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นายรุ่งธรรม เกษโกวิท

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IDENTIFICATION OF PORCINE CIRCOVIRUS TYPE 3 (PCV3) IN THAILAND AND ITS ASSOCIATED SYNDROME

Mr. Roongtham Kedkovid

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Pathobiology
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รุ่งธรรม เกษโกวิท : การตรวจพบเซอร์โคไวรัสชนิดที่ 3 (พีซีวี 3) ในประเทศไทยและกลุ่มอาการที่เกี่ยวข้อง (IDENTIFICATION OF PORCINE CIRCOVIRUS TYPE 3 (PCV3) IN THAILAND AND ITS ASSOCIATED SYNDROME) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, 103 หน้า.

เซอร์โคไวรัสชนิดที่ 3 ในสุกร (พีซีวี 3) คือไวรัสที่ได้รับการค้นพบใหม่ที่ยังไม่ทราบความสามารถในการก่อโรค ปัจจุบันพีซีวี 3 แพร่กระจายไปในหลายประเทศดังนั้นจึงควรมีการศึกษาถึงความสำคัญของไวรัสชนิดนี้อย่างเร่งด่วน เพื่อให้เข้าใจถึงอิทธิพลของพีซีวี 3 ที่อาจมีต่ออุตสาหกรรมการผลิตสุกรได้ดียิ่งขึ้นควรพิจารณาไวรัสนี้ในหลายแง่มุมของวงจรการผลิตสุกร การศึกษาในครั้งนี้มีการตรวจพบพีซีวี 3 เป็นครั้งแรกในประเทศไทยและยังแสดงให้เห็นด้วยว่าไวรัสนี้สามารถพบในประเทศไทยได้ตั้งแต่ปี พ.ศ. 2548 เพื่อให้เข้าใจถึงความสามารถในการก่อโรคของพีซีวี 3 มากยิ่งขึ้น การศึกษาในครั้งนี้ได้ทำการสำรวจบทบาทของพีซีวี 3 ในระยะต่างๆ ของวงจรการผลิตสุกร ผลการศึกษาพบว่าการตรวจพบพีซีวี 3 มีความเกี่ยวข้องกับการแท้งของสุกรแม่พันธุ์ นอกจากนี้การติดเชื้อในสุกรแม่พันธุ์ยังทำให้เกิดการถ่ายทอดพีซีวี 3 ผ่านไปยังลูกสุกรทางนมน้ำเหลืองได้อีกด้วย ซึ่งการศึกษานี้เป็นครั้งแรกที่มีการแสดงให้เห็นว่าพีซีวี 3 สามารถแพร่กระจายผ่านทางนมน้ำเหลืองได้ การแพร่กระจายของพีซีวี 3 สู่ลูกสุกรอาจส่งผลให้ลูกสุกรเหล่านี้แพร่กระจายไวรัสสู่สุกรตัวอื่นต่อไปในอนาคตได้ ซึ่งในการศึกษาในครั้งนี้ได้แสดงให้เห็นว่าการติดเชื้อในสุกรมีความเกี่ยวข้องกับการเกิดโรคระบบทางเดินหายใจแบบซับซ้อนในสุกรด้วย สรุปคือการศึกษานี้แสดงให้เห็นถึงบทบาทของพีซีวี 3 ในช่วงต่างๆ ของวงจรการผลิตสุกร นอกจากนี้การศึกษานี้ยังมีการอภิปรายถึงการใช้การจัดการในส่วนของสุกรทดแทนเพื่อควบคุมปัญหาจากพีซีวี 3 อีกด้วย

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

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KEYWORDS: PORCINE CIRCOVIRUS TYPE 3 / THAILAND / ABORTION / PORCINE RESPIRATORY DISEASE COMPLEX / COLOSTRUM

ROONGTHAM KEDKOVID: IDENTIFICATION OF PORCINE CIRCOVIRUS TYPE 3 (PCV3) IN THAILAND AND ITS ASSOCIATED SYNDROME. ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D., DTBVP., 103 pp.

Porcine circovirus type 3 (PCV3) is a recently identified swine virus with currently unknown pathogenesis. To date, PCV3 has been found in many countries, indicating the wide spread of the virus. Therefore, there is an urgent need to clarify the detrimental role of PCV3. To better understand how PCV3 might influence the pig industry, various perspectives in swine production cycle should be considered. In this study, firstly, PCV3 was identified for the first time in Thailand. The study also showed that PCV3 has existed in the Thai pig population as early as 2005, suggesting that the virus might have long been a part of swine health problems in Thailand. To gain more information about PCV3 pathogenesis, a series of investigation in different phases of swine production cycle were conducted. The results showed that in gestating pigs, the presence of PCV3 was associated with abortion. Moreover, sow infection could also lead to vertical transmission of multiple routes. PCV3 shedding in colostrum was identified for the first time in this study. Consequently, early infection in piglets by vertically transmitted PCV3 might also lead to horizontal transmission later. An investigation on a farm with early PCV3 infection showed that PCV3 was associated with porcine respiratory disease complex (PRDC) in grower pigs. In conclusion, this study demonstrated that PCV3 could show different roles in each phase of the swine production cycle. Management of gilt/primiparous sows as an initial control strategy against PCV3 is also discussed in this study.

Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
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LIST OF ABBREVIATIONS

ADV	Aujeszky's disease virus
BA	born alive
CU-ACUC	Chulalongkorn University Animal Care and Use Committee
CU-EIDAs	Center of Excellence in Emerging Infectious Diseases in Animals, Chulalongkorn University
CU-VDL	Chulalongkorn University-Veterinary Diagnostic Laboratory
DIG	digoxigenin
FFPE	formalin-fixed paraffin-embedded
HE	hematoxylin and eosin
IBC	Institutional Biosafety Committee
ISH	*in situ* hybridization
MCMC	Markov chain Monte Carlo
MEM	modified Eagle’s medium
MF	mummified fetuse
MLV	modified-live vaccine
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PCV	porcine circovirus
PCV1	porcine circovirus type 1
PCV2	porcine circovirus type 2
PCV2-LD	PCV2-associated lung disease
PCV2-SD	PCV2-associated systemic disease

PCV3	porcine circovirus type 3
PCVAD	porcine circovirus associated disease
PCVD	porcine circovirus disease
PDNS	porcine dermatitis and nephropathy syndrome
PMWS	post-weaning multisystemic syndrome
PNP	proliferative necrotizing pneumonia
PPV	porcine parvovirus
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
SB	stillborn
TB	total born
tMRCA	time to the most recent common ancestor
UN Comtrade	United Nation Commodity Trade Statistics Database

CHAPTER I

INTRODUCTION

Importance and Rationale

Porcine circovirus type 3 (PCV3) is a recently discovered swine virus that attracting attention from pig producers worldwide. Although PCV3 has been identified in pigs with various clinical outcomes, the pathogenesis of the virus is currently unknown. This is mainly due to most of the studies so far were case reports rather than systematic studies. The virus might be pathogenic or it is possible that the virus is non-pathogenic. Therefore, the impact of PCV3 on pig industries is not yet known. Since the virus has been found in many countries, indicating wide spread of the virus, there is an urgent need to clarify the roles of PCV3 in the swine industry.

To better understand how PCV3 could influence the pig industries, various aspects of the virus in swine production cycle should be considered. Due to the nature of the swine production cycle, it is possible that the pathogen could show different roles/effects in each groups of pigs; eg sows, boars, and grower pigs. Infection in sows leading to vertical transmission to the piglets and disease initiation in these infected piglets is a well-known pattern and consequently, found the virus circulation in the swine herds, horizontally. Unfortunately, the roles of PCV3 in swine production cycle were not well-understood. Previously, PCV3 has been identified from aborted sows and the fetuses. Not only that, PCV3 has also been identified from pigs suffering from porcine respiratory disease complex (PRDC). However, as previously mentioned, associations between PCV3 and the disease outcomes have never been elucidated.

Regarding vertical transmission, transplacental and direct-contact transmission PCV3 could be suspected. However, virus transmission through colostrum (trans-colostrum), which has been shown to be an important route of PCV2, has not yet been studied. In conclusion, a better understanding about the impact of PCV3 in the swine industry could be partly reached by fulfilling these knowledge gaps.

For Thailand, the situation was more ambiguous since the information regarding PCV3 status was not available. Whether the virus has already been introduced to Thailand was not known. Moreover, whether the virus has long been circulating in Thai pigs or recently introduced to Thailand was not known either.

In this study, firstly, PCV3 identification was done in Thai pigs. A retrospective study was conducted to identify PCV3 from swine tissue samples previously collected during 2005 – 2008. Then, a series of investigation regarding the roles of PCV3 in swine production cycle were conducted. Firstly, association between PCV3 and abortion in gilts was investigated. Secondly, shedding of PCV3 in sow colostrum was studied. Finally, association between PCV3 and PRDC in grower pigs was determined.

Understanding the roles of PCV3 in swine production cycle could be one of the initial steps in understanding the effect of PCV3 in swine industries and business. It could also help in further studies on PCV3 pathogenesis and the control strategies of the virus.

Objectives of the Study

- To identify PCV3 from Thai pigs in 2017
- To identify PCV3 from tissue samples collected from gilts during 2005 – 2008
- To investigate the association of PCV3 infection and abortion in gilts
- To investigate PCV3 shedding in sow colostrum
- To investigate the association of PCV3 infection and PRDC in grower pigs

Ethical Issues

Animal experiments in this study were approved by Chulalongkorn University Animal Care and Use Committee (CU-ACUC), approval number 1731077 and 1731064. Experiments involving viruses in this study were reviewed and approved by the Institutional Biosafety Committee (IBC) of Faculty of Veterinary Science, Chulalongkorn University (CU-VET-BC), approval number IBC1831016.

Literature Review

Introduction

Porcine circovirus (PCV) is a member of the family Circoviridae, genus *Circovirus*. PCV is a non-enveloped DNA virus with approximately 12 to 23 nm virion diameter (Rodriguez-Carino and Segales, 2009). There are two major open reading frames (ORFs) in PCV genomes including ORF1 (*rep* gene), coding for replicase proteins involving in virus replication; and ORF2 (*cap* gene), coding for capsid proteins serving as the only structural protein of the virion.

Before the discovery of PCV3, there are two known groups of PCV which are PCV1 and PCV2. PCV1 is generally considered as a non-pathogenic virus for pigs. The virus was discovered in 1974 in pig kidney cell lines (Tischer et al., 1974). Later in 1998, PCV2 has been isolated and characterized (Ellis et al., 1998). In contrast to PCV1, PCV2 is one of the most important swine pathogens causing the so-called 'porcine circovirus disease (PCVD)' leading to economic losses to the pig industry worldwide. Although PCV2 was first isolated in 1998, the virus has existed for a much longer time. The earliest PCV2 has been identified by PCR from a sample collected in 1962 (Jacobsen et al., 2009). The recently discovered PCV3 has been firstly reported in 2016 (Palinski et al., 2017; Phan et al., 2016). The existence of PCV3 was further confirmed to be far earlier than 2016, similar to the case of PCV2. Therefore, PCV3 might not be a forthcoming problem of the swine industry. Rather, it possibly has long been a part of swine health management obstacles.

In this literature review section, the emergence of PCV3 will be discussed followed by the speculation of PCV3 in Thailand whether it is a new virus or not. Then, to better understand the potential deleterious effects of PCV3 to the swine industry, the roles of the virus are examined in three consecutive stages of swine production cycle including the gestation period, the farrowing period, and the growing period. In general, infection (of any virus) in gilts/sows could lead to both 1) the disease in these pigs and 2) vertical transmission. Consequently, infection of the piglets early in their lives (suckling period) by the vertically transmitted pathogen could lead to both the disease in the younger ages or the later stages of the growing period, horizontally. Therefore, the current knowledge about PCV3 regarding abortion, vertical transmission, and PRDC are discussed.

The Emergence of PCV3

In late 2016, two reports from separated groups have shown that a novel virus was found in the US pig population (Palinski et al., 2017; Phan et al., 2016). The novel viruses from both reports were genetically related to the previously known circoviruses and those were referred to PCV3 but distantly related to PCV1 and PCV2. Later, it has been shown that PCV3 can be found in various countries. Consequently, this wide spread distribution of PCV3 has globally drawn the swine producers' attention towards the deleterious potential of the virus to the swine industry.

Currently, PCV3 research is growing and expanding. However, the knowledge on PCV3 pathogenesis was limited. Therefore, estimating the potential risk of PCV3 to the swine business is challenging.

Although the spectrum of disease found in PCV3-infected pigs is diverse, whether PCV3 is the actual causative agent in these pigs was not confirmed. In the first two reports of PCV3 (Palinski et al., 2017; Phan et al., 2016), the virus was found in pigs showing myocarditis, reproductive failure, and porcine dermatitis and nephropathy syndrome (PDNS). In the study by Phan and colleagues (2016), three separated cases of pigs of approximately 3 to 10 weeks of age were investigated for unexplained myocarditis. Initially, the major clinical signs of these pigs were 1) swollen joints and anorexia, 2) respiratory signs and rectal prolapse, and 3) dyspnea and neurologic signs. Myocarditis was later observed and became the major focus of the study. Lesions of other tissues were also found; however, the suspected causative pathogens were identified (mostly). The major lesions found in the hearts of these pigs were from microscopic examination, including 1) lymphoplasmacytic/histiocytic myocarditis and arteriolitis, 2) mural arteriolitis with pyknotic nuclear debris and endothelial cells hypertrophy/hyperplasia, and 3) interstitial lymphocytic myocarditis. By using *in situ* hybridization, PCV3 mRNA was found in the myocardiocytes, arterioles, and inflammatory cells in the myocardium. PCV2, which is well-known for its roles in inducing myocarditis and vascular lesions (Brunborg et al., 2007; Marks et al., 2016; Marks et al., 2010; Resendes and Segales, 2015; Yang et al., 2017), was negative in these animals. It is very interesting whether these PCV2 and PCV3 could induce similar lesions or not. However, it cannot be concluded that PCV3 was the causative agent in those cases. It is still possible that the lesions were caused by other pathogens and PCV3 did not involve in this scenarios at all. In the study by Palinski and colleagues (2017), an investigation was done on a swine farm showing increasing sow mortality and abortion. The affected sows showed PDNS-like lesions; including 1) necrotizing vasculitis and

transmural neutrophilic infiltration of dermis and subcutis, and 2) proliferative glomerulonephritis. Lesions of other organs were also observed in these sows; including 1) bronchointerstitial pneumonia with lymphocytic/plasmacytic perivascular cuffing, 2) lymphoid depletion with granulomatous lymphadenitis. By using immunohistochemistry, PCV3 antigen was identified in the lymphocytes and macrophages. The positive signal was also observed in the tubular epithelium of the kidneys. Lesions in aborted fetuses were not mentioned in this study. Whether myocarditis and vascular lesions can be observed in these aborted fetuses is very interesting. Again, PCV2 was not found in this study. However, as in the previously mentioned study, it cannot be implied that PCV3 involved in the observed lesions. Further studies are needed to clarify this issue.

