สถานภาพทางซีรัมวิทยาของฝูงสุกรที่ติดเชื้อเซอร์โคไวรัส

นายเชิดพงษ์ ภูพลผัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

SEROLOGICAL STATUS OF PCV2-INFECTED SWINE HERDS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2017

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เชิดพงษ์ ภูพลผัน : สถานภาพทางซีรัมวิทยาของฝูงสุกรที่ติดเชื้อเซอร์โคไวรัส (SEROLOGICAL STATUS OF PCV2-INFECTED SWINE HERDS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.คมกฤช เทียนคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.สว่าง เกษแดงสกลวุฒิ, 66 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาพลวัตของการตอบสนองทางภูมิคุ้มกันที่มีต่อเชื้อเซอร์โคไวรัสชนิดที่ 2 ของฝูงสุกรที่แสดงอาการและไม่แสดงอาการทางคลินิกในภาคสนาม โดยใช้วิธีอีไลซาร่วมกับภาวะการมีไวรัสในเลือดโดยใช้วิธีเรียลไทม์พีซีอาร์ ทำการคัดเลือกฟาร์มสุกรโดยอาศัยข้อมูลอาการทางคลินิก แบ่งฟาร์มสุกรออกเป็น 2 กลุ่ม ได้แก่ กลุ่ม A เป็นฟาร์มสุกรที่แสดงอาการ (5 ฟาร์ม) มีอัตราการสูญเสียสุกรหลังหย่านมและขุนมากกว่าร้อยละ 5 และทำวัคซีนป้องกันโรคเซอร์โคไวรัสชนิดที่ 2 สุกรกลุ่ม B ไม่แสดงอาการของการติดเชื้อเซอร์โคไวรัสชนิดที่ 2 (5 ฟาร์ม) มีอัตราการสูญเสียน้อยกว่าร้อยละ 5 ภายในกลุ่ม B แบ่งเป็นกลุ่มย่อย 2 กลุ่ม ได้แก่ กลุ่มที่ทำวัคซีนป้องกันโรคเซอร์โคไวรัสชนิดที่ 2 (B-Vac, 2 ฟาร์ม) และกลุ่มที่ไม่ทำวัคซีน (B-non-Vac, 3 ฟาร์ม) ทำการเก็บตัวอย่างซีรัมรวมทั้งสิ้น 500 ตัวอย่าง ได้จากแม่สุกรลำดับท้องที่ 1, 3 และ 5 และลูกสุกรที่อายุ 3, 5, 9, 13, 17, 21 และ 25 สัปดาห์ จากผลการทดลองระดับภูมิคุ้มกันต่อเชื้อเซอร์โคไวรัสชนิดที่ 2 เป็นไปในทิศทางเดียวกันทั้ง 2 กลุ่ม พบว่าในแม่สุกรทุกลำดับท้องมีระดับภูมิคุ้มกันที่สูง แต่อย่างไรก็ดีแม่สุกรลำดับท้องที่ 1 และ 5 ของกลุ่ม A มีระดับภูมิคุ้มกันสูงกว่ากลุ่ม B อย่างมีนัยสำคัญ ($p<0.05$) ส่วนในลูกสุกรพบว่าระดับภูมิคุ้มกันสูงในช่วงอายุ 3 สัปดาห์และลดลงในช่วง 5 ถึง 9 สัปดาห์ ซึ่งอาจเป็นการลดลงของภูมิคุ้มกันที่ได้รับจากแม่ ในลูกสุกร พบระดับภูมิคุ้มกันของทั้ง 2 กลุ่มเริ่มสูงขึ้นตั้งแต่อายุ 9 สัปดาห์จนถึงสุกรขุน ร่วมกับพบระดับไวรัสในเลือดที่สูงโดยลำดับ และสุกรแสดงอาการป่วยและตายมากขึ้น ซึ่งบ่งบอกได้ว่าสุกรติดเชื้อโดยธรรมชาติ สุกรในกลุ่ม A ที่อายุ 17 สัปดาห์มีระดับภูมิคุ้มกันสูงกว่ากลุ่ม B ในช่วงอายุเดียวกันอย่างมีนัยสำคัญ ($p<0.05$) ภายในกลุ่ม B ลูกสุกรช่วงอายุ 3 ถึง 13 สัปดาห์ของกลุ่มที่ทำวัคซีนระดับภูมิคุ้มกันสูงกว่ากลุ่มที่ไม่ได้ทำวัคซีนอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ผลการตรวจปริมาณไวรัสในเลือดพบว่ากลุ่มที่แสดงอาการมีระดับไวรัสในเลือดเฉลี่ยสูงกว่ากลุ่มที่ไม่แสดงอาการทางคลินิก แต่ไม่มีมีนัยสำคัญทางสถิติ ภายในกลุ่ม B พบว่ากลุ่มที่ทำวัคซีนมีระดับไวรัสในเลือดเฉลี่ยน้อยกว่ากลุ่มที่ไม่ทำวัคซีนอย่างมีนัยสำคัญ ($p<0.05$) ซึ่งบ่งชี้ว่าการใช้วัคซีนในกลุ่มที่ไม่มีอาการจะช่วยลดปริมาณไวรัสในเลือดและลดความเสี่ยงในการเกิดโรคได้ การศึกษาครั้งนี้แสดงให้เห็นว่าการตรวจวัดระดับภูมิคุ้มกันด้วยวิธีอีไลซาที่ผลิตขึ้นมาใช้เองร่วมการตรวจปริมาณเชื้อไวรัสในเลือดและอาการทางคลินิกของสุกร จะสามารถบอกถึงพลวัตของการติดเชื้อเซอร์โคไวรัสภายในฟาร์มและเป็นประโยชน์ต่อการควบคุมโรคได้เป็นอย่างดี

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5775305931 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: PORCINE CIRCOVIRUS TYPE 2, IN-HOUSE INDIRECT ELISA, SEROLOGICAL PROFILE, VIRAL LOAD

CHERDPONG PHUPOLPHAN: SEROLOGICAL STATUS OF PCV2-INFECTED SWINE HERDS. ADVISOR: ASST. PROF. KOMKRICH TEANKUM, D.V.M., M.Sc., Dr. med. vet., DTBVP, CO-ADVISOR: ASST. PROF. SAWANG KESDANGSAKONWUT, D.V.M, MSc., Ph.D., DTBVP, 66 pp.

This study aimed to determine the dynamics of serological and virological profiles against Porcine Circovirus type 2 (PCV2) infection in Thai swine herds using the in-house indirect ELISA and real-time PCR techniques. A total of 10 swine herds in Thailand were divided into two groups according to their clinical history. Group A (n=5) had clinical signs of PCV2, and was routinely vaccinated by PCV2 vaccines with production loss more than 5%. Group B (n=5) had no clinical signs of PCV2 with production loss less than 5%. Group B consisted of two subgroups: PCV2-vaccinated herds (B-Vac, n=2) and non-vaccinated herds (B-non-Vac, n=3). Serum samples (n=500) were collected from parity 1, 3 and 5 sows and at 3, 5, 9, 13, 17, 21 and 25 weeks of pigs (n=5 per age group). The result of seroprofiles of group A and B revealed similar pattern in which high antibody titers in all parity sows. However, PCV2 antiboby titers of group A at parity 1 and 5 sows were significantly higher than those of group B (p<0.05). In piglets, the titers were high at 3 weeks and gradully decreased at 5 to 9 weeks of age indicating the decline in maternal immunity. After 9 weeks, PCV2 antibodies were gardually increased until fattening period coincided with high viral loads and high percentage of production loss indicating natural infection. At 17 weeks of age, the titers of group A were significantly higher than those of group B (p<0.05). In group B, the antibody levels at 3 to 13 weeks of B-Vac were significantly lower than those of B-non-Vac. The detection of PCV2 load revealed that mean viral load of group A was higher than group B but it was not significantly different. Within group B, the PCV2 DNA load in B-Vac group was significantly lower than B-non-Vac group indicating that vaccination in non-clinical pigs could reduce viral load and risk of clinical disease. This study showed that in-house ELISA and detection of viral DNA together with PCV2 clinical expressions of pigs could indicate the dynamic of PCV2 infection within swine herds and could be beneficial for disease control.

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

ACKNOWLEDGEMENTS

This study was carried out at the Veterinary Diagnostic Laboratory, Large Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakorn Prathom province.

I would like to express my appreciation and grateful thanks to my advisors and co-advisor, Asst. Prof. Dr. Komkrich Teankum and Asst. Prof. Dr. Sawang Kesdangsakonwut from the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, for their guidance, helpful suggestions and comments throughout this research study.

I would like to offer my special thanks to Dr. Pornchalit Assavacheep, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, for his help in selection of pig farms and sample collection.

I am particularly grateful for assistance with the statistical work given by Asst. Prof. Dr. Chaidate Inchaisri, Department of Veterinary Medicine and Prof. Dr. Padet Tummaruk, Department of Obstetrics, Gynaecology and Reproduction.

Assistance provided by Dr. Rachod Tantilertcharoen, Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University was greatly appreciated.

It is also important for me to thank Dr. Suphattra Jittimanee, Department of Veterinary Pathology, Faculty of Veterinary Medicine, Khon Kaen University, for her great suggestions in laboratory methods.

In addition, I would like to express my deep thanks to Dr. Roongtham Kedkovit, Ph.D. candidate in the Pathobiology Program, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, for his kindness help and encouragement.

I am additionally grateful to Mrs. Sukuma Samngarmnim, Mrs. Panida Khanda, Miss Siraprapa Krethathorn, Miss Suwannee Tongmalee and other members of the CUVDL NP, for their help and suggestions in laboratory work.

Finally, I would like to express my deepest appreciation to my parents, Mr. Manop and Mrs. Chuanchom Phupolphan, my grandmother, Mrs. Siriluck Thanthaluck and my friends, Mr. Witawas Handee and Mr. Arnon Mongkhonlaksamee, who always provide cheerful and helpful support and believe in my ability to achieve my goals.

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LIST OF ABBREVIATIONS

ADWG	=	Average daily weight gain
BSA	=	Bovine serum albumin
CD4+	=	cluster of differentiation 4+
CD8+	=	cluster of differentiation 8+
CSFV	=	Classical swine fever virus
DAB	=	Diaminobenzidine
DNA	=	Deoxyribonucleic acid
ELISAs	=	Enzyme-linked immunosorbent assays
E. coli	=	*Escherichia coli*
FMD	=	Foot and Mouth Disease
GST	=	Glutathione S-transferase
HRP	=	Horseradish peroxidase
IgG	=	Immunoglobulin G
IIFA	=	Indirect immunofluorescence assays
IFN-α	=	Interferon-alpha
IL-10	=	Interleukin 10
IPMA	=	Immunoperoxidase monolayer assays
IPTG	=	Isopropyl-D-1-thiogalactopyranoside
KDa	=	Kilodalton
LAH-CU-VDL	=	Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University
LB	=	Luria-Bertani
MCP-1	=	Monocyte chemoattractant protein-1
MDA	=	Maternal-derived antibodies
mm	=	Millimeter
mM	=	Millimolar
NIPC	=	Natural interferon producing cell
NLS	=	Nuclear localization signal
nm	=	Nanometer
OD	=	Optical density
ORFs	=	Open reading frames
PBMC	=	Peripheral blood mononuclear cells
PBS	=	Phosphate buffered saline

PCR	=	Polymerase chain reaction
PCV	=	Porcine circovirus
PCVD	=	Porcine circovirus type 2 diseases
PCVAD	=	Porcine circovirus type 2 associated diseases
PCV1	=	Porcine circovirus type 1
PCV2	=	Porcine circovirus type 2
PCV2-LD	=	PCV2-lung disease
PCV2-ED	=	PCV2-enteric disease
PCV2-RD	=	PCV2-reproductive disease
PCV2-SI	=	PCV2-subclinical infection
PCV2-SD	=	Porcine circovirus type 2– systemic disease
PDNS	=	Porcine dermatitis and nephropathy syndrome
PDS	=	Postpartum dysagalactia syndrome
PEDV	=	Porcine Epidemic Diarrhea Virus
PK-15 cell	=	Porcine kidney – 15 cell
PMWS	=	Post weaning multisystemic wasting syndrome
PRDC	=	Porcine respiratory disease complex
PRRSV	=	Porcine reproductive and respiratory virus
PRV	=	Pseudorabies virus
TNF-α	=	Tumor necrosis factor-alpha
qPCR	=	Quantitative polymerase chain reaction
rntCap	=	Recombinant nuclear localization signal truncated capsid
S/P ratio	=	Sample / positive ratio
SIV	=	Swine influenza virus
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	=	Tris-buffered saline with Tween-20

CHAPTER 1

INTRODUCTION

Porcine circovirus type 2 diseases (PCVD) or porcine circovirus type 2 associated diseases (PCVAD), is currently considered as one of the important infectious diseases with serious economic losses to swine industry worldwide. PCVAD composed of mainly manifestation such as post weaning multisystemic wasting syndrome (PMWS), Porcine circovirus type 2 (PCV2) – systemic disease (SD), etc. Moreover, it is commonly associated with many severe clinical diseases including porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, and enteritis.

PCV2 infection commonly occurred at 3 or 5 weeks of age depending on co-factors as well as the characteristics of pig flow within the farm. PCV2 affected in all farm system industry. The disease has been reported in all sizes of farm. PCV2-SD occasionally induced low morbidity with high mortality cases (Segales and Domingo, 2002). The disease is characterized by progressive weight loss, skin paleness, jaundice respiratory distress, diarrhea and generalized lymphadenopathy.

Although the pathogenesis of PCV2-SD is still unclear, the disease is believed to be mediated by host immune responses. After 2 weeks of virus exposure, PCV2 access to blood circulation and infects susceptible lymphocytes, histiocytes and dendritic cells leading to viremia, leukopenia and rise in anti-PCV2 antibody (seroconversion). Consequently, the development of the total PCV2-antibodies co-incides with PCV2-neutralizing antibodies and both are important defense mechanism against viral infections in pigs. Moreover, grower pigs can recover from the disease, and this virus can persist in lymphoid tissues of the infected pigs for prolonged period. Therefore, the infected animals should be monitored for the status of PCV2 infection in herds by serology assay.

According to clinical and subclinical (reduce growth without evidence of clinical signs) PCV2 infection in swine herds, further antemortem investigations are additionally used, including polymerase chain reaction (PCR) and serology assays. It will be helpful for monitoring of viral persistence in swine herds, if PCV2 investigation can be performed using both pathogen detection and serological profiles of PCV2 infection.

Recently, several commercial enzyme-linked immunosorbent assays (ELISAs) for detecting PCV2 have been developed. In previous reports, mostly in-house ELISA assays based on a recombinant capsid protein expressed in baculovirus or bacterial expression systems are practically convenient. Some based on cell-culture propagated PCV2 and PCV2 monoclonal antibodies have been developed which greater technically difficult and expensive. In previous study, a recombinant nuclear localization signal truncated capsid (rntCap) indirect ELISA for PCV2 antibody detection have been developed by using the rntCap protein expressed in the *Escherichia coli (E. coli)* system which effectively used as a serodiagnostic tool for PCV2 antibody detection in swine herds (Jittimanee et al., 2012). The rntCap indirect ELISA is useful for detecting maternal-derived antibodies (MDA), determining the infection periods and possibly measuring the antibody levels post-vaccination or infection. Serological profiles from ELISA assay is essential data for management, monitoring production parameters and preventing the disease in swine herds, thus this assay could be a valuable test for routine monitoring in large-scale population.

