ประสิทธิภาพในการลดรอยโรคทางพยาธิวิทยา ด้วยวัคซีนเซอร์โคไวรัสชนิดที่ 2 ชนิดเชื้อตาย ในภาคสนาม

นายเติมสิทธิ ปภาวสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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A FIELD TRIAL ON EFFICACY OF KILLED PCV-2 VACCINE

IN DECREASING PATHOLOGICAL LESIONS

Mr. Termsitthi Paphavasit

A Thesis Submitted in Partial Fulfillment of the Requirements

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เดิมสิทธิ ปภาวสิทธิ์ : ประสิทธิภาพในการลดรอยโรคทางพยาธิวิทยา ด้วยวัคซีนเซอร์โคไวรัสชนิดที่ 2 ชนิดเชื้อตาย ในภาคสนาม. (A FIELD TRIAL ON EFFICACY OF KILLED PCV2 VACCINE IN DECREASING PATHOLOGICAL LESIONS) อ.ที่ปรึกษา: รศ.น.สพ.ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาร่วม: อ.น.สพ.ดร. คมกฤช เทียนคำ, จำนวนหน้า 59 หน้า

Suvaxyn® PCV2 (Fort Dodge Animal health, USA) เป็นวัคซีนเซอร์โคไวรัสชนิดที่ 2 ชนิดเชื้อตายผลิตโดยเทคโนโลยีการตัดต่อโครงสร้างของเซอร์โคไวรัสชนิดที่ 2 เข้าไปในโครงสร้างของเซอร์โคไวรัสชนิดที่ 1 ที่ไม่ก่อโรคในสัตว์ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อทดสอบประสิทธิภาพในการลดรอยโรคทางพยาธิวิทยาและภาวการณ์ติดเชื้อในกระแสเลือดในฟาร์มสุกรที่พบปัญหาการติดเชื้อเซอร์โคไวรัสในประเทศไทย ทำการคัดเลือกฟาร์มสุกรขนาด 3,200 แม่โดยอาศัยประวัติความสูญเสียจากการติดเชื้อเซอร์โคไวรัสชนิดที่ 2 ในอดีตในช่วง 1 ปีที่ผ่านมา ผลการศึกษาทางซีรัมวิทยาและผลการชันสูตรซาก ลูกสุกรหย่านมอายุ 3 สัปดาห์ จำนวน 200 ตัว แบ่งกลุ่มออกเป็น 2 กลุ่ม A และ B โดยทำการสุ่มแบ่งแต่ละคอกๆ ละ 10 ตัวจำนวน 20 คอกทำการสลับคอกในแต่ละกลุ่มในโรงเรือนเดียวกันที่อายุ 4 สัปดาห์ ฉีดวัคซีนชนิดเชื้อตาย Suvaxyn® PCV2 ปริมาณ 2 มิลลิลิตรเข้ากล้ามเนื้อขณะที่สุกรกลุ่มควบคุมได้รับการฉีดน้ำเกลือปริมาณ 2 มิลลิลิตร เจาะเลือดสุกรทดลองจำนวน 2 ตัว/คอก รวมเป็นกลุ่มละ 20 ตัวอย่าง ในช่วงอายุ 4, 5, 7, 9, 12 และ 15 สัปดาห์ เพื่อศึกษาทางซีรัมวิทยาและตรวจหาเชื้อเซอร์โคไวรัสชนิดที่ 2 โดยวิธีปฏิกิริยาลูกโซ่โพลิเมอร์เรส ชันสูตรซากสุกรจำนวน 20 ตัวในแต่ละกลุ่มเมื่อสุกรอายุ 16 สัปดาห์ จากผลการทดลองพบว่าระดับการตอบสนองทางภูมิคุ้มกันต่อเชื้อเซอร์โคไวรัสชนิดที่2 ในสุกรทั้งสองกลุ่มสูงในช่วงอายุ 4 สัปดาห์และเริ่มลดลงในช่วง 5 ถึง 7 สัปดาห์ ซึ่งน่าจะเป็นผลจากการลดลงของภูมิคุ้มกันถ่ายทอดจากแม่สุกร แต่เมื่อสุกรมีอายุ 9 สัปดาห์สุกรกลุ่มที่ได้รับวัคซีนมีการตอบสนองของระดับภูมิคุ้มกันที่สูงขึ้น ในขณะที่สุกรกลุ่มควบคุมที่ไม่ได้รับวัคซีนจะมีการตอบสนองของระดับภูมิคุ้มกันที่ช้ากว่าโดยเริ่มที่ 12 สัปดาห์ซึ่งการตอบสนองในกลุ่มที่ได้รับวัคซีนน่าจะเป็นผลมาจากการได้รับวัคซีนชนิดเชื้อตายเมื่ออายุ 4 สัปดาห์ ขณะเดียวกันผลการตรวจหาเชื้อเซอร์โคไวรัส ชนิดที่ 2 ในซีรัมพบว่าสุกรกลุ่มที่ได้รับวัคซีนตรวจไม่พบเชื้อเซอร์โคไวรัส ชนิดที่ 2 จนถึงอายุ 15 สัปดาห์ แต่ในสุกรกลุ่มควบคุมสามารถตรวจพบเชื้อเซอร์โคไวรัส ชนิดที่ 2 ได้ในทุกกลุ่มอายุ การศึกษาน้ำหนักสัมพัทธ์ของต่อมน้ำเหลืองพบว่าในสุกรกลุ่มที่รับวัคซีนมีแนวโน้มที่ค่าน้ำหนักสัมพัทธ์ของต่อมน้ำเหลือง (38.5×10^{-5}) จะน้อยกว่าสุกรกลุ่มควบคุม (45.4×10^{-5}) นอกจากนี้รอยโรคทางจุลพยาธิวิทยาของต่อมน้ำเหลืองในสุกรที่ได้รับวัคซีนมีระดับความรุนแรงน้อยกว่าสุกรกลุ่มควบคุมที่ไม่ได้รับวัคซีน จากผลการทดลองพบว่าวัคซีนชนิดเชื้อตาย เซอร์โคไวรัส ชนิดที่ 2 มีประสิทธิภาพในการลดการติดเชื้อในเลือดของสุกรและมีแนวโน้มในการลดความรุนแรงของรอยโรคทางพยาธิวิทยาได้เมื่อทำการทดสอบในภาคสนาม

ภาควิชา พยาธิวิทยา
สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: PORCINE CIRCOVIRUS TYPE2/ CHIMERIC VACCINE/ EFFICACY / LYMPHOID DEPLETION/ VIREMIA

TERMSITTHI PAPHAVASIT: A FIELD TRIAL ON EFFICACY OF KILLED PCV2 VACCINE IN DECREASING PATHOLOGICAL LESIONS. THESIS ADVISOR: ASSOC.PROF. ROONGROJE THANAWONGNUWECH, D.V.M., PH.D., THESIS CO-ADVISOR: KOMKRICH TEANKUM, D.V.M., DR.MED.VET, 59 pp

Suvaxyn® PCV2 (Fort Dodge Animal health, USA) is the Chimeric PCV1-2 vaccine containing immunogenic capsid gene cloned of PCV2 into the backbone of the nonpathogenic PCV1. The objective of this study was to investigate the efficacy of Suvaxyn® PCV2 on decreasing pathological lesions and PCV-2 viremic condition in a PCVAD-affected herd in Thailand. A PCVAD-affected herd (3,200-sow herd) was selected by previous history, necropsy reports and serology. Two hundred 3-week-old weaners were equally divided into two groups: A and B. At 4 weeks of age, group-A pigs were vaccinated with 2 ml of Suvaxyn® PCV2 vaccine, whereas, pigs in group B were injected with 2 ml of normal saline. Serum samples were collected from 20 pigs per group at 4, 5, 7, 9, 12 and 15 weeks of age for serological examination (2 pigs/pen), and polymerase chain reaction (PCR). The average serological titers were high at 4 weeks of age and then declined at about 5 weeks in both groups indicating the waning of the maternal derived antibodies between 5 and 7 weeks old. The seroconversion was observed in vaccinated pigs at 9 weeks of age suggesting of vaccination-induced antibody titers. In non-vaccinated pigs, PCV-2 seroconversion was detected at 12 weeks of age, probably due to the natural PCV2-infection after weaning. None of PCV2 DNA was detected in vaccinated pigs before 15 weeks of age, while it was detected in the sera of non-vaccinated pigs at every time point. The average of lymph node/body weight ratio in vaccinated pigs ($38.5x10^{-5}$) was lower than those in non-vaccinated pigs ($45.4x10^{-5}$), but it was not statistically significant. Histopathologically, lymph nodes had less severe lesions in the vaccinated pigs. The results suggest that Suvaxyn® PCV2 is able to induce PCV2 antibody and subsequently, reduce PCV2 viremia and pathological lesions.

Department: Pathology Student's signature: T Paphavasit

Field of study: Veterinary pathobiology Advisor's signature: .....

Academic year: 2007 Co-advisor signature: .....

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LIST OF ABBREVIATIONS

AASV	American association of swine veterinarians
ADWG	Average daily weight gain
AMs	Alveolar macrophages
BCS	Body condition scores
BSA	Bovine serum albumin
cap gene	Capsid gene
CBC	Complete blood count
CpG-ODNs	Cytosine-phosphorothioate-guanin Oligodeoxynucleotides
ºC	Degree Celsius
DAB	3,3'-diaminobenzidine-4HCl
DC	Dendritic cells
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FFPE	Formalin-fixed paraffin-embedded
g	Gram (s)
HR	Histiocytes replacement
IHC	Immunohistochemistry
IFN	Interferon
kg	Kilogram (s)
LD	Lymphoid depletion
LN	Lymph node
µl	Microlitre
min	Minutes
n	Number of samples
NK cells	Natural killer cells

nm	Nanometre
ORF	Open reading frame
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus type 1
PCV2	Porcine circovirus type 2
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Postweaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
rep gene	Replication gene
s	Seconds
SIV	Swine influenza virus
Th lymphocytes	Helper T lymphocytes
TLRs	Toll-like receptor
TNF	Tumor necrotic factor
TUNEL	Terminal deoxynucleotidyl tranferase-Mediated dUTP-nick end labeling
US	United State of America
wks	Weeks

CHAPTER I

INTRODUCTION

Porcine circovirus (PCV) belong to the family *Circoviridae* is a small non-enveloped virus with a capsid size of 17 nm. Porcine circovirus type 1 (PCV1) and type 2 (PCV2) are widespread in commercial swine populations worldwide. However, PCV1 does not produce clinical disease and is generally considered to be non-pathogenic. In contrast, PCV2 is the causative agent of postweaning multisystemic wasting syndrome (PMWS), a multi-factorial new emerging disease in swine (Mankertz et al., 2004). PCV2 has also been associated with several pathological conditions in pigs including porcine dermatitis and nephropathy syndrome (PDNS), reproductive failures, porcine respiratory disease complex (PRDC), proliferative and necrotizing pneumonia and congenital tremor (Segales et al., 2004). PMWS or currently known as porcine circovirus associated disease (PCVAD) is a disease of pigs first recognized in the North America in 1991. Later, PMWS has been reported worldwide in the swine raising areas especially in Europe and Asia (Allan and Ellis, 2000; Fenaux et al., 2004; Chae, 2005). Clinical signs of PMWS include progressive weight loss, dyspnea, enlargement of superficial inguinal lymph nodes, pallor, jaundice and diarrhea (Darwich et al., 2004; Segales et al., 2004).

In Thailand, a retrospective study on PCV2 antigen detection using immunohistochemistry found the first PCV-infected cases as early as in 1993. Later, using formalin-fixed, paraffin-embedded (FFPE) tissues between 2000-2002 demonstrated 38.76 % (50/129) having PCV2 infection in suspected cases. In addition, the presence of PCV-2 antigen was primarily seen about 40.70% in the lymph nodes (Banlunara et al., 2002). Recent data based on swine diagnosis annual report in 2006 from the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University found an increasing incidence of PMWS and PDNS in the swine systemic infection cases (58.23 %, n = 79). Therefore, this indicated that PCV2 is one of the major causative agents in the Thai swine industry.

Killed PCV2 vaccine (Suvaxyn® PCV2, Fort Dodge Animal health, USA) is a Chimeric PCV1-2 vaccine containing immunogenic capsid gene cloned of PCV2 into the backbone of the nonpathogenic PCV1 (Fenaux et al., 2004). Vaccination with the Chimeric PCV1-2 significant demonstrated reducing viremia and decreasing risk of clinical diseases experimentally (Fenaux et al., 2004). Suvaxyn® PCV2 when administered 1-shot to 3 week-old pigs was also able to prevent PCV2 viremia experimentally. It was able to prevent the development of microscopic lesions in the lymphoid tissues when pigs were challenged 4 months after vaccination (Urniza et al., 2006). However, swine farms in Thailand had different conditions and management from other countries. The field trial in Thailand should be performed before the implementation of PCV vaccine in the farm management.

The objective of this study was to investigate the efficacy of Suvaxyn® PCV2 on pathological lesions and viremic condition in a PCV2-affected farm in Thailand.

CHAPTER II

REVIEWS OF THE LITERATURES

Porcine circovirus associated disease (PCVAD)

A. Histological background and Etiology

Porcine circovirus (PCV), a small, nonenveloped, single-stranded DNA virus with a circular genome, was first recognized as a contaminant of the continuous porcine kidney cell line PK-15 (ATCC CCL31) in 1974 (Allan and Ellis, 2000). In the late 1990s, a variant strain of PCV was associated with a newly emerged disease syndrome in pigs which became known as postweaning multisystemic wasting syndrome (PMWS). After the description of PMWS, new studies were shown that two types of PCV existed, PCV1 and PCV2. PCV1 does not produce clinical disease and PCV1 is thus generally considered to be non-pathogenic. In contrast, PCV2 is finally proved to be the causative agent of PMWS by many researchers (Allan et al., 1998; Meehan et al., 1998; Allan and Ellis, 2000).

