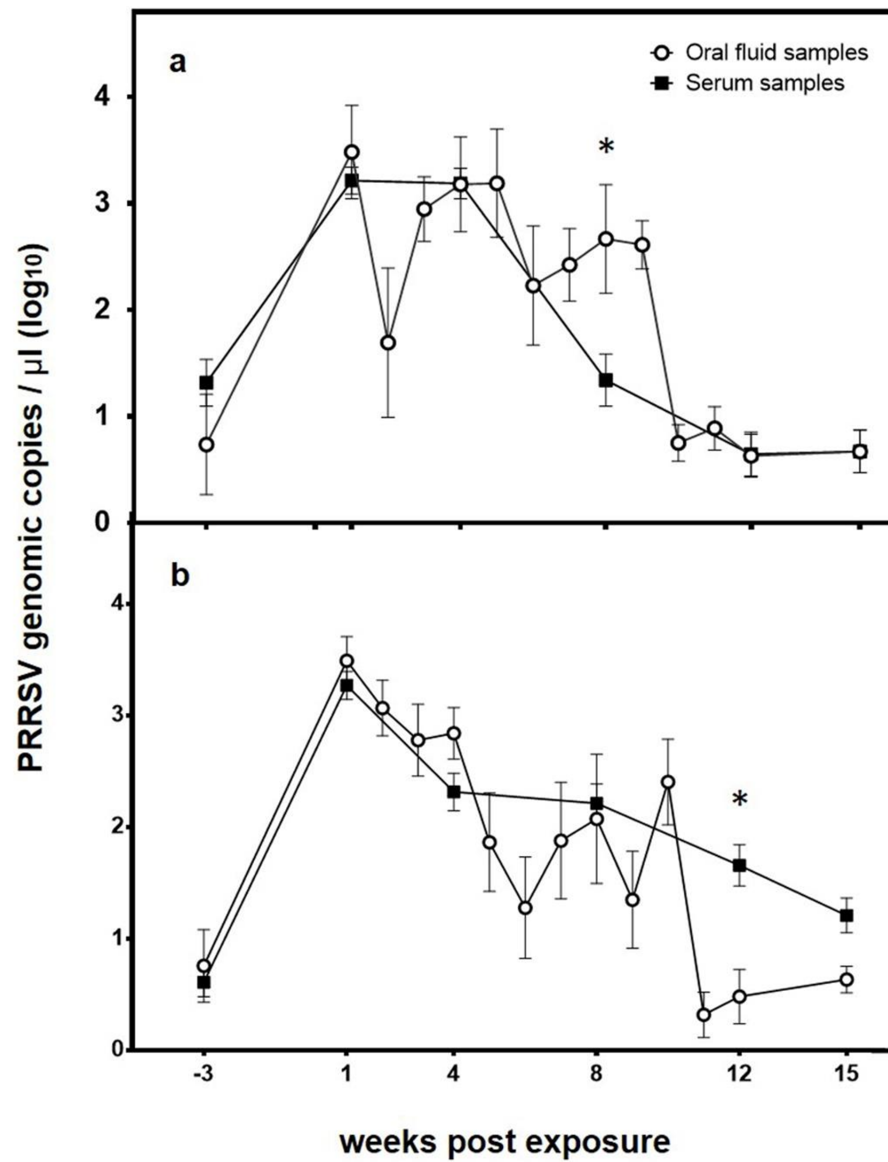

Figure 3.1 PRRSV genomic copies/µl ($\log_{10}$) in serum and oral fluid samples of Farm A (a) and Farm B (b) during the acclimatization periods. The Asterisks indicate significant differences between sample types at the same time point ($p < 0.05$).

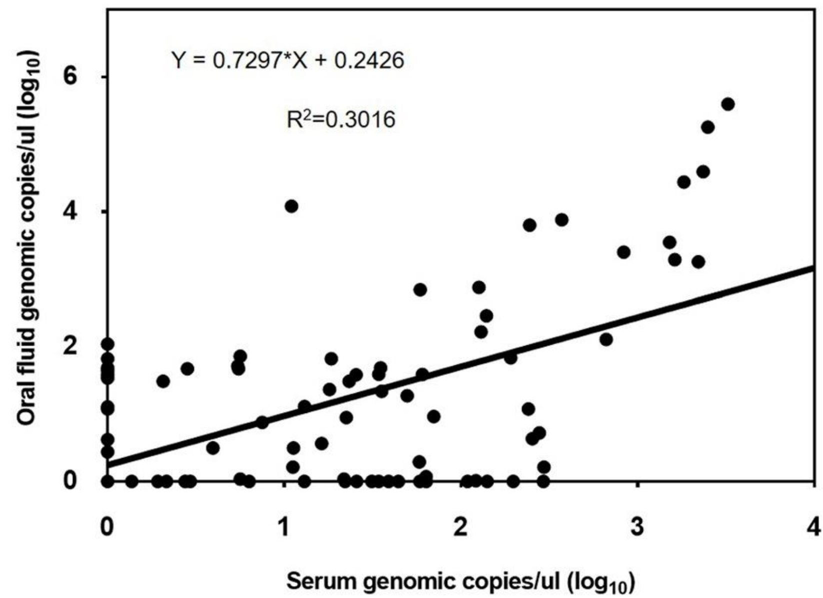

Figure 3.2 Correlation between paired serum and oral fluid samples on PRRSV genomic copies/µl (log_{10}) (Pearson's correlation coefficient r = 0.55; $p < 0.0001$).

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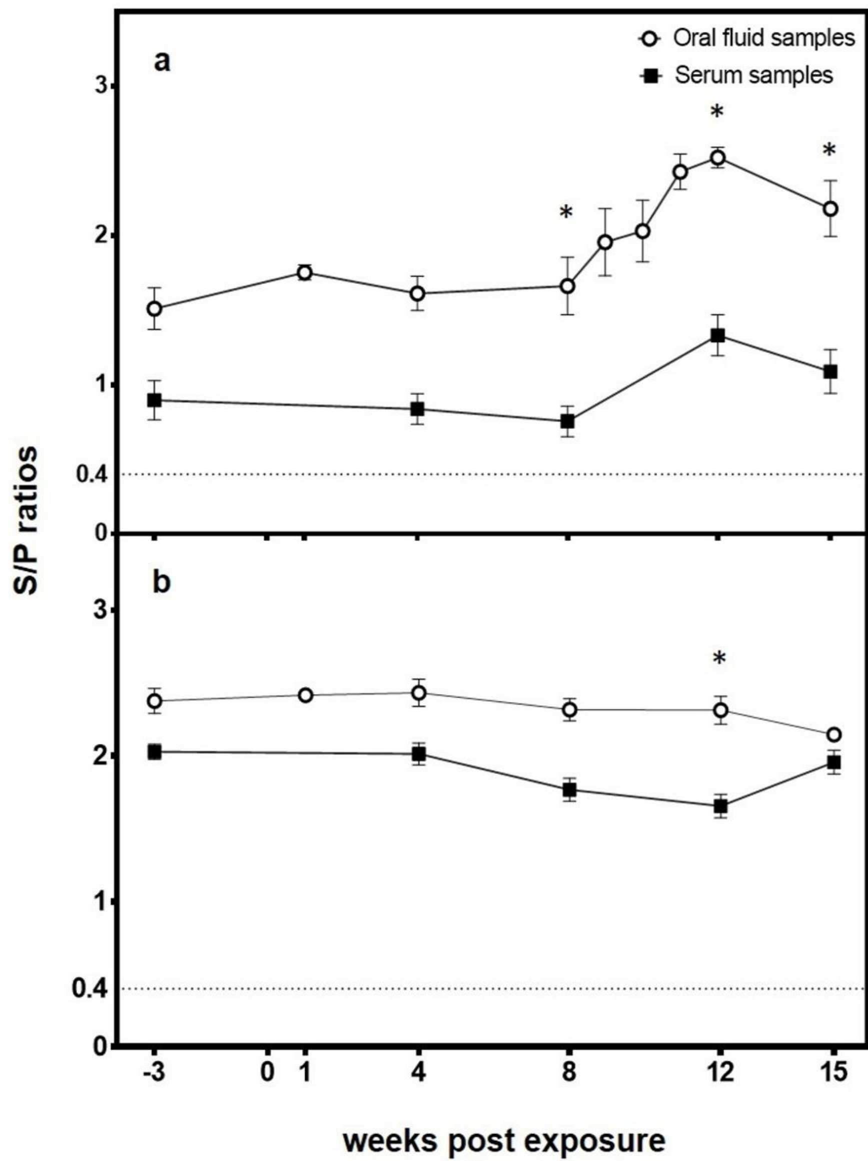