Necropsy data from Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University (LAH-CU-VDL), demonstrated that PCV2 infection in pigs was diagnosed in 120 cases of 293 cases (40.96%) during 2015 and 69 cases 310 cases (54.52%) during 2016. PCV2 infection seems to be increased and widely spread in recent year and also has crucial impacts on economy of swine industry.

Vaccine against PCV2 is widely used in swine herds reduce this incidence of PCV2-SD in herds (Opriessnig et al., 2010). However, PCV2 vaccination programs have been used in different ages depending on the infection periods. PCV2 vaccination failure has been more complained from swine practitioner. The major issue was the PCV2 vaccinations might not provide prolonged immunity to control PCV2 infection until finishing period probably due to several factors such as unsuitable managements or inappropriate vaccine administration. To solve this vaccine failure problem, more information about vaccine program, serological status, PCV2 infection status is needed to be investigated by using ELISA and PCR.

Objectives

To investigate the relation of the serological status of PCV2 infection in both Thai swine farms with and without clinical signs of PCVAD using an in-house indirect ELISA in relation to clinical signs, viremic status, and antibody titers

Hypothesis

Serological profiles of swine herds with clinical PCV2 infection are different from herds without clinical signs.

CHAPTER 2

LITERATURE REVIEW

2.1 Porcine circovirus (PCV)

Porcine circovirus (PCV) was first identified in the 1970s as a contaminant of porcine kidney cell lines (Tischer et al., 1982). PCV is a member of the genus Circovirus, family Circoviridae, which has been known as the smallest viruses that infect vertebrates. PCV was classified into 2 type; PCV1 and PCV2. PCV1 is a persistent contaminant of PK-15 cell lines, which has been considered as a nonpathogenic virus (Tischer et al., 1982). In contrast, PCV2 is identified as virulent pathogen (Allan et al., 1998; Meehan et al., 1998). The virus particle is non-enveloped, 15–24 nm in diameter and single strand circular DNA genome of 1.76–2.31 kb (Todd et al., 1991). Recently, PCV2 are classified into four genotypes, including PCV2a (Group 2), PCV2b (Group 1), PCV2c (has been described only in Denmark), and PCV2d (Guo et al., 2010). In Thailand, there have been reported that PCV2b and PCV2d were widely spread (Jantafong et al., 2011; Jittimanee et al., 2011; Thangthamniyom et al., 2017).

2.2 Characterization of PCV2

PCV has six potential open reading frames (ORFs) (Mankertz et al., 1997). There are three major ORFs; ORF1 encodes a replication-associated protein (Ilyina and Koonin, 1992), ORF2 encodes viral capsid protein (Nawagitgul et al., 2000) and ORF3 encodes apoptotic protein (Liu et al., 2006) (Figure 1). Recently, ORF4 was determined and associated with the regulation of CD4+ and CD8+ T-lymphocytes during PCV2 infection (He et al., 2013). The capsid protein, the major immunogenic protein of PCV2, is commonly used for reconstructive phylogenetic tree.

The ORF2 gene of PCV2 encodes a putative capsid protein with a size of approximately 30 kDa. The expression of ORF2-recombinant proteins in insect cells (Nawagitgul et al., 2000) and *E. coli* (Wu et al., 2008) evidenced self-assembles to form virus-like particles. Since the capsid protein has a unique epitope that can be used to distinguish between PCV2 (pathogenic) and PCV1 (non-pathogenic) (Mahe et al., 2000), most studies have used the capsid proteins for the induction of antibody of which it is important for prevention or elimination of PCV2. The antibody specifically induced

from the PCV2 capsid protein does not cross-react with the PCV1 capsid protein. Recently, the PCV2 protein has been used for developing PCV2 vaccines and serological diagnostic reagents such as ELISA.

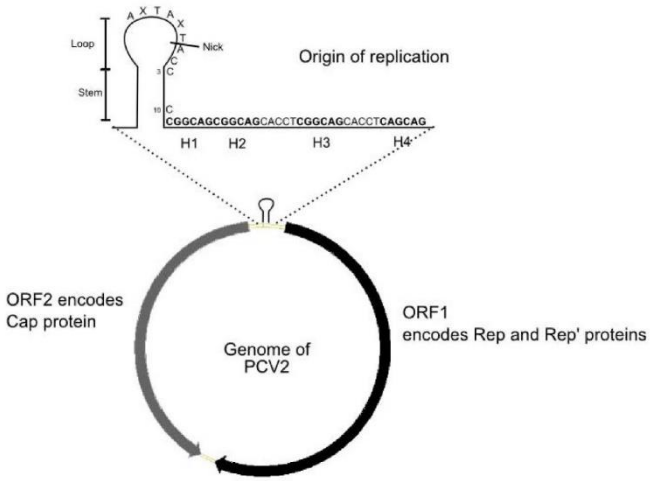

Figure 1 Representation of PCV2 genome; Black arrow: ORF1, located on the positive strand which encodes Rep and Rep' protein. Grey arrow: ORF2, located on the negative strand, which encodes Cap protein. Between ORF1 and ORF2 are intergenic regions. The origin of replication is located in the intergenic region between the beginnings of the two ORFs. H1, H2, H3 and H4 are hexamers (adapted from (Faurez et al., 2009).

2.3 PCV2 in Thailand

PCV2 has been reported in many countries since 1991. In Thailand, PCV2 was firstly reported in 1999 in 7-9 weeks-old pigs that had the lesion consisted with PMWS and detected the antigen and viral particle by immunohistochemistry and electron microscope respectively (Tantilertcharoen et al., 1999). The microscopic findings revealed that depletion of lymphoid tissues and basophilic intracytoplasmic inclusion bodies were found in macrophages in tonsil, lymph nodes, and Peyer's patch. The inclusion bodies were investigated by immunohistochemistry and electron microscopy which showed virus-like particles with the size of 14-18 nm in diameter.

In 2002, a retrospective study using nested PCR technique for the detection of PCV2 DNA taken from paraffin-embedded tissues revealed that the virus at least has been in existance in Thailand since 1993 (Kiatipattanasakul-Banlunara et al., 2002). This indicates that the virus may have been long present in Thai swine herds.

Early studies based on field isolations of PCV2 in Thailand revealed that all of the PCV2 virus were classified only as the PCV2b subtype (Jantafong et al., 2011; Jittimanee et al., 2011). However, in the present day, more cases of PCV2d infection has been frequently recognized. The displacement from PCV2b to PCV2d was clearly occured during 2013–2014 (Thangthamniyom et al., 2017).

Recently, necropsy results from Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakorn Prathom province, demonstrated that PCV2 infection in pigs was increased in prevalence year by year and diagnosed for 48 out of 164 cases (29.27%) during 2013, 69 out of 138 cases (50%) during 2014, 120 out of 293 cases (40.96%) during 2015 and 69 out of 310 cases (54.52%) during 2016 (data not yet published). Based on this data, PCV2 infection has been increasing and widely spread in recent years and also has crucial impacts to the economy of the swine industry. In addition, PCV2 was detected in both clinical and subclinical signs.

2.4 Transmission and pathogenesis

The main transmission route is fecal-oral route. The pigs expose the virus by a direct contact with the discharge from shedding pigs or contaminated environment. After the infection, the virus initially replicates in lymphocytes in lymphoid tissues such as the tonsil, the Peyer's patches, or draining lymph nodes near the infection area. In addition, the viral replication can also occur in peripheral blood mononuclear cells (PBMC) in a short period. These infected cells are presumed as carriers for circulating virus through the whole host body (Darwich and Mateu, 2012; Yu et al., 2007). In the subsequent stage, virus is spread via hematogenous route called viremia which can be detected within 1-2 post-exposure week. At week 2-3 after the infection, viral shed is observed via feces, oral secretion and semen. The leucopenia can be identified since the first stage of the infection, predominantly indicated by lower B and T lymphocyte (Lin et al., 2008). Coincidentally, histiocytic infiltration is observed in lymphoid follicles leading to lymphoid depletion, granulomatous inflammation and multinucleated giant cells in various lymphoid organs. The reduction of lymphocytes is proportionately significant at which the structures of lymphoid tissues are disintegrated. It is postulated that PCV2 could activate the production of monocyte chemoattractant protein-1 (MCP-1) which is a chemokine for attracting other monocytes to the affected organs especially lymphoid tissues (Tsai et al., 2010). The latest duration period is approximately 3-30-week post infection (Chae, 2005).

The pathogenesis of PCV2-SD and PDNS remains undetermined. Although PCV2 is associated with both PCV2-SD and PDNS, the relationship between PCV2-SD and PDNS in the affected herds is appeared to be indirect. The pigs with PCV2-SD will never progress into PDNS as well as the pigs with PDNS also will never progress into PCV2-SD. However, pathogenicity of PCV2-SD is linked with the interaction between the virus and the host immune system. The host immune responses to PCV2 infection and the subsequent immunological suppression of the host have been major subjects of investigation (Darwich et al., 2004).

2.5 PCV-associated disease: clinical signs and lesions

PCV2 was first described in 1991 in Canada (Harding and Clark, 1997) and has spread into swine farms throughout the world with seriously economic impacts on the swine industry.

PCV2 infection has been associated with a variety of syndromes, including post weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis and congenital tremors (Allan and Ellis, 2000).

Recently, the terminology of PCV2 infection was re-statement. These have been called porcine circovirus type 2 – systemic disease (PCV2-SD). The following terms PCV2-systemic disease (PCV2-SD), PCV2 lung disease (PCV2-LD), PCV2 enteric disease (PCV2-ED) and PCV2 reproductive disease (PCV2-RD) have been proposed to replace PMWS, PRDC, PCV2-associated enteritis and PCV2-associated reproductive failure, respectively. The PDNS is also considered as a PCVAD, although its pathogenesis is still unclear about antigen linked with an immune complex-mediated mechanism. PRDC is one of the PCV2 infections associated with other viruses, such as PRRSV and swine influenza virus (SIV), as well as respiratory bacteria including *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida* and *Haemophilus parasuis* (Chae, 2005). Moreover, in the case of PCV2-subclinical infection (PCV2-SI) which is a serious pig disease, it is coupled with growth retardation without overt clinical signs of PCV2. PCV2-SD and PCV2-SI are considered severe PCVDs of swine industries in many countries. However, the use of PCV2 vaccine can reduce clinical signs and production costs (Segales, 2012).

2.5.1 PCV2-systemic disease (PCV2-SD)

The PCV2-SD infected herds are observed in many groups from nursery and grower pig operations, farrow-to-finish, farrow-to feeder, and also are varied in sizes from small to large production systems (Allan and Ellis, 2000). This syndrome mostly occurs at 5–12-week-old piglets with clinical signs of progressive weight loss, fever, tachypnea, dyspnea, anemia and jaundice (Ellis et al., 1999) (Figure 2A). However, the PCV2 infection alone is not enough to trigger clinical signs. Other factors, such as co-infections (bacteria and/or other viruses), poorly environmental conditions and management practices can lead to higher stress and/or increased infection rates and manifestations. Other clinical symptoms may be due to co-infections caused by immunosuppression, such as arthritis and seizures from *Streptococcus suis* and *Haemophilus parasuis* infection. Moreover, other risk factors from host such as age or route of infection can affect the pathogenicity and clinical manifestations of PCV2 infection. Morbidity and mortality are often low but it can increase more than 50% in severe cases depending on the presence of co-factors (Harding and Clark, 1997). Postmortem examinations of affected piglets reveal lymphadenopathy, interstitial pneumonia, and occasionally hepatitis or nephritis (Allan and Ellis, 2000) (Figure 2B). Histopathological lesions contain proliferation of lymphohistiocytic cells in various organs (Allan and Ellis, 2000). Multinucleated giant cells are occasionally observed in lymphoid tissues. Inclusion bodies are typically observed within the multisystemic lesions in affected piglets (Chae, 2005).

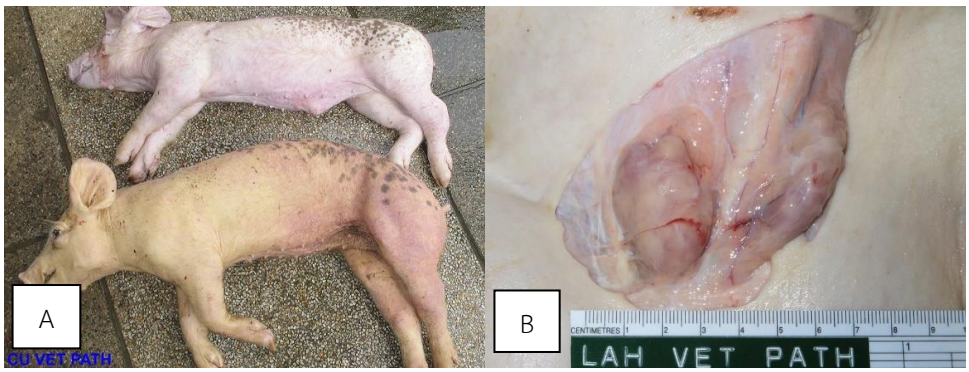

Figure 2 A: Nursery pigs or growing pigs with PCV2-SD syndrome show progressive weight loss, respiratory distress, pallor and commonly with jaundice. Other systemic symptoms such as diarrhea, arthritis and seizures from secondary infections are also observed. Most clinical PCV2-SD signs are similar to those of PRDC but with higher morbidity and mortality rates. **B:** Superficial inguinal lymph nodes are enlarged and clearly noticed. This lesion is a relatively specific marker for this disease, but it can be atrophied at the later stage. (courtesy of Dr. Sawang Kesdangsakonwut)

2.5.2 Porcine dermatitis and nephropathy syndrome (PDNS)

PDNS mostly occurs in older pigs started at 12 to 14 weeks of age until the finishing pigs before the slaughter period as well as in replacement gilts (Chae, 2005). The prevalence of PDNS is very low (approximately 1%), but in a severe case, pigs usually die rapidly within 2-3 days after showing PDNS clinical signs. Mortality rate of infected pigs is approximately 50% for pigs in nursery to growing and 100% for fattening peroid. Clinical signs will disappear within 7-10 days after the illness. Skin lesions were multifocal and raised with dark red to purple macules and papules with the size of 1–20 mm in diameter. This is due to dermal necrosis and hemorrhage of the skin associated with vascular damages or necrotizing vasculitis (Figure 3A). The skin lesions are usually observed at all limbs, ventral abdomen and often at thorax, flank or ears. After the appearance of these lesions, pigs may develop depression, anorexia, fever and progressive weight loss. Subsequently, the kidneys are enlarged with pale renal cortex and multiple red hemorrhagic foci. Mucosal edema of ureter and renal pelvis are additionally observed along with enlarged inguinal lymph nodes (Allan and Ellis, 2000). The microscopic findings revealed that the most significant lesions were non-suppurative interstitial nephritis and fibrinonecrotizing vasculitis in various organs (such

as dermis, subcutis, kidney, lymph nodes, stomach, spleen, and liver). Numerous multinucleated giant cells are often seen in the renal cortex and paracortex. This vasculitis is associated with the mechanism of the immune system (Thibault et al., 1998).