Both PCV1 and PCV2 belong to the family *Circoviridae*. The *Circoviridae* family contains two genera. The *Gyrovirus* genus is represented by chicken anemia virus (CAV). The *Circovirus* genus contains porcine circovirus (PCV), beak and feather disease virus (BFDV) and Columbid circovirus of pigeons (Mankertz et al., 2000; Chae, 2004). Viruses belonging to the *Circoviridae* family have characteristic virions that exibit icosahedral symmetry and lack of an envelope. The genomes are covalently closed, circular, single strand DNA molecules which range in size from 1.8 to 2.3 kilobase (kb). The genomes of PCV1 and PCV2 showed a high degree of homology. The overall DNA sequence homology between PCV1 and PCV2 is 76% (Fenaux et al., 2000). PCV2 genome contains six open reading frames (ORFs). The three major structure is ORF1 encoding a replication protein (rep protein) essential for replication viral DNA. ORF2 encodes major structural immunogenic capsid protein (Nawagitgul et al., 2000). ORF3 is essential for virus-induced apoptosis (Liu et al., 2006). PCV1 and PCV2 are widespread in commercial swine populations worldwide (Allan and Ellis, 2000). Phylogenetic analyses of PCV1, avian circovirus, plant geminiviruses and nanoviruses classified PCV1 as the most closely related to the BFDV and were intermediated between the two plant viral groups. Furthermore, it has been proposed that predecessor of PCV1 and BFDV may have originated from a plant

nanovirus (Opriessnig et al., 2007). Nevertheless, phylogenetic analysis of the genome clearly indicated that PCV2 sequences can be divided into two major groups. Both PCV2 groups are homogeneous and have several marker positions, mainly located in the *cap* gene. PCV2 group 1 and PCV2 group 2 can be further divided into clusters. PCV2 group 1 can be divided into 3 clusters (1A-1C) and PCV2 group 2 can be divided into 5 clusters (2A-2E). Interestingly, genomes of group 1 were mainly published in NCBI after 2003 (87 out of 94) and genomes of group 2 were mainly published before 2003 (33 out of 53), indicating that group 1 could be more recent than those of group 2 (Olvera et al., 2007). At the end of 2004, the swine industry in the province of Quebec, Canada experienced a significant increase in mortality rate related to PCV2. Concurrently, many North American laboratories started to group PCV2 field isolates as North American-like isolates or PCV2a and as European-like isolates or PCV2b (Gagnon et al., 2007). Historically, many swine herds in the U.S. have been infected with the PCV2a genotype. However, since 2005, PCV2b has been identified in North America. The construction of phylogenetic trees using whole genome sequences from diagnostic submissions at Kansas Veterinary Diagnostic Laboratory showed that one isolate, 0737A, was only loosely associated with other PCV2b isolates. Analysis of the variable sites between representative PCV2a and PCV2b DNA sequences and the 0737A sequence, showed that 0737A was a mosaic sequence, with the ORF1 region from PCV2a and ORF2 from PCV2b. This study demonstrates that pigs can be naturally infected with multiple PCV2 genotypes and that PCV2a/PCV2b recombination events might occur in the field (Hesse et al., 2008).

B. Postweaning multisystemic wasting syndorme (PMWS)

Postweaning multisystemic wasting syndrome (PMWS) or currently known as porcine circovirus associated disease (PCVAD) is a disease of pigs first recognized in North America in 1991 caused by porcine circovirus type 2. PMWS has been reported worldwide in North America, Europe and Asia (Allan and Ellis, 2000; Fenaux et al., 2004; Chae, 2005). PMWS primarily affects pigs between 25 and 120 days of age, with most cases occurring between 60 and 80 days of age (Kim et al., 2002).

Clinical signs of PMWS are nonspecific and variable in both field and experimental observations (Chae, 2005). In weaned pigs, PMWS is characterized by progressive weight loss,

dyspnea, enlargement of superficial inguinal lymph nodes, pallor, jaundice, diarrhea and marked increase in mortality rate from single or multiple concurrent bacterial infections (Kim et al., 2002; Darwich et al., 2004; Segales et al., 2004). PMWS is oftenly seen in combination with other viruses or bacteria such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), *Haemophilus parasuis (H. parasuis)* , *Actinobacillus pleuropneumoniae*(APP), *Streptococcus suis (S.suis)* and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) (Kim and Chae, 2002; Pallares et al., 2002; Rovira et al., 2002; Chae, 2004). Furthermore, the prevalence of co-infections appears to vary in different countries. PRRSV and PPV are the most common pathogens co-infected with PCV2 in the Republic of Korea. In contrast, PPV in the United States is less common (Kim et al., 2002; Pallares et al., 2002). *H. parasuis* was the most prevalent bacterial co-infection in Korea, while *S. suis* is the most prevalent co-infection in the US (Kim et al., 2002; Pallares et al., 2002). The fact that many different infectious agents are isolated in cases of PMWS strongly supports the view that a variety of pathogens may share a common mechanism in affecting the immune system and allow progression of PCV2 infection to PMWS (Krakowka et al., 2001; Krakowka et al., 2002; Chae, 2004). Alternatively, PCV2 induces lymphoid depletion in the lymphoid organs, resulting in an increased susceptibility to other viral or bacterial infection. This immunosuppression has been confirmed in the PMWS-affected pigs (Darwich et al., 2003). However, many non-infectious factors are also involved in PMWS pathogenesis. PMWS is able to reproduce in PCV2-inoculated piglets which were immunostimulated with keyhole limpet hemocyanin in incomplete Freund's adjuvant (Krakowka et al., 2002). However, recent reports demonstrated that not all adjuvants used in the commercial swine vaccines are capable to trigger the mechanisms for PMWS (Resendes et al., 2004).

Generally, pigs with PMWS are wasting with or without respiratory signs, diarrhea, paleness of the skin, icteric mucous membrane, generalized lymphadenopathy and lack of lung collapse (Chae, 2004; Darwich et al., 2004). Less frequently, PMWS-affected pigs show hepatitis and nephropathy lesions (Rosell et al., 2000; Chae, 2005). Ulceration of pars esophageal part of

stomach has been reported in the affected pigs. Gastric ulcer is correlated to paleness of carcasses and anemic condition in PMWS affected pigs (Darwich et al., 2003). Histopathologiccally, typical lesions of lymphoid tissues in PMWS affected pigs are characterized as (i) granulomatous inflammation with variable degree of lymphoid depletion and (ii) the presence of intracytoplasmic inclusion bodies. Intracytoplasmic inclusion bodies are large, multiple, basophilic to amphophilic grape-likes structure in cytoplasm of histiocytes (Chae, 2004; Darwich et al., 2004). The granulomatous lymphadenitis is characterized by infiltrates of histiocytes, epithelioid cells and multinucleated giant cells. The granulomatous inflammation could be observed in the lymph nodes, liver, spleen, tonsil, thymus, lung and Peyer's patches, but it occurs consistently in superficial inguinal lymph nodes (Chae, 2004). Lymph nodes and Peyer's patch showed depletion and coagulative necrosis of the follicular centers. However, the presence of intracytoplasmic inclusion bodies may be not present in all PMWS cases. Only 27.8% of PMWS examined in Korea had inclusion bodies (Kim et al., 2002). In contrast, 97% of PMWS cases examined had granulomatous inflammation in the lymphoid tissues. The presence of granulomatous inflammation is therefore very useful as an indicator of PMWS diagnosis (Kim et al., 2002; Chae, 2004). Features of the histopathological lesions suggest that monocytes/macrophages infiltration may closely relate to the pathogenesis and progression of PMWS (Chae, 2004; Chae, 2005). The characteristic granulomatous inflammatory lesions are immune mediated (Krakowka et al., 2002). It has been suggested that monocyte chemoattractant protein-1 (MCP-1) expression may play a role in the pathogenesis of granulomatous inflammation in PMWS affected pigs (Kim and Chae, 2003). A correlation between the presence of MCP-1 by mononuclear cells in response to PCV2, indicates that PCV2 plays an important role in initiating granulomatous inflammation (Kim and Chae, 2003). Hepatic lesions are characterized by lymphocytes and histiocytes infiltration in portal area, some hepatocellular vacuolation and swelling with perilobular fibrosis (Rosell et al., 2000; Chae, 2004). In kidneys, there are multifocal lymphohistiocytic interstitial nephritis and pyelitis. The inflammatory foci are surrounded by zone of fibroblast proliferation (Chae, 2004). A hallmark of pulmonary lesions are characterized by moderate thickening of the alveolar septa due to the infiltration of mononuclear

cells (primarily macrophages and lymphocytes and occasionally multinucleated giant cells) and pneumocyte type II hyperplasia (Kim et al., 2002; Chae, 2004).

PMWS caused by PCV2 should be differentiated from PRRSV infection. Both PMWS and PRRSV are characterized by lymphohistiocytic interstitial pneumonia (Halbur et al., 1995; Chae, 2004). Depletion of lymphoid tissues and replacement by macrophages and multinucleated giant cells are the hallmark of PMWS (Kim et al., 2002; Kim et al., 2003; Darwich et al., 2004; Chae, 2005). Generally, PRRSV induces marked follicular hyperplasia of lymphoid tissues (Halbur et al., 1995).

Diagnostic criteria of PMWS should include (i) the presence of compatible clinical signs (ii) the presence of characteristic microscopic lesions, and (iii) the presence of PCV2 within these lesions (Chae, 2004). Since clinical signs of PMWS are nonspecific and variable, the presence of PCV2 DNA or antigen in lymphoid tissues, together with lymphoid depletion and/or granulomatous inflammation are also used as the criteria for the PMWS diagnosis (Chae, 2004).

C. Porcine circovirus associated disease (PCVAD)

Currently, PMWS described only a portion of the PCV2-associated diseases. PCV2 has also been associated with a number of pathological conditions of pigs, including porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex (PRDC), proliferative and necrotizing pneumonia and congenital tremor (Segales et al., 2004). In March 2006, the American Association of Swine Veterinarians (AASV) proposed the name of porcine circovirus associated diseases (PCVAD) to describe the different diseases attributed to porcine circovirus. It has recently been hypothesized that different types of PCV2 may be responsible for different disease presentations. Several studies have suggested that PCV2 isolated from reproductive failure and PDNS may be phenotypically or genetically different from PCV2 associated with PMWS (Meehan et al., 2001; O' Connor et al., 2001). However, PCV2 isolates from different clinical disease manifestations have been sequenced and all are highly homologous (overall >90–96%). Most of these studies have found minor differences in the

respective PCV genomes (Meehan et al., 2001; O'Connor et al., 2001). Although PMWS is considered the major disease presentation of PCV2 infection, a number of other disorders have been linked to infection with this virus and some of these should be considered as porcine circovirus associated diseases (PCVADs).

PCV2 is now recognized as a causal agent of reproductive disorders in pigs (West et al., 1999). The case definition for PCV2-associated reproductive problems should include three main criteria : (i) abortions and/or stillbirths and/or mummified fetuses (ii) the presence of fetal heart lesions characterized by extensive fibrosing and/or necrotizing myocarditis (iii) the presence of PCV2 in the myocardial lesions and other fetal tissues (Allan and McNeilly, 2006). Furthermore, PCV2 antigen has been demonstrated abundantly in lung lesions from pigs with proliferative and necrotizing pneumonia (Allan and Ellis, 2000), and PCV2 is also considered to associated with porcine respiratory disease complex (PRDC) (Kim et al., 2003; Segales et al., 2004).

Recent field investigations (Kim et al., 2003; Segales et al., 2004) and case trend analysis at the US diagnostic laboratories (Opriessnig et al., 2007) suggest that PCV2 may play an important role in the porcine respiratory disease complex (PRDC). PRDC is a condition observed mainly in 8- to 26-week-old pigs and is associated with multiple respiratory pathogens including PRRSV, SIV, and *M. hyopneumoniae*. PRDC is characterized by decreased rate of growth, decreased feed efficiency, anorexia, fever, cough and dyspnea. There may be diagnostic overlap between PMWS and PCV2-associated respiratory disease. The presence of prolonged and unusually severe clinical respiratory disease, granulomatous bronchointerstitial pneumonia with bronchiolitis and bronchiolar fibrosis, and abundant PCV2 antigen associated with the lesions is suggestive that PCV2 definitely plays a role in the PRDC problem. PCV2-associated pneumonia reported in the cases of PMWS is characterized by lymphohistiocytic to granulomatous interstitial pneumonia, peribronchiolar fibroplasia and mild-to severe necrotizing and ulcerative bronchiolitis (Chae, 2005). The PCV2-associated bronchiolitis lesions can resemble to those induced by swine influenza virus or porcine respiratory coronavirus (Opriessnig et al., 2007). It is currently not possible to definitively outline the role of PCV2 infection in some of these disease complexes as

experimental reproduction of the diseases has not been carried out with an inoculums containing PCV2 (Allan and Mcneilly, 2006).

