การกระจายตัวของแอนติเจนของเชื้อเซอร์โคไวรัสชนิดที่ 2 และพยาธิสภาพในอัณฑะ เซมินอลเวสสิเคิล และหนังหุ้มลึงค์ ของพ่อสุกรคัดทิ้ง

นางสาวณัฐฐ์ธวรรณ โสภิพันธ์

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Distribution of porcine circovirus type 2 (PCV2) antigens and pathological changes in testes, seminal vesicles, and prepuce of culled boars

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
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ณัฐฐ์ธวรรณ โสภิพันธ์ : การกระจายตัวของแอนติเจนของเชื้อเซอร์โคไวรัสชนิดที่ 2 และพยาธิสภาพในอัณฑะเซมินอลเวสสิเคิล และหนังหุ้มลึงค์ ของพ่อสุกรคัดทิ้ง (Distribution of porcine circovirus type 2 (PCV2) antigens and pathological changes in testes, seminal vesicles, and prepuce of culled boars) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. คมกฤช เทียนคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สพ.ญ ดร. สุภัทตรา จิตติมณี{, 53 หน้า.

เซอร์โคไวรัสชนิดที่ 2 ในสุกรเป็นไวรัสที่ทำให้เกิดโรค porcine circovirus associated disease (PCVAD) ปฏิสัมพันธ์ระหว่างเซอร์โคไวรัสชนิดที่ 2 และอวัยวะระบบสืบพันธุ์ของพ่อสุกรเป็นสิ่งที่น่าสนใจเป็นอย่างมาก เนื่องจากสามารถตรวจพบไวรัสได้ในน้ำเชื้อของพ่อสุกร และไวรัสอาจใช้อวัยวะระบบสืบพันธุ์ของพ่อสุกรเป็นแหล่งรังโรคได้อีกด้วย นอกจากนี้ยังไม่เป็นที่แน่ชัดว่าเซอร์โคไวรัสชนิดที่ 2 สามารถก่อให้เกิดพยาธิสภาพในอวัยวะระบบสืบพันธุ์ของพ่อสุกรได้หรือไม่ การศึกษาในครั้งนี้เป็นการศึกษาอุบัติการณ์ของเซอร์โคไวรัสชนิดที่ 2 ในอวัยวะระบบสืบพันธุ์ของพ่อสุกร และการกระจายตัวของแอนติเจนดังกล่าวด้วย ตัวอย่างที่ใช้ในการศึกษา ได้แก่ อัณฑะ ต่อมเซมินอลเวสสิเคิล และหนังหุ้มลึงค์ จากพ่อสุกรคัดทิ้งจำนวน 59 ตัว จากการศึกษาพบสารพันธุกรรมของเซอร์โคไวรัสชนิดที่ 2 ในระบบสืบพันธุ์ของสุกรจำนวน 13 ตัว การศึกษาการกระจายตัวของแอนติเจนพบว่าอัณฑะมีอัตราการตรวจพบสูงสุด (ร้อยละ 76.9) หนังหุ้มลึงค์มีอัตราการตรวจพบในลำดับถัดมา (ร้อยละ 38.5) และต่อมเซมินอลเวสสิเคิลมีอัตราการตรวจพบต่ำสุด (ร้อยละ 30.8) โดยที่อัตราการตรวจพบในอัณฑะสูงกว่าในต่อมเซมินอลเวสสิเคิลอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เซลล์ที่ให้ผลบวกในอัณฑะ ได้แก่ เซลล์เลย์ดิก (Leydig cell) เซลล์เซอร์โทไล (Sertoli cell) และเซลล์สืบพันธุ์ อย่างไรก็ตามไม่พบความสัมพันธ์ระหว่างอัตราการตรวจพบแอนติเจนในอัณฑะและการเกิดการเสื่อมของอัณฑะ เซลล์ที่ให้ผลบวกในต่อมเซมินอลเวสสิเคิลคือเซลล์เยื่อบุผิวของต่อม ในขณะที่เซลล์ที่ให้ผลบวกในหนังหุ้มลึงค์คือลิมโฟไซต์ภายในเนื้อเยื่อน้ำเหลืองบริเวณเยื่อเมือก โดยสามารถพบภายในเนื้อเยื่อน้ำเหลืองที่เกิดพยาธิสภาพด้วย การศึกษานี้เป็นครั้งแรกที่ตรวจพบแอนติเจนของเซอร์โคไวรัสชนิดที่ 2 ในหนังหุ้มลึงค์ของพ่อสุกร การศึกษาในครั้งนี้แสดงถึงข้อมูลด้านการกระจายตัวของแอนติเจนของเซอร์โคไวรัสชนิดที่ 2 ในอวัยวะระบบสืบพันธุ์ของพ่อสุกร ซึ่งบ่งชี้ถึงความสำคัญของอัณฑะ ต่อมเซมินอลเวสสิเคิล และหนังหุ้มลึงค์ ต่อการแพร่กระจายเชื้อไวรัสทางน้ำเชื้อ

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สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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NATTHAWAN SOPIPAN: Distribution of porcine circovirus type 2 (PCV2) antigens and pathological changes in testes, seminal vesicles, and prepuce of culled boars. ADVISOR: ASST. PROF. DR. KOMKRICH TEANKUM, CO-ADVISOR: DR. SUPHATTRA JITTIMANEE{, pp.

Porcine circovirus type 2 (PCV2) is a swine pathogen causing porcine circovirus associated disease (PCVAD). The interaction between PCV2 and boar's reproductive organs is of interest. It has been shown that PCV2 could be shedded via semen and the virus might use the boar's reproductive organs as reservoir. Moreover, PCV2 could induce in reproductive organ is not known. In the present study, we determined the prevalence of PCV2 in boar's reproductive organs. The antigen distribution in boar's reproductive organs was also characterized. Testes, seminal vesicles, and prepuces were collected from 59 culled. Thirteen boars contained PCV2 DNA in the reproductive organs. PCV2 antigen distribution study revealed that the prevalence of PCV2 antigen was highest in the testis (76.9%), followed by the prepuce (38.5%), and then the seminal vesicle (30.8%). The testis showed significantly higher prevalence than the seminal vesicle ($p < 0.05$). Various cell types, including Leydig cells, Sertoli cells, and germinal epithelial cells, showed positive signal of PCV2 antigen. However, these findings were not related with testicular degeneration. In the seminal vesicles, the antigen was found only in the glandular epithelial cells. In the prepuces, the antigen was detected in lymphocytes in the mucosal lymphoid follicles with lymphoid depletion. This is the first time that PCV2 antigen was identified in boar's prepuces. Our study provided knowledge of PCV2 antigen distribution in boar's reproductive organs indicating an impact of these organs for PCV2 spreading via semen.

Department:	Veterinary Pathology	Student's Signature .....
Field of Study:	Veterinary Pathobiology	Advisor's Signature .....
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ABC	=	Avidin-biotin complex
ADV	=	Aujeszky's disease virus
AI	=	Artificial insemination
cap	=	capsid
CSFV	=	Classical swine fever virus
CU-VDL	=	Veterinary Diagnostic Laboratory of Chulalongkorn University
DAB	=	3, 3'-Diaminobenzidine
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
dpi	=	Day post infection
FMDV	=	Foot-and-mouth disease virus
H&E	=	Hematoxylin and eosin
HIV	=	Human immunodeficiency virus
IHC	=	Immunohistochemistry
IR	=	Intergenic region
ISH	=	*In situ* hybridization
ISRE	=	Interferon-stimulated response element
JEV	=	Japanese encephalitis virus
LN-DNA-neg	=	PCV2-DNA negative in the lymph node
LN-DNA-pos	=	PCV2-DNA positive in the lymph node
ODN	=	Oligodeoxynucleotide
ORF	=	Open reading frame
PCR	=	Polymerase-chain reaction
PCV	=	Porcine circovirus

PCV2-ED	=	PCV2 enteric disease
PCV2-LD	=	PCV2 lung disease
PCV2-RD	=	PCV2 reproductive disease
PCV2-SD	=	PCV2 systemic disease
PCVAD	=	Porcine circovirus associated disease
PCVD	=	Porcine circovirus disease
pDC	=	Plasmacytoid DC
PDNS	=	Porcine dermatitis and nephropathy syndrome
PEV	=	Porcine enterovirus
PMWS	=	Postweaning multisystemic wasting syndrome
PPV	=	Porcine parvovirus
PRDC	=	Porcine respiratory disease complex
PRRSV	=	Porcine reproductive and respiratory syndrome virus
rep	=	Replicase
Rep-DNA-neg	=	PCV2-DNA negative in the reproductive organs
Rep-DNA-pos	=	PCV2-DNA positive in the reproductive organs
SL	=	Stem-loop
SVDV	=	Swine vesicular disease virus
$TCID_{50}$	=	Tissue culture infective dose

CHAPTER 1

1.1 Introduction

Porcine circovirus type 2 (PCV2) is a single stranded DNA virus belongs to the family *Circoviridae* and genus *Circovirus*. There are two genotypes of porcine circovirus; PCV1 and PCV2. PCV1 is non-pathogenic while PCV2 is pathogenic. Target cells of PCV2 are lymphocytes and mononuclear phagocytes. Symptoms caused by PCV2 infection are called porcine circovirus associated disease (PCVAD) or porcine circovirus disease (PCVD).

