การพัฒนาซับยูนิตวัคซีนและวิธีวินิจฉัยโรคเซอร์โคไวรัสชนิดที่สองในสุกร

นางสาวสุภัทตรา จิตติมณี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2556

DEVELOPMENT OF A SUBUNIT VACCINE AND DIAGNOSTIC ASSAYS AGAINST PORCINE CIRCOVIRUS TYPE 2

Miss Suphattra Jittimanee

A Dissertation Submitted in Partial Fulfillment of Requirements

for the Degree of Doctor of Philosophy Program in Veterinary Pathobiology

Department of Veterinary Pathology

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2013

Thesis Title	DEVELOPMENT OF A SUBUNIT VACCINE AND DIAGNOSTIC ASSAYS AGAINST PORCINE CIRCOVIRUS TYPE 2
By	Miss Suphattra Jitiimanee
Field of Study	Veterinary Pathobiology
Thesis Advisor	Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.
Thesis Co-advisor	Assistant Professor Komkrich Teankum, D.V.M., M.Sc., Dr.Med.Vet.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

…….Dean of the Faculty of Veterinary Science
(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

……..Chairman
(Associate Professor Anudep Rungsipipat, D.V.M., Ph.D.)

……..Thesis advisor
(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

…….Thesis co-advisor
(Assistant Professor Komkrich Teankum, D.V.M., M.Sc., Dr.Med.Vet.)

…….Examiner
(Associate Professor Sanipa Suradhat, D.V.M., Ph.D.)

…….Examiner
(Associate Professor Wijit Banlunara, D.V.M., Ph.D.)

…….External examiner
(Associate Professor Porntippa Lekcharoensuk, D.V.M., M.Sc., Ph.D.)

สุภัทตรา จิตติมณี : การพัฒนาซับยูนิตวัคซีนและวิธีวินิจฉัยโรคเซอร์โคไวรัสชนิดที่สองในสุกร (DEVELOPMENT OF A SUBUNIT VACCINE AND DIAGNOSTIC ASSAYS AGAINST PORCINE CIRCOVIRUS TYPE 2) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร.คมกฤช เทียนคำ, 98 หน้า

กลุ่มอาการพีซีวีเอดีซึ่งมีเชื้อเซอร์โคไวรัสชนิดที่สองในสุกรเป็นสาเหตุ พบการระบาดครั้งแรกในประเทศแคนาดาปี พ.ศ. 2534 ซึ่งปัจจุบันก่อปัญหาต่อสุขภาพของสุกรหลายระบบและก่อให้เกิดผลเสียหายทางเศรษฐกิจต่อระบบการเลี้ยงสุกร การศึกษาทางด้านไวรัสวิทยา ซีรัมวิทยาของเชื้อเซอร์โคไวรัสชนิดที่สอง รวมไปถึงการพัฒนาวิธีตรวจวินิจฉัยโรคและการพัฒนาวัคซีนจึงมีความสำคัญที่จะช่วยเพิ่มประสิทธิภาพในการป้องกันและควบคุมโรคได้ การศึกษาครั้งนี้ประกอบด้วย 3 ส่วนคือ ลักษณะทางพันธุกรรมของเชื้อเซอร์โคไวรัสชนิดที่สองจากสุกรที่ป่วยด้วยกลุ่มอาการพีซีวีเอดีในประเทศไทยในช่วงปี พ.ศ. 2550 ถึง พ.ศ. 2553 ผลพบเชื้อที่พบทั้งหมดจัดอยู่ในจีโนไทป์ 1 หรือจีโนไทป์บี (PCV2b) โดยกลุ่มย่อย 1A/B เป็นสายพันธุ์ที่พบมากที่สุด ทำการศึกษาพัฒนาชุดทดสอบอีไลซาโดยใช้รีคอมบิแนนท์แคปซิดโปรตีนของเชื้อเซอร์โคไวรัสชนิดที่สองจีโนไทป์บีที่ผลิตด้วยระบบ *Escherichia coli* มาเป็นแอนติเจนของชุดทดสอบ เมื่อเปรียบเทียบประสิทธิภาพของชุดทดสอบอีไลซากับไอพีเอ็มเอซึ่งเป็นวิธีมาตรฐานทางซีรัมวิทยากับตัวอย่างซีรัมสุกร พบว่าชุดทดสอบที่ผลิตขึ้นนี้มีความไวร้อยละ 98.33 ความจำเพาะร้อยละ 93.33 และความแม่นยำร้อยละ 96.67 ส่วนการศึกษาพัฒนาซับยูนิตวัคซีนจากรีคอมบิแนนท์แคปซิดโปรตีนของเชื้อเซอร์โคไวรัสชนิดที่สองจีโนไทป์บีและทดสอบประสิทธิภาพในการกระตุ้นภูมิคุ้มกันชนิดสารน้ำและช่วยลดปริมาณของเชื้อในกระแสเลือดของสุกรในฟาร์มที่ประสบปัญหาพีซีวีเอดีนั้น พบว่าซับยูนิตวัคซีนที่ผลิตขึ้นนี้สามารถกระตุ้นภูมิคุ้มกันชนิดสารน้ำที่จำเพาะได้ แต่สามารถควบคุมปริมาณของเชื้อในกระแสเลือดได้เพียงบางส่วน ซึ่งผลการทดลองที่เกิดขึ้นนี้อาจเป็นผลเนื่องมาจากปริมาณของโปรตีนที่ใช้ยังไม่เพียงพอ รวมไปถึงชนิดของสารสื่อที่ใช้นั้นอาจยังไม่เหมาะสม ซึ่งจำเป็นต้องทำการศึกษาเพิ่มเติมเพื่อปรับปรุงให้ซับยูนิตวัคซีนที่ผลิตขึ้นนี้มีประสิทธิภาพที่สูงขึ้นต่อไป

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา พยาธิวิทยาชีววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

517595931: MAJOR VETERINARY PATHOLOGY
KEYWORD: PORCINE CIRCOVIRUS TYPE 2 / GENETIC CHARACTERIZATION / SEROLOGY ASSAYS / VACCINE / THAILAND

SUPHATTRA JITTIMANEE: DEVELOPMENT OF A SUBUNIT VACCINE AND DIAGNOSTIC ASSAYS AGAINST PORCINE CIRCOVIRUS TYPE 2.
ADVISOR: PROF. ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D,
CO-ADVISOR: ASST. PROF. KOMKRICH TEANKUM, D.V.M., M.Sc., Dr.Med.Vet.
98pp.

Porcine circovirus type 2 (PCV2) was first recognized as a causative agent of porcine circovirus associated diseases (PCVAD) in Canada in 1991. Subsequently, PMWS has become a major economic problem to the swine industry worldwide including Thailand. Importantly, virological, serological study and also development of vaccine and serological assay are useful for prevention and control of PCVAD. Genetic characterizations of PCV2 isolates from Thai pigs with PCVAD during 2007-2010 revealed that all recent Thai PCV2 sequences belong to genotype 1 (PCV2b) and subgroup 1A/B was predominated in this study. Later, an indirect ELISA was developed and validated for PCV2 antibody detection using a recombinant truncated capsid (rntCap) protein expressed in *E. coli* system. Swine serum samples were obtained and tested using the indirect ELISA comparing with IPMA as a gold standard for serological assay. The results demonstrated that the diagnostic sensitivity, specificity and accuracy of a developed indirect ELISA are 98.33%, 93.33% and 96.67%, respectively. Finally, the efficacy of a developed subunit PCV2b vaccine using a rntCap protein expressed in *E. coli was* determined in PCVAD-affected farm. The results indicated that vaccination by a developed subunit PCV2b vaccine could induce specific antibody and partially control PCV2 viral load in serum suggesting that using higher protein concentration dose and/or using an appropriate adjuvant could enhance the better efficacy.

Department : Pathology Student's Signature…….
Field of Study : Pathobiology Advisor's Signature…….
Academic Year : 2013 Co-advisor's Signature…….

ACKNOWLEDGMENTS

I would like to express my appreciation and grateful thanks to my advisors, Prof. Dr. Roongroje Thanawongnuwech and Asst. Prof. Dr.Komkrich Teankum for their guidance and helpful suggestion throughout this work as my mentor. And I would like to express my appreciation thanks to committee members for their suggestion to my work.

I would like to thank Drs. Suparlark Nuntawan Na Ayudhya, Donruethai Sreta, Roongtham Kedkovid, Thanin Mulaseewa for their assistant in vaccination and sample collection. I am grateful to all my peer graduate students and all the members of the Veterinary Diagnosis Laboratory, Pathology, Virology and Microbiology department, Faculty of Veterinary Science, Chulalongkorn University, for their warmhearted, kindness helps and encouragement.

I do appreciate the financial support of this work by grants from National Research Council of Thailand and The National Research University from Chulalongkorn University (Health Cluster-HR1164A7). I would like to thank to the grant supporting for my Doctor of Philosophy Program in Veterinary Pathobiology study by the Royal Golden Jubilee Ph.D. (RGJ) Program from the Thailand Research Fund (TRF) and Chulalongkorn University (PHD/0252/2550).

Finally, I would like to express my sincerely thank to my parents (Mr.Surapon and Mrs.Suporn Jittiimanee) and my sisters (Ms.Jutharat and Ms.Suthasinee Jittimanee) for their support throughout my Doctor of Philosophy study.

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AEC	=	3-amino-9-ethylcarbazole
BSA	=	Bovine serum albumin
CIAP	=	Calf intestinal alkaline phosphatase
dpv	=	Day post vaccination
DC	=	Dendritic cells
ELISA	=	Enzyme -Linked Immunosorbent Assay
GS4B	=	Glutathione-Sepharose 4B
GST	=	Glutathione S-transferase
HRP	=	Horseradish Peroxidase
IL	=	Interleukin
IFA	=	Indirect fluorescent antibody assay
IPMA	=	Immunoperoxidase monolayer assay
IPTG	=	Isopropyl thiogalactopyranoside
LB	=	Luria-Bertani
NLS	=	Nuclear localizing signal
ntCap	=	NLS truncated capsid
ORF	=	Open reading frame
PBS-T	=	Phosphate buffer saline - 0.5% Tween-20
PCR	=	Polymerase chain reaction
PCV2	=	Porcine circovirus type 2
PCVAD	=	Porcine circovirus associated diseases
PDNS	=	Porcine dermatitis & nephropathy syndrome
PK-15 cells	=	Porcine kidney-15 cells line
PMWS	=	Postweaning multisytemic wasting syndrome
PRRSV	=	Porcine reproductive and respiratory syndrome virus
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
rntCap	=	Recombinant NLS truncated capsid

S/P ratio	=	Sample / positive ratio
TBS-T	=	Tris-buffered saline – 0.5% Tween 20
TMB	=	Tetramethylbenzidine
qPCR	=	Quantitative polymerase chain reaction

CHAPTER I

1.1 INTRODUCTION

Porcine circovirus type 2 (PCV2) has been recognized as a causative agent of postweaning multisystemic wasting syndrome (PMWS), a multi-factorial disease in swine (Harding and Clark, 1997). PCV2 has also been associated with several clinical and pathological conditions in pigs including porcine respiratory disease complex (PRDC), reproductive failures, porcine dermatitis and nephropathy syndrome (PDNS), proliferative and necrotizing pneumonia and congenital tremor (Darwich et al., 2004; Fenaux et al., 2004; Chae, 2005). Currently, porcine circovirus associated diseases (PCVAD) was commonly used and previously was first recognized in Canada in 1991. PCVAD has subsequently been observed in almost all intensive pig production regions of the world (Allan and Ellis, 2000; Chae, 2005). However, major problems of PCV2-affected herds are mostly due to co-infection with other pathogens such as porcine reproductive and respiratory syndrome (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), *Streptococcus suis* and *Mycoplasma hyopneumoniae*.

Although, PCVAD can be subclinical or severe clinical manifestations depending on four main factors consist of virus, host, co-infections and immune modulation (Opriessnig et al., 2007). The morbidity rate varies from 4% to 30%, according to the health status of the affected herd, but the mortality rate of affected pigs is high (70-80%) (Harding and Clark, 1997). As a result, PCV2 is one of the major causative agents leading to economic loss in the swine industry worldwide. The economic impact of PCVAD in the United States (US) has cost producers an average of 3-4 dollars per pig and up to 20 dollars per pig in peak losses (Gillespie et al., 2009). Factors underlying the economic losses consist of lesser amount of pigs at slaughter, reduced feed conversion rates, increased costs for medication and management of poorly pigs and

costs of other diseases following PCV2-associated immunosuppression (Segalés et al., 2004[a]).

In Thailand, PMWS was firstly reported in 1998 (Tantilertcharoen et al., 1999) and a PCV2 retrospective study using nested PCR from formalin-fixed tissues, paraffin-embedded (FFPE) of PMWS pigs in Thailand identified PCV-infected case occurring as early as in 1993 (Kiatipattanasakul-Banlunara et al., 2002). Recently, data from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) based on swine diagnosis annual report in 2009 (unpublished data) found increasing incidence of PCVAD in the cases of swine systemic infection (41.62%, n = 197) and the number of cases seemed to increase in 2010 (47.06%, n = 304) and 2011 (41.89%, n = 592). This report indicates that PCV2 is one of the major causative agents of Thai swine industry.

PCV2 can be divided into 2 genotypes: PCV2a and PCV2b (Cheung et al., 2007; Takahagi et al., 2008; Kim et al., 2009[b]). In addition, PCV2c genotype has also been described and found only in Denmark (Segalés et al., 2008). Recently a new type of PCV referred to PCV1/2a was found in Canada in 2009 and has raised the concern on its origin, possibly from the chimeric killed vaccine. In Thailand, it should be noted that only one isolate of Thai PCV2 has been submitted to GenBank (AY864814) in 2004 and the genetic information of PCV2 in Thai swine herds are needed.

Traditionally, virus isolation is regarded as a gold standard technique in viral diagnosis. However, PCV2 virus isolation is difficult and time consuming because PCV2 does not produce cytopathic effects and having some difficulties to passage through infected-cells after treated with d-glucosamine. PCVAD can be recognized by expression of hallmark lesions associated with PCV2 antigen or DNA in the particular organs. Currently, immunohistochemistry (IHC), *in situ* hybridization (ISH) and a rapid PCR technique are considered as alternative diagnostic assays for PCVAD (Kim et al.,

2009[a]). However, a rapid and sensitive assay is not suitable for large-scale population-based screening of swine herds. Therefore, to develop serological techniques that can accurately detect the presence of PCV2 antibody in swine sera is necessary. Routine serological tests for detection the antibodies against PCV2 are currently performed by indirect fluorescent antibody (IFA) tests and immunoperoxidase monolayer assays (IPMA) which are highly time-consuming and labor intensive. An indirect enzyme-linked immunosorbent assay (ELISA) is much simpler to produce and perform, time-saving, low cost and suitable for large scale surveys of PCV2 infection. It is also good for evaluation of the efficacy of various vaccines against PCV2. This ELISA test would be beneficial for obtaining epidemiological information on the prevalence of PCV2 in the herds, and providing the data for the better control and management of the disease.

Extensive researches have focused on the development of vaccines against PCV2 and several commercial vaccines are now available. The first available one is an inactivated PCV2 oil adjuvant vaccine used for sows and gilts to protect the piglet through passive transfer of PCV2-protective maternal antibody via colostrums. The other vaccines are recommended for piglets, one is killed chimera PCV combining the antigenic capsid gene of PCV2 into the PCV1 backbone (Fenaux et al., 2004) and others are subunit vaccines from a recombinant capsid protein expressed in baculovirus (Fachinger et al., 2008). Experimental results indicate that vaccines against PCV2 are helpful in reducing viremia and PCV2-associated lesions in challenge models (Patterson et al., 2008; Fort et al., 2009; Opriessnig et al., 2010[a]). From the previous studies, the capsid protein is the preferred antigen for both serological tests and subunit vaccine candidate because of its strong immunoreactivity (Blanchard et al., 2003[b]; Zhou et al., 2005). The difficulties in expressing ORF2 protein in *Escherichia coli* cells are most likely due to the specific amino acid composition of nuclear localization signal (NLS). As a result, the fusion of ORF2 with glutathione-S-transferase (GST) allowed expression in

minute amounts. Accordingly, the expression of recombinant capsid protein was successful primarily in the baculovirus expression system (Fan et al., 2007). Nevertheless, the problem of expression in the eukaryotic system is somewhat expensive. Recently a recombinant capsid and a recombinant NLS truncated capsid (rntCap) protein has successfully been expressed in the codon optimized *E. coli* cells and it was reacted with PCV2 positive swine serum in immunoblot (Trundova and Celer, 2007; Marcekova et al., 2009). The rntCap protein retains antigenic properties indicating some functionally important epitopes, and thus be interesting for further studies to develop serological diagnostic assays and vaccines against PCV2 associated diseases.

In our preliminary study on the efficacy of a developed subunit vaccine on immunological response in pigs revealed that the rntCap protein expressed in the *E. coli* system could react with anti-PCV2 antibody and also induce the specific antibody responses in the experimental pigs especially in the two shot vaccinations (Jittimanee et al., 2010). Interestingly, our developed subunit vaccine could be used as a potential vaccine candidate in the future. However, the efficacy of this vaccine in a PCV2-affected Thai swine herd is undergoing investigation.

1.2 OBJECTIVES The objectives of this study are:

1. To investigate the genetic characterizations of current Thai PCV2 isolates
2. To develop an in-house indirect ELISA for PCV2 diagnosis in Thailand.
3. To develop a subunit vaccine against PCV2 and investigate the efficacy of this vaccine on enhancing specific antibodies and reducing viremia in a PCV2-affected swine herd.

1.3 LITERATURE REVIEW

1.3.1 Porcine circovirus type 2 (PCV2)

Porcine circovirus (PCV) belonging to the family *Circoviridae*, is a small, non-enveloped, single-stranded DNA virus enclosing a circular genome of 1767-1768 nucleotides as shown in Figure 1.1 (Hamel et al., 1998). The *Circoviridae* contains two genera. The *Gyrovirus genus* is represented by chicken anemia virus (CAV). The *Circovirus genus* contains porcine circovirus with other animal viruses consisting of beak and feather disease virus of psittacine birds and columbid circovirus of pigeon (Mankertz et al., 2000; Chae, 2004). PCV is classified into 2 groups, which are antigenically and genetically different from each other (Mahé et al., 2000). Porcine circovirus type 1 (PCV1) was firstly identified as a persistent non-cytopathic contaminant of the porcine kidney (PK-15) cell line (Tischer et al., 1982). On the other hand porcine circovirus type 2 (PCV2) has been isolated from both healthy and clinically diseased pigs associated with post-weaning multisystemic wasting syndrome (PMWS) and porcine circovirus associated disease (PCVAD) (Allan et al., 1998[b]; Ellis et al., 1998; Morozov et al., 1998).

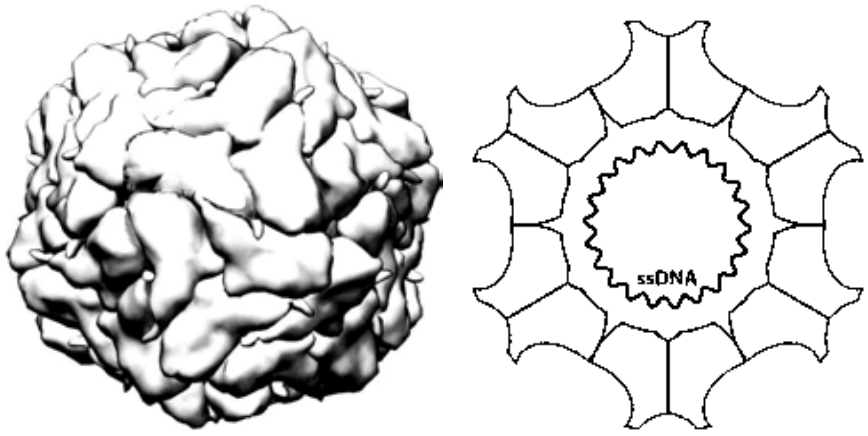

Figure 1.1: Morphology of Circovirus particles (adapted from Saha et al., 2012)

The genome of PCV2 contains 3 major open reading frames (ORFs). ORF1 encodes a replication protein of 35.7 kDa involved in virus replication which is highly conserved in all circoviruses (Mankertz et al., 1998). ORF2 encodes a major structural capsid protein of 27.8 kDa (Nawagitgul et al., 2000). ORF3 is essential for virus-induced apoptosis (Liu et al., 2006). The origin of replication is located in the intergenic region between the beginnings of ORF1 and ORF2 as shown in Figure 1.2 (Faurez et al., 2009).

Recently, a novel ORF4 gene was discovered and it was about 180 bp in length, located within ORF3 in the same direction (Figure 1.3). The functions of ORF4 involved in suppressing caspase activity and regulating CD4+ and CD8+ T lymphocytes during PCV2 infection (He et al., 2013).

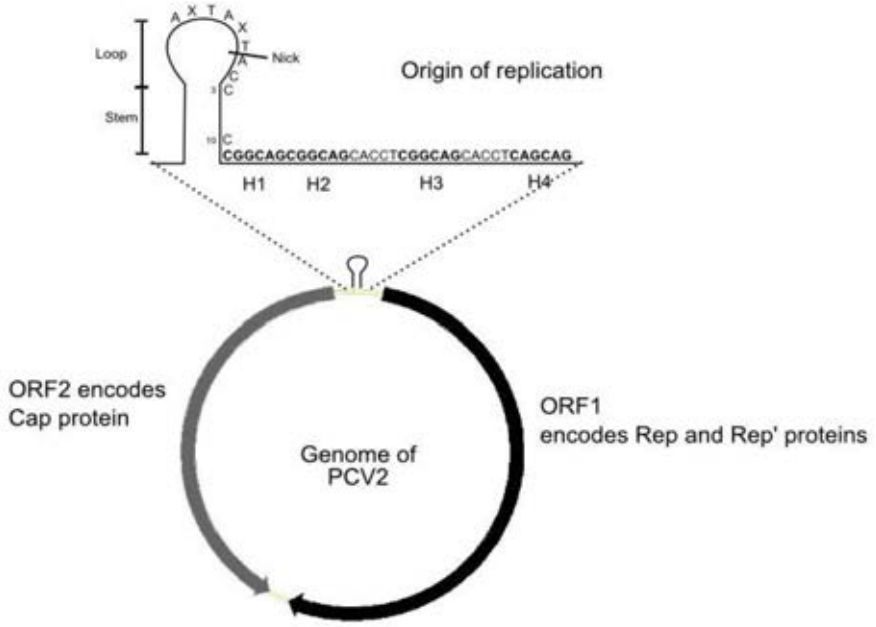

Figure 1.2: Demonstration of PCV2 genome; Black arrow: ORF1, located on the positive strand which encodes Rep and Rep' protein. Grey arrow: ORF2, located on the negative strand, which encodes Cap protein. Between ORF1 and ORF2 are intergenic regions. The origin of replication is located in the intergenic region between the beginnings of the two ORFs. H1, H2, H3 and H4 are hexamers (adapted from Faurez et al., 2009).