PDNS is often fatal disease that primarily affects recently weaned and feeder pigs from 1.5 to 4 months of age (Smith et al., 1993). The syndrome was first recognized in the UK in 1993 (Smith et al., 1993). Since then, it has been reported in several countries including Korea and North American countries (Rosell et al., 2000; Choi and Chae, 2001). PDNS is generally sporadic. In fatal cases, cutaneous lesions consist of severe necrotizing vasculitis affecting the dermis and subcutis, characterized by leukocytoclastic (the presence of neutrophils with nuclear fragments) inflammation involving capillaries, small and medium sized venules and arterioles, accompanied by epidermal necrosis and ulceration and dermal hemorrhage (Choi and Chae, 2001; Chae, 2005). Significant gross lesions are present mostly in the skin and kidneys of swine with PDNS, although other organs may also be affected (Choi and Chae, 2001). Gross skin lesions consist of multiple rounded to irregularly shaped red to purple macules and papules that coalesce over the perineum and distal limbs to form large irregular patches. The skin lesions are usually first noted over the hind-quarters, limbs and abdomen but may progress to involve the thorax, flank or ears. The kidneys are enlarged and have pale cortices with multiple red circular haemorrhagic cortical foci measuring 2-4 mm. in diameter. Renal and inguinal lymph nodes are usually enlarged and red (Ramos-Vara et al., 1997). Microscopically, the most significant lesion is the presence of severe, fibrinoid, necrotizing vasculitis in the dermis, subcutis, kidney, lymph nodes, stomach, spleen and liver. Other renal lesions consist of exudative glomerulonephritis and interstitial nephritis. In lymph nodes, there is lymphoid depletion and occasional necrosis of lymphocytes in both the cortex and paracortex. Numerous multinucleated giant cells are often scattered in the cortex and paracortex. Positive hybridization signals for PCV2 can be detected in renal tubular epithelial cells, fusiform interstitial cells, and macrophage-like cells. These cells usually localized around vessels of the renal pelvis and among infiltrating non-positive mononuclear cells in the interstitium of the renal cortex and medulla (Choi and Chae, 2001). Distinct positive labelling has been found scattered throughout the cortex and paracortex of the

lymph node (Choi and Chae, 2001; Choi et al., 2002). The vasculitis occurring in PDNS is though to be associated with an immune-mediated mechanism (Rosell et al., 2000).

D. Pathogenesis and Immunology of PCV2 infection

PCV2 is widespread in most pig populations and has repeatedly confirmed by several serological surveys. Infection with the virus appears to induce an antibody-mediated immunity. Certainly, PCV2-infected pigs developing PCVAD or PMWS would suggest an immunopathological disorder. Interestingly, the enlargement of lymph nodes and lymphoid depletion in lymphoid organs are normally seen in PCVAD-affected pigs (Rosell et al., 2000; Chae, 2004; Darwich et al., 2004; Krakowka et al., 2005). The development of leukopenia is also the character of PCVAD-affected pigs in the fields (Segales et al., 2001; Darwich et al., 2004). Nielsen et al. (2003) reported leukopenia in PMWS-affected pigs. The leukopenia can be identified early post-infection, particularly with the B lymphocytes, followed by the T lymphocytes. The depletion of memory Th lymphocytes is particularly discernible. Naïve Th lymphocytes, cytotoxic T lymphocytes, gd T lymphocytes, Natural killer cells and mature granulocytes are also affected. To disclose the mechanism of cellular injury by PCV2, Shibahara et al. (2000) examined lymphoid tissue for the apoptosis of lymphocytes by the terminal deoxynucleotidyl tranferase-mediatrd dUTP-nick end labeling (TUNEL) method and immunohistochemistry (IHC). The study showed marked apoptosis of lymphocytes, lymphocytes depletion and macrophages with multinucleated giant cells containing numerous PCV2 inclusion bodies with or without apoptotic bodies. The immunohistochemical detection showed many lysozyme-positive macrophages in the lymphoid follicle but CD79a-positive B lymphocytes were scanty. PCV2 antigens were found mainly in the cytoplasm of macrophages and also in the nuclei of macrophages and apoptotic lymphocytes. These results suggest that lymphoid depletion and apoptotic cells death of B lymphocytes caused by PCV2 infection. This hypothesis was also supported by Kiupel et al. (2005), who found that PCV2 replicated and associated with apoptosis in spleens, lymph nodes and Peyer ‘s patches of infected BALB/c mice. However, some studies seem to contradict with this hypothesis. Resendes et al. (2004) reported the lower levels of

apoptotic cells observed in B lymphocytes area of PMWS-affected pigs when compared to healthy pigs. Moreover, Mandrioli et al. (2004) stated that apoptosis did not seem important in the pathogenesis of cell depletion in PMWS-affected pigs. The results of this study showed that the apoptotic index of PMWS cases was lower than controls both in the lymphoid follicle and medulla-like tissue. From the results the authors suggested that the lymphoid depletion in PMWS-affected pigs is mainly related to decrease proliferative activity in lymphoid tissue and a long-standing absence of lymph nodes positive growth factors (mainly cytokine) produces by lymphocytes activation. It is therefore quite likely that the cell death observe in PMWS is not due to PCV2-induce apoptosis but occurring by indirect effect (Allan and Ellis, 2000).

Based on histopathological and immunohistochemical studies, detection of PCV2 antigen is mainly found in the cytoplasm of macrophages, multinucleated giant cells and other monocytes/macrophage lineage cells such as pulmonary alveolar macrophages, Kupffer cells and follicular dendritic cells (Allan and Ellis, 2000; Gilpin et al., 2003). Furthermore, it is possible to detect the virus antigen in the cytoplasm of the renal and pulmonary epithelial cells, vascular endothelial cells, lymphocytes, smooth muscle cells, hepatocytes and enterocytes (McNeilly et al., 1999; Rosell et al., 2000; Sirinarumitr et al., 2000; Darwich et al., 2004). However, in PCVAD-affected pigs, PCV2 antigen-positive lymphocytes are rarely seen (Rosell et al., 1999; Shibahara et al., 2000; Nielsen et al., 2002). The cellular distribution of PCV2 related with the age of pigs. Fetuses inoculated with PCV2 at 57 days of gestation had high amounts of virus in cardiomyocytes. Virus could also be found in macrophages and hepatocytes. At 1 day after birth, virus was found mostly in macrophages and also found in T lymphocytes (Sanchez et al., 2003). These data suggest a role for virus infection of monocytes/macrophage lineage cells in pathogenesis of the disease. The role for monocytes/macrophage lineage cells in PCV2 infection was studied by several authors. Recently, Chang et al. (2006[a]) reported the effects of PCV2 inoculation on swine pulmonary alveolar macrophages (PAMs) in the *in vitro* system. The PCV2-inoculated AMs decrease in the production of O_2 and H_2O_2 caused reduced phagocytosis and microbicidal capability. This indicated that PCV2-containing AMs may favor the survival and spread of PCV2. In PCV2-inoculated AMs, the levels of tumor necrosis-a (TNF-a), the mRNA

expression levels of alveolar macrophage-derived neutrophil chemotactic factors-II (AMCF-II), granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1) and IL-8 were strongly up-regulated. In addition, the co-infection with bacteria and activation of immune system are suggested of promoting viral replication. Lipopolysaccharide (LPS) from gram-negative bacteria may also be an important factor in promoting PCV2 replication (Chang et al., 2006[b]). Binding and entering characteristics of PCV2 in monocyte/macrophage lineage cells were studied in 2 theories. (i) PCV2 is found in the cytoplasm of phagocytic cells as a results of the clearance of other infected cells or by endocytosis (Kiupel et al., 2001; Vincent et al., 2003; Chang et al., 2006[a]; Chang et al., 2006[b]). PCV2, then enters monocyte/macrophage lineage cells via clathrin-mediated endocytosis and requires an acidic environment (Misinzo et al., 2005). (ii) PCV2 penetrates in the cytoplasm of macrophages via PCV2-specific receptor at the surface of macrophages. Misinzo et al. (2006) reported that PCV2 used heparan sulfate and chondroitin sulfate B on the surface of monocytes/macrophages lineage cells as receptors for its attachment to the host cells. However, PCV2 does not encode its polymerase. The replication of PCV2 depends on the cell polymerases found in the nucleus of cells during the S phase of cell cycle. This indicated that the efficient cells for replication of PCV2 would be those with a high mitotic activity such as fetal myocardiocytes (Sanchez et al., 2003). Viral antigen is not found in cells with lower mitotic activity such as resting lymphocytes of adult pigs (Gilpin et al., 2003). Interestingly, phagocytic properties of macrophages are responsible for the presence of virus in the cytoplasm of monocytes/macrophages lineage cells and these cells are not the primary cells that support PCV2 replication (Gilpin et al., 2003). In addition, PCV2 antigen has been detected most frequently in the nucleus of hepatocytes and other epithelial cells (Rosell et al., 2000). Similarly, another circovirus such as psittacine beak and feather disease virus (PBFD) is an epitheliotropic virus, targeting the basal epithelial layer of the feather and feather follicles (Todd, 2004). Therefore, it is suggested that epithelial cells could be the primary cells for PCV2 replication in pigs.

PCV2 does not induce cell death in dendritic cells nor in lymphocytes co-culture with the infected dendritic cells (Vincent et al., 2003). There are no association with these dendritic cells

and lymphocytes and no evidence of virus transmission from dendritic cells to lymphocytes. This association of PCV2 with monocytic cells could explain the leukopenia due to aberrant signaling from infected cells and interference with homeostasis. The presence of PCV2 in dendritic cells, thus does not impair their immunological interaction with the lymphocytes (Vincent et al., 2005) and the dendritic cells remain processing and presenting antigen. PCV2 can escape the cellular endosomal processing system and extensive degradation. Low level of degradation occurs in the infected dendritic cells and is able to maintain the high levels of virus antigen in the dendritic cells. The majority of PCV2 within the dendritic cells clearly evade degradation, indicating to the prolonged persistence of antigen indicating the delay of an anti-PCV2 immune response. Furthermore, this PCV2-dendritic cells interaction does not induce or inhibit dendritic cells differentiation. The ability to process and present antigen to T lymphocytes remain intact in the presence of PCV2. Nevertheless, PCV2 is immunomodulatory through the reaction of natural interferon-producing cells (NIPCs). Myeloid dendritic cells maturation was clearly impaired by the presence of PCV2, caused by PCV2-induced inhibition of interferon alpha (IFN-α) and tumor necrotic factor alpha (TNF-α) by cytosine-phosphorothioate-guanin oligodeoxynucleotides (CpG-ODNs) (Vincent et al., 2005). The pathogen-recognition process mediated through Toll-like receptors (TLRs) are particularly sensitive. The most sensitive cells within the innate immune defense are the plasmacytoid dendritic cells which responsible for the production of the interferon IFN and TNF maturation factors essential for myeloid dendritic cells maturation. The impairment of the plasmacytoid dendritic cells maturation factor production by PCV2 infection will effectively prevent efficient immune responses developing against other pathogens (Vincent et al., 2003; Vincent et al., 2005; McCullough et al., 2007).

E. Outbreak of PMWS/PCVAD in Asia

To date, outbreaks of PMWS/PCVAD have been reported in China, Korea, Japan, Philippines, Taiwan, and Thailand (Kawashima et al., 2003). The first description of a disease similar to PMWS was in Taiwan in 1995. The description of PMWS-characteristic lesions and the electron microscopical identification of PCV2 were published in 1997. In Japan, the outbreak of PMWS disease was reported in 1997. A retrospective study in Japan was observed in paraffin-embedded tissues of pigs in 1989. The first diagnosis of PMWS in Korea was reported in 2000. This report described the characteristic lesions of PMWS and the detection of PCV2 by

immunohistochemical and polymerase chain reaction (PCR) methods (Choi et al., 2000). In Thailand, the description of PMWS-characteristic lesions and the detection of PCV2 were reported in 1999 (Tantilertcharoen et al., 1999). A retrospective study of PMWS suggested that the first case of PCV2-associated diseases in Thailand was in the year of 1993 (Kiatipattanasakul-Banlunara et al., 2002). The description of PMWS and detection of PCV2 in China were reported in 2001. The presence of PMWS and PCV2 in the Philippines was reported in 2002. The seroprevalence of PCV2 in Asia was also found in Japan and China. In 1999, in Japan PCV2 antibodies were examined from 643 pigs (sows and finisher pigs) from 149 farms by indirect immunofluorescent method. PCV2 antibodies were found in 608 pigs (94.6 %) from 144 farms (96.6 %). In China, PCV2 antibodies were detected in 38 of 64 pigs (59.4%) in year 2002. A survey of prevalence of PCV2 by PCR methods was done in Taiwan, Korea and Japan (Kawashima et al., 2003). In Taiwan, 319 pigs with suspected PMWS signs (4-12 weeks of age) from 60 farms were examined in 2001. Tests for PCV2 were positive in 225 pigs (70.5%) and 48 farms (80.0%). In Korea, 369 suckling pigs with wasting disease (3-17 weeks of age) were investigated through PCR. The prevalence of PCV2 infection from1999 to 2003 was 40.4% (109/270 farms) in farms and 59.1% (218 pigs from 369 pigs with suspected PMWS) in pigs. In Japan, 312 post-weaning pigs suffering from wasting diseases were collected from 56 farms in 2000 and 2001. There was 85.3% (266 from 312 suspected pigs) PCV2 positive and 96.4% (54/56) was positive from farm submitted. These results demonstrate that PCV2 and PMWS/PCVAD have been widespread in the pig population in Asian countries.