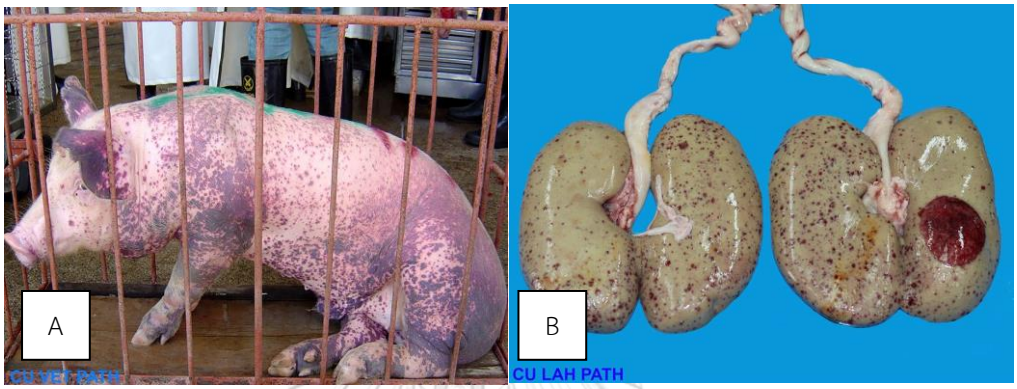

Figure 3 A: The clinical signs of PDNS. Infected pigs exhibit depression, anorexia, fever and multifoci with raised dark-red to purple macules and papules on skin especially at all limbs, ventral abdomen, perianal area and often at thorax, flank or ears, **B:** Red spots or necrotic foci caused by non-suppurative interstitial nephritis which are a specific lesion to PDNS. Edema of the urethra can be also detected. (courtesy of Dr. Sawang Kesdangsakonwut)

2.5.3 PCV2-associated porcine respiratory disease complex (PRDC) and PCV2 lung disease (PCV2-LD)

PRDC remains a serious health problem for swine productions in the world. It mostly occurs in pigs during growing to finishing periods. PRDC is a co-infection between viruses and bacteria. The examples of the viral agents are PRRSV, classical swine fever virus (CSFV), swine influenza virus, pseudorabies (PRV) or Aujeszky's disease, and also PCV2, whereas the bacterial pathogens include *Mycoplasma hyopneumoniae*, *Pasteurella multocida, Bordetella bronchiseptica, Haemophilus parasuis, Streptococcus suis,* and *Actinobacillus pleuropneumoniae* (Brockmeier et al., 2002; Chae, 2005) (Figure 4A). Because PRDC infection is caused by various agents, the clinical signs are mostly non-specific and variable. The most common agent that causes over 55% of the PRDC test results is the co-infection of PRRSV and PCV2 (Kim et al., 2003).

Although PCV2 does not induces PRRSV to increase higher severity in term of lesions and duration but PRRSV certainly aggravate the action of PCV (Allan and Ellis, 2000). PCV2-associated PRDC symptom is characterized by slow growth, prolong cough, fever, anorexia, lethargy, decreased feed efficiency and dyspnea. PCV2 and PRRSV both have an ablililty to suppress the immune system which could induce severe lesions and clinical signs. Pigs co-infected with PCV2 and PRRSV mostly develop a bronchointerstitial pneumonia which is a typical lesion found in PRDC. The main microscopical lesions of PCV2-associated PRDC revealed that bronchointerstitial pneumonia is often associated with peribronchiolar cuffing (Figure 4B). Moderate to severe multifocal peribronchial and peribronchiolar fibrosis are also frequently observed (Kim et al., 2003).

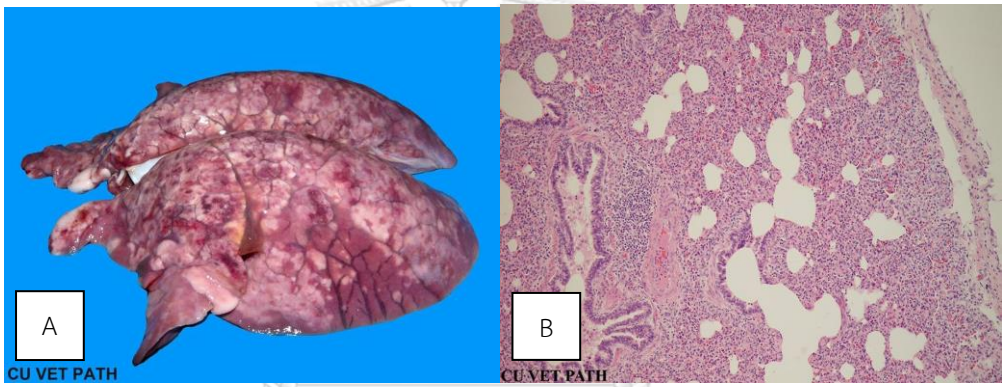

Figure 4 A: Severe respiratory signs and pulmonary lesions in PCV2-associated PRDC occured with concurrent infections of PCV2, PRRSV and bacteria. It is characterized by mottled lung with red to gray hepatization due to the infection of Mycoplasma hyopneumoniae, and multiple hemorrhagic spots spread throughout the lungs from the infection of *Streptococcus suis*, **B:** Infiltration of lymphocytes and macrophages in the lung interstitium is frequenly diagnosed with co-infection of PCV2 and PRRSV. Neutrophils may be presented in the case of bacterial co-infections such as *Hemophilus parasuis* and *Pasteurella multocida*, H&E staining, 4x. (courtesy of Dr. Sawang Kesdangsakonwut)

2.5.4 PCV2-subclinical infection (PCV2-SI)

In addition, PCV2-SI infection is significantly enhanced from of PCV2 infection in swine producing countries worldwide but it does not show obvious clinical observations and infected pigs may remain healthy. However, the pigs may reduce average daily weight gain (ADWG) even without overt clinical signs. Microscopic investigation could exhibit lymphoid lesions similarly found in pigs with PCV2-SD but with a mild degree (Segales, 2012).

2.5.5 PCV2 reproductive disease (PCV2-RD)

Reproductive failure caused by PCV2 infection in field cases is uncommon because most replacement pigs are immunocompetent due to previous natural infection. PCV2-RD is characterized by late-term abortions, stillbirths and fetal mummification (Ritzmann et al., 2005). Affected piglets will show weakness and congenital tremors or "dancing pig", especially piglets from the first parity of sows. Other reproductive pathogens of maternal infection such as classical swine fever virus and/or organophosphate show the same clinical signs as PCV2 infection.

2.5.6 PCV2 enteric disease (PCV2-ED)

Most clinical manifestations of PCV2-ED are diarrhea in 8- to 16-week-old pigs in field cases. Gross lesions and microscopic findings in intestines are overlap between PCV2-SD and subacute to chronic ileitis from *Lawsonia intracellularis* infection (Opriessnig et al., 2007). The intestinal mucosa is thickened and mesenteric lymph nodes are enlarged. Microscopic examination reveal the presence of granulomatous inflammation with lymphoid depletion in Peyer's patches but no evidence in other lymphoid tissues and moderate to high amount of PCV2 in intestinal mucosa and Peyer's patches.

2.6 Immunity

In a PCV2 infected herd, the seropositive is more likely to be found in pigs in the stage of viremia because of natural infections. Interestingly, neutralizing antibody mechanism is crucial to against the PCV2 infection. The antibody production is rapidly induced by the virus which is coincided with raising total PCV2-antibodies after 2-3-week post-exposure (Meerts et al., 2006). In a previous study, it was shown that some PCV2-affected pigs had lower neutralizing antibody titers than subclinical ones. On the

other hand, high neutralizing antibody titers are inversely correlated with PCV2 loads in blood circulation, i.e., some PCV2-infected animals had high antibody titres but low or absent neutralizing antibody. These observations might indicate either that some pigs had developed a humoral response lacking neutralizing antibody or that neutralizing antibody was developed far later than the non-neutralizing antibody (Fort et al., 2007).

In previous study, in the PCV2-negative pigs, the levels of neutralizing antibody titers demonstrate higher variation based of the age of pigs. In piglets aged 2 and 3 months show low neutralizing antibody titers which could due to the fact that this age group still hold some maternal immunity or contain a very recent seroconversion to PCV2. On the other hand, duing fattening period (older than 3 months), pigs display higher level of neutralizing antibody titers suggesting that they have had the infection but was able to eliminate the PCV2 infection (Fort et al., 2007).

The lower level of viral loads is commonly noticed when the neutralizing antibody titers are high, based on the results from the serological and real time PCR profile. Actually, if the animals show a slight lower response of the neutralizing antibody than the total antibodies, their viremia would drop only after the pig seroconverted for neutralizing antibody. These observations suggest that viral particles in blood circulation is reduced because of the antibody-mediated neutralization which is presumably the important mechanism for the viral clearance and recovery from the infection (Fort et al., 2007).

The result of highly damaged B cell follicles in animals with PCV2-SD is correlated with the evidence that these animals are frequently accompanied by aberrant production of neutralizing antibodies against PCV2. Even though infected animals with no PCV2 signs can commonly produce neutralizing antibodies that could overcome the disease, the level of neutralizing antibodies is inversely correlated with viral amount detected in blood circulation (Fort et al., 2007). It is noticed the PCV2 is well reproduced in dividing cells (Darwich et al., 2004) or activated lymphocyte (Lin et al., 2008; Yu et al., 2007). This may explain a case in which the replication capacity of PCV2 is increased if swine are co-infected with other virus or bacteria. The co-infection intensifies PCV2 signs and may increase mortality rate from 1-2% to 10-15% (Chae, 2004).

According to innate immune response, the viral infection into dendritic cells is considered crucial for the antigen presenting process. While the virus cannot replicate in them, the infected dendritic cells still has complete potentiality to serve as antigen presenting cell for T lymphocytes. However, it influentially triggers to decrease efficacy and number of innate cytokines including interferon-alpha (IFN-α) and tumor necrosis factor-alpha (TNF-α) (NIPC; Natural interferon producing cell). NIPC works as a scrutinizing cell for virus via Toll-like receptor, which results in inhibition of adaptive immunity (Ito et al., 2003). It may also indirectly interfere with immune responses to other pathogens. Though, the roles of cellular immune responses against PCV2 infection have not been clarified yet, it has been well known that CD4+ and CD8+ T cells are obviously involved in this response (Koinig et al., 2015). Moreover, PCV2 infection could increase interleukin 10 (IL-10) production, especially in those infected swine with PCV2 without showing signs. IL-10 is also found in the blood circulation leading to immunosuppression of the host. Although at present, there is no definitive information about the induction profile of cytokines of monocyte/macrophage. However, the pigs with wasting syndrome which associated with IL-10 production demonstrate chronic stage that leads to failure of immune system (Darwich et al., 2008). The infected pigs from this results were poor feed efficiency, decrease of average daily gain and overall health.

2.7 PCV2 diagnosis

The PCV2-SD diagnosis is based on the presence of PCV2 clinical signs, characteristic histopathological lesions and detection of PCV2 agent and antibodies response. The data from clinical signs, necropsy reports and histopathology examinations can indicate if pigs are infected with PCV2. However, PCV2 clinical signs are non-specific and often unclear which can be difficult to diagnose. There are other advanced techniques for identifying PCV2-SI infected pigs that do not exhibit PCV2 signs. These guidelines include: 1) decrease of average daily gain without clear PCV2 clinical signs, 2) no or mild degree of histiopathological findings in tissues and 3) none or low amount of PCV2 in lymphoid tissues (Segales, 2012). Differential diagnosis of PDNS depends on the significant pathologic lesions on skin and in kidneys. Skin lesions may be similar to classical swine fever, septicemic salmonellosis, swine erysipelas, *Actinobacillus suis* infection, porcine stress syndrome or other bacterial septicemias. The significant diagnoses for kidney lesions also include classical swine fever, septicemic salmonellosis and swine erysipelas (Chae, 2005). However, with PDNS serum

biochemical test of urea and creatinine concentrations show marked increases in both. Therefore, these inceases may help differentiate PDNS from other diseases (Segales, 2012).

The definitive diagnosis for PDNS includes two main criteria: 1) presence of hemorrhagic and necrotizing dermatitis, mainly located on the hindlimbs and perineal area, and/or swollen and pale kidneys with multifocal petechial hemorrhage at cortex, and 2) presence of severe, fibrinoid, necrotizing vasculitis and glomerulonephritis (Chae, 2005; Thibault et al., 1998).

There are a lack of overt clinical signs in PCV2-SI. Veterinarians mainly use the PCR technique to demonstrate of infection which is enough to establish such a diagnosis. In previous research, values of $<10^5$ or 10^6 PCV2 genome copies/ml of serum have usually been observed with subclinical pigs (Brunborg et al., 2004).

The diagnosis of PCV2-RD could be based on the following criteria: 1) late-term abortions and stillbirths, sometimes with hypertrophy of heart, 2) extensive areas of myocardial degeneration and/or necrotizing myocarditis as well as mild fibrosis, and 3) high viral concentrations in the myocardial lesions and other fetal tissues. However, there are no formal criteria to diagnose, because the differential diagnoses for PCV2-RD are the same as PRRS, porcine parvovirus, pseudorabies (Aujeszky's disease), leptospirosis, and other diseases that cause late-term abortions, stillbirths and weak piglets. The occurrence of such signs together with evidence of viral circulation during the clinical episode should be demonstrated to accurately diagnose PCV2-RD (Segales, 2012).

Opriessnig et al. (2007) suggested that diagnosis of PCV2-ED occurs only in the presence of 1) diarrhea, 2) lymphoid depletion in Peyer's patches but not in other lymph nodes and 3) PCV2 antigen within the lesions.

PCV2 can be detected by using several techniques such as immunohistochemistry (Rosell et al., 1999), *in situ* hybridization (Nawagitgul et al., 2000), indirect immunofluorescence (Allan and Ellis, 2000), PCR (Meehan et al., 1998) and virus isolation (Tischer et al., 1982). In blood sample, such as a whole blood and serum, PCR technique is usually used for detection of PCV2 and in tissues can be performed by *in situ* hybridization, immunohistochemistry and PCR.

2.7.1 Detection of genomic DNA

2.7.1.1 Conventional and Quantitative Polymerase Chain Reaction (PCR and qPCR)

PCR is a technique for increasing the amount of DNA by a DNA replication process, which is the synthesis of the new DNA strands from a DNA template. PCR assay is common in laboratory for detecting the antigen of PCV2. The technique is specific to detecting ORF1 or ORF2 of PCV2 virus, which can be conducted in both fresh specimens and paraffin blocks (paraffin-embedded) of lymphoid tissues such as lymph nodes, tonsil, spleen, as well as serum. Other infected organs such as lung, liver, kidneys are unsuitable for the diagnosis because they have fewer viral particles (Morozov et al., 1998).

Currently, there are two types of PCR including conventional and real-time or quantitative PCR. Conventional PCR estimates that the temperature must be adjusted several times to obtain sufficient DNA products to detect by gel electrophoresis. However, real-time PCR technique can monitor all temperature shifting phases in progress and obtain information for an analysis of DNA products. In addition, it can exactly be verified product amounts by the melting curve analysis during reactions. Applying this technique can measure the viral DNA products with highly specificity and reproducibility. It has more rapid detection ability than the conventional PCR, but its cost is not significantly higher. There are several techiniques for qPCR including 1) probes-based methods which uses double-stranded DNA labeled with fluorophore such as Taqman Probes (Heid et al., 1996) and 2) generic dye-based method which commonly used SYBR Green I as a dye (Ririe et al., 1997). The qPCR methods have become the most widely used technique for generic detection of amplified DNA because of its cost efficiency and the ability to differentiate PCR products by melting curve analysis (Gudnason et al., 2007).

2.7.2 Serological assay

Serological assay for detecting antibody response are essential to determine the prevalence of PCV2 infection and describe PCV2-SD disguise in herds. Most serological diagnostic methods for detecting PCV2 antibodies, including indirect immunofluorescence assays (IIFA), immunoperoxidase monolayer assays (IPMA) and

enzyme-linked immunosorbent assays (ELISAs). All techniques have been developed by preparing live virus as a diagnostic antigen in cell culture.