After these two initial PCV3 studies were recognized, PCV3 investigation had been conducted in various countries using aborted sows/fetuses as the target population. This is possibly due to the more obvious clinical presentation of abortion over myocarditis. PCV3 was subsequently identified in many countries around the world (Table 1). Moreover, more clinical signs/diseases have been suspected to be associated with PCV3 (Chen et al., 2017; Faccini et al., 2017; Klaumann et al., 2018; Ku et al., 2017; Shen et al., 2018; Sun et al., 2018; Tochetto et al., 2018; Wang et al., 2017; Ye et al., 2018; Zhai et al., 2017). Additionally, PCV3 has been detected from clinically healthy pigs (Kwon et al., 2017; Ye et al., 2018), further complicating the understanding of PCV3 pathogenesis.

Table 1 Countries reporting PCV3 identification

North America	South America	Europe	Asia
US	Brazil	Germany	China
		Italy	South Korea
		Poland	
		Spain	
		Sweden	
		UK	

PCV3 in Thailand: a Newly Introduced or an Endemic Virus?

PCV3 was firstly reported in 2016 in the US. However, it is not yet clear how long the virus has been circulating in the pigs. Many studies have shown or suggested that the virus was already present decades before 2016. In Thailand, a preliminary study showed that PCV3 was found in Thai pigs in 2017. Whether PCV3 is newly introduced to Thailand or not is a question yet to be clarified. The answer could lead to a better understanding of PCV3 epidemiology (especially in Thailand). Moreover, it might assist optimizing awareness of PCV3 among Thai swine producers as well.

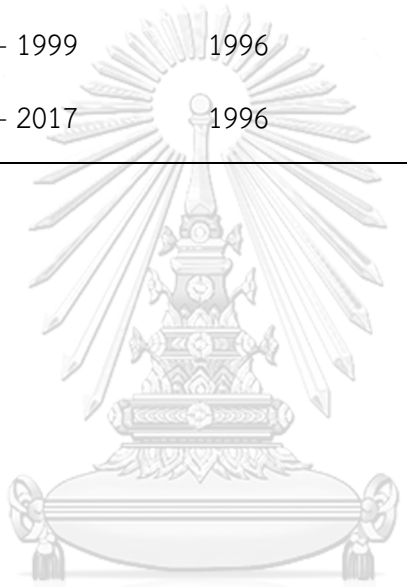
PCV3 has been shown to predate the first report (in 2016) in various countries. PCV3 DNA has been identified since 1993 in Sweden (Ye et al., 2018) and since 1996 in China (Sun et al., 2018) and Spain (Klaumann et al., 2018) (the virus has also been found in samples collected during 2000 – 2016 in many countries). It should be noted that these studies used samples collected from 1990s onward (Table 2). The emergence of PCV3 might possibly occur before that. However, sample availability could be the major obstacle. In fact, in the studies from Sweden (Ye et al., 2018) and Spain (Klaumann et al., 2018), PCV3 could be identified from the earliest samples collected (Table 2). Therefore, it is possible that the virus might be circulating in Sweden and Spain prior to 1993 and 1996, respectively. Nevertheless, it is clear that PCV3 already exists in both Europe and Asia at least since 1993 – 1996.

PCV3 DNA sequence analysis also supported that the virus might emerge before 1993. Two studies have shown very similar results that the origin of PCV3 was predicted approximately 1966 and 1967 (Fu et al., 2018; Saraiva et al., 2018). By using Bayesian Markov chain Monte Carlo (MCMC) to estimate time to the most recent common

ancestor (tMRCA), Saraiva and colleagues (2018) have shown that origins of recent PCV3 strains from various regions of the world (mainly collected during 2015 - 2017) were 1946 – 1987 with the mean tMRCA estimated at 1967. With the same approach, the work from Fu and colleagues (2018) has demonstrated that the origins of PCV3, mainly Chinese strains (collected during 2015 – 2017), were 1911.5 – 1996.8 with the mean tMRCA estimated approximately at 1966. It should be noted that, in both of these studies, higher number of PCV3 sequences from China was used (possibly due to high number of available PCV3 sequences from China). This could affect the overall PCV3 origins calculation (Saraiva et al., 2018). Therefore, more sequences from other countries are needed for further clarification.

Table 2 PCV3 identification in 1990s

Country	Sample Collection year	Earliest PCV3 identification (collection year)	References
Sweden	1993 – 2007	1993	Ye et al., 2018
China	1990 – 1999	1996	Sun et al., 2018
Spain	1996 – 2017	1996	Klaumann et al., 2018



The fact that PCV3 can be identified in different countries since 1993 – 1996 (Klaumann et al., 2018; Sun et al., 2018; Ye et al., 2018) and that the origins of the virus might be 1966 – 1967 (Fu et al., 2018; Saraiva et al., 2018) is not sufficient by itself to suggest that the virus might be introduced to Thailand prior to 2017 (the first PCV3 identification in Thailand from our preliminary study). However, with data from epidemiological studies of other swine pathogens in Thailand, it could be conceivable.

It has been shown previously that Thai pigs were not an isolated population. Pathogen could be introduced to Thailand through various possible routes, although control strategies and regulations have been applied. Trading of live pigs (both legally and illegally), pig's products, and fomites could be important routes of the transmission. According to the United Nation Commodity Trade Statistics Database (UN Comtrade) recorded during 1990 - 2016, live pigs from various countries have been imported to Thailand (Table 3). Furthermore, transmissions of several viruses between Thailand and other countries have been speculated in various studies. Epidemiology studies of previously known porcine circovirus, PCV2, could provide an excellent example linking animal movement and the global emergence of PCVD (Vidigal et al., 2012). PCV2 can be classified into four major groups based on their genetics including PCV2a, PCV2b, PCV2c, and PCV2d. In Thailand, a recent study in 2017 has shown that PCV2a, PCV2b and PCV2d were circulating in Thailand during 2009 - 2015 (Thangthamniyom et al., 2017). By using haplotypes network analysis together with data regarding swine importation to Thailand (UN Comtrade), it has been suggested that PCV2a strains in Thailand were introduced from various regions including USA, Europe, and Japan. The ancestor of Thai PCV2b was suggested to be of the Netherlands origin. It has been suggested that the virus spread from the Netherlands to China. Then

the virus was circulating in China and further spread to other Asian countries including Thailand (Thangthamniyom et al., 2017). PCV2d was suggested to originate from China (Xiao et al., 2015) and most of Thai PCV2d strains were also speculated to evolve from the Chinese strains as well (Thangthamniyom et al., 2017). This data indicating that introduction of viruses from other countries to Thailand is highly possible.

Table 3 Live-pig importation to Thailand during 1990 to 2016

1990 - 1994	1995 - 1999	2000 - 2004	2005 - 2009	2010 - 2014	2015 - 2016
Australia	Australia	Belgium	China	Belgium	Canada
Belgium	Belgium	Denmark	Denmark	Canada	China
Brazil	Denmark	France	Germany	Denmark	Denmark
Canada	Finland	Ireland	Ireland	Ireland	USA
Denmark	Germany	Norway	Norway	Japan	
Finland	Indonesia	USA	South Korea	New Zealand	
Germany	Iran		UK	Norway	
Ireland	Ireland		USA	Philippines	
Malaysia	Israel			USA	
Netherlands	Japan				
Norway	Netherlands				
Singapore	Norway				
Sweden	Poland				
UK	Sweden				
USA	UK				
	USA				

To determine whether PCV3 has been introduced to Thailand before 2017, many factors should be taken into consideration. Some of the important factors include 1) the expected prevalence, 2) target populations, 3) types of the collected tissues, 4) tissue storage conditions, 5) virus detection methods, and 6) preliminary studies. In brief, expected prevalence determines the sample size. It requires a large sample size to identify the low prevalence virus. Choosing the right target population can further increase the successful virus detection. It is possible that the pathogens of interest could be found at higher rates in certain groups of pigs; eg pigs of specific sex, age, clinical signs etc. Types of the tested tissue could affect the virus detection in the same manner as the target population selection. Virus might not be equally accumulated in all pig tissues eg some virus might be found at higher prevalence in the lymph node, therefore, this should be the tissue of choice in the investigation. The results could be affected by the tissue storage conditions and the detection methods as well. Certain types of storage condition might not be efficiently preserved the target molecules, thus, requiring detection methods with higher sensitivity. In general, highly sensitive detection methods are always preferable in various situations. Finally, results from preliminary studies are undoubtedly valuable for optimizing the study designs.

In the preliminary study, it has been shown that PCV3 was found in Thai pigs in 2017. In this study, to identify whether PCV3 has existed in Thailand long before 2017, formalin-fixed paraffin-embedded (FFPE) uterine tissues from aborted gilts collected during 2005 – 2008 from participated farms in Thailand were used to detect the PCV3 DNA by real time PCR. PCV3 has been found in aborted pigs in various reports (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Tochetto et al., 2018; Wang et al., 2017; Zheng et al., 2017). Using uterine tissues could be challenging because PCV3

has never been reports in this tissue before. However, PCV2 has been successfully detected from pig's uterus (Pearodwong et al., 2015). FFPE tissue processing is well-known for inducing DNA damage (Grierson et al., 2004), thus, reducing the chance of target DNA detection. Using real time PCR as the detection method could overcome this obstacle by targeting small DNA fragment with high detection sensitivity. A previously established real time PCR assay for PCV3 target only 78 bp of the ORF2 region of PCV3 with the detection limit of 2 log DNA copies/PCR reaction (Wang et al., 2017).

PCV3 and Abortion

PCV3-induced abortion has been one of the major concerns about PCV3 since the virus was discovered. This might be in part due to the fact that one of the first two reports about PCV3 showed that the virus was identified from aborted fetuses. Subsequently, several studies have successfully identified the virus from both aborted sows and aborted fetuses. However, studies regarding the pathogenesis are still lacking. Most importantly, whether PCV3 actually involved in these abortion cases was not known.

Although the association between PCV3 and abortion (and other sow reproductive failures) is unknown, it is notable for PCV2. Currently, fetal infection is thought to play a major role in PCV2 induce sow reproductive failure. Myocarditis characterized by infiltration of mononuclear cells is one of the most prominent lesions in the affected fetuses/piglets (Brunborg et al., 2007). It has also been proposed that the criteria to diagnose PCV2-associated reproductive failure includes: 1) late-term abortions and stillbirths, 2) the presence of fibrosis and/or necrotizing myocarditis in

the fetuses, and 3) presence of PCV2 in the myocardial lesions and other fetal tissues (Sanchez et al., 2003; Sanchez et al., 2001; Segales et al., 2005). Porcine fetuses have been speculated to be immunocompetent to PCV2 at around 57 – 75 days of gestation (Sanchez et al., 2001). Around these ages, fetuses could be able to produce anti-PCV2 antibody in response to the infection. PCV2 titers were much lower in fetuses infected after 75 days of gestation. It has been hypothesized that decreasing susceptibility of cardiomyocytes (reducing mitotic activity) was also responsible for the decreasing PCV2 titers in older fetuses (Sanchez et al., 2003). It can be seen that one of the key factors inducing PCV2-associated reproductive failures could be the viral load. This is also shown in farm situations. In general virus-host interaction, it could be expected that after the infection, the virus titers would be higher in naïve animals, comparing with the previously exposed counterparts. In the case of PCV2-associated reproductive failure, interestingly, most of the affected herds frequently contained a large population of naïve animals (Brunborg et al., 2007; Opriessnig et al., 2007; Oropeza-Moe et al., 2017; Togashi et al., 2011; West et al., 1999); eg startup herds, re-established herd etc. It has also been shown that high PCV2 titers were observed in the aborted fetuses. From a previous study, an investigation was done on a newly established herd experiencing high rates of mummification, stillbirth, and neonatal mortality (Brunborg et al., 2007). The results showed that high PCV2 titers of over 7 log DNA copies/500 ng DNA were observed in the aborted fetuses. The titers were extremely high in the hearts and livers, ranged from 9 to 12 log DNA copies/500 ng DNA.

Studies on reproductive failure in PCV2-infected sows have been mainly focused on fetus infection. Less is known about PCV2-inducing uterine pathology. On the other hand, it has been shown in porcine reproductive and respiratory syndrome

virus (PRRSV) (another important swine pathogen capable of inducing reproductive failure in sows) that events at uterine compartment, as well as maternal-fetal interface and fetuses; might play important roles in reproductive failure of late gestation gilts (Harding et al., 2017; Novakovic et al., 2016). An excellent study by Novakovic et al., was done using late gestation gilts model (Novakovic et al., 2016). PRRSV-naïve pregnant gilts (n = 114) were intramuscularly inoculated with PRRSV at 85 day-of-gestation. Dams and their litters were euthanized at 21 days after the PRRSV inoculation. It was found that lymphohistiocytic endometritis (mostly moderate degree) was observed in 99.6% of the examined uterine tissue samples of the infected animals. This was observed in only 0.9% of samples from negative control animals. It has been hypothesized that the inflammation of endometrium might disrupt hematotrophic nutrient transfer to the fetuses, consequently resulting in reproductive failure. Therefore, it would be interesting whether other viruses, especially PCV2 and PCV3, could be found in endometrium and/or induce endometritis or not.

For PCV3, the association between the infection and reproductive failure is currently unknown. PCV3 DNA has been detected in mummified and stillborn fetuses as previously mentioned. Myocarditis has been observed from PCV3-positive piglets (Phan et al., 2016). It is possible that PCV3 infection could directly induce the lesions in myocardium, as in the case of PCV2. However, a study demonstrating association between PCV3 and reproductive failure/myocarditis are still needed. Moreover, whether PCV3 could induce uterine pathology in pregnant sows is not known.

One approach to clarify whether PCV3 could induce reproductive failure is by using an animal inoculation experiment eg inoculating purified PCV3 to naïve pregnant

pigs etc. However, to minimize the animal usage, prior to this kind of experiments other approaches should be considered according to the principles of the 3Rs: replacement, reduction, and refinement. In this study, FFPE uterine tissue of gilts from a PCV3-infected farm in Thailand was used to determine the association between the presence of PCV3 in the uterine tissue and abortion. The samples were from both culled aborted gilts and gilts culled due to non-reproductive problems eg lameness etc. Therefore, samples from the later group of gilts could be used as a control group. Although PCV3 has never been identified from uterine tissues before, a previous PCV2 study showed that the virus could be found in this tissue (Limsaranrom et al., 2015; Pearodwong et al., 2015). Moreover, our preliminary study showed that PCV3 could be detected from uterine tissues of aborted gilts as well. In this study, real time PCR was used to detect PCV3 DNA in the sample. The high sensitivity of real time PCR could, therefore, increase the chance of successful detection when the virus titers were low which could be expected when using FFPE tissue samples. In conclusion, this study would be the first to associate PCV3 with abortion in those gilts.