Figure 3.3 Means S/P ratios in oral fluid and serum samples of Farm A (a) and Farm B (b) during the acclimatization program. The Asterisks indicate significant differences between sample types at the same time point ($p < 0.05$).

CHAPTER 4

DETECTION OF PRRSV CIRCULATION USING ORAL FLUID SAMPLES FOR NURSERY MANAGEMENT IN ENDEMICALLY PRRSV-INFECTED FARMS

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) has become a major swine disease worldwide. Relevant management strategies and diagnostic assays are of importance for PRRS virus (PRRSV) control. The objective of this study was to determine the use of oral fluids for PRRSV monitoring in endemically PRRSV-infected herds. PRRSV RNA and PRRSV-specific antibodies were monitored using oral fluid samples and serum samples in two conventional swine farms in Thailand ('Farm A', a one-site conventional system and 'Farm B', a one-site P0-P1 segregation system) during farrowing to nursery periods. Both PRRSV RNA and PRRSV antibodies were detected from 3 to 9 weeks of age in both sample types. Pen-based oral fluid samples were detected positive over 71% when the prevalence of serum PRRSV-positive pigs in the pens was at least 40%. Mean S/P ratios of oral fluid samples showed significantly higher levels but had similar pattern to the seroprofile of blood samples. Increased levels of PRRSV antibodies were detected in all groups at 5 to 9 weeks of age. Overall, the positive correlation of both sample types was 0.65 ($p < 0.001$). It should be noted that Farm B had higher production losses in the farrowing and nursery unit, concurrently, with higher levels of PRRSV load in both sample types. Oral fluid testing provided convenient and economical approach, better welfare, and satisfied performance to determine the PRRS status, especially, in the nursery period when having moderate to high PRRSV prevalence. Achievement of this objective could be better and beneficial to the practitioners by using oral fluid testing together with other measurements.

Keywords: management, monitoring, nursery pigs, oral fluid, PRRSV, swine

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4.1 Introduction

Porcine reproductive and respiratory syndrome (PRRS), caused by PRRS virus (PRRSV), has become one of the most important swine diseases since the first report in the United State (Christianson, 1994). Economic losses in all production stages from PRRSV circulations within and between farms could be observed worldwide (Baron et al., 1992; Hirose et al., 1995; Holtkamp et al., 2011). According to the disease characteristics involving both reproductive and respiratory problems, outbreaks in swine breeding herds could be extremely severe and are usually followed by porcine respiratory disease complex (PRDC) in growing pigs. Although establishment of different PRRS control protocols has been conducted, ongoing problems still occur. The impacts of PRRS monitoring strategies on the successful of PRRS controls should not be overlooked. Recognition on the benefits of oral fluid use for PRRS monitoring has recently become apparent due to the advantages over the use of serum samples. Implementation of oral-fluid-based PRRS monitoring during the disease controls in the breeding herds and various stages of swine production should be explored for the valued information benefiting to the control strategies.

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Improvement of PRRS monitoring strategies could be a crucial factor in effective PRRS control program in the breeding herds. Utilizations of various strategies in the sow herd including parity segregation, gilt acclimation and gilt/sow vaccination have been suggested to be effective. Briefly, parity segregation involves minimizing

production losses from PRRSV infection in gilts/primiparous sows by separating their gestation and farrowing units from other multiparous pigs (Moore et al., 2005). Similarly, those gilt management strategies aim to reduce the PRRSV shedding and horizontal transmission before moving those acclimatized gilts to the sow herd and choosing effective monitoring strategies can be vital in PRRS control. The best method used for monitoring must demonstrate better precise information on PRRS status. Besides, clinical observation, PRRS monitoring program usually involves the degree and timing of PRRSV spreading in the herd. Although sensitivity and specificity are essential, those values are not the only key determinants in choosing the monitoring method. Simplicity, cost and consideration on animal welfare should also be taken into account since those measurements could affect the overall efficacy of the management strategies.

Oral fluid sample is an alternative diagnostic sample for PRRS monitoring and control program in the breeding herds (Kittawornrat et al., 2014; Trang et al., 2014). Previously, serum samples have long been used in PRRS monitoring. Several studies suggested that using oral fluids could gain a better benefit in general PRRS surveillance (Cuong et al., 2014; De Regge and Cay, 2016). Previous data indicated that oral fluids provided a longer detection period and yielded a better sensitivity for PRRSV detection than other methods (Kittawornrat et al., 2010; Goodell et al., 2013). Blood collection is invasive and could cause traumatic tissue injury to the animals, while oral fluid

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collection hardly causes tissue injury. However, specific protocol for using oral fluid samples to monitor PRRSV in the farrowing to nursery units is necessary to yield the better use for PRRS control program in nursery pigs.

In this study, comparing the results obtained from oral fluids- and serum-based methods was investigated. Quantitative PRRSV RNA and PRRSV-specific antibody parameters were compared and analyzed. The comparison was done in two selected farms with different management systems with and without parity segregation system.

4.2 Material and Methods

Ethics statement:

The study obtained ethical approval from Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC number 1531020).

Trial farms:

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The study was conducted at 2 farrowing to finishing swine farms (Farm A and Farm B) using crossbred (Large White x Landrace x Duroc) pigs. The selected farms located in the intensive farming density in central part of Thailand and were diagnosed as PRRSV-positive farms, based on PRRSV-specific antibody responses by ELISA.

Farm A is a one-site conventional system (without P0-P1 segregation system) having approximately 1,300 sows. Farrowing barn had natural-ventilation facility with 120 farrowing crates. Warming laying area was provided for lactation pigs. Nursery units were designed with evaporative cooling system (EVAP), and stocked 600 pigs (20-25

animals per pen) in each barn. During the farrowing periods, both primiparous (P1) sows and multiparous (P2+) sows were kept in the same barn. After weaning at 24-28 days of age, those sows were mixed in the same nursery barn (Nursery A) until 10 weeks old.