At present, virus spreading via semen is of major interest, since artificial insemination plays a major role in swine production. It has been demonstrated that PCV2 could be transferred via artificial insemination (Allan et al., 2007; Schmoll et al., 2008) and it has been shown that PCV2 infection via intrauterine route can result in infection of both the inseminated sows and their piglets (Madson et al., 2009a), resulting in reproductive failure and mummified fetus. Recently, it has been revealed that PCV2 shedding in semen could be significantly reduced using the commercial PCV2 vaccine (Seo et al., 2013). However, vaccination could not completely eliminate the virus shedding. One possibility explaining the ongoing semen shedding is that, after reaching the reproductive organ via viremia, PCV2 might still remain at the site with the presence of vaccine-induced anti-PCV2 antibody.

Other than lymphocytes and macrophages, PCV2 can be identified in other cell types. By immunohistochemistry (IHC) technique, PCV2 antigen can be demonstrated in testicular germinal epithelial cells in a 2-day-old-piglet model (Kennedy et al., 2000), in testicular germinal epithelial cells and fibroblast-like cells in the seminal vesicle of a mature boar (Opriessnig et al., 2006b). Furthermore, PCV2 DNA can be detected in testis and semen using nested and real-time PCR (Ritterbusch et al., 2012). Thus, boar's reproductive organs especially the testis might play an important role in viral spreading.

Preputial mucosa generally contains mucosal lymphoid tissues which normally appeared as organized lymphoid follicles, playing an important role of

mucosal defense mechanism. Thus, it is another interesting tissue in PCV2 antigen distribution study. As in case of human patient immunodeficiency virus (HIV) is hypothesized to infect human foreskin by targeting local lymphocytes (Hirbod et al., 2010).

Although PCV2 antigen was previously identified in the testis and seminal vesicle of a mature boar, it is interesting that whether variation of antigen distribution in boar reproductive tract could be observed or not. In this study, we determine PCV2 antigen distribution as well as related pathological findings in testis, seminal vesicle, and prepuce of culled boars.

In our study, boar's reproductive tissues (testis, seminal vesicle and prepuce) and superficial inguinal lymph node of 59 culled boars were collected from slaughter houses in the western region of Thailand, during 2007 – 2014. The prevalence of PCV2 carrying culled boars was done using polymerase-chain reaction (PCR) and immunohistochemistry (IHC). Cells with positive signal for IHC were then described. Pathological lesions of the reproductive organs from PCV2 positive and PCV2 negative boars were compared.

1.2 Objectives

1. To determine the prevalence of PCV2 DNA in testes, seminal vesicles and prepuces of culled boars by PCR technique
2. To describe PCV2 antigen distribution in testes, seminal vesicles and prepuces of culled boars by Immunohistochemistry
3. To describe related pathological changes of reproductive organs of PCV2-infected boars

CHAPTER 2

2.1 Literature review

2.1.1 Virology

PCV belongs to the family *Circoviridae* genus *Circovirus*. The virus is non-enveloped with circular single stranded DNA genome of 1.7 kb. PCV can be divided into two genotypes, PCV type 1 (PCV1) and PCV type 2 (PCV2). PCV1 is non-pathogenic in swine, whereas PCV2 can induce many symptoms in swine; postweaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC) and reproductive failure. Nowadays, pathogenesis of PCVADs has not yet been completely clarified. However, interferences with swine immune system could be one of the major problems caused by PCV2 infection (Darwich and Mateu, 2012).

PCV2 genome is circular, single-stranded DNA of 1.7 kb. The genome contains at least 4 open reading frames (ORFs), including ORF1 (replicase gene, *rep*), ORF2 (capsid gene, *cap*), ORF3 and ORF4 (Lv et al., 2014) (Fig 1). ORF1 (945 nt) encodes protein associated mainly with viral replication. ORF1 also contains interferon-stimulated response element (ISRE)-like sequences in the promoter, which plays a role in viral transcription (Gu et al., 2012). ORF2 (702 bp) encodes capsid protein which is a major structural protein of PCV2, and involves mainly in viral entry. ORF3 (315 bp), according to the ability to induce apoptosis of its encoding protein, was thought to play a role in PCV2 pathogenesis and virulence. However, the exact role of ORF3 encoding protein is still elusive. ORF4 (180 bp) locates within ORF3. It is still under debate whether ORF4 is expressed during PCV2 infection or not. However, it has been shown that ORF4 protein showed apoptosis suppressing activity.

PCV2 can be genetically divided into 5 genotypes, PCV2a – e. In general, PCV2a and PCV2b are considered to be the major causes of PCVADs worldwide. PCV2c has been reported only from Denmark. PCV2d and PCV2e are the two newest

discovered genotypes found in China. To date, the relationship between genetic variation and virulence could not be demonstrated.

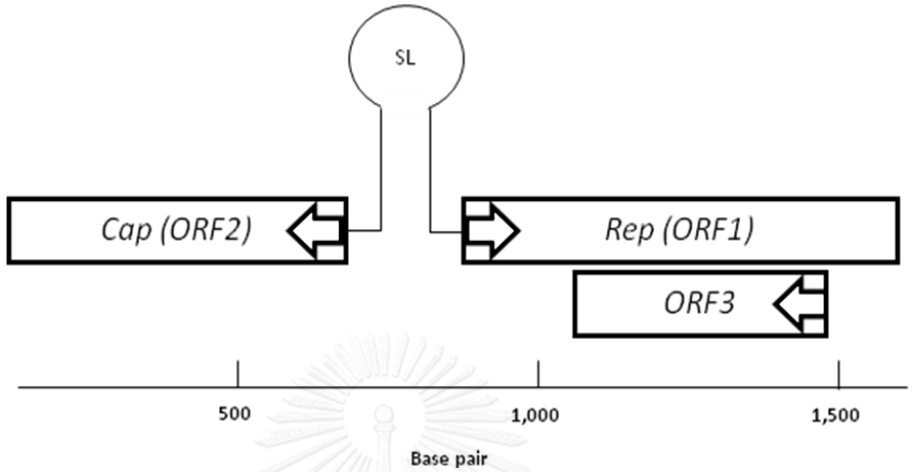

Figure 1. Genome organization of PCV2. Relative position of ORF1-3 is illustrated. SL, stem-loop; IR, intergenic region.

2.1.2 Epidemiology

2.1.2.1 General epidemiology

The epidemiology of PCV2 showed some interesting aspects. The virus showed certain degree of genetic variation. Presently, distribution patterns of PCV2 genotypes were observed in different location. Interestingly, genotype shift of the virus were shown worldwide.

Among PCV2a-e, PCV2a and PCV2b are the major genotypes. Recently, PCV2b was shown to predominate over PCV2a in the swine industry, especially in North America and Europe. Studies from many countries showed that PCV2b was presented in a high frequency. For example, a study in Canada (during 2005 – 2006) showed that PCV2b was identified in 79.5% (66/83) of PCV2 isolates (Gagnon et al., 2007).

The wide spread of PCV2b was from genotype shift of the virus. Studies from many countries showed that PCV2a dominated in the past. Thereafter, PCV2b was predominates (Olvera et al., 2007). The time point of the shifting could be varied in

different countries. Globally, the shifting was hypothesized to occur in or prior to 2003 (Dupont et al., 2008). Data on genetic variation also support the later occurrence of PCV2b since PCV2b, which showed a lesser degree of nucleotide variability (Grau-Roma et al., 2008).

2.1.2.2 PCV2 in Thailand

In Thailand, the first case of PCVAD was reported in 1998 from Nakorn Pathom province, where nursery pigs at approximately 8 weeks old were shown to be affected by PMWS (Tantilertcharoen et al., 1999). However, PCV2 identification could be dated back to 1993, PCV2 DNA was detected by nested PCR from the formalin-fixed, paraffin-embedded tissue of pig (Kiatipattanasakul-Banlunara et al., 2002). Since then PCV2 has been circulated in Thailand.

Recent data from the Veterinary Diagnostic Laboratory Chulalongkorn University (Nakorn Pathom) during 2013 - 2014 showed an increasing ratio of PCV2 infection. During January to September in 2013, PCV2 infection was identified by pathological examination and viral DNA identification using PCR in 22.4% (28/127) of the necropsy cases. The percentage was increased to 47.52% (48/101) during the same period in 2014. Moreover, 73.73% (73/99) of the directly submitted sera and tissue samples was positive for PCV2 DNA during January to September in 2014. These data indicate that the prevalence of PCV2 infection in Thai swine industry. Therefore, studies involving in viral transmission are necessary.