Colostrum Shedding of PCV3

PCV3 infection in sows has been the major focus of PCV3 pathogenesis studies since the virus discovery. Although PCV3 has been found in diseased sows in various studies, whether the virus is the causative agent is still not yet determined. Together with the disease induction, infection in sows could also lead to another issue of equal importance, which is vertical pathogen transmission. It has been shown in various infectious diseases that early infection could greatly affected the overall herd health

status. Consequently, limiting the vertical transmission is one of the major goals in disease control strategies.

In general, infection of piglets during suckling period or in the farrowing units could occur via transplacental and direct-contact routes. For PCV3, it could be speculated that both transplacental and direct-contact transmission could occur since 1) the virus has been found in both aborted sows and mummified/still born fetuses (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Tochetto et al., 2018; Zheng et al., 2017), suggesting transplacental infection, and 2) PCV3 can be detected from oral fluids (Kwon et al., 2017), suggesting that direct-contact transmission is highly possible. For PCV2, it has been shown that other than transplacental and direct-contact transmission, vertical transmission could occur vertically via transcolostrum route as well. Therefore, it is interesting whether PCV3 could also shed via this route. Virus shedding in the colostrum/milk could be critical since suckling piglets normally receives milk from sows approximately 20 times per day. The lactation period could last for approximately 21 days or more. Therefore, suckling piglets are highly exposed to the virus shedding from milk. Unfortunately, PCV3 shedding in sow milk has never been studied.

Although PCV2 has long been one of the major swine pathogens, PCV2 shedding in sow colostrum was not recognized since 2006 (Shibata et al., 2006). After that, various interesting aspects of PCV2 colostrum shedding have been found. Firstly, it has been shown that PCV3 shed in the colostrum and milk was infectious and was able to induce PCVD (Ha et al., 2010). Although PCV2 shedding in milk can last until 27 days of lactation (Ha et al., 2009), it has been demonstrated that in the farm situations,

the prevalence of PCV2 in the colostrum were higher than in the milk (Gerber et al., 2011). Regarding swine farm management and disease control, one of the most important issues about PCV3 shedding in colostrum/milk is that vaccination could only reduce rather than completely eliminate the virus shedding. In an investigation in PCV2 infected herds by Gerber and colleagues (2011), it has been shown that 35.0% (7/20) of vaccinated sows still shed infectious PCV2 in the colostrum, comparing with 71.4% (15/21) of unvaccinated sows (Gerber et al., 2011). It should be noted that high levels of neutralizing antibody against PCV2 were detected in all of these studied sows. Another study by Madson and colleagues (2009) also showed similar results using virus inoculation experiment. Sows oronasally inoculated with PCV2 at 56 days of gestation showed higher PCV2 DNA in the colostrum, comparing with the inoculated sows previously vaccinated at 28 days of gestation (4.68 vs 2.41 log DNA copies/ml colostrum, respectively) (Madson et al., 2009). In some farm situations, PCV2 shedding in colostrum (and other routes) can be even more complicated. A study by Dvorak and colleagues (2013) demonstrated that vaccination status and maternal immunity against PCV2 was not associated with PCV2 shedding through various route, including colostrum (Dvorak et al., 2013). In the study, highest PCV2 titers in the colostrum were found in the sow vaccinated farm with the average PCV2 titers of approximately 7 log DNA copies/ml. PCV2 replication can occur in the mammary gland (Park et al., 2009), possibly inside the monocytes/macrophages. A study by Park and colleagues (2009) showed that both PCV2 DNA and antigen was found in macrophage-like cells mainly located in the glandular lumina, suggesting that the virus replication occurred. Whether this virus replication inside the monocytes/macrophages involves in the failure of

vaccine (and maternal antibody) to completely eliminate PCV2 shedding is very interesting.

In this study, PCV2 shedding in sow colostrum was determined for the first time. Colostrum samples from a PCV3 infected farm in Thailand were used. Real time PCR was used to measure PCV3 DNA titers in the colostrum. PCV3 DNA detection was used rather than the virus isolation mainly because PCV3 isolation methods were not established yet. Moreover, real time PCR showed high sensitivity which could be a great advantage in detecting virus shedding in low amount. Since virus shedding could be affected by parity number, colostrum samples were collected from sows of parity 1 to 5. Information from this investigation could be further used in more detailed colostrum/milk shedding studies.

PCV3 and PRDC

Since the first identification of PCV3 in pigs with myocarditis, PDNS, and reproductive failure; several studies have shown that PCV3 could be found in pigs with respiratory problems as well. Therefore, it is very interesting whether PCV3 could involve in PRDC or not. The proposed association between PCV3 and PRDC is not unexpected since PCV2 is considered to be one of the major pathogens in PRDC. Moreover, PRDC is an important syndrome affecting global swine industries.

The term PRDC is used to describe the clinical presentation of respiratory signs with retarded growth in grower pigs. The disease is multifactorial and morbidity and mortality of affected pigs could be greatly varied. Various pathogens could induce PRDC. In farm situations, combinations of pathogens, rather than a single agent, are normally found in PRDC-affected pigs. PRRSV and PCV2 have been shown to be the

major viral respiratory pathogens in PRDC. However, other viruses (eg swine influenza etc.) and bacteria (*Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Streptococcus suis* etc) could also involve in the disease.

A new aspect of PCV2 on PRDC has recently been proposed by Tico and colleagues (2013). Previously, it has been thought that PCV2 could be associated with PRDC in two different patterns, 1) PCV2-associated systemic disease (PCV2-SD), and 2) PCV2-associated lung disease (PCV2-LD). In general, PCV2-SD could be considered as a more serious health problem than PCV2-LD, since PCV2-SD involves lymphoid destruction. Interestingly, it has been recently suggested that PCV2 might frequently contribute to PRDC as PCV2-SD (Tico et al., 2013). PCV2-SD is a multifactorial disease involving PCV2 infection and other contributing factors. Three criteria are needed for PCV2-SD diagnosis (Segales, 2012): 1) clinical signs including weight loss and pale skin, respiratory signs maybe present; 2) lymphocyte depletion with granulomatous inflammation of lymphoid tissues; and 3) detection of PCV2 in the affected tissues. Diagnosis of PCV2-LD is made when there are 1) respiratory signs; 2) lymphohistiocytic/granulomatous interstitial pneumonia or broncho-interstitial pneumonia or proliferative necrotizing pneumonia (PNP); and 3) detection of PCV2 in the lung; 4) absent of lymphocyte depletion with granulomatous inflammation of lymphoid tissues and absent of PCV2 in the lymphoid tissues. It can be seen from these criteria of PCV2-SD and PCV2-LD that PCV2-LD will become PCV2-SD when the lesion or PCV2 is observed in lymphoid tissues. The investigation of 226 PRDC-affected pigs with PCV2 infection in Spain by Tico et al. showed that PCV2 could be found in the lymphoid tissues of all of these studied pigs. None showed only PCV2 found in the

lung. This could mean that in farm situation (at least in this study), PCV2 was hardly restricted to the lung. Rather, the immune system of PRDC-suffering PCV2-infected pigs might always be affected (by PCV2 infection in lymphoid tissues).

PCV2 and PRRSV have been generally considered to be the most frequently detected viral pathogens in PRDC cases. It is possible that when this co-infection occurs, pigs might develop both PRDC and PCV2-SD, not just PRDC alone (Chae, 2016). This is plausibly due to the interaction between these two pathogens. At the herd level, PRRSV infection has been suggested as a risk factor for PCV2-SD (Rose et al., 2003; Wellenberg et al., 2004). It has also been shown that PRRSV, especially type 2 PRRSV and highly-pathogenic PRRSV, could enhance PCV2 viremia and PCV2-associated lesions (Fan et al., 2013; Harms et al., 2001; Park et al., 2014; Rovira et al., 2002; Sinha et al., 2011). The exact mechanism is currently unknown. PRRSV-induced innate immunity suppression has been proposed to play a part in PRDC (Niederwerder et al., 2015).

Vaccination has long been one of the control strategies in various swine infectious diseases, including PRDC. For PRDC causing by PCV2-PRRSV coinfection, vaccination might be a little more complicated. It has been accepted that PCV2 infection alone might not cause severe disease (eg only subclinical infection), while PRRSV is pathogenic by itself. Consequently, attempted has been made to reduce the PCV2-PRRSV coinfected PRDC by applying only PRRSV vaccination. Unfortunately, this strategy might not be effective (Chae, 2016). It has been demonstrated that, not only the virulence PRRSV that could enhance PCV2 replication and PCV2-associated lesions, modified-live PRRSV vaccine (PRRSV-MLV) could also show this feature (Niederwerder

et al., 2015; Park et al., 2013). The mechanism could resemble PRRSV-induced PCV2 replication. Moreover, PRRSV-MLV could induce lymphocyte activation (Ferrari et al., 2013) which consequently could induce PCV2 replication (Gu et al., 2012; Lin et al., 2008; Ramamoorthy et al., 2011).

For PCV3, it has been demonstrated that the virus could be found in pigs with respiratory disease (Fux et al., 2018; Phan et al., 2016; Shen et al., 2018). From a study by Phan and colleagues (2016), it has been shown that lymphohistiocytic interstitial pneumonia was found in the PCV3 infected pigs. However, association between PCV3 and the disease has never been established. It is very interesting whether PCV3 could induce PRDC similar to PCV2 or not.

To better understand the association between PCV3 and PRDC, in this study, field observation approach was conducted. PCV3 infection was investigated in a farm suffering from PRDC. Information from this study could be used for further studies, especially for answering various important questions including, 1) what is the mechanisms of PCV3-associated PRDC? 2) could PCV3 induce disease similar to PCV2-SD?, 3) could PRRSV and PRRSV-MLV enhance PCV3 replication and PCV3-associated diseases? As found in PCV2 etc.

CHAPTER II

PORCINE CIRCOVIRUS TYPE 3 (PCV3) DETECTION IN FORMALIN-FIXED PARAFFIN-EMBEDDED (FFPE) UTERINE TISSUES FROM ABORTED GILTS IN THAILAND, 2005 – 2008

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Abstract

The major objectives of this study were to 1) investigate the presence of porcine circovirus type 3 (PCV3) in Thailand during 2005 - 2008, and 2) investigate an association between PCV3 and gilt abortion in a farm in Thailand. Formalin-fixed paraffin-embedded (FFPE) uterine tissue samples, from gilt during 2005 - 2008, from the previous study by Tummaruk and colleagues [*Theriogenology*, volume 71, issue 2 (2009)] were used. To investigate the presence of PCV3 during 2005 - 2008, the samples of 13 gilts were used. PCV3 DNA was found in seven gilts. The PCR result was confirmed by DNA sequencing. The PCV3 positive samples were from 2005, 2006 and 2008. Since the earliest samples used in this study were from 2005, therefore it is possible that PCV3 might exist in Thailand before 2005. To investigate an association between PCV3 and abortion in gilts, PCV3 DNA was determined from the uterine samples of aborted gilts (n = 22) and control gilts (n = 21) from a farm in Thailand. The results showed that the prevalence of PCV3 DNA in the aborted gilts (45.45%, 10/22) was significantly higher than that of the control gilts (4.76%, 1/21). For aborted gilts, prevalence of lymphocytic endometritis in PCV3-positive gilts (n = 10) and PCV3-negative gilts (n = 12) was further analyzed. The results showed that the prevalence of the lesion in the PCV3-positive gilts (60.00%, 6/10) was not different from the PCV3-negative gilts (58.33%, 7/12). In conclusion, this study showed that PCV3 could be found in Thai pigs as early as 2005 and the presence of PCV3 in uterine tissues was associated with abortion. However, the interaction between PCV3 and other pathogens in abortion induction, and PCV3-associated lymphocytic endometritis should be further clarified.

Keywords: abortion, PCV3, uterine tissue, Thailand

Introduction

Porcine circovirus type 3 (PCV3) is newly identified virus in pigs. The pathogenesis of PCV3 is unknown. However, the virus has been detected in pigs with several clinical outcomes including reproductive failure in sows (Palinski et al., 2017; Tochetto et al., 2018; Wang et al., 2017), aborted/stillborn/mummified fetuses (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017). Other diseases found in PCV3-positive pigs include myocarditis (Phan et al., 2016), porcine dermatitis and nephropathy syndrome (PDNS) (Palinski et al., 2017; Wang et al., 2017), diarrhea (Zhai et al., 2017), respiratory problems (Kedkovid et al., 2018; Phan et al., 2016; Shen et al., 2018), and neurologic signs (Chen et al., 2017; Phan et al., 2016).

PCV3 was firstly reported in 2016 from pigs in the US (Palinski et al., 2017; Phan et al., 2016). However, various retrospective studies have later shown that PCV3 has been circulating in pigs for a very long time. Studies from Sweden (Ye et al., 2018), Spain (Klaumann et al., 2018), and China (Sun et al., 2018) showed that the virus might have existed in these countries at least since 1993, 1996, and 1996, respectively.

In Thailand, it has been demonstrated previously that PCV3 could be found in Thai pigs in 2017 (Kedkovid et al., 2018). However, it is possible that the virus has been introduced into Thailand before 2017. In this study, previously collected formalin-fixed paraffin-embedded (FFPE) uterine tissues collected during 2005 to 2008 from aborted gilts in Thailand were used to 1) determined whether PCV3 could be found in Thailand during 2005 to 2008, and 2) determined whether the presence of PCV3 in the uterine tissues associated with abortion in these gilts or not.

Materials and Methods

Tissue Samples and Study Design

Tissue samples used in this study were FFPE uterine tissues of gilts in Thailand collected during 2005 to 2008. These samples were received from the previous study (Tummaruk et al., 2009). To determine whether PCV3 could be found in Thai pigs during 2005 to 2008, a total of 49 FFPE uterine tissue samples from 13 aborted gilts from three farms located in Udonthani and Ratchaburi were used. Within these studied samples, there were 2 pigs from 2005, 10 pigs from 2006, and 1 pig from 2008. One of these three farms was further selected to determine whether the presence of PCV3 in uterine tissues could be associated with abortion during 2005 to 2008 or not. A total of 194 FFPE blocks from 43 gilts were used. Twenty two gilts were culled due to abortion (ABORT) group and 21 gilts served as control animals (CONTROL) group since they were culled due to non-reproductive problems eg leg problems.

Virus Detection

Viral DNA was extracted from the FFPE blocks using QIAamp DNA FFPE Tissue kit (Qiagen, Germany) according to the manufacturer's manual. Viral DNA was extracted from each individual FFPE block using three sections of 10 μm/FFPE block. PCR assays were then performed to detect swine viruses, including PCV3, PCV2, Aujeszky's disease virus (ADV), and porcine parvovirus (PPV). For PCV3 and ADV detection, previously established TaqMan-based real time PCR assays for each virus were used (Ma et al., 2008; Wang et al., 2017). For PCV2, SYBR green real-time PCR was used (Jittimanee et al., 2013). For PPV, conventional PCR was used (Veterinary Diagnostic Laboratory,

Chulalongkorn University). To confirm the negative result in PPV conventional PCR, two rounds of PCR were performed. PCR products from the first round were used as DNA samples for the second round PCR.