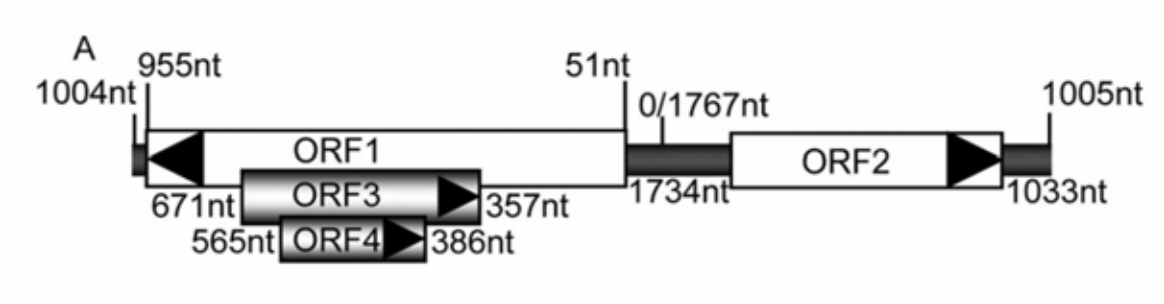

Figure 1.3: Genetic map of PCV2; The ORF2 ORF3 and ORF4 genes are transcribed leftward and the ORF1 gene is transcribed rightward (He et al., 2013).

Porcine circovirus associated diseases (PCVAD)

Postweaning multisystemic wasting syndrome (PMWS) or recently known as porcine circovirus associated disease (PCVAD) and PCV2-systemic disease (PCV2-SD), a multi-factorial disease of swine caused by porcine circovirus type 2 (PCV2) was firstly observed in Canada in 1991 (Harding and Clark, 1997). After initial reports, the huge spread of PMWS occurred in nearly all pig-producing countries around the world (Allan and Ellis, 2000). A summary of the historical events related with the information on porcine circoviruses are shown in Table 1.1 (Segalés et al., 2013).

PCVAD affected pigs of 7 to 15 weeks of age were clinically characterized by progressive weight loss, respiratory signs, lymph nodes enlargement, pallor, jaundice and diarrhea (Darwich et al., 2004; Segalés et al., 2004[b]). Generally, PCVAD is often co-infected with other pathogens such as porcine reproductive and respiratory syndrome (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), *Hemophillus parasuis, Actinobacillus pleuropneumoniae, Streptococcus suis* and *Mycoplasma hyopneumoniae* (Kim and Chae, 2002; Rovira et al., 2002; Chae, 2004). PCV2 induces lymphoid depletion in lymphoid organ, resulting in an increased susceptibility to other pathogens infection. This immunosuppression has been proved in PCV2-affected pigs (Darwich et al., 2003).

Table 1.1: Summary of historical events related with the information on porcine circoviruses (adapted from Segalés et al., 2013).

Year	Event	References
1962	Earliest retrospective evidence of porcine circovirus type 2 (PCV2) detection by PCR	(Jacobsen et al., 2009)
1982	First description of porcine circovirus (nowadays PCV1) as a very small porcine virus with circular single-stranded DNA	(Tischer et al., 1982)
1985	Earliest retrospective evidence of PCV2-systemic disease (PCV2-SD)	(Jacobsen et al., 2009)
1996	First description of a new, sporadic disease named postweaning multisystemic wasting syndrome (PMWS) – initial cases from 1991 to 1994. Association with a porcine circovirus-like agent (subsequently identified as PCV2)	(Harding and Clark, 1997)
1997	First descriptions of PCV2-SD as severe disease outbreaks in Europe	(Segalés et al., 1997)
1998	First isolation and characterization of PCV2 isolates	(Allan et al., 1998[b]; Ellis et al., 1998)
1998	First sequences of complete PCV2 genome available	(Morozov et al., 1998; Meehan et al., 2001)
1997 - 1999	First descriptions of PMWS as severe disease Outbreaks in Asia	(Choi et al., 2000)
1998	First case of PMWS observed in Thailand	(Tantilertcharoen et al., 1999)
1999	First experimental reproduction of PMWS by means of PCV2 and porcine parvovirus	(Allan et al., 1999[a])

co-inoculation

Year	Event	References
2004	First PCV2 vaccine available in France and Germany; inactivated, adjuvant product temporarily licensed for its use in sows	(Segalés et al., 2005)
2004 - 2005	First descriptions of PCVAD as severe disease outbreaks in North-America	(Carman et al., 2006)
2006	First year of availability of PCV2 vaccines in North-America; products licensed for using in piglets (USA and Canada) and sows (Canada)	(Opriessnig et al., 2007)
2007	First year of general availability of PCV2 vaccines all over the world	(Chae, 2012)
2008	Formal proposal of nomenclature and definition of PCV2 genotypes by a EU consortium on PCVD	(Segalés et al., 2008)

However, wasting in weaning and growing pigs are the most typical appearance and additional pathological conditions of PCV2-affected pigs can be observed, including porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), proliferative and necrotizing pneumonia, reproductive failures and congenital tremor (Segalés et al., 2004[b]; Chae, 2005). Gross lesions of PCVAD are wasting with or without respiratory disorder, diarrhea, jaundice and generalized lymphadenopathy (Chae, 2004; Darwich et al., 2004). However, a few PCV2-affected pigs present nephropathy, hepatitis, gastric ulcer and anemia (Chae, 2005).

Typical microscopic lesions of PCVAD in lymphoid tissue are characterized by variable degree of lymphoid depletion, granulomatous lymphadenitis (Figure 1.4 A) with multinucleated giant cells and the present of basophilic intracytoplasmic inclusion bodies in histiocytes (Chae, 2004; Darwich et al., 2004). Hepatic lesions in PCV affected pigs are observed with lymphoid and histiocytes infiltration in portal area with perilobular fibrosis. PDNS cases are characterized by a diffuse necrotizing vasculitis and fibrinonecrotic glomerulonephritis shown in Figure 1.4B. These lesions are also observed in subcutis and dermis (Chae, 2005). Microscopic lesions of PCV2-associated PRDC are bronchointerstitial pneumonia with peribronchiolar fibrosis, thicken alveolar septum caused by infiltration of macrophages and hypertrophy of pneumocytes type 2 (Kim et al., 2002).

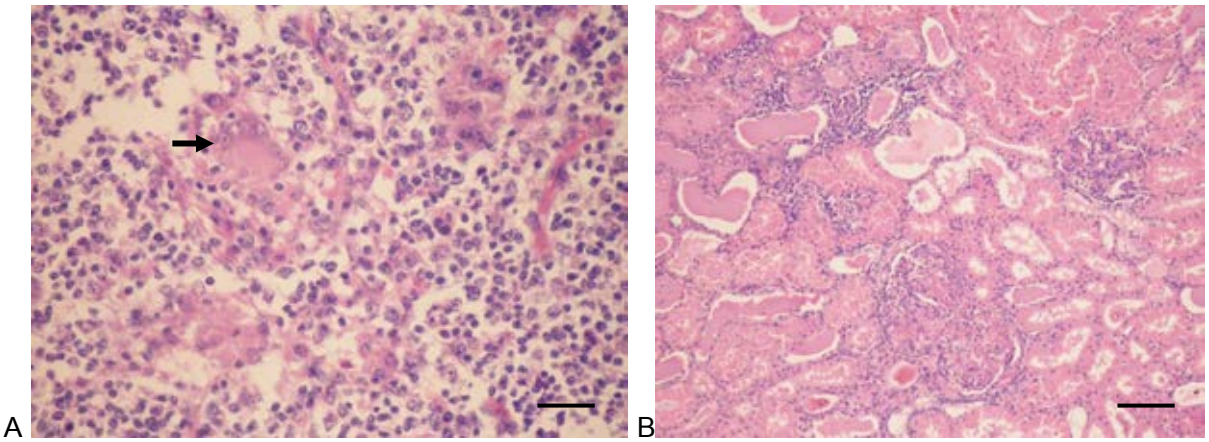

Figure 1.4: Typical microscopic lesions of PCVAD and PDNS field case. **A**: lymph node of PCVAD case revealed lymphoid depletion and histiocytic to granulomatous replacement of the follicle with multinucleated giant cells (arrow) (Hematoxylin and eosin, bar = 40 µm); B: kidney of PDNS case characterized by a diffuse necrotizing vasculitis and fibrinonecrotic glomerulonephritis (Hematoxylin and eosin, bar = 4 µm).

1.3.2 Immunology of PCV2 infection

PCV2 spread via infected peripheral blood mononuclear cells (PBMC). Consequently, the virus referentially replicate in B and T lymphocytes leading to lymphoid depletion. As a result, leukopenia can be identified early in PCVAD-affected pigs characterized by marked apoptosis of lymphocytes or lymphocyte depletion (Shibahara et al., 2000; Nielsen et al., 2003; Darwich et al., 2004) but less found of depletion in monocytic/ macrophage lineage and in dendritic cells (DC) (Vincent et al., 2005; McCullough et al., 2007). In addition, the macrophages infected with PCV2 produce high amounts of tumor necrotic facto -alpha (TNF-α), interleukin-8 (IL-8) and present upregulation of neutrophil chemotactic factor-II and monocyte chemotactic protein-1 (MCP-1) level which associated in the pathogenesis of granulomatous inflammation in PCVAD-affected pigs (Kim and Chae, 2003). The damage of lymphoid follicle by PCV2 infection was resulting in abnormal of neutralizing antibodies (Fort et al., 2007). However, the presence of PCV2 in dendritic cells does not impair their immunological interaction with the lymphocytes (Vincent et al., 2005; McCullough et al., 2009). The dendritic cells remain processing and presenting antigen. Nevertheless, PCV2 is the immunomodulatory being effect through the reaction of natural interferon-producting cells (NIPCs). Myeloid DC maturation was clearly impaired, caused by PCV2-induced inhibition of interferon alpha (IFN-α) and tumor necrosis factor (TNF-α) (Vincent et al., 2005; McCullough et al., 2009). Double-strand DNA replicative form of PCV2 are manifested as implement of danger recognition (via Toll-like receptors; TLR9) by antigen presenting cells of the innate defenses (McCullough et al., 2009).

1.3.3 Genetic diversity of PCV2

Several studies suggested that PCV2 could be divided into 2 major genotypes referred to PCV2a and PCV2b (Carman et al., 2006; Cheung et al., 2007; Ma et al., 2007; Takahagi et al., 2008; Kim et al., 2009[b]). However, PCV2c genotype has been described, but it has only been found in Denmark in the past (Segalés et al., 2008). Currently, two novel genotypes referred to PCV2d and PCV2e genotype were reported in China (Wang et al., 2009; Guo et al., 2010).

It should be noted that the signature motif in ORF2 can be used to differentiate between 2a and 2b. PCV2a viruses, the motif is 1480-1469 ACC/AAC/AAA/ATC [amino acid sequence TNKI] and PCV2b viruses, the motif is 1479-1468 TCA/AAC/CCC/CG(T)C [amino acid sequence SNPR(L)] (Cheung et al., 2007). Previously, the presence of both PCV2a and PCV2b DNA in tissues of the same infected swine was reported indicating that the pigs was co-infected with both PCV2 subgroups and PCV2 can even undergo recombination (Cheung, 2009; Lefebvre et al., 2009). However, the virulence of PCV2a and PCV2b isolates was similar in the conventional SPF pig model but the virulence of isolates within the same cluster was different (Opriessnig et al., 2008[b]). Alternatively, PCV2 can be classified into 8 subgroups 1A to 1C and 2A to 2E (Olvera et al., 2007) but those were not associated with disease condition or geographic area. The nucleotide substitution rate of PCV2 is high among single-stranded DNA viruses, and it was estimated to be approximately $1.2x10^{3}$ substitutions/site/year (Firth et al., 2009). Therefore, the emerging of novel PCV2 genotypes is possible in the future.

Recently, a new type of PCV referred to PCV1/2a was identified in Canada in 2009. Based on sequence analyses, this new PCV genome contains the ORF1 of PCV1 and the ORF2 of PCV2a and its entire viral genome nucleotide identity compared to PCV1, PCV2a and 2b are 86.4%, 88.7% and 86.5%, respectively (Gagnon et al., 2010).

1.3.4 PCV2 in Thailand

The first PMWS case of 7 to 9 week-old pigs from Rachaburi province, Thailand was reported in 1998 (Tantilertcharoen et al., 1999). A retrospective study of Thai PCV2 detection using nested PCR from formalin-fixed tissue, paraffin-embedded (FFPE) found the earliest PCV-infected case in 1993 (Kiatipattanasakul-Banlunara et al., 2002). An IHC investigation on FFPE tissues from suspected cases occurring during 2000-2002 demonstrated approximately 38.76% of PCV2 infection (50/129) (Bunlunara, et al., 2002). Recently, the seroprevalence in year 2007 (unpublished data from CU-VDL) was performed in 316 pigs (sows and finishers) from 21 farms using commercial competitive blocking ELISA (SERELISA® PCV2 Ab Mono Blocking, Synbiotics, France). PCV2 antibodies were found in 220 pigs (69.6%) from 21 farms. Currently, data from CU-VDL based on swine diagnosis annual report in 2009 (unpublished data) found increasing incidence of PCVAD in the cases of swine systemic infection (41.62%, n = 197) and the number of cases tend to increase in 2010 (47.06%, n = 304) and 2011 (41.89%, n = 592). This report indicates that PCV2 is one of the major causative agents of swine diseases in the Thai swine industry.

It should be noted that only one isolate of Thai PCV2 has been submitted to GenBank (AY864814) in 2004 and it was classified into 1C subgroups (Manokaran et al., 2008; Wiederkehr et al., 2009). Genetic information about PCV2 in Thai swine herds is still not available and needed further investigation.

1.3.5 Diagnosis

PCV2 can be found in both clinically healthy and diseased pigs (Allan and Ellis, 2000). A PCVAD pig is diagnosed by the presences of microscopic lesions incorporated with PCV2 antigen in the respective organs (Darwich et al., 2004; Chae,

2005). Laboratory tests for PCV2 diagnosis are the important tools, both direct and indirect methods. The direct methods compose of virus isolation, IHC, ISH, PCR, real time PCR and electron microscope (EM). However, viral DNA or viral antigens are much preferred over an indirect method such as PCV2-specific antibody detection. Nevertheless, antibody detection can be a very useful tool in epidemiological information on the prevalence of PCV2 in swine herds. For the indirect methods, several serological assays; IFA, IPMA, SN and ELISA have been used for detecting antibody against PCV2. Among these assays, ELISA test has been the most commonly used in veterinary diagnostic laboratories to detect anti-PCV2 antibodies since this test is simple to produce and perform, time-saving, and suitable for large scale surveys of PCV2 infection at low cost. The development of the appropriated ELISA test would be useful to obtain epidemiological information on the prevalence of PCV2 in the herds.

1.3.6 PCV2 vaccines

Prior to the availability of PCV2 vaccines, no specific treatment is available for PCVAD. Primarily treatment and control of PCVAD had focused on good production practices, minimizing co-infections and eliminating potential factors that trigger progression of PCV2 infection to PCVAD. The unsatisfactory situation creates a good rationale for the development of effective vaccines against PCV2 (Fachinger et al., 2008). The first commercial PCV2 vaccines for use in breeding stock pigs become available in Europe and Canada in 2006 (Opriessnig et al., 2007). Currently, five commercial vaccines against PCV2 used in both breeding stock pigs and piglets are now available (Table 1.2). Vaccination of sows and gilts are used for inducing PCV2 antibody titers in serum and colostrums in order to protect the piglets through passive transfer of maternal antibody against the development of PCVAD (Opriessnig et al.,

2009). Piglets are vaccinated after 3 weeks of age when maternal antibodies wane. This will elicit a neutralizing antibody response and will reduce or delay PCV2 infection during weaning or fattening (Fort et al., 2008; Opriessnig et al., 2009).

Table 1.2: Details of commercial porcine circovirus type 2 (PCV2) vaccines currently available (adapted from Grau-Roma et al., 2010)

Vaccine	Company	Antigen	Administration	Licensed for
Ingelvac circoflex	Boehringer Ingelheim	PCV2 ORF2 protein	1 ml IM single dose	Piglets (>2 wks of age)
Fostera (Suvaxyn)	Zoetis (Fort Dodge Pfizer)	Inactivated PCV1-2 Chimera	2 ml IM single dose	Piglets (>4 wks of age)
Porcilis PCV (EU)	Intervet–Schering Plough Animal Health	PCV2 ORF2 protein	2 ml IM 1 or 2 doses/2 doses	Piglets (>3 days/ >3 wks of age)
Circumvent PCV(US)				
Cirvovac	Merial	Inactivated PCV2	2 ml IM 2 doses	sows and gilts

All of these vaccines have proved effective under both experimental and field trial, reducing the incidence of PMWS and improving average daily weight gain and feed conversion ratios (Fachinger et al., 2008; Horlen et al., 2008; Pejsak et al., 2009; Segalés et al., 2009). Furthermore, vaccination reduces the number of co-infections (Kixmöller et al., 2008) and the severity of lesions in lymphoid tissues (Segalés et al., 2009). Other experimental works have also suggested that vaccines (either single or

double doses) reduce the PCV2 viremia and the severity of microscopic lesions, even in the presence of maternal antibodies (Fort et al., 2008; Opriessnig et al., 2008[a]).

In Thailand, a field trial with killed PCV1-2 chimera vaccine in a PCV2-affected herd has also shown that vaccine reduce the level of PCV2 viremia and the severity of gross lesions (Paphavasit et al., 2009).

Due to strong immunoreactivity, the capsid protein is the preferred antigen for serological tests and vaccine candidate (Nawagitgul et al., 2002; Blanchard et al., 2003[b]; Racine et al., 2004). This protein has been identified as the major immunogenic and protective protein of PCV2. The expression of recombinant capsid protein was successful primarily in the baculovirus system (Nawagitgul et al., 2000) and the expressed protein assembled into virus-like particles (VLPs) structures. In addition, the others developed vaccines include viral-vectored vaccines (Wang et al., 2007; Fan et al., 2008[a]) and DNA vaccines (An et al., 2008; Shuai et al., 2013) were further reported. However, the production of VLPs for vaccination in eukaryotic systems and the other type of PCV2 vaccine are costly for veterinary applications. On the other hand, heterologous protein expression in *E. coli* is in focus as an alternative choice of capsid protein production (Liu et al., 2001[b]). Nevertheless, the difficulties of ORF2 protein expressed in *E. coli* are likely due to the specific amino acid sequence at NLS position. Arginine displays significant differences in codon usage between viral and *E. coli*. For this reason, the fusion of the whole ORF2 with GST allowed expression in less amounts. Recently, recombinant NLS-truncated capsid protein and full-length capsid protein have been expressed in optimized *E. coli* (Zhou et al., 2005; Trundova and Celer, 2007) and react with PCV2 positive swine sera in immunofluorescent test and Western blot (Zhou et al., 2005; Marcekova et al., 2009).

Several PCV2 vaccine approaches have been tested under experimental conditions in mice and/or pigs including a killed PCV2vaccine and an ORF2 subunit vaccine (Blanchard et al., 2003[a]; Fenaux et al., 2004). The most successful vaccine candidates were those based on the induction of an active immune response against the capsid protein of PCV2. The vaccine candidates reduced PCV2 viremia and histopathological lesions following experimental challenge. Moreover, field studies conducted on the efficacy of vaccine demonstrated decreasing PCV2 viremia and improving overall growth performance (Fachinger et al., 2008; Kixmöller et al., 2008).

Available data on efficacy of a developed subunit PCV2 vaccine using recombinant capsid protein expressed in *E. coli* expression system are still inconclusive. Therefore, the objectives of this study are to develop a subunit vaccine against PCV2 and to investigate the efficacy of this vaccine on enhancing specific antibodies and reducing PCV2 viremia in a PCV2-affected swine herd under natural condition.

CHAPTER II

Genetic Characterization and Phylogenetic Analysis of Porcine Circovirus Type 2 in Thai Pigs with Porcine Circovirus Associated Diseases (PCVAD) during 2007-2010

2.1 Introduction

Porcine circovirus type 2 (PCV2) was first recognized as a causative agent of postweaning multisystemic wasting syndrome (PMWS), a multi-factorial disease in swine in Canada in 1991 (Harding and Clark, 1997). Subsequently, it has been reported in almost all intensive pig production countries worldwide (Allan and Ellis, 2000; Chae, 2004). PCV2 causes several clinical and pathological conditions in pigs including porcine respiratory disease complex (PRDC), reproductive failures, porcine dermatitis and nephropathy syndrome (PDNS), proliferative and necrotizing pneumonia and congenital tremor (Darwich et al., 2004; Chae, 2005). Currently, these associated diseases and conditions linked to PCV2 are called porcine circovirus associated diseases (PCVAD). PCV2 belonging to the family *Circoviridae*, is a smallest mammalian, non-enveloped, single-stranded DNA virus enclosing a circular genome about 1.76 kb (Mankertz et al., 1997). The genome of PCV2 contains 3 major open reading frames (ORFs): ORF1, ORF2 and ORF3. The Cap protein is the main structural and major immunogenic of PCV2, which is encoded by ORF2. As a result, ORF2 is commonly used for reconstruction of the phylogenetic tree similar to the whole PCV2 genome study (Olvera et al., 2007).