2.1.2.3 Transmission

PCV2 gain entry into pigs mainly via oronasal route and the infected pigs can shed the virus via many routes. PCV2 could be shedded in nasal fluid, saliva, feces and semen. Previous studies showed that PCV2 infected pigs show viremia up to 9 days post infection (dpi). Interestingly, the virus could shed in feces, semen and oronasal fluid for a long period approximately 70 dpi (Caprioli et al., 2006; Shibata et al., 2003)

For horizontal transmission of PCV2, quantitative information has already been described. Serial transmission experiments (Andraud et al., 2009) using direct contact model showed that the mean disease generation time was 18.4 days. Pigs at 39 dpi could still be able to transmit the virus. However after 55 dpi, even in the presence of viremia, the probability of transmission was minor.

Vertical transmission such as transplacental transmission from viremic sow could be demonstrated (Ha et al., 2008). A previous study showed that 32.3% of viremic new-born piglets were seronegative, indicating that the infection could occur prior to the immune competence development (Shen et al., 2010). Fetal infection could result in abortion. During fetal development, cardiomyocytes were the main target cells and myocarditis in fetus was observed (Sanchez et al., 2003).

2.1.3 Pathology

Although most PCV2 infections are asymptomatic, however in some cases pathological changes can be found (Yu et al., 2007). Major lesions of PCVAD are characterized by lymphoid depletion and histiocytic infiltration of lymph nodes or lymphoid follicles. At the present, PCV2 was thought to cause immunosuppression rendering the pigs susceptible to PCVAD development. Thus, other factors other than PCV2 itself are required for clinical signs to occur. The mechanism of immunosuppression by PCV2 is not fully understood. However, impaired or altered functions of PCV2-infected cells might play a part in this phenomenon.

Recently, PCV2 infection could be characterized into two major types, subclinical infection, and clinical infection (Segales, 2012). It is generally considered that subclinical infection is the major form of PCV2 infection. For clinical infection, at least 5 forms of disease were suggested based on types of affected tissue/organ, including PCV2 systemic disease (PCV2-SD), PCV2 lung disease (PCV2-LD), PCV2 enteric disease (PCV2-ED), PCV2 reproductive disease (PCV2-RD) and porcine dermatitis and nephropathy syndrome (PDNS).

2.1.3.1 Pathogenesis

PCVAD could be ranged from specific organ involvement or systemic disease. Naturally, PCV2 might enter the pigs mainly via oronasal exposure. After that, virus could spread to other sites via viremia, using both cell-free and cell-associated mechanism. The major target of PCV2 replication sites are lymphoid tissues. Subsequently, lymphoid depletion and histiocytic replacement of the lymphoid tissues were occurred as a hallmark lesion. In general, the spreading pattern and the location of affected lymphoid follicles are in part correlated with different outcomes of PCVADs. If lymphoid follicles of multiple tissues are involved, it could be resulted in PCV2-SD. However, if only lymphoid follicles of a single tissue are involved, the diseases might be of specific organ.

During viremia, PCV2 could also replicate in the PBMC especially in B and T lymphocytes and monocytes. However, B and T lymphocyte of lymphoid follicles were shown to be the major targets (Yu et al., 2007). It has also suggested that PCV2 use conventional dendritic cells (DC) to facilitate virus transmission as well, although DC functions were not altered by the infection (Vincent et al., 2003). It is interesting that PCV2 could use these migrating immune cells to evade the antiviral immunity and to spread at the same time.

Hallmark lesion of PCVAD is lymphoid depletion and histiocytic replacement of the lymphoid tissues leading to immunosupression. However the mechanism of the lymphoid depletion is poorly understood. It could be that lymphoid depletion was a result of PCV2-induced apoptosis since lymphoid depletion in PCVAD was shown to be linked with PCV2 replication and PCV2-induced apoptosis of lymphocytes could be observed (Meng, 2013).

PCV2 infection of the immune cells was thought to be a major factor leading to immunosuppression that rendering pigs to secondary infection. Though, it is clear that the virus replicates in B and T lymphocytes and mononuclear phagocytes,

infection in certain population of the mononuclear phagocytes and dendritic cells (DCs) is considered to play a pivotal role in PCV2 infection and PCVAD development.

Since DCs are very important immune cells linking innate and adaptive immune responses and the fact that PCV2 could infect DCs, interfering DC functions by PCV2 is of major interest. It has been shown that PCV2 could infect DCs without viral replication. At least from *in vitro* studies, PCV2 could alter DC functions (Balmelli et al., 2011; Baumann et al., 2013). Not only that, PCV2 through a yet unknown mechanism, could also inhibit cytokine production of plasmacytoid DC (pDC) an important type I interferon producer (Balmelli et al., 2011; Vincent et al., 2007).

Recently, information on the interaction between PCV2 components and host molecules was advancing. The expression of host molecules involving in innate immune defense, immunosuppression, proinflammatory signal and fasting process were shown to alter in PCV2 infected pigs. Both DNA and protein of PCV2 were capable of interacting with host molecules. However, the outcome of the interaction is still unclear. It has been shown that PCV2 genome contains oligodeoxynucleotide (ODN) containing CpG motifs and interferon-stimulated response element (ISRE) sequences. These 2 DNA fragments might be involved in modulating cytokines production and interferon-mediated enhancement of PCV2 replication, respectively (Hasslung et al., 2003; Mankertz and Hillenbrand, 2002; Ramamoorthy et al., 2009; Ramamoorthy et al., 2011; Wikstrom et al., 2007). Viral capsid protein is mainly linked with PCV2 entry by interacting with viral receptor. Replicase is mainly involved in virus replication. Interestingly, capsid, replicase and ORF3 protein were all shown to interact with host protein involving in apoptosis. Further study should be done to clarify the mechanism of the interaction between PCV2 and host molecules.

2.1.3.2 Factors promoting PCVAD

PCV2 infection alone might not be enough for PVCAD development. There are 4 major factors influencing the disease outcome including host, husbandry, co-infection status and genetic of PCV2. For host factor, most of the studies are mainly

focusing on both pig genetics and the immune status of the infected pigs. Although the effect of pig breeds on susceptibility is still controversial, partly due to the multi-factorial nature of PCVADs, it has been shown that Landrace was more susceptible than Pietrain, Duroc, and Large White (Opriessnig et al., 2006a; Opriessnig et al., 2009).

For immune status, the effect of immunostimulation on increasing PCV2 infection is an interesting issue. However, solid evidence supporting this theory is still lacking. It was thought that certain types of immunostimulation that promote lymphocyte replication, which is one of the PCV2 target cells, might enhance the PCV2 replication rate (Allan et al., 2007; Ha et al., 2009; Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). From *in vitro* studies, it was shown that PCV2 could replicate more in activated T lymphocytes, using Concanavalin A stimulation (Yu et al., 2007). It has been suggested that the enhanced PCV2 replication was not due to an increasing cellular replication, since PCV2 replication was also elevated in the resting T lymphocytes (that was stimulated with Concanavalin A) (Yu et al., 2009).

Swine husbandry and co-infection status have also been linked with PCVAD. The housing system, vaccination schedule, hygiene practices and biosecurity are of major concern (Dewey et al., 2006; Lopez-Soria et al., 2005; Rose et al., 2012; Woodbine et al., 2007). In addition, many swine pathogens were demonstrated to promote PCVAD, including PRRSV (Allan et al., 2000; Harms et al., 2001), *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004) and porcine parvovirus (Allan et al., 1999), etc.

2.1.3.3 Pathology of boar reproductive system

Testicular atrophy is one of the commonly observed pathological changes in culled boars (Teankum et al., 2008). In general, testicular atrophy is a result of germ cell degeneration. Aging, heat stress (Kanter et al., 2013), hormonal disturbance (Sofikitis et al., 2008), toxic substance (Boekelheide, 2005; Kim et al., 2003a) (e.g. zearalenone poisoning) and infection can lead to testicular degeneration and testicular atrophy. Chronic inflammation of the boar reproductive system is

commonly observed along with the testicular atrophy (Noguchi et al., 2013; Teankum et al., 2013).

Additionally, fibrosis is usually seen on cut-surface of the atrophic testes. Degeneration of seminiferous tubules with interstitial fibrosis were often observed by histology. Lymphocyte infiltration in the interstitial tissue was also seen (Teankum et al., 2013).

Various viruses were reported to infect boar reproductive tracts and caused many serious outcomes (Fig 2). Semen shedding, sows and fetal infection (from contaminated semen) and pathological changes in boar reproductive organs were shown in some swine viral models. Although many viruses can be shedded in semen, data on viral distribution in boar's reproductive organs remains elusive. Rubulavirus, classical swine fever virus, foot-and-mouth disease virus, Japanese encephalitis virus, porcine enterovirus, porcine parvovirus, porcine reproductive and respiratory syndrome virus, Aujeszky's disease virus and swine vesicular disease virus could be detected in semen (Maes et al., 2008).