F. PCV2 in Thailand

The first case of PMWS in 7 to 9 week-old pigs was reported in Rachaburi province, Thailand in 1998 (Tantilertcharoen et al., 1999). The pigs submitted for examination had typical microscopic findings of PMWS characterized by diffuse lymphoid depletion and the presence of amphophilic intracytoplasmic inclusion bodies in the infiltrating histiocytes or in the multinucleated giant cells. PCV2 antigen was detected in various tissues of the infected pigs by immunohistochemical staining (IHC). A retrospective study on PCV2 detection using immunohistochemistry found the earliest PCV-infected cases in 1993 and later was performed using formalin-fixed, paraffin-embedded (FFPE) tissues between 2000-2002. The later study showed the incidence of PCV2 infection in suspected cases 38.76 % (50/129). The presence of

PCV-2 antigen was primarily seen in the lymph node 40.70% (Banlunara et al., 2002). Based on the data from the Veterinary Diagnosis Laboratoty, Chulalongkorn University, the incidence of PCV2 infection during the years from 2000 to 2002 are 13/273 (4.76 %), 33/248 (13.31 %) and 55/231 (21.63%), respectively. Most of the pigs age were between 7 and 14 weeks (Thanawongnuwech et al., 2003). Currently, based on swine diagnosis annual report in 2006 from the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University found increasing incidence of PMWS and PDNS in swine systemic infection cases (58.23 %, n = 79). In 2007 the seroprevalence was performed in 316 pigs (sows and finishers) from 21 farms by commercially available competitive blocking ELISA (SERELISA® PCV2 Ab Mono Blocking, Synbiotics, Europe, Lyon, France). PCV2 antibodies were found in 220 pigs (69.6%) from 21 farms (100%) (Chulalongkorn University, Veterinary Diagnostic Laboratory, unpublished data). This indicated that PCV2 is one of the major causative agents in the Thai swine industry.

G. PCV2 vaccines

Treatment of PMWS or PCVAD can only focus on good production practices and on reducing co-infections since there are no commercially available vaccines in the market before 2007. Those control and treatment of the disease often requires the increased use of antibiotic therapy. This unsatisfactory situation creates a good rationale for the development of effective vaccines against PCV2 (Farchinger et al., 2008). Commercial PCV2 vaccines for use in growing pigs and breeding-age animals became available in the North America in 2006 (Opriessnig et al., 2007). Recently, several kinds of PCV2 vaccines have been developed such as a chimeric PCV1-2 virus with the immunogenic capsid gene of PCV2 cloned into the backbone of PCV1 (Suvaxyn® PCV2 , Fort Dodge Animal Health) (Fenaux et al., 2004), PCV2 ORF2 protein expressed in baculovirus (Ingelvac® CIRCOFLEX™, Boehringer Ingelheim Vetmedica Inc.) (Blanchard et al., 2003), PCV2 expressed in inactivated baculovirus (Porcillis® PCV2, Intervet) and the inactivated, oil-adjuvanted PCV2 vaccine (CIRCOVAC®, Merial). The Americans and Canadians described a variety of trials with both the killed PCV2 sow vaccine and three various killed vaccines for piglets and their results are discussed. Plourde and Machell (2007) described the response to the

sow vaccine CIRCOVAC® (Merial) in Canada in 77 farms. On average, the mortality was running at 12.6% (11.8 – 13.3%) before vaccination and 5.2% (5.0 – 5.4%) following vaccination giving a 7.4% drop in mortality. Desrosiers et al. (2007) reported on a blinded, controlled study in Canada with Ingelvac® CIRCOFLEX™ (Boehringer Ingelheim Vetmedica Inc.) on a 1300 sow unit. The herd was enzootic pneumonia and PRRS free and there was a low mortality (0.4%) in the nursery but PCVAD developed normally 3-4 weeks into the finishing barn. The trial involved 3850 pigs. The combined mortality in the unvaccinated placebo controls averaged at 9.5% and the vaccinated group at 2.4% with a reduction of 7.1%. De Grau et al. (2007) carried out a trial in Canada (Quebec and Ontario) with Porcilis PCV2 (Intervet Inc.) using a two-shot killed vaccine. The trial was designed as a multi-centred, randomised study involving 21 farms. The pigs were vaccinated initially at 3-5 weeks of age and given a booster 3 weeks later. The overall mortality in the unvaccinated pigs was 9.3% and in the vaccinated pigs 2.1% with a fall of 7.2%. Connor and Elsenier (2007) described a series of trials with Suvaxyn® PCV2 (Fort Dodge Animal Health) in the US, where the product is now licensed. The average of the 6 trials showed a reduction of mortality from 7.7% to 1.8. Interestingly, PCV2 viremia was noted between 8-10 weeks of age and the disease signs started 1-2 weeks later. Previously, vaccination in the nursery pigs with this chimeric vaccine shown significantly viremic reduction and the risk of clinical diseases were decreased (Fenaux et al., 2004). Suvaxyn® PCV2 when administered 1-shot to 3 week-old pigs was able to prevent PCV2 viremia and the development of microscopic lesions in lymphoid tissues when pigs were challenged 4 months after vaccination (Urniza et al., 2006). Since swine farms in Thailand have different conditions and management system from the US, the field trial in Thailand should be performed in order to compare the results to the previous reports (Fenaux et al., 2004) before PCV2 vaccine implementation in the Thai farms. Therefore, the efficacy of killed PCV2 vaccine (Suvaxyn® PCV2) in a PCV2-affected herd in Thailand was conducted in this study.

CHAPTER III

MATERIALS AND METHODS

Herd status before animal experiment

Based on history (since October 2006) and necropsy reports, a PCV2-affected herd with 3,200 sows in Prachinburi province, Thailand was selected for this experiment. Three pigs (9-week-old, *n* = 1 and 16-week-old pigs, *n* = 2) submitted to the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University were diagnosed as PCVAD on October 6, 2006. To examine the serological status of this herd, preliminary survey was performed in January 2007, serum samples (sows, *n* = 20; nursery, *n* = 40 and finisher *n* = 40) were tested by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (Fort Dodge Animal health, Biological Research & Development and Regulatory Affairs).

Animal experiment

Two hundred, 3-week-old weaned piglets were randomly divided into two groups, (Group A and Group B; 100 pigs per group). The pigs from each group were equally divided into ten subgroups (A1 - A10 and B1- B10) and were housed in small pens in the same building. The distribution of the subgroups was designed as in Fig. 1. At 4 weeks of age, group-A pigs were vaccinated intramusculary by 2 ml of killed PCV2 vaccine (Suvaxyn® PCV2, Fort Dodge Animal Health) and group-B pigs were injected intramusculary with 2 ml of normal saline. All pigs were clinically examined by an animal husbandman on the farm once weekly without knowing of group affiliation. Additionally, classical swine fever vaccine was given at 3 and 5 weeks of age, and pseudorabies vaccine was given at 6 and 8 weeks of age.

A1	**B2**	**A3**	**B4**	**A5**	**B6**	**A7**	**B8**	**A9**	**B10**
B1	**A2**	**B3**	**A4**	**B5**	**A6**	**B7**	**A8**	**B9**	**A10**

Figure 1 Animal housing model; 10 pigs/pen (A = vaccinated pigs, B = non-vaccinated pigs)

Clinical parameters

For the production parameter, individual live body weights of the study pigs were measured at 4 and 16 weeks of age. Average daily weight gain (ADWG) was calculated (as the difference between the body weights between two weighting time points divided by the number of days between these two weighting time points) (Farchinger et al., 2008). All animals were monitored weekly for clinical signs of porcine respiratory disease complex (PRDC), and the severity was ranged from 0-6 (0 = normal and 6 = severe) as described previously (Halbur et al., 1995). The body condition score based on the degree of fat cover were observed and ranged from 1-6 (1 = thin and 6 = fat) (Straw et al., 1999). The ill pigs were given with a single dose of long acting amoxycillin (15 mg per kg bodyweight).

Hematological study

EDTA-stabilized blood samples were done from 10 pigs per group (1 pig/pen) at 4, 5, 7, 9, 12 and 15 weeks of age for complete blood count (CBC).

Serological examination

Serum samples were collected from 20 pigs per group (2 pigs/pen) at 4, 5, 7, 9, 12 and 15 weeks of age for serological examination. PCV2 antibodies were detected by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (Fort Dodge Animal health, Biological Research & Development and Regulatory Affairs). One hundred µl of capture antigen diluted in coating buffer (1:1,000) was added to the 96-well plates as follows: negative capture antigen control (Sf9 cells) in Row "H", wells 7-12, positive capture antigen control (capsid protein-Baculovirus) in the remaining wells that will have a test serum. The serum samples and the swine serum control were diluted by diluent reagent (1:6,000). The sera were bound with primary antibody anti-IgG (1:500). Bound antibodies were detected with TMB peroxidase substrate (KPL©, MD, USA). The absorbance of each well was read by spectrophotometer at 650 nm. Sera that give corrected sample compare to positive (S/P) ratio over

0.070 were considered as positive. Antibodies against PRRSV in the same sera were detected using a commercial ELISA test kit (IDEXX Labs, Inc., U.S.A.).

Postmortem examination

During the study, the animals showing clinical signs and died during the experiment were necropsied and organ samples were collected for histopathology, immunohistochemistry, bacteriology and PCR detection for PCV2. At 16 weeks of age, the trial was terminated and twenty pigs per group were euthanized using pentobarbital and later with saturated magnesium sulfate. Complete necropsy was performed and gross lesions were recorded. Pulmonary lesions were observed and scored (Thanawongnuwech, 2005). The degree of lymph nodes enlargement (superficial inguinal lymph nodes, tracheobronchial lymph nodes and mesenteric lymph nodes) ranged from 0-3 (0 = normal size, 1 = one time larger than normal, 2 = two time larger than normal, 3 = three times larger than normal) was estimated (Fenaux et al., 2004). Weight of the superficial inguinal lymph nodes (both side) was measured and lymph node/ body weight ratio was calculated for individual necropsied pig. Organ samples including lymphoid tissue (tracheobronchial lymph nodes, mesenteric lymph nodes, superficial inguinal lymph nodes, spleen and ileum), lung, liver and kidney were collected for histopathology, immunohistochemistry, bacteriology and PCR detection of PCV2.

Histopathology

The collected tissues were routinely processed for histopathology. The sections from lung, lymph nodes and Peyer's patch were scored for the severity of lesions according to Halbur et al. (1995) and Fenaux et al. (2004). Lung scores were ranged from 0-4 (0 = normal, 1 = mild interstitial pneumonia, 2 = moderate multifocal interstitial pneumonia, 3 = moderate diffuse interstitial pneumonia and 4 = severe diffuse interstitial pneumonia). Depletion of lymphoid tissue was observed and scored ranging from 0-3 (0 = no lymphoid depletion, 1= mild lymphoid depletion, 2 = moderate multifocal lymphoid depletion and 3 = severe lymphoid depletion). The

degree of histiocytic replacement (HR) in the follicles was scored ranging from 0-3 (0 = no replacement to, 1 = small amount, 2 = moderate amount, 3 = large amount) (Fenaux et al., 2004).

Detection of PCV2 antigen in lymphoid tissue by immunohistochemistry (IHC)

Paraffin sections from lymphoid tissues (superficial inguinal lymph nodes, tracheobronchial lymph nodes, mesenteric lymph nodes and ileum) of the studied pigs were screened for the presence of PCV2 antigen. The sections were cut at 4 micron and were placed on 3-aminopropyltriethoxysilan treated slides and were then incubated at 60 °C for 10 min. After deparaffinization, the sections were treated with 0.1% trypsin at 37 °C for 30 min and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity in tissue section was eliminated by using 0.3% H_2O_2 in methanol (2 ml: 200 ml) for 30 min at room temperature and washed in PBS. Blocking of non-specific reactions was performed by using 0.01 % bovine serum albumin (BSA) in humidified chamber at 37 °C for 30 min and then washed in PBS. The sections were then incubated with primary antibody, 1:500 dilution of polyclonal rabbit anti-PCV-2 antibody (Fort Dodge Animal health, Biological Research & Development and Regulatory Affairs) then incubated at 4 °C overnight. After washing, the slides were incubated with 1:400 a biotinylated goat anti-rabbit IgG antibody (Dako, Denmark) at 37 °C for 35 min, followed by incubation with avidin-biotin complex peroxidase solution (ABC, Dako, Denmark) at 37 °C for 35 min. The immunoreactivity was detected in 3, 3'-diaminobenzidine –4HCl (DAB) substrate (Sigma, USA). Sections were counterstained with hematoxylin. The immunohistochemistry was scored (average from 5 areas/slide) ranging from 1-3 (1 = < 10 positive cells/HPF, 2 = 10-20 positive cells/HPF, 3 = >20 positive cells/HPF) (Banlunara et al., 2002).

Detection of PCV2 in organ and serum samples by polymerase chain reaction (PCR)

DNA extraction

Organ samples ; From necropsied pigs (*n*=20), superficial inguinal lymph node, mesenteric lymph node and tracheobronchial lymph nodes from each pig were pooled as one samples. Organ samples including lung, spleen, liver and ileum from each pig were pooled as one

sample. Extraction of DNA from pooled organs and pooled lymph nodes was performed using a commercial DNA extraction kit (ChargeSwitch® gDNA Tissue Kits, Invitrogen©, California, USA). ***Serum samples*** ; pooled sera were collected from 20 pigs per group at 4, 5, 7, 9, 12 and 15 weeks of age (2 pigs/pen) as mentioned above and serum samples from the same pen (n = 2) were pooled as one sample. DNA was extracted from pooled serum samples using a commercial DNA extraction kit (Viral Nucleic Acid Extraction Kit, RBC Bioscience©, Taiwan).

PCR detection of PCV2 DNA

To detect PCV2 DNA in organ and serum samples, the paired primers, forward and reverse primers were designed to amplify product of the ORF gene specific for the Open reading frame 1(ORF1), which encoded for the replication protein. The amplification was performed in 20 µl reaction mixture containing 10 µl of a commercial master mix (Go taq© Green Master Mix, Promega©, Madison, USA), 0.5 µl of each forward primer (ATG CCC AGC AAG AAT GGA AGA AG) and reverse primers (AGG TCA CTC CGT TGT CCT TGA GAT C), 3 µl of DNA template and distilled water 6 µl to yield a final volume of 20 µl. Amplification conditions were 1 cycle with initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 20 s, and extension at 72 °C for 20 s, with a final extension at 72 °C for 2 min. PCR products (10 µl) were separated by electrophoresis by 1.5% agarose gel. The gels were stained with 10% ethidium bromide and visualized under UV transilluminator for bands of expected size 300-400 bp.