2.7.2.1 Immunoperoxidase monolayer assays (IPMA)

Both IIFA and IPMA are the longitudinal standard techniques for detecting antibody titer in scientific research and clinical laboratory studies worldwide. IIFA uses fluorescent-labeled antibodies in which a primary antibody (unlabeled antibody) binds to the target antigen and then a secondary antibody is used to detect the first antibody (Odell and Cook, 2013). For IPMA, enzyme-labeled antibodies are used to detect specific target antigens using two-step antibody bindings. The monoclonal mouse-anti-PCV2 antibody is added as the primary antibody followed by the HRP-conjugated goat anti-mouse Ig polyclonal antibody as the secondary antibody into a 96-well plate which is coated with PK-15 cells. It is a "Gold Standard" technique and has been widely used to measure antibody titers of various pathogens in several research studies and also for PCV2 (Fort et al., 2007; Pileri et al., 2014). However, these method also require experience for interpreting the results. Moreover, in both techniques require expensive equipment, facilities, experienced technicians and time-consuming. Therefore, IIFA and IPMA are not suitable for large-scale surveys in swine herds.

2.7.2.2 Enzyme-linked immunosorbent assays

In contrast, ELISAs can be automated, rapid detection of antibodies in a wide range of sample and also decrease the potential bias that may occur with the interpretation of IIFA or IPMA results. ELISAs are good diagnostic assays which can be applied for interpretation of the antibody profiles at farm levels. The ELISA results are also useful for monitoring antibody titers of PCV2 infection and response after vaccination. According to the study from Pileri et al. (2014), three commercial ELISA test kits were analyzed for antibody level against PCV2: Synbiotics, Ingenasa and Biochek combination with the IPMA method. Their results were similar to that of IPMA. Currently, there are commonly three commercial ELISAs: 1) SERELISA PCV2 Ab Mono Blocking (Synbiotics), 2) Ingezim Circo IgG 11. PCV.K1 (Ingenasa), and 3) PCV2 ELISA SK105 (Biochek).

Several ELISA's techniques for detecting PCV2 infection have been developed. Some examples are using cell-culture-propagated PCV2 with specific PCV2 monoclonal antibodies (Walker et al., 2000), baculovirus-expressed recombinant Cap proteins (Nawagitgul et al., 2002) or bacterial expression systems (Marcekova et al., 2009). The

recombinant Cap protein reacts strongly with antigen from the serum of infected pig, suggesting its possibility to use as a diagnostic assay (Nawagitgul et al., 2002). However, the production of recombinant proteins in eukaryotic cell-culture and its expression system are expensive. In many countries, studies have been conducted to develop an ELISA test kit by applying protein coding portion of the recombinant protein to produce in *E. coli* (Marcekova et al., 2009; Shang et al., 2008; Wu et al., 2008). This method provided the same high specificity and accuracy compared to those of produced by insect cell systems. But the production cost is lower with faster and easier protocols.

The antigenic epitopes of PCV-2 capsid proteins are at amino acid residues. Previous reports demonstrated that the recognition of epitope was also located in the nuclear localization signal (NLS) of the capsid protein (Guo et al., 2011). In Thailand, a study by Jittimanee et al. (2012) has developed the protein for an ELISA assay by using a recombinant NLS truncated capsid (rntCap) of PCV2 expressed in *E. coli* compared with the IPMA method using 90 sera samples. The result displayed high sensitivity, specificity and accuracy of 98.33%, 93.33 and 96.67%, respectively. However, the number of serum samples used in these tests was too small and had low reliability. Moreover, this technique has not been applied to high number of field samples from farms with known history of PCV2 infection. This in-house-ELISA, therefore, is used in this study to determine the duration of infection and the immune status of swine with PCV2 in various ages in both clinical and subclinical farms. It is expected that the serological data obtained by this ELISA method can indicate decreasing time of maternal immunity and immune seroconversion when an infection in the herds is present. If success, it will be very useful for planning vaccination program and control management for this disease in the future.

Oliver-Ferrando et al. (2016) investigated the serologic test and immune response by an ELISA technique of PCV2-infected pigs that did not displaying clinical signs. Two groups of swine were compared; one is vaccinated piglets for circovirus type 2 at 3, 6 and 10 weeks of age and another group with no vaccination. The sera and saliva samples were collected and examined. It found that both groups had high viral particles in circulatory system and saliva at the age of 10–14 weeks but gradually decreased until the selling age. This result indicated that the viral infection occurred in piglets prior to 10-week old. The lower immunity of both groups was observed during age 3-6 weeks but raising after 10-week old. This concluded that the proper vaccinated time for PCV2 was 3- or 6-week old pigs. These findings suggested that serological

examination and PCR were among the effective methods for determining potent treatments for diseases in farming.

The advantages of the in-house ELISA assay are low cost, easy production, and suitable application for large-scale surveys of PCV2 infection. This in-house ELISA assay can be performed to monitor the levels of anti-PCV2 antibodies in different ages of pigs within the same herd. These serological data are useful for developing a strategic plan to control PCV2 infection.

2.8 PCV2 vaccination

Although PCV2 could not be easily eliminated from pig farms, but vaccination is a helpful tool to control the effect of PCV2-SD. Currently, commercial vaccines originated from various antigen production systems are available such as subunit of ORF2, inactivated whole PCV2 virus and inactivated chimeric PCV1-2 (Martelli et al., 2011). All types of vaccines have been established based on the target animals such as sow and/or piglet (Opriessnig et al., 2010). PCV2 vaccination in the sow protected the piglets against a PCV2 challenge up to 8 weeks of age (Opriessnig et al., 2010). Consequently, sow and piglet vaccination protocols can prevent PCV2 infection from weaning to early weaned periods by the passive transfer of maternally-derived antibodies from vaccinated sows (Table 1).

Table 1 Types of PCV2 vaccines available in Thailand

Manufacturer	Antigen	Age of the pigs	indications
CIRCOVAC® (CEVA)	Whole virus Inactivated PCV2	Sows and gilts / piglets > 3-wk-old	Two injections 3-4 weeks apart, at least 2 weeks before mating
Circumvent® (Intervet : MSD)	PCV2a capsid protein	Piglets > 3-wk-old	One or two injections 3 weeks apart
Porcillis®PCV (Intervet : MSD)	PCV2a capsid protein	Piglets > 3-wk-old	Single dose
Fostera™ (Zoetis) (formerly Suvaxyn® PCV2)	Chimeric PCV1-2a (inactivated, attenuated)	Piglets > 3-wk-old	Single dose
Ingelvac CircoFLEX® Boehringer Ingelheim	PCV2a capsid protein	Piglets > 2-wk-old	Single dose

Previous studies have illustrated the effect of vaccination to immunity profiles and viral loads in blood. Oh et al. (2012) analyzed innate immunity levels that transfer from sow to their piglets via colostrum. The piglets consumed secreted milk and colostrum from vaccinated and unvaccinated sows were analyzed for innate immunity status. The outcome clearly stated that only piglets taking milk from vaccinated sows could develop immunity indicating that the immune activation was occurred via sow secretions. Moreover, it could protect against PCV2-SD and subclinical infection of PCV2 and may control PCV2 infection. Another study determined antibody levels against PCV2 after vaccinated with 3 available vaccines in the market. By applying to both sow and piglets, they all showed the same level of immune responses after 25 weeks of vaccination (Oh et al., 2014). Thus, it is crucial to obtain immunity information from pigs up until before slauhtering period.

Larochelle et al. (2003) studied farms in which infected pigs either showed or did not showed PCV2 signs along with testing co-infection such as PRRSV and parvovirus. The results demonstrated that pigs started to display PCV2-SD signs after the weaning as well as positive test to PRRSV in every farm, but to parvovirus in some farms. The immunologic tests revealed that pigs from all farms had reduced immunity in the early period and progressively increased at week 11 of age up until they were sold. The finding suggested that the coinfection of PCV2 with other viruses could lead to recognizable ailing clinical signs along with positive blood test. However, showing PCV2-SD signs can be varied depending on production facillities and condition of the vaccines as well as co-infection status of PCV2-infected pigs.

In field condition, efficacy of PCV2 vaccination is sometimes not enough to control the clinical disease of PCV2-SD. To solve this particular problem, a lot of information on management, dynamic of PCV2 infection, time of vaccination and co-infection is needed to be investigated. In fattening pigs, high prevalence of PCV2 infection has been complained from farmers. In some herds, pigs were vaccinated at 3 weeks of age. It is possible that PCV2 vaccination at this time might not provide prolonged immunity to control PCV2 infection until slaughtering period. The high levels of PCV2 viremia in fattening pigs also support the epidemiology of viral circulation in fattening period leading to vaccine failures (Puvanendiran et al., 2011).

2.9 Pig production systems

Production system is very important factor for the transmission of PCV2 within swine herds. Currently, there are three systems of pig production; one-site, two-site and three-site characterized by stages of production or age groups which reared on separate sites and locations. In 1970s, many farmers reared pigs on one location under one roof which was reffered as one-site production (breeding/gestation/farrowing complex). By this mean, workers could move pigs from one site to another easily. The biggest disadvantage of the one-site production is that infectious pathogens can spread from one infected age group to the others. The piglets can receive the diseases from infected sows and subsequently these growing pigs could re-infect the sows (Figure 5A). Alternatively, farmers can separate the breeding production stage from the nursery-grower-finisher production stages to another site or the breeding and nursery production stage from finisher production stages. These systems are typically refered as two-site isowean (breeding/gestation/farrowing and nursery/grower/finisher) and traditional two-site production (breeding/gestation/farrowing/nursery (Figure 5BA) and grower/finisher) (Figure 5BB), respectively. Biosecurity of both one-site and two-site systems is considered as poor prevention of the diseases leading to high death loss and low pig production which disrupts the farm economy. The all-in/all-out system seems to be the solution for eliminating the widespread of the diseases from one-site and two-site operations without depopulation.

Multi-site production is defined as any pig farm in which a various stages of age groups are reared on seperated buildings and locations (breeding/gestation/farrowing + nursery + growing/finishing). The advantage of multi-site production systems is the elimination of infectious agents from breeding/gestation/farrowing buildings by early weaning. This system is suitable for a large number of breedings and slaughter productions. Multi-site production is developed for high–health herd status of breeding pigs (Figure 5C). This production type is an important method for limiting the transmission and outbreak of diseases in a pig farm (Harris, 2008; McGlone and Pond, 2002)

In Thailand, the common pig production types are one-site, two-site and three-site systems. Many factors such as overcrowding, poor ventilation, comingling of different age groups, co-infection of other viruses and bacteria and other stress factors may trigger more prevalence of PCV2-SD in swine herds. Some fattening pig farms import pigs from various sources resulting in more prevalence of PCV2-SD in the herds. Even though, it is known that PCV2 could be eliminated from pigs by early weaning, it

is unclear whether PCV2-SD can be eliminated or controlled effectively in the multi-site systems.

To gain a better understanding of dynamic of PCV2 infection, serological surveillance using the in-house indirect ELISA together with detection of PCV2 in blood circulation will be useful tools for control the disease.

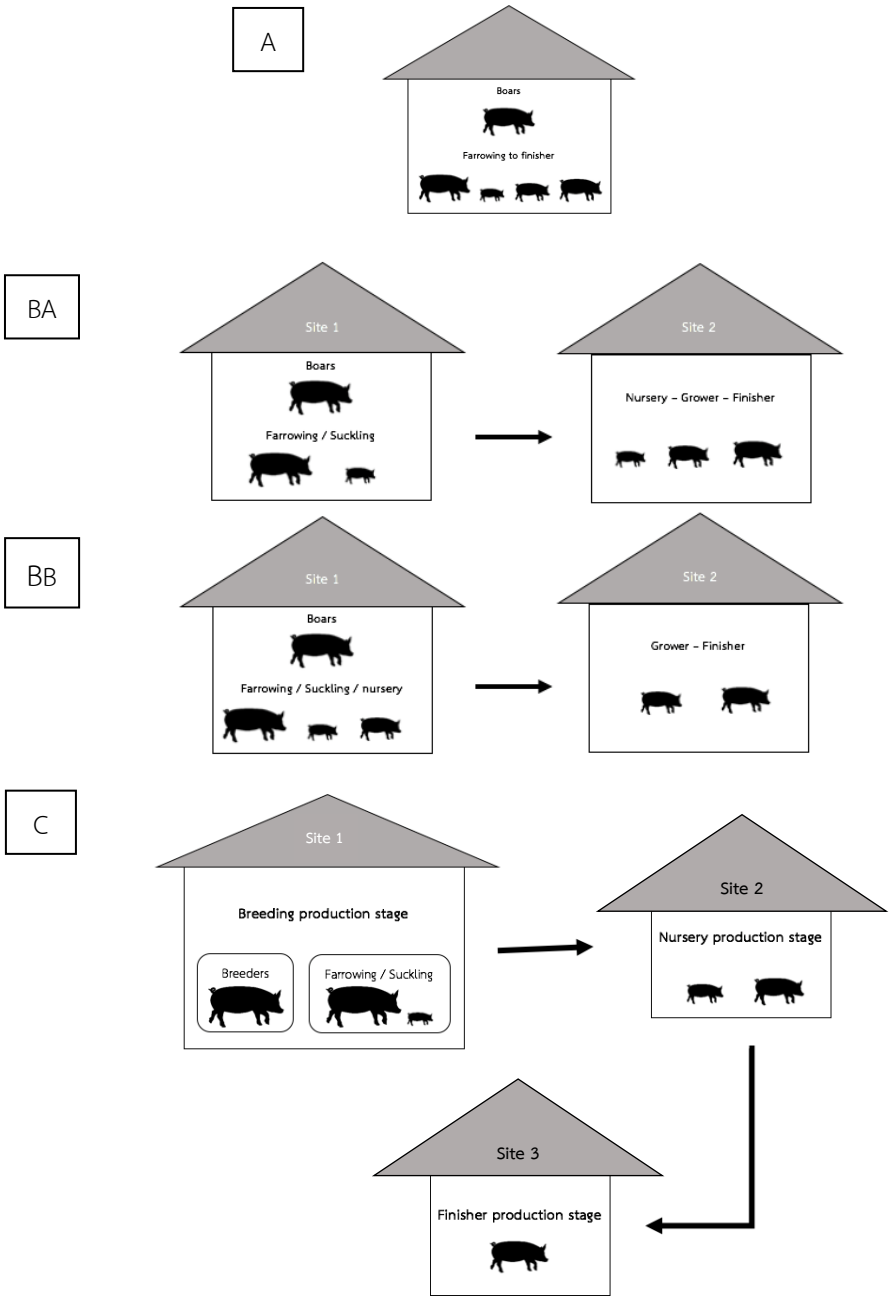

Figure 5 A: One – site system; Pigs of all age groups were placed in one location under the same roof which reffered as the one-site production (breeding/gestation/farrowing complex). **BA:** Two – site pig isowean. The nursery/grower/finisher building is isolated from the farrow-to-feeder pig facilities; the operations were called a two-site farm. **BB:** The Traditional two-site system: The breeding and nursery production stages are located on site 1 with one or more loci, and the finisher production stages are placed on site 2 with one or more loci. **C:** Three – site pig production; This swine production system is a multiple site; buildings and rooms are divided into different age groups and functions.

CHAPTER 3

MATERIALS AND METHODS

3.1 Farm selection and animals

The sampled pig farms were divided into 2 groups: A and B, as shown in Table 2. All herds (n=10) were either one-site and/or multi-site located in the eastern, central or western regions of Thailand, and contained approximately 200-5,000 breeding sows.

