PREVALENCE AND GENETIC CHARACTERIZATION OF PORCINE CIRCOVIRUSES IN THAI SWINE FARMS DURING 2019-2020

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ปัจจุบันเชื้อเซอร์โคไวรัสในสุกร (พีซีวี) พบการแพร่กระจายไปทั่วโลกโดยเฉพาะพีซีวีสองและพีซีวีสาม ในขณะที่สามารถพบพีซีวีสี่ในทวีปเอเชียเท่านั้น ในการศึกษาครั้งนี้มีจุดประสงค์เพื่อสำรวจความชุกและลักษณะทางพันธุกรรมของเชื้อเซอร์โคไวรัสในฟาร์มสุกรในประเทศไทยระหว่างปี 2562-2563 โดยผลการศึกษาในครั้งนี้พบเชื้อพีซีวีสองมีความชุกมากที่สุดตามด้วยเชื้อพีซีวีสามและพีซีวีสี่ จากการวิเคราะห์รหัสพันธุกรรมพบเชื้อพีซีวีสองกลุ่มใหม่ที่มีการกลายพันธุ์ลำดับของกรดอะมิโน (133HDAM136) ในส่วนของโออาร์เอฟสอง เป็นจำนวนมากที่สุดในฟาร์มสุกรในประเทศไทยซึ่งการกลายพันธุ์ของลำดับกรดอะมิโนในตำแหน่งนี้ไม่เคยตรวจพบในประเทศไทยมาก่อน นอกจากนี้ การศึกษาครั้งนี้ยังพบเชื้อไวรัสสายพันธุ์ใหม่ที่เกิดการรวมกันของสารพันธุกรรมของพีซีวีสองสายพันธุ์บี และ พีซีวีสองสายพันธุ์ดี เป็นครั้งแรกในประเทศไทย ส่วนผลการวิเคราะห์รหัสพันธุกรรมของเชื้อพีซีวีสาม พบว่าเชื้อพีซีวีสามถูกจัดอยู่ในกลุ่มพีซีวีสามเอซึ่งพบว่ามีความเสถียรทางพันธุกรรมกันระหว่างเชื้อไวรัสพีซีวีสามที่พบในประเทศไทย ที่น่าสนใจการศึกษาในครั้งนี้มีการตรวจพบเชื้อพีซีวีสี่ครั้งแรกในประเทศไทย และผลการถอดรหัสพันธุกรรมของเชื้อพีซีวีสี่ พบว่าเชื้อไวรัสพีซีวีสี่เป็นสายพันธุ์บี ซึ่งมีความใกล้เคียงกับเชื้อพีซีวีสี่ในประเทศจีน อีกทั้งการศึกษาในครั้งนี้ยังสามารถพบการกระจายตัวของสารพันธุกรรมของไวรัสพีซีวีสี่ ในเซลล์เยื่อบุหลอดลม ด้วยเทคนิคการตรวจวิธีอินไซตูไฮบริไดเซชั่น ซึ่งอาจมีผลต่อกระบวนการก่อโรคของเชื้ออีกด้วย การศึกษาครั้งนี้ยังได้มีการพัฒนาวิธีการตรวจหาสารพันธุกรรมในสภาพจริงต่อเชื้อไวรัสทั้งสามชนิด ซึ่งให้ผลเป็นที่น่าพอใจในหลายด้านๆ เช่น ประหยัดเวลาในการตรวจ มีความไวรับและความจำเพาะสูง อีกทั้งยังสามารถคำนวณหาปริมาณสารพันธุกรรมของเชื้อแต่ละชนิดได้อีกด้วย ซึ่งเหมาะกับการนำไปตรวจวินิจฉัยและการศึกษาทางระบาดวิทยาในอนาคต การศึกษาในครั้งนี้สามารถยืนยันการตรวจพบเชื้อพีซีวีทั้งสามชนิดในประเทศไทยและยังเน้นย้ำความสำคัญของการเฝ้าระวังโรคและการวิจัยเพิ่มเติมเพื่อทำความเข้าใจเกี่ยวกับไวรัสเหล่านี้และพัฒนากลยุทธ์การป้องกันและควบคุมที่มีประสิทธิภาพสำหรับอุตสาหกรรมสุกร

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Chaitawat Sirisereewan : PREVALENCE AND GENETIC CHARACTERIZATION OF PORCINE CIRCOVIRUSES IN THAI SWINE FARMS DURING 2019-2020. Advisor: Prof. ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D., DTBVP Co-advisor: ROONGTHAM KEDKOVID, M.Sc., Ph.D.