Statistical analysis

Statistical analysis for the histopathology and immunohistochemistry (non-parametric data) was performed using Mann-Whitney rank sum test analysis. Average daily weight gain and mean S/P ratio from ELISA technique were analyzed using unpaired t-test.

CHAPTER IV

RESULTS

Herd status before the experiment

Three pigs (9-week-old, *n* = 1 and 16-week-old pigs, *n* = 2) were submitted for necropsy revealing generalized lymphadenopathy (2 times) and icteric mucous membrane. Histopathology revealed severe lymphoid depletion in the lymphoid tissues with the presence of intracytoplasmic inclusion bodies in macrophages and giant cells in the germinal center of those lymphoid follicles. The diagnosis of these cases was PMWS associated with PCV-2 infection (Table 1). In addition, the serological results from this farm revealed high prevalence of PCV-2 infection both in the sows (90%, 18/20) and in the growing pigs (40/40 = 100%). In piglets, the average PCV serological titers were high possibly due to maternal-derived antibody at 3 weeks of age and then declined at about 5 weeks. The titers were again gradually increased from 9 weeks to 24 weeks of age (Figure 2) due to natural PCV-2 infection.

Table 1 Necropsy results from the 9-week-old and 16-week-old pigs before the experiment in the selected farm

Ages	Gross lesions	Histopathology (lymphoid tissue)
9 weeks (*n* = 1)	Mild generalized lymphadenopathy Mild multifocal to diffuse pneumonia Mild chronic focal erosive gastritis Severe diffuse catarrhal enterocolitis Icterus	Tonsil : mild lymphoid depletion Ileum : mild catarrhal enteritis and lymphoid depletion Lymph node : mild lymphoid depletion
16 weeks (*n* = 2)	Moderate multifocal to diffuse pneumonia (2/2) Chronic ulcerative gastritis (1/2) Severe diffuse catarrhal enterocolitis (2/2) Severe generalized lymphadenopathy (2/2)	Colon : catarrhal colitis with intracytoplasmic inclusion bodies in Peyer's patch , lymphoid depletion and granulomatous lymphadenitis (2/2) Lymph node : lymphoid depletion and intracytoplasmic inclusion bodies in macrophages (2/2)

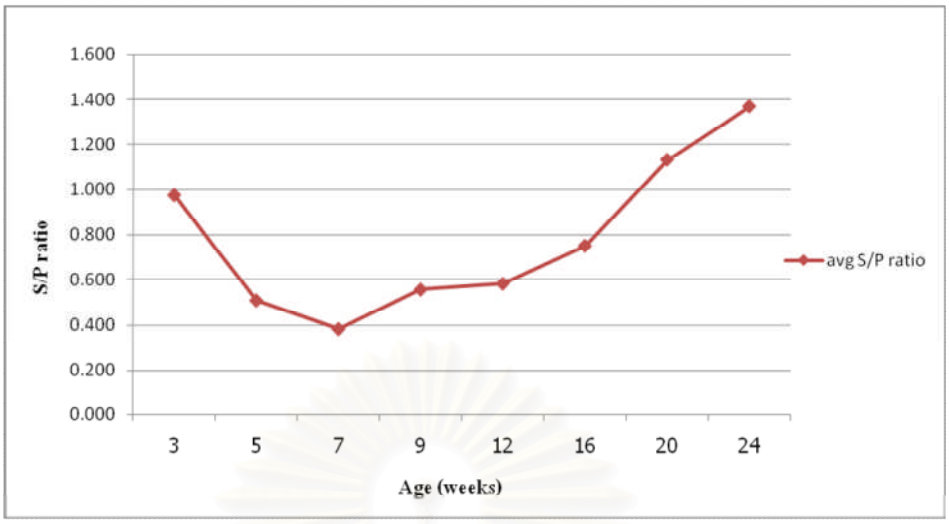

Figure 2 Average PCV2 S/P ratio in nursery and growers as monitored in January 2007.

S/P ratio = sample/positive ratio.

Respiratory problems, body condition scores (*n* = 40), average daily weight gain (ADG) (*n* = 20/group) and mortality rate of the experimental pigs

In both groups, respiratory signs (coughing and dyspnea) were more frequent during 5 to 9 weeks of ages, and were then subsided until the end of the experiment. The body condition scores (BCS) were similar in both groups as seen in Table 2. Average daily weight gain (ADWG), measured during the experimental period was 380 g/d in both groups. In this experiment, total mortality rate for vaccinated pigs was 23% versus 22% for non-vaccinated pigs. The mortality rates for both groups of pigs were high during 5 to 9 weeks of age due to secondary bacterial infection and then decreasing for about one month. At 14 weeks of age, some pigs from both groups were died from salmonellosis and Hemoyltic *E.coli* infection based on the bacteriological results (Figure 3 and Table 5).

Hematological results

Erythrocytes count did not show the sign of anemia in both groups at the time of experiment. The total number of white blood cell count (WBC) was similar in both groups (data

not shown). However, decreasing of WBC or leukopenia (WBC < 9,000 cells/μl) was found in a few pigs during the experiment (Table 3).

Table 2 Respiratory signs and mean body condition scores (BCS) in the experimental pigs (Mean ± S.E.)

Age	vaccinated (*n* = 20)		Non-vaccinated (*n* = 20)	
(weeks)	respiratory signs	mean BCS	respiratory signs	mean BCS
5	2.45 ± 0.43	2.45 ± 0.18	2.58 ± 0.37	2.74 ± 0.25
6	2.40 ± 0.45	2.30 ± 0.20	1.74 ± 0.35	3.05 ± 0.19
7	2.11 ± 0.54	2.26 ± 0.21	1.72 ± 0.42	2.83 ± 0.18
8	2.94 ± 0.52	2.33 ± 0.21	1.38 ± 0.18	2.69 ± 0.12
10	1.41 ± 0.35	2.88 ± 0.18	0.88 ± 0.38	3.38 ± 0.18
11	0.77 ± 0.21	3.47 ± 0.27	0.73 ± 0.40	4.06 ± 0.24
12	1.00 ± 0.32	3.53 ± 0.21	0.47 ± 0.16	3.40 ± 0.21
13	0.65 ± 0.29	3.17 ± 0.21	0.33 ± 0.12	4.06 ± 0.36
14	0.33 ± 0.12	4.07 ± 0.31	0.20 ± 0.14	4.20 ± 0.17
15	0.33 ± 0.21	3.67 ± 0.27	0.13 ± 0.09	3.53 ± 0.16

Values are expressed as mean score ± S.E. (standard error). BCS = Body condition scores (A = vaccinated pigs; B = non-vaccinated pigs).

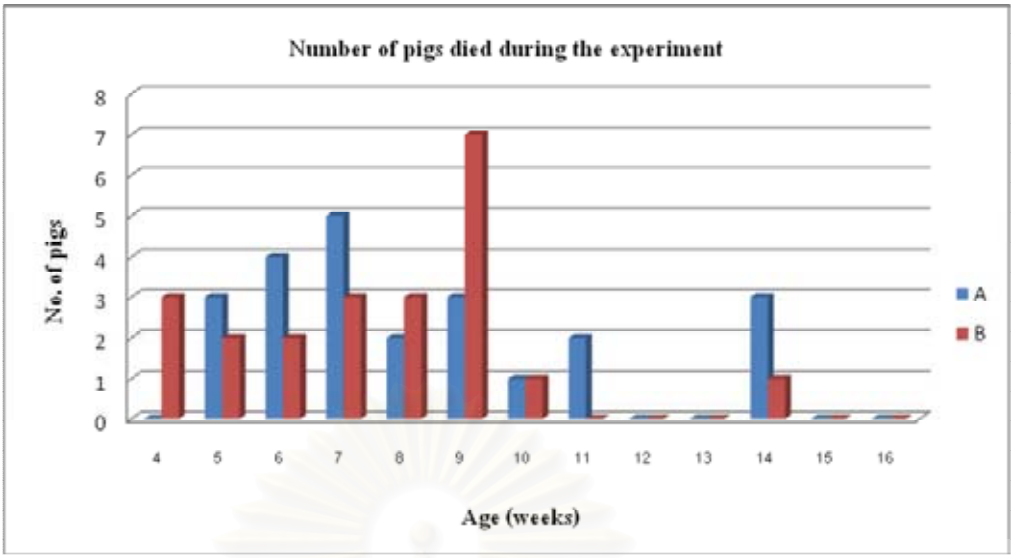

Figure 3 Number of pigs died during the experiment (A = vaccinated pigs, B = non-vaccinated pigs).

Table 3 The number of pigs with leukopenia (WBC < 9,000 cells/μl) during the experiment from 4 weeks to 12 weeks of age (*n* = 10 / group; *n* = 6 at 4 weeks of age)

Group	No. of pigs having leukopenia (*n* = 10)				
	Age (wks)				
	4	5	7	9	12
Vaccinated	1	0	0	1	0
Non-vaccinated	2	0	0	1	0

Serological results

PCV2 antibodies; The results of PCV2 antibodies testing are shown in Figure 4. The average serological titers were high at 4 weeks of age and then declined at about 5 weeks in both groups indicating the declining of the maternal derived antibodies between 4 and 7 weeks of age. The seroconversion was observed in vaccinated pigs at 9 weeks of age possibly due to the response of PCV2 vaccination. In non-vaccinated pigs, PCV-2 seroconversion was detected at 12 weeks of age due to the PCV2 natural infection in the endemic farm.

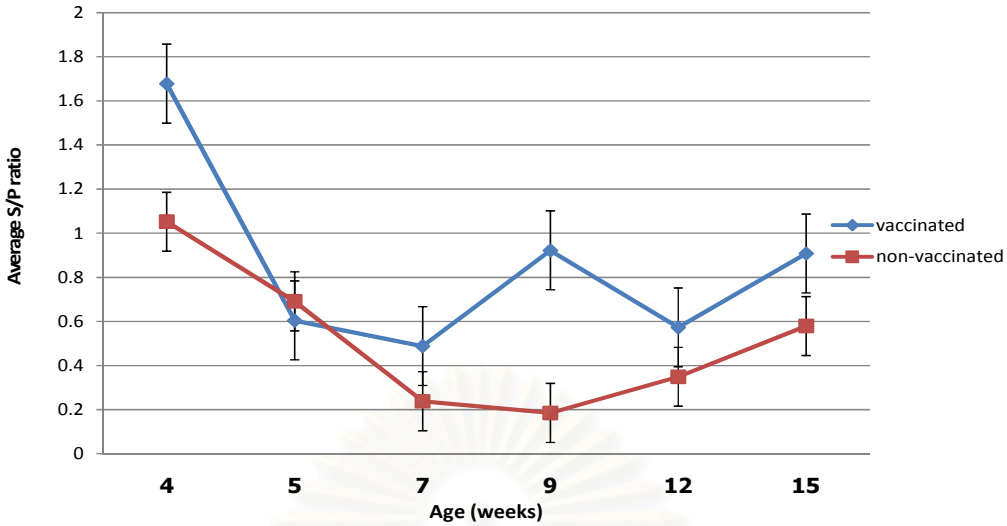

Figure 4 Average S/P ratio of PCV-2 ELISA in the experimental pigs. There was statistically significant difference between the two groups at 9 weeks of age.

PRRSV antibodies; The results of PRRSV antibodies testing are shown in Figure 5. Seroconversion to PRRSV antibodies was observed at 5 weeks of age in both groups and remained seropositive through the end of the experiment.

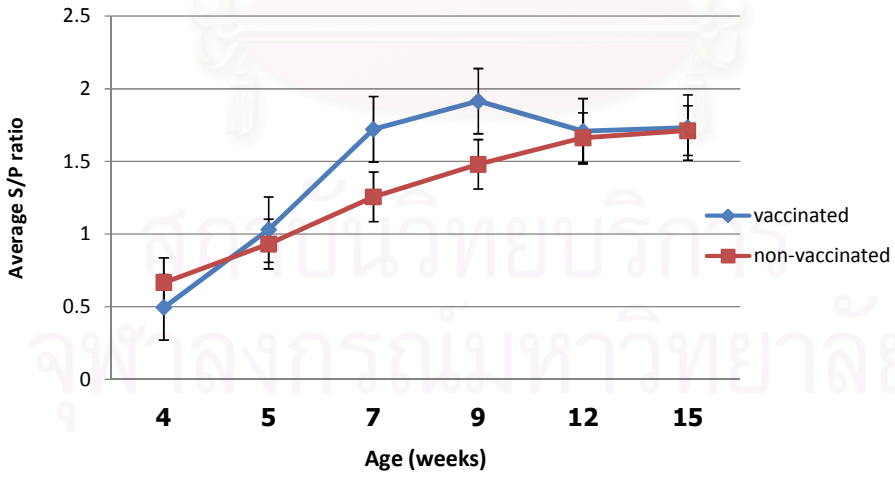

Figure 5 Average S/P ratio of PRRSV ELISA in the experimental pigs.

Postmortem examination

Necropsy of pigs that died at 7 and 8 weeks of age showed generalized lymphadenopathy and severe fibrinopurulent polyserositis in both groups as shown in Table 4. *Pasteurella multocida, Streptococcus suis* , Hemolytic *E. coli* and *Salmonella* spp. were isolated from organ samples in both groups during the experiment (Table 5).