Farm B is a one-site P0-P1 segregation system having approximately 5,000 sows. The ventilation of all farrowing and nursery facilities was controlled by the EVAP. Each farrowing barn consisted of 160 farrowing crates with a warming box for piglets on each crate. The nursery barn stocked 700-800 pigs with 20-25 pigs in a pen. P1 and P2+ sows were kept in separate barns during the farrowing periods. After weaning at 24-28 days of age, the weanling piglets of P1 sows were placed in a separate EVAP barn from the piglets of P2+ sows (Nursery B-P1 and Nursery B-P2+, respectively) until 10 weeks old.

P1 sows (n = 11) and P2+ sows (n = 15) from Farm A, and P1 sows (n = 15) and P2+ sows (n = 15) from Farm B that farrowed within the same week were selected and participated in the study. After parturition, all piglets were vaccinated with a modified live virus (MLV) PRRS vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim) at 2 weeks old in both farms. For further sample collection in the nursery units in the study, 12 nursery pens were randomly selected as the monitoring nursery pens for the Nursery A. In Farm B, 6 monitoring nursery pens were selected in Nursery B-P1 and Nursery B-P2+ (12 monitoring pens in Farm B).

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Oral fluids and serum collections:

Oral fluid and serum collections were done in the same manner for both farms (Figure 4.1). In the farrowing units, oral fluid and serum samples were individually collected from sows at one and 3 week-post farrowing (WPF). Only serum samples were collected from the piglets (32 pigs on each sow group) at 3 weeks of age. For individual oral fluid sampling, 100% cotton ropes of 2.0-cm diameter were hung in each stall at the shoulder height of the sows for 20-30 minutes. The rope was carefully collected to prevent cross-contamination from the other pigs. The oral fluids were extracted from the ropes by mechanical compression and then transferred into 50-ml tubes. Blood samples were collected by single-use blood collection systems (Monovette® 9ml Z, Sarstedt AG & Co, Germany) from the jugular vein. All samples were stored at -80°C until assayed.

In the nursery units, pen-based oral fluid samples and individual serum samples were collected at 5, 7, 9 weeks of age from pigs in the assigned monitoring nursery pens. Pen-based oral fluid collection was done by hanging 2 cotton ropes (100%) of 1.0-cm diameter in the monitoring pens. Oral fluids were then extracted from the ropes and stored as previously described. Blood samples were collected from 5 pigs in each monitoring pen.

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Quantification of PRRSV RNA:

PRRSV RNA was extracted from serum and oral fluid samples using NucleoSpin® RNA virus kit (MACHEREY-NAGEL GmbH &Co. KG, Germany). Viral RNA extraction from serum samples were done as described in the manufacturer's instructions, whereas, the extraction from oral fluid samples was performed with a modified method by using larger volumes of 200 µl, instead of 150 µl. Finally, copy number of viral RNA was examined using previously described TaqMan® probe-based qRT-PCR, with primers and probes specific for PRRSV nucleoprotein gene (ORF7) (Egli et al., 2001). RT-PCR mixture (25 µl) was based on SuperScript™ III Platinum® One-step Quantitative RT-PCR system (Invitrogen, Carlabed, Califormia,USA); RT-PCR were performed on Corbett Rotor-Gene™ 6000 (Qiagen) real-time PCR machine. Copy number of the viral RNA was calculated using standard curve method. For each test, samples with a Ct < 33 were considered positive (Sirisereewan et al., 2017).

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Detection of PRRSV-specific antibody:

Anti-PRRSV antibody detection was performed on serum samples using IDEXX PRRS X3 ELISA test kit (IDEXX laboratories, Inc., Westbrook, ME, USA). Oral fluid samples were tested by a commercial PRRSV Antibody Test Kit for Oral Fluids (IDEXX PRRS OF, IDEXX laboratories, Inc., The Netherlands). ELISA was performed as described in the manufacturer's instructions. S/P ratio value of greater than 0.4 was considered positive.

Performance monitoring of sows and nursery pigs:

The reproductive performance parameters of the sows and performance index of nursery pigs were recorded on a computerized recording system for swine herds (PigLive® software, Kasetsart University) (Udomprasert et al., 1993). Production parameters related to PRRSV infection were monitored from farrowing until weaning.

Data analysis:

Statistical analyses were done using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical analysis of variance (ANOVA) following by Tukey's multiple comparison tests was done to compare each variable of interest between monitoring periods and sample types. Viral titers and S/P ratios of oral fluid and serum samples of sows were presented as copy number means from individual sows. In nursery unit, the oral fluid results were calculated from pen-based oral fluid samples of the monitoring pens, and the serum analysis were calculated in all monitoring pens of the same nursery unit. Association between PRRSV RNA detection rate from pen-based oral fluid samples and positive percentages based on five serum samples in the monitoring pen was done using data from all 3 nursery units. The Pearson's correlation coefficient was used to determine the relationship of virus concentration and average S/P ratios yielded from serum and from oral fluid samples. Statistical significance was set at $p < 0.05$.

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4.3 Results

Detection of PRRSV RNA in oral fluid and serum samples:

An overview of PRRSV concentration and the proportion of PRRSV-positive in serum and oral fluid specimens on each farm are displayed in Figure 4.2. PRRSV RNA quantification from oral fluid and serum samples of the lactating sows were done twice, at 1 and 3 weeks after parturition. In both farms, viral RNA was not detected in all tested samples from the sows. PRRSV was first detected in serum samples at 3 weeks of ages (pre-weaning period). For PRRSV monitoring in nursery pigs, the direct comparison between oral fluid and serum samples were done in three nursery units; Nursery A, Nursery B-P1, and Nursery B-P2+ (Table 4.1). Pen-based oral fluid samples showed significantly higher viral titers than those of serum samples in Nursery B-P1 at 5 weeks old and Nursery B-P2+ at 5 and 9 weeks old. However, serum sample showed significantly higher viral titers in Nursery A at 7 weeks old, and Nursery B-P1 at 7 and 9 weeks old. In addition, positive correlations were estimated between both sample types in Nursery-A ($r = 0.57$; $p < 0.001$), Nursery-B-P1 ($r = 0.80$; $p < 0.001$) and Nursery-B-P2+ ($r = 0.75$; $p < 0.001$). Moreover, no differences of the proportion of PRRSV positive were found between pen-based oral fluid and serum samples over the monitoring periods. The association between PRRSV detection rate of pen-based oral fluid sample and the percentages of serum-positive pigs within a pen is shown in Table 4.2. The detection rate of the pen-based oral fluids increased when the serum prevalence increased. Over 71% of the PRRSV-positive pens were identified by pen-

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based oral fluids when the serum percentages of PRRSV-positive pigs in the pens had at least 40%.