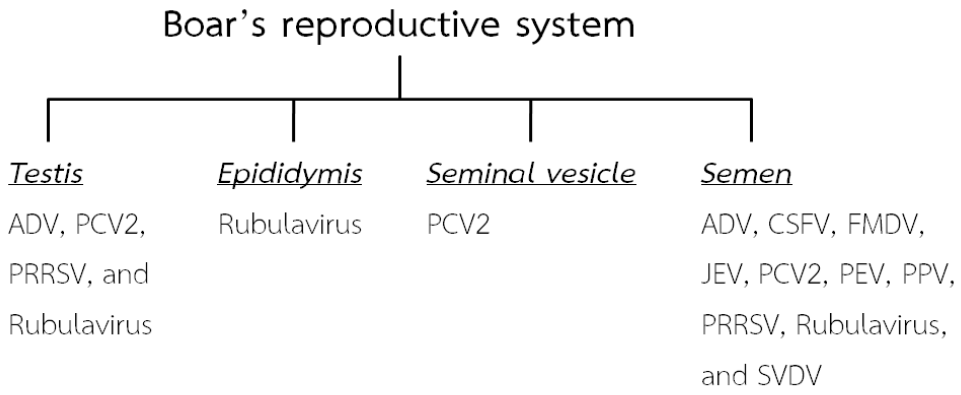

Figure 2. Swine virus reported in specific part of the boar's reproductive organ. PCV2; porcine circovirus type 2; ADV, Aujeszky's disease virus; PRRSV, porcine reproductive and respiratory syndrome virus; CSFV, classical swine fever virus; FMDV, foot-and-mouth disease virus; JEV, Japanese encephalitis virus; PEV, porcine enterovirus; PPV, porcine parvovirus; SVDV, swine vesicular disease virus (modified from Maes et al., 2008).

In a certain aspect, PRRSV could be a good model for PCV2, since both viruses could infect macrophages. Several studies on viral distribution centered on testis, since it has been linked with pathological changes of this area. It was thought that the virus could gain access to boar genital tract through viremia and virus dissemination within the genital tract could be macrophage-associated (Han et al., 2013; Sur et al., 1997).

PRRSV could be found in testis and epididymis of infected boars, in which macrophages and germ cells were the major positive cell types. An experiment performed by Sur and colleague (1997) using IHC and *in situ* hybridization (ISH) showed very interesting results. Pigs of 5-6 months old were inoculated with a virulent strain of PRRSV (12068-96). The pigs were euthanized and testis and epididymis were collected during 7 – 60 days post infection (dpi). IHC and ISH were

used to characterize the viral distribution. PRRSV could be readily detected in both testis and epididymis at 7 dpi. In testis, PRRSV was revealed in macrophages at the interstitium and in germ cells of the seminiferous tubules. In epididymis, the virus was mainly found in macrophages at the connective tissue between ducts.

Hypospermatogenesis after PRRSV infection was shown to be manifested by pathological changes of germ cells (e.g. desquamation and multinucleated giant cell formation). The complete absence of mature spermatids, and greatly reduced germ cells could be observed after PRRSV infection. Although the testicular lesion was highest at 35 DPI (the last necropsy date of the study), the lesion was readily detected as early as 14 DPI (the first necropsy date). Semen shedding of PRRSV is mainly cell-associated with sperms and macrophages as the major target cells. Moreover, PRRSV could also be detected in the connective tissue of epididymis, ductus deferens, and prostate gland.

2.1.4 PCV2 shedding in semen

It is clear that PCV2 can be shedded via semen. The virus could be detected in semen using both molecular technique (PCR) and virology technique (virus isolation) (Larochelle et al., 2000; Schmoll et al., 2008). Interestingly, PCV2 DNA could be found mainly in seminal fluid in the non-sperm cell fraction (Kim et al., 2003b). Shedding of PCV2 in the semen poses 2 major concerns, including 1) the spreading of the virus via artificial insemination (AI) and 2) the ability of boar's reproductive organs to serve as viral reservoirs.

PCV2 transmission via AI is an interesting issue. Many studies have been carried out in many other countries including Australia, Canada, Germany and Korea. For example; previous report from Austria/Germany showed that PCV2 was identified in semen of 18-30% of boars, using PCR (Schmoll et al., 2008). A study in Canada also showed that PCV2 DNA could be detected in semen of 30.2% of naturally infected boars. These reports highlight a considerably high prevalence of AI-mediated PCV2 transmission. PCV2 infection in sows by PCV2-containing semen could result in reproductive failure and fetus infection. The previous study in sows artificially

inseminated with PCV2-spiked semen showed viremia in sow last as long as 112 DPI. Mummified fetuses and stillborn piglets were observed from vertical transmission (Madson et al., 2009a). PCV2 antigen was detected in myocardium of mummified fetuses, stillborn piglets, and live born piglets. This study showed that the presence of PCV2 in semen could lead to both sow and fetus infection. Thus PCV2 shedding via boar semen is an important mode of viral transmission (Madson et al., 2009a).

Some studies indicated that PCV2 could use boar's reproductive organs as viral reservoirs. It has been shown that PCV2 shedding in the semen might occur after the viremia. Hence, boar reproductive organs might possibly be a reservoir. In one study, PCV2 was detected in semen as long as 27.3 weeks (McIntosh et al., 2006). According to the study in Austria and Germany (Schmoll et al., 2008), from 80 PCV2 semen-shedding boars, there were 58 boars that showed no detectable PCV2-specific nucleic acids in serum. Thus, boar's reproductive organ might serve as reservoir for PCV2. Further study should be done to clarify this hypothesis.

2.1.5 PCV2 infection in male genital tract

Previously, the studies on PCV2 distribution within tissues in boar genital tract, using staining methods (such as immunohischemistry and in situ hybridization) rather than PCR, were very limited. Nevertheless, detailed studies on boar genital tract pathology after PCV2 infection were also still limited.

In an early study suggesting an importance of PCV2 in male genital tract was done using PCV2-parvovirus coinfection model in 1-2 days old piglets (Kennedy et al., 2000). In the study, PCV2 antigen distribution was also done using immunohistochemistry (rabbit anti-PCV2 polyclonal antibody). After the co-infection, numerous amounts of PCV2 antigen were detected in cytoplasm and nuclei of both lymphoid and non-lymphoid tissues. In the lymphoid tissue, positive cells were mainly mononuclear cells. Lymphoid follicles of the ileal Peyer's patches were also contained positive macrophages. In testis, the majority of PCV2 antigen was detected in infiltrating macrophages in the tunica albuginia. To a lesser extent, the antigen was also found in germinal epithelial cells and infiltrating macrophages in the epididymis.

Noted that PCV2 antigen was also observed in a single PCV2 infection in this study, however in a much lesser degree. Histopathologically, mild to moderate granulomatous orchitis was observed. In this study, 1-2 days old piglets were used and PCV2 antigen distribution was done only in the testis. The question may arise, whether PCV2 infection could lead to pathological changes in other genital organs resulting in infertility of boars.

Another study on PCV2 antigen distribution using immunohistochemistry technique was done on the ill boar (Opriessnig et al., 2006c). An 11-month-old boar with a reduced sperm production and poor sperm motility over the 12-week period was examined. The affected boar had fever with labored breathing. Semen was positive for PCV2-specific nucleic acids. Upon necropsy, gross lesions were restricted to respiratory and genital tract. The lungs were failed to collapse and mottled-tan in color. Macroscopically, severe edema around the head and the body of the epididymides was seen. Interestingly, immunohistochemical staining against PCV2 antigen showed that bulbourethral glands, epididymides, testes, and seminal vesicles were positive, while prostate gland was negative. The staining was primarily detected in the cytoplasm of macrophages and fibroblast-like cells, especially in the interstitium of accessory sex glands and the seminal vesicles. This study provided more information on the antigen distribution and histopathology in mature boar genital tract. However, the information was obtained using only one boar, thus variation in the antigen distribution could not be inferred.

CHAPTER 3

3.1 Materials and methods

3.1.1 Boars and sample collection

Reproductive organs and superficial inguinal lymph nodes of culled boars (n = 59) were collected during 2007 - 2014 from slaughter houses in the western region of Thailand. Each tissue type was collected as both fresh frozen tissue and formalin-fixed tissues (10% neutral buffered formalin) and then submitted to the Department of Veterinary Pathology, Chulalongkorn University, Thailand. According to standard methods, the formalin-fixed tissues were paraffin-embedded and cut into 4 μm sections.

Pathological findings of boar reproductive organs were observed and recorded. The frequency of the testicular degeneration, testicular fibrosis, and the enlargement of spermatic cord lymph node were analyzed. Severity of testicular degeneration and testicular fibrosis were scored (+1: mild, +2: moderate, and +3: severe) (Teankum et al., 2013). Enlargement of spermatic cord lymph node was also recorded. For histopathology, tissue sections were stained with hematoxylin and eosin (H&E). Microscopic findings were examined under a light microscope.