Table 4 Gross pathology from experimental pigs (necropsy at 7 and 8 weeks of age)

Group	Fibrinopurulent Polyserositis	Cranioventral pneumonia	Polyarthritis	Generalized lymphadenopathy
Vaccinated	5/6	5/6	2/6	4/6
Non-vaccinated	5/9	6/9	1/9	4/9

* vaccinated pigs: 1 specimen was autolysis

Table 5 Bacterial culture from the experimental pigs (*n* = number of sample submitted to laboratory)

	Pigs died during 5 to 11 weeks of age (*n* = 12 ; both group)					**Pigs died at 14 weeks of age (*n* = 3 ; vaccinated)**				
	S. suis	***H. parasuis***	***P. multocida***	***Salmonella* spp.**	***Hemolytic E. coli***	***S. suis***	***H. parasuis***	***P. multocida***	***Salmonella* spp.**	***Hemolytic E. coli***
Vaccinated	**5/12**	0/12	**4/12**	1/12	1/12	0/3	0/3	0/3	**3/3**	**3/3**
Non-vaccinated	**5/12**	0/12	**5/12**	2/12	2/12	NA	NA	NA	NA	NA

NA = Not examined

Pathological findings at 16 weeks of age

At the end of experiment, necropsy was performed on previously selected 20 pigs per group. Gross lesions of the lung showed mild degree of cranioventral pneumonia (vaccinated pigs , 5/20; non-vaccinated pigs, 4/20) and mild to moderate chronic pleuritis in both group of pigs (vaccinated pigs, 4/20; non-vaccinated pigs, 2/20) as seen in Figure 5. The average bacterial pneumonic lung scores did not differ between both groups (vaccinated pigs = 1.05 ± 2.94 and non-vaccinated pigs = 1.15 ± 2.90). The enlargement of superficial inguinal lymph nodes of vaccinated pigs was ranged from 1 to 2 times enlargement, mainly at one time (9/20, 45 %). In contrast to vaccinated pigs, enlargement of superficial inguinal lymph nodes of non-vaccinated pigs were ranged from 1 to 3 times enlargement, especially about 2 times (8/20, 40%) (Table 6). Additionally, the average of lymph nodes/body weight ratio in vaccinated pigs (38.5×10^{-5}) was lower than those in non-vaccinated pigs (45.4×10^{-5}), but it was not statistically significant. Interestingly, one pig from vaccinated pigs (No.7A1) had severe edema of perirenal and periureteral areas with moderate diffuse petechial hemorrhagic nephritis (Figure 5A-B). The bacterial culture from lung of this animal revealed *Bordetella bronchiseptica* and *Streptococcus suis*, whereas non-specific bacteria were found in other pigs. One pig from non-vaccinated pigs (No. 5B1) had mild enlarged kidney with multifocal white foci (Figure 5D).

Table 6 Number of pigs with enlargement of superficial inguinal lymph nodes

	Superficial inguinal lymph node			
Enlargement	**x 1**	**x 1.5**	**x 2**	**x 3**
vaccinated (*n* = 20)	**9/20**	7/20	4/20	0/20
Non-vaccinated (*n* = 20)	4/20	7/20	**8/20**	**1/20**

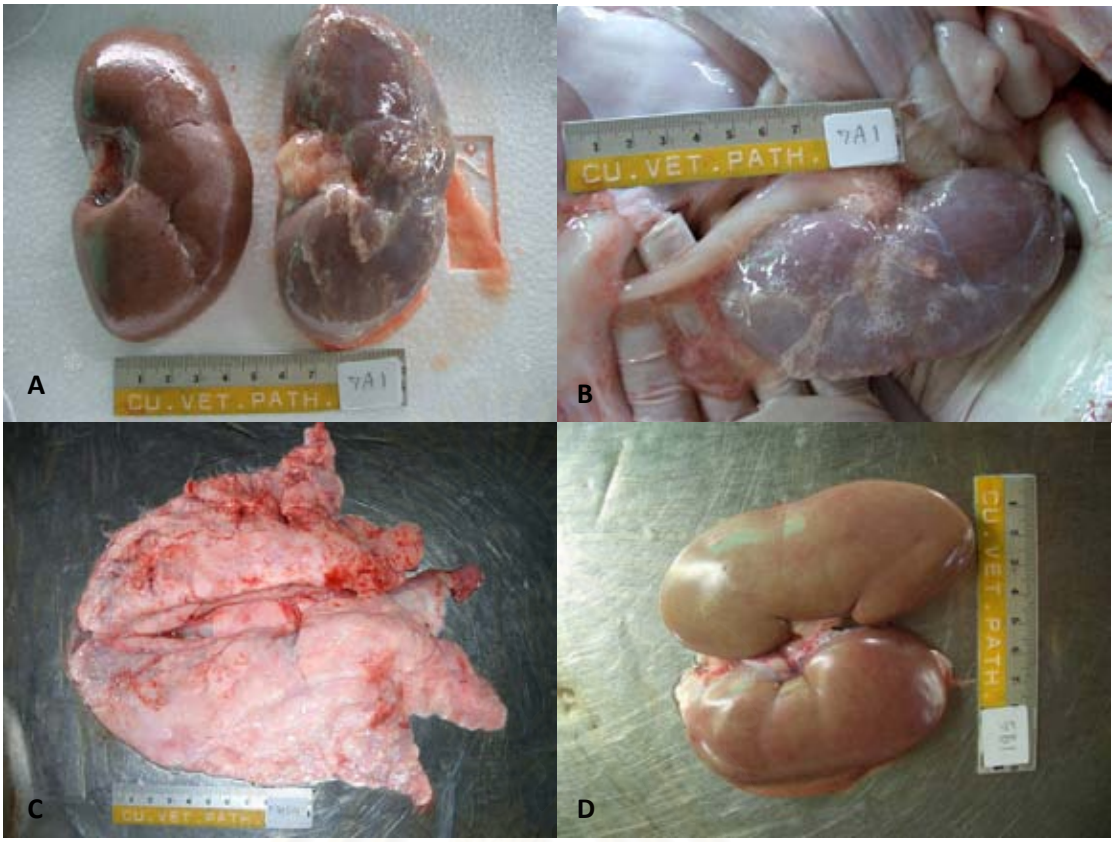

Figure 6 Gross lesions of the experimental pigs. A, B: Perirenal and periureteral edema with hemorrhagic nephritis (group-A pig, No. 7A1), C: Severe chronic diffuse fibrinous pleuritis (group-B pig, No9B2), D: Mild enlarged kidney with multifocal to diffuse white foci (Group B pig, No.5B1).

Histopatholgy

Microscopic examination of lymphoid tissue revealed variable degree of lymphoid depletion, histiocytic replacement and granulomatous inflammation in both groups. Inclusion bodies characteristic of PCV2 viral inclusion could not be observed in all samples. In vaccinated pigs, mean scores of lymphoid depletion and histiocytic replacement in lymphoid organs were lower than non-vaccinated pigs, especially in the mesenteric lymph node and Peyer's patches (Figure 6A). Microscopic lymphoid depletion and histiocytic infiltration results are shown in Table 7. Microscopic lesions in the kidney of pigs No. 7A1 and 5B1 showed diffuse lymphocytic interstitial nephritis. In the lungs, variable degrees of peribronchiolar cuffing were observed in both groups (group A, 8/20; group B, 7/20). Interestingly, interstitial pneumonia with

granulomatous inflammation was prominently seen in non-vaccinated pigs (8/20, 40%), whereas this lesion was found only in 3/20 (15%) of vaccinated pigs (Figure 6B).

Table 7 Mean scores of lymphoid depletion and histiocytosis in the experimental pigs (Mean ± S.E.)

Mean scores ± standard error of lymphoid depletion in lymphoid organs				
	Superficial inguinal ln	**Tracheobronchial ln**	**Mesenteric ln**	**Peyer's patches**
Group A	2.75 ± 0.09*	1.70 ± 0.16	1.10 ± 0.14[a]	0.65 ± 0.16[a]
Group B	2.70 ± 0.10	2.00 ± 0.162	1.90 ± 0.19[b]	1.40 ± 0.15[b]
Mean scores ± standard error of histiocytic replacement in lymphoid organs				
	Superficial inguinal ln	**Tracheobronchial ln**	**Mesenteric ln**	**Peyer's patches**
Group A	2.45 ± 0.13	1.80 ± 0.15	1.20 ± 0.17[a]	0.75 ± 0.20[a]
Group B	2.75 ± 0.09	2.15 ± 0.16	1.85 ± 0.16[b]	1.65 ± 0.16[b]

*standard error, [a,b]different superscripts in the same column means statistically different ($P < 0.05$), ln = lymph nodes (A = vaccinated pigs; B = non-vaccinated pigs). Statistical analysis of non-parametric data using Mann-Whitney rank sum test.

Detection of PCV2 antigen in lymphoid tissue by immunohistochemistry

At necropsy, PCV2 antigen was detected in the lymphoid tissue in both groups. In vaccinated pigs, mild degree (+1) of positive labeling was observed in the superficial inguinal lymph nodes, tracheobronchial lymph nodes and mesenteric lymph nodes (7/20, 35%), and in Peyer's patches (3/20, 15%). In non-vaccinated pigs, mild to moderate degree of PCV2-positive cells (+1 and +2) was seen in the superficial inguinal lymph nodes (12/20, 60%) (Figure 8). Low amount of PCV2 positive cells (+1) was detected in tracheobronchial lymph nodes (7/20, 35%),

mesenteric lymph nodes (8/20, 40%) and Peyer's patches (5/20, 25%). Mean scores of PCV2 detection in lymphoid organs are shown in Table 8.

Table 8 Mean scores of PCV2 detection by immunohistochemistry (IHC) in lymphoid organs and numbers of pigs with positive IHC (n = 20) (Mean ± S.E.)

Mean scores ± standard error of PCV2 detection in lymphoid organs by IHC				
	Superficial inguinal ln	**Tracheobronchial ln**	**Mesenteric ln**	**Peyer's patches**
Group A	0.35 ± 0.10*	0.35 ± 0.10	0.35 ± 0.10	0.15 ± 0.08
Group B	0.65 ± 0.13	0.35 ± 0.10	0.40 ± 0.11	0.25 ± 0.09
No. of pigs with positive IHC				
Group A	7/20 (35%)	7/20 (35%)	7/20 (35%)	3/20 (15%)
Group B	12/20[a] (60%)	7/20 (35%)	8/20 (40%)	5/20 (25%)

*standard error; No a statistically significant difference between groups, [a] one pig in group B revealed moderate degree of PCV2 antigen detection (score = 2), ln = lymph nodes (A = vaccinated pigs; B = non-vaccinated pigs). Statistical analysis of non-parametric data using Mann-Whitney rank sum test.

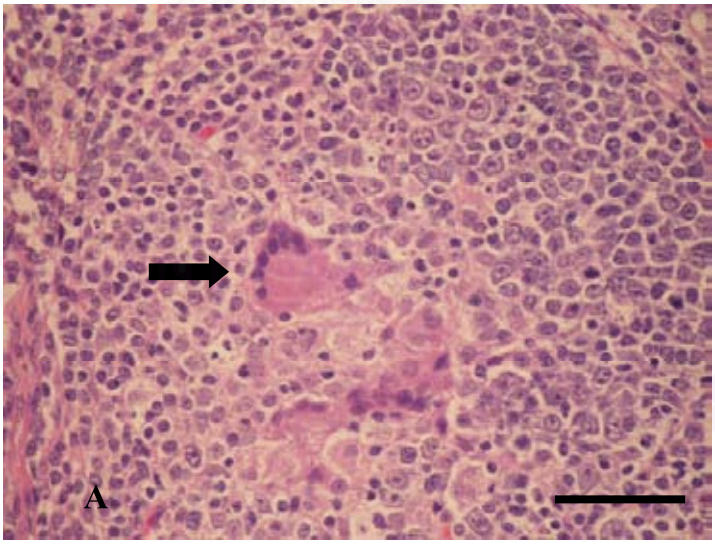

Figure 7A Mesenteric lymph node of non-vaccinated pigs (group-B pig; No. 2B2); Severe histiocytic replacement in lymphoid follicle with the presence of multinucleated giant cells (arrow), bar = 20 µm.

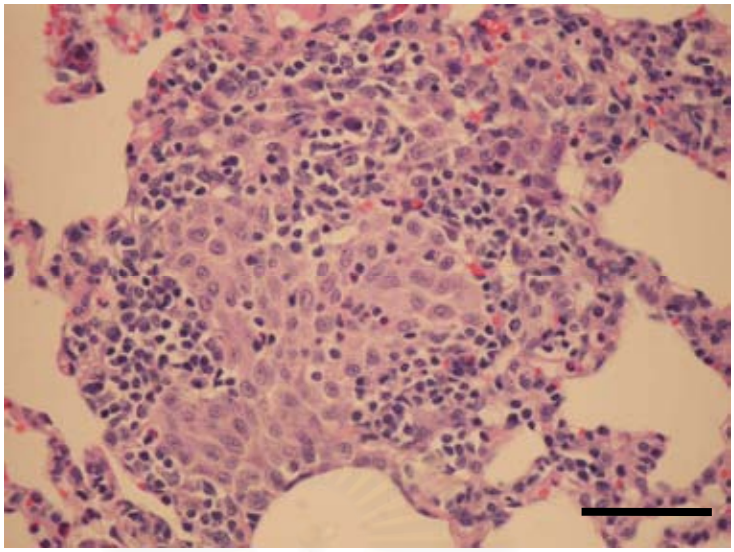

Figure 7B Interstitial pneumonia with granulomatous inflammation in non-vaccinated pig (3B2), bar = 20 µm.