Serology test:

An overview of the antibody titers and the proportion of positive results in serum and oral fluid on each farm are displayed in Figure 4.3. Mean S/P ratios of oral fluid samples showed significantly higher but having similar pattern than those of the serum samples. Similarly, both sample types had average S/P ratio of sows declining significantly from 1 to 3 week-post farrowing. In the nursery period, a total of 360 pigs from 72 pens were tested by ELISA and the results indicated that pigs had PRRSV antibodies increased at 5 to 9 weeks of age. All pen-based oral fluid samples were found positive to PRRSV corresponding well with the serum results depending on the percentage of serum positive-pigs in each pen. Correlations between average S/P ratios in serum and oral fluid samples using Pearson correlation coefficient were 0.85, 0.87, and 0.72; $p < 0.001$ in Farm A, Farm B-P1, and Farm B-P2+, respectively.

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Production parameters:

Computerized performance parameters of the sows, pre-weaning piglets, and nursery pigs are summarized in Table 4.3. In Farm A, following the farrowing period, two enrolled P1 sows became sick by post-partum dysgalactia syndrome and were culled before weaning. In Farm B, one enrolled P1 sow was also culled before weaning. Percentages of stillbirth were increased significantly in Farm B-P1 sows comparing to

the data of Farm A sows. Pre-weaning mortality was high in all sow groups of both farms. Lactation length and wean-to-first-service interval were longer in Farm B due to the clinical condition of the animals. Data yielded from Nursery A were in acceptable ranges. In contrast to Farm A, clinical signs were observed in nursery pigs from both P1 and P2+ sows of Farm B, including depression, respiratory distress and emaciation correlated well with the higher viral loads shown in both oral fluid and serum samples (Figure 2). ADG, FCR, FCG and mortality rates of Farm B nursery pigs were also below the base-line data.

4.4 Discussion

As expected, oral fluid collection was easy to perform in the farrowing sows and the nursery pigs. The workers could handle by themselves without causing stress or injury to the animals. It should be noted that failure to collect the oral fluid samples from the suckling pigs was probably related to the untrained younger age piglets. Previous studies described 97% success rate on oral fluid collection from 3-week-old, trained pigs (Kittawornrat et al., 2014; White et al., 2014). In nursery period, pen-based oral fluid collection could be collected as frequently as needed after being trained. In addition, pen-based collection costs less associated with using less sample number and also increased the sensitivity of the samples

After farrowing, no evidence of PRRSV viremia or shedding was found in all sows tested. However, viremia was found in 3-week-old piglets in all groups. It should be

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speculated that sample collection in the absence of clinical signs might have low sensitivity comparing to the collection from the sick sows. In addition, PRRSV viremia in sows is difficult to detect because PRRS viremic duration was rather short in immunized animals, particularly in the repeatedly immunized sows. Low proportion of PRPSV positive piglets in Farm A might be related to the MLV vaccination at 2 weeks of ages or possibly the presence of low level of vertical transmission (Balka et al., 2016) However, Farm B pigs had higher viral loads possibly due to having higher levels of both vertical and horizontal transmission together with MLV vaccination with the presence of clinical diseases

In the nursery period, oral fluid samples could be very promising for PRRSV monitoring, especially when the prevalence of PRRSV infection is moderate to high. It has been suggested previously that the prevalence of PRRSV infection is a crucial factor for a successful use of oral fluids in PRRSV monitoring (Strugnell, 2010; Olsen et al., 2013b; De Regge and Cay, 2016). This is also true in our situations. We found that over 70% of PRRSV-infected pen can be detected by pen-based oral fluid samples when at least 40% of the pigs in the pen showing viremia. On the other hand, when the prevalence of PRRSV is low, such as in Farm A, pen-based oral fluid might be insufficient. In our study, approximately 42% of PRRSV-infected pen (mostly from farm A) showed negative result using pen-based oral fluid samples. However most of these pens (approximately 80%) had low prevalence of viremic pigs (20%, 1 in 5). Detection

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rate of oral fluid samples in our study seems to be lower comparing with other studies. De Regge and Cay (2016) demonstrated that when the serum prevalence within pens exceding 30%, detection rate in oral fluids could reach 100%. The major reason explaining the difference in the detection rates could be the difference of the phase of infection. In our study, we focused on 3 – 7 weeks old piglets, representing an early phase of infection, while the other study was covering approximately 8 – 28 weeks of age including a late phase of infection. It has been shown previously that during the later phase of PRRSV infection, the viurs could be found frequently more in the oral fluids (Decorte et al., 2015). Therefore, when viremia is declining together with high virus shedding in oral fluids, detection rate of the oral fluid in infected pen could be higher. Our study indicated that in the early phase of infection, oral fluids could still be useful with maximum benefit when the prevalence of viremic pigs is moderate to high.

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Previous studies have already demonstrated the positive correlation of the ELISA results between individual serum and pen-based oral fluid samples (Decorte et al., 2015; Kuiek et al., 2015; De Regge and Cay, 2016). Similar to those previous studies, means S/P ratios in oral fluid samples showed significantly higher levels than those found in serum samples. However, both sample types had similar pattern. The higher levels of S/P ratios in oral fluid samples were reported in association with the IDEXX PRRS Oral Fluids Ab Test (IDEXX, 2013).

PRRSV antibodies in the studied sows showed declining in S/P ratios from 1 to 3 WPF possibly due to the recovery from stress after farrowing. Generally, vertical transmission and evidence of positive weaning pigs should not be seen in the absence of new or re-infections in sows (Cano et al., 2008). However, some sows in Farm B-P1 and P2+ had S/P ratios increasing at 3 WPF (25% and 37.5% in oral fluid samples; 12.5% in serum samples of both groups). Those sows might have concurrent infections causing PRRSV circulation in the farrowing unit, especially, in Farm B situation. Additionally, their litters had PRRSV RT-PCR-positive (50 - 63%) associated with the production losses in the farrowing units comparing to Farm A data. However, litters should not be used as the only sample size because the prevalence of infection within litter varies (Graham et al., 2013). The production parameters showed higher pre-weaning mortality and had higher numbers of culled sows after weaning associated with the increased levels of average wean-to-first-service interval and sows in heat by 7 days after weaning. Concurrently, high detection levels of PRRSV RNA and PRRS antibodies were found both in serum and oral fluid samples at 5 to 9 weeks of age. It could be speculated that post-weaning infection occurred concurrently with production loss and increased feed costs in nursery periods. Albina et al. (1994) has shown that maternal antibodies persisted until 4-8 weeks. Therefore, it should be speculated that confounding factors from concurrent infection with PRRSV and other diseases might affect the production performances in both Farm B-P1 and P2+ groups.

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Based on the objective of this study, P0-P1 segregation and conventional management farms were chosen to determine the use of oral fluid testing for PRRSV monitoring before and after weaning. This study demonstrated that PRRSV status could be monitored using oral fluid samples for both ELSA and RT-PCR tests in case of persistent infection when having moderate to high prevalence. Unexpectedly, the detection of virus circulation and herd immunity were higher in the P0-P1 segregation than found in the mixed parity management farm corresponding with the poor production parameters. It was possible due to poor biosecurity management, lack of workers and short distance between each group of Farm B (Madec et al., 2001; Papatsiros, 2012). In addition, P0-P1 segregation needs high layer, costs and biosecurity program (Dee, 1997). These data suggested that disease prevalence surveys for monitoring disease problem is necessary in the field situation together with other parameters.