Several studies suggested that PCV2 could be divided into 2 major genotypes (Carman et al., 2006; Cheung et al., 2007; Ma et al., 2007; Takahagi et al., 2008; Kim et al., 2009[a]). Recently, both genotypes were proposed and referred to PCV2a (PCV2-genotype 2) and PCV2b (PCV2-genotype 1). However, PCV2c genotype has been

described, but only found in Denmark (Segalés et al., 2008). Interestingly, the virulence of PCV2a and PCV2b isolates was similar in the conventional SPF pig model, but the virulence of the isolates within the same cluster differed (Opriessnig et al., 2008[b]). Alternatively, PCV2 can be classified into 8 subgroups 1A to 1C and 2A to 2E (Olvera et al., 2007), but those were not associated with the disease conditions or geographic areas. Recently, a new type of PCV referred to PCV1/2a was reported and later found to be a chimeric virus containing ORF1 of PCV1 and ORF2 of PCV2a in Canada in 2009 (Gagnon et al., 2010).

In Thailand, PMWS caused by PCV2 was firstly reported in 1998 (Tantilertcharoen et al., 1999) and a PCV2 retrospective study in Thailand identified PCV-infected case occurring as early as in 1993 (Kiatipattanasakul-Bunlunara et al., 2002). It should be noted that only one Thai PCV2 isolate from our group has been submitted to GenBank (AY864814) in 2004 and it was classified into subgroup 1C (Manokaran et al., 2008; Wiederkehr et al., 2009). However, genetic information about PCV2 in Thai swine herds has still been unavailable. Therefore, the objective of this study was to determine the genetic characterizations of ORF2 genome of current 12 PCV2 isolates from Thai pigs with PCVAD during 2007-2010.

2.2 Materials and Methods

2.2.1 Field samples

Clinical samples (serum or lymphoid tissue) from different farms in high pig density provinces of Thailand submitted to Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) and Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University during 2007 - 2010 were included in this study. These samples were kept in -80°C until performing DNA extraction and PCR. Viral DNA was extracted from lymphoid tissue homogenates and serum samples using NucleoSpin

Extract Viral DNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.2.2 PCR amplification of ORF 2 gene

A full-length ORF2 gene of PCV2 was amplified by PCR with forward primer, PCV2-f1 (5'-CCATGCCCTGAATTTCCATA-3') and reverse primer PCV2-r1 (5'-ACAGCG CACTTCTTTCGTTT-3') previously published (Takahagi et al., 2008), in a 50 µl reaction mixture. The amplification reaction was performed with an initial step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The PCV2 positive samples of 702 nucleotides were used for DNA sequencing.

2.2.3 Viral sequences and phylogenetic analysis

The PCR products were separated by 1.5% agarose gel electrophoresis and purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) for viral sequences. DNA sequencing was carried out by 1st BASE Company (Singapore) with primers used in the previous PCR reaction. A total of 12 ORF2 sequences from Thai pigs with PCVAD during 2007-2010 were obtained and translated into amino acid sequences for analysis. The 12 Thai PCV2 sequences were analyzed together with 19 representative ORF2 sequences reported in GenBank including 16 reference strains of PCV2a and PCV2b, the former Thai isolate in 2004 (THA_01NP1, AY864814), PCV2c ORF2 sequence (EU148503) and PCV1 (AY193712). A phylogenetic trees was constructed by MEGA 4 software (Tamura et al., 2007) based on the sequence of ORF2 to determine the distribution and evolutionary trend of PCV2 in Thailand using the neighbor-joining (NJ) method with 1000 bootstrapping replicates (Saitou and Nei, 1987).

2.3 Results

2.3.1 Genetic characterization

All 12 Thai ORF2 of PCV2 sequences in this study had a genome length of 702 nucleotides and revealed nucleotide identities ranged between 97.7-99.8% (Table 2.1), indicating no significant differences between PCV2 genotype. However, the nucleotide substitutions in the ORF2 gene among the 12 Thai PCV2 sequences compare to the former Thai PCV2 sequences in 2004 (THA_01NP1, AY864814) were observed. These substitutions led to amino acid changes located between amino acid positions 46-90, 121-131 and 190-191 as shown in Figure 2.1.

2.3.2 Phylogenetic analyses

The phylogenetic analysis in this study reconstructed from the 12 Thai ORF2 sequences during 2007-2010 along with 19 ORF2 sequences published in GenBank database representing all PCV2 genotypes shown in Figure 2.2. All 12 Thai PCV2 sequence belonged only to genotype 1 (PCV2b) according to the classification proposed previously (Grau-Roma et al., 2008). Based on the subgroup terminology described previously (Olvera et al., 2007), nucleotides 262-267 and amino acids 88-89 of ORF2 were compared and classified. The nucleotide sequences “CCCCGC”, “CCCCTC” and “AAAATC” are the signatures motif for PCV2b subgroup 1A/B, 1C and PCV2a, respectively. The amino acid “PR” was enclosed with subgroup 1A/B, while the PL and KI were related with subgroup 1C and PCV2a. All 12 Thai PCV2 genotype 1 were divided into 2 subgroups consisting of 1A/ B (10/12, 83.33%) and 1C (2/12, 16.67%). This results indicates that the predominant Thai PCV2 in this study is genotype 1 (100%) particularly subgroup 1A/B (83.33%). It should be noted that previously found subgroup 1C (01NP1) in 2004 was still observed (09NP165 and 09NP290) in 2009 in the same province.

Table 2.1: Classification of 12 Thai PCV2 isolates compared with 8 PCV2 reference strains in the same group by nucleotide and deduced amino-acid sequences of ORF2

Accession number	Nucleotide sequence (262-267)	Amino acid sequence (86-87)	Genotype	subgroup	Country/area	% NT identity[2]
FJ905468	**CCCCGC**	**PR**	**PCV2b**	**1A/B**	**Korea**	**100**
AY484409	**CCCCGC**	**PR**	**PCV2b**	**1A/B**	**Netherland**	**100**
THA_07NP88	CCCCGC	PR	PCV2b	1A/B	Nakhon Pathom	98.9/98.6
THA_07NP144	CCCCGC	PR	PCV2b	1A/B	Nakhon Pathom	99.6/99.6
THA_08NP113	CCCCGC	PR	PCV2b	1A/B	Nakhon Pathom	99.8/99.8
THA_08NP266	CCCCGC	PR	PCV2b	1A/B	Nakhon Pathom	99.8/99.8
THA_10SB01	CCCCGC	PR	PCV2b	1A/B	Saraburi	99.6/99.6
THA_10SB02	CCCCGC	PR	PCV2b	1A/B	Saraburi	99.8/99.4
THA_10SB03	CCCCGC	PR	PCV2b	1A/B	Saraburi	99.4/99.4
THA_10SB04	CCCCGC	PR	PCV2b	1A/B	Saraburi	99.1/99.1
THA_10NP01	CCCCGC	PR	PCV2b	1A/B	Nakhon Pathom	99.5/99.5
THA_10AY01	CCCCGC	PR	PCV2b	1A/B	Phra Nakhon Si Ayutthaya	99.3/99.3
AY864814 (01NP1)	**CCCCTC**	**PL**	**PCV2b**	**1C**	**Thailand**	**100**
EU302140	**CCCCTC**	**PL**	**PCV2b**	**1C**	**Indonesia**	**100**
THA_09NP165	CCCCTC	PL	PCV2b	1C	Nakhon Pathom	98.4/98.6
THA_09NP290	CCCCTC	PL	PCV2b	1C	Nakhon Pathom	97.7/98.1
AY256455	**AAAATC**	**KI**	**PCV2a**	**2C**	**Hungary**	**100**
AY322004	**AAAATC**	**KI**	**PCV2a**	**2D**	**France**	**100**
EU148503	**CCCCTC**	**PL**	**PCV2c**	**-**	**Denmark**	**100**
AY193712	**ATCTTC**	**IF**	**PCV1**	**-**	**China**	**100**

[1]Boldface letters are reference strains

[2]Compared with the reference nucleotide sequences in the same group

2.4 Discussion

Porcine circovirus type 2 (PCV2) is divided into 2 major genotypes based on

sequencing analysis. However, several nomenclature have been used such as group I and II, pattern 321 and 422, SG3 and SG1/SG2, group 1 and 2, and Group A and B (Olvera et al., 2007; Segalés et al., 2008; Timmusk et al., 2008). Recently, both genotypes were proposed and referred to PCV2a and PCV2b (Segales et al., 2008). In Thailand, PMWS was firstly reported in 1998 (Tantilertcharoen et al., 1999). It should be noted that only one Thai PCV2 isolate belonging to genotype 1 was submitted to GenBank (AY864814) in 2004. In this study, we characterized and reconstructed phylogenetic analysis of 12 Thai ORF2 of PCV2 sequences collected from pigs with PCVAD during 2007-2010 to accurately determine the cluster relationships. All 12 Thai PCV2 isolates in this study were closely related (The nucleotide identities ranged between 97.7-99.8%) based on ORF 2 sequence. The main position of amino acid replacements among Thai ORF2 sequences in this study were located at amino acid positions 46-90, 121-131 and 190-191 within heterogenic regions (Fig. 2.1) similar to previous reports (Larochelle et al., 2002; Grau-Roma et al., 2008). The phylogenetic analysis of the 12 Thai PCV2 isolates revealed that all studied Thai PCV2 sequences belonged to genotype 1 (Fig. 2.2), according to previous classification (Grau-Roma et al., 2008).

Previous studies revealed that both genotypes were associated with PCVAD-affected and non-affected herds (Cheung et al., 2007; Carman et al., 2008; Grau-Roma et al., 2008; Horlen et al., 2008; Wiederkehr et al., 2009). However PCV2b is currently prevailing in naturally occurring infections (Takahagi et al., 2008; Timmusk et al., 2008; Cortey et al., 2011[b]). Although the virulence of PCV2a and PCV2b isolates was similar in the SPF pig model, the virulence of isolates within the same cluster was different (Opriessnig et al., 2008[a]). On the other hand, many recent publications have reported a shift from PCV2a to PCV2b genotype that might be related to the occurrence of PMWS

outbreaks in Canada (Gagnon et al., 2007), Sweden (Timmusk et al., 2008), Switzerland (Wiederkehr et al., 2009) and Spain (Cortey et al., 2011[b]), indicating that PCV2b may be more virulent than PCV2a.

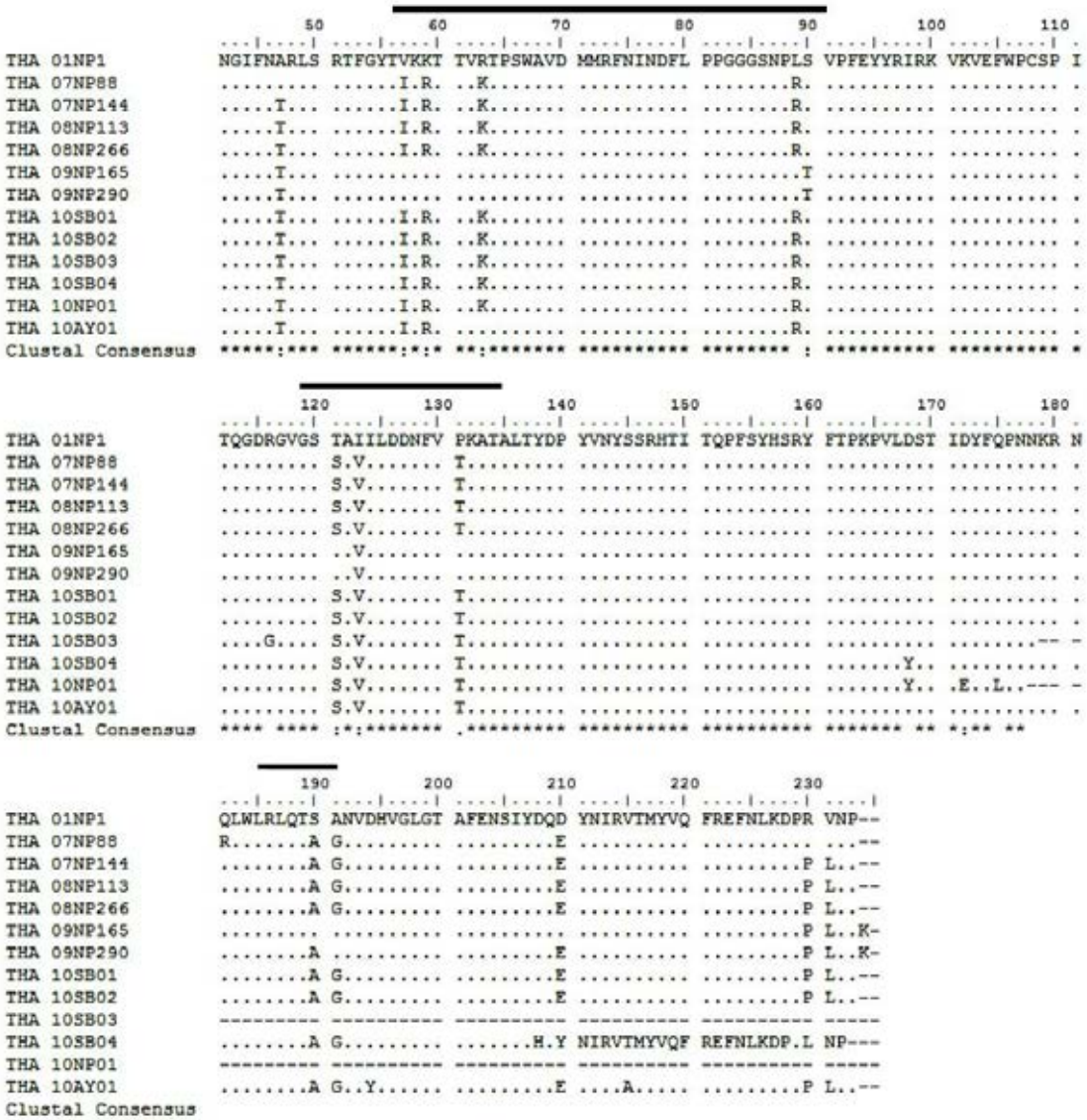

Figure 2.1: Alignment of amino acids predicted from the ORF2 nucleotide sequences of the 12 Thai PCV2 sequences during 2007-2010 compared with the former Thai sequences in 2004 (01NP1) revealing amino acid changes located between amino acid positions 46-90, 121-131 and 190-191. Regions reported by Grau-Roma et al. (2008) as more heterogenic are highlighted in black lines.

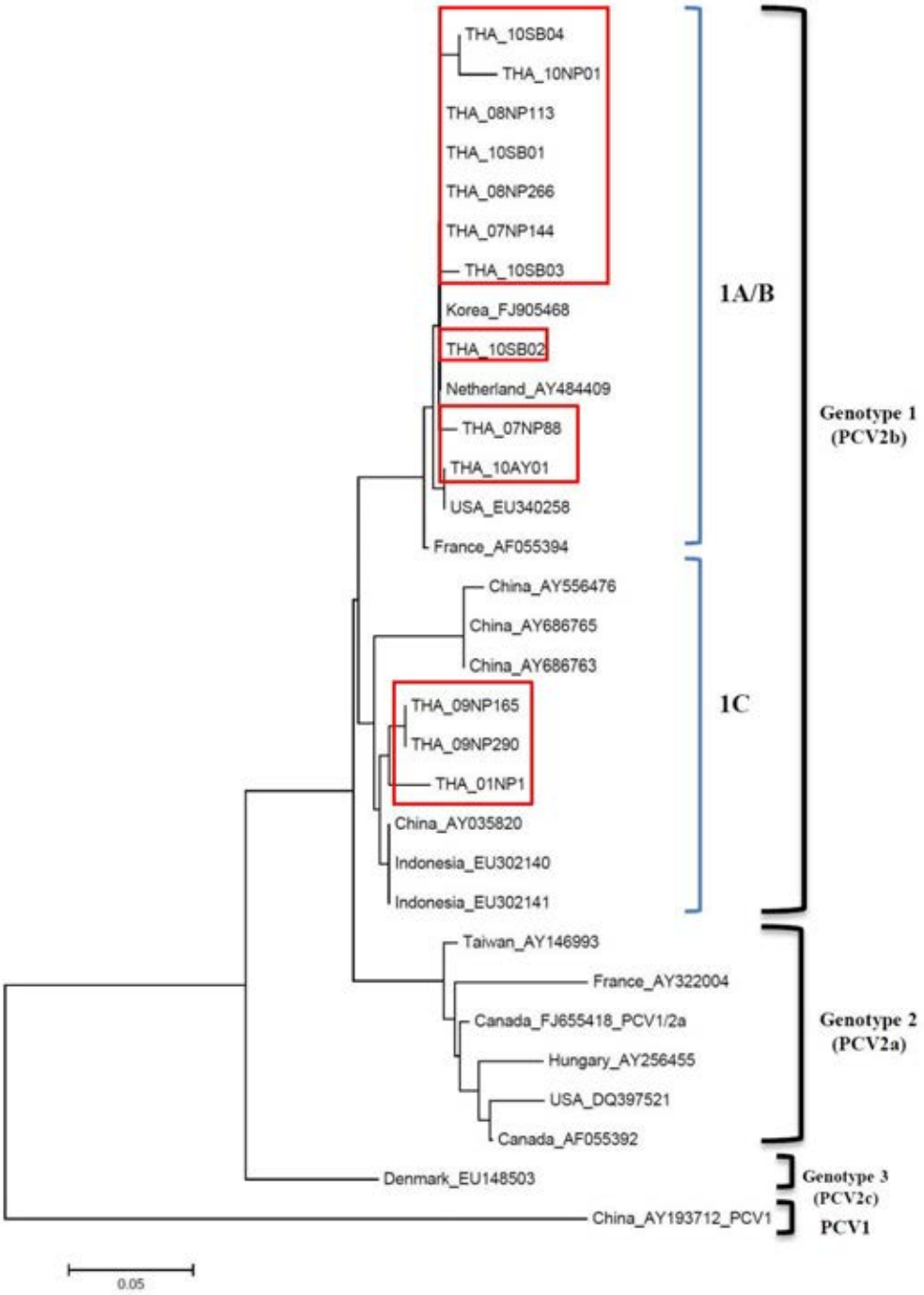

Figure 2.2: Phylogenetic tree based on neighbor-joining (NJ) method is constructed from the 12 Thai PCV2 sequences during 2007-2010 in this study with 19 ORF2 of PCV2 sequences reported in GenBank including 16 reference strains of PCV2a and PCV2b, the former Thai isolate (THA_01NP1), PCV2 group 3 (PCV2c) sequence (EU148503) and PCV1 (AY193712) strain.

All 12 Thai PCV2 isolates in this study were classified within only one PCV2 genotype (genotype 1: 1A/B and 1C) similar to several countries such as Cuba (genotype 1: 1A) and Indonesia (genotype 1: 1C). Interestingly 10 Thai PCV2 sequences were located within subgroup A/B (10/12, 83.33%) together with the other PCV2 sequences from Korea (FJ905468), the Netherlands (AY484409) and USA (EU340258). These PCV2 isolates subgroup A/B were found in PCVAD pigs from Nakhon Pathom (2007, 2008 and 2010), Saraburi (2010) and Phra Nakhon Si Ayutthaya province (2010). The other 2 Thai PCV2 sequences (THA_09NP165 and THA_09NP290) from Nakhon Pathom province were classified within subgroup 1C (2/12, 16.67%) similar to the previous Thai PCV2 isolate (THA_01NP1, AY864814), a Chinese isolate (AY035820) and 2 Indonesian isolates (EU302140 and EU302141) which were found in Indonesian pigs imported to Singapore (Manokaran et al., 2008).

Nevertheless, the sequence and phylogenetic analyses performing in this study did not show any evidence of recombination as reported in PCV type 2 isolated in Hong Kong, Korea and USA (Ma et al., 2007; Choi and Chae, 2008; Horlen et al., 2008). However, a few amino acid replacements among Thai ORF2 sequences in this study were observed. Due to the high nucleotide substitution rate of PCV2 compared to other single-stranded DNA viruses, it was estimated approximately $1.2x10^{3}$ substitutions/site/year (Firth et al., 2009). Therefore, the emerging of any new PCV2 genotype is possible in the future. Since the samples in this study were collected from the highest pig density provinces located in central Thailand, the results yielded in this study can demonstrate at least 2 introductions of PCV2 genotype 1 into Thailand. Imported swine breeders and semen appear to be the major route or transmission. Another evidence of introducing new virus strain into the swine herds is using improper killed chimeric vaccine in Canada (Gagnon et al., 2010). It should be noted that no chimeric virus or no PCV2a

was found in this study even though that killed chimeric vaccine has been commercially available in Thailand a few years ago (Paphavasit et al., 2009). In order to yield the accurate data, more PCV2 strains from different parts of the country should be examined for more epidemiological information of PCV2 in the Thai swine herds.

In summary, based on the sequences and phylogenetic analysis of all current 12 Thai PCV2 isolates, two subgroups: 1A/B and 1C of genotype 1 were found in Thailand. A few specific substitution patterns in each subgroup were observed in amino acid positions. This report revealed that PCV2 genotype 1 was predominated in Thai pigs with PCVAD. This finding provides some useful information about PCV2 molecular epidemiology in Thailand with at least 2 introductions into the country.

CHAPTER III

Development of an indirect enzyme-linked immunosorbent assay using a recombinant truncated capsid protein of porcine circovirus-2

3.1 Introduction

Porcine circovirus (PCV) is a non-enveloped, single-stranded, circular-genome DNA virus with a diameter of 17 nm, belonging to the family *Circoviridae*. Porcine circovirus type 1 was first isolated as a nonpathogenic contaminant virus from a porcine kidney cell line (Tischer et al., 1982; Allan et al., 1995). In contrast, porcine circovirus type 2 (PCV2) was first recognized as a causative agent of postweaning multisystemic wasting syndrome (PMWS), a multi-factorial disease in swine firstly identified in Canada in 1991(Harding and Clark, 1997). Subsequently, PCV2 has been reported in most intensive pig producing countries worldwide including Thailand (Allan et al., 1998[a]; Balasch et al., 1999; Tantilertcharoen et al., 1999; Allan and Ellis, 2000; Darwich et al., 2004; Prickett et al., 2011). PCV2 presently causes several clinical and pathological conditions in pigs composing of porcine respiratory disease complex (PRDC), reproductive failures, porcine dermatitis and nephropathy syndrome (PDNS) and congenital tremor which currently known as porcine circovirus associated diseases (PCVAD) (Darwich et al., 2004; Chae, 2005). The genome of PCV2 contains 3 open reading frames (ORFs): ORF1, ORF2 and ORF3. ORF1 encodes the Rep proteins of 35.7 kDa involving in viral replication, while ORF2 encodes the structural capsid protein of 30 kDa involving in immunogenicity of PCV2 (Nawagitgul et al., 2000; Mankertz et al., 2004) and ORF3 protein is involving in PCV2-induced apoptosis(Liu et al., 2005).