3.1.2 Experimental design

All frozen superficial inguinal lymph nodes were screened for PCV2 DNA using polymerase chain reaction (PCR). At this step, boars with positive result were designated as “LN-DNA-pos” boars. Boars with negative result were designated as “LN-DNA-neg” boars. The reproductive tissues from the LN-DNA-pos boars were then further screened for PCV2 DNA using PCR. Boars with positive result (in at least one reproductive organ) were designated as “Rep-DNA-pos” boars. Boars with negative result (in every organ) were designated as “Rep-DNA-neg” boars. IHC studies were done in all reproductive organs from Rep-DNA-pos boars. Pathological studies were

done in all 59 boars. Positive and negative controls for IHC studies were kindly provided by the Department of Veterinary Pathology, Chulalongkorn University.

3.1.3 DNA extraction and PCR

PCV2 DNA extraction was done from frozen tissues. Commercially available silica membrane-based extraction kit (NucleoSpin®, Machery-Nagel, Germany) was used. PCR protocol was derived from the standard protocol of the Veterinary Diagnostic Laboratory, Chulalongkorn University (CU-VDL). PCR mixture (25-µl reaction) were as follow; 1) 1x Go taq®green master mix, 2) PCV2-specific primers 0.5 µM [forward primer 5'-ATGCCCAGCAAGAAGAATGGAAGAAG-3' and reverse primer 5'-AGGTCACTCCGTTGTCCTTG AAGTC - 3'] and 3) DNA template (5 µl). The final volume of the mixture was adjusted using nuclease-free water. For temperature and duration of incubation in PCR reaction, the reaction begins with the initial denaturation step at 95°C for 2 min. Then, 35 rounds of DNA amplification stage were performed [95°C for 30 s, 55°C for 30 s, and, 72°C for 30 s]. Final extension step was done at 75°C for 5 min. Agarose gel electrophoresis was done to determine the size of PCR product. The amplicon size is 356 base pair.

3.1.4 Pathological study

Histopathological lesion evaluations of testes and seminal vesicle were done as previously described (Teankum et al., 2013) with minor changes. Briefly, the presence of testicular degeneration, testicular fibrosis, and seminal vesiculitis were observed. Severity of the lesions were scored (+1: mild, +2: moderate, and +3: severe). For prepuces, the evaluation of lymphoid depletion was modified from the previous study (Opriessnig et al., 2004). The presence of lymphoid depletion of the mucosal lymphoid follicles was evaluated. Severity of the lesions were scored (+1: mild, +2: moderate, and +3: severe). The presence of each specific lesion was scored as 1 and the absence was scored as 0.

3.1.5 Immunohistochemistry (IHC)

To identify the PCV2 antigen in testis, seminal vesicle and prepuce of Rep-DNA-pos boars, IHC (ABC system) was used, as previously described (Pearodwong et al., 2015). Polyclonal antibody against PCV2 capsid protein was used to target PCV2 antigen (GeneTex Inc., USA, CA). In brief, the paraffin-embedded tissues were deparafinized. The antigen was retrieved using citric acid (pH6), microwave method (750 wt, 5 min, 2 times). Endogenous peroxidase blocking was done using 3% H_2O_2 (10 min incubation at room temperature). Non-specific staining blocking was done by goat serum (30 min incubation at room temperature). Anti-PCV2 antibody (1:200) was added to the slides and then incubated at 4°C, overnight. Secondary antibody (Goat anti-rabbit antibody) was added and incubated at room temperature for 30 min. Then, the slides were incubated with ABC for 30 min at room temperature and incubated for another 25 s with DAB substrate. The slides were counter stained with hematoxylin at room temperature for 4 s. Slides were mounted and examined under a light microscope. The presence of PCV2-positive cells was evaluated. Reproductive organs with positive signal were scored as 1. Reproductive organs without positive signal were scored as 0. Cell types that gave positive signal were identified, according to their morphology.

3.1.6 Statistical analysis

Frequency analysis was used to analyze the prevalence of PCV2 DNA and PCV2 antigen detection in each organ of the boars. McNemar test was used to determine the difference among 1) percentage of PCR-positive sample of each reproductive organ and 2) percentage of IHC-positive sample of each reproductive organ. Fisher's exact test was used to compare the percentage of positive lesion in 1) LN-DNA-pos and LN-DNA-neg boars and 2) Rep-DNA-pos and Rep-DNA-neg boars. The significant was set at $P < 0.05$.

CHAPTER 4

4.1 Results

4.1.1 PCV2 DNA prevalence in boar's reproductive organs

PCR for PCV2 was done from superficial inguinal lymph nodes of culled boars (n = 59). LN-DNA-pos boars were identified in 37.3% (22/59) of the tested boars. PCR for PCV2 was further done from the reproductive organs (testis, seminal vesicle and prepuce) of the LN-DNA-pos boars. Rep-DNA-pos boars were identified in 59.1% (13/22) of LN-DNA-pos boars.

The prevalence of PCV2 DNA in the testes, the seminal vesicles and the prepuces were not significantly difference. The prevalences were ranged from 10 – 45.5%. The prevalence was highest in the seminal vesicle (45.5%, 10/22), followed by the prepuce (40.9% 9/22) and then the testis (22.7%, 5/22). We could also observe multi-organ involvement of the reproductive tissues. Approximately, half of the Rep-DNA-pos boars (7/13) showed positive results in at least 2 organs (Table 1).

Table 1 Summary of PCR and IHC results from Rep-DNA-pos boars (n =13)

Boar no.	PCR [a]			IHC [a]			Lymph node enlargement [b] (times)
	T	S	P	T	S	P	
1	+	+	+	-	-	+	3
2	+	+	+	+	+	-	3
3	-	+	-	+	-	+	1
4	-	-	+	-	-	+	1
5	+	+	+	+	+	+	2
6	-	+	+	+	-	+	1
7	-	-	+	+	-	-	2
8	+	-	+	+	-	-	NA
9	+	+	+	+	-	-	NA
10	-	+	+	+	+	-	2
11	-	+	-	+	+	-	5
12	-	+	-	-	-	-	2
13	-	+	-	+	-	-	NA

[a] T = testis, S = seminal vesicle and P = prepuce; (+) = positive and (-) = negative

[b] NA = data not available

4.1.2 PCV2 antigen distribution in boar's reproductive organs

The prevalences of PCV2 antigen in the reproductive organs were ranged from 30.8% - 76.9%. The testis showed the highest prevalence. Multi-organ involvement could also be demonstrated. Rep-DNA-pos boars (n = 13) were used in the study. Reproductive tissue sections were stained using IHC technique. PCV2 antigen was targeted using anti-PCV2 capsid antibody. The results showed that, 92.3% (12 boars) was IHC positive in at least one reproductive organ. The prevalence was highest in the testis (76.9%, 10/13), followed by the prepuce (38.5%, 5/13) and then the seminal vesicle (30.8%, 4/13) (Fig 3). The testis showed a significantly higher prevalence than the seminal vesicle ($p < 0.05$). Multi-organ involvement was demonstrated by approximately half of the Rep-DNA-pos boars showed positive signal in at least two reproductive organs (6/13) (Table 1).

According to cell morphology, Leydig cells, Sertoli cells, germinal epithelial cells, fibroblasts of blood vessels and fibroblasts at the basement membrane of seminiferous tubules (Fig 4) were shown to give positive signal. These signals were mainly intracytoplasmic. Interestingly, the major cell type containing positive signal was Leydig cell.

In the seminal vesicles, the signals were found in the seminal vesicle epithelial cells. The signal was mainly intracytoplasmic. In the prepuces, the signals were found mostly in the lymphocytes. The signals were found as both intranuclear and intracytoplasmic.

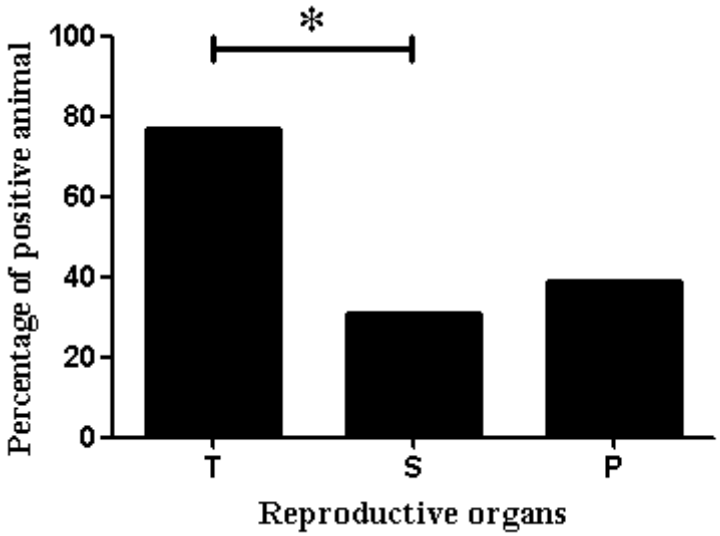

Figure 3. The percentages of PCV2 positive samples by IHC in the Rep-DNA-pos boars (n = 13). *; statistically significant ($p < 0.05$). T, testis; S, seminal vesicle; P, prepuce.