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4.5 Conclusion

The present study demonstrated that oral fluid samples could be used for monitoring PRRSV infection status for planning the management strategies in both P0-P1 segregation and conventional management farms. Oral fluid testing provides better economical approach, cost effective and animal welfare. Oral fluid samples could be used to determine the timing of infection in the sow herd and in the nursery period based on the results from ELISA and RT-PCR tests. However, sensitivity of PRRSV

detection is acceptable with some limitation comparing to the use of serum samples. Achievement of this objective could be better and benefit the practitioners by using oral fluid testing together with other measurements.

4.6 Acknowledgements

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Tables & Figures

Table 4.1 The number of serum and oral fluid samples positive in qRT-PCR for PRRSV within each sampling pen by age of pigs (weeks).

Farm	Pen	**Detection of PRRSV using qRT-PCR by age of pigs (weeks)**					
		5		**7**		**9**	
		Oral fluid	Serum	Oral fluid	Serum	Oral fluid	Serum
A	1	+	0/5	+	2/5	-	1/5
	2	+	2/5	-	1/5	-	0/5
	3	-	0/5	-	0/5	-	1/5
	4	-	0/5	-	1/5	+	0/5
	5	-	0/5	-	0/5	-	0/5
	6	-	1/5	-	0/5	-	0/5
	7	-	0/5	-	1/5	-	1/5
	8	-	0/5	-	0/5	+	2/5
	9	-	1/5	-	0/5	-	0/5
	10	+	0/5	-	0/5	-	1/5
	11	-	0/5	-	1/5	-	0/5
	12	+	1/5	+	1/5	-	0/5
B-P1	1	+	2/5	-	1/5	-	1/5
	2	+	2/5	+	2/5	-	1/5
	3	+	3/5	-	2/5	+	2/5
	4	-	3/5	+	3/5	+	2/5
	5	+	1/5	+	3/5	+	3/5
	6	+	4/5	+	4/5	-	2/5
B-P2+	1	+	1/5	+	1/5	-	1/5
	2	+	1/5	+	2/5	-	0/5
	3	+	2/5	+	2/5	+	1/5
	4	+	1/5	-	1/5	-	0/5
	5	+	1/5	-	1/5	+	2/5
	6	-	2/5	+	3/5	+	0/5

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Table 4.2 Percentages of PRRSV detection by a modified qRT-PCR using pen-based oral fluid samples based on the prevalence of serum PRRSV-positive pigs within a pen*

Prevalence of serum PRRSV-positive pigs within a pen (%)	**PRRSV positive pen-based oral fluid samples**[†]			
	Farm A (n=36)	Farm B-P1 (n=18)	Farm B-P2+ (n=18)	**Total**
0	14.29% (3/21)	0	33% (1/3)	16.67% (4/24)
20 (1+)	16.67% (2/12)	25.00% (1/4)	67% (6/9)	36.00% (9/25)
40 (2+)	100.00% (3/3)	71.43% (5/7)	80% (4/5)	80.00% (12/15)
60 (3+)	NA	80.00% (4/5)	100% (1/1)	83.33% (5/6)
80 (4+)	NA	100.00% (2/2)	NA	100.00% (2/2)
100 (5+)	NA	NA	NA	NA

* Data were evaluated based on the samples from nursery periods at 5, 7, 9 weeks of age.

[†] NA = not available

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Table 4.3 Performance indices of pigs in the farrowing and nursery units (mean ± SEM)*

Farm units	Parameters	Farm A[†]	Farm B	
		Mixed parity	P1	P2+
Farrowing	No. of total born/litter	11.19±0.90	11.75±1.15	11.75±1.10
	No. of born alive litter size	10.55±0.93	10.45±1.03	10.80±0.98
	Stillborn (%)	4.77±1.83[a]	10.46±3.28[b]	5.91±1.93[a,b]
	Mummies (%)	0.91±0.50	0.57±0.57	2.21±1.28
	No. of pigs weaned/litter	9.53±0.53	9.30±0.50	9.54±0.54
	Weaning weight (kg)	6.93±0.19	7.43±0.31	8.77±0.18
	Pre-weaning mortality (%)	9.70±2.47	11.02±3.72	11.59±4.04
	Lactation length (days)	25.80±0.23	29.27±0.22	28.13±0.39
	Wean-to-first-service interval (days)	4.84±0.41	5.67±0.72	7.88±1.87
Nursery	ADG (g)	428.00	180.00	194.00
	FCR	1.26	1.97	1.82
	FCG (THB)	29.00	43.64	40.80
	Culled and Mortality (%)	1.90	14.70	11.10

* Statistical analyses were performed among Farm A, Farm B-P1 and Farm B-P2+ at the same parameter (within a row) in the farrowing unit with different superscript letters (a and b) ($p < 0.05$).

[†] Nursery pigs in Farm A were from sows of mixed parity.

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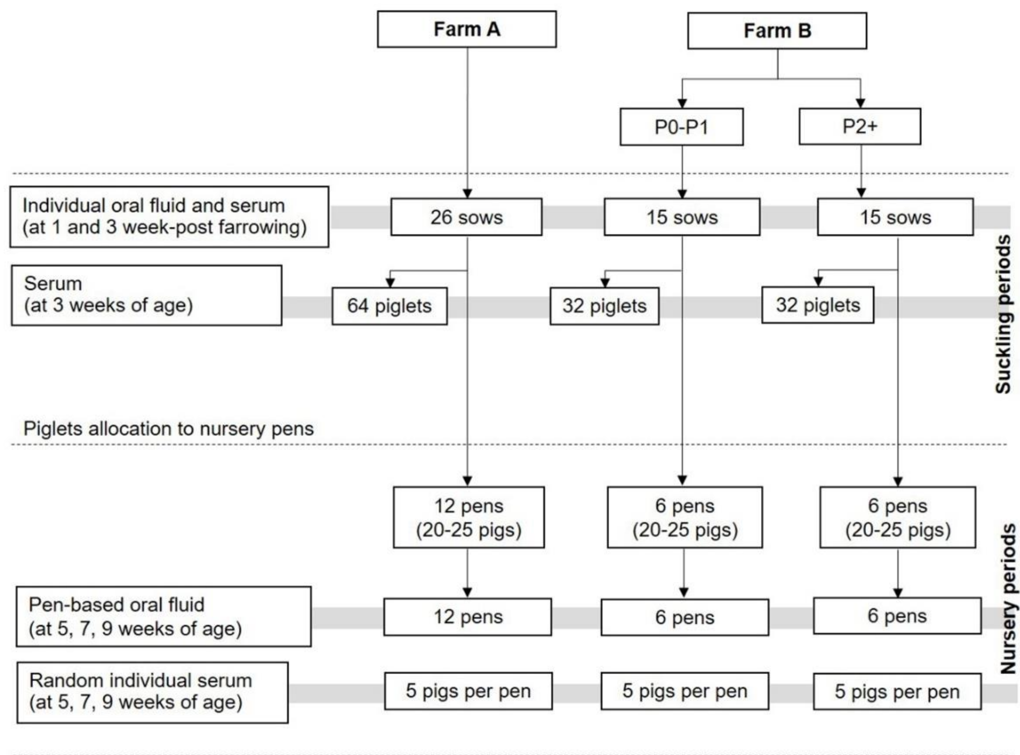