PCV3 Sequencing

PCR assay for partial ORF2 PCV3 sequencing was performed using primers newly designed in this study. Forward primer: 5' CCATGAACGTCATTTCCGTTGG 3' and reverse primer: 5' AGAAGAGGCTTTGTCCTGGG 3' were targeting an ORF2 region of nt 134 to 493 (360 bp amplicon). DreamTaq PCR Master Mix (Thermo Scientific, USA) was used for the PCR reactions. PCR products were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and submitted to First BASE Laboratories Sdn Bhd (Malaysia) for sequencing. The sequences were analyzed using BioEdit 7.0.9.0 and MEGA 5.2.

Histological Examination

The FFPE tissue samples from ABORT group were processed for hematoxylin and eosin (HE) staining and examined under the light microscope. The presence of lymphocytic endometritis, characterized by lymphocyte infiltration in the endometrium, was recorded.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Prevalence of gilts with PCV3-positive uterine tissue samples from ABORT and CONTROL groups were compared using Fisher's exact test. In ABORT group, gilts were subsequently divided to two groups according to PCV3 status including ABORT-PCV3-POS (aborted gilts found

PCV3 DNA in at least one of the FFPE tissue samples) and ABORT-PCV3-NEG (aborted gilts found no PCV3 DNA in none of the FFPE tissue samples). Prevalence of gilts showing lymphocytic endometritis from ABORT-PCV3-POS and ABORT-PCV3-NEG were compared using Fisher's exact test. Statistical significance was set at $p < 0.05$.

Results

PCV3 Found in Thai Pigs as Early as 2005

To determine the presence of PCV3 in Thai pigs during 2005 to 2008, FFPE uterine tissue samples from 13 aborted gilts from three farms in Thailand collected during that period were tested for PCV3 DNA by real-time PCR. PCV3 DNA was identified from gilts of all three farms. From 13 tested gilts, seven were positive. According to the sample collection year, all samples (2/2) from 2005, four of ten samples from 2006, and the one sample from 2008 were found positive. PCR for partial ORF2 DNA sequencing was further attempted from all positive samples. PCR product from the sample in 2008 (from a farm in Udonthani) showed the most intense band after 3 rounds of PCR and was then submitted for sequencing. This PCV3 strain was named 'PCV3/Thailand/UD01/08'. BLAST (https://blast.ncbi.nlm.nih.gov/) result based on the partial ORF2 sequences demonstrated that PCV3/Thailand/UD01/08 showed highest nucleotide identity of 99.30% with various previously reported PCV3 strains including Thai PCV3 collected in 2017 from a farm in Prachinburi, PCV3/Thailand/PB01/17 (GenBank accession no. MG310152).

The Presence of PCV3 in Uterine Tissues Associated with Abortion in Gilts

To identify an association between PCV3 and abortion, FFPE uterine tissue samples from gilts in one farm were further investigated. The results showed that the prevalence of gilts with PCV3-positive FFPE tissue samples from ABORT group was significantly higher than CONTROL group (Figure 1).

In ABORT group, prevalence of other viruses and prevalence of endometritis were further studied in ABORT-PCV3-POS (n = 10) and ABORT-PCV3-NEG (n = 12). The results showed that PCV2, ADV, and PPV were not found in those samples except one sample showing PPV positive result. This gilt was in ABORT-PCV3-NEG. Prevalence of lymphocytic endometritis in ABORT-PCV3-POS and ABORT-PCV3-NEG were not different; 60.0% (6/10) and 58.3% (7/12). Most of the lymphocytic endometritis lesions were of mild degree.

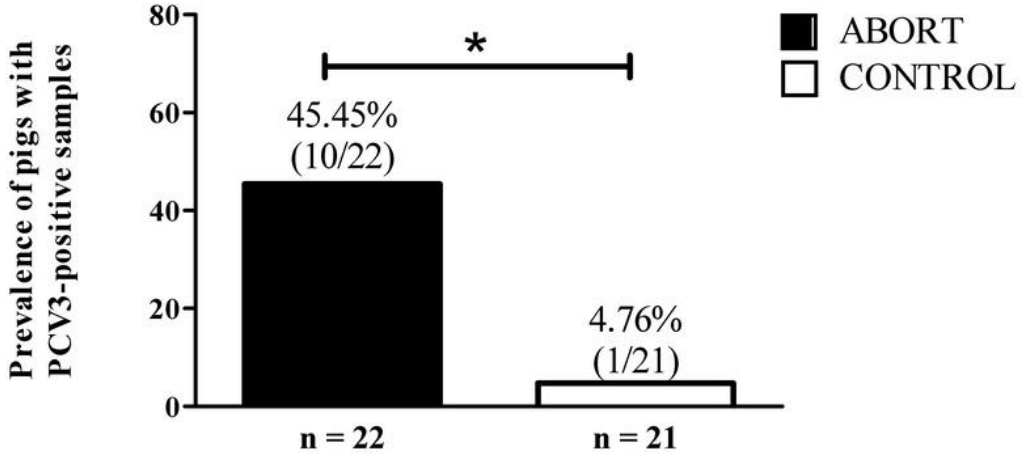

Figure 1 PCV3 detection from FFPE uterine tissue samples of aborted gilts (ABORT) and control gilts (CONTROL).

The prevalence of gilts with at least one PCV3-positive sample was determined using real-time PCR. Percentages of PCV3-positive gilts are shown. Number of positive animals and total animals are shown in the parenthesis.

Discussion

This study was conducted to investigate 1) the presence of PCV3 in Thailand during 2005 to 2008, and 2) an association between the presence of PCV3 in the uterine tissues and abortion. In the study, FFPE uterine tissue samples from gilts in Thailand collected between 2005 and 2008 were used.

Previously, it has been shown that PCV3 could be found in Thailand in 2017 (Kedkovid et al., 2018). However, it is possible that the virus has long existed in the Thai pig population. Retrospective studies in other countries have already shown that the virus has existed since at least 1990s (Klaumann et al., 2018; Sun et al., 2018; Ye et al., 2018). In this study, PCV3 DNA was identified from the uterine tissues of aborted Thai pigs collected in 2005, 2006, and 2008. The earliest collected tissues of the study were from 2005. Therefore, it is possible that PCV3 might have been circulating in Thailand before 2005.

In this study, BLAST result showed that the partial DNA sequence of PCV3/Thailand/UD01/08 (collected in 2008) showed high nucleotide identity with the recently identified Thai PCV3 from 2017, PCV3/Thailand/PB01/17 and also PCV3 from other countries. It is very interesting that whether PCV3/Thailand/UD01/08 could be the ancestor of PCV3/Thailand/PB01/17 and this PCV3 strain has been circulating in Thailand since 2005. More sequences and whole genome data are needed to clarify this issue.

This study also demonstrated for the first time that PCV3 can be identified from uterine tissues. This finding is not unexpected since PCV2 can be found in the uterus

as well (Limsaranrom et al., 2015; Pearodwong et al., 2015). It is interesting whether uterine tissues are a major target organ of PCV3 or not. More studies are needed.

Interestingly, in this study, the presence of PCV3 in the uterine tissues was shown to be associated with abortion. Previously, various investigations have shown that PCV3 could be identified from aborted sows/fetuses. This study demonstrated that the virus was associated with abortion. However, coinfection with other pathogens, especially PRRSV, could not be ruled out in this study. Only FFPE uterine tissues were used in this study. Therefore, the negative results against PCV2, PPV, and ADV did not confirm that the infection with these pathogens did not occur. For PRRSV, since FFPE tissue procession is well-known for RNA destruction PRRSV detection was not attempted in this study.

It has been suggested previously that (lymphocytic) endometritis following PRRSV infection could be one of the key mechanisms for abortion induction (Novakovic et al., 2016). In this study, lymphocytic endometritis could be observed in the PCV3-positive uterine tissues from aborted gilts as well. However, lymphocytic endometritis could also be found in PCV3-negative uterine tissues. It is still possible that lymphocytic endometritis in the PCV3-negative uterine tissues was induced by other pathogens or even by PCV3. Further studies using other tissue types should be done to clarify this issue.

Conclusion

In conclusion, this study showed that PCV3 could be found in Thailand as early as 2005. The results also indicated that the presence of PCV3 in the uterine tissues involved with abortion. Further studies on the mechanism of PCV3-associated abortion are needed, especially the role of coinfection with other pathogens.

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Conflict of Interest Statement

Conflict of interest: none

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CHAPTER III

PORCINE CIRCOVIRUS TYPE 3 (PCV3) SHEDDING IN SOW COLOSTRUM

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Abstract

The major objective of this work was to investigate the shedding of porcine circovirus type 3 (PCV3) in sow colostrum. PCV3 titers in the serum and colostrum samples of 38 sows were determined using qPCR. Interestingly, this is the first report regarding the identification of PCV3 from the colostrum samples. In the studied farm, the prevalence of PCV3 in the colostrum samples was 44.74% (17/38). When sows were grouped based on the PCV3 titers in the serum into the “High-viremic”, “Low-viremic” and “Non-viremic” sows, it was shown that the High-viremic sows showed significantly higher PCV3 colostrum prevalence (100%; 9/9) with the PCV3 titers ranging from 4.01 to 7.33 genomic copies/mL. The results indicated that PCV3 in the colostrum might be partly influenced by the viremic stage of the infection. However, the results also showed that approximately 41% of sows shedding PCV3 with low titers in the colostrum (7/17) were non-viremic sows. In conclusion, this study identified the presence of PCV3 in sow colostrum. Clinical impacts and mechanisms of colostrum shedding of PCV3 should be further investigated.

Keywords: colostrum, porcine circovirus type 3, sow, Thailand

Introduction

Porcine circovirus type 3 (PCV3) is a newly emerging virus in pigs, which has been reported worldwide. Although the pathogenesis of PCV3 is still unknown, the virus has been detected in pigs with several clinical outcomes including reproductive failure in sows (Palinski et al., 2017; Tochetto et al., 2018; Wang et al., 2017), aborted/mummified/stillborn fetuses (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017), myocarditis (Phan et al., 2016), porcine dermatitis and nephropathy syndrome (PDNS) (Palinski et al., 2017; Wang et al., 2017), diarrhea (Zhai et al., 2017), respiratory disease (Kedkovid et al., 2018; Phan et al., 2016; Shen et al., 2018), and neurologic disease (Chen et al., 2017; Phan et al., 2016). The virus can also be found in clinically healthy pigs (Kedkovid et al., 2018; Zheng et al., 2017).

PCV3 has been detected in pigs of different ages (Kedkovid et al., 2018; Kwon et al., 2017; Palinski et al., 2017; Stadejek et al., 2017). However, PCV3 transmission in the infected herds has not been well-characterized. In general, vertical transmission of the virus could be extremely crucial to maintenance and spread within a herd. Previously, it has been shown that PCV2 transmission via colostrum has played a major role for the infection of suckling piglets and the virus could consequently be found in weanling pigs later via horizontal transmission (Dvorak et al., 2013; Ha et al., 2010).

It should be noted that PCV3 shedding via the colostrum has not previously been reported. In this study, we describe the first known identification of PCV3 from colostrum samples of sows in a PCV3-infected herd in Thailand. The relationship of PCV3 titers in serum and colostrum samples was investigated and discussed

Materials and Methods

Samples and Data Collection

Matched serum and colostrum samples for this study were used with permission from a previous study (Juthamanee et al., 2017). The samples were collected cross-sectionally from 38 sows of parity 1–6, from a farm in Ratchaburi province, Thailand, in 2017. Both serum and colostrum samples were collected within 6 h after parturition. The sample collection protocol has been reviewed and approved by Chulalongkorn University Animal Care and Use Committee (Animal use protocol number: 1731064). In this farm, porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 are endemic and sow vaccinations against these viruses are routinely performed. PCV3 was previously identified in this farm by PCR during farm monitoring. Reproduction parameters of the studied sows including total born (TB), born alive (BA), stillborn (SB), and mummified fetuses (MF), were retrieved from the farm records.

Virus Detection and Quantification

Virus nucleic acid was extracted from the serum and colostrum samples using NucleoSpin RNA Virus Kit (Macherey-Nagel, Germany). Prior to the extraction, the fat layer was removed from the colostrum samples by centrifugation at 900gfor 10 min, as previously described (Ha et al., 2010). The serum was used directly for the extraction. For each extraction reaction, 150 μL of the samples were used. For PCV3 detection and quantification, a previously published TaqMan-based qPCR assay (Wang et al., 2017) targeting nt 1351 to 1428 of PCV3 genome (ORF2) was used with minor modification. Briefly, the PCR reaction was performed using QuantiFast Probe PCR Kit (Qiagen, Germany) and MyGo Pro (IT-IS Life Science, Ireland) instrument. DNA fragment

covering nt 601 to 1626 of PCV3 genome was generated using previously published PCR assay (Palinski et al., 2017) and used in the standard curve generation. This DNA fragment was also used as a positive control for the qPCR assay. The standard curve was generated using 10-fold serial dilutions ranging from 10^8 to 10^1 copies/μL of the positive control DNA fragment. Copy number calculation of the target DNA was done using the internal software of the instrument. The detection limit of the assay was 4 log genomic copies/mL. PCV2 detection was done using a previously published conventional PCR assay targeting PCV2 ORF1 (Paphavasit et al., 2009) with minor modification. The PCR reaction was performed using DreamTaq Green PCR MasterMix (Thermo Fisher Scientific, USA). The PCR product of 356 bp was determined using 1.5% agarose-gel electrophoresis.

To confirm the presence of PCV3 in the colostrum sample, DNA sequencing was performed. A colostrum sample showing high PCV3 titer was randomly selected. PCR targeting partial ORF2 region (nt 1 to 363 of the full 645 nt ORF2) of PCV3 was done using a previously reported assay (Palinski et al., 2017). The PCR product was submitted to First BASE Laboratories Sdn Bhd (Malaysia) for sequencing. The sequence was analyzed using BioEdit 7.0.9.0. The sequence of this Thai PCV3 was named PCV3/Thailand/RB01/17 and deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) with the accession number MH158731. Nucleotide identity calculation and cladogram generation were done using MEGA 5.2. Previously reported ORF2 DNA sequences of PCV3 (n = 177), PCV2 (GenBank accession number AF027217), and PCV1 (GU799575) were retrieved from GenBank for nucleotide identity calculation. For cladogram, PCV3 ORF2 sequences used (n = 51) were from 1) PCV3 previously characterized into four subgroups: a1, a2, b1, and b2 (n = 40) (Fux et al., 2018); 2) PCV3 strains showing high

nucleotide identity (over 99.00%) with PCV3/Thailand/RB01/17 (n = 4); 3) a previously reported Thai PCV3 (n = 1); and 4) recently reported PCV3 strains from Italy and Denmark (n = 5).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. The overall prevalence and virus titers of PCV3 between the serum and colostrum samples were compared using McNemar test (for prevalence comparison) and Wilcoxon signed rank test (for titers comparison). The relationship between the PCV3 titers in the serum and colostrum samples was determined using Spearman's rank correlation test. The strength of the correlation was interpreted from the Spearman's rho values as follows: 0.00 to 0.19, very weak; 0.20 to 0.39, weak; 0.40 to 0.59, moderate; 0.60 to 0.79, strong; 0.80 to 1.00, very strong. Pigs were divided into three groups based on the PCV3 titers in the serum including "Nonviremic" (PCV3 was not detected), "Low-viremic" (PCV3 titers below the median of the positive serum titers), and "High-viremic" (PCV3 titers above the median of the positive serum titers). Prevalence and titers of PCV3 in the colostrum were compared among these three groups using Fisher's exact test (for prevalence comparison) and Kruskal-Wallis H test (for virus titers comparison). Comparisons of reproduction parameters between PCV3 positive and negative animals were done using Mann Whitney U test. Statistical significance was set at $p < 0.05$. Mean values were reported as mean ± standard deviation.