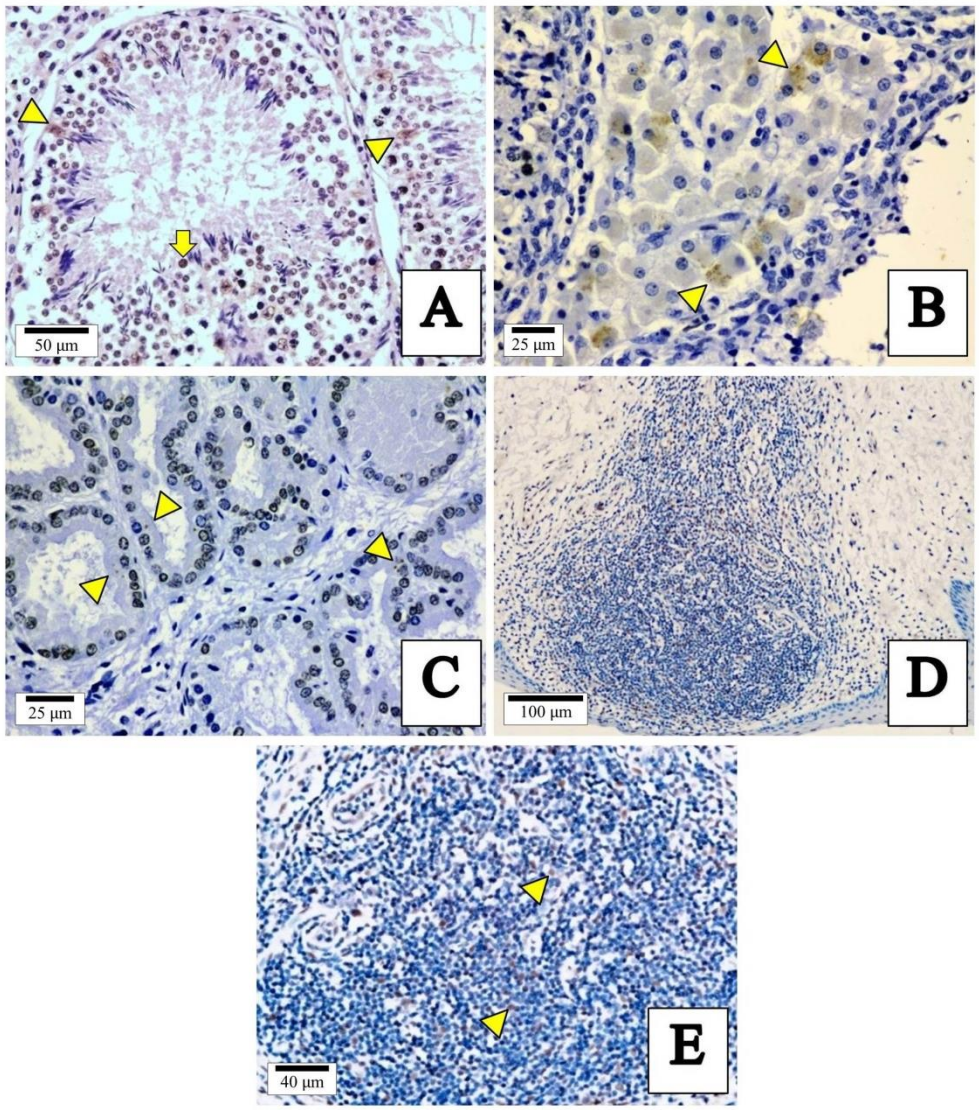

Figure 4. IHC study from the reproductive organs of Rep-DNS-pos boars. Testes: germinal epithelial cells (A, arrows), Sertoli cells (A, arrowheads), and Leydig cells (B, arrowheads). In the seminal vesicles (C), signals are shown in seminal vesicle epithelial cells (arrowheads). In the prepuces (D, E), signals are shown in lymphocytes in the lymphoid follicles (arrowheads).

4.1.3 Macroscopic examination and histopathology

Firstly, macroscopic and histopathological lesions were compared between LN-DNA-pos and LN-DNA-neg boars. There was no significant difference between prevalences of pathological changes (both macroscopic and histopathological changes) in the tested organs.

Testicular degeneration and fibrosis, and enlargement of spermatic cord lymph node were the most remarkable lesions found in both groups (Fig 5, Table 2). In the testes, various degrees of testicular degeneration and fibrosis were observed (Table 2). However, the prevalences were not significantly different. The prevalence of spermatic cord lymph node enlargement in the Rep-DNA-pos boars was significantly higher than the Rep-DNA-neg boars [70.0% (7/10) in the Rep-DNA-pos boars, compared with 14.3% (1/7) in the Rep-DNA-neg boars]. The enlargement ranged from 2 – 5 times of the normal size and was mainly found as 2 times enlargement (Table 1). Histopathologically, we observed lymphoid depletion of the spermatic cord lymph nodes from the Rep-DNA-pos boar with 5 times spermatic cord lymph node enlargement (Boar No. 11). For the seminal vesicles and prepuces, no remarkable gross lesion was found.

Histopathological lesions between the Rep-DNA-pos and the Rep-DNA-neg boars were summarized in Table 2. In the testes, in accordance with macroscopic lesions, testicular degeneration (characterized by the loss of the germinal epithelial lining) and interstitial fibrosis could also be detected (Table 2 and Fig 6). However, no statistical difference was found for both lesions. The severity of the testicular degeneration and fibrosis ranged from mild to severe in both Rep-DNA-pos and the Rep-DNA-neg boars.

Seminal vesiculitis of mild degree, characterized by lymphocyte infiltration in the interstitium, was found in only one boar from the Rep-DNA-pos group (Table 2 and Fig 6). In the prepuce, no significant difference of the prevalence of lymphoid depletion was found. Only Rep-DNA-pos boars showed lymphoid depletion (Fig 6) with the prevalence of 30.8% (4/13). Three of them showed a mild degree of

lymphoid depletion while the other one showed a severe lesion. Only one of these boars contained PCV2 antigen in the prepuce (from IHC study).

Table 2 Number of boars with histopathological changes of the reproductive organs

Boar group	Testis [a] (degeneration)				Testis [a] (fibrosis)				Seminal vesicle [a]		Prepuce [a]			
	0	1	2	3	0	1	2	3	0	1	0	1	2	3
LN-DNA-pos	4	5	4	8	6	5	3	7	19	1	17	3	0	1
LN-DNA-neg	10	10	8	7	13	9	6	7	26	4	34	1	0	0
Rep-DNA-pos	3	4	2	4	4	3	3	3	11	1	9	3	0	1
Rep-DNA-neg	1	1	2	4	2	2	0	4	8	0	8	0	0	0

[a] Lesions observed; Testis = testicular degeneration and testicular fibrosis, seminal vesicle = seminal vesiculitis, prepuce = lymphoid depletion of the mucosal lymphoid follicle; Severity, 0 = no remarkable lesion, 1 = mild, 2 = moderate, and 3 = severe