Group A represented a one-site and multi-site systems. In this group, the vaccination programs were applied for the protection against PCV2 in sows and 3-week-old piglets. In addition, other vaccines including PRRSV, classical swine fever, Pseudorabies (Aujeszky's disease), and foot and mouth disease (FMD) vaccines were taken by the time suggested from the Department of Livestock Development and/or the vaccine manufacturers. The designated clinical signs of PCVAD at the time of sample collection consists of wasting syndrome, skin paleness and/or jaundice, respiratory distress, diarrhea and inguinal lymph node enlargement as described in the previous study (Segales and Domingo, 2002). The total mortality rate from the weaning to finishing pigs must higher than 5% along with positive PCV2 outcomes by PCR detection and/or by pathological investigation for the last 1 month.

Herds in group B were multi-site pig farms and were divided into 2 subgroups: B-Vac (PCV2 vaccination in piglets, B1 and B2), B-non-Vac (PCV2 non-vaccinated herds, B3, B4 and B5). Additional vaccination programs were applied as described above. The pigs in these herds must show no overt clinical PCV2 signs with the mortality from the weaning to the finishing pigs lower than 5% and with negative results by PCR and/or necropsy report for the last 1 year.

Table 2 Groups of swine herds with/without clinical signs of PCVAD

Group	Clinical PCV2 signs (at least 1 year)	PCV2 vaccine	No. of farms	No. of serum samples
A	Present	Yes	3	150
B-Vac	None	Yes	2	100
B-non-Vac	None	No	3	150

3.2 Clinical data

Historical details of the pig herds were collected including clinical signs of PCVAD, status of PRRSV, morbidity and mortality rate, vaccination programs, medical history and further informations about PCV2-infected cases with co-infections such as bacterial and/or viral infections from the last 1 year were analyzed by the Livestock Animal Hospital, Faculty of Veterinary Medicine, Chulalongkorn University in Nakorn Prathom.

3.3 Study design

The cross-sectional design for sample collection was used in this experiment. In group A and B, serum samples were collected randomly from 5 animals in each age and devided into 2 groups; sows at parity 1, 3, and 5 and piglets at 3, 5, 9, 13, 17, 21 and 25 weeks of age (Figure 6). Serum samples were measured for PCV2 antibody levels by the in-house ELISA technique and detected for the presence of PCV2 DNA using real-time PCR in order to investigate maternal immunity transmitted to their piglets and to examine antibody levels in pigs from weaning until before slaughterhouse periods.

3.4 Sample collections

The blood samples were collected from jugular vein about 4-5 mL per pig in collection tubes (serum tubes) and placed for clotting. The tubes were then centrifuged to separate the serum and the others at 3,000 rpm at room temperature for 5 minutes. Subsequently, serum samples were reserved for qPCR and in-house indirect ELISA analysis by stored in a freezer at -20°C.

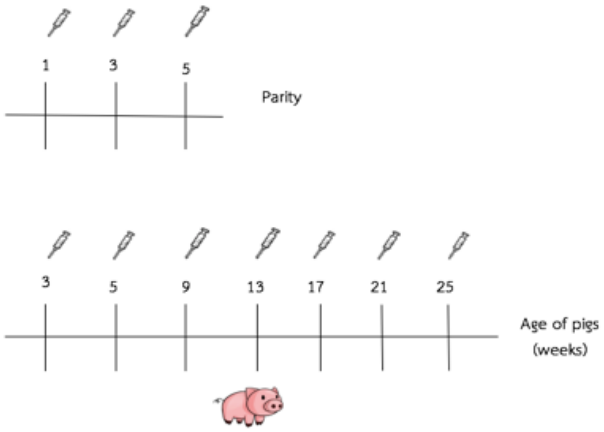

Figure 6 The sampling ages of the parity and the pigs in the study (weeks)

3.5 Serologic analyses

All serum samples were analyzed by serological assay against PCV2 using an indirect in-house rntCap ELISA (Jittimanee et al., 2012). Briefly, ELISA plate containing recombinant capsid proteins of PCV2 (0.5 µg/well expressed from *E. coli*. Serum samples were diluted 1:100 with serum diluent (1% bovine serum albumin (BSA) in 0.01 M PBS). Then 100 µL of diluted samples were aliquoted into 96-well plate with antigen coating, and then incubated at 37°C for 30 min. After the incubation, the ELISA plates were washed 4 times with washing buffer. Subsequently, 100 µL of goat anti-swine IgG antibody (Horseradish Peroxidase, diluted 1:10,000 with conjugate diluents) were added. The plate was incubated 37°C for 30 minutes and again washed for 4 times. The 100 µl of ELISA substrate (SureBlue Reserve™ TMB 1-Component Microwell Peroxidase Substrate, KPL: Product code 52-00-00, USA) were added per well, and the plate were further incubated for 60 min at 37°C.

The enzyme-substrate reaction was stopped and measured for optical density by spectrometer at 650 nm. A detected value of sample/positive (S/P) ratio above 0.30 were considered as positive.

Interpretation:

S/P ratio $\geq$ 0.3 = Positive

S/P ratio $\leq$ 0.3 = Negative

3.6 Quantification of genomic PCV2 DNA

In each age group, the pooled sera (5 samples for each age group) were detected for the presence of PCV2 DNA. Viral DNA were extracted from 200 µL of the serum using Invisorb® DNA extraction kits (STRATEC Molecular GmbH). The extracted DNA were investigated by real-time PCR assay using PCV2-specific primers

ORF1-F: 5'-ATg CCC AgC AAg AAg AAT ggA AgA Ag-3'
ORF1-R: 5'-Agg TCA CTC CgT TgT CCT TgA gAT C-3'

Five µL of DNA sample, RNase-free water 2.2 µL and 1.4 µL (10 µM) of each primer were added to a PCR mixture (QuantiNova™ SYBR® Green PCR kit (QIAGEN®)) to obtain 20 µL using a real-time PCR cycler (Roter-Gene® Q, Qiagen). Amplification were achieved by a hold single cycle at 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 52°C for 15 sec and 72°C for 20 sec. Finally, the second hold at 95°C for 30 sec and the third hold at 65°C for 30 sec (Table 3).

The standard for qPCR optimization was prepared from the PCV2 DNA of a strong postitive PCR result (from ORF1-PCV2-specific primers). The DNA amounts in milligram were obtained from a nanodrop measurement. Later, they were used to calculate copy numbers of the DNA standards by the formula in Appendix C. The DNA was serially diluted ten-fold to create a standard curve. In this experiment, the standard DNA of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copy number/microliter were used to optimize qPCR data. Finally, amounts of DNA from all samples were presented as a logarithmic number of copy numbers per mL.

Table 3 Cycling condition for real-time PCR amplifying of ORF1 gene of PCV2

Process	Temperature (°C)	Time	Cycles
Hold 1	95	2 min	1 cycle
Cycling	95	15 sec	40 cycles
Cycling	52	15 sec	
Cycling	72	20 sec	
Hold 2	95	30 sec	1 cycle
Hold 3	65	30 sec	1 cycle

3.7 Statistical analysis

The statistical analyses was carried out by using SAS (SAS version 9.0, SAS Inst., Cary, NC, USA). Multiple analysis of variance (ANOVA) was performed to analyse the continuous variables (i.e, S/P ratio and qPCR). The statistical models included the effect of clinical signs (clinical signs versus non-clinical signs), herd nested within clinical signs (A, B-Vac and B-non-Vac), age group (sows at parity 1, 3, and 5 and piglets at 3, 5, 9, 13, 17, 21 and 25 weeks of age) and interaction between vaccination. Least squares means were obtained from each class of the variable and were compared by using least significat difference (LSD) test. The values with *P* value <0.05 were considered to be statistically significant.

CHAPTER 4

RESULTS

4.1 Farms, animals and clinical disease

Pig farms in group A (clinical PCV2) were one-site and multi-site production systems. The archival records (2016-2017) of the Veterinary Diagnostic Laboratory, Large Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University (Nakorn Pathom, Thailand) were evaluated for clinical symptoms compatible with PCV2-SD, including progressive weight loss, pale yellow skin, dyspnea, diarrhea and superficial inguinal lymph node enlargement. Microscopic observations revealed lymphoid depletion, histiocytosis, multinucleated giant cell and/or inclusion body in lymphoid tissues and also with positive PCR and/or qPCR of PCV2.

Based on the clinical history, pigs in group A showed wasting, pale yellow, dyspnea and diarrhea in all herds ranging from 5-40% of pigs. However, only in the A3 herd demonstrated enlarged lymph nodes by 10% of its population. These symptoms were not observed in A1 and A2. Weight loss was the most common feature found in all herds. The other less common symptoms were as follows: diarrhea, respiratory distress, pale to yellow skin and mucous membrane, long rough hair and enlargement of superficial inguinal lymph nodes, respectively (Table 4).

The morbidity rate in the nursery to finishing pigs ranged from 5-20%. The high rates were found predominantly in the growers and the finishers but none in sows (Table 5).

Both sows and piglets were routinely vaccinated against PCV2 (A1, A2 and A3). Pigs in A1 and A2 herds were vaccinated twice at age 3 and 10 weeks and in A3 herd at 4 and 7 weeks (Table 5).

All swine herds in group B (non-clinical PCV2) were a two-site system. The pigs in this group had less than 5% of PCV2 clinical signs and the history of conventional PCR were negative for PCV2 within 1 year before the blood collection period.

The B2 herd had found mainly a respiratory problem with 1-3% in all age along with 1% of long rough hairs. In B1 herd, only 1% of wasting condition in fattening pigs

was present. Approximately 1% of sows in B4, B5, and B6 herds were displayed dystocia and/or lack of maternal behavior and mortality (Table 4 and 5).

Table 4 Characteristics of PCV2 signs in studied herds

Group		PCV2 clinical signs (%)					
		Wasting	Long rough hair	Pallor /Jaundice	Respiratory distress	Diarrhea	Superficial inguinal lymph node enlargement
A (clinical PCV2)	A1	5	5	5	5	10	N/A
	A2	5	5	5	5	10	N/A
	A3	40	10	10	10	30	10
B (non-clinical PCV2)	B1	1	-	-	-	-	-
	B2	-	1	-	3	-	-
	B3	-	-	-	-	-	-
	B4	-	-	-	-	-	-
	B5	-	-	-	-	-	-

N/A; no data available

Table 5 Production system, PCV2 vaccination programs, morbidity and mortality rate and PRRS status of the herds

Group		Production system	PCV2 vaccination (wk)		Morbidity (%)				PRRSV status
			Sow	Pig	Nursery	Grower	Finisher	Sow	
A (clinical PCV2)	A1	Three	Yes	3, 10	5	20	20	-	Stable
	A2	Three	Yes	3, 10	5	20	20	-	Stable
	A3	One	Yes	4, 7	10	10	5	-	Stable
B (non-clinical PCV2)	B1	Two	-	3	-	-	1	-	Stable
	B2	Two	-	3	1	3	1	-	Stable
	B3	Two	-	-	-	-	-	1	Stable
	B4	Two	-	-	-	-	-	1	Stable
	B5	Two	-	-	-	-	-	1	Stable

4.2 Serologic test results

In group A, 101 samples (67.33%) were positive and 108 samples (43.20%) of group B were positive. The results showed that the number of positive pigs in group A had significantly than that of group B ($p<0.05$) as seen in Table 6.

Table 6 Percentage of PCV2-positive pigs in this study

Group	Negative (No.) (%)	Positive (No.) (%)	Total (%)
A (clinical PCV2)	49 (32.66)	101 (67.33)	150
B (non-clinical PCV2)	142 (56.80)	108 (43.20)	250
Total	191 (47.75)	209 (52.25)	400

A statistical individual analysis of S/P ratio was performed between group A and B. The antibodiy titers of pigs against PCV2 included clinical signs ($p=0.001$) and age of pigs ($p<0.001$); interactions between clinical signs and age of pigs ($p=0.003$) were all significantly influenced.

Most of the seroprofiles of the five herds in group A followed the same pattern. The pigs showed a high S/P ratio at 3 weeks and a lower S/P ratio at 5 weeks (Figure 7). The seroconversion to PCV2 was observed once at age 13 weeks in all clinical herds (Appendix B, Table B-2).

In group B, the serologic profiles were similar to group A with the level of PCV2 antibodies being initially high at 3 weeks and then declining at 5 weeks (Figure 7). The antibody titers gradually increased from week 9-21 in the B1, B3, B4 and B5 herds. In contrast, seroconversion was observed at 5 and 13 weeks in the B2 herd (Appendix B, Table B-2).

To compare the seroprofiles between the two groups, the proportion outcome of seronegative pigs in group B was significantly higher than group A, especially in nursery and grower pigs. On the other hand, in group A, parity 1, 5, and 17 weeks-old-piglets showed significantly higher S/P ratio than those of group B in the same age groups ($p<0.05$) (Figure 7). All parity sows displayed positive results in both groups.

The comparison of PCV2 antibody titers between farms in group A (A1-A3) and group B (B1-B2, PCV2 vaccinated) revealed similar outcomes but the titers in group A

was slightly higher than in group B. However, significantly differences were observed at 17 and 25 weeks (Figure 8 and and 11).

The comparison within the non-PCV2 vaccinated herds [A and B-non-Vac (B3-B5)] found a similar trend of serological profiles. The antibody levels in group A were statistically higher than B-non-Vac at various time points (Figure 9 and 11).