The most reliable serological diagnostic assays for antibody detection against PCV2 consist of immunoperoxidase monolayer assays (IPMA) (Allan et al., 1999[a]) and

indirect immunofluorescent antibody assay (IFA) (Allan et al., 1999[b]). However, These conventional assays are expensive, highly time-consuming and carry the risk of virus contamination whereas commercially available Enzyme-Linked Immunosorbent Assay (ELISA) based on recombinant capsid protein expressed in baculovirus expression systems (Blanchard et al., 2003[b]; Liu et al., 2004) or bacterial expression systems (Wu et al., 2008; Marcekova et al., 2009; Sun et al., 2010; Yin et al., 2010) are more convenient. However, the production of recombinant proteins in the eukaryotic expression system is costly. It should be noted that several antigenic epitopes of PCV2 capsid protein were demonstrated at amino acid residues 47-85, 165-200, 230-233 (Lekcharoensuk et al., 2004) and 65-87, 113-147, 157-183, 193-207 (Mahé et al., 2000). On the other hand, a recent report (Guo et al., 2011) demonstrated that the recognition epitope was also located in the nuclear localization signal (NLS) of capsid protein at amino acid residues 26-36. Nevertheless, most information of antigenic epitopes of PCV2 suggested that 41 amino acid residues located in the NLS region might not be the major domains for the conformational epitopes. As a result, in this present study a recombinant NLS truncated capsid (rntCap) protein of PCV2 expressed in *Escherichia coli* system was used as an antigen for an indirect ELISA development. The objectives of this study were to establish and validate the rntCap indirect ELISA diagnostic assay to detect anti-PCV2 antibodis.

3.2 Materials and Methods

3.2.1 PCV2 genomic DNA and sera

The genomic DNA obtained from the purified PCV2-infected PK15 cell lysate of the PCV2 isolate THA_07NP88; GenBank accession number JQ866913 (Jittimanee et al., 2011) was used as a template PCR. This Thai PCV2 (genotype 1 or PCV2b) was

originally isolated from a lymph node tissue of a pig having PCVAD in 2007. A total of 90-field serum samples (30 IPMA-negative and 60 IPMA-positive) were used to evaluate the performance of the established indirect ELISA assays.

3.2.2 Construction of the rntCap in expression plasmids

The PCR was amplified to generate the NLS truncated capsid (ntCap) fragment of PCV2. The forward (5' GCGGCCGCAATGGCATCTTCAACGC 3') and reverse (5' GCGGCCGCTTAGGGGTTAACTCGGGGGTC 3') primers were designed to incorporate the *NotI* restriction sites (underlined) at both ends of the PCR product. The purified PCR products of ntCap gene were cloned into the pGEM®-T-easy vector (Promega Corporation, WI, USA) according to the manufacturer's instructions. Subsequently, the pGEM/ntCap plasmids were transformed into *E. coli* JM109 for plasmid amplification and further extracted with NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany). Consequently, the purified pGEM/ntCap plasmids were digested by *NotI* endonuclease (New England Biolabs Inc., MA, USA), separated by 1.5% agarose gel electrophoresis and purified with NucleoSpin Extract II. The expression plasmid, pGEX-5x-3 (GE Healthcare Limited, Buckinghamshire, UK) was also digested with *NotI* endonuclease and dephosphated by using calf intestinal alkaline phosphatas (CIAP) (New England Biolabs Inc., MA, USA). The digested pGEM/ntCap fragments were transferred into the digested pGEX-5x-3 using T4 ligase enzyme (Promega Corporation, WI, USA) resulted in the recombinant pGEX-pGEM/ntCap (rntCap) plasmid.

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

The rntCap plasmids were transformed into the *E. coli* BL21-Rosetta™ 2 (Merck KGaA, Darmstadt, Germany) using the $CaCl_2$-induced transformation method. For recombinant protein expression, a fresh overnight culture of *E. coli* carrying the rntCap

plasmids were diluted 1:50 (v/v) into Luria-Bertani (LB) broth medium containing 100 mg/ml of ampicillin and 20 mg/ml of chloramphenicol and then allowed to grow at 37°C to an optical density between 0.6 and 0.8 at 600 nm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega Corporation, WI, USA), 0.1 mM were added and incubated at 30°C for 6 h (Liu et al., 2001[a]; Trundova and Celer, 2007). The rntCap protein was purified from the bacterial lysate by using a glutathione (GST) affinity purification module (GE Healthcare Limited, Buckinghamshire, UK).

3.2.4 Protein expression analysis

The purified rntCap proteins were separated by 12% SDS polyacrylamide gel (SDS-PAGE). For Western blotting, proteins were blotted onto a nitrocellulose membrane (Pall Corporation, NY, USA) at 110 V for 70 min. As previously described (Liu et al., 2004) with some modifications, the nitrocellulose strips 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 h. The strips were then incubated with monoclonal mouse anti-PCV2 antibodies (Rural Technologies, Inc., SD, USA) for 1 h and later incubated with HRP-labeled rabbit anti-mouse IgG antibodies (SouthernBiotech, AL, USA) for 1 h. The reaction was finally developed using 3', 3'-diaminobenzedine (DAB) substrate (Merck Millipore, MA, USA).

3.2.5 Optimization of rntCap indirect ELISA working conditions

The rntCap protein was used as the coating antigen for this established indirect ELISA. A final antigen concentration, serum dilution, conjugate-antibody concentration and incubation period were determined by the checkerboard titration procedures (Crowther, 2002). Briefly, the rntCap proteins were coated in different concentrations

from 0.1 µg/ml to 10 µg/ml to set the optimal concentration. Subsequently, 100 µl of swine PCV2 positive serum and negative serum control were diluted in a 2-fold dilution manner from 1:100 to 1:1600 (in 1% BSA in PBS) to determine the optimization of serum dilution. In order to determine the final concentration of conjugate, HRP-labeled goat anti-swine IgG antibodies (Kirkegaard & Perry Laboratories, MD, USA) were diluted in a serial 2-fold dilution manner from 1:500 to 1:64,000. After adding TMB substrate for 15, 30, 60 and 90 min, the colorimetric reactions were developed. The conditions yielding the highest OD650 ratio between positive and negative sera (P/N value) and the OD650 value of positive serum close to 1.0 were scored as the optimal working conditions.

3.2.6 Immunoperoxidase monolayer assay (IPMA)

IPMA was used as a reference method for detecting the PCV2 antibodies in pig sera. IPMA was performed in duplication as previously described (Liu et al., 2004) with some modification. Briefly, 96 well flat bottom plates (eBioscience Inc., CA, USA) containing the PK15/PCV2-infected cells and the negative control cells (mock-infected PK15 cells) were fixed in 4% formalin in PBS for 30 min at room temperature. Following 3 washes with PBS-T, diluted-field serum samples (1:400) and positive control (1:500, monoclonal mouse anti-PCV2 antibodies, Rural Technologies Inc., SD, USA) was added and then incubated at 37˚C for 1 h. After 3 washes, 1:300 dilution of HRP-goat anti-swine IgG antibodies (New England Biolabs Inc., MA, USA) and 1:300 of HRP-conjugated rabbit anti-mouse Ig antibodies (SouthernBiotech AL, USA) were added. Following 1 h of incubation at 37˚C, plates were washed 3 times and 3-amino-9-ethyl-carbazol (AEC) solution (Merck Millipore, MA, USA) was added and incubated for 30 min at 37˚C. Color developed from substrate was removed and then the plate was

washed by tap water. The IPMA plate was examined for dark red staining nuclei under an inverted light microscope.

3.2.7 Evaluation of assay performance

To evaluate the performance of the rntCap indirect ELISA, 90-field sera tested by IPMA were run in duplication. Diagnostic accuracy of this assay was determined by calculating the diagnostic sensitivity (Dsn) and specificity (Dsp). The Dsn and Dsp of this indirect ELISA calculated using the following formulas: Dsn = TP/(TP + FN) x 100 and Dsp = TN/(TN + FP) x 100 (TP, FN, TN and FP are indicated true-positive, false-negative, true-negative and false-positive, respectively).

3.2.8 Confirmation of the positive-negative cutoff

Ninety-field serum samples (30 IPMA-negative and 60 IPMA positive sera) were used for the rntCap indirect ELISA validation. The optical density (OD650) values obtained from 90 field serum samples were compared with the IPMA results. To determine a positive–negative cutoff and the diagnostic performance for this assay, a receiver operating characteristic (ROC) curve analysis was performed. A cutoff value was determined using maximized diagnostic sensitivity and specificity and minimized the number of false-negative and false-positive results.

3.2.9 Evaluation of the assay repeatability

Evaluation of the assay repeatability within and between assay were performed as previously suggested (Jacobson, 1998). Eight-positive sera and 8-negative sera were designated for the repeatability test. For intra-plate repeatability, 3 replication of each serum sample were run in the same plate. For inter-plate repeatability, 3

replication of each sample were consigned in different plates. Mean OD ratio and coefficient of variation (CV) of each test were analyzed.

3.2.10 Evaluation of the correlation between ELISA and IPMA

The rntCap indirect ELISA OD values obtained from 16 positive serum samples of known PCV2-infected pigs were compared with the antibody titers determined by IPMA on PK15-PCV2-infected cells, as previously described (Truong et al., 2001). The IPMA was performed on serial dilutions of the corresponding sera from 1:100 to 1:51,200. A correlation between the IPMA titers and the OD ratio was determined by the correlation coefficient.

3.3 Results

3.3.1 Expression and Analysis of PCV2 rntCap protein

The NLS truncated capsid gene fragments containing several immunoreactive ORF2 epitopes of PCV2 were amplified by PCR. Consequently, the amplified 579 nucleotides encoding the 42-234 peptide of ORF2 protein were sequenced and cloned into the expression vector pGEX-5-3. The rntCap protein was expressed as GST-tagged fusion protein and purified by affinity chromatography. The purified rntCap protein was analyzed by SDS-PAGE and Western blotting. The presence of the rntCap protein in the bacterial cell lysate after induction and purification was revealed (Figure 3.1). Approximately, 40 kDa of rntCap protein from the purified protein on lane 4 strongly reacted with the monoclonal mouse anti-PCV2 antibodies in Western blotting. The results indicated that this rntCap protein expressed in *E. coli* could be used as an antigen for the detection of specific antibodies against PCV2.

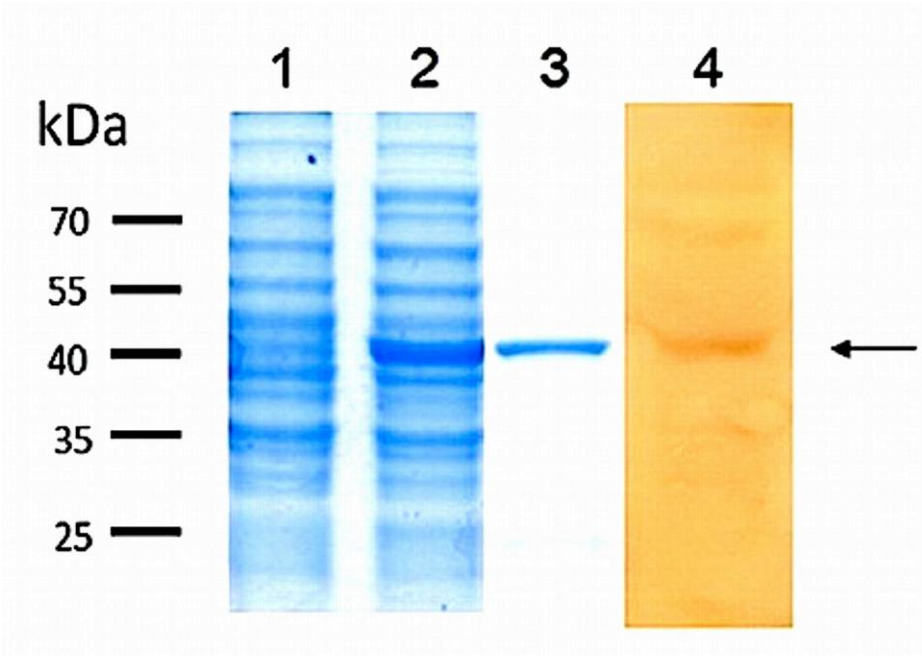

Figure 3.1: Expression of the recombinant nuclear localization signal truncated capsid (rntCap) protein was analyzed by SDS-PAGE (lanes 1–3) and Western blot (lane 4). A clear band of 40 kDa (arrow) after purification is demonstrated. Lane 1: crude protein from noninduced *E. coli* cell lysate; lane 2: crude protein from IPTG-induced *E. coli* cell lysate; lane 3: purified rntCap protein from IPTG-induced *E. coli* cell lysate; lane 4: purified rntCap proteins reacting with monoclonal mouse anti-PCV2 antibody.

3.3.2 Optimization of the rntCap indirect ELISA

The optimal working condition of this assay was performed with the final rntCap protein concentration of 5 µg/ml in 0.05 M sodium carbonate buffer (pH 9.6). The optimal diluted serum (1:100) was added with further incubation at 37°C for 30 min. The final dilution of the HRP-labeled goat anti-swine IgG antibodies was done at 1:10,000 with further incubation at 37°C for 30 min. After TMB were added and incubated in the dark for 1 h, the OD was measured at 650 nm to determine serum/positive (S/P) ratio.

Additionally, to determine whether the GST tag interfered with the rntCap indirect ELISA, the established indirect ELISA was performed using either purified GST protein

or rntCap protein as the coating antigen. Plates were measured at 650 nm OD after tested with 8-positive and 8-negative known in duplication (Figure 3.2).

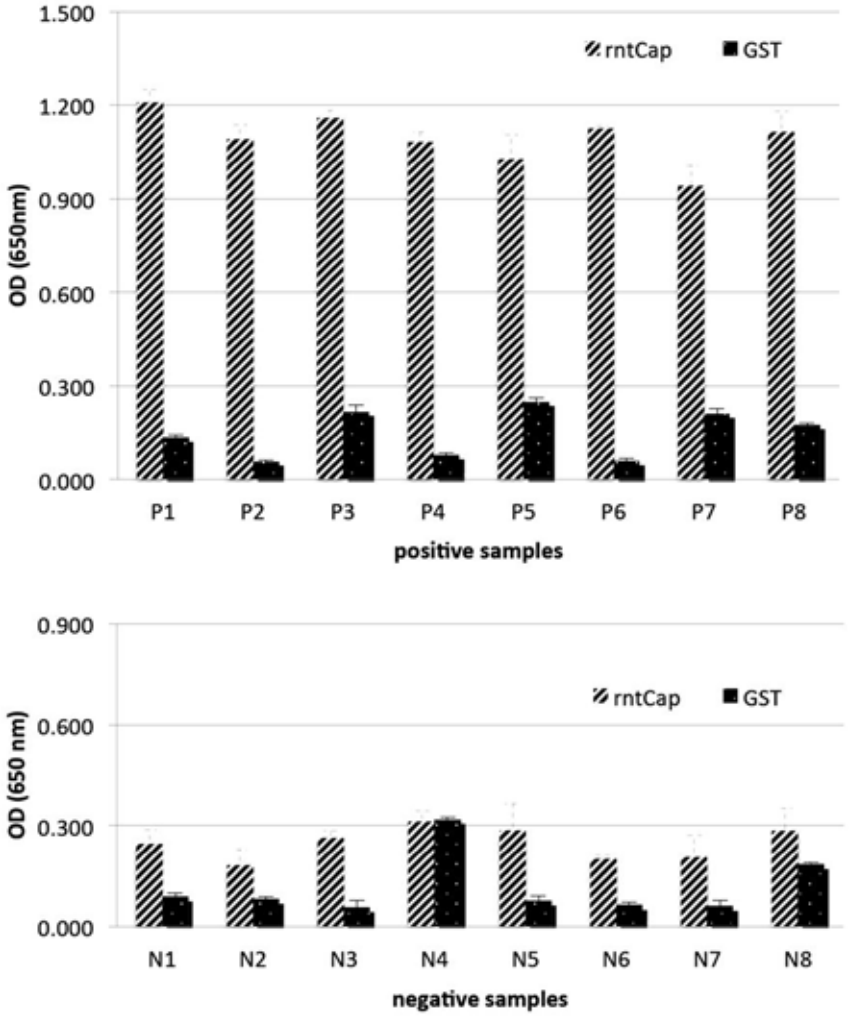

Figure 3.2: Comparing ELISA OD values obtained from the rntCap protein and GST coating protein. Sixteen-serum samples including 8-positive and 8-negative sera were used. Each serum sample was run in quad replication, two on the rntCap protein and two on the GST antigen coated wells. Plates were included with both positive and negative control sera.

The statistical analysis (paired *t*-test) between average OD values of the positive and negative sera tested by both assays (GST alone or rntCap) revealed that the mean

OD values of the positive sera tested by the rntCap indirect ELISA was significantly different compared to the indirect ELISA using the GST alone ($p<0.01$). The average OD values of the negative sera tested by the rntCap indirect ELISA was also significantly different ($p<0.05$). Moreover, the average OD values tested on the indirect ELISA using the GST alone was not significantly different among the positive sera and negative sera ($p>0.05$).

3.3.3 The positive-negative cutoff and the assay performance

Based on ROC analysis of the rntCap indirect ELISA, the OD value of 90-field serum samples varied from a minimum of 0.105 to a maximum of 0.551 for negative sera (n=30) and the OD value ranged from 0.312 to 1.091 for positive sera (n=60). A cutoff OD of 0.330 was determined an optimal result with a Dsn of 98.33% and a Dsp of 93.33% whereas the accuracy was 96.67% (Table 3.1).

Tables 3.1:Comparison of area under the receiver operating curve (AUC) values (±standard deviation), sensitivity, specificity and accuracy between the rntCap indirect ELISA and the IPMA test on different cut off OD value

Cutoff OD*	AUC	Sensitivity (%)	Specificity (%)	Accuracy (%)
0.300	0.988 ±0.02	100.00	86.67	95.6
0.315	0.988 ±0.01	98.33	90.00	95.6
0.330	0.988 ±0.01	98.33	93.33	96.7
0.360	0.987 ±0.01	95.00	93.33	94.4
0.375	0.987 ±0.01	95.00	96.67	95.6
0.400	0.988 ±0.01	90.00	96.67	92.2

*Cutoff determined by receiver operating characteristic (ROC) analysis (n=90).

3.3.4 The assay repeatability

The intra-plate repeatability test was performed by comparing of the OD ratios results in triplication of each sample tested in the same plate. The inter-plate repeatability was also done in different plates at different times. The results from 8-negative sera revealed the intra-plate CV ranged from 0.37% to 19.86%, with a median value of 3.74%, whereas those of the positive serum samples ranged from 0.91% to 7.68%, with a median value of 3.82%. The inter-plate CV for the negative sera was between 5.55% and 30.60%, with a median value of 21.10%, while the CV of the positive serum samples was between 7.18% and 17.69%, with a median value of 12.21%. These results indicated that the rntCap indirect ELISA was repeatable.

3.3.5 The correlation between the ELISA and IPMA tests

To determine the correlation between the IPMA titers and the OD ratios of the rntCap indirect ELISA, endpoint IPMA titers of 16-positive serum samples with variation of PCV2 antibody levels against the OD ratios of the tested serums were plotted as shown in Figure 3.3. The correlation between the IPMA titers and the OD ratios of the rntCap indirect ELISA was revealed the linear regression (Spearman's correlation coefficient = 0.772, $p<0.05$) within a minimum and a maximum limited range, respectively (2.90 and 3.80 for the IPMA and 0.31 and 0.94 for the OD ratios). As a result, the OD ratios obtained from the rntCap indirect ELISA could be reliably used to estimate the IPMA titers.

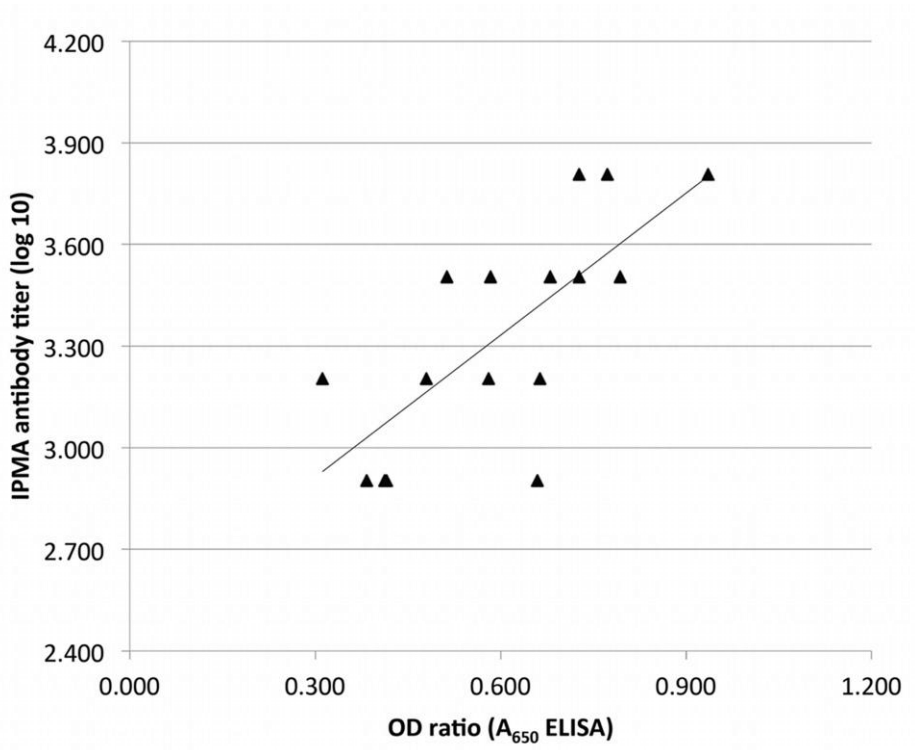

Figure 3.3: Correlation between log10 IPMA titers of 16 known serum samples and OD ratios from the rntCap indirect ELISA. The relationship between the IPMA titers and OD ratios yielded a linear progression (Spearman's correlation coefficient of 0.772, $p<0.05$).