Results

PCV3 Prevalence in Serum and Colostrum Samples Were Not Different

The prevalence of PCV3 in the serum and colostrum samples was determined to provide an initial data on PCV3 viremia and colostrum shedding status. The numbers of PCV3-positive serum and colostrum samples using qPCR from all sows are shown in Table 4. The prevalence of the PCV3-positive serum and colostrum samples were not statistically different (Figure 2A). The virus titers between these sample types were not statistically different either. The average PCV3 titers in the serum and colostrum of the positive animals were 5.06 ± 0.44 (n = 18) and 5.02 ± 1.08 (n = 17) log genomic copies/mL, respectively. When taking both types of samples into account, the prevalence of PCV3-positive animals (in at least one type of the samples) increased to 65.79% (25/38). However, it was not significantly different from the single sample prevalence. PCV3 prevalence in the sows of each parity is shown in Figure 2B.

Table 4 PCV3 DNA detection results from the serum and colostrum samples

		Serum		
		Positive	Negative	Total
Colostrum	Positive	10	7	17
	Negative	8	13	21
	Total	18	20	38

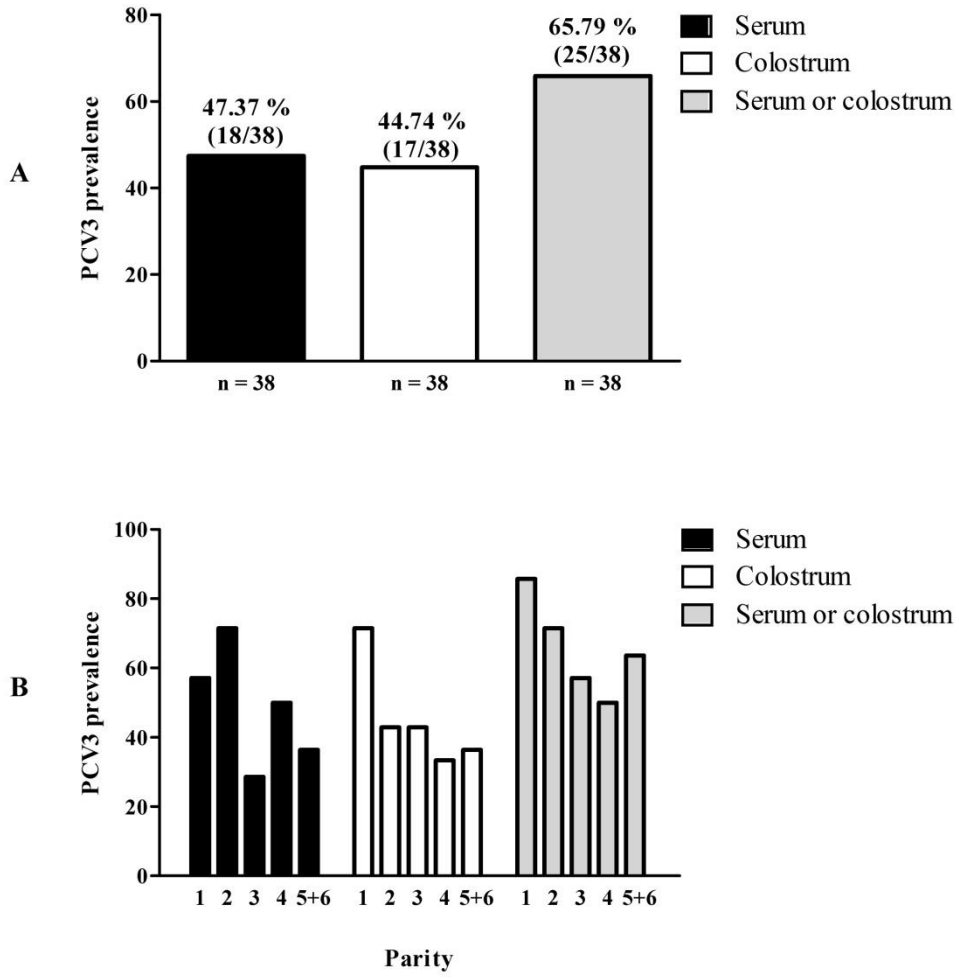

Figure 2 Prevalence of PCV3 in the serum and colostrum samples of sows.

Overall prevalence of PCV3 in each sample type (A) and PCV3 prevalence in different parities of sows (B) were measured using qPCR. Percentages of PCV3 positive samples are shown above the graph. Number of positive animals and total animals are shown in the parenthesis.

The presence of PCV3 in the colostrum samples was confirmed by DNA sequencing. Partial ORF2 DNA sequences of PCV3/Thailand/ RB01/17 showed 95.04 to 99.17% nucleotide identity with the other PCV3 strains. However, the virus showed 54.41 and 52.92% nucleotide identity with PCV2 and PCV1, respectively. PCV3/Thailand/RB01/17 showed 97.80% nucleotide identity with the previously reported Thai PCV3. The cladogram showed that PCV3/Thailand/RB01/17 clustered in PCV3a subgroup (Figure 3).

PCV3 Titers in the Serum and Colostrum Were Positively Correlated

To gain more information regarding the relationship between PCV3 in the serum and colostrum samples, correlation between the PCV3 DNA from both sample types was determined. From the data of all sows, a significant positive correlation of PCV3 titers between the serum and colostrum samples was found, with the correlation coefficient of 0.49. The colostrum shedding was then characterized in more detail regarding to the PCV3 viremia level.

Sows were further divided into three groups based on the viremia status, including High-viremic, Low-viremic and Non-viremic sows. The serum virus titers of the serum-positive animals ranged from 4.36 to 6.15 log genomic copies/mL with the median of 5.04 log genomic copies/mL. The prevalence and titers of PCV3 in the colostrum samples of the Non-viremic, Low-viremic, and High-viremic sows are shown in Figure 4. The PCV3 titers in the colostrum of the non-viremic and viremic pigs ranged from 4.06 to 4.42 and 4.01 to 7.33 log genomic copies/mL, respectively. The prevalence and titers of PCV3 in the colostrum samples of the High-viremic sows were significantly higher than the other groups (Figure 4A and 4B, respectively). It should be noted that PCV2 was not detected in any of the colostrum samples tested.

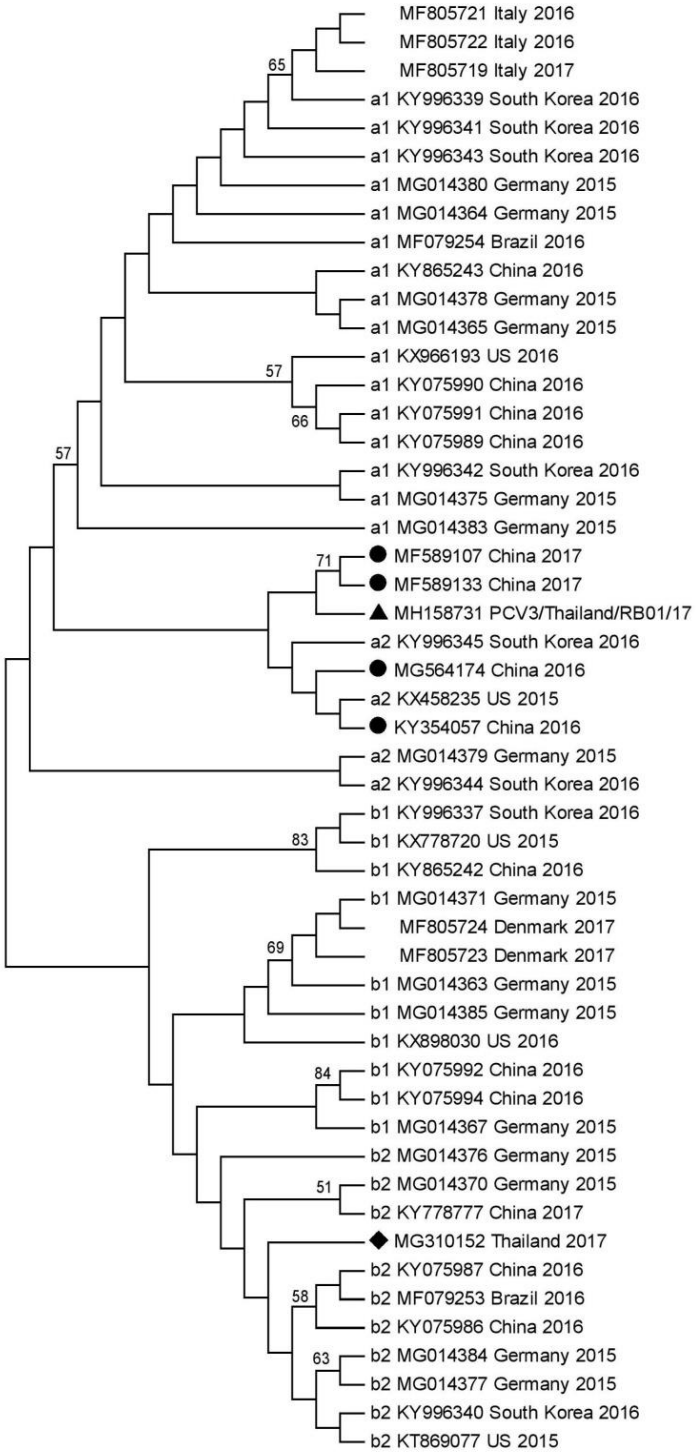

Figure 3 Maximum likelihood cladogram of PCV3/Thailand/RB01/17 and other PCV3 strains based on partial ORF2 nucleotide sequences.

The tree was constructed based on the general time reversible model with G + I. Bootstrap values (1,000 replicates) for each node are displayed next to the branch if > 50%. PCV3 strains are shown with the GenBank accession numbers, the country of origin, and the collection year. PCV3/Thailand/RB01/17 is labeled with a black triangle. The previous Thai PCV3 is labeled with a black diamond. PCV3 strains with high nucleotide identity (> 99.00%) with PCV3/Thailand/RB01/17 are labeled with black circles. PCV3 strains previously classified into subgroups; a1, a2, b1, and b2 (Fux et al., 2018); are shown with prefixes indicating the subgroup.

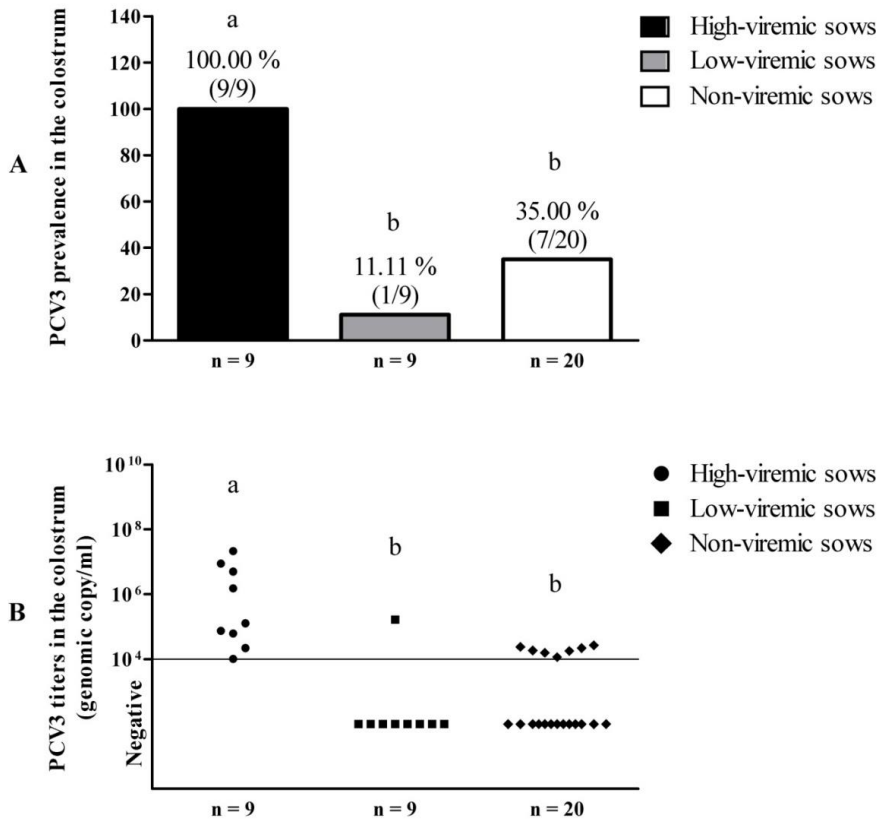

Figure 4 PCV3 detection from the colostrum of sows with different PCV3 viremia statuses.

PCV3 was measured in the colostrum samples of the High-viremic, Low-viremic, and Non-viremic sows. Prevalence (A) and virus titers (B) of PCV3 were determined using qPCR. Percentages of PCV3 positive samples are shown above the graph. Number of positive animals and total animals are shown in the parenthesis. A horizontal line (B) indicates the detection limit of the qPCR at 10^4 genomic copies/ml samples. Lower case letters above the graphs indicate statistically significant difference using Fisher's exact test (A) and Kruskal-Wallis H test (B) with $p < 0.05$.

Performance of Sows with Different PCV3 Statuses Were Not Different

To describe the clinical impact of PCV3 viremia/shedding status in this farm, sows' reproduction parameters were determined. The numbers of TB and BA were not significantly different between the PCV3-viremic sows (12.94 ± 2.78 and 12.61 ± 2.75, respectively) and nonviremic sows (12.30 ± 4.26 and 10.85 ± 3.36, respectively). The numbers of TB and BA in the sows with PCV3-colostrum shedding (12. 53 ± 3.41 and 11.59 ± 2.62, respectively) were not significantly different from the non-shedding sows (12.67 ± 3.83 and 11.76 ± 3.62, respectively).

Discussion

This study was conducted to investigate the colostrum shedding of PCV3 in sows from a PCV3-infected herd in Thailand. An association between colostrum shedding and viremia status, and the colostrum shedding patterns of sows of each parity were also described.