To date, porcine circoviruses (PCVs), particularly PCV2 and PCV3, have been identified globally, while PCV4 is mainly found in Asia. This study aimed to investigate the prevalence and genetic characterization of PCVs in Thai swine farms during 2019-2020. Results showed PCV2 is the predominant species, followed by PCV3 and PCV4. For genetic analysis, the dominant novel PCV2d cluster with amino acid mutation (133HDAM136) in ORF2 was found in Thai swine farms that had not been detected in Thailand before. Moreover, inter-genotypic recombinant PCV2b/d was first identified in Thailand. The genetic analysis of PCV3 showed that PCV3 was grouped into PCV3a with high genetic stability among Thai PCV3 strains. Notably, this study presents the first report of PCV4 in Thailand. Genetic analysis revealed that Thai PCV4 strains belong to the PCV4b classification and are closely related to PCV4 strains from China. Furthermore, our research identified the viral tropism of PCV4 in bronchial epithelial cells using *in situ* hybridization, shedding light on its pathogenesis. Furthermore, we developed a triplex real-time PCR assay (tqPCR) for simultaneous detection. The tqPCR assay offers several advantages, including reduced testing time, high sensitivity and specificity, and the ability to conduct quantitative analyses, making it a valuable tool for clinical diagnostics and epidemiological studies of PCV2, PCV3, and PCV4 in the future. In conclusion, our study confirms the presence of PCV2, PCV3, and PCV4 in Thai swine farms. These findings emphasize the importance of ongoing surveillance and research efforts to gain a deeper understanding of these viruses and to develop effective prevention and control strategies for the swine industry.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
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		Co-advisor's Signature .....

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CHAPTER 1

INTRODUCTION

Importance and Rationale

The emergence of porcine circovirus 2 (PCV2) a few decades ago led to substantial economic losses to the swine industry worldwide. Significant characteristics of PCV2 are associated with numerous syndromes and different clinical manifestations in terms of porcine circovirus diseases (PCVDs), including PCV2 systemic disease (PCV2-SD), porcine dermatitis and nephropathy syndrome (PDNS), PCV2 reproductive disease (PCV2-RD), and PCV2-subclinical infection (PCV2-SI) (Segales, 2012; Opriessnig and Langohr, 2013; Segales and Sibila, 2022). At present, PCV2 commercial vaccines have been extensively used in the field for the improvement of production parameters and reduction of viremia and viral shedding (Segales, 2015). However, those cannot provide sterile immunity, and their efficacy remains questionable due to several factors of vaccine failure in the field (Seo et al., 2014).

Several porcine circoviruses have been discovered, bringing the total number of recognized PCVs to four: PCV1, PCV2, PCV3, and PCV4. Regarding the pathogenicity, PCV1 is a non-pathogenic one found as the contaminant of cultured porcine kidney cell line (Tischer et al., 1974). PCV2 had been identified in pigs with postweaning multisystemic wasting syndrome (PMWS) in the late 1990s (Allan et al., 1995; Clark, 1996; Harding, 1996; Nayar et al., 1997; Allan et al., 1998; Ellis et al., 1998; Harding et

al., 1998). In 2015, PCV3 was first identified in the USA linked to PDNS, reproductive failure, and multisystemic infection (Phan et al., 2016; Palinski et al., 2017). PCV4 was later discovered in China in 2019 with different clinical presentations (Zhang et al., 2020), and little is known about the potential threat to the swine industry and limited geographical transmission. Interestingly, the substitution rates of circovirus, ssDNA virus, appear to be similar to those of RNA viruses (Duffy et al., 2008). Thus, the continued evolution of PCV may contribute to the emergence of new variant strains probably causing sporadic outbreaks due to vaccine failure.