3.4 Discussion

Porcine circovirus type 2 (PCV2) was first recognized as a causative agent of PMWS in Canada in 1991(Harding and Clark, 1997). Subsequently, the incidence of PMWS in the swine herds has become a major economic impact to the swine industry worldwide (Allan et al., 1998[a]; Tantilertcharoen et al., 1999; Allan and Ellis, 2000; Segalés and Domingo, 2002). Traditionally, virus isolation is regarded as a gold standard technique in most viral diagnosis. However, PCV2 virus isolation is difficult and time-consuming since PCV2 does not produce cytopathic effects and is unable to grow in cell culture with a high virus titer. Previously, several serological diagnostic methods have been used for PCV2 antibody detection such as IFA and IPMA assays

(Allan et al., 1999[a]; Allan et al., 1999[b]). In addition, these assays are time-consuming, labor-intensive and not suitable for large-scale surveys. It should be noted that using whole viral particles from PCV2-infected cells could generate cross-reactivity between the rep proteins of PCV1 and PCV2 when using PCV1 contaminated cell line leading to false-positive results (Magar et al., 2000). However, no cross-reactivity is found between the ORF2 capsid proteins of PCV1 and PCV2 when using the rntCap indirect ELISA of PCV2 (Mahé et al., 2000).

Based on previous studies, the capsid protein of PCV2 is the preferred antigen for serological tests due to its strong immunoreactivity (Blanchard et al., 2003[b]; Zhou et al., 2006). In addition, the antigenic epitopes of PCV2 capsid protein demonstrated that the amino acid residues located in the NLS region of PCV2 did not involve in the conformational epitopes (Mahé et al., 2000; Lekcharoensuk et al., 2004).

In this study, the ntCap gene was expressed in the *E. coli* as the rntCap with GST-tagged protein and used as an antigen for the developed rntCap indirect ELISA. Since the rntCap protein was a recombinant GST-tagged protein possibly interfering with the results. To resolve that matter, the mean OD values of 8-positive and 8-negative serum samples were test and the results demonstrated that the GST-tagged protein in the rntCap protein was not interfering with this assay. It should be noted that both Dsn and Dsp of this established assay yielded higher values than those of previous reports (Shang et al., 2008; Wu et al., 2008). However, the high sensitivity obtained from this assay might derive from using the non-purified rntCap protein antigen which could generate false-positive results. Nevertheless, the results from the established rntCap indirect ELISA demonstrated the appropriate accuracy rate (96.67%) with the IPMA results when tested with the 90-field swine serum samples. More field samples are needed to confirm the reliable of this indirect ELISA.

The repeatability of this assay also revealed a low variability. The intra-plate test showed minor value differences when compared to the inter-plate test, suggesting that the stability of the rntCap antigen might not be completed. However, the CV of the negative and positive serum samples in both assays indicated that the variability of this established rntCap indirect ELISA was acceptable. The correlation between the rntCap indirect ELISA and the IPMA demonstrated the linear regression indicating that the OD ratios from the rntCap indirect ELISA could be used to predict the IPMA titers. Again, more samples are needed to enhance the diagnostic sensitivity, specificity and accuracy rate.

Additionally, based on the results from 59-positive sera (cutoff 0.330) tested by the rntCap indirect ELISA and collected from vaccinated and non-vaccinated pigs (data not shown) indicated that the established rntCap indirect ELISA could detect the antibodies against PCV2 both from naturally infected pigs and vaccinated pigs. It would be of interest to observe whether the rntCap indirect ELISA could be used for PCV-2 antibody detection in oral fluid samples as oral fluid sample is currently widely used for major swine pathogen detection, including PCV-2, in the United States (Ramirez et al., 2012). Another additional advantage of using the rntCap protein is possibly using for the development of other diagnostic tests such as a rapid strip test.

In conclusion, the development of the rntCap indirect ELISA for PCV2 antibodies detection by using the rntCap protein expressed in the *E. coli* in this report is able to use as a serodiagnostic tool for PCV2 antibody detection in the swine herds. The applications of using this ELISA include seromonitoring on NLS-capsid of PCV2 antibodies from maternal derived antibodies, determining the time of infection in the production cycle and possibly measuring the levels of the antibody level after vaccination. Those mentioned advantages can be analyzed in the future after gathering

more data from the field uses. In addition, this assay could be a valuable test for the routine diagnostic tool for the large-scale samples and could possibly be produced for commercial purpose.

CHAPTER IV

Enhancing specific antibodies using a developed subunit PCV2b vaccination in a PCV2-affacted herd

4.1 Introduction

Porcine circovirus type 2 (PCV2) has been identified as a causative agent of porcine circovirus associated diseases (PCVAD) in pigs, an important swine disease having a huge impact on swine industry worldwide (Allan and Ellis, 2000; Chae, 2005). Currently, epidemiological studies indicate that PCV2b is the predominant genotype in the global pig population (Allan et al., 2007; Patterson and Opriessnig, 2010; Cortey et al., 2011[b]) including Thailand (Jittimanee et al., 2011). The first available commercial PCV2 vaccine is an inactivated PCV2 oil adjuvant vaccine used for breeders (Reynaud et al., 2004). On the other hand, other three commercial vaccines are recommended for piglets. One is a killed chimera vaccine combining the antigenic capsid gene of PCV2 into the PCV1 backbone (Fenaux et al., 2004). The others are subunit vaccines from a recombinant capsid protein expressed in baculovirus system (Fachinger et al., 2008). All of commercially available vaccines are based on PCV2a genotype. Although previous experimental results indicated that current commercial vaccines based on PCV2a could confer cross protection against PCV2b in challenge models (Patterson et al., 2008; Fort et al., 2009; Opriessnig et al., 2010[b]). Recently, a PCV2 vaccine trial concurrent with PCV2, PRRSV and PPV infection mimicking field situation revealed that a PCV2b vaccine was more effective on reducing viremia than the PCV2a vaccine when challenged with only PCV2b or both PCV2a and PCV2b (Opriessnig et al., 2013). Additionally, novel vaccine prototypes based on PCV2b genotype such as subunit

vaccines or modified live-attenuated vaccines are being developed and may provide better protection and lower vaccine costs. These developed vaccines include recombinant capsid protein in different bacterial systems (Liu et al., 2001b; Wang et al., 2008), viral-vectored vaccines (Wang et al., 2007; Fan et al., 2008[a]) and DNA vaccines (An et al., 2008; Shuai et al., 2013). Therefore, most of these studies were performed only in experimental models (mice or pigs). Moreover, it would be interesting to find protection efficacy of the PCV2 prototype vaccine under field condition.

Based on preliminary results revealed that a developed subunit PCV2b vaccine using a recombinant truncated capsid protein expressed in *Escherichia coli* could induce the specific antibody responses in the experimental pigs especially when vaccinated twice (Jittimanee et al., 2010). However, the efficacy of this vaccine trial under the field condition is undergoing investigation. Therefore, the objective of this study was to investigate the efficacy of a developed PCV2b vaccine in a PCV2-affected herd based on serology and viremic parameters.

4.2 Materials and Methods

4.2.1 Subunit vaccine preparation

A subunit PCV2b vaccine was prepared using a purified recombinant truncated Cap (rntCap) protein based on PCV2b genotype (GenBank accession no. JQ866913) expressed in *E. coli* system as previously described (Jittimanee et al., 2012). A developed subunit PCV2b vaccine was mixed with a commercial water based adjuvant (MONTANIDE™ GEL-01, SEPPIC Inc., France) to make final concentrations of 50 µg/ 2mL/dose (final concentrations was based on the preliminary results, data shown in appendix E).

4.2.2 Farms, housing and study designs

The field trial was conducted on a selected farm containing 1000-sows with multi-site production and all-in/all-out production system. The experimental farm was seropositive to porcine reproductive and respiratory syndrome virus (PRRSV) with no history of PRRS vaccination. This farm had continuous losses in recent months due to porcine circovirus associated diseases (PCVAD) without using PCV2 vaccination previously. The clinical signs were observed at approximately 12-18 weeks of age and preliminary PCV2 DNA was found in five-serum pooled samples from 12, 15, 18, 21 and 24-week–old-pig by a routine PCR. One hundred and ten-5-week old pigs were randomly allocated into 2 groups; a vaccinated group (n = 55) was received two-shot vaccinations and a control group (n = 55) was received only an adjuvant. Both studied group were in separated pens in the same building. Vaccination was done twice on day 0 and day 14 when pigs at 5 and 7 weeks of age. Serum samples were collected from randomly selected 10 pigs of each group (n=10) before vaccination as the baseline and at 14, 28, 77 and 119 dpv for serological examination and quantitative PCR.

4.2.3 Serology

4.2.3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

All serum samples were tested in duplication by an indirect rntCap ELISA assay as described previously (Jittimanee et al., 2012). Briefly, Flat-bottom 96 well plates (eBioscience Inc., CA, USA) were coated with the rntCap protein and blocked with 1% BSA in PBS. Subsequently, 100 µl/well of the 1:100 serum diluted were added and incubated at 37^{o}C for 30 min. Following 4 washes with phosphate buffer saline - 0.5% tween-20 (PBS-T), the 1:10,000 dilution of the horseradish peroxidase-labeled goat anti-swine IgG antibodies (Kirkegaard & Perry Laboratories, MD, USA) were added with further incubated at 37^{o}C for 30 min. The wells were again washed for 4 times and the

reaction was developed using 100 µl of tetramethylbenzidine solution (Sigma-Aldrich, MO, USA). After incubation for 1 h at room temperature in the dark, the optical density was measured at 650 nm.

4.2.3.2 Immunoperoxidase monolayer assay (IPMA)

IPMA was conducted for detecting the neutralizing PCV2 antibodies in pig sera. IPMA was performed in duplication as previously described (Liu et al., 2004) with some modification. Briefly, 96 well flat bottom plates containing the PK15/PCV2-infected cells and the negative control cells (mock-infected PK15 cells) were fixed in 4% formalin in PBS for 30 min at room temperature. Following 3 washes with PBS-T, 2-fold diluted-field serum samples and positive control (monoclonal mouse anti-PCV2 antibodies, Rural Technologies Inc., SD, USA) were added and then incubated at 37°C for 1 h. After 3 washes, 1:300 dilution of HRP-goat anti-swine IgG antibodies (New England Biolabs Inc., MA, USA) and 1:300 of HRP-conjugated rabbit anti-mouse Ig antibodies (SouthernBiotech, AL, USA) were added. Following 1 h of incubation at 37°C, plates were washed 3 times and 3-amino-9-ethyl-carbazol (Merck Millipore, MA, USA) solution was added and incubated for 30 min at 37°C. Color developed from substrate was removed and then the plate was washed by tap water. The IPMA plate was examined for dark brown staining of PCV2-infected cells under an inverted light microscope.

4.2.4 Quantification of PCV2 DNA in serum samples

DNA extraction from serum samples (at 0, 14, 28, 77 and 119 dpv) was performed using the NucleoSpin Extract Viral DNA Kit (Macherey-Nagel, Düren, Germany) for quantification of PCV2 genomic DNA copy numbers by a SYBR green real-time PCR as described previously (McIntosh et al., 2009) with some modification.

Briefly, The PCR reaction was performed in a 25 µl volume containing: 12.5 µl QuantiTect SYBR Green PCR Kit (QIAGEN, USA), 150 nM of each forward and reverse primer (F: 5'ATGCCCAGCAAGAAGAGTGGAAGAAG3', R:5'AGGTCACTCCGTTGTCCTTGAGATC3') and 3 µl of sample DNA extracts or 10-fold dilutions of standard PCV2 DNA respectively. The amplification was performed under the conditions at 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 45 sec each. Means of viral loads were calculated from the individual viral load (PCV2 DNA genomic copies per ml of serum) of all analyzed animals of each respective sampling day.

4.2.5 Statistical analysis

Values of the IPMA titers and viral loads in serum were transformed to log2 and log10 values, respectively. Mean comparison of parameters (IPMA, ELISA and viral load) between vaccinated and control group was analyzed by unpaired *t*-test. The significance level (*p*-value) for all parameters was set at 0.05.

4.3 Results

4.3.1 Serology

IPMA and indirect ELISA were performed in duplication on total serum samples as the serological assays for this study. Mean log2 IPMA titers, the prevalence of PCV2 seropositive pigs and mean group S/P ratios between vaccinated and control groups of different sampling days were summarized in Table 4.1. Pigs vaccinated with a developed subunit PCV2b vaccine had better anti-PCV2 IgG response compared to the control pigs. The IPMA titers in the vaccinated pigs (mean titers: 4.90 ± 0.99 log2) were significantly higher than those of titers in the control pigs (mean titers: 2.50 ± 0.85 log2) at 28 dpv ($p < 0.05$). However, at the time of PCV2 vaccinations (on day 0 and 14) and at 77 and 119 dpv, no significant differences of IPMA titers were detected between two

groups (Fig. 4.1) but, the ELISA results revealed significant differences ($p < 0.05$) in mean S/P ratios between vaccinated and control groups at 28 dpv (0.688 ± 0.270 and 0.200 ± 0.17) and at 119 dpv (1.440 ± 0.38 and 0.808 ± 0.47) as shown in Figure 4.2.

Moreover, the prevalence of ELISA seropositive pigs at 14, 28 and 77 dpv of the vaccinated group were 70 % (7/10), 100 % (10/10) and 80% (8/10), respectively, whereas only 30 % (3/10), 20 % (2/10) and 66.67% (6/9) of the control group had seropositive to PCV2 (Table 4.1)

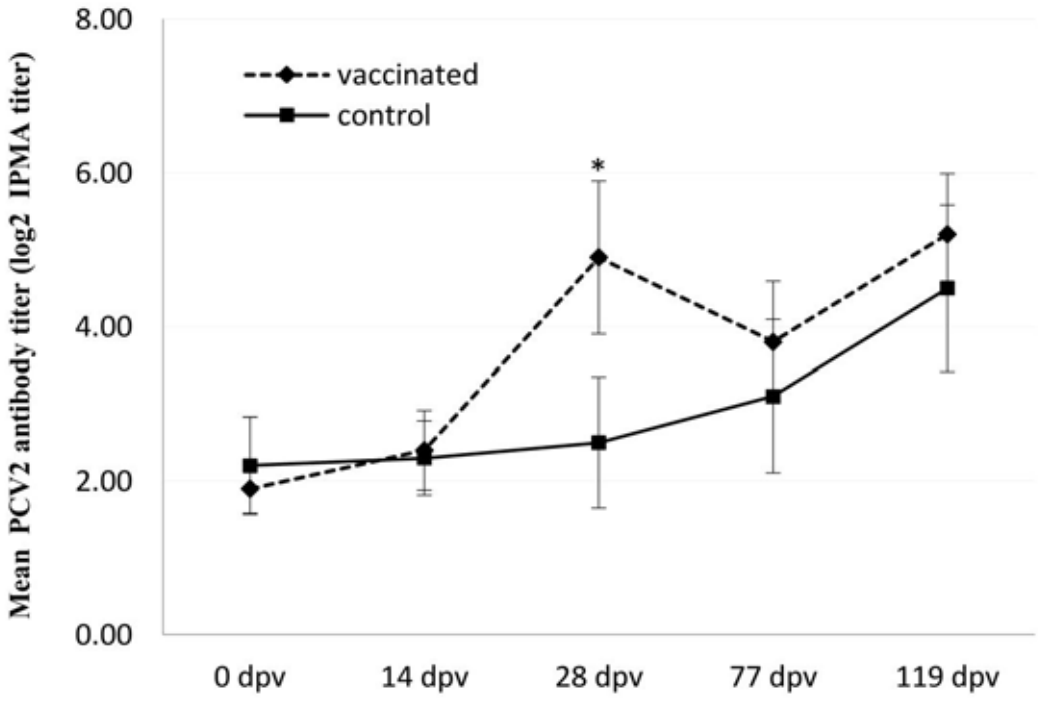

Figure 4.1: Mean group of log2 serum antibody titers measured by IPMA between the vaccinated group (n = 10) and the control group (n = 10) on different trial days (at 0, 14, 28, 77, and 119 dpv). Asterisks (*) indicating significant differences ($p < 0.05$). Error bars represent standard errors.

Table 4.1: Results of IPMA, indirect ELISA assays and quantitative PCR. Gray shaded areas indicate the PCV2 seropositive pigs from the indirect ELISA test (S/P ratio > 0.3).

Sampling days	IPMA		ELISA		qPCR	
	Mean log2 PCV2 titer ± SD		Prevalence (mean S/P ratio ± SD)		Mean log10 PCV2 (copies/ml ± SD)	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
0	1.90 ± 0.32	2.20 ± 0.63	2/10 (0.224 ± 0.17)	5/10 (0.303 ± 0.21)	4.12 ± 0.34	4.17 ± 0.29
14	2.40 ± 0.52	2.30 ± 0.48	7/10 (0.368 ± 0.15)	3/10 (0.242 ± 0.15)	3.64 ± 0.27	3.77 ± 0.30
28	4.90 ± 0.99*	2.50 ± 0.85	10/10 (0.688 ± 0.27)*	2/10 (0.200 ± 0.17)	4.97 ± 0.25*	5.20 ± 0.10
77	3.80 ± 0.79	3.10 ± 0.99	8/10 (0.500 ± 0.21)	6/9 (0.373 ± 0.20)	4.71 ± .0.38*	6.03 ±1.41
119	5.20 ± 0.79	4.50 ± 1.08	10/10 (1.440 ± 0.38)*	9/9 (0.808 ± 0.47)	4.26 ± 0.25*	5.03 ± 0.67

* Indicating significant ($p < 0.05$) differences between the vaccinated and control groups.

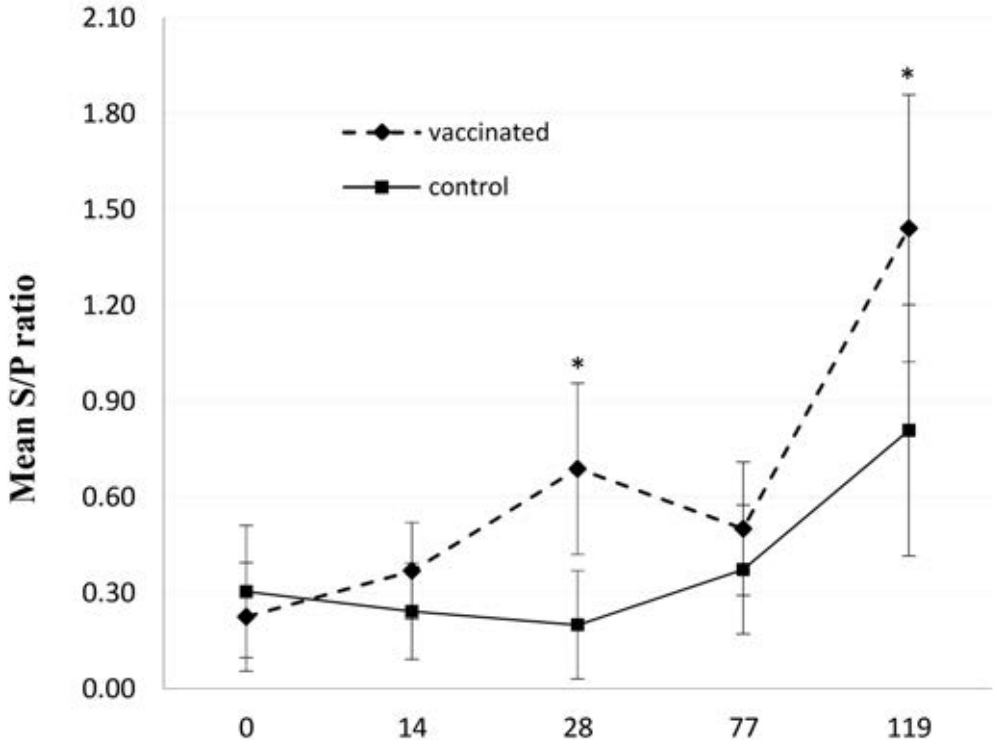

Figure 4.2: Mean group of sample-to-positive (S/P) ratios in serum samples obtained from the vaccinated group (n = 10) and the control group (n = 10) on different trial days (at 0, 14, 28, 77, and 119 dpv). An S/P ratio of 0.3 is considered positive. Asterisks (*) indicating significant differences ($p < 0.05$). Error bars represent standard errors.

4.3.2 Quantification of PCV2 DNA in serum samples

Quantification of PCV2 DNA in serum samples was performed in duplication using a modified SYBR green real-time PCR. The group means of log10 of PCV2 DNA genomic copies per ml of serum were calculated from the individual copy number of PCV2 genomic from each group transformed into log10 and summarized in table 1. No statistical differences in means of PCV2 viral load in sera were observed between the vaccinated and the control pigs at both vaccination days (day 0 and day 14). Therefore, means of PCV2 viral load in sera of vaccinated group (viral load of 4.97 ± 0.25, 4.71 ±

0.38 and 4.26 ± 0.25 log10) were significantly ($p < 0.05$) lower when compared to means of the control group (5.20 ± 0.10, 6.03 ±1.41 and 5.03 ± 0.67 log10) at 28, 77 and 119 dpv, respectively (Fig. 4.3).