At present, vertical transmission of PCV3 is not well characterized. From previous studies, two routes including trans-placenta and direct-contact transmission could be implied. It has been shown that PCV3 could be detected from sows showing reproductive failure and also from the mummified and stillborn fetuses (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Tochetto et al., 2017), suggesting transplacental transmission. Direct-contact transmission might occur from the virus shedding via oral fluids. A work from Kwon and colleagues showed that PCV3 can be found in the oral fluid samples of naturally infected pigs (Kwon et al., 2017). Additionally, PCV3 DNA can also be detected in the salivary gland (Kedkovid et al., 2018). In this study, we showed the first evidence of shedding of PCV3 via colostrum.

Interestingly, there was a relationship between the colostrum shedding and the viremia status. In the studied farm, the PCV3 prevalence in the colostrum was not different from the serum (approximately 45 to 47%). The results further showed that the virus titers in the serum and colostrum were positively correlated. These results indicated that, as could be expected, PCV3 shedding in the colostrum might in part be influenced (directly or indirectly) by the virus in the blood circulation. However, the degree of correlation between the colostrum and serum titers was found to be only 'moderate'. This could be partly explained by the pattern of PCV3 shedding in the colostrum observed in this study. Apparently, when the serum titers were above the median value (5.04 log genomic copies/mL), all sows shed PCV3 in the colostrum. When the serum titers were below the median value, the colostrum virus titers, if present, dropped rapidly to the baseline level, rather than gradually decreasing. It was also shown in this study that approximately 41% of pigs (7/17) showing PCV3 in the colostrum were non-viremic pigs. All of these pigs shed low amount of PCV3 in the colostrum. Similarly, shedding by non-viremic pigs could also be observed in PCV2-infected pigs (Schmoll et al., 2008), and could occur in the late or recovery stage of sows, before farrowing. However, the mechanism for this has not yet been clarified. For PCV3, further studies should also be conducted to investigate the mechanisms of colostrum shedding, especially in the non-viremic pigs.

The colostrum titers of PCV3 observed in this study is comparable to a previous study of PCV2. Dvorak and colleagues have shown that in five studied farms, average PCV2 titers in the colostrum ranged from approximately 4 to 7 genomic copies/mL (Dvorak et al., 2013). In the present study, PCV3 titers in the colostrum also ranged from 4.01 to 7.33 genomic copies/mL. In this study, the prevalence of PCV3 shedding

in the colostrum of the primiparous sows was approximately 71% while the multiparous sows showed approximately 33 to 43%. Not only that, lower parity sows tend to have more positive animals than higher parity sows based on the presence of PCV3 in the serum and colostrum. This is in line with our previous study (Kedkovid et al., 2018). In PCV2, the clinical impacts of primiparous sows were also reported (Fraile et al., 2009). It was shown that piglets from primiparous sows had higher risk of PCV2-PRRSV coinfection compared with the multiparous sows. The impacts of PCV3 shedding in primiparous sows should be further investigated so effective management and control can be identified in the future.

Clinical impact of PCV3 shedding in the colostrum was not observed in this study. The sow performance parameters were not different among sows with different PCV3 statuses. It is possible that the number of sows used in this study is not sufficient to detect a small or infrequent effect. More importantly, it should be noted that data regarding PCV3 (and other pathogens) identification and clinical signs in the suckling piglets of these sows were not available in this study. Pathological study of PCV3 infected pigs during suckling period is still limited. In one study, PCV3 has been identified in a 19 days old pig (Phan et al., 2016). Clinical signs of the pigs included severe dyspnea and neurologic disease. Microscopic examination showed interstitial lymphocytic myocarditis, histiocytic interstitial pneumonia and acute bronchitis. Together with PCV3, porcine astrovirus 4 and equine hepacivirus were also identified from that pig using metagenomic approach. It is not clear whether PCV3 was the major pathogen (or a pathogen) in the diseased pig or not.

It is also possible that protective antibody against PCV3 is present in the colostrum. It is well-known that PCV2 maternal immunity protects the piglets against porcine circovirus associated disease (PCVAD), but not the PCV2 infection. PCVAD can become apparent after the decline of the maternally derived immunity (Calsamiglia et al., 2007; Dvorak et al., 2013; Madson and Opriessnig, 2011; McKeown et al., 2005). Early PCV3 infection (from PCV3 in the colostrum) might affect pigs at later stages of production. Recently, it has been shown that PCV3 could be involved in porcine respiratory disease complex (PRDC) in grower pigs (Kedkovid et al., 2018). In the previous study, PCV3 infection occurred as early as 5 weeks of age (the earliest age of the study). Subclinical infection of PCV3 was also identified in that study. Therefore, PCV3 infection during suckling period might result in a more severe outcome in the nursery and grower periods.

Colostral shedding of PCV2 was not observed in this study while the shedding of PCV3 was found in approximately 45% of the tested sows. Interestingly, it has been previously shown that coinfection between PCV3 and other viruses especially PCV2 could be observed (Ku et al., 2017; Zhang et al., 2018; Zheng et al., 2018). The absence of PCV2 in the colostrum in this study may be partly due to the routine sow vaccination against PCV2 (Madson et al., 2009). It has been shown that PCV2 dynamics could be affected by co-infection with other pathogens (Sinha et al., 2011). It is unknown whether PCV3 shedding pattern would be altered or not if animals were co-infected with other pathogens. Further studies are needed to clarify the effects of co-infection on PCV3 shedding.

Previously, it has been suggested that PCV3 can be classified into two major groups PCV3a and PCV3b (Fux et al., 2018). PCV3 identified in this study belongs to PCV3a as the virus clustered with the previously proposed PCV3a (Fux et al., 2018). It would be interesting to determine whether PCV3 genetic variation could affect colostrum shedding or not. Further studies are needed to clarify this issue.

Conclusion

In this study, the results showed that PCV3 can be shed in the sow colostrum. In the studied farm, 44.74% of the studied sows shed PCV3 in the colostrum. PCV3 titers in the serum and colostrum samples were positively correlated. However, it should be noted that non-viremic sows also shed low levels of PCV3 in the colostrum. The clinical impact of PCV3 shedding in the colostrum should be further investigated.

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Conflict of Interest Statement

Conflict of interest: none

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CHAPTER IV

PORCINE CIRCOVIRUS TYPE 3 (PCV3) INFECTION IN GROWER PIGS FROM A THAI FARM SUFFERING FROM PORCINE RESPIRATORY DISEASE COMPLEX (PRDC)

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Abstract

Porcine circovirus type 3 (PCV3) is a newly emerging virus with unknown pathogenesis. The major objective of this study was to investigate the presence of PCV3 in pigs from a farm in Thailand suffering from porcine respiratory disease complex (PRDC). Initially, a Thai PCV3 strain (PCV3/Thailand/PB01/17) was identified from a pig originated from a farm with PRDC problem during grower period and whole genome analysis showed that the Thai PCV3 shared highest nucleotide identity of 99.60% with the South Korean strain PCV3/KU-1602. The presence of PCV3 infection in PRDC-affected pigs was then investigated in this farm. Serum samples from clinically healthy pigs and pigs showing PRDC-related clinical signs during 5 to 18 weeks were used in PCV3 detection by PCR. The results showed that the PRDC-affected pigs exhibited higher prevalence of PCV3 infection and higher PCV3 titers comparing with the clinically healthy pigs. These results confirmed the presence of PCV3 in a Thai farm with PRDC problem. The pathogenesis of PCV3 on PRDC should be clarified in further studies.

Keywords: porcine circovirus type 3, porcine respiratory disease complex, Thailand

Introduction

Circoviruses are non-enveloped viruses with circular, single stranded DNA genomes of the family *Circoviridae*. Circoviruses contain two major open reading frames (ORFs) including ORF1 and ORF2, coding for replicase and capsid protein, respectively. Currently, there are three circoviruses in pigs including porcine circovirus (PCV) 1, PCV2, and recently discovered PCV3. The pathogenesis of PCV3 is yet to be elucidated. Since 2016, PCV3 has been identified in the US (Palinski et al., 2017; Phan et al., 2016), Brazil (Tochetto et al., 2017), the UK (Collins et al., 2017), Italy (Faccini et al., 2017), Poland (Stadejek et al., 2017), China (Chen et al., 2017; Fan et al., 2017; Ku et al., 2017; Shen et al., 2017; Zhang et al., 2017; Zheng et al., 2017), and South Korea (Kwon et al., 2017). Previously, PCV3 has been found in pigs with porcine dermatitis and nephropathy syndrome (PDNS) (Palinski et al., 2017; Stadejek et al., 2017), reproductive failure (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Stadejek et al., 2017), and congenital tremor (Chen et al., 2017). It is possible that PCV3 could induce a broad range of diseases similar to PCV2.

Porcine respiratory disease complex (PRDC) is a major respiratory disease affecting the swine industry worldwide. The disease is characterized by reduced growth and feed efficiency with clinical signs including cough, dyspnea, fever and anorexia (Chae, 2016). Co-infection of various pathogens is the major cause of PRDC. In Thailand, PRRSV (Thanawongnuwech et al., 2004), PCV2 (Jantafong et al., 2011), and SIV (Arunorat et al., 2016) are commonly found causing PRDC. The present study firstly identified PCV3 in Thailand and determined the presence of PCV3 infection in PRDC-affected pigs in a Thai swine herd.

Materials and Methods

Tissue Samples

Tissue samples used in this study were from the tissue collection bank of the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). All samples were collected during July and September 2017 from a 3000-sow herd in Prachinburi province, Thailand. PRRSV is endemic and PRDC in grower pigs, especially during 18 weeks of age, is the major health problem in this farm. The samples include fresh frozen tissue and formalin-fixed paraffin-embedded (FFPE) tissue samples of various organs from 8 necropsied pigs of 5 to 7 weeks old and serum samples from 50 grower pigs of 5 to 18 weeks old. The serum samples were from both clinically healthy pigs and pigs showing PRDC-related signs (n = 25, each). The serum samples from 20 sows of parity 1 to 4 (n = 5, each) were also collected.

Virus Nucleic Acid Extraction

For each nucleic acid extraction reaction, 30 mg of the solid tissue or 150 µl of the serum samples were used. The solid tissues were homogenized in modified Eagle's medium (MEM) prior to the extraction process while the serum samples were used directly. Virus nucleic acid was extracted using NucleoSpin RNA Virus Kit (Macherey-Nagel, Germany).

PCV3 Genome Sequencing

PCR assay for PCV3 genomic sequencing was performed using previously published primers (Palinski et al., 2017). DreamTaq PCR Master Mix (Thermo Scientific, USA) was used for the PCR reactions. PCR products were purified using NucleoSpin Gel

and PCR Clean-up Kit (Macherey-Nagel, Germany) and submitted to First BASE Laboratories Sdn Bhd (Malaysia) for sequencing. DNA sequencing of each DNA fragment was done twice from two separated PCR reactions. The sequences were analyzed using BioEdit 7.0.9.0. The sequence of the Thai PCV3 named PCV3/Thailand/PB01/17 in this study was deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) with the accession number MG310152. Whole genome sequences of other PCV3 from GenBank were retrieved for comparisons (n = 30). MEGA 5.2 was used for nucleotide identity calculation and phylogenetic tree construction. The trees were generated using the maximum-likelihood method with 1000 bootstrapping replications. SimPlot 3.5.1 was used to identify recombination events in the Thai strain.

Detection and Quantification of PCV3

PCV3 titration was done using TaqMan-based qPCR with a previous published assay (Wang et al., 2017) with some modifications. Briefly, the PCR reaction was carried out using QuantiFast Probe PCR Kit (Qiagen, Germany) with MyGo Pro (IT-IS Life Science, Ireland) instrument. Copy number of the target DNA was calculated using internal software of the instrument. The detection limit of the assay were 3 log genomic copies/g and 3 log genomic copies/ml for solid tissue and serum samples, respectively.

Histological Examination

The FFPE tissue samples were processed for hematoxylin and eosin (HE) staining as the standard protocol of the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. The slides were examined under the light microscope. *In situ* hybridization (ISH) was performed from the lung tissue of LK003 using the standard protocol of the Department of Pathology, Faculty of Veterinary

Medicine, Kasetsart University. Briefly, the digoxigenin (DIG) probe was prepared using PCR DIG Labeling Mix (Roche, Switzerland) with primers targeting PCV3 ORF1; primer 1: 5′ ATACTGCAGGCATCTTCT- CCG 3′ and primer 2: 5′ TATTGTGGAGTGTGGAGGCAGT 3′ (PCR product size of 336 bp). Anti-Digoxigenin-AP, Fab fragments (Roche, Switzerland)was used for targeting the DIG probe. Hybridization signal was detected as deep purple colorimetric staining of NBT/BCIP with Nuclear Fast Red (Sigma) counterstaining. ISH based on lung tissue section from PCV3-negative pig (by PCR) and ISH based on LK003 lung tissue section without the DIG probe were used as control.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Prevalence of PCV3 infections of the clinically healthy and PRDC-affected pigs were compared using Fisher's exact test. Virus titers based on qPCR in the serum of the clinically healthy grower pigs and pigs with PRDC were log transformed and compared using t-test. Statistical significance was set at $p < 0.05$. Virus titers were expressed as the mean value ± standard deviation.

Results

PCV3 Was Identified from PRDC-Affected Pigs from a Farm in Thailand

From eight necropsied pigs showing PRDC-related clinical signs, PCV3 DNA was identified in the lung and lymph node samples of five pigs, LK000–LK004. The common macroscopic lesions of the PCV3-positive animals included redden and firm lungs together with enlargement of both tracheobronchial and superficial inguinal lymph nodes. Microscopic examination showed that broncho-interstitial pneumonia, and

lymphoid necrosis and histiocytic proliferation of the lymph nodes were the common lesions. LK003 showed the most severe lesions with severe accumulation of cellular debris in the alveolar space and diffuse interalveolar septal thickening by mononuclear cells, resembling proliferative and necrotizing pneumonia (PNP). The PNP-related lesions were not observed in other pigs. PRRSV and PCV2 were identified in only LK003 by PCR from pooled lung/lymph node tissues. Alpha-hemolytic streptococcus and *Pasteurella multocida* was also identified in LK003 and some other animals. It should be noted that these pigs were treated with antibiotics prior to the necropsies. ISH of the lung tissue of LK003 showed that lymphocytes, especially infiltrating lymphocytes in the alveolar space, were the major cellular population with positive signals (Figure 5). Positive signal was not observed from the ISH using the lung tissue of LK003 without addition of the DIG probe and the ISH using the lung tissue of the PCV3-negative pig.

The Thai PCV3 DNA Sequence is Highly Similar to Other PCV3 Strains

Genomic DNA sequencing was performed from the lung tissue of LK000. This PCV3 strain was named ‘PCV3/Thailand/PB01/17′. The Thai PCV3 showed highest genomic nucleotide identity (99.60%) with the Korean strain PCV3/KU-1602 (KX828229). The ORF1 and ORF2 of the Thai PCV3 showed 100.00% to 98.32% and 99.53% to 96.90% nucleotide identity with the other PCV3 strains, respectively. Phylogenetic tree based on the genome sequences are shown in Figure 6. Phylogenetic analysis based on ORF1 and ORF2 yielded similar results. Recombination is not detected in the Thai PCV3 genome.