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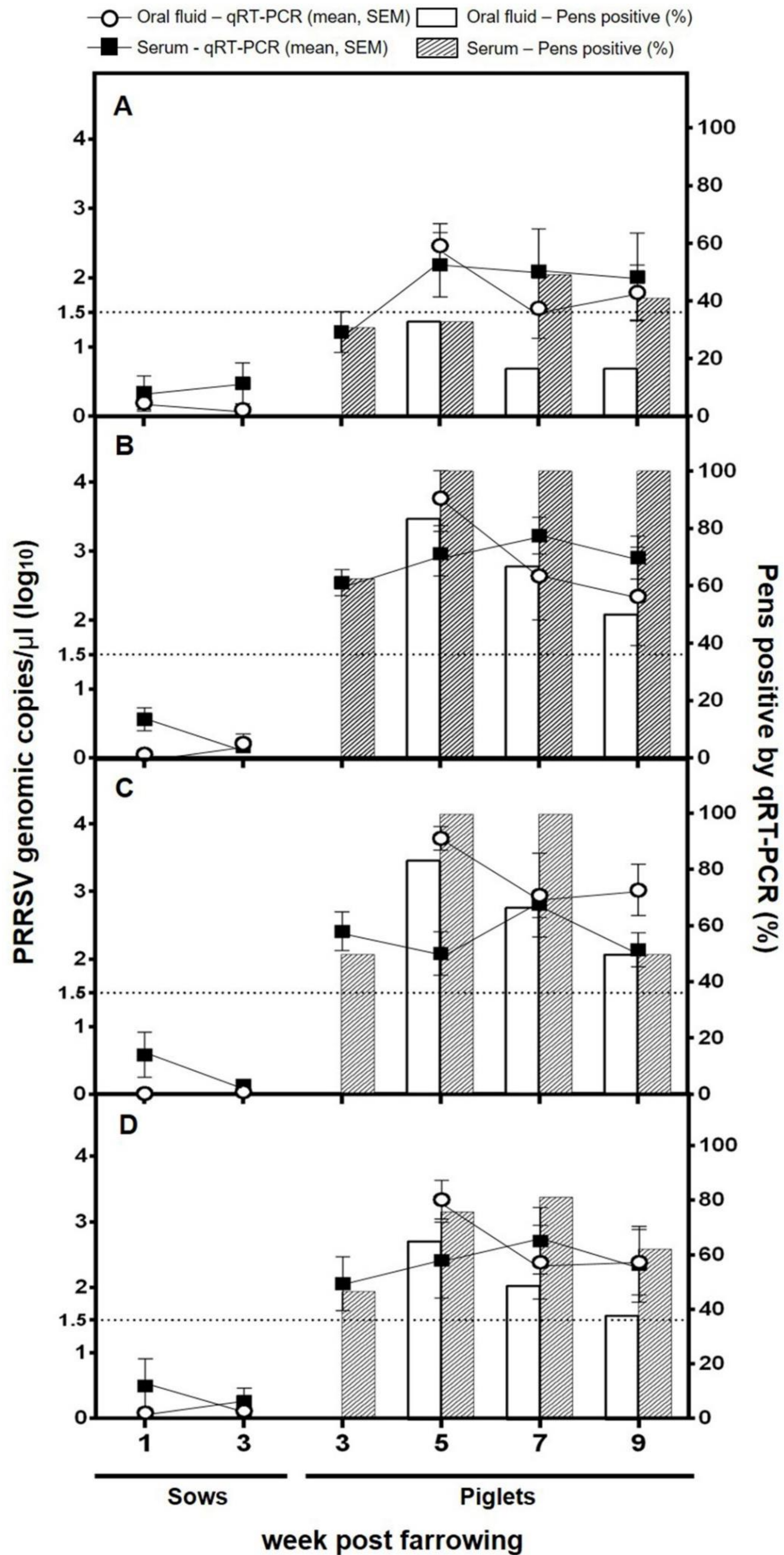
Figure 4.1 Flow diagram summarizing sample collection in suckling and nursery periods.



Oral fluid – qRT-PCR (mean, SEM)
Oral fluid – Pens positive (%)
Serum - qRT-PCR (mean, SEM)
Serum – Pens positive (%)
A
B
C
D
PRRSV genomic copies/µl (log10)
Pens positive by qRT-PCR (%)
Sows
Piglets
week post farrowing

Figure 4.2 PRRSV quantitative RT-PCR ($\log_{10}$ genomic copies per µl) of Farm A (a), Farm B-P1 (b), Farm B-P2+ (c) and the overall samples (d) in sows and their piglets from serum and oral fluid samples. All results are expressed as the pen-based results for the qRT-PCR test. The horizontal line at 1.5 $\log_{10}$ genomic copies per µl represents the cut-off for positive samples (Ct of <33).

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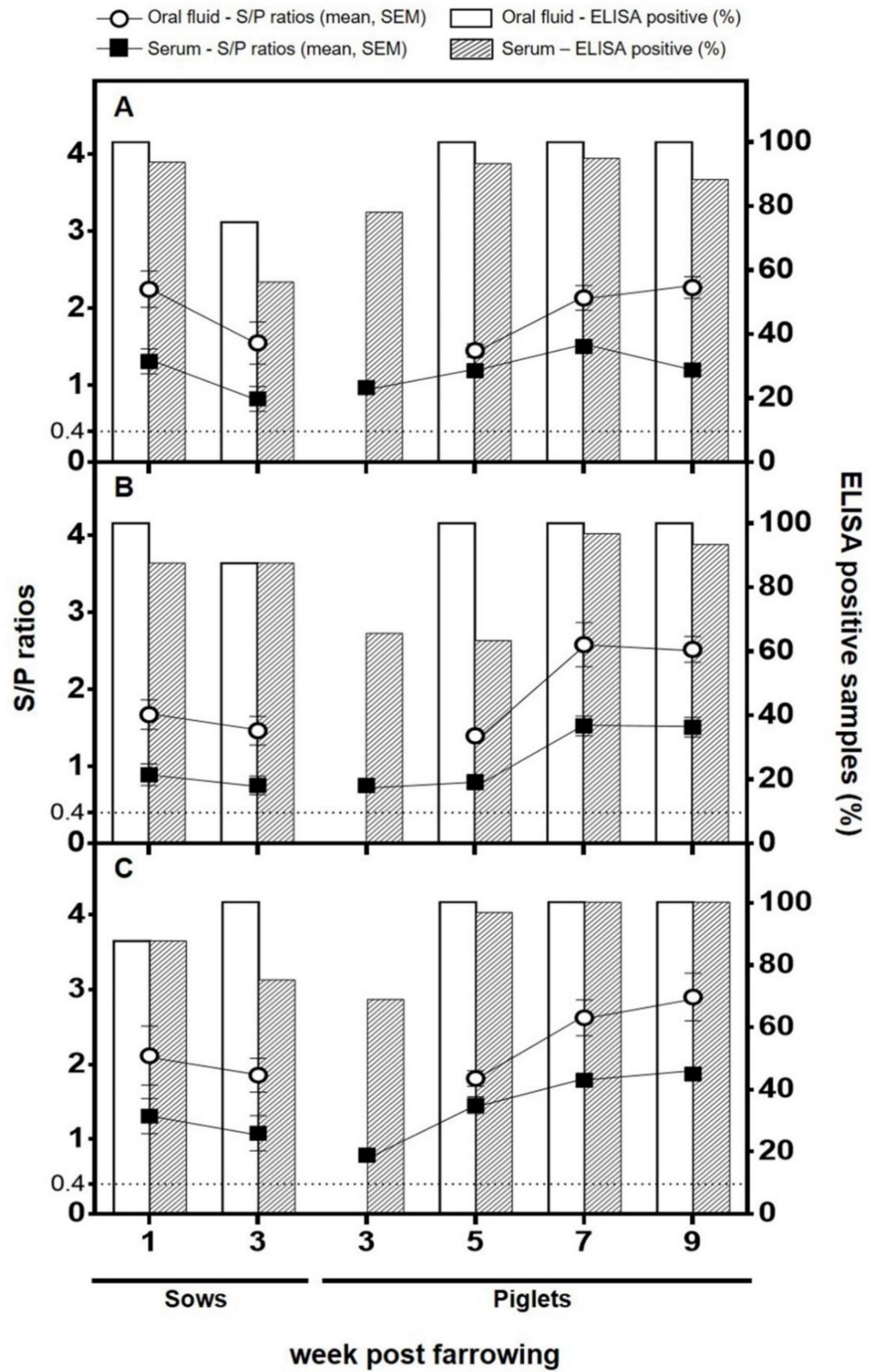