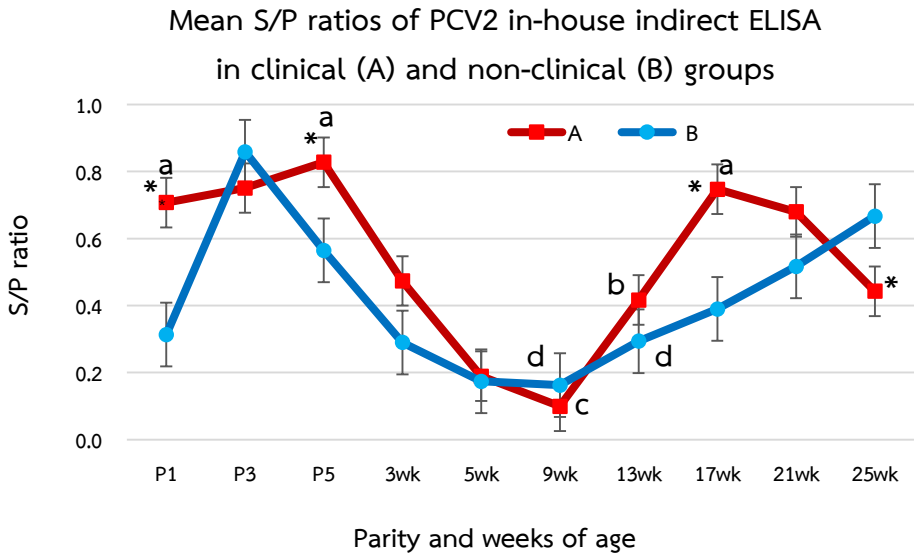

Figure 7 Comparison of mean S/P ratios of PCV2 in-house indirect ELISA with standard error in clinical (A; square) and non-clinical PCV2 (B; circle) groups. (S/P ratio ≥ o.3 is positive)
Different letters mean significantly differences within the same group (p<0.05)
* Significantly differences between group A and B (p<0.05)

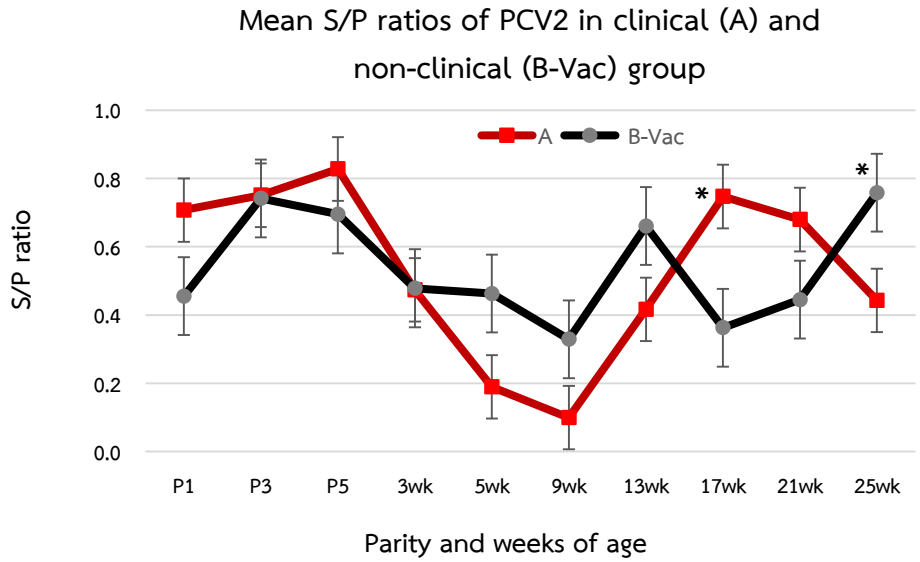

Figure 8 Comparison of mean S/P ratios of PCV2 in-house indirect ELISA with standard error in clinical (A; square) and non-clinical PCV2 (B-Vac; circle) groups.
* Significantly differences between group A and B-Vac (p<0.05)

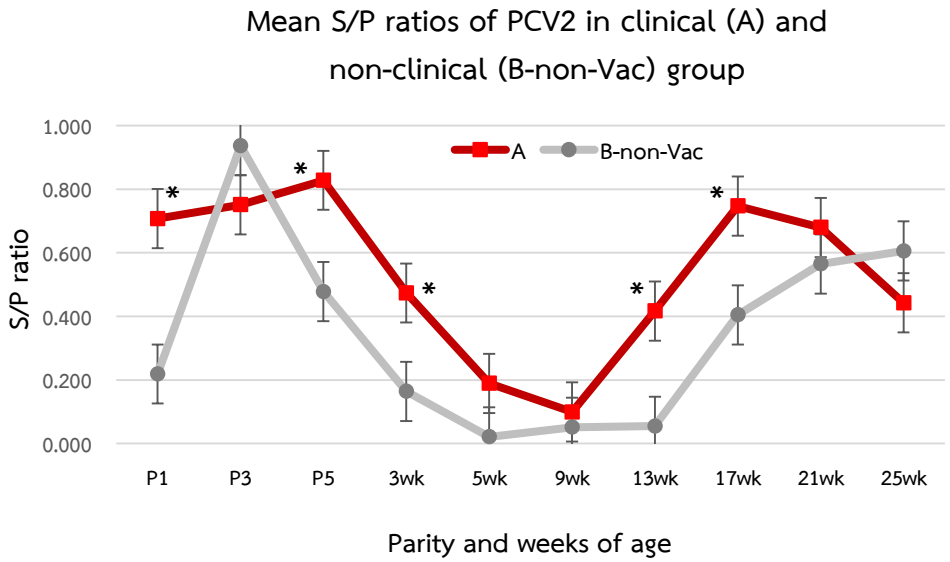

Figure 9 Comparison of mean S/P ratios of PCV2 in-house indirect ELISA with standard error in clinical (A; square) and non-clinical PCV2 (B-non-Vac; circle) groups.
* Significantly differences between group A and B-non-Vac (p<0.05)

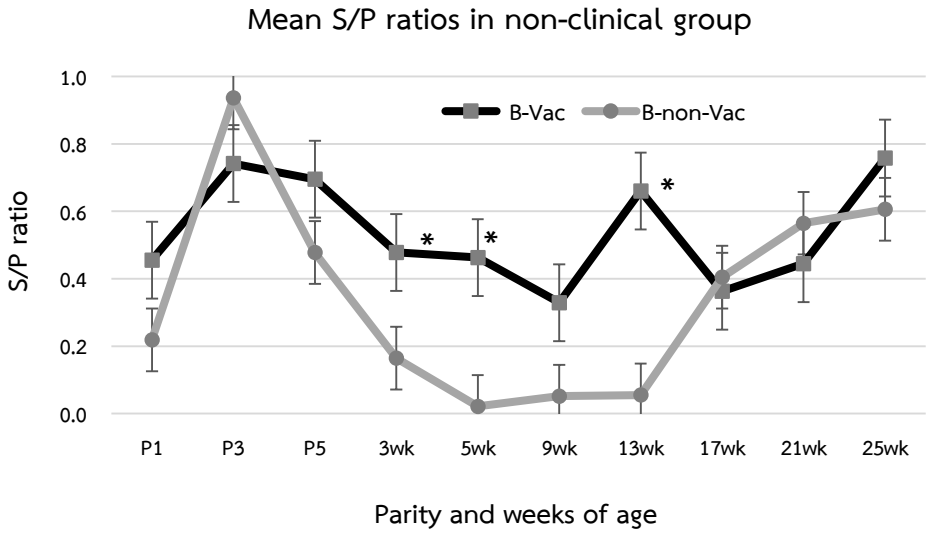

Figure 10 Serologic profiles of PCV2 in-house indirect ELISA with standard error in non-clinical PCV2 signs group (B-Vac; sqaure and B-non-Vac; circle).

* Significantly differences between B-Vac and B-non-Vac groups (p<0.05)

The seroprofiles of B-Vac and B-non-Vac groups demonstrated the decrease of immunity in piglets at the age of 3-5 weeks due to the probability of lower maternal-dirived antibodies (Figure 10). The antibody levels of B-Vac group were significantly higher than B-non-Vac group at 3, 5 and 13 weeks (p<0.05). In addition, the piglets in B-Vac group showed significantly higher antibody levels than those of B-non-Vac group (p<0.05) (Table 7).

Table 7 Mean S/P ratio of PCV2 ELISA in this study

Group	Mean	Standard error	Total
A (clinical PCV2)	0.533[a]	0.030	150
B (vaccination)	0.533[a]	0.037	100
B (non-vaccination)	0.350[b]	0.030	150

[a,b] Differences letters mean significantly differences (p<0.05)

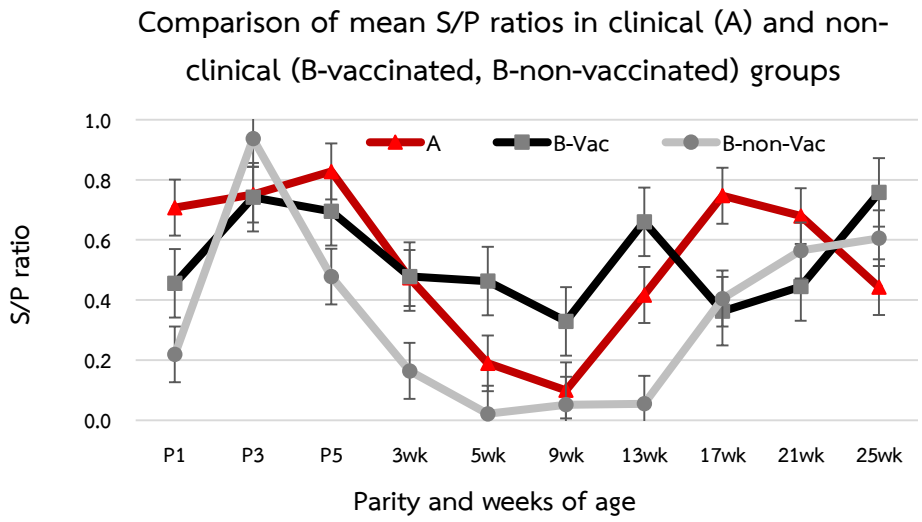

Figure 11 Serologic profiles of PCV2 in-house indirect ELISA with standard error in clinical (A; triangle) and non-clinical PCV2 signs group (B-Vac; sqaure and B-non-Vac; circle).

4.3 PCV2 viremia and genomic copy numbers

Viremia was detected by qPCR in the pooled sera from 5-to-1 samples in each age group. The mean numbers of PCV2 genomic copies in serum are shown in Appendix Table C-1. A statistically significant difference was observed between clinical signs (p=0.021) and age of pigs (p=0.006) and interactions between clinical signs and age of pigs (p=0.013)

Most herds in group A (A1-A3) had viral loads of 10^7, 10^6, 10^5 and 10^4 PCV2 genomes per mL serum. All herds had the highest viral load numbers from 13 to 21 weeks. The higher viral loads were consistent with the clinical expressions as shown in Table 5 and varied according to the S/P ratio observed in each age range.

On the other hand, the results showed 10^6, 10^5 and 10^4 viral load per mL serum in all herds in group B. The highest viral load levels (10^6 PCV2 genomes per mL serum) were observed in B-non-Vac herd in parity 1, 3 and 5 sows and pigs at 13-25 weeks.

The mean levels of viral loads from both groups were similar in parity 1, 3 and 5 sows, and in piglets aged 3-9 weeks. On the other hand, high viral loads were observed in piglets aged 13-21 weeks. In group A, pigs at 13 and 17 weeks of age had the highest viral load in the sera compared to the other same or different age groups as shown in Figure 12.

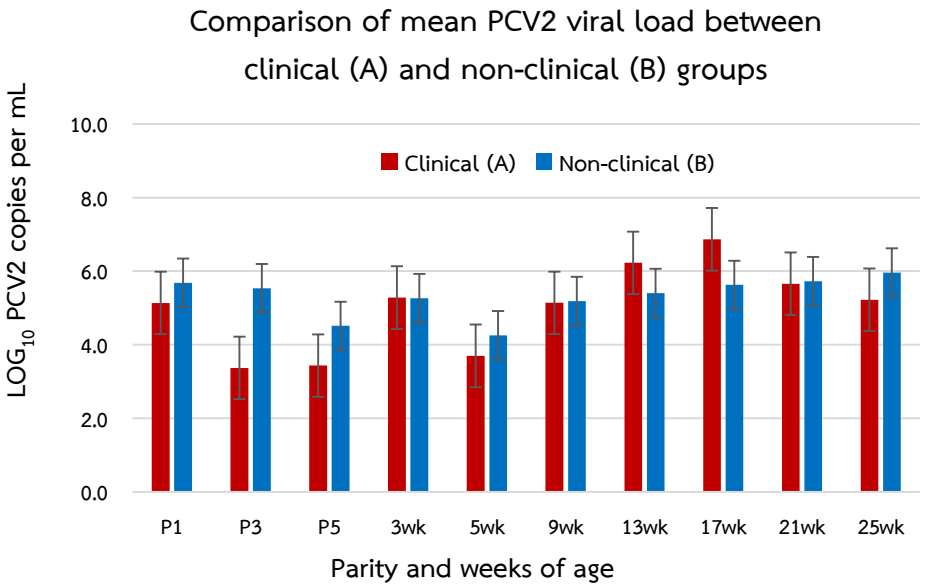

Figure 12 Comparison of copy number of PCV2 DNA per mL with standard error from aninals in different ages in group A (clinical PCV2) and B (non-clinical PCV2) by qPCR.

Table 8 Mean qPCR PCV2 positive pigs in sera in group A and B

Group	No. in herd	Total PCV2 copies per mL, mean ± S.E. in group A and B Mean	± S.E.
A (clinical)	30	5.004	0.276
B (non-clinical)	50	5.219	0.218

The detection of PCV2 viral load from pig sera by qPCR method showed that PCV2 infection in the late nursery pigs had low level but subsequently increased in grower pigs. The highest level of the virus was found at 17 weeks in group A. In addition, most of group B had a lower viral load than that of group A. In general, group A had an average of 10^6 viral load serum while group B had 10^5. The data in Table 8 showed that the high viral loads were consistent with overt PCV2 symptoms and followed by high level of immunity. In group A, pigs at 13 and 17 weeks of age had significantly higher PCV2 viral load than those of group B as shown in Figure 12.

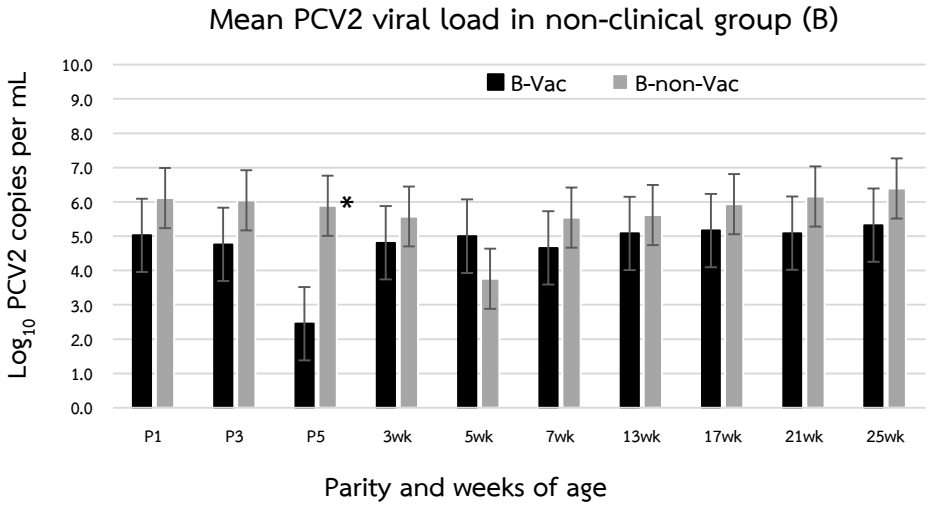

Figure 13 Virologic profiles of non-clinical PCV2 signs group (B-Vac; B1 and B2, B-non-Vac; B3, B4, B5).

* Significantly differences between B-Vac and B-non-Vac groups (p<0.05)

The comparison of viral loads within all group (Figure 14) revealed that the B-Vac had significantly lower viral loads in all age groups of pigs and sows, especially at age of 17, 21 and 25 weeks old. The B-Vac group which had a vaccination demonstrated 10 times lower viral loads than those of the B-non-Vac (Table 9). However, the viral load in group A (5.004) was not significantly different with B-Vac (4.736) (Table 9, Figure 14).

Table 9 Mean qPCR PCV2 in sera in all group

Total PCV2 copies per mL, mean ± S.E.			
Group	**No. in herd**	**Mean**	**± S.E.**
A	30	5.004[ab]	0.269
B-Vac	20	4.736[b]	0.330
B-non-Vac	30	5.703[a]	0.269

[a,b] Differences letters mean significant differences (p<0.05)

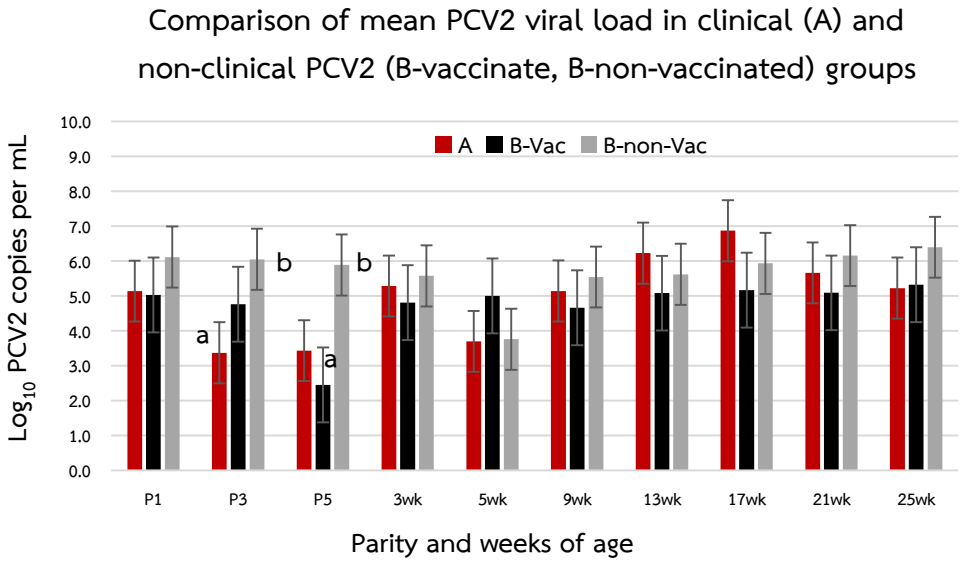

Figure 14 Virologic profiles of clinical (A; A1, A2, A3) and non-clinical PCV2 signs group (B-Vac; B1 and B2, B-non-Vac; B3, B4, B5).