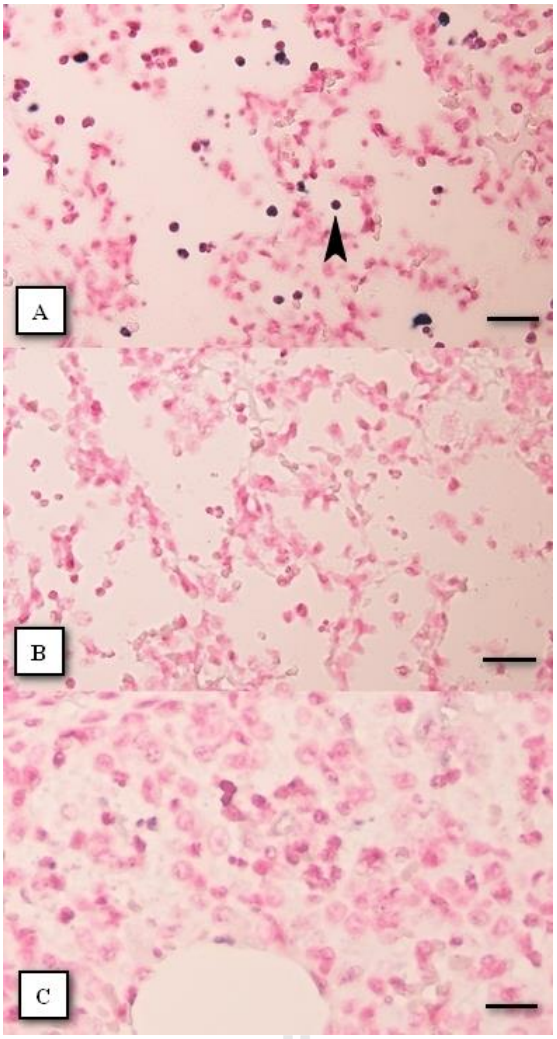

Figure 5 *In situ* hybridization results from the lungs of PRDC-affected pigs.

A PRDC-affected pig with positive PCV3 signals found in lymphocytes (arrow head) (A). The tissue sections were incubated with (A) or without (B) DIG probe targeting PCV3 ORF1 (no-probe control) and from a PRDC-affected pig with negative PCV3-PCR result (negative control) (C). Anti-DIG-AP, Fab fragments was used to target the DIG probe. Hybridization signal was detected as deep purple staining of NBT/BCIP. Counterstaining was done with Nuclear Fast Red. Bars are equivalent to 20 μm.

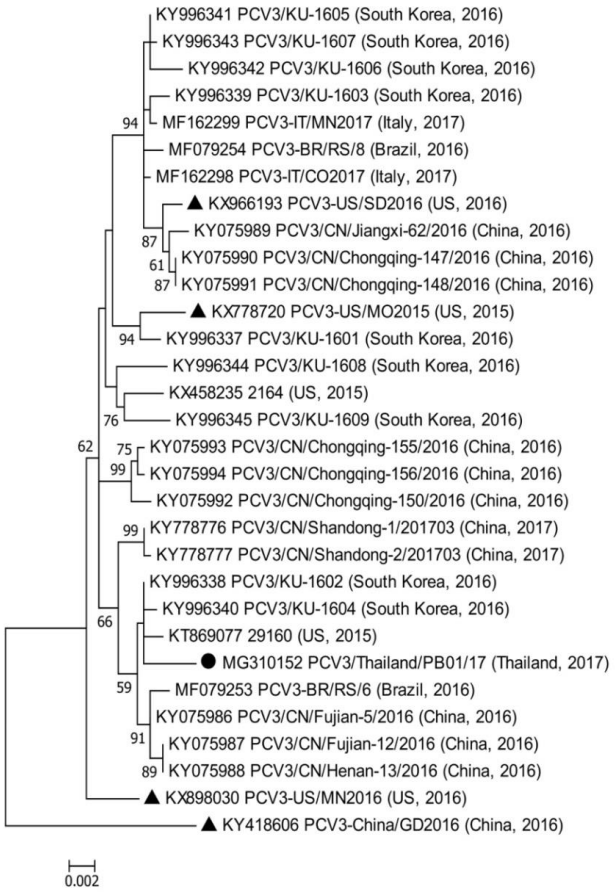

Figure 6 Maximum likelihood phylogenetic tree of PCV3/Thailand/PB01/17 and the other PCV3 strains based on full-genome nucleotide sequences.

The tree was constructed based on the general time reversible model with G + I. Bootstrap values (1,000 replicates) for each node are displayed next to the branch if > 55%. PCV3 strains are shown with the GenBank accession numbers and the country of origin and the collection date are in parenthesis. PCV3/Thailand/PB01/17 is labeled with black circle and previously reported PCV3 strains from pigs with respiratory problems are labeled with black triangle.

PRDC-Affected Grower Pigs Showed Higher PCV3 Infections

After the identification of PCV3 from pigs in this farm along with the ongoing PRDC problem in the grower pigs, PCV3 infection in PRDC-affected pigs was further investigated in this farm. The prevalence of PCV3 viremia in the PRDC-affected pigs (60%, 15/25 animals) was significantly higher than that of the clinically healthy pigs (28%, 7/25 animals) (Figure 7). Overall, PCV3 titers in the PRDC-affected pigs (3.24 ± 2.80 log genomic copies/mL) were also significantly higher than those of the clinically healthy pigs (1.61 ± 2.67 log genomic copies/mL). Dynamic of PCV3 infections in grower pigs are shown in Figure 7. Prevalence of PRRSV and PCV2 viremia were also determined from the serum of the PRDC-affected pigs. PRRSV was found at only 16 and 18 weeks of age (100% prevalence, 5/5 animals, in both age group) while PCV2 was not detected in any age groups (Figure 7).

PCV3 Showed Higher Virus Titers in the Lungs and Lymph Nodes

PCV3 titration was further done from serum, lung, and lymph nodes of LK001 to LK004 to investigate more on PCV3 distribution in PRDC-affected pigs. PCV3 titers were found higher in the lung and pooled lymph node samples in all animals, comparing with the serum samples (Figure 7). PCV3 was observed in the serum of only one pig, LK003. The virus titer was further investigated in seven additional tissues of LK003, including heart, tracheobronchial lymph node, spleen, liver, tonsil, kidney and salivary gland. PCV3 was found in all tested tissues with the titers ranging from 8.00 (salivary gland) to 9.85 (heart) log genomic copies/g.

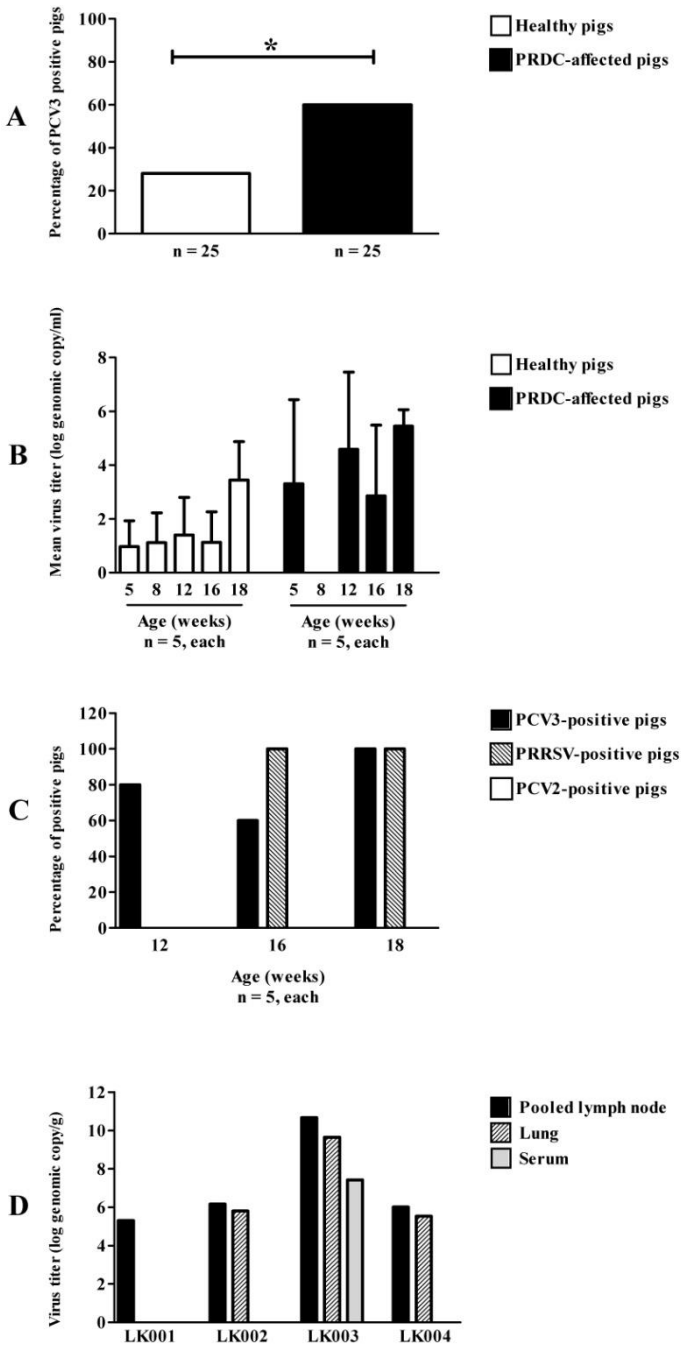

Figure 7 PCV3 prevalence and titers in pigs from a PRDC-affected farm.

PCV3 was quantified from healthy and PRDC-affected pigs. Overall prevalence of PCV3 infection (A) and PCV3 titers at each age (B) were measured using qPCR from the serum samples. The prevalence of PCV3 was from qPCR data regardless of the virus titers. Prevalence of PCV3, PRRSV and PCV2 viremia were evaluated from PRDC-affected pigs (C). PCV3 titers were evaluated in pooled lymph node, lung, and serum samples of pigs showing PRDC-related pathological lesions (D). Error bars represent standard deviation values. A star indicates statistically significant difference using Fisher's exact test with $p < 0.05$.

Low Parity Sows Showed Higher PCV3 Viremia

PCV3 DNA was quantified from the serum samples of the sows of parity 1 to 4. Overall, PCV3 was detected from 60% (12/20 animals) of the tested sows. The virus titer was peaked at the parity 2 (6.05 ± 0.79 log genomic copies/mL) and then, continuously declined at the parity 4 (1.07 ± 2.40 log genomic copies/mL).

Discussion

This study was carried out to investigate the presence of PCV3 in the grower pigs from the PRDC-affected farm in Thailand. Genetics of the Thai PCV3 strain and initial data on virus distribution in the PRDC-affected pigs were also described in this study.

In this study, PCV3 was firstly recognized in Thailand. The genetics of the Thai PCV3 is closely related with certain PCV3 strains such as the South Korean strain (eg PCV3/KU-1602), the US strain (29160), the Chinese strain (eg PCV3/CN/Fujian-12/2016), and the recently reported Brazilian strain (PCV3-BR/RS/6). However, the phylogenetic analysis showed low bootstrap support of the clustering. Since phylogenetic analysis using ORF1 and ORF2 sequences yielded similar clustering patterns as the genomic sequences and recombination events were not detected in the Thai PCV3 genome, this finding could represent that the low bootstrap value was in part due to 1) the unique evolution of PCV3 in Thailand suggesting that the virus might be introduced into Thailand for a period of time, or 2) the Thai virus might originate from other unreported PCV3 strain. More sequences, from Thailand and other regions, are needed to clarify the clustering of the virus. It should also be noted that the Thai strains might have multiple origins and the sequence of PCV3/Thailand/PB01/17 alone might not be

enough to answer this question. It has been shown previously, and also from this study, that PCV3 strains of the same country might be distantly located on the phylogenetic tree (Ku et al., 2017; Kwon et al., 2017; Tochetto et al., 2017). Genetic variation of PCV3 in Thailand should, therefore, be studied.

This work demonstrated PCV3 infection in PRDC-affected pigs at the farm level. Previously, PCV3 was found in pigs showing PDNS (Palinski et al., 2017; Stadejek et al., 2017) and reproductive failure (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017;Stadejek et al., 2017). In this study, prevalence of PCV3 infections and PCV3 titers were greater in the pigs with PRDC-related clinical signs comparing to the clinically healthy pigs. The association between PCV3 and PRDC should be further studied whether 1) PCV3 infections directly induce PRDC, 2) PCV3 infections render the pigs more susceptible to PRDC development or 3) other factors in the PRDC-affected pigs promote PCV3 replication, similar to PCV2 infection (Patterson et al., 2015). It is also possible that PCV3does not have any critical role in PRDC. In the present study, it is not surprised that the healthy grower pigs showed extremely low PCV3 titers in the serum. In the PRDC-affected pigs, although the virus titer was significantly higher than the clinically healthy pigs, they can still be considerably low. This could be partly due to the fact that PCV3 was mainly found in the later phase of the grower period, especially at 18 weeks of age, which is when PRDC prevalence is high in this farm. Not only that, PCV3 was also found in non-viremic pigs (LK001, LK002 and LK004). In this study, PCV3 infection at 12 weeks of age was followed by PRRSV infection at 16 weeks of age, concurrently with the high PRDC prevalence at 18 weeks of age. The association between these two viruses should be further studied. Additionally, having higher virus titers in lymph nodes, showing the presence of the virus in lymphocytes, and having

lymphoid necrosis in PCV3-positive pigs, association between PCV3 and pig's immune system should be studied. PCV3 antigen was previously identified in lymphocytes in lymph nodes using immunohistochemistry (Palinski et al., 2017). However, comparing PCV2, the major cellular population in lymph nodes showing positive signal against the virus was suggested to be follicular dendritic cells (Hansen et al., 2010) rather than lymphocytes. Cellular tropism of PCV3 should be further studied.

It should be noted that severe proliferative necrotizing pneumonia in pig LK003 was probably a result of co-infection of various pathogens combined with high PCV3 titer. It is also possible that LK003 might mainly suffer from post-weaning multisystemic syndrome (PMWS) due to the observed pathological lesions and the presence of PCV2 in this animal. Previously, PCV3 has also been found in pigs with myocarditis (Phan et al., 2016). In this study, high titer of PCV3 was found in the heart tissue. However, no significant lesions could be identified either macroscopically or microscopically. It could be that the lesion in the heart might not uniformly distribute or the lesions were not located in the heart tissue section.

This study also suggested that both sows and nursery pigs could play a role in the PRDC problems in grower pigs of this farm. The results showed that high PCV3 viremia could be observed from sows, especially of lower parity. This suggested that sow-to-piglet transmission is highly possible. High PCV3 titers were then observed in the PRDC-affected pigs at 5 and 12 weeks of age possibly due to having PCV3 unstable sow herd. This might also be due to the changing environment during early nursery and early grower periods where pigs from different units/pens were regrouped together

along with decreasing maternal immunity causing the virus to spread horizontally to the naïve pigs.