Oral fluid - S/P ratios (mean, SEM)
Serum - S/P ratios (mean, SEM)
Oral fluid - ELISA positive (%)
Serum – ELISA positive (%)
A
B
C
S/P ratios
ELISA positive samples (%)
Sows
Piglets
week post farrowing

Figure 4.3 Mean S/P ratios of Farm A (a), Farm B-P1 (b) and Farm B-P2+ (c) in sows and their piglets from serum and oral fluid samples. Sow oral fluid and serum ELISA results are presented as the mean of individual samples. In nursery units, mean S/P ratios of oral fluid samples were expressed as the pen-based results. S/P ratios of > 0.4 are considered positive.

CHAPTER 5

CONCLUSION

5.1 Research summary

The transition of livestock production to large-scale confinement has created the chances for several disease outbreaks and spreading to the others particularly in the high density of livestock population. Because of these challenges, monitoring and surveillance program are very important for disease prevention and control. Recently, oral fluid samples have shown to be a useful biological specimen for the swine diagnosis. Oral fluids collection is an efficient, non-invasive and convenient method using in many countries. Moreover, pen-based oral fluid specimens can represent a larger number of animals with fewer samples than using serum or other sample types. The research presented in this thesis reviewed that many factors of oral fluid specimens could affect the results of oral fluid testing. Although clean oral fluid samples have the potential to be used effectively for laboratory diagnostic methods, the contaminated materials in the dirty field samples might have an impact on PCR analysis. Therefore, the improvement in sensitivity of PRRSV detection using oral fluid samples should be evaluated.

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The first research objective was to evaluate the effect of different post-collection processing on PRRSV qRT-PCR detection for swine oral fluid samples. Initially, the finding demonstrated that the sensitivity of PRRSV detection by qRT-PCR were improved in RNA extraction from a larger sample volume. The possible reason for this finding is that the larger initial sample volume might obtain a higher eluted RNA and improve detection by qRT-PCR. However, an excess of oral fluid sample volume might affect the capacity of column based extraction kit and increased more endogenous inhibitors. Therefore, larger input volume followed by increasing the lysis buffer and PCR enzyme concentration should be concern in the future evaluation. Other procedures, i.e., centrifugation, NaOH treatment might also have an impact on the target antigen yielded. That is, PRRSV viral RNA in swine oral fluid samples might be trapped and hampered by the organic matters that could be eliminated together with the pellet during centrifugation. Interestingly, the modified pre-extraction using NaOH treatment may possibly be the method to reduce PCR inhibitors and recover the target antigen. However, the degree of degradation might be a major problem for NaOH treatment. The present study indicated that all three procedures can you list them here (increasing input volume, treated with NaOH, centrifugation) have yielded variable results for swine oral fluid samples. Based on the results from the study, increasing sample input volume (200 µl) were the best simple method for enhancing the qRT-PCR PRRSV detection by column based extraction kit.

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Currently, oral fluids are the new diagnostic samples using for swine diagnosis commonly representing pen-based analysis. Therefore, it is important to understand the applications and limitations of the diagnostic samples collected from the field. The second research presented in this thesis examined the utilization of oral fluid specimens for PRRS status determining in commercial swine production systems. Because PRRSV acclimatization is an important management to achieve PRRS stability within the herd. Thus, the evaluation of the efficacy of PRRSV acclimatization protocol must be performed. The comparison of the results from oral fluid samples with individual serum samples as a gold standard for defining PRRSV status was determined. In gilt acclimatization period, the present study demonstrated that PRRSV detection revealed a significant positive correlation between oral fluid and serum qRT-PCR results. Moreover, these findings suggested that oral fluid monitoring associated with the management and the clinical outcomes in each phase through PRRSV acclimatization period. The PRRSV could be detected by qRT-PCR from oral fluids at a longer period than serum corresponding in the same pen. However, prolong viremia was detected in a few serum samples from Farm B at the end of acclimatization, but not in the oral fluid samples. The higher PRRSV detection levels in serum might correspond to an early phase of infection in Farm B. For this finding, it might possibly be due to re-infection in the gilt pool and could represent the failure of cooling down period.

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The present study demonstrated that the S/P ratios of oral fluids detected by IDEXX PRRS Oral Fluids Ab Test had higher levels than serum, but the antibody response found in both sample types did have the similar pattern. In PRRSV-vaccinated endemic herds, detection of PRRSV antibody response during acclimatization could not be used to differentiate the response from between infection and vaccination when exposure to the homologous strain (McCaw, 2002). Similar to the serum response, the increasing of PRRSV antibody level could be detected in oral fluid samples when pigs exposed with a heterologous strain of PRRSV.

Oral fluid testing is an effective, practical and economical method. Interestingly, pen-based oral fluid samples could provide larger population associated with lower number of samples. Then, increasing sample size of oral fluids should be applied to increase sensitivity test in low prevalence situation such as at the end of acclimatization. These results suggested that oral fluid samples could be applied for gilt acclimatization monitoring in PRRSV endemic farms especially for PRRSV detection. It would benefit the most when using with other parameters, e.g., production performance index and clinical outcomes.

Following the second research, the ability of oral fluid testing to detect PRRSV status were evaluated in farrowed to nursery period in the third study. To examine the efficacy of oral fluid testing in the different management farm situations, P0-P1 segregation and conventional systems were evaluated in this study. The study showed

that oral fluid collection was more convenient in farrowing sows and nursery pigs, but failed in suckling pig. Therefore, the animals should be trained before sampling to ensure the success oral fluid collection particularly in the younger age piglets.