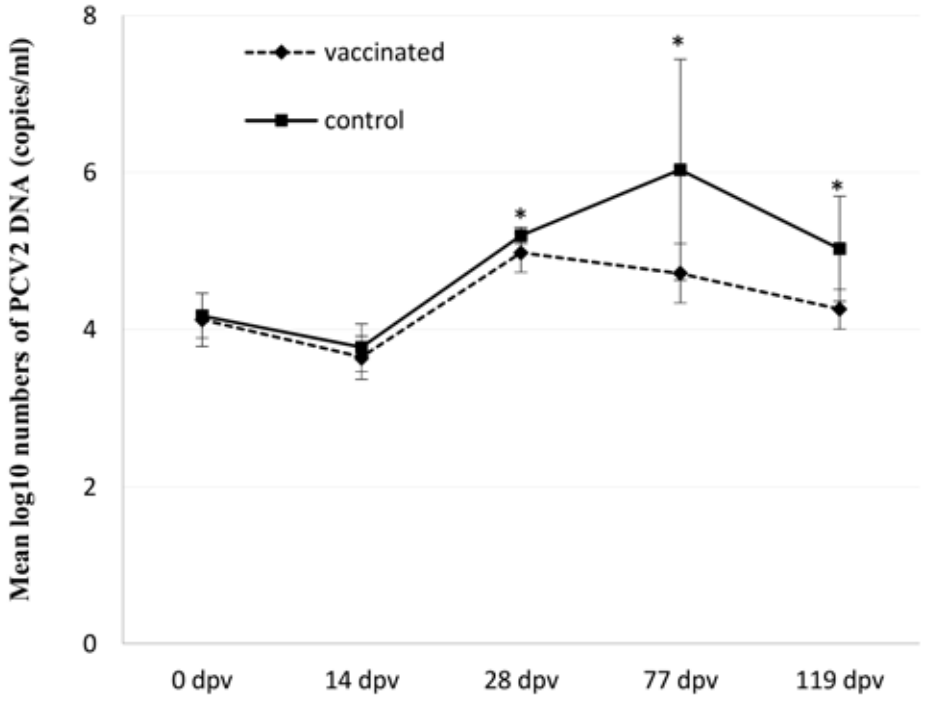

Figure 4.3: Mean group of log10 PCV2 DNA copies in serum samples obtained from the vaccinated group (n = 10) and the control group (n = 10) at 0, 14, 28, 77, and 119 dpv. Asterisks (*) indicating significant differences ($p < 0.05$). Error bars represent standard errors.

4.4 Discussion

Since PCV2 has been identified as a causative agent of PCVAD in pigs, the incidence of PCVAD in the swine herds has become a major economic impact to the swine industry worldwide (Allan et al., 1998[a]; Allan and Ellis, 2000; Segalés et al., 2002). Currently, epidemiological studies indicate that PCV2b is the predominant genotype causing major losses in the global pig population (Patterson and Opriessnig, 2010; Cortey et al., 2011[b]) including Thailand (Jittimanee et al., 2011). While, several

commercial PCV2 vaccines based on PCV2a are still widely used for the protection against PCVAD, cross protection against PCV2b has been demonstrated in experimental models (Patterson et al., 2008; Fort et al., 2009; Opriessnig et al., 2010[b]). However, PCVAD outbreaks in vaccinated pig herds still occurred. Recently, a PCV2 vaccine trial concurrently with PCV2, PRRSV and PPV infections mimicking field situation revealed that a PCV2b vaccine is more effective on reducing viremia than a PCV2a vaccine when challenged with PCV2b or combined PCV2a/2b (Opriessnig et al., 2013). The result suggested that a new prototype vaccine based on PCV2b genotype might be useful and necessary.

Several previous studies of capsid protein expressed in *E. coli* suggested that this particular recombinant capsid protein had a potential as a vaccine candidate (Zhou et al., 2005; Fan et al., 2008b; Marcekova et al., 2009). However, the efficacy of a subunit vaccine trail using a recombinant capsid protein is still needed. Moreover, it would be of interest to find protection efficacy of a subunit PCV2 vaccine prototype tested under the field condition.

This study investigated the efficacy of a developed subunit PCV2 vaccine based on PCV2b recombinant capsid protein expressed in *E. coli* on enhancing humoral antibodies and reducing viremia in a PCV2-affected farm. The results from IPMA and indirect ELISA tests revealed that pigs vaccinated with a developed subunit PCV2b vaccine had better anti-PCV2 IgG response compared to the control pigs.

However, the significant differences between the vaccinated and control groups were observed only at 28 dpv by IPMA and at 28 dpv and 119 dpv by the indirect ELISA (Table 4.1). No significant differences between the two groups were shown at 77 dpv. This result indicated that vaccination by a subunit PCV2b vaccine might induce a moderate level of IPMA titer (mean titer: 4.90 ± 0.99 log2) and might partially control PCV2 infection measured by viremic reduction and the PCV2 antibody seemed to rise

again after pigs exposed to PCV2 naturally at 119 dpv. The short protection period of this developed subunit PCV2b vaccine might be due to having insufficient recombinant capsid protein or using an inappropriate adjuvant type. Moreover, the percentages of seropositive pigs in the vaccinated group were higher than those of the control group at 14, 28 and 77 dpv.

The qPCR results revealed that means of PCV2 viral load in sera of the vaccinated group were significantly lower than those of the control group at 28, 77 and 119 dpv. These results indicated that a developed subunit PCV2b vaccine could reduce viral load in serum samples of naturally infected pigs. Nevertheless, the vaccinated group showed lower viral load than that of the control group. However, viral load within the vaccine group seemed to increase at 28 dpv onward possibly due to after the natural infection. As a result, a subunit PCV2b vaccination could partially reduce the PCV2 viremia or improving the clinical signs caused by PCV2 infection.

In summary, a developed subunit PCV2b vaccine could enhance specific antibody and reduce the PCV2 viral load in serum samples in the studied PCV2-affected farm. Therefore, humoral immune responses induced by a developed subunit PCV2b vaccine could partially contribute to the PCV2 control and clearance of PCV2 clinical signs under field conditions. In addition, increased concentration dose of protein used and/or using an appropriate adjuvant could enhance better efficacy of this developed subunit vaccine. This study demonstrated that a developed subunit PCV2b vaccine has a potential use as a future vaccine candidate. However, more filed trials and other measured parameters such as production parameter, cell mediated immune response, viral shedding from excretion or organs are needed for the better evaluation of the efficacy.

CHAPTER V

CONCLUSION

This dissertation was conducted to investigation of three main objectives. The first objective was to genetically characterize the current Thai PCV2 isolates during 2007-2010. The second objective was to develop an in-house indirect ELISA for PCV2 diagnosis. And the last objective was to develop a subunit vaccine against PCV2 and investigate the efficacy of this vaccine on enhancing specific antibodies and reducing viremia in a PCV2-affected swine herd.

In Thailand, PMWS was first reported in 1998. However, only one isolate of Thai PCV2 from our group has been submitted to GenBank (AY864814). The first objective of this dissertation was to determine the genetic characterizations of the current Thai PCV2 isolates from clinical PCVAD pigs located in the high pig density provinces during 2007-2010. Twelve ORF2 of Thai PCV2 sequences were analyzed together with 19 representative ORF2 sequences. Interestingly, all 12 recent Thai PCV2 sequences belonged to two subgroups referred to 1A/B (10/12, 83.33%) and 1C (2/12, 16.67%) of PCV2b (genotype 1) similar to the previous Thai PCV2 isolate (AY864814) that also belong to subgroups 1C. Based on genetic characterization and phylogenetic analysis, all Thai PCV2 isolates in this study were closely related (the nucleotide identities ranged between 97.7-99.8%). Only a few specific substitution patterns were observed in amino acid positions and PCV2b genotype, subgroup 1A/B was predominated in Thai pigs with PCVAD in this study. Since all samples in this study were collected from the highest pig density provinces located in central and western part of Thailand (Phra Nakhon Si Ayutthaya, Saraburi and Nakhon Pathom province), imported swine breeders and semen could be at least 2 major routes of introductions of PCV2b genotype into Thailand.

In addition, another PCV2 study in PMWS-positive farms in Thailand were found 7 from total 10 PCV2 isolates belong to PCV2b cluster 1A/B similar to our study. While, three remaining isolates were grouped in the new subtype called PCV2e (Jantafong et al., 2011).

Previously, only three PCV2 genotypes were accepted from the available sequences in GenBank using the criteria for PCV2 genotype definition (Grau-Roma et al., 2008; Segalés et al., 2008). Recently, a novel genotype referred to PCV2d genotype was reported in China (Wang et al., 2009; Guo et al., 2010). Lastly, a fifth genotype called PCV2e have also been reported in China (Wang et al., 2009), Thailand (Jantafong et al., 2011) and wild boars from Europe and South America in preliminary data (Cortey et al., 2011[a]).

Nowadays, epidemiological studies indicate that genotype PCV2b is the predominant genotype in the global pig population (Allan et al., 2007; Patterson and Opriessnig, 2010; Cortey et al., 2011[b]) including Thailand (Jittimanee et al., 2011). Up to date, no evident of PCV2a, PCV2c and PCV2d genotypes were observed in Thailand. Therefore, continuous monitoring for PCV2 infection is needed to investigate the existence of the other genotype and the emerging of any new PCV2 genotype is possible in the future.

The second objective was to develop an indirect ELISA assay for PCV2 infection in Thailand. In general, IPMA and IFA assays are the standard serological diagnostic assays for antibody detection against PCV2 (Allan et al., 1999[a]). However, these conventional assays are expensive, time-consuming and not suitable for large-scale surveys of PCV2 infection in Thailand. For the second objective, we establish and validate an indirect ELISA for PCV2 antibody detection using a recombinant truncated capsid (rntCap) protein expressed in *Escherichia coli* system as a coating antigen. Then, 90-field swine serum samples were obtained and tested using the developed

rntCap indirect ELISA and IPMA induplicate. The diagnostic sensitivity (Dsn) and specificity (Dsp) of the rntCap indirect ELISA are 98.33% and 93.33%, respectively when comparing to the IPMA. It should be noted that both Dsn and Dsp of this established assay yielded higher values than those of previous reports (Shang et al., 2008; Wu et al., 2008). However, the high sensitivity obtained from this assay might derive from using the non-purified rntCap protein antigen which could generate false-positive results since the rntCap protein was a recombinant GST-tagged protein. However, the results from 8-positive and 8-negative serum samples demonstrated that the GST-tagged protein in the rntCap protein was not interfering with this assay. Moreover, the results from the established rntCap indirect ELISA demonstrated the appropriate accuracy rate (96.67%) with the IPMA results. The correlation between the established rntCap indirect ELISA assay and IPMA demonstrated as the linear regression (Spearman's correlation coefficient = 0.772, $p<0.05$) indicating that the optical density ratio obtained from rntCap indirect ELISA could be used to predict the levels of IPMA titers.

We suggest that the development of the rntCap indirect ELISA for PCV2 antibodies detection by using the rntCap protein expressed in the *E. coli* is able to use as a serodiagnostic tool for PCV2 antibody detection in the swine herds. The applications of using this indirect ELISA include seromonitoring on PCV2 antibodies from maternal derived antibodies, determining the time of PCV2 infection and possibly measuring and monitoring the levels of the antibody after vaccination. It would be of interest to observe whether the established indirect ELISA could be used for PCV-2 antibody detection in oral fluid samples as oral fluid sample is currently widely used for major swine pathogen detection, including PCV-2, in the United States (Ramirez et al., 2012). Additional advantage of using the rntCap protein is possibly using for the development of other diagnostic tests such as a rapid strip test and this assay could

possibly be produced for commercial purpose. In addition, those mentioned advantages can be analyzed in the future after gathering more data from the field uses.

The third objective was to develop a subunit vaccine against PCV2 and investigate the efficacy of this vaccine on enhancing specific antibodies and reducing viremia in a PCV2-affected swine herd. Several previous studies of capsid protein expressed in *E. coli* suggested that this particular recombinant capsid protein had a potential as a vaccine candidate (Zhou et al., 2005[b]; Fan et al., 2008[b]; Marcekova et al., 2009). However, the efficacy of a subunit vaccine trail using a recombinant capsid protein is still needed. Currently, several available commercial PCV2 vaccines based on PCV2a are widely used for the protection against PCVAD. Even through cross protection against PCV2b using PCV2a vaccination have been reported in challenge models (Fort et al., 2009; Opriessnig et al., 2010[a]), PCVAD outbreaks in vaccinated pig herds still occurred (Opriessnig et al., 2013) due to many factors involving in vaccination failure. This report suggested that a new prototype vaccine based on PCV2b genotype might be necessary.

For the third objective of this dissertation, we determine the efficacy of a developed subunit PCV2b vaccine using a recombinant truncated capsid protein expressed in *Escherichia coli* in a pig farm affected by PCVAD. Five-week old conventional pigs were allocated into 2 groups referred to the vaccinated and the negative control group. IPMA and in-house indirect ELISA were used to evaluate antibody level and viral load of PCV2 in serum was determined by a modified real-time PCR. The IPMA and indirect ELISA results revealed that vaccinated pigs had significantly higher antibody level than the control group. The PCV2 viral loads in vaccinated pigs were also revealed significant lower levels than those of in the control pigs. However, after the vaccinated pig having the exposure with the natural PCV2 infection, the viral loads were rather high from 28 day post first vaccination onward.

These results demonstrated that humoral immune responses induced by our developed subunit PCV2b vaccine could partially contribute to the PCV2 viral load control and clearance of PCV2 clinical signs under field conditions. The short protection period of our developed subunit PCV2b vaccine are possibly due to insufficient amount of recombinant capsid protein or using an inappropriate adjuvant type. We suggest further investigation to optimize a concentration dose of protein used and/or using an appropriate adjuvant are needed to enhance better efficacy of this developed subunit vaccine.

In conclusion, this study indicated that only two subgroups referred to 1A/B and 1C of PCV2b genotype had been observed in pigs with PCVAD in Thailand during 2007-2010 and subgroup 1A/B was predominated with a few specific substitution patterns were observed in amino acid positions. Currently, five PCV2 genotypes have been reported (PCV2a to PCV2e) and PCV2b genotype is the predominant genotype in the global pig population including Thailand. Up to date, no evident of PCV2a, PCV2c and PCV2d genotypes were observed in Thailand. The nucleotide substitution rate of PCV2 is approximately $1.2x10^{3}$ substitutions/site/year (Firth et al., 2009) and it was high among single-stranded DNA viruses. Therefore, continuous monitoring for PCV2 infection is needed to investigate the existence of other genotypes, and the emerging of the novel PCV2 genotype is possible in the future. In addition, monitoring of PCV2 serological surveillance also should be continuous for evaluation of the PCV2 status in swine herds. Hence, the PCV2 serological status can be helpful for prevention and control program of PCV2 infection in swine herds. Moreover, due to PCV2b genotype is the predominant genotype in Thai swine herd. As a results, the developed indirect ELISA using PCV2b recombinant protein as a coating antigen could be appropriated use as a routine serodiagnostic assay for PCV2 serological surveillance in Thailand. However, comparing a developed indirect ELISA with the other available commercial ELISAs is

needed to investigate a true efficacy of our indirect ELISA. Prevention and control of PCVAD on pig farms depend on good management, biosecurity which avoiding the introduction of carrier animals and vaccination program. It should be noted that several PCV2a commercial vaccines are widely used for prevention and control PCVAD in the markets. However, PCVAD outbreaks in vaccinated herds still occurred. As a result, a new prototype vaccine based on PCV2b genotype might be necessary. Interestingly, our developed subunit PCV2b vaccine using rntCap protein expressed in *E. coli* could enhance specific antibody and reduce the PCV2 viral load in serum samples in the studied PCV2-affected farm and it has a potential use as a future vaccine candidate. However, more filed trials and other measured parameters such as production parameter, cell mediated immune response, viral shedding from excretion or organs are needed for the better evaluation of the efficacy.

Moreover, besides PCV2b spreading and the international trade of pig genetic lines might have contributed a novel PCV2 genotype in the future. As a result, continuous monitoring for PCV2 infection is still needed in the future. Additionally, more focus on specific antigenic epitope of capsid protein and its functional might be useful for further studies to develop an effective serological diagnostic assays and vaccines.

References

Allan G, Meehan B, Todd D, Kennedy S, McNeilly F, Ellis J, Clark EG, Harding J, Espuna E, Botner A and Charreyre C. 1998[a]. Novel porcine circoviruses from pigs with wasting disease syndromes. Vet Rec. 142(17): 467-468.

Allan GM and Ellis JA. 2000. Porcine circoviruses: a review. J Vet Diagn Invest. 12(1): 3-14.

Allan GM, Kennedy S, Mc Neilly F, Foster JC, Ellis JA, Krakowka SJ, Meehan BM and Adair BM. 1999[a]. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J Comp Pathol. 121(1): 1-11.

Allan GM, Mc Neilly F, Meehan BM, Kennedy S, Mackie DP, Ellis JA, Clark EG, Espuna E, Saubi N, Riera P, Botner A and Charreyre CE. 1999[b]. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet Microbiol. 66(2): 115-123.

Allan GM, McNeilly F, Cassidy JP, Reilly GA, Adair B, Ellis WA and McNulty MS. 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet Microbiol. 44(1): 49-64.

Allan GM, McNeilly F, Kennedy S, Daft B, Clarke EG, Ellis JA, Haines DM, Meehan BM and Adair BM. 1998[b]. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest. 10(1): 3-10.

Allan GM, McNeilly F, McMenamy M, McNair I, Krakowka SG, Timmusk S, Walls D, Donnelly M, Minahin D, Ellis J, Wallgren P and Fossum C. 2007. Temporal distribution of porcine circovirus 2 genogroups recovered from postweaning

multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern Ireland. J Vet Diagn Invest. 19(6): 668-673.

An DJ, Song DS and Park BK. 2008. Systemic cytokine profiles of mice vaccinated with naked DNAs encoding six open reading frame antigens of porcine circovirus type 2 (PCV2). Res Vet Sci. 85(3): 503-509.

Balasch M, Segalés J, Rosell C, Domingo M, Mankertz A, Urniza A and Plana-Durán J. 1999. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. J Comp Pathol. 121(2): 139-148.

Blanchard P, Mahé D, Cariolet R, Keranflec'h A, Baudouard MA, Cordioli P, Albina E and Jestin A. 2003[a]. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine. 21(31): 4565-4575.

Blanchard P, Mahé D, Cariolet R, Truong C, Le Dimna M, Arnauld C, Rose N, Eveno E, Albina E, Madec F and Jestin A. 2003[b]. An ORF2 protein-based ELISA for porcine circovirus type 2 antibodies in post-weaning multisystemic wasting syndrome. Vet Microbiol. 94(3): 183-194.

Carman S, Cai HY, DeLay J, Youssef SA, McEwen BJ, Gagnon CA, Tremblay D, Hazlett M, Lusis P, Fairles J, Alexander HS and van Dreumel T. 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. Can J Vet Res. 72(3): 259-268.

Carman S, McEwen B, DeLay J, van Dreumel T, Lusis P, Cai H and Fairles J. 2006. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Can Vet J. 47(8): 761-762.

Chae C. 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Vet J. 168(1): 41-49.

Chae C. 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet J. 169(3): 326-336.

Chae C. 2012. Commercial porcine circovirus type 2 vaccines: efficacy and clinical application. Vet J. 194(2): 151-157.

Cheung AK. 2009. Homologous recombination within the capsid gene of porcine circovirus type 2 subgroup viruses via natural co-infection. Arch Virol. 154(3): 531-534.

Cheung AK, Lager KM, Kohutyuk OI, Vincent AL, Henry SC, Baker RB, Rowland RR and Dunham AG. 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch Virol. 152(5): 1035-1044.

Choi C, Chae C and Clark EG. 2000. Porcine postweaning multisystemic wasting syndrome in Korean pig: detection of porcine circovirus 2 infection by immunohistochemistry and polymerase chain reaction. J Vet Diagn Invest. 12(2): 151-153.

Choi KS and Chae JS. 2008. Genetic characterization of porcine circovirus type 2 in Republic of Korea. Res Vet Sci. 84(3): 497-501.

Cortey M, Olvera A, Grau-Roma L and Joaquim S. 2011[a]. Further comments on porcine circovirus type 2 (PCV2) genotype definition and nomenclature. Vet Microbiol. 149(3-4): 522-523.

Cortey M, Pileri E, Sibila M, Pujols J, Balasch M, Plana J and Segales J. 2011[b]. Genotypic shift of porcine circovirus type 2 from PCV-2a to PCV-2b in Spain from 1985 to 2008. Vet J. 187(3): 363-368.

Crowther JR. 2002. The ELISA Guidebook. Methods in molecular biology. Totowa, New Jersey: Humana press. 1-413 p.

Darwich L, Pie S, Rovira A, Segales J, Domingo M, Oswald IP and Mateu E. 2003. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. J Gen Virol. 84(Pt 8): 2117-2125.

Darwich L, Segalés J and Mateu E. 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by porcine circovirus 2: an immune riddle. Arch Virol. 149(5): 857-874.

Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D and Haines D. 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J. 39(1): 44-51.

Fachinger V, Bischoff R, Jedidia SB, Saalmüller A and Elbers K. 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine. 26(11): 1488-1499.

Fan H, Ju C, Tong T, Huang H, Lv J and Chen H. 2007. Immunogenicity of empty capsids of porcine circovius type 2 produced in insect cells. Vet Res Commun. 31(4): 487-496.

Fan H, Pan Y, Fang L, Wang D, Wang S, Jiang Y, Chen H and Xiao S. 2008[a]. Construction and immunogenicity of recombinant pseudotype baculovirus expressing the capsid protein of porcine circovirus type 2 in mice. J Virol Methods. 150(1-2): 21-26.

Fan H, Xiao S, Tong T, Wang S, Xie L, Jiang Y, Chen H and Fang L. 2008[b]. Immunogenicity of porcine circovirus type 2 capsid protein targeting to different subcellular compartments. Mol Immunol. 45(3): 653-660.

Faurez F, Dory D, Grasland B and Jestin A. 2009. Replication of porcine circoviruses. Virol J. 6: 60.

Fenaux M, Opriessnig T, Halbur PG, Elvinger F and Meng XJ. 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol. 78(12): 6297-6303.

Firth C, Charleston MA, Duffy S, Shapiro B and Holmes EC. 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. J Virol. 83(24): 12813-12821.

Fort M, Olvera A, Sibila M, Segales J and Mateu E. 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Vet Microbiol. 125(3-4): 244-255.

Fort M, Sibila M, Allepuz A, Mateu E, Roerink F and Segales J. 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine. 26(8): 1063-1071.