[a,b] Differences letters mean significantly differences (p<0.05)

CHAPTER 5

DISCUSSION

PCVAD is a multi-factorial disease that has negative impact on morbidity, mortality leading to economic loss on pig production worldwide for more than 20 years (Allan and Ellis, 2000; Chae, 2005). There have been good managements and vaccination programs to control the disease, but loss of piglets is still occurred. To mornitor PCV2 infection in swine herds, serological and virological methods have been used in both clinical and subclinical PCV2-infected herds (Grau-Roma et al., 2009; Larochelle et al., 2003; Podgorska and Stadejek, 2011; Sibila et al., 2004). This study compared serological and virological status of PCV2 infection in Thai swine herds with and without clinical signs.

Considering the picture of the serological profiles of clinically affected herds (group A) and non-affected herds (group B) reveals the same pattern which is in accordance with previous studies (Larochelle et al., 2003 and Sibila et al., 2004). In group A, there were high proportion of seropositive pigs for more than 90% of all parity sows (P1, P3 and P5), this might be due to the responses of PCV2 vaccination or subclinical infection in sows.

In piglets, antibody titers decreased continuously from 3 to 9 weeks, which was likely due to the decreasing maternal immunity. Even though vaccination was done at week 3 on A1 and A2 herds and at week 4 on the A3 herd, the level of antibodies did not increase. This was might be due to vaccinating too early which interfering maternal immunity (Fraile et al., 2012; Martelli et al., 2011). However, seroconversion was observed at week 13 until the ending stage (21-25 weeks) for all three herds. This seroconversion supports the intensive infection of PCV2 in these pigs coincided with high rate of clinical signs of PCVAD during this period. In addition, a second PCV2 vaccine was given to the A3 herd at week 7 and to the A1 and A2 herds at week 10. Although the second PCV2 vaccine was administered, pigs were still infected with PCV2 (Oh et al., 2014; Puvanendiran et al., 2011; Seo et al., 2014). However, it should be noted that this ELISA method could not distinguish between antibodies responses from vaccination or infection (Jittimanee et al., 2013; Jittimanee et al., 2012) The previous experiment of PCV2 vaccination showed that PCV2 vaccine could effectively reduce

viremia even in PCV2-viremic piglets or with MDA (Seo et al., 2014). In contrast with this field study, PCV2 vaccination was not enough to control clinical disease in the studied herds. This might be due to coinfection by PRRSV, poor biosecurity or inappropriate production management.

In general, the seroprofile of pigs in non-clinical group was lower than clinical group, but still be the same pattern. The sows in non-clinical group had PCV2 titers in P1 and P5 were significantly lower because no vaccination against PCV2 in sows. However, the highest level of antibodies was found in P3, indicating more exposure of virus in older sows. In weaned pigs, the antibody titers of group A increased dramatically at 13 weeks of age. The titers of group B, however, only slightly increased at this time point. This was likely due to lower infection (low PCV2 load) in group B as similar to (Brunborg et al., 2010) which observed that healthy herds (free from signs of PCVAD) had lower levels of immune responses and lower viral loads than PCV2 affected herds. Seroconversion that observed at 13 weeks of both groups was due to exposure of weaned pigs to natural infection. The decline of MDA in this period and mixing of piglets from various sows also accelerated the infection. The infection might occur 2 weeks before the seroconversion as shown in increased viral load levels in those periods.

By comparison within PCV2-vaccinated herds, the antibody titer was gradully declined from 3 to 9 weeks of age. Thereafter, the titers of group A were increased until 17 weeks while the titers in group B-Vac was significantly lower than group A. This could be explained by more intense natural infection in group A.

In this study, the viral loads increased at 13 weeks of age, and reached the highest level at 17 weeks especially in clinically affected group. When compared to the other ages within group A, the pigs exhibited more clinical symtoms of PCVAD at the grower and finishing stage. The viral load was then diminished at late stage of finishing pigs. In addition, the clinically affected pigs showed significantly higher antibody titers than those of non-clinical pigs. Although this increase of antibody might be due to vaccination or natural infection, but high viral load during late nursery to growing stage indicated the intensive PCV2 infection during this period.

In non-clinical pigs, detection of the PCV2 DNA copies in this group revealed low amounts in most pigs. However, the highest values ranged from 10^5-10^6 copies/ml in the pigs at 21 to 25 weeks of age. These data were in accordance with previous

study suggesting that the viral levels of PCV2 genome above 10^6 copies/ml were usually identified in PCV2-affected herds; on the other hand, the viral levels lower than 10^5 or 10^6 copies/ml were commonly found in subclinical herds (Brunborg et al., 2004).

Within non-clinical group, the seroprofiles of the vaccinated group had higher titers than the non-vaccinated group, however, no statistically significant was observed. On the other hand, the PCV2 copies of the vaccinated non-clinical group were 10 times less than the non-vaccinated one. This indicated that the PCV2 vaccine could reduce viral load in non-clinical pigs and reduce risk of clinical disease (Fort et al., 2008).

Normally, one-site production system could show higher morbidity and mortality rates than three-site system (Harris, 2008). On the other hand, the three-site system in this study showed higher percentage of production loss than in the one-site herd. This might be due to the mixing of pigs during nursery and fattening periods. This poor managment could enchance PCV2 spreading within the herd leading to a higher percentage of PCV2-infected pigs.

In conclusion, this study is able to demonstrate that our in-house ELISA kit is suitable for a large number of serum samples in monitoring of serological profiles of PCV2 infection. Investigation of viral load in serum as well as PCV2 clinical expressions of pigs could indicate the dynamic of PCV2 infection within swine herds and could be beneficial for disease controls. In addition, the information obtained in the study including the dynamic of PCV2 infection and serological surveillance using the in-house indirect ELISA together with the detection of PCV2 in blood circulation are remarkably useful for planning vaccination programs in a swine farm. As aforementioned, PCV2 prevalence is one of the most common problem in many areas in Thailand, and its treatment is difficult to accomplish. This approach will provide potential means for a swine farmer to take control the infection at early stages.

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APPENDIX A

Protein preparation

Expression and purification of rntCap proteins in *E. coli*

The expression vector used in this study was introduced by Jittimanee et al. (2012) using rntCap plasmids into *E. coli* strain BL21-Rosetta™ (Merck KGaA, Darmstadt, Germany). The bacterial rntCap protein reserved in the -80°C was thawed out and subcultured on Luria-Bertani (LB) agar plates containing 100 mg/ml of ampicillin and 25 mg/ml of chloramphenicol. The plates were incubated at 37°C overnight, and a single colony was transfered into 50 ml LB broth and incubated at 37°C overnight. This culture then seeded 40 ml into 2 liters of LB (1:50 subcultivation) and incubated at 37°C C for 2.5 hours. Subsequently, 0.1 mM Isopropyl-D-1-thiogalactopyranoside (IPTG) (Promega Corporation, WI, USA) were added and the incubation was continued at 37°C for 4 hours. The bacteria were harvested by centrifugation at 2,000 rpm at 4°C for 45 minutes. Purification of the rntCap protein from the bacterial lysate by using a glutathione (GST) affinity purification module (GE Healthcare Limited, Buckingham, shire, UK).

Protein expression analysis

The rntCap proteins were separated by running 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were detected via Western blotting as previously described (Guo et al., 2010). In brief, proteins were blotted onto a nitrocellulose membrane (Pall Corporation, NY, USA) at 110 V for 70 min. The membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBS-T; 25 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 8.0) for 2 hours. The strips were then incubated with monoclonal mouse anti-PCV2 antibodies (Rural Technologies, Inc., SD, USA) for 1 hours and later incubated with HRP-labeled rabbit anti-mouse IgG antibodies (SouthernBiotech, AL, USA) for 1 hours. The final reaction was performed by adding 3', 3'-diaminobenzidine (DAB) as a substrate (Merck Millipore, MA, USA). Protein concentration was measured by Bradford assay according to the manufacturer's instructions using a spectrophotometer (BioPhotometer®, Eppendorf. AG, Germany)

Appendix B

In-house ELISA

Detection of PCV2 antibody by in-house indirect ELISA

This in-house indirect ELISA was based on a recombinant capsid protein expressed in *E.coli* system which was coated on a 96-well plate (0.5 ug/well). When 1:100 dilutions of serum samples from pigs infected with PCV2 were added, the antibody against PCV2 reacted with the recombinant protein. Then the second antibody, the conjugate peroxidase-labeled anti-pig immunoglobulin G diluted 1:10,000, was placed in each well for binding the anti-PCV2 antibody. Finally, the peroxidase substrate was added to test for an enzyme-substate reaction. This induced changes of color in some samples which indicated that there were reactions between the anti-PCV2 antibody and recombinant protein.

Coating plate

- the recombinant proteins were diluted with coating buffer (0.015 M Na_2 CO_3 + 0.035 M $NaHCO_3$, ph 9.6).
- incubated at 4°C, overnight
- after incubation, washed four times with washing buffer (0.01M phosphate buffer saline - 0.5% Tween 20)

Blocking plate

- added blocking solution (1% Bovine Serum Albumin (BSA) in 0.01 M PBS) to each well
- closed the cover and incubated at room temperature for 1 hour
- washed four times using PBST washing buffer
- dried the plate at room temperature and then stored at 4°C

Serum dilution

- serum samples were diluted 1 to 200 in serum diluent (1% BSA in 0.01 M PBS).
- transferred diluted serum 100 μl to the PCV2 ELISA plate
- closed the cover and incubated at 37°C for 30 minutes
- washed four times using PBST washing buffer

Conjugate addition (Goat anti-swine IgG antibody, Horseradish peroxidase (HRP) conjugate)

- diluted the conjugate 1 to 10,000 in diluents (1% BSA in 0.01 M PBS)
- added 100 μl of diluted conjugate to the plate
- closed the cover and incubated at 37°C for 30 minutes
- washed the plate four times using PBST washing buffer

Substrate addition

- added 100 μl of substrate (tetramethylbenzidine(TMB), SureBlue Reserve ™)
- incubated at room temperature in a dark area for 1 hour
- the color of some samples changed to blue and measured at 650 nm using ELISA reader (Biochrom EZ Read 800 Plus ELISA microplate reader, UK) to calculate the optical density

A	P											
B	P											
C	N											
D	N											
E	S1											
F	S2											
G	S3											
H	S4											
		1	2	3	4	5	6	7	8	9	10	11	12

ABBREVIATION: S = sample, P = positive, N = negative

$$\text{Average positive} = \frac{[\text{OD650 (A1)} + \text{OD650 (B1)}]}{2}$$

$$\text{Average negative} = \frac{[\text{OD650 (C1)} + \text{OD650 (D1)}]}{2}$$

Calculation of S/P ratio:

$$\textbf{S/P ratio} = \frac{\left[\text{OD650 (Sample)} - \text{OD650 (Average Negative)}\right]}{\left[\text{OD650 (Average Positive)} - \text{OD650 (Average Negative)}\right]}$$

Interpretation:

S/P ratio $\geq$ 0.3 = Positive

S/P ratio $\leq$ 0.3 = Negative

Table B-1 S/P ratios of PCV2 in-house ELISA with standard error in individual pig in group A (clinical PCV2) (n = 5)

In-house ELISA results (S/P ratio) in group A										
Herd	Parity sows			Weaned pigs (weeks)						
	1	3	5	3	5	9	13	17	21	25
A1-1	0.789	0.687	0.843	0.493	0.393	0.094	0.125	0.402	1.120	0.208
A1-2	0.755	0.863	1.011	0.504	0.248	0.034	1.074	0.929	0.969	0.271
A1-3	0.627	0.687	0.934	0.160	0.236	0.046	0.048	0.775	0.519	0.279
A1-4	1.014	0.863	0.661	0.228	0.379	0.097	0.117	0.889	0.165	0.721
A1-5	0.835	0.920	0.863	0.422	0.481	0.162	0.379	1.140	0.650	0.094
mean.A1	0.804	0.804	0.863	0.361	0.348	0.087	0.349	0.827	0.684	0.315
± SD	0.141	0.110	0.131	0.158	0.104	0.051	0.425	0.272	0.377	0.239
A2-1	0.398	0.897	0.670	0.042	0.069	0.000	0.835	0.510	0.291	0.057
A2-2	0.257	0.747	0.648	0.513	0.034	0.000	0.218	0.287	0.077	0.406
A2-3	0.330	0.613	0.893	0.142	0.146	0.157	0.567	0.456	0.536	0.088
A2-4	0.648	0.418	0.425	0.444	0.061	0.023	0.107	0.720	0.441	0.586
A2-5	1.023	0.602	0.444	0.169	0.046	0.084	0.031	0.782	0.471	0.333
mean.A2	0.531	0.655	0.616	0.262	0.071	0.053	0.352	0.551	0.363	0.294
± SD	0.312	0.179	0.191	0.205	0.044	0.068	0.339	0.201	0.184	0.222
A3-1	0.670	1.020	0.988	0.751	0.046	0.042	0.996	0.851	0.694	0.612
A3-2	0.934	0.515	0.771	0.755	0.400	0.292	0.875	0.942	0.636	0.759
A3-3	0.581	0.883	1.141	0.791	0.163	0.093	0.692	1.163	1.058	0.682
A3-4	0.795	0.710	0.928	0.972	0.101	0.312	0.030	0.501	1.175	0.654
A3-5	0.956	0.837	1.195	0.718	0.036	0.054	0.155	0.861	1.392	0.889
mean.A3	0.787	0.793	1.004	0.797	0.149	0.159	0.550	0.864	0.991	0.719
± SD	0.163	0.191	0.170	0.101	0.149	0.132	0.433	0.238	0.322	0.109

S/P ratio ≥ 0.3 = Positive

Table B-2 S/P ratios of PCV2 in-house indirect ELISA with standard error in individual pig in group B (non-clinical PCV2) (n = 5)