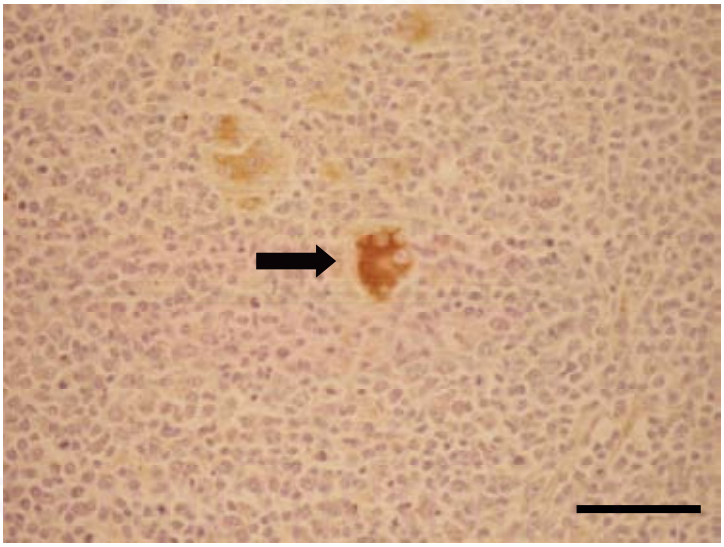

Figure 8 Immunohistochemical detection of PCV2 in superficial inguinal lymph nodes from non-vaccinated pigs (No. 3B2). Positive labeling (brown staining) (arrow) was observed in multinucleated giant cells and histiocyte in germinal center of lymphoid follicle. (IHC-DAB, hematoxylene counterstain, bar = 20 µm).

Detection of PCV2 antigen in organ and serum samples by polymerase chain reaction (PCR)

PCR detection in pooled sera

Interestingly, none of PCV2 DNA was detected in pooled sera of vaccinated pigs until 15 weeks of age when one sample had positive PCV2 DNA. In contrast to the vaccinated pigs, PCV2 DNA was detected in the non-vaccinated pigs at 4 weeks (2/10, 20%), 5 weeks (7/10, 70%), 7 weeks (5/10, 50%), 9 weeks (10/10, 100%), 12 weeks (3/10, 30%) and 15 weeks of age (3/10, 30%) (Figure 9).

PCR detection in pooled lymph nodes and pooled organs

PCR detection of PCV2 DNA in the pooled lymph nodes revealed 75% (15/20) positive in vaccinated pigs but 100% (20/20) in the non-vaccinated pigs at necropsy. In addition, PCR detection of PCV2 in pooled organs revealed 70% (14/20) positive in the vaccinated pigs and 90% (18/20) in the non-vaccinated pigs.

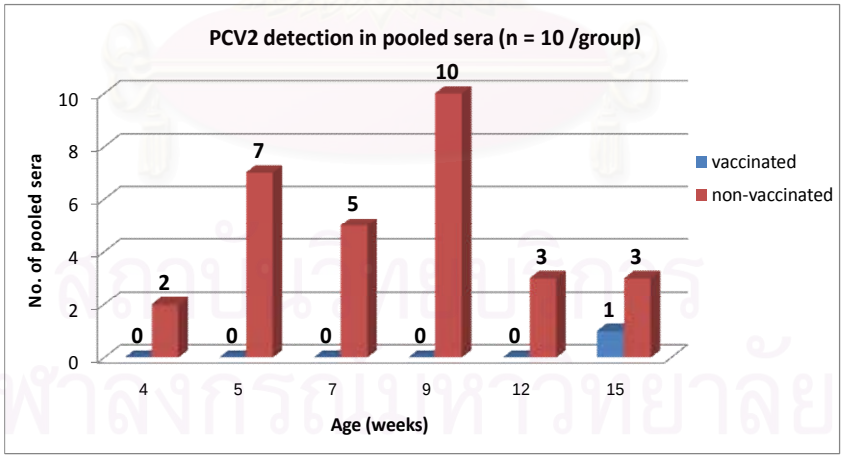

Figure 9 PCR detection of PCV2 in pooled sera from the experimental pigs.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Post-weaning multisystemic wasting syndrome (PMWS), also known as porcine circovirus type 2 associated diseases (PCVAD), is now considered as one of the most important disease complexes in pig industry worldwide. Since the identification of PCV2 and its association with post-weaning multisystemic wasting syndrome (PMWS), PCV2 has been increasingly isolated from pigs affected with various clinical manifestations. PMWS is the most important clinical manifestation of PCVAD causing significant economic losses. In addition, PCV2 is associated with granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis and reproductive failures (Chae, 2005). Currently, based on the swine diagnosis annual report in 2006 from the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand, the increasing incidence of PMWS and PDNS in swine systemic infection cases (58.23 %, n = 79) was observed. From all evidence mentioned above, PCV2 definitely has an economic impact for the Thai swine industry. Prevention and control strategy of PCVAD in the farms have been implemented. The improvement of management and control of other disease seem to give good results. However, the economical impact remains serious. Several kinds of PCV2 vaccines have been developed such as an inactivated PCV2 vaccine for sows (CIRCOVAC®, Merial) and a chimeric PCV1-2 vaccine recommending for piglets (Suvaxyn® PCV2, Fort Dodge Animal Health). Previous reports of the Suvaxyn® PCV2 efficacy test was conducted in the specific pathogen free pigs revealed that significant reduction of viremia and the risk of clinical diseases were observed (Fenaux et al., 2004). Field trial performed in the US also showed the promising results (Connor and Elsener, 2007). However a field trial in Thailand was conducted in this study before the implementation of the PCV2 vaccines in the Thai swine industry.

The preliminary study of disease status before the experiment has shown that the selected pig farm has been affected with PCVAD with occasional development of PMWS characterized by wasting with or without respiratory signs, diarrhea, paleness of the skin or icterus (Chae, 2004). The high prevalence of PCV2 infection of this herd was also confirmed by serological profile in all pigs including the sows, nursery and growing pigs. Evidently, the averaged S/P ratio and seroprevalence were high in the gilts probably due to the gilt acclimatization process in the endemic area. In the piglets, the declining of the maternal derived antibodies was seen between 3 to 7 weeks of age. The seroconversion was later observed from 9 weeks to 24 weeks of age indicating that the piglets were naturally infected with PCV2 between 5-9 weeks of age when their maternal immunity was declined. The same pattern of maternal immunity decay was also observed previously (McKeown, et al., 2005). Similar to the study in a large Canadian farrow to finishing barn, seroconversion of the piglets to PCV2 was observed during 10 and 15 weeks of age indicating the transmission during the nursery period (MacIntosh et al., 2006). High seroprevalence of PCV2 in the grower and finisher pigs and the presence of PCV2 DNA in sera or the viremic condition at 9, 12, 16 and 24 weeks of ages were well correlated to the clinical signs of PMWS observed in this farm.

During the experiment, respiratory signs (coughing and dyspnea) were more frequently observed in both groups of pigs during 5 to 9 weeks of ages, when the animals were susceptible for PCV2 and PRRSV infection resulting in the increasing of secondary bacterial infection and mortality rate at this time. The evidence of PCV2 infection in these affected pigs was confirmed by PCR. During this time (9 week-old) the PCV2 DNA detection in the pooled sera (n = 10) were found 100% positive in the non-vaccinated pigs and these may correlate to the clinical signs and mortality rate at this age. Several previous studies showed that PCV2 vaccination could reduce the clinical diseases and mortality rate significantly (Fenaux et al., 2004; Urniza et al., 2006; Connor and Elsener, 2007). However, the improvement of the clinical diseases in the vaccinated pigs was not strongly evident in this study. Complicated factor such as PRRSV and secondary bacterial infection (Streptococcosis, Pasteurellosis, Salmonellosis and Colibacillosis) may play

the important role to produce the PMWS clinical signs and mortality rate of the experimental pigs as previous study (Kim and Chae, 2002; Pallares et al., 2002; Rovira et al., 2002; Chae, 2004). Co-infections of PRRSV and PCV2 result in a synergistic effect on transient decrease in immune cells in the peripheral blood of piglets (Shi et al., 2007). Moreover, the insufficiency of antibiotic treatment may not overcome the secondary bacterial infection occurring in the experimental pigs. These porcine respiratory disease complexes in the nursery resulted in poor body condition scores and high mortality rate in both groups.

Hematological studies in this experiment did not show statistically significant in both groups of the experimental pigs. Leukopenia in both groups were seen in small number, indicating that the time point collecting the sample did not relate to the onset of leukopenia (normally 7-10 day post infection) (Segales et al., 2004; Shi et al., 2007), and the secondary bacterial infection in the experimental pigs during the experiment.

Similar to the preliminary data, the waning pattern of the maternal derived antibodies PCV2 titers were similarly seen in both groups. After vaccination, the seroconversion was observed in vaccinated pigs at 9 weeks of age suggesting the induction of PCV-2 antibodies within 4-5 weeks post vaccination. The similar pattern was also seen in another study when the vaccinated pigs with Suvaxyn® PCV2 showed seroconversion to PCV2 within 4 to 6 weeks post vaccination (Fenaux et al., 2004). In non-vaccinated pigs, PCV2 seroconversion was detected later at about 12 weeks of age. This might be due to the natural PCV2-infection after weaning as previously seen in the preliminary study of this farm in January 2007.

Before the experiment in this studied herd, PCV-2 viremia was demonstrated in the grower and finisher pigs indicating the active circulation of PCV2 in the herd. Interrestingly, after vaccination, none of PCV2 DNA was detected in the pooled sera of vaccinated pigs before 15 weeks of age, implying that vaccine-induced antibody could successfully reduce PCV-2 viremia. In contrast, PCV2 DNA was detected in non-vaccinated pigs since the beginning of the experiment until 15 weeks of age. The similar results of the PCV2 viremic reduction were also

demonstrated in the earlier studies (Fenaux et al., 2004: Urniza et al., 2006; Opriessnig et al., 2007; Opriessnig et al., 2008).

Macroscopically, the enlargement of superficial inguinal lymph nodes of vaccinated pigs was smaller than those in non-vaccinated pigs. Moreover, the average of lymph node/body weight ratio in vaccinated pigs was tended to lower than those in non-vaccinated pigs but not statistically different. This result suggested that the vaccinated pigs might have lesser lesions in the lymph nodes similar to the previous report (Fenaux et al., 2004; Urniza et al., 2006). The pneumonic lung scores did not differ in both group based on the recovery and chronic lesions in the lung. Interestingly, one vaccinated pigs showed severe edema of perirenal and periureteral areas with moderate diffuse lymphohistiocytic interstitial nephritis. These lesions were related to porcine dermatitis and nephropathy syndromes (PDNS) (Choi and Chae, 2001; Chae, 2005). The kidney lesions observed in this animal could be the result of PCV2 infection concurrent with PRRSV and other bacteria as similar to other studies (Choi and Chae, 2001; Chae, 2005; Opriessnig et al., 2007).

Histopathologically, PMWS has main characteristic lesions including lymphoid depletion and histiocytosis produce granulomatous lymphadenitis. Those two characteristic lesions were observed in both groups of the experimental pigs. However, mean scores of lymphoid depletion and histiocytosis in those lymphoid organs of the vaccinated pigs were lower than those in the non-vaccinated pigs, especially in the mesenteric lymph node and Peyer's patches. Interestingly, granulomatous pneumonia was more frequently observed in non-vaccinated pigs than in vaccinated pigs. These results showed that histological lesions of the vaccinated pigs were less severe than non-vaccinated pigs. These results were similar to the previous studies in which vaccination with Suvaxyn® PCV2 demonstrated significantly decreased lymphoid depletion and histiocytosis in the vaccinated pigs compared to the non-vaccinated pigs (Fenaux et al., 2004; Urniza et al., 2006; Opriessnig et al., 2007; Opriessnig et al., 2008).

Numbers of pigs with positive immunohistochemical detection in the superficial inguinal lymph node in vaccinated pigs (7/20) were lower than those in non-vaccinated pigs (12/20). Although it was not statistically significant, the mean IHC scores of PCV2 detection in vaccinated

pigs were lower than those in the non-vaccinated pigs. In addition, PCR detection of PCV2 in the pooled lymph nodes in vaccinated pigs also revealed the lower percentages. These results suggested that the vaccinated pigs may be able to clear the viral burden in their lymphoid tissues leading to the reduction in severity of the lymph nodes. However, the IHC detection of PCV2 may not be a good tool for evaluating the severity of the PCVAD since low amount of PCV2 antigen could be found in the chronic stage of infection represented by the granulomatous inflammation in lymphoid tissue (Opriessnig et al., 2007).

Conclusion

In conclusion, this study demonstrated that the efficacy of Suvaxyn® PCV2 is able to induce PCV2 antibody, reduce PCV2 viremia and decrease pathological lesions in the field conditions. However, the PMWS clinical signs and mortality rate did not differ between these two groups. The co-infection with PRRSV and other secondary bacterial infection in the experimental pigs still play the important roles in the PMWS producing in this farm. These results confirm that good management practices and control of co-infections are more important in reducing impact of severe PCVAD. The American Association of Swine Veterinarians (AASV) has recommended 20 Madec's principles complied by Dr. Francois Madec (National Pork Board, 2006). These measures were designed to reduce infection pressure in regard to PCV2, control other infections, improve hygiene and reduce stress at the different production stages. In our study, strict biosecurity, effective control management practices, and utilizing strategic medication and vaccination to control co-infections should be considered as the first priority. The veterinarians may consider recommending the use of a circovirus vaccine based on the situation and herd prevalence in farms. The serological profiles of PCV2 should also be evaluated before vaccine implementation.