Fort M, Sibila M, Pérez-Martín E, Nofrarías M, Mateu E and Segalés J. 2009. One dose of a porcine circovirus 2 (PCV2) vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. Vaccine. 27(30): 4031-4037.

Gagnon CA, Music N, Fontaine G, Tremblay D and Harel J. 2010. Emergence of a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant. Vet Microbiol 144(1-2): 18-23.

Gagnon CA, Tremblay D, Tijssen P, Venne MH, Houde A and Elahi SM. 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can Vet J. 48(8): 811-819.

Gillespie J, Opriessnig T, Meng XJ, Pelzer K and Buechner-Maxwell V. 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J Vet Intern Med. 23(6): 1151-1163.

Grau-Roma L, Crisci E, Sibila M, Lopez-Soria S, Nofrarias M, Cortey M, Fraile L, Olvera A and Segales J. 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol. 128(1-2): 23-35.

Guo L, Lu Y, Huang L, Wei Y and Liu C. 2011. Identification of a new antigen epitope in the nuclear localization signal region of porcine circovirus type 2 capsid protein. Intervirology. 54(3): 156-163.

Guo L, Lu Y, Wei Y, Huang L and Liu C. 2010. Porcine circovirus type 2 (PCV2): genetic variation and newly emerging genotypes in China. Virol J. 7(1): 273.

Hamel AL, Lin LL and Nayar GPS. 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J Virol. 72(6): 5262-5267.

Harding JCS and Clark EG. 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome(PMWS). Swine Health Prod. 5: 201-203.

He J, Cao J, Zhou N, Jin Y, Wu J and Zhou J. 2013. Identification and functional analysis of the novel ORF4 protein encoded by porcine circovirus type 2. J Virol. 87(3): 1420-1429.

Horlen KP, Dritz SS, Nietfeld JC, Henry SC, Hesse RA, Oberst R, Hays M, Anderson J and Rowland RR. 2008. A field evaluation of mortality rate and growth performance in pigs vaccinated against porcine circovirus type 2. J Am Vet Med Assoc. 232(6): 906-912.

Jacobsen B, Krueger L, Seeliger F, Bruegmann M, Segalés J and Baumgaertner W. 2009. Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities in Northern Germany. Vet Microbiol. 138(1-2): 27-33.

Jacobson RH. 1998. Validation of serological assays for diagnosis of infectious diseases. Rev Sci Tech. 17(2): 469-526.

Jantafong T, Boonsoongnern A, Poolperm P, Urairong K, Lekcharoensuk C and Lekcharoensuk P. 2011. Genetic characterization of porcine circovirus type 2 in piglets from PMWS-affected and -negative farms in Thailand. Virol J. 8(1): 88.

Jittimanee S, Kedkovid R, Teankum K, Suradhat S and Thanawongnuwech. 2010. Heterologous expression of nuclear localization signal (NLS) truncated capsid protein of porcine circovirus type 2 (PCV2) in *Escherichia coli* and its potential of the specific antibodies induction. Proceeding of the 21st International Pig Veterinary Society Congress Vancouver, British Columbia, Canada. July, 18-21: 311.

Jittimanee S, Nuntawan Na Ayudhya S, Kedkovid R, Teankum K, Suradhat S and Thanawongnuwech R. 2012. An indirect enzyme-linked immunosorbent assay using a recombinant truncated capsid protein of porcine circovirus-2. J Vet Diagn Invest. 24(6): 1129-1132.

Jittimanee S, Nuntawan Na Ayudhya S, Teankum K, Kedkovid R and Thanawongnuwech R. 2011. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 in thai pigs with porcine circovirus associated diseases (PCVAD) during 2007-2010. Thai J Vet Med. 41(2): 163-169.

Kiatipattanasakul-Bunlunara W, Tantilertcharoen R, Suzuki K, Albarenque SM, Thanawongnuwech R, Nakayama H and Doi K. 2002. Detection of porcine circovirus 2 (PCV-2) DNA by nested PCR from formalin-fixed tissue of post-

weaning multisystemic wasting syndrome (PMWS) pig in Thailand. Thai J Vet Med 65(5): 449-452.

Kim D, Ha Y, Lee YH, Chae S, Lee K, Han K, Kim J, Lee JH, Kim SH, Hwang KK and Chae C. 2009[a]. Comparative Study of In situ hybridization and immunohistochemistry for the detection of porcine circovirus 2 in formalin-fixed, paraffin-embedded tissues. J Vet Med Sci. 71(7): 1001-1004.

Kim HH, Park SI, Hyun BH, Park SJ, Jeong YJ, Shin DJ, Chun YH, Hosmillo M, Lee BJ, Kang MI and Cho KO. 2009[b]. Genetic diversity of porcine circovirus type 2 in Korean pigs with postweaning multisystemic wasting syndrome during 2005-2007. J Vet Med Sci. 71(3): 349-353.

Kim J and Chae C. 2002. Double *in situ* hybridization for simultaneous detection and differentiation of porcine circovirus 1 and 2 in pigs with postweaning multisystemic wasting syndrome. Vet J. 164(3): 247-253.

Kim J and Chae C. 2003. Expression of monocyte chemoattractant protein-1 but not interleukin-8 in granulomatous lesions in lymph nodes from pigs with naturally occurring postweaning multisystemic wasting syndrome. Vet Pathol. 40(2): 181-186.

Kim J, Chung HK, Jung T, Cho WS, Choi C and Chae C. 2002. Postweaning multisystemic wasting syndrome of pigs in Korea: prevalence, microscopic lesions and coexisting microorganisms. J Vet Med Sci. 64(1): 57-62.

Kixmöller M, Ritzmann M, Eddicks M, Saalmüller A, Elbers K and Fachinger V. 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine. 26(27-28): 3443-3451.

Larochelle R, Magar R and D'Allaire S. 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Res. 90(1-2): 101-112.

Lefebvre DJ, Van Doorsselaere J, Delputte PL and Nauwynck HJ. 2009. Recombination of two porcine circovirus type 2 strains. Arch Virol. 154(5): 875-879.

Lekcharoensuk P, Morozov I, Paul PS, Thangthumniyom N, Wajjawalku W and Meng XJ. 2004. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol. 78(15): 8135-8145.

Liu C, Ihara T, Nunoya T and Ueda S. 2004. Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. J Vet Med Sci. 66(3): 237-242.

Liu J, Chen I, Du Q, Chua H and Kwang J. 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis *in vivo*. J Virol. 80(10): 5065-5073.

Liu J, Chen I and Kwang J. 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J Virol. 79(13): 8262-8274.

Liu Q, Tikoo SK and Babiuk LA. 2001[a]. Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. Virology. 285(1): 91-99.

Liu Q, Willson P, Attoh-Poku S and Babiuk LA. 2001[b]. Bacterial expression of an immunologically reactive PCV2 ORF2 fusion protein. Protein Expr Purif. 21(1): 115-120.

Ma CM, Hon CC, Lam TY, Li VY, Wong CK, de Oliveira T and Leung FC. 2007. Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. J Gen Virol. 88(6): 1733-1737.

Magar R, Muller P and Larochelle R. 2000. Retrospective serological survey of antibodies to porcine circovirus type 1 and type 2. Can J Vet Res. 64(3): 184-186.

Mahé D, Blanchard P, Truong C, Arnauld C, Le Cann P, Cariolet R, Madec F, Albina E and Jestin A. 2000. Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. J Gen Virol. 81(7): 1815-1824.

Mankertz A, Caliskan R, Hattermann K, Hillenbrand B, Kurzendoerfer P, Mueller B, Schmitt C, Steinfeldt T and Finsterbusch T. 2004. Molecular biology of porcine circovirus: analyses of gene expression and viral replication. Vet Microbiol. 98(2): 81-88.

Mankertz A, Domingo M, Folch JM, LeCann P, Jestin A, Segales J, Chmielewicz B, Plana-Duran J and Soike D. 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. 66(1): 65-77.

Mankertz A, Mankertz J, Wolf K and Buhk HJ. 1998. Identification of a protein essential for replication of porcine circovirus. J Gen Virol. 79 (Pt 2): 381-384.

Mankertz A, Persson F, Mankertz J, Blaess G and Buhk HJ. 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J Virol. 71(3): 2562-2566.

Manokaran G, Lin Y-N, Soh M-L, Lim EA-S, Lim C-W and Tan B-H. 2008. Detection of porcine circovirus type 2 in pigs imported from Indonesia. Vet Microbiol 132(1-2): 165-170.

Marcekova Z, Psikal I, Kosinova E, Benada O, Sebo P and Bumba L. 2009. Heterologous expression of full-length capsid protein of porcine circovirus 2 in *Escherichia coli* and its potential use for detection of antibodies. J Virol Methods. 162(1-2): 133-141.

McCullough KC, Ruggli N and Summerfield A. 2009. Dendritic cells-at the front-line of pathogen attack. Vet Immunol Immunopathol. 128(1-3): 7-15.

McCullough KC, Vincent IE, Summerfield A, Krakowka S, Ellis JA, Segalés J and Allan GM. 2007. The immunology of PCV2 infections. Proceedings of the 38th annual meeting of american association of swine veterinarians. Florida, USA. March, 3-6: 497-503.

McIntosh KA, Tumber A, Harding JC, Krakowka S, Ellis JA and Hill JE. 2009. Development and validation of a SYBR green real-time PCR for the quantification of porcine circovirus type 2 in serum, buffy coat, feces, and multiple tissues. Vet Microbiol. 133(1-2): 23-33.

Meehan BM, McNeilly F, McNair I, Walker I, Ellis JA, Krakowka S and Allan GM. 2001. Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. Arch Virol. 146(4): 835-842.

Morozov I, Sirinarumitr T, Sorden SD, Halbur PG, Morgan MK, Yoon KJ and Paul PS. 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J Clin Microbiol. 36(9): 2535-2541.

Nawagitgul P, Harms PA, Morozov I, Thacker BJ, Sorden SD, Lekcharoensuk C and Paul PS. 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin Diagn Lab Immunol. 9(1): 33-40.

Nawagitgul P, Morozov I, Bolin SR, Harms PA, Sorden SD and Paul PS. 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol. 81(9): 2281-2287.

Nielsen J, Vincent IE, Botner A, Ladekaer-Mikkelsen AS, Allan G, Summerfield A and McCullough KC. 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet Immunol Immunopathol. 92(3-4): 97-111.

Olvera A, Cortey M and Segales J. 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology. 357(2): 175-185.

Opriessnig T, Meng X-J and Halbur PG. 2007. Porcine Circovirus Type 2 associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J Vet Diagn Invest. 19(6): 591-615.

Opriessnig T, O'Neill K, Gerber PF, de Castro AMMG, Gimenéz-Lirola LG, Beach NM, Zhou L, Meng X-J, Wang C and Halbur PG. 2013. A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. Vaccine. 31(3): 487-494.

Opriessnig T, Patterson AR, Elsener J, Meng XJ and Halbur PG. 2008[a]. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental Infection with PCV2. Clin Vaccine Immunol. 15(3): 397-401.

Opriessnig T, Patterson AR, Madson DM, Pal N and Halbur PG. 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine. 27(7): 1002-1007.

Opriessnig T, Patterson AR, Madson DM, Pal N, Ramamoorthy S, Meng XJ and Halbur PG. 2010[a]. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Vet Microbiol. 142(3-4): 177-183.

Opriessnig T, Prickett JR, Madson DM, Shen HG, Juhan NM, Pogranichniy RM, Meng XJ and Halbur PG. 2010[b]. Porcine circovirus type 2 (PCV2)-infection and re-

inoculation with homologous or heterologous strains: virological, serological, pathological and clinical effects in growing pigs. Vet Res. 41(3): 31.

Opriessnig T, Ramamoorthy S, Madson DM, Patterson AR, Pal N, Carman S, Meng XJ and Halbur PG. 2008[b]. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. J Gen Virol. 89(10): 2482-2491.

Paphavasit T, Lehrbach P, Navasakuljinda W, Kedkovid R, Lacharoje S, Thanawongnuwech R and Teankum K. 2009. Efficacy of a chimeric PCV2 vaccine: a field trial. Thai J Vet Med. 39(2): 145-155.

Patterson AR, Johnson J, Ramamoorthy S, Meng X-J, Halbur PG and Opriessnig T. 2008. Comparison of three enzyme-linked immunosorbent assays to detect porcine circovirus-2 (PCV-2)-specific antibodies after vaccination or inoculation of pigs with distinct PCV-1 or PCV-2 isolates. J Vet Diagn Invest. 20(6): 744-751.

Patterson AR and Opriessnig T. 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). Anim Health Res Rev. 11(02): 217-234.

Pejsak Z, Podgorska K, Truszczynski M, Karbowiak P and Stadejek T. 2009. Efficacy of different protocols of vaccination against porcine circovirus type 2 (PCV2) in a farm affected by postweaning multisystemic wasting syndrome (PMWS). Comp Immunol Microbiol Infect Dis.

Prickett JR, Johnson J, Murtaugh MP, Puvanendiran S, Wang C, Zimmerman JJ and Opriessnig T. 2011. Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation. Transbound Emerg Dis.

Racine S, Kheyar A, Gagnon CA, Charbonneau B and Dea S. 2004. Eucaryotic expression of the nucleocapsid protein gene of porcine circovirus type 2 and use of the protein in an indirect immunofluorescence assay for serological

diagnosis of postweaning multisystemic wasting syndrome in pigs. Clin Diagn Lab Immunol. 11(4): 736-741.

Ramirez A, Wang C, Prickett JR, Pogranichniy R, Yoon K-J, Main R, Johnson JK, Rademacher C, Hoogland M, Hoffmann P, Kurtz A, Kurtz E and Zimmerman J. 2012. Efficient surveillance of pig populations using oral fluids. Prev Vet Med 104(3-4): 292-300.

Reynaud G, Brun A, Charreyre C, Desgouilles S and Jeannin P. 2004. Safety of a repeated overdose of an inactivated adjuvanted PCV2 vaccine in conventional pregnant gilts and sows. Proceeding of the 18th International Pig Veterinary Society Congress Hamburg, Germany. June 27- July 2: 88.

Rovira A, Balasch M, Segales J, Garcia L, Plana-Duran J, Rosell C, Ellerbrok H, Mankertz A and Domingo M. 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J Virol. 76(7): 3232-3239.

Saha D, Huang L, Bussalleu E, Lefebvre DJ, Fort M, Van Doorsselaere J and Nauwynck HJ. 2012. Antigenic subtyping and epitopes™ competition analysis of porcine circovirus type 2 using monoclonal antibodies. Vet Microbiol. 157(1-2): 13-22.

Saitou N and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4(4): 406-425.

Segalés J, Allan GM and Domingo M. 2005. Porcine circovirus diseases. Anim Health Res Rev 6(02): 119-142.

Segalés J, Calsamiglia M, Rosell C, Soler M, Maldonado J, Martin M and Domingo M. 2002. Porcine reproductive and respiratory syndrome virus (PRRSV) infection status in pigs naturally affected with post-weaning multisystemic wasting syndrome (PMWS) in Spain. Vet Microbiol. 85(1): 23-30.

Segalés J and Domingo M. 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet Q. 24(3): 109-124.

Segalés J, Domingo M, Chianini F, Majó N, Domínguez J, Darwich L and Mateu E. 2004[a]. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. Vet Microbiol. 98(2): 151-158.

Segalés J, Kekarainen T and Cortey M. 2013. The natural history of porcine circovirus type 2: from an inoffensive virus to a devastating swine disease? Vet Microbiol. 165(1-2): 13-20.

Segalés J, Olvera A, Grau-Roma L, Charreyre C, Nauwynck H, Larsen L, Dupont K, McCullough K, Ellis J, Krakowka S, Mankertz A, Fredholm M, Fossum C, Timmusk S, Stockhofe-Zurwieden N, Beattie V, Armstrong D, Grassland B, Baekbo P and Allan G. 2008. PCV-2 genotype definition and nomenclature. Vet Rec. 162(26): 867-868.

Segalés J, Rosell C and Domingo M. 2004[b]. Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. Vet Microbiol. 98(2): 137-149.

Segalés J, Sitjar M, Domingo M, Dee S, Del Pozo M, Noval R, Sacristan C, De las Heras A, Ferro A and Latimer KS. 1997. First report of post-weaning multisystemic wasting syndrome in pigs in Spain. Vet Rec 141: 600-601.

Segalés J, Urniza A, Alegre A, Bru T, Crisci E, Nofrarías M, López-Soria S, Balasch M, Sibila M, Xu Z, Chu HJ, Fraile L and Plana-Duran J. 2009. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine. 27(52): 7313-7321.

Shang S-B, Li Y-F, Guo J-Q, Wang Z-T, Chen Q-X, Shen H-G and Zhou J-Y. 2008. Development and validation of a recombinant capsid protein-based ELISA for detection of antibody to porcine circovirus type 2. Res Vet Sci. 84(1): 150-157.

Shibahara T, Sato K, Ishikawa Y and Kadota K. 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. J Vet Med Sci. 62(11): 1125-1131.

Shuai J, Zhang X, Chen W, Li K, Wu S, He Y and Fang W. 2013. *In vivo* characterization of chimeric PCV DNA clones containing heterogeneous capsid protein nuclear localization signals (NLS). Virol J. 10(1): 16.

Sun SQ, Guo HC, Sun DH, Yin SH, Shang YJ, Cai XP and Liu XT. 2010. Development and validation of an ELISA using a protein encoded by ORF2 antigenic domain of porcine circovirus type 2. Virol J. 7: 274.

Takahagi Y, Nishiyama Y, Toki S, Yonekita T, Morimatsu F and Murakami H. 2008. Genotypic change of porcine circovirus type 2 on Japanese pig farms as revealed by restriction fragment length polymorphism analysis. J Vet Med Sci. 70(6): 603-606.

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 24(8): 1596-1599.

Tantilertcharoen R, Kiatipattanasakul W and Thanawongnuwech R. 1999. Report of circovirus infection in pig in Thailand. Thai J Vet Med. 29: 73-83. (in Thai).

Timmusk S, Wallgren P, Brunborg I, Wikström F, Allan G, Meehan B, McMenamy M, McNeilly F, Fuxler L, Belák K, Põdersoo D, Saar T, Berg M and Fossum C. 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). Virus Genes. 36(3): 509-520.

Tischer I, Gelderblom H, Vettermann W and Koch MA. 1982. A very small porcine virus with circular single-stranded DNA. Nature. 295(5844): 64-66.

Trundova M and Celer V. 2007. Expression of porcine circovirus 2 ORF2 gene requires codon optimized E. coli cells. Virus Genes. 34(2): 199-204.

Truong C, Mahe D, Blanchard P, Le Dimna M, Madec F, Jestin A and Albina E. 2001. Identification of an immunorelevant ORF2 epitope from porcine circovirus type 2 as a serological marker for experimental and natural infection. Arch Virol. 146(6): 1197-1211.

Vincent IE, Carrasco CP, Guzylack-Piriou L, Herrmann B, McNeilly F, Allan GM, Summerfield A and McCullough KC. 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. Immunology. 115(3): 388-398.

Wang F, Guo X, Ge X, Wang Z, Chen Y, Cha Z and Yang H. 2009. Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res. 145(1): 151-156.

Wang K, Huang L, Kong J and Zhang X. 2008. Expression of the capsid protein of porcine circovirus type 2 in Lactococcus lactis for oral vaccination. J Virol Methods. 150(1-2): 1-6.

Wang X, Jiang P, Li Y, Jiang W and Dong X. 2007. Protection of pigs against post-weaning multisystemic wasting syndrome by a recombinant adenovirus expressing the capsid protein of porcine circovirus type 2. Vet Microbiol. 121(3-4): 215-224.

Wiederkehr DD, Sydler T, Buergi E, Haessig M, Zimmermann D, Pospischil A, Brugnera E and Sidler X. 2009. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet Microbiol. 136(1-2): 27-35.

Wu PC, Chien MS, Tseng YY, Lin J, Lin WL, Yang CY and Huang C. 2008. Expression of the porcine circovirus type 2 capsid protein subunits and application to an indirect ELISA. J Biotechnol. 133(1): 58-64.

Yin SH, Yang SL, Tian H, Wu JY, Shang YJ, Cai XP and Liu XT. 2010. An ELISA based on a truncated soluble ORF2 protein for the detection of PCV2 antibodies in domestic pigs. Virol Sin. 25(3): 191-198.

Zhou JY, Chen QX, Ye JX, Shen HG, Chen TF and Shang SB. 2006. Serological investigation and genomic characterization of PCV2 isolates from different geographic regions of Zhejiang province in China. Vet Res Commun. 30(2): 205-220.

Zhou JY, Shang SB, Gong H, Chen QX, Wu JX, Shen HG, Chen TF and Guo JQ. 2005. In vitro expression, monoclonal antibody and bioactivity for capsid protein of porcine circovirus type II without nuclear localization signal. J Biotechnol. 118(2): 201-211.

APPENDICES

APPENDIX A

Criteria of sample collection and farm selection

1. **Sample collection**
   - Serum samples or lymphoid tissue (lymph node and tonsil) samples from clinical PCVAD submitted to Chulalongkorn University - Veterinary Diagnostic Laboratory (CU-VDL) and Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University during year 2007 - 2010 were included in chapter II of the study. The samples were kept in - 80°C until performing DNA extraction and PCR. Serum samples were also kept in - 20°C for an indirect ELISA development in chapter III of the study.

2. **Farm selection**
   - The field trial study in chapter IV was performed on a farm contains at least 500-sow herds with all-in/all-out production system.
   - Farm had a history of continuous losses in several recent months due to porcine circovirus associated disease (PCVAD) with or without PCV2 vaccination.
   - Farm had seropositive status by ELISA cross-sectional survey at least 2-month-period before experimental start.
   - The clinical signs of PCVAD should be observed in weaning or/and finishing pigs and the necropsy examination or PCR were also performed to confirm a PCV2-affected status.