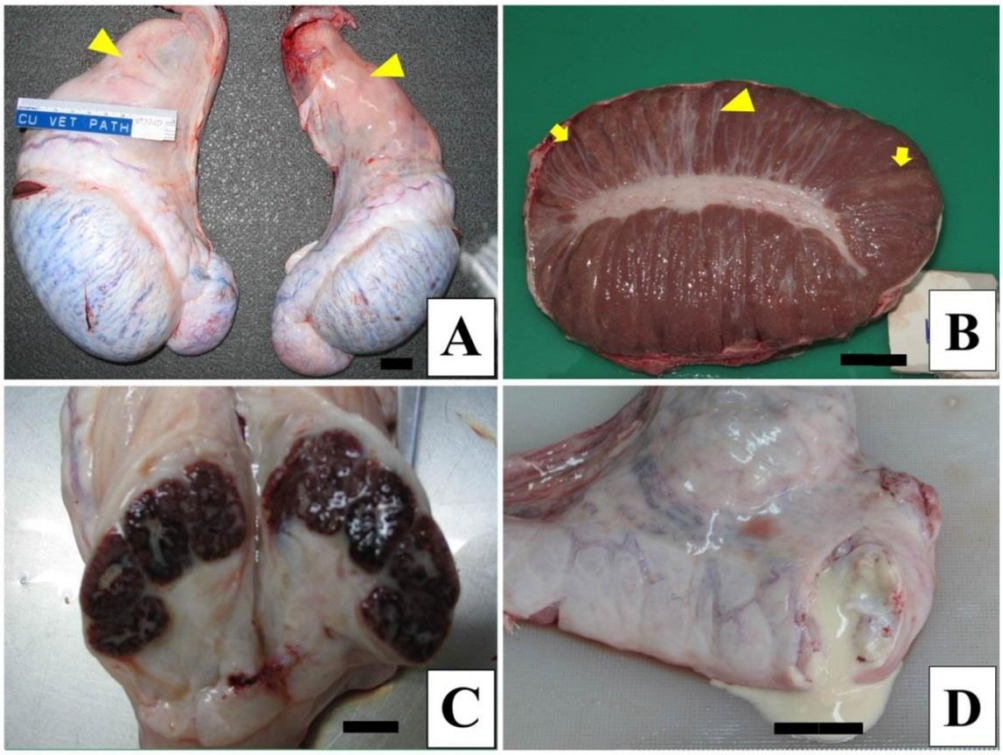

Figure 5 Macroscopic lesions from a Rep-DNA-pos boar showing mild atrophy of right testis with 5 times enlargement of spermatic cord lymph nodes (A, arrowheads, Boar no. 11); multifocal testicular degeneration revealed by tan compressed areas (B, arrows, Boar no. 6), (B, arrows), fibrosis appears as white strips (B, arrowhead). Cross section of the enlarged spermatic cord lymph nodes (C, Boar no. 11), sperm granuloma of epididymis (D, Boar no. 6), (Bar = 2 cm).

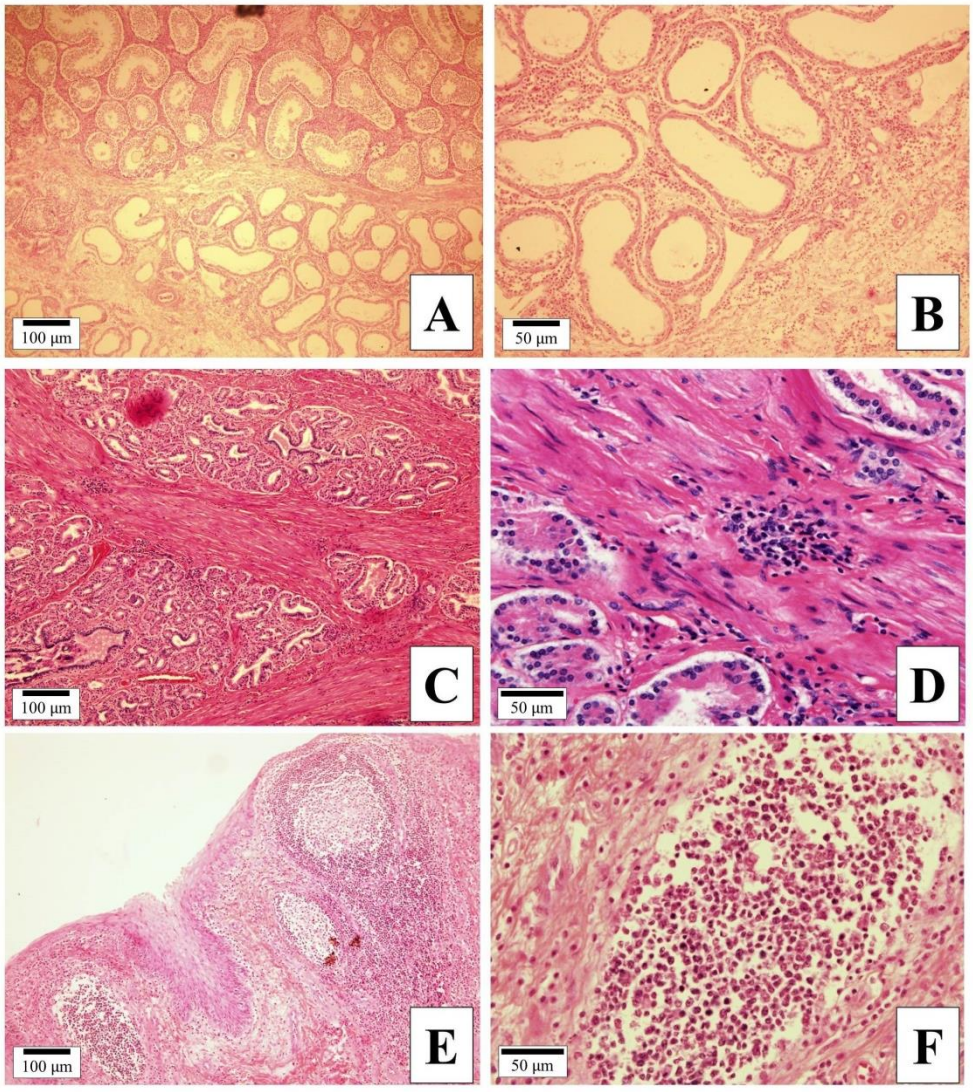

Figure 6. Histopathology of IHC-positive boars. Testicular degeneration revealed by loss of grerminal epithelium by 1-2 cell layers (A and B). Mild interstitial fibrosis with multifocal lymphocytic infiltration in the seminal vesicle (C and D). Histopathological lesion observed in the testes, the seminal vesicles, and the prepuces of the culled boars. Severe multifocal testicular degeneration and fibrosis (A and B). Mild chronic focal seminal vesiculitis (C and D). Multifocal lymphoid depletion of lymphoid follicles of the prepuce mucosa (E and F).

CHAPTER 5

5.1 Discussion

This study provides the pivotal information on both PCV2 prevalence and PCV2 antigen distribution in reproductive organs of culled boars in Thailand. From 59 culled boars, PCV2 DNA was detected in the superficial inguinal lymph node of 37.3% (22/59) of the boars. Of those positive boars, 13 boars (59%) were shown to contain PCV2 DNA in the reproductive organs. Of these 13 boars, 12 boars contain PCV2 antigen in their reproductive organs. By IHC method, PCV2 antigen could be detected in all tested organs, including testes, seminal vesicles and prepuces.

PCV2 infection in sows is well characterized, however, a study in boars is limited. Fetal death was observed after PCV2 infection of pregnant sows. PCV2 antigen could be demonstrated in the affected fetuses (Farnham et al., 2003; Ladekjaer-Mikkelsen et al., 2001; O'Connor et al., 2001; West et al., 1999). PCV2 in the viremic sows can cross the placenta and infected the fetuses (Park et al., 2005). Depending on the gestation phase, PCV2 preferentially infects different tissues. The virus mainly infects myocardial cells of the fetus during early- to mid-gestation (Park et al., 2005). However, the virus mainly infects lymphoid tissues during late gestation (Sanchez et al., 2003). Moreover, PCV2 could replicate in the porcine embryo and leads to embryonic death (Mateusen et al., 2007).

In the reported cases of sow reproductive failure, the major source of PCV2 is elusive. In recent years, the interest on the semen shedding issue of PCV2 is growing. Although PCV2 is shedded in the semen, the amount of the virus could be low (Madson et al., 2008). Thus virus transmission via artificial transmission might not occur (Madson et al., 2009b). However, it is clear that insemination with PCV2-spike semen leads to reproductive failure in dams (Madson et al., 2009a). Semen shedding is reduced by vaccination (Opriessnig et al., 2011; Seo et al., 2013), however, low amount of virus is still presence in the semen (Seo et al., 2013). Moreover, in the

PCV2-spike semen insemination model, vaccine could not prevent fetal infection as well. Accordingly, basic knowledge on PCV2 and boar's reproductive organs is crucial.

In this study, the prevalence of PCV2 DNA in the boar's reproductive organs of the infected culled boars was high, indicating that PCV2 was accumulated at quite high frequency in the boar's reproductive organs. The previous study demonstrated that PCV2 DNA could be found in the semen of serological negative boars (Schmoll et al., 2008) implying the persistent infection of the virus in the boar's reproductive organs. Moreover, the prevalence of PCV2 in boar's semen was shown to be 18.2% in Austria and Germany (Schmoll et al., 2008), 20.4% in Korea (Kim et al., 2003b) and 30.2% in Canada (McIntosh et al., 2006). These findings emphasized an important role of boar's reproductive system in PCV2 spreading. PCR and IHC results were inconsistent in the testes. This might be partly due to prolonged preservation of the frozen tissues. For IHC, PCV2 antigen was frequently observed in the germinal epithelial cells and Sertoli cells. In accordance with the previous reports using IHC method, germinal epithelial cells were shown to carry PCV2 antigen. In a coinfection model using PCV2/porcine parvovirus combination in 1-2 days old piglets (Kennedy et al., 2000), PCV2 antigen was observed in the germinal epithelial cells and the infiltrating macrophages. Germinal epithelium necrosis was also detected.