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APPENDICES

Appendix A

Table A1 White blood cell count (x 1000 cells/µl) in the experimental pigs

	Age (weeks)				
Group	4	5	7	9	12
1A	19.2	22.0	20.0	12.5	19.4
2A	11.6	21.3	15.4	4.7*	35.5
3A	10.6	25.5	15.6	15.5	NA
4A	10.7	24.2	17.0	24.0	24.3
5A	8.8*	19.0	16.6	20.4	16.9
6A	13.0	22.7	17.6	10.8	61.2
7A	NA	13.7	19.0	17.7	29.0
8A	NA	19.5	18.2	32.3	21.2
9A	NA	31.1	11.9	21.4	28.2
10A	NA	11.6	17.4	15.2	15.8
1B	9.1	16.0	16.4	17.1	41.8
2B	7.3*	15.9	18.9	25.5	33.0
3B	9.9	12.8	17.6	18.9	15.2
4B	3.6*	17.5	17.4	14.2	19.9
5B	13.8	15.3	46.5	17.2	29.9
6B	9.1	27.2	13.8	14.0	21.6
7B	NA	13.7	19.5	7.8*	15.1
8B	NA	17.7	18.4	28.0	33.4
9B	NA	9.7	17.0	14.9	14.6
10B	NA	22.8	18.7	24.0	16.8

* Leukopenia

NA = not examined

Table A2 Body weight and average daily weight gain from the experimental pigs (n = 20/group)

	D0 : 4 weeks (kg)	D84 : 16 weeks (kg)	ADG (kg/day)		D0 : 4 weeks (kg)	D84 : 16 weeks (kg)	ADG (kg/day)
1A1	6.0	37.0	0.37	**1B1**	6.4	38.2	0.38
1A2	6.2	46.0	0.47	**1B2**	5.0	39.4	0.41
2A1	7.2	31.0	0.28	**2B 4394**	5.4	35.2	0.35
2A2	6.2	36.6	0.36	**2B2**	5.0	38.0	0.39
3A1	5.0	30.8	0.31	**3B 4248**	6.0	36.6	0.36
3A 4309	5.5	42.6	0.44	**3B2**	5.0	36.0	0.37
4A1	7.0	41.2	0.41	**4B1**	8.0	41.8	0.40
4A2	7.2	35.8	0.34	**4B2**	7.2	47.0	0.47
5A1	5.9	40.4	0.41	**5B1**	6.0	35.6	0.35
5A2	6.1	43.0	0.44	**5B 4464**	6.3	41.6	0.42
6A1	5.4	34.8	0.35	**6B 4239**	6.2	46.0	0.47
6A2	6.2	56.0	0.59	**6B 4420**	6.2	37.2	0.37
7A1	6.4	23.6	0.20	**7B1**	7.3	40.8	0.40
7A2	5.0	29.4	0.29	**7B2**	7.0	37.4	0.36
8A 4621	5.6	39.0	0.40	**8B1**	5.6	43.6	0.45
8A 4450	6.4	37.6	0.37	**8B2**	6.0	48.8	0.51
9A 4636	6.5	41.8	0.42	**9B1**	5.9	28.2	0.27
9A2	6.8	41.0	0.41	**9B 4159**	5.0	29.6	0.29
10A 4398	6.0	40.6	0.41	**10B1**	5.8	35.2	0.35
10A2	5.6	24.4	0.22	**10B2**	4.2	19.8	0.19

Table A3 PCR detection of PCV2 DNA in pooled sera

	4 wks	5 wks	7 wks	9 wks	12 wks	15 wks
1A	-	-	-	-	-	-
2A	-	-	-	-	-	-
3A	-	-	-	-	-	-
4A	-	-	-	-	-	-
5A	-	-	-	-	-	-
6A	-	-	-	-	-	+
7A	-	-	-	-	-	-
8A	-	-	-	-	-	-
9A	-	-	-	-	-	-
10A	-	-	-	-	-	-
	4 wks	5 wks	7 wks	9 wks	12 wks	15 wks
1B	-	+	-	+	+	-
2B	-	+	+	+	-	-
3B	-	+	+	+	-	-
4B	-	+	-	+	-	-
5B	-	+	+	+	+	+
6B	-	+	-	+	-	-
7B	+	+	+	+	-	+
8B	-	-	-	+	+	+
9B	+	-	+	+	-	-
10B	-	-	-	+	-	-

+ Positve , - Negative

Table A4 PCR detection of PCV2 DNA in pooled organs and pooled lymph nodes

ID	lymph node	organ	ID	lymph node	organ
1A1	+	+	1B1	+	+
1A2	+	+	1B2	+	+
2A1	+	+	2B1	+	+
2A2	+	+	2B2	+	+
3A1	+	+	3B1	+	+
3A2	+	+	3B2	+	-
4A1	+	-	4B1	+	+
4A2	-	-	4B2	+	+
5A1	-	-	5B1	+	-
5A2	-	-	5B2	+	+
6A1	+	+	6B1	+	+
6A2	-	-	6B2	+	+
7A1	+	+	7B1	+	+
7A2	-	-	7B2	+	+
8A1	+	+	8B1	+	+
8A2	+	+	8B2	+	+
9A1	+	+	9B1	+	+
9A2	+	+	9B2	+	+
10A1	+	+	10B1	+	+
10A2	+	+	10B2	+	+

+ Positve , - Negative

Table A5 Lung scores and enlargement of lymph nodes in the experimental pigs (group A)

	Superficial inguinal ln		Tracheobronchial ln	Lung	Mesenteric ln
	Enlargement	Weight (g)	Enlargement	Scores	Enlargement
1A1	1	15	0	0.5	2
1A2	1	13	0	0	2
2A1	1	12	0	0	1
2A2	1	9	0	0	2
3A1	1.5	15	0	0	2
3A 4309	1.5	15	0	0	2
4A1	2	14	0	0	1.5
4A2	1.5	14	0	0	2
5A1	1.5	12	0	0	2
5A2	1	11	0	0	1.5
6A1	1.5	15	0	0	1
6A2	2	22	0	0	2
7A1	1.5	14	0	11	2
7A2	1.5	13	0	8	2
8A 4621	2	26	0	0	2
8A 4450	1	12	0	0	2
9A 4636	1	15	0	0	2
9A2	2	21	0	0	2
10A 4398	1	9	0	0.5	2
10A2	1	8	0	1	2

ln = lymph nodes

Table A6 Lung score and enlargement of lymph nodes in the experimental pigs (group B)

	Superficial inguinal ln		Tracheobronchial ln	Lung	Mesenteric ln
	Enlargement	Weight (g)	Enlargement	Scores	Enlargement
1B1	2	22	0	0	1.5
1B2	3	32	0	0	2
2B 4394	2	21	0	0	2
2B2	1.5	12	0	0	2
3B 4248	1.5	14	0	0	2
3B2	1	18	0	7	2
4B1	2	17	0	0	1.5
4B2	1	11	0	0	2
5B1	1.5	18	0	0	2
5B 4464	2	19	0	0	2
6B 4239	2	16	0	0	2
6B 4420	2	24	0	0	2
7B1	1.5	15	1	11	2
7B2	1.5	21	0	1	2
8B1	1	11	0	0	2
8B2	1.5	12	0	0	2
9B1	2	14	0	0	2
9B 4159	1.5	11	0	4	2
10B1	2	18	0	0	2
10B2	1	10	0	0	2

ln = lymph nodes

Table A7 Histopathological scores and immunohistochemistry scores in the experimental pigs (group A)

	Superficial inguinal ln			Tracheobronchial ln			Mesenteric ln			Peyer 's patches		
	IHC score	LD	HR	IHC score	LD	HR	IHC score	LD	HR	IHC score	LD	HR
1A1	1	3	1	1	2	1	0	1	2	0	1	2
1A2	0	3	3	0	2	2	0	1	1	0	0	0
2A1	0	3	2	0	2	2	0	1	1	0	0	0
2A2	0	3	2	0	1	2	1	1	1	0	0	0
3A1	0	3	3	1	2	1	1	1	1	0	1	1
3A 4309	0	3	3	1	2	2	1	2	2	0	1	1
4A1	1	3	3	0	1	1	0	1	1	0	2	2
4A2	0	3	3	0	2	2	0	2	3	1	0	0
5A1	1	3	3	1	2	2	0	2	2	1	2	2
5A2	1	2	2	0	2	2	0	2	2	0	1	1
6A1	0	2	2	1	0	0	0	1	1	0	1	1
6A2	0	3	3	0	2	2	0	1	1	0	1	1
7A1	1	3	3	0	3	3	1	1	1	0	2	3
7A2	0	3	2	0	1	2	1	0	0	0	0	0
8A 4621	0	3	2	1	2	2	0	2	2	0	0	0
8A 4450	0	2	2	1	1	2	1	0	0	0	1	1
9A 4636	1	2	2	0	1	1	1	1	1	0	0	0
9A2	0	3	3	0	1	2	0	1	1	0	0	0
10A 4398	0	2	2	0	2	2	0	1	1	0	0	0
10A2	1	3	3	0	3	3	0	0	0	1	0	0

IHC = immunohistochemistry LD = lymphoid depletion HR = histiocytic replacement

Table A8 Histopathological scores and immunohistochemistry scores in the experimental pigs (group B)

	Superficial inguinal ln			Tracheobronchial ln			Mesenteric ln			Peyer 's patches		
	IHC score	LD	HR	IHC score	LD	HR	IHC score	LD	HR	IHC score	LD	HR
1B1	1	3	3	0	1	2	1	3	2	0	2	2
1B2	1	2	3	0	2	2	1	3	3	0	2	2
2B 4394	1	2	3	0	3	3	0	3	3	1	1	2
2B2	0	3	3	0	2	3	0	1	1	0	2	2
3B 4248	1	3	3	1	2	2	0	2	2	0	2	2
3B2	1	3	3	1	1	2	1	2	2	0	2	3
4B1	1	2	2	0	1	1	1	2	2	0	1	1
4B2	2	2	3	0	1	1	0	2	1	1	1	1
5B1	0	2	3	0	3	2	0	1	1	0	1	2
5B 4464	0	3	2	1	3	3	1	3	2	0	2	2
6B 4239	1	3	3	1	2	2	0	1	1	0	1	1
6B 4420	0	3	3	0	2	2	1	1	2	1	3	3
7B1	1	3	2	0	2	2	0	0	0	0	0	0
7B2	1	3	3	1	2	3	0	2	2	0	1	2
8B1	1	3	3	0	2	2	0	1	2	1	1	1
8B2	1	2	2	0	3	3	0	2	2	0	2	2
9B1	0	3	3	1	2	3	0	2	2	1	1	2
9B 4159	0	3	3	0	2	1	1	2	2	0	1	1
10B1	0	3	2	1	3	3	0	3	3	0	1	1
10B2	0	3	3	0	1	1	1	2	2	0	1	1

IHC = immunohistochemistry LD = lymphoid depletion HR = histiocytic replacement

Appendix B

Reagent for a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (Fort Dodge Animal health, Biological Research & Development and Regulatory Affairs)

A. ELISA Immunoplate ; 96 well NUNC Maxi Sorp

B. Plate sealers

C. Coating buffer ; 0.05 M Sodium carbonate\Sodium bicarbonate

   a. 1.59 g Na_2CO_3

   b. 2.93 g $NaHCO_3$

   c. Add a distilled water to yield 1 L

   d. pH 9.70 ± 0.10

   e. store at 2 to 7 ºC (use within 5 days)

D. 0.01 M Phosphate buffer saline (PBS)

   a. 8.5 g NaCl

   b. 0.253 g NaH_2PO_4

   c. 1.19 g Na_2HPO_4

   d. pH 7.2 ± 0.10

   e. Add a distilled water to yield 1 L

   f. Store at 2 to 7 ºC (use within 30 days)

E. 0.3 % Tween/PBS Wash buffer and reagent

   a. Dissolve 3 ml of Tween 20 in 1 L of 0.o1 M PBS pH 7.2 pH 7.2 ± 0.10

   b. Store wash buffer at 15 to 30 ºC (use within 30 days)

   c. Store reagent at 2 to 7 ºC (use within 30 days)

F. Blocking reagent : Non-fat dry milk (NFDN)

   a. Dissolve 5.75 g of NFDM in coating buffer 500 ml

   b. Filter with Schleicher and Schuell#588 Filters or equivalent

   c. Store reagent at 2 to 7 ºC (use within 5 days)

G. Diluent reagent : 1.15% NFDM in 0.3% Tween/PBS

   a. Dissolve 5.75 g of NFDM in 0.3% Tween/PBS 500 ml

   b. Filter with Schleicher and Schuell#588 Filters or equivalent

   c. Store reagent at 2 to 7 ºC (use within 5 days)

H. Swine serum control : PCV2 swine serum, Fort Dodge Animal Health Lot No. 2117-89-15Jun04

I. Conjugate : Peroxidae-conjugated AffiPure Goat Anti-swine IgG, Jackson ImmunoResearch Laboratories, Cat. No. 114-035-003

J. TMB Substrate Kit for peroxidase, Kirkegaard & Perry Laboratory (KPL)

   a. Solution A (TMB Peroxidase Substrate) Cat. No. 50-76-02

   b. Solution B Cat. No. 50-65-02

   c. Store at 2 to 7 ºC

K. PCV2 Positive capture antigen control : rBacV-PCV2 capsid, Fort Dodge Animal Health Lot No. 2256-44-12May05

L. PCV2 Negative capture antigen control : Sf 9 cells, Fort Dodge Animal Health Lot No. 2256-45-12May05

BIOGRAPHY

Mr. Termsitthi Paphavasit was born in 1979 at Bangkok, Thailand. He received Doctor of Veterinary Medicine from Chulalongkorn University in 2003. His major of interesting is swine pathology. He worked at Veterinary Diagnostic Laboratory, Livestock Animals Hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakon-pathom, Thailand for three year. At present, he studied and worked as teacher assistant in Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.