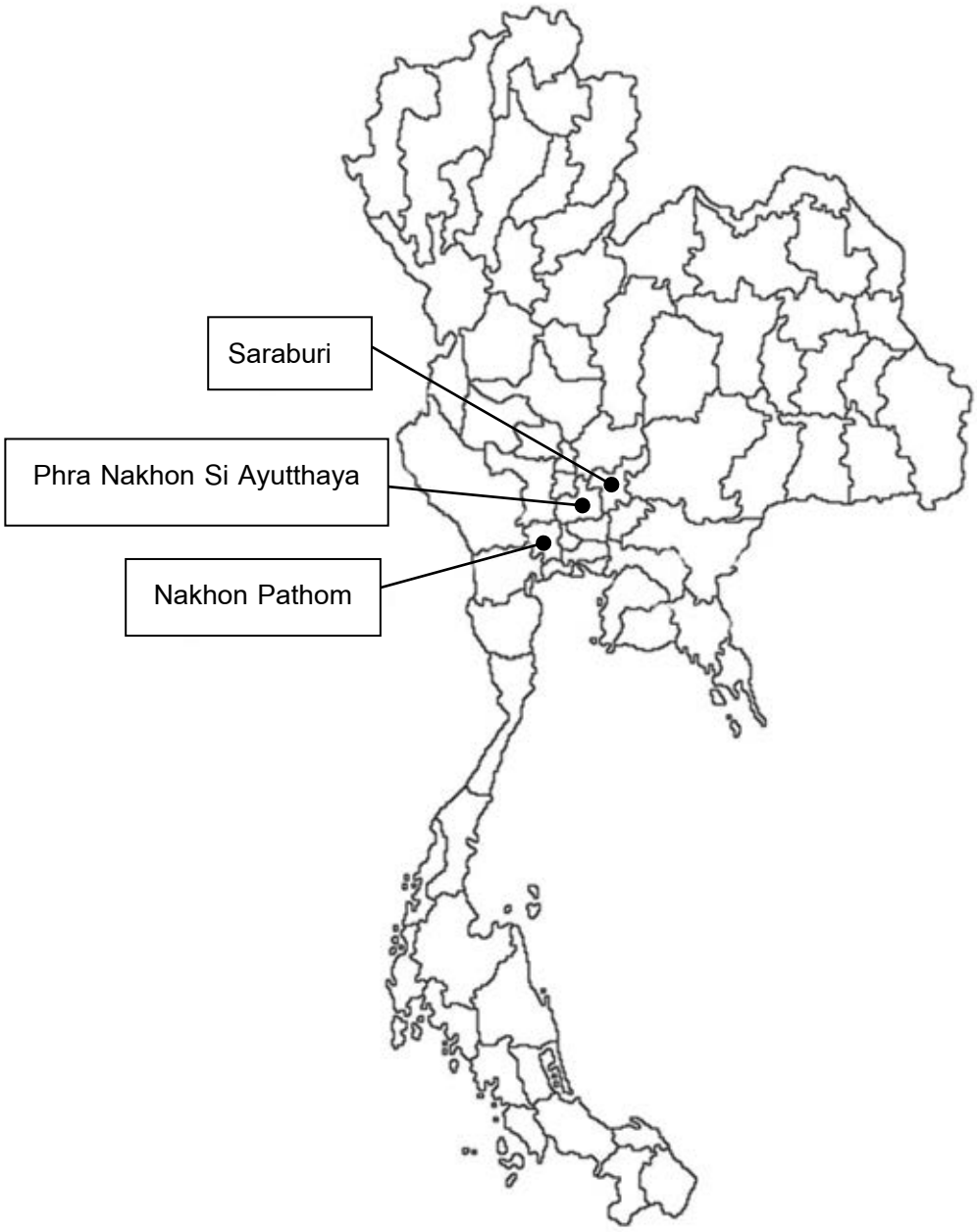

Figure A.1: Thailand map show the three provinces that had high pig density which all 12 sequences in this study were collected during 2007 - 2010. Those provinces include Saraburi, Phra Nakhon Si Ayutthaya and Nakhon Pathom Province from the central part of Thailand.

APPENDIX B

Protocol for PCV2 detection and ORF2 genetic characterization

1. **Primers used in this study**

   For screening PCR and qPCR

Primer name	Sequence (5' – 3')
ORF1 PCV2 F	ATGCCCAGCAAGAAGAGTGGAAGAAG
ORF1 PCV2 R	AGGTCACTCCGTTGTCCTTGAGATC

   For capsid gene sequencing (Takahagi et al., 2008)

Primer name	Sequence (5' – 3')
ORF2 PCV2 F	CCATGCCCTGAATTTCCATA
ORF2 PCV2 R	ACAGCGCACTTCTTTCGTTT

2. **Protocol for PCR and qPCR of partial ORF1 gene**

   Three microliters of extracted DNA in a 25 µl reaction mixture contains 10 µM/µl of forward and reverse primers, 12.5 µl of 2x GoTaq® Green Master Mix (Promega, USA) and nuclease-free water to make up the final volume. The amplification reaction was performed in a thermocycler with an initial step at 94°C for 2 minutes, followed by 35 cycles consisting denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes.

3. **Protocol for Genetic Characterization of ORF2 gene**

Five microliters of extracted DNA in a 50 µl reaction mixture contains 10 µM/µl of forward and reverse primers published by (Takahagi et al., 2008), 25 µl of 2x GoTaq® Green Master Mix (Promega, USA) and nuclease-free water to make up the final volume. The amplification reaction was performed in a thermocycler with an initial step at 94°C for 2 minutes, followed by 35 cycles consisting denaturation at 94°C for 30 seconds, primer annealing at 60°C for 45 seconds and extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes.

APPENDIX C

Nucleotide sequences

Porcine circovirus 2 isolate THA_07NP88 capsid protein gene, partial cds

GenBank: JQ866913.1

AATGGCATCTTCAACGCCCGCCTCTCCCGCACCTTCGGATATACTATCAAGCGAACCA
CAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATATTAATGACTTTCT
TCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAG
AAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGTGACAGGGGAGT
GGGATCCAGTGCTGTCATTCTAGATGATAACTTTGTAACAAAGGCCACAGCCCTCACC
TATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCCCTTCTCCTACC
ACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATTACTTCCAACCA
AACAACAAAAGAAATCGGCTGTGGCTGAGACTACAAACTGCTGGAAATGTAGACCAC
GTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATACAATATCCGT
GTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCGAGTTAACCCCTA
A

Porcine circovirus 2 isolate THA_07NP114 capsid protein gene, complete cds

GenBank: JQ387582.1

ATGACGTATCCAAGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTAT
CAAACGAACCACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATAT
TAATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGT
GACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCA
CAGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCC
CTTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATT
ACTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAA
TGTAGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATA
CAATATCCGTGTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCAC
TTAACCCTTAA

Porcine circovirus 2 isolate THA_08NP113 capsid protein gene, complete cds

GenBank: JQ866914.1

ATGACGTATCCAAGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTAT
CAAGCGAACCACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATAT
TAATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGT
GACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCA
CAGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCC
CTTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATT
ACTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAA
TGTAGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATA
CAATATCCGTGTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCAC
TTAACCCTTAA

Porcine circovirus 2 isolate THA_08NP266 capsid protein gene, complete cds

GenBank: JQ866915.1

ATGACGTATCCAAGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTAT
CAAGCGAACCACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATAT
TAATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGT
GACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCA
CAGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCC
CTTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATT
ACTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAA
TGTAGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATA
CAATATCCGTGTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCAC
TTAACCCTTAA

Porcine circovirus 2 isolate THA_09NP165 capsid protein gene, partial cds

GenBank: JQ866916.1

ATGACGTATCCAAGGAGGCGTTTCCGCAGACGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAAAAGAAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTGT
GAAGAAAACCACAGTCAGAACGCCCTCCTGGGCGGTGGACATGATGAGATTTAATAT
TAACGATTTCCTTCCCCCAGGAGGGGGCTCAAACCCCCTCACTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCAATCACCCAGGGTG
ACAGGGGAGTTGGATCCACTGCTGTTATTCTAGATGATAACTTTGTACCAAAGGCCAC
AGCCCTGACTTATGATCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCCC
TTCTCCTACCACTCCCGGTACTTTACCCCCAAACCTGTTCTTGATTCCACTATTGATTAC
TTCCAACCAAATAACAAAAGGAATCAGCTTTGGCTGAGGCTACAAACCTCTGCAAATG
TGGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGACTACA
ATATCCGTGTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCACTTA
ACCCTAAA

Porcine circovirus 2 isolate THA_09NP290 capsid protein gene, partial cds

GenBank: JQ866917.1

ATGACGTATCCAAGGAGGCGTTTCCGCAGACGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAAAAGAAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTGT
GAAGAAAACCACAGTCAGAACGCCCTCCTGGGCGGTGGACATGATGAGATTTAATAT
TAACGATTTCCTTCCCCCAGGAGGGGGCTCAAACCCCCTCACTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCAATCACCCAGGGTG
ACAGGGGAGTTGGATCCACTGCTGTTATTCTAGATGATAACTTTGTACCAAAGGCCAC
AGCCCTGACTTATGATCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCCC
TTCTCCTACCACTCCCGGTACTTTACCCCCAAACCTGTTCTAGATTCCACTATTGATTAC
TTCCAACCAAACAACAAAAGGAATCAGCTTTGGCTGAGGCTACAAACCGCTGCAAAT
GTGGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATAC
AATATCCGTGTAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCACTT
AACCCTAAA

Porcine circovirus 2 isolate THA_10SB01 capsid protein gene, complete cds

GenBank: JQ866918.1

ATGACGTATCCAAGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTAT
CAAGCGAACCACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATAT
TAATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGT
GACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCA
CAGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCC
CTTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATT
ACTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAA
TGTAGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATA
CAATATCCGTGTAACCATGTACGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCAC
TTAACCCTTAA

Porcine circovirus 2 isolate THA_10SB02 capsid protein gene, complete cds

GenBank: JQ866919.1

ATGACGAAATCAGGGAGGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCT
TGGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGTTACCGCT
GGAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTAT
CAAGCGAACCACAGTCAAAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATAT
TAATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATAC
TACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGT
GACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCA
CAGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCC
CTTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATT
ACTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAA
TGTAGACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATA
CAATATCCGTGTAACCATGTACGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCAC
TTAACCCCTAA

Porcine circovirus 2 isolate THA_10AY01 capsid protein gene, complete cds

GenBank: JQ866920.1

ATGACGTATCCAAGAGGCGTTACCGCAAGAAGAAGACACCGCCCCCGCAGCCATCTT
GGCCAGATCCTCCGCCGCCGCCCCTGGCTCGTCCACCCACGCCACCGTTACCGCTG
GAGAAGGAAAAATGGCATCTTCAACACCCGCCTCTCCCGCACCTTCGGATATACTATC
AAGCGAACCACAGTCAGAACGCCCTCCTGGGCGGTGGACATGATGAGATTCAATATT
AATGACTTTCTTCCCCCAGGAGGGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACT
ACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCGATCACCCAGGGTG
ACAGGGGAGTGGGCTCCAGTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCAC
AGCCCTCACCTATGACCCCTATGTAAACTACTCCTCCCGCCATACCATAACCCAGCCC
TTCTCCTACCACTCCCGCTACTTTACCCCCAAACCTGTCCTAGATTCCACTATTGATTA
CTTCCAACCAAACAACAAAAGAAATCAGCTGTGGCTGAGACTACAAACTGCTGGAAAT
GTATACCACGTAGGCCTCGGCACTGCGTTCGAAAACAGTATATACGACCAGGAATAC
AATATCCGTGCAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCACT
TAACCCTTAA

APPENDIX D

Protocol for PCV2 isolation in porcine kidney-15 (PK-15) cells

Preparation of PK-15 cells for PCV2 isolation

1. PK-15 cells - free of PCV1 were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics containing penicillin (100 U/ml) and streptomycin (100 mg/ml) in 25 cm2 flasks, then the culture flash was incubated at 37°C in 5% CO2 atmosphere for 48 hours.
2. For sub-cultivation, after remove MEM and following 3 washed with PBS , a monolayer - confluent of PK-15 cells were digested by trypsin-versene (TV) at 37°C for 10 min. Then 3 ml of MEM with 10% FBS were added following flushed a monolayer of PK-15 cell into a single cell.
3. Each ml of suspended PK-15 cells was added to another 25 cm^2 flasks , then 10 ml MEM with 10% FBS and antibiotics were added and then incubated for a further 48 hours.
4. PK-15 cells were passaged every 2 days for further use.

PCV2 isolation by co-cultivation method (adapted from Morozov et al., 1998)

1. Tissues from lung and lymph node of the pig with PCVAD (positive from PCR screening test) were pooled and homogenized in MEM containing penicillin (100 U/ml) and streptomycin (100 mg/ml) to make an approximately 10% suspension.
2. Serum or tissue homogenized sample were filtered through a 0.22 mm-pore-size filter and used as an inoculum for PCV2 isolation.
3. Two 25-cm^2 flasks of suspended PK-15 cells in 10 ml MEM with 5% FBS and antibiotics were inoculated with 1ml of inocula and incubated at 37°C in 5%CO2 atmosphere for 24 hours for virus adsorption.
4. Following incubation, a semi-confluent PK-15 cells were treated with 1 µl of 300 mM D-glucosamine for 30 min and then incubated for a further 48 hours until cells were 100% confluences.
5. One flask was used to confirm the PCV2 isolation by IPMA assay.
6. Another flask was passaged into fresh 25-cm^2 flasks for further propagation by freezing and thawing in - 80^o C three times.
7. PK-15-PCV2 infected cells were sub-cultivation and passaged every 2 days and treated with D-glucosamine 24 h after each passage.
8. The propagated PCV2 virus was stored at - 80^o C for viral titration and further used.

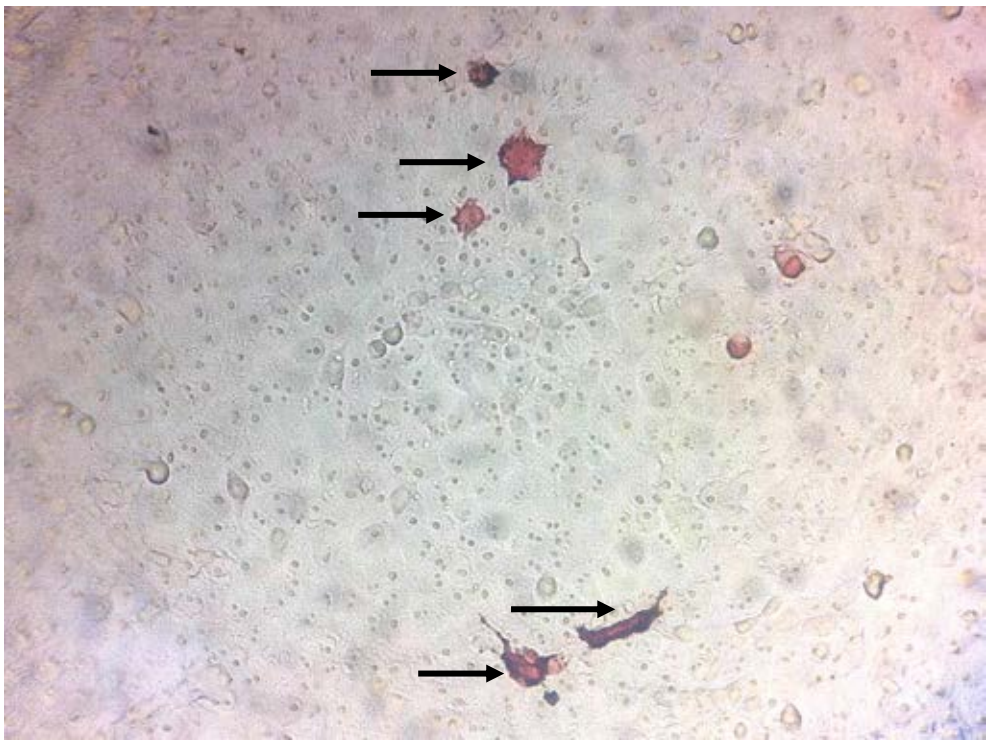

Figure D.1: Positive result from Immunoperoxidase monolayer assay (IPMA). Positive PK-15 cells (arrows); brown color in cytoplasm of PCV2-infected PK-15 cells were observed.

APPENDIX E

Preliminary study: Vaccine trial in a PCV2-free swine herd

This preliminary study was performed to evaluate the optimal dose of the vaccine in pigs free from PCV2 infection.

Herd status before animal experiment:

A randomized serum samples of selected PCV2-free swine herd (sow, n = 20; nursery, n = 40; finisher, n = 40) were tested by an indirect ELISA and PCR against PCV2 DNA (5-sample–pooled sera) to confirm the negativity of the herd in the one-month-period prior to the first vaccination.

Experimental pigs:

Sixteen-weaned, 4-week-old pigs were randomly allocated into 4 groups and were housed in small pens in the same building. Dose of vaccine in this experiment was modified from the results of our previous study (Jittimanee et al., 2010) as shown in table E.1. All groups were receiving two shot vaccinations within 2 weeks interval.

Table E.1: Detail of concentrations dose of vaccine and number of vaccination among experimental group.

Group	(n)	concentrations of protein/ dose (μg)	Number of vaccination
A	4	100	2
B	4	50	2
C	4	25	2
D	4	Adjuvant	2

Serological examination:

The sera were collected on day 0 before the vaccination (3 weeks of age) as the baseline and at 1, 2, 4, 6, 8 and 12 weeks after first vaccination (wpv) for serological examination. Antibodies against recombinant NLS truncated capsid (rntCap) protein was detected using an indirect ELISA.

Statistical analysis:

One-way ANOVA and the Tukey's test were used for mean S/P ratio comparison of the normally distributed variables between experiment groups.

Results:

No significantly different of mean S/P ratio among 4 groups was observed at 0, 1, 2 and 8 WPV as shown in figure E.1. However, all experimental groups (A, B and C) revealed significantly higher of mean S/P ratio than the control group D at 4 WPV ($p<0.05$). Nevertheless, at 6 WPV two experimental groups (A and B) showed significantly different from the experimental group C and the control group D ($p<0.05$). These results indicated that a subunit vaccine containing 50 and 100 µg of PCV2 rntCap protein could induce the specific antibodies higher than that of 25 µg, but there was no difference between 50 and 100 µg dose of vaccine.

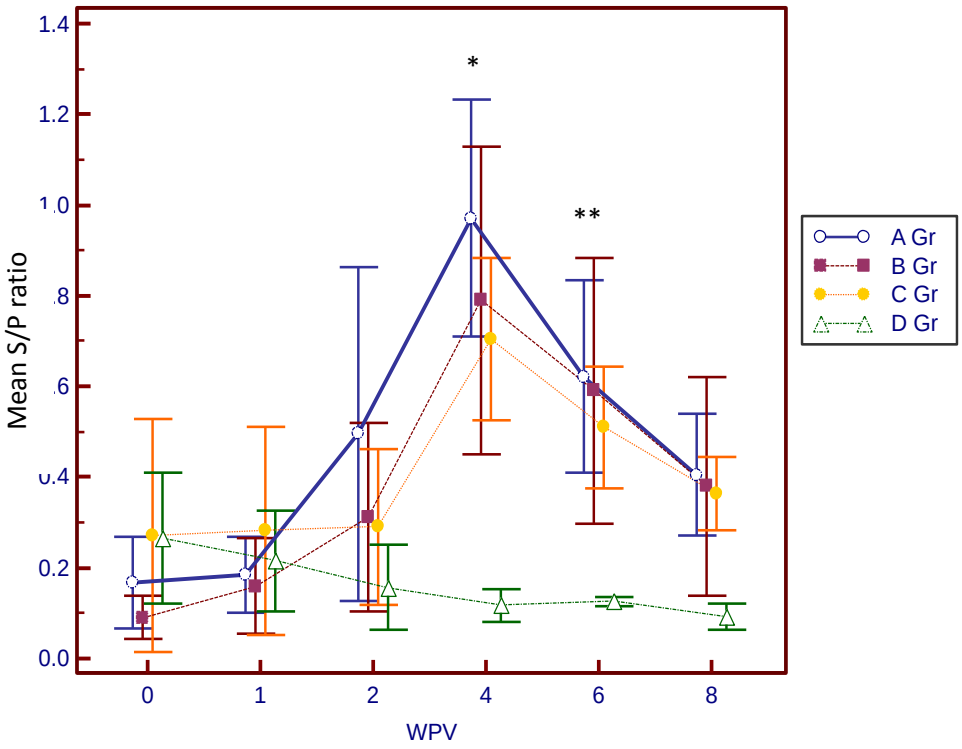

Figure E.1: Mean S/P ratio obtained from 4 groups on different trial weeks (0, 1, 2, 4, 6, 8 and 12 wpv). Group A, B and C were received 100, 50 and 25 µg of rntCap protein per dose, respectively and group C was received only adjuvant. Asterisks (*, **) indicating significant differences ($p < 0.05$).

BIOGRAPHY

AUTHOR Miss Suphattra Jittimanee

DATE OF BIRTH 18 August 1980

PLACE OF BIRTH Nakhon Ratchasima, Thailand

EDUCATIONAL CREDENTIALS

Faculty of Veterinary Medicine, Khon Kaen University (second class honors), 1998-2004

Doctor of Veterinary Medicine

Faculty of Veterinary Sciences, Chulalongkorn University, 2008-2013

Doctor of Philosophy

SCHOLARSHIP

The Royal Golden Jubilee Ph.D. Program (RGJ) from the Thailand Research Fund (TRF) and Chulalongkorn University (PHD/0252/2550)

ACADEMIC PUBLICATION

Jittimanee S, Nuntawan Na Ayudhya S, Kedkovid R, Teankum K, Suradhat S and Thanawongnuwech R. 2012. An indirect enzyme-linked immunosorbent assay using a recombinant truncated capsid protein of Porcine circovirus-2. J Vet Diagn Invest. 24(6): 1129-1132.

Jittimanee S, Nuntawan Na Ayudhya S, Teankum K, Kedkovid R and Thanawongnuwech R. 2011. Genetic Characterization and Phylogenetic Analysis of Porcine Circovirus Type 2 in Thai Pigs with Porcine Circovirus Associated Diseases (PCVAD) during 2007-2010. Thai J Vet Med. 41(2): 163-169.

CURRENT WORK POSITION AND OFFICE

A faculty member in Department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University.