Conclusion

In conclusion, PCV3 was identified from Thai pigs in the PRDC-affected farm in 2017. This is the first report of PCV3 in Thailand and is also the first study demonstrating PCV3 infection in PRDC-affected pigs at the farm level. Larger scale studies should be conducted to provide crucial information on disease spectrums of PCV3 and the impact of PCV3 to the swine industry.

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Conflict of Interest Statement

Conflict of interest: none

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CHAPTER V

OVERALL DISCUSSION

Previously, PCV3 has been identified in pigs of various clinical presentations as well as clinically healthy pigs. However, studies that associate PCV3 with diseases are still lacking. Nevertheless, due to its wide spread, there is an urgent need to clarify the significant of PCV3 in the swine industry. In this study, the presence of PCV3 in Thailand and its roles in various phases of swine production cycles were demonstrated.

Firstly, PCV3 has been identified in Thailand for the first time in this study. Moreover, the results also showed that PCV3 could be found in the samples collected in 2005. The data indicates that PCV3 might have been circulating in Thailand as early as 2005. The roles of PCV3 in pig production cycle were determined. The results firstly showed that PCV3 was associated with abortion. The virus was also found in sow colostrum. Finally, the virus was associated with PRDC in grower pigs. Taken together, the roles of PCV3 in pig production cycle is proposed and shown in Figure 8. Infection in gilts/sows could lead to abortion. Endometritis from PCV3 infection might play a role in this situation. Sow infection during this phase could also result in vertical infection including transplacental, direct contact, and transcolostrum transmission. Early infection in suckling piglets could then lead to disease in this period. However, PCV3-associated disease in these pigs was not included in this study. Instead, the study showed that the infection could result in PRDC in the grower period. It can be seen that PCV3 could have different roles for each phase of the production cycle.

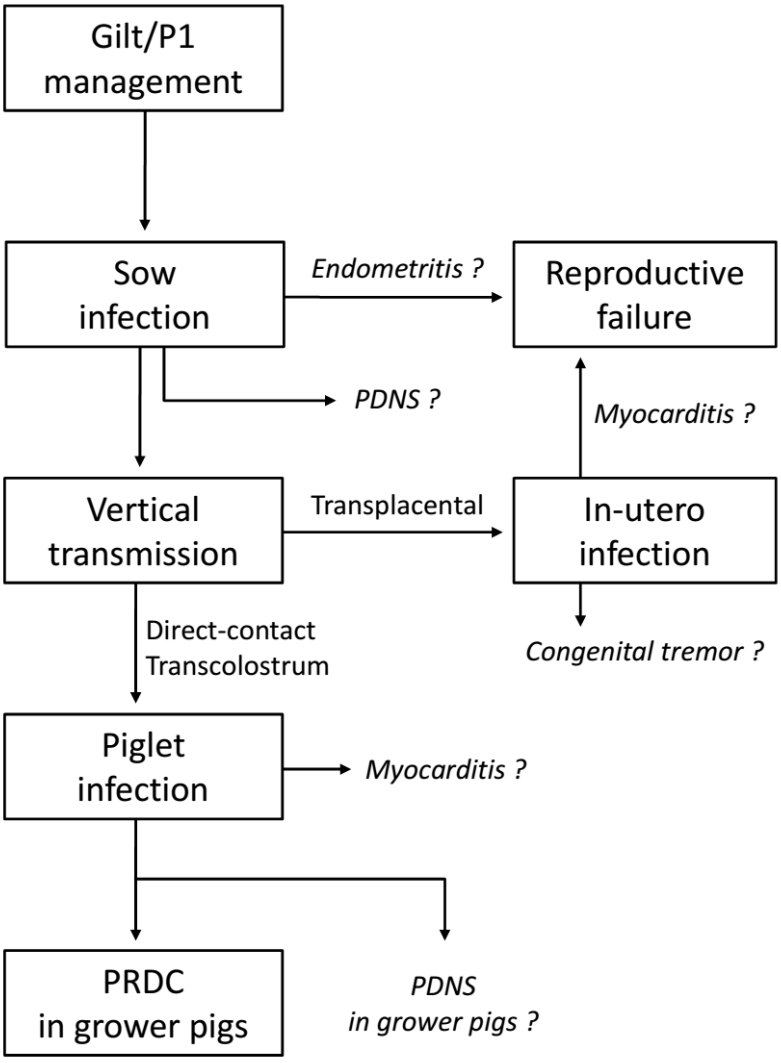

Figure 8 Diagram of possible roles of PCV3 in pig production cycle.

Gilt and primiparous sow (P1) management could affect infection during gestation period and overall sow infection. Infection in sows could result in reproductive failure due to vertical transmission, including transplacental, direct-contact, and transcolostrum. PRDC in grower pigs could be subsequently developed. Clinical signs or lesions have not yet been found associated with PCV3, including endometritis, PDNS, myocarditis and congenital tremor, are shown with a question mark (?).

The results from this study showed that gilt and primiparous sow management could have a very important role in PCV3 disease occurrence and also in initial control strategies against PCV3. This might not be surprised as these pigs (gilts and primiparous sows) were possibly involved in other swine infectious diseases such as PRRSV and PCV2 (Fraile et al., 2009). It has been shown that PCV3 viremia and colostrum shedding tend to be found higher in the lower parity sows. This might be due to lower immunity against PCV3 in these pigs from incomplete gilt acclimatization. Therefore, gilt acclimatization could be of importance in reducing overall PCV3 load in the herd similar to other swine pathogens (Corzo et al., 2010; Garza-Moreno et al., 2018; Roos et al., 2016). Gilts that developed high immunity against PCV3 during acclimatization might be protected against abortion in the gestation period. Moreover, these gilts might shed lower PCV3 titers in the farrowing units. Finally, PCV3-associated PRDC could also be reduced due to decreased virus titers in the grower period (Figure 8).

Since immunity and viral load could play a critical role in disease occurrence, it could also be speculated that the pattern of PCV3-associated syndrome could be influenced by PCV3 status of the herds. When PCV3 is introduced to a naïve herd, it is possible that PCV3-associated abortion (or other reproductive failure) could be dominant. However, in PCV3 endemic farm, PCV3-associated PRDC in the grower pigs could be more obvious. Reproductive problems might be limited to lower parity sows, especially when gilt acclimatization was not done effectively or to the higher parity sows in the unstable sow herd. Further studies are needed to confirm this hypothesis.

CHAPTER VI

CONCLUSION

In conclusion, this study firstly showed that PCV3 could be found in Thailand. The first Thai PCV3 (PCV3/Thailand/PB01/17, MG310152) was reported in the study. The results also showed that PCV3 might have existed in this country since as early as 2005. Thai PCV3 strains showed genetic heterogeneity since both PCV3a and PCV3b were identified. Regarding viral pathogenesis, this study demonstrated that PCV3 has multiple roles in pig production cycle. During gestation period, PCV2 was associated with abortion. In the farrowing period, the vertical transmission could occur via various routes. PCV3 shedding in sow colostrum was identified for the first time in this study. In the growing period, PCV3 was associated with PRDC in grower pigs. The results from this study also suggested that gilt management could be significant in the occurrence of PCV3-associated diseases. Therefore, control strategies for gilts, such as gilt acclimatization, might be valuable for controlling PCV3 similar to PRRSV and other reproductive pathogens.

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APPENDIX

Whole Genome Sequence of PCV3/Thailand/PB01/17

TATTTATACGCTATGGGCGGGGTTTGCGTGATTTTTGCGGGGTGATGGGGTTGGGTAAAC

CGCGTGATTTTAAAACTGAAGTTTATGTTTTTATTGGTCCTCCAGGATGCGGGAAAACGC

GGGAAGCTTGTGCGGATGCGGCTGCGCGGGAATTGCAGTTGTATTTCAAGCCACGGGGGC

CTTGGTGGGATGGTTATAATGGGGAGGGTGCTGTTATTCTGGATGATTTTTATGGGTGGG

TTCCATTTGATGAATTGCTGAGAATTGGGGACAGGTACCCTCTGAGGGTTCCTGTTAAGG

GTGGGTTTGTTAATTTTGTGGCTAAGGTATTATATATTACTAGTAATGTTGTACCGGAGG

AGTGGTATTCATCGGAGAATATTCGTGGAAAGTTGGAGGCCTTGTTTAGGAGGTTCACTA

AGGTTGTTTGTTGGGGGGAGGGGGGGGTAAAGAAAGACATGGAGACAGTGTATCCAATAA

ACTATTGATTTTATTTGCACTTGTGTACAATTATTGCGTTGGGGTGTGGGAATTTATTGG

GAGGGTGGGTGGGCAGCCCCCTAGCCACGGCTTGTCGCCCCCACCGAAGCATGTGGGGGA

TGGGGTCCCCACATGCGAGGGCGTTTACCTGTGCCCGCACCCGAAGCGCAGCGGGAGCGC

GCGCGAGGGGACACGGCTTGTCGCCACCGGAGGGGTCAGATTTATATTTATTTTCACTTA

GAGAACGGACTTGTAACGAATCCAAACTTCTTTGGTGCCGTAGAAGTCTGTCATTCCAGT

TTTTTCCGGGACATAAATGCTCCAAAGCAGTGCTCCCCATTGAACGGTGGGGTCATATGT

GTTGAGCCAGGGGGTGGGTCTGGAGAAAAAGAAGAGGCTTTGTCCTGGGTGAGCGCTGGT

AGTTCCCGCCAGAAGTGGTTTGGGGGTGAAGTAACGGCTGTGTTTTTTTTTAGAAGTCAT

AACTTTACGAGTGGAACTTTCCGCATAAGGGTCGTCTTGGAGCCAAGTGTTTGTGGTCCA

GGCGCCGTCTAGATCTATGGCTGTGTGCCCGAACATAGTTTTTGTTTGCTGAGCCGGAGA

AATTACAGGGCTGAGTGTAACTTTCATTTTTAGTATCTTATAATATTCAAAGGTAATTGC

AGTTTCCCATTCGTTTAGGCGGGTAATGAAGTGGTTGGCGTGCCAGGGCTTGTTATTCTG

AGGGGTTCCAACGGAAATGACGTTCATGGTGGAGTATTTCTTTGTGTAGTATGTGCCAGC

TGTGGGCCTCCTAATGAATAGTCTTCTTCTGGCATAGCGCCTTCTGTGGCGTCGTCGTCT

CCTTGGGCGGGGTCTTCGTCTGAATATAGCTCTGTGTCTCATTTTGGTGCCGGGCTAGTA

TTACCCGGCACCTCGGAACCCGGAACCACGGAGGTCTGTAGGGAGAAAAAGTGGTATCCG

ATTATGGATGCTCCGCACCGTGTGAGTGGATATACCGGGCAGTGGATGATGAAGCGGCCT

CGTGTTTTGATGCCGCAGGACGGGGACTGGATAACTGAGTTTTTGTGGTGCTACGAATGT

CCTGAAGATAAGGACTTTTATTGTCATCCTATTCTAGGTCCGGAGGGAAAGCCCGAAACA

CAGGTGGTGTTTTACGATAAACAACTGGACCCCGACCGAGTGGGAATCTATTGTGGAGTG

TGGAGGCAGTATAGCGAGATACCTTATTATCGGCAAAGAGGTTGGAAAAAGCGGTACCCC

ACACTTGCAAGGGTACGTGAATTTCAAGAACAAAAGGCGACTCAGCTCGGTGAAGCGCTT

ACCCGGATTTGGTCGGGCCCATCTGGAGCCGGCGAGGGGGAGCCACAAAGAGGCCAGCGA

GTATTGCAAGAAAGAGGGGGATTACCTCGAGATTGGCGAAGATTCCTCTTCGGGTACCAG

ATCGGATCTTCAAGCAGCAGCTCGGATTCTGACGGAGACGTCGGGAAATCTGACTGAAGT

TGCGGAGAAGATGCCTGCAG

Partial ORF2 Sequence of PCV3/Thailand/RB01/17

ATGAGACACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGCCGACGCCACAGA

AGGCGCTATGTCAGAAGAAAACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACA

AAGAAATACTCCACCATGAACGTCATTTCCGTTGGAACCCCTCAGAATAATAAGCCCTGG

CACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTAGCTTTGAATAT

TATAAAATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACA

AAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGG

CTC

Partial ORF2 Sequence of PCV3/Thailand/UD01/08

CACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAG

ATACTAAAAATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACT

ATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACGACAAACACTTGGCTCCAA

GACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGC

CGTTACTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCT

Primers for ORF2 Sequencing from FFPE Samples

Primer pair	Sequence (5'->3')	Nucleotide position[a]	Amplicon size (bp)
1	CTACAGACCTCCGTGGTTCC TCTTCTGGCATAGCGCCTTC	580 - 716	137
2	GGTAATACTAGCCCGGCACC GGGTTCCAACGGAAATGACG	616 - 798	183
3	TTCATTAGGAGGCCCACAGC TTCCCATTCGTTTAGGCGGG	723 - 857	135
4	CCATGAACGTCATTTCCGTTGG GAGCCGGAGAAATTACAGGGC	772 - 930	159
5	CACTTCATTACCCGCCTAAACG GAACTTTCCGCATAAGGGTCG	828 - 1027	200
6	CTGGACCACAAACACTTGGC AGAAGAGGCTTTGTCCTGGG	980 - 1131	152
7	ACTTCTGGCGGGAACTACC TCCAAACTTCTTTGGTGCCG	1085 - 1260	176
8	GCTTTGGAGCATTTATGTCCCG ACATGCGAGGGCGTTTACC	1193 - 1390	198

[a] Based on a PCV3 genome sequence (GenBank: MG310152)

VITA

Roongtham Kedkovid was born on 30 January 1982, Bangkok (Thailand). He earned his DVM in 2007 from Faculty of Veterinary Science, Chulalongkorn University. He completed an MSc in Pathobiology in 2010 from Faculty of Veterinary Science, Chulalongkorn University. His research, supervised by Professor Dr. Roongroje Thanawongnuwech, is 'NSP2 gene variation of the North American genotype of the Thai PRRSV in central Thailand'. The research involves investigating the genetic variation of porcine reproductive and respiratory syndrome virus (PRRSV), one of the most important swine viruses in Thailand and also in other swine producing countries around the world. The research focuses on nonstructural protein 2 (NSP2) gene of the virus which, at that time, has been linked with the virulence of the virus. After finishing his MSc degree, he was working as a researcher at Betagro Science Center for 3 years. He began his PhD in Pathobiology in 2013. His dissertation research, also supervised by Professor Dr. Roongroje Thanawongnuwech, involves identifying porcine circovirus type 3 (PCV3) in Thailand for the first time and investigating its roles in different stages of swine production cycle. Roongtham Kedkovid is interested in virology, pathology, immunology, and molecular biology. His interests also extended to swine genetics. He is currently working as a researcher at Research and Development Center for Livestock Production Technology, Faculty of Veterinary Science, Chulalongkorn University. He can be contacted at Roongthamk@gmail.com.