In-house indirect ELISA results (S/P ratio) in group B										
Herd	Parity sows			Weaned pigs (weeks)						
	1	3	5	3	5	9	13	17	21	25
B1-1	0.811	1.301	0.282	0.000	0.031	0.011	0.690	0.172	0.127	0.276
B1-2	0.107	0.611	0.397	1.034	0.000	0.901	0.828	0.197	0.121	0.262
B1-3	0.037	0.856	0.301	0.000	1.121	0.806	1.282	0.079	0.248	0.296
B1-4	0.062	1.211	1.592	1.020	0.056	0.000	0.992	0.113	0.192	0.180
B1-5	0.146	1.073	1.507	0.000	0.014	0.000	0.431	0.797	0.130	0.276
mean.B1	0.233	1.011	0.816	0.411	0.245	0.344	0.845	0.272	0.163	0.258
± SD	0.326	0.279	0.672	0.562	0.490	0.467	0.319	0.298	0.055	0.045
B2-1	0.523	0.646	0.651	0.518	0.087	0.149	0.473	0.183	0.930	1.346
B2-2	0.695	0.450	0.105	0.783	1.138	0.166	0.273	0.223	1.161	1.448
B2-3	0.544	0.301	0.393	0.000	0.423	0.389	0.132	0.515	0.338	0.676
B2-4	0.826	0.234	0.803	0.234	0.242	0.420	1.234	1.172	0.752	1.715
B2-5	0.801	0.734	0.920	1.194	0.921	0.448	0.270	0.177	0.451	1.104
mean.B2	0.678	0.473	0.574	0.546	0.681	0.314	0.477	0.454	0.726	1.258
± SD	0.141	0.215	0.328	0.467	0.449	0.145	0.440	0.425	0.338	0.392
B3-1	0.074	0.938	0.296	0.099	0.000	0.000	0.086	0.080	0.963	0.889
B3-2	0.253	0.568	0.784	0.105	0.068	0.000	0.000	0.000	0.796	0.784
B3-3	0.086	0.105	0.346	0.235	0.173	0.025	0.031	0.000	0.858	0.574
B3-4	0.222	1.253	1.809	0.167	0.000	0.037	0.031	0.056	0.481	1.025
B3-5	0.160	0.457	0.296	0.031	0.000	0.043	0.000	0.265	0.086	0.414
mean.B3	0.159	0.664	0.667	0.127	0.048	0.021	0.031	0.016	0.673	0.737
± SD	0.080	0.407	0.686	0.077	0.076	0.020	0.035	0.036	0.290	0.245
B4-1	0.019	0.920	0.685	0.648	0.000	0.222	0.327	0.247	0.451	1.385
B4-2	0.173	0.049	1.068	0.228	0.000	0.000	0.000	1.420	1.235	1.020
B4-3	0.222	1.309	0.488	0.451	0.000	0.000	0.012	0.259	0.676	0.758
B4-4	0.025	0.019	0.000	0.000	0.006	0.142	0.160	0.309	1.119	0.775
B4-5	0.025	1.667	0.191	0.000	0.000	0.012	0.074	0.932	0.697	0.348
mean.B4	0.093	0.793	0.486	0.265	0.001	0.075	0.115	0.633	0.835	0.857
± SD	0.097	0.741	0.419	0.284	0.003	0.102	0.135	0.525	0.329	0.381
B5-1	0.098	0.164	0.090	0.471	0.008	0.000	0.004	1.082	0.336	0.000

B5-2	1.020	1.381	0.074	0.000	0.037	0.078	0.000	1.016	0.029	0.123
B5-3	0.549	3.057	1.020	0.000	0.020	0.143	0.000	0.164	0.066	0.811
B5-4	0.197	1.012	0.070	0.029	0.000	0.070	0.008	0.287	0.406	0.107
B5-5	0.160	1.152	0.148	0.000	0.008	0.000	0.082	0.275	0.094	0.074
mean.B5	0.405	1.353	0.280	0.100	0.015	0.058	0.019	0.565	0.186	0.223
± SD	0.386	1.058	0.415	0.208	0.014	0.060	0.035	0.445	0.172	0.332

S/P ratio ≥ 0.3 = Positive

Table B-3 Mean S/P ratios of PCV2 in-house indirect ELISA with standard error in each age in clinical (A) (n = 15) and non-clinical PCV2 (B) groups (n = 25)

S/P ratio, ± standard error (S.E.)										
Group	**Parity sows**			**Weaned pigs (weeks)**						
	1	**3**	**5**	**3**	**5**	**9**	**13**	**17**	**21**	**25**
A	0.707	0.751	0.828	0.474	0.189	0.099	0.417	0.747[a]	0.680	0.443
± S.E.	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093
B	0.313	0.859	0.565	0.290	0.174	0.162	0.294	0.390	0.517	0.667[b]
± S.E.	0.074	0.074	0.074	0.074	0.074	0.074	0.074	0.074	0.074	0.074

[a,b] Statistically significant differences between group A and B ($p<0.05$)

Table B-4 Mean S/P ratios of PCV2 in-house indirect ELISA with standard error in each age in clinical (A) (n = 15) and subgroup B; B-Vac (PCV2 vaccinated group, n = 10) and B-non-Vac (no PCV2 vaccinated group, n = 15)

S/P ratio, standard error (S.E.)										
Group	**Parity sows**			**Weaned pigs (weeks)**						
	1	**3**	**5**	**3**	**5**	**9**	**13**	**17**	**21**	**25**
A	0.707[a]	0.751	0.828[a]	0.474[a]	0.189	0.099	0.417[a]	0.747[a]	0.680	0.443
± S.E.	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093
B-Vac	0.455	0.742	0.695	0.478[a]	0.403[a]	0.329	0.661[a]	0.363	0.445	0.758
± S.E.	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.114
B-non-Vac	0.219[b]	0.937	0.478	0.164[b]	0.021[b]	0.051	0.050[b]	0.408[b]	0.565	0.606
± S.E.	0.093	0.093	0.093[b]	0.093	0.093	0.093	0.093	0.093	0.093	0.093

Appendix C

Quantitative PCR

Preparation of standard qPCR standard

The DNA concentration from the PCR product obtained from conventional PCR using 356 nucleotide PCV2 primers (ORF1) was measured using a NanoDrop™ spectrophotometer (Thermo Scientific™). The absorbance at 260 nm was used to calculate the concentration of nucleic acids. The amount of genomic DNA was calculated to determine the number of copies of the PCV2 template by the nanogram/microliter converted to copy number/microliter as follows:

$$\textbf{Number of copies} = (\textbf{Amount} \times 6.022\times10^{23}) / (\textbf{Length}*1\times10^{9}*650)$$

(Avogadro's constant = 6.022×10^{23}, length = DNA template length (in bp), average DNA weight of 1 base pair (bp) = 650 daltons)

Website: http://www.uri.edu/research/gsc/resources/endna.html

The number of genomic DNA after calculation was 6.64×10^{10} copy number/microliter. Then the copy DNA number was diluted in a ten-fold dilution series to create the standard curve. In this experiment, the DNA templates of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copy number/microliter were used for standard qPCR (Figure C-1).

Because difference extraction kitsuse different volumes, the results must be calculated a secone time based on the actual volume used in the extraction kit (Invisorb® Spin DNA Extraction Kit) as follows:

$$\textbf{Copies/ml} = (\textbf{Result} \times \textbf{Elution volume}) / \textbf{Sample volume}$$

(sample volume = 200 microliter, elution volume = 100 microliter)

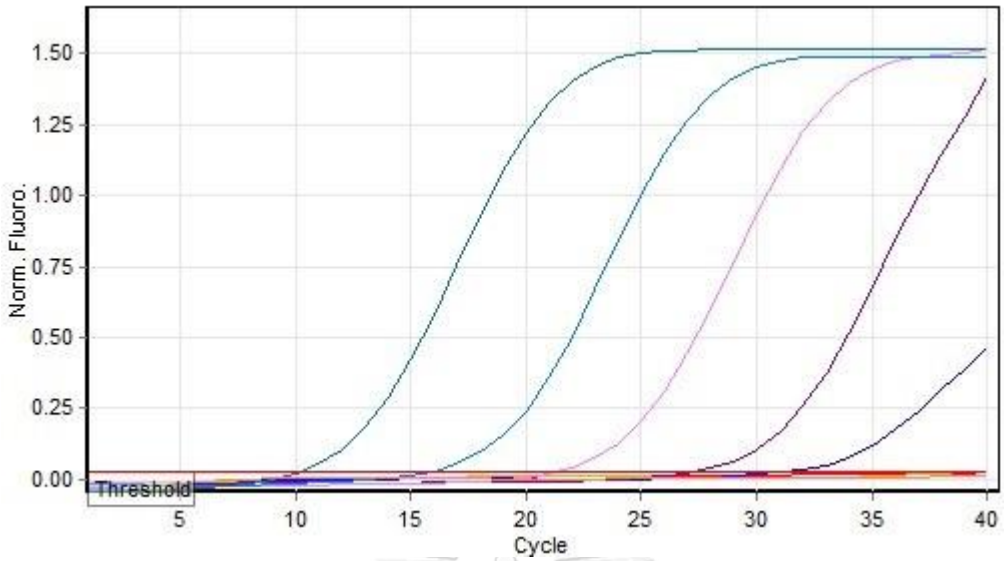

Figure C-1: Quantitation data of standards for Cycling A.Green

Melting curve analysis (T_m)

Melting curve analysis is frequently used as a diagnostic tool for assessing qPCR product length with SYBR Green I dye. In this analysis, a real-time PCR cycler (Roter-Gene® Q, Qiagen) was used to run in the programmed qPCR to produce the melting curve after the amplification cycles were completed. At the end of the qPCR run, the thermal cycler started ramping up the temperature from 65°C to 95°C, rising by 0.5°C in each step and monitoring the fluorescence signal for detection and quantification of the presence of nucleic acid sequences.

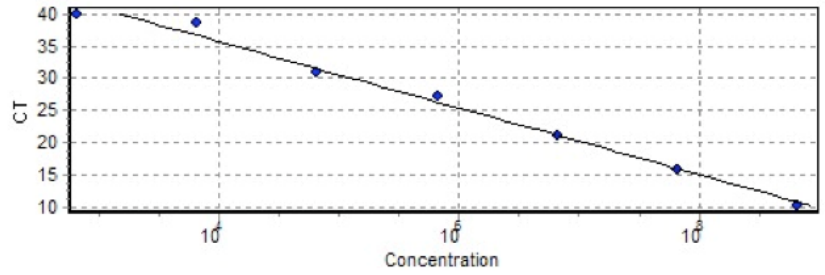

Figure C-2: Standard curve of DNA-based qPCR

From the results, the standard curves had an R^2 value = 0.99 (regression coefficient) which is considered an acceptable qPCR efficiency (Figure C-2). The correct PCR product showed a single dissociation peak at high temperature as shown in Figure C-3. On the other hand, the non-specific product (artifact) or primer-dimer were longer

or shorter than the specific product. Therefore, the melting curve analysis of qPCR product was used to confirm the desired product. In this study, the melting peak was observed at approximately 81.65 °C.

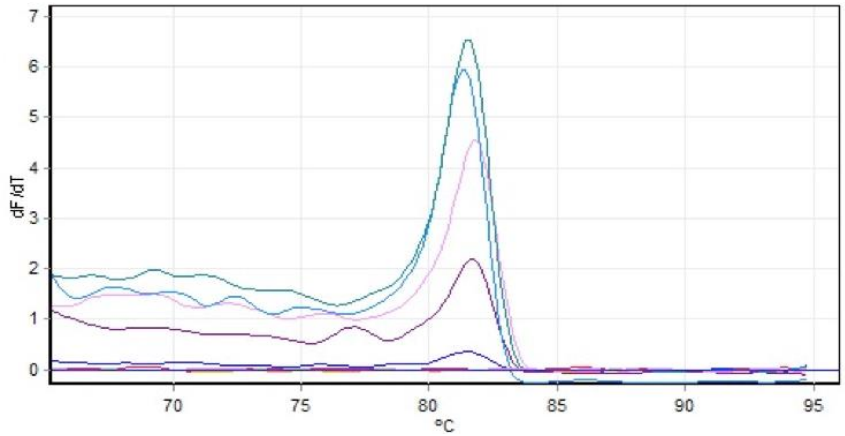

Figure C-3: Melting point of products generated during qPCR amplification using PCV2 ORF1 primers

Table C-1 Mean log_{10} levels of PCV2 DNA titers per mL serum and **mean ± SD** by real-time PCR in herds A (clinical) and B (non-clinical).

	PCV2 titers (log DNA copy/mL), mean ± SD									
Herd	**Parity sows**			**Weaned pigs (weeks)**						
	1	**3**	**5**	**3**	**5**	**9**	**13**	**17**	**21**	**25**
A1	4.41	4.60	0	4.53	0	4.14	4.19	7.14	4.22	4.03
± SD	0.000	0.000	0.000	0.000	0.000	0.005	0.009	2.463	0.005	0.000
A2	5.51	0	5.01	5.83	5.48	5.06	7.41	5.72	5.68	5.29
± SD	0.004	0.000	0.000	0.116	0.092	0.057	0.312	0.084	0.017	0.024
A3	5.49	5.51	5.29	5.49	5.61	6.22	7.08	7.74	7.08	6.35
± SD	0.099	0.152	0.093	0.011	0.069	0.057	0.481	2.058	0.202	0.213
B1	5.35	5.22	4.89	5.37	5.25	5.48	5.35	5.53	5.27	5.71
± SD	0.097	0.056	0.000	0.009	0.018	0.171	0.114	0.017	0.027	0.052
B2	4.71	4.30	0	4.24	4.76	3.84	4.81	4.80	4.90	4.93
± SD	0.021	0.000	0.000	0.000	0.005	0.000	0.017	0.003	0.013	0.019
B3	6.09	6.08	5.69	5.84	0	5.15	4.90	5.48	6.10	6.71
± SD	0.058	0.253	0.015	0.163	0.000	0.050	0.000	0.165	0.332	0.165
B4	6.02	5.91	5.90	5.42	5.39	5.73	5.71	5.60	6.50	6.57
± SD	0.063	0.053	0.153	0.131	0.083	0.120	0.011	0.000	0.308	0.491
B5	6.23	6.16	6.08	5.46	5.89	5.74	6.25	6.72	5.87	5.90
± SD	0.208	0.060	0.159	0.107	0.295	0.000	0.415	0.125	0.058	0.175

0 = Not detected

Table C-2 Mean of $\log_{10}$ (levels of PCV2 DNA copy number per mL) in serum and mean ± S.E. by qPCR in group A (clinical PCV2) (n=5) and B (non-clinical PCV2) (n=5).

Mean PCV2 titers (log DNA copy/mL), mean ± S.E.										
Group	Parity sows			Weaned pigs (weeks)						
	1	3	5	3	5	9	13	17	21	25
A	5.137	3.370	3.433	5.283	3.697	5.140	6.227	6.867	5.660	5.223
± S.E.	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874
B	5.572	5.405	4.168	5.189	4.383	5.100	5.350	5.549	5.621	5.857
± S.E.	0.691	0.691	0.691	0.691	0.691	0.691	0.691	0.691	0.691	0.691

Table C-3: Mean of $\log_{10}$ (levels of PCV2 DNA copy number per mL) in serum and mean ± S.E. by qPCR in group A and subgroup B (B-Vac; vaccinated and B-non-Vac; non-vaccinated).

Mean PCV2 titers (log DNA copy/mL), mean ± S.E.										
Group	Parity sows			Weaned pigs (weeks)						
	1	3	5	3	5	9	13	17	21	25
A	5.137	3.370[a]	3.433	5.283	3.697	5.140	6.227	6.867	5.660	5.223
± S.E.	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874
B-Vac	4.805	5.005	4.560[a]	0.013	0.012	0.016	5.080	5.165	5.085	5.320
± S.E.	1.070	1.070	1.070	1.070	1.070	1.070	1.070	1.070	1.070	1.070
B-non-Vac	6.113	6.050[b]	5.890[b]	5.573	3.760	5.540	6.113	5.933	6.157	6.393
± S.E.	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874	0.874

[a,b] Differences letters mean significantly differences (p<0.05)

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