In farrowing period, the PCR and ELISA results of individual sow oral fluid samples correlated well with the results of individual serum sample. In nursery period, some processes of pen-based oral fluid collection including oral fluid volume, number of participated pigs and timing of collection might affect the viral RNA concentration in oral fluid samples, and therefore the PRRSV detection levels might exhibit varied levels comparing to the serum results. The finding demonstrated that PRRSV detection in pen-based oral fluid samples was associated to the current prevalence of PRRSV. In these situations, the results indicated that over 70% of PRRSV-infected pens could be detected by pen-based oral fluid samples when the serum prevalence within pens exceeded 40%. In the present study, PRRSV detection rate of oral fluid samples seems lower than in the previous study (De Regge and Cay, 2016). The major reason explaining was the difference of the phase of PRRSV infection. That is, the viurus could be more frequently found in the oral fluid samples during the chronic phase of PRRSV infection. Accordingly, it has been suggested that oral fluids could still be useful with maximum benefit when the prevalence of viremic pigs is moderate to high in the early phase of infection.

Furthermore, the S/P values of PRRSV antibodies in oral fluids were higher than those of the serum samples, with the similar pattern throughout the monitoring periods, which similar to the results found in the second study. On the other hand, the individual oral fluid testing could be used to determine health status of sows after farrowing. The seroconversion that were detected from 1 to 3 WPF might be due to the concurrent PRRSV infection in the farrowing unit. For pen-based levels, the increasing of average S/P ratios were detected in both oral fluid and serum samples at 5 to 9 weeks of age associated with the timing of PRRSV infection in piglets. Therefore, PRRSV-specific antibody titer of oral fluid results could be used to monitor disease-related change of the herd immunity. However, the results of ELISA positive percentages in pen-based oral fluid samples might not be useful to determine the alteration of antibody, because pen-based oral fluid samples would show 100% positive ELISA results when vaccinated with MLV PRRSV vaccine in piglets.

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These findings indicated that oral fluid testing could be applied for monitoring PRRS status in the commercial farms. The workers could easily handle this method themselves without pain and distress from animals. The present study demonstrated that oral fluid samples offered the valuable samples of choice over the serum samples in case of chronic infection. Oral fluid samples could be used to determine the timing of infection, the transition of PRRS status in the gilt pool, in the sow herd and in the nursery period. However, the results showed that the disease

status is one factor that might have an impact on pen-based oral fluid detection. This study demonstrated that the detection rates of oral fluid testing were lower than found in individual serum samples in acute phase of infection or when having low disease prevalence. Therefore, the defining PRRSV status using oral fluid samples can exhibit the most benefit in case of persistent infection or having moderate to high prevalence. Accordingly, increasing in number of pen-based oral fluid samples should be suggested to cover the larger production area and to increase the sensitivity of test which would be beneficial to the proper prevention and control program.

5.2 Research limitation and further investigation

The oral fluid sample is the new diagnostic sample in Thailand's pig industry. A baseline for oral fluid testing has not previously been created for the field situation in Thailand. There are different and various conditions applied in conventional swine farms such as vaccination protocol, farm management as well as disease status, therefore different field application and outcomes should be concerned comparing to the experimental-challenged models. Furthermore, unexpected disease outbreaks occurring during the study might lead to the production losses in the experimental groups. The interpretation of oral fluid testing would be beneficial when using with other parameters. Accordingly, oral fluid testing might be mis-diagnosed if the sample collection method is insufficient. Therefore, a new baseline and the application for oral fluids in field situation should be established. This study would provide useful

information for oral fluid-based testing and would benefit the farmers for implementation the appropriate management protocol for effectively control and prevent transmission of PRRSV in the field situation.

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Chulalongkorn University

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APPENDIX

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Appendix A: Oral fluid collection

1. Prepare highly absorbent rope, 100% cotton rope is recommended. Note: use; 1.0-cm diameter for nursery pigs; 2.0-cm diameter for grow-finish pigs.
2. Hang rope at shoulder height of pigs. Place in a clean area easily accessible to pigs. Note: for pen sampling, two or more ropes were recommended when having 25 or more pigs per pen; for individual sampling, be careful to prevent cross-contamination from the other gilts.
3. Take 20-30 minutes allowing the pigs to chew the rope. Note: the younger piglets should be trained prior to the collection ensuring that the majority of pigs chewed on the rope.
4. Insert the wet end of the rope into a clean plastic bag. Extract oral fluid samples by squeezing the rope. Note: Recommended minimum sample of 2.5 ml for optimal resolution. Do not pool oral fluid samples from different pens.
5. Discard used ropes after collection.
6. Stored at -80°C until assayed.

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Appendix B: Virus titration and Immunoperoxidase monolayer assay (IPMA)

1. Prepare monolayer of MARC-145 cells in 96-well plate
2. Wash with 1X PBS 3 times
3. Put 100 µl of the 10-fold dilution of samples into each well (four replicate wells per dilution)
4. Add 100 µl/well of MEM complete medium containing 3% fetal bovine serum
5. Incubate at 37°C for 72 hours
6. Discard culture media and add 50 µl/well of fixation (4% formaldehyde in PBS-0.5% tween) for 25 min at room temperature
7. Wash with PBS-0.5% tween 3 times
8. Apply primary antibody (mAb SDOW 17 (1:1000), in 1% BSA in PBS-0.5%tween) 30 µl/well, incubation for 1 hour at room temperature
9. Wash with PBS-0.5% tween 3 times
10. Apply secondary antibody (polyclonal rabbit anti-mouse HRP conjugate (1:300), in 1% BSA in PBS-0.5% tween), 30 µl/well, incubate 1 hour at room temperature
11. Wash with PBS-0.5% tween 3 times
12. Apply AEC peroxidase substrate 50 µl/well, incubate for 10 min at room temperature
13. Wash in tap water 3 times and dry plate
14. Calculate by Reed and munch method

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CHULALONGKORN UNIVERSITY

VITA

Miss Yonlayong Woonwong was born on January 9, 1986 in Chon buri, Thailand. She graduated with Doctor of Veterinary Medicine (DVM), second class honor, from the Faculty of Veterinary Medicine, Kasetsart University in academic year 2009. After graduated, she was a veterinary clinician at Kamphaengsaen Veterinary Diagnostic Laboratory, Kasetsart University for 2 years. She enrolled in the Ph.D program in Veterinary Pathobiology program since 2012. During her Ph.D program, she was an awardee from Elanco Animal Health (Thailand) to practice the swine production medicine at The Swine Medicine Education Center, College of Veterinary Medicine, Iowa State University, USA for 1 month.

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The publication from her thesis:

Woonwong Y, Kedkovid R, Arunorat J, Sirisereewan C, Nedumpun T, Poonsuk K, Panyasing Y, Poolperm P, Boonsoongnern A and Thanawongnuwech R. 2017. Oral fluid samples used for PRRSV acclimatization program and sow performance monitoring in endemic PRRS-positive farms. Trop Anim Health Prod. DOI 10.1007/s11250-017-1428-z.

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