In this study, degenerative changes in the testes were also observed. This may be partly related with the presence of PCV2 antigen in multiple cell types in the testes. However, PCV2 antigen was also observed in the non-degenerative seminiferous tubules as well. Interestingly, the antigen was also demonstrated in the cytoplasm of Leydig cells. Thus, due to the infection, some function of these cells might be altered, such as the defensive mechanisms and the testosterone production (Dejucq et al., 1995), as seen in Mumps virus infection in human (Beard et al., 1977). Other causes of testicular degeneration could not be excluded in this study. The boars used in this study were from PRRSV endemic area. PRRSV, can also cause testicular degeneration (Han et al., 2013). Therefore, PRRSV could contribute to those pathological changes as well.

Previously, PCV2 antigen was observed in the fibroblast-like cells in interstitium of seminal vesicles (Opriessnig et al., 2006b). In this study, PCV2 positive antigen was detected in glandular epithelial cells, implying viral contamination in seminal fluid. This finding support the evidence of PCV2 shedding in non-sperm cell fraction (Kim et al., 2003b). This should be further confirmed, since transmission of virus via semen is usually occur as cell association, as seen in PRRSV infection (Sur et al., 1997).

This study revealed a striking evidence of PCV2 infection in the lymphoid follicles of preputial mucosa. However the pathological lesion of lymphoid depletion in those follicles was not obvious. Interestingly, this is the first report identifying PCV2 antigen in boar prepuces. It is possible that PCV2 might use lymphoid follicles in this area for replication and shedding into semen similar to human immunodeficiency virus (HIV) that was hypothesized to use human prepuce mucosa as viral replication site as well (Hirbod et al., 2010).

5.2 Conclusion

PCV2 antigen prevalence in Thai culled boars and PCV2 antigen distribution in their reproductive organs were provided in this study. The testicular tissue showed the highest prevalence of PCV2 antigen, over the seminal vesicle, and the prepuce. Moreover, this study is the first to detect PCV2 antigen in boar's prepuces. Therefore, the boar's reproductive organs might play an essential role on PCV2 semen spreading. Viral inoculation experiment should be done to clarify the PCV2 pathogenesis in the boar's reproductive organs.

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APPENDIX

Appendix A

Immunohistochemistry for PCV2 antigen identification

1. Deparaffinization
    - Incubate the slides in solutions as follows
        i. Xylene I for 5 min
        ii. Xylene II for 5 min
        iii. Absolute ethanol I for 10 strokes
        iv. Absolute ethanol II for 10 strokes
        v. 95% ethanol I for 10 strokes
        vi. 95% ethanol II for 10 strokes
        vii. 70% ethanol for 10 strokes
        viii. D.W. for 5 min
2. Antigen retrieval
    - Put the slides in a jar of citric acid (pH 6)
    - Put the jar in the microwave and set the microwave to 750 watts
    - Incubate for 5 min for 2 times
    - Cool down at room temperature for 20 min
3. Washing
    - Wash the slides in PBS for 3 times
4. Endogenous peroxidase blocking
    - Incubate the slides in a fresh 3% H_2O_2 solution [3% H_2O_2 in absolute methanol] at room temperature for 10 min
5. Washing
    - Wash the slides in PBS for 3 times
6. Non-specific staining blocking
    - Incubate the slides with normal goat serum solution [1.5% goat serum in PBS] at room temperature for 30 min
7. Primary antibody incubation

- Incubate the slides with the primary antibody solution [0.5% v/v antibody in PBS] at 4 $^{\circ}$C for 18-24 h

8. Washing
    - Wash the slides in PBS for 3 times
9. Secondary antibody incubation
    - Incubate the slides with the secondary antibody solution [0.5% v/v antibody in PBS] at room temperature for 18-24 h
10. Washing
    - Wash the slides in PBS for 3 times
11. ABC incubation
    - Incubate the slides with the ABC solution at room temperature for 30 min
12. Washing
    - Wash the slides in PBS for 3 times
13. DAB substrate incubation
    - Incubate the slides with the DAB solution [3% DAB substrate in reagent solution] at room temperature for 30 s or until the brown color is developed
14. Reaction stopping
    - Wash the slides in PBS for 3 times
15. Counterstaining
    - Incubate the slides with hematoxylin 30 s – 1 min
    - Wash the slides in PBS for 3 times
16. Dehydration
    - Incubate the slides in solutions as follows
        i. D.W. for 5 min
        ii. 70% ethanol for 2 min
        iii. 95% ethanol for 2 min
        iv. 95% ethanol for 2 min
        v. Absolute ethanol for 2 min

vi. Absolute ethanol for 2 min
vii. Xylene for 3 min
viii. Xylene for 5 min
ix. Xylene for 10 min

- Mounting

Appendix B

Table **3 Summary of gross examination results from Rep-DNA-pos boars (n = 13)**

Boar no.	Testicular degeneration score	Testicular fibrosis score	Spermatic cord lymph node enlargement (times)
1	0	0	3
2	0	0	3
3	3	3	0
4	0	2	0
5	2	1	2
6	1	1	0
7	0	0	2
8	NA	NA	NA
9	NA	NA	NA
10	0	0	2
11	2	2	5
12	3	3	2
13	NA	NA	NA

NA = not available

Table **4 Summary of gross examination results from Rep-DNA-neg boars (n = 9)**

Boar no.	Testicular degeneration score	Testicular fibrosis score	Spermatic cord lymph node enlargement (times)
14	1	0	0
15	3	3	Non-developed
16	2	1	2
17	2	1	Non-developed
18	0	1	Non-developed
19	1	1	Non-developed
20	2	3	NA
21	0	0	NA
22	2	3	Non-developed

NA = not available

Table **5 Summary of gross examination results from LN-DNA-neg boars (n = 37)**

Boar no.	Testicular degeneration score	Testicular fibrosis score	Spermatic cord lymph node enlargement (times)
23	1	0	non-developed
24	0	0	NA
25	0	0	5
26	1	2	non-developed
27	0	1	2
28	3	2	non-developed
29	1	1	2
30	0	1	NA
31	3	3	NA
32	0	1	NA
33	2	0	non-developed
34	1	1	non-developed
35	3	3	non-developed
36	2	0	NA
37	0	1	non-developed
38	0	0	non-developed
39	1	0	non-developed
40	3	2	2
41	1	0	non-developed
42	0	0	2
43	1	2	2
44	3	3	2
45	2	2	0
46	NA	NA	NA
47	0	0	2
48	2	2	2
49	1	1	non-developed
50	0	1	2
51	0	0	4
52	1	2	non-developed
53	0	0	0
54	3	3	0
55	0	1	non-developed
56	3	3	non-developed
57	0	1	2
58	NA	NA	NA
59	NA	NA	NA

NA = not available

Table **6** Summary of microscopic examination results from Rep-DNA-pos boars (n = 13)

Boar no.	Testicular degeneration score	Testicular fibrosis score	Seminal vesiculitis score	Preputial lymphoid depletion score
1	0	1	1	0
2	0	0	0	0
3	2	0	0	0
4	1	0	0	1
5	3	2	0	0
6	1	1	0	0
7	0	0	0	0
8	1	2	0	0
9	3	3	NA	3
10	3	2	0	1
11	2	3	0	1
12	3	3	0	0
13	1	1	0	0

NA = not available

Table **7** Summary of microscopic examination results from Rep-DNA-neg boars (n = 9)

Boar no.	Testicular degeneration score	Testicular fibrosis score	Seminal vesiculitis score	Preputial lymphoid depletion score
14	2	0	0	0
15	1	0	0	0
16	2	1	0	0
17	3	3	0	0
18	0	1	0	0
19	3	3	0	0
20	3	3	0	0
21	NA	NA	NA	NA
22	3	3	0	0

NA = not available

Table **8** Summary of microscopic examination results from LN-DNA-neg boars (n = 37)

Boar no.	Testicular degeneration score	Testicular fibrosis score	Seminal vesiculitis score	Preputial lymphoid depletion score
23	1	0	0	0
24	0	0	0	0
25	0	0	0	0
26	1	0	0	0
27	0	0	0	0
28	3	2	0	0
29	2	2	NA	0
30	NA	NA	NA	NA
31	3	3	0	0
32	1	1	0	0
33	0	0	0	0
34	1	3	0	0
35	2	2	NA	0
36	2	1	0	0
37	NA	NA	NA	NA
38	0	0	0	0
39	1	0	0	0
40	3	0	1	0
41	1	0	0	0
42	0	0	0	0
43	3	3	0	0
44	3	3	1	0
45	1	2	0	0
46	2	3	0	0
47	2	1	NA	0
48	3	1	NA	0
49	1	2	NA	0
50	0	1	1	0
51	0	0	0	0
52	2	1	0	0
53	0	1	0	0
54	2	3	0	0
55	3	3	0	0
56	2	2	1	1
57	0	1	0	0
58	1	0	0	0
59	1	1	0	0

NA = not available

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ACADEMIC PUBLICATION

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