Within the first decade after its discovery, significant genetic diversity of PCV2 was recognized (Olvera et al., 2007; Kim et al., 2009a). Based on an updated classification, PCV2 genotypes were well defined into at least nine genotypes (PCV2a to PCV2i) (Franzo and Segales, 2018; Wang et al., 2020). A detailed timeline for major PCV2 genotypes revealed that PCV2a was the most common genotype from 1996 to the early 2000s, then the first genotype shifted to PCV2b between roughly 2003 to 2006 (Patterson and Opriessnig, 2010), after which PCV-2d predominated as the second genotype shift between 2010-2015 (Xiao et al., 2015; Franzo and Segales, 2018). In contrast, PCV3 has limited genetic diversity, especially when compared to PCV2, suggesting being only one genotype based on the new unified classification criteria (Franzo et al., 2020a). However, the phylogenetic tree is based on the complete genome of PCV3 strains; they are divided into two groups, termed PCV3a

and tentative PCV3b (Franzo et al., 2018). Recently, a novel PCV4 has emerged, and the genomic sequence information is insufficient, so there are no conclusions on PCV4 diversity.

PCV2 and PCV3 have been reported in Thailand (Tantilertcharoen et al., 1999; Jantafong et al., 2011; Thangthamniyom et al., 2017; Kedkovid et al., 2018; Sukmak et al., 2019); however, PCV4 status was not yet revealed. Recently, previous studies of Thai PCV2 strains showed that PCV2d had been recognized as the predominant genotype, followed by PCV2b since 2013 (Thangthamniyom et al., 2017). However, no reports on the prevalence and genetic diversity have been published in the last five years. In comparison, the recent study of PCV3 in Thailand revealed that the viruses were grouped in PCV3a with a minor variation (Visuthsak et al., 2021). However, the prevalence and genetic sequence data of PCV3 are still limited. Lastly, PCV4 has never been reported in Thailand. Hence, it is critical to keep track of these viruses for disease prevention and control.

Real-time PCR (quantitative PCR, qPCR) is frequently used to detect various microbial agents in veterinary diagnosis (Kralik and Ricchi, 2017). However, only a few multiplex qPCR assays for the simultaneous detection of all three PCV species have been developed. In this study, a novel diagnostic assay based on multiplex qPCR will be developed for the differential identification of those three PCV strains which will be useful for diagnosis and surveillance. Then, a series of investigations of the

prevalence of PCV2, PCV3, and PCV4 will be conducted on submitted samples from Thai swine farms. In addition, the genetic characteristics of these viruses will be performed in understanding the genetic diversity in the Thai pig population.

Objectives of Study

1. To determine the prevalence of porcine circoviruses in Thailand during 2019-2020
2. To identify PCV4 from Thai pigs during 2019-2020
3. To determine the genetic diversity and characteristics of PCV2, PCV3, and PCV4
4. To establish a novel multiplex qPCR for PCV2, PCV3, and PCV4 detection

CHAPTER 2

LITERATURE REVIEW

Circoviruses

Circoviruses (CVs) is a non-enveloped, single-stranded circular DNA (ssDNA) virus belonging to the family *Circoviridae*, genus Circovirus. This virus has a genome size of less than 2 kb, making it the smallest known animal virus. CVs have the capability to infect a broad spectrum of hosts such as pigs, dogs, and various others as extensively reviewed elsewhere (Opriessnig et al., 2020). Notably, most ssDNA viruses are classified as eukaryotic circular rep-encoding single-stranded DNA (CRESS DNA) viruses. Among the thirteen ssDNA viruses acknowledged by the International Committee on the Taxonomy of Viruses (ICTV, http://ictv.global/report), eleven exhibit circular genomes, while *Parvoviridae* and *Bidnaviridae* deviate from this pattern (Zhao et al., 2019). In general, CRESS DNA viruses typically possess two primary open reading frames (ORFs): one for replicase (rep) protein and another for capsid (cap) protein. The group of viruses is held together by the conserved rep, whereas the capsid protein displays notable divergence (Zhao et al., 2019).

Porcine circoviruses (PCVs) are non-enveloped single-stranded circular DNA (ssDNA) with approximately 15 to 25 nm virion diameter (Rosario et al., 2017). At present, four types of porcine circoviruses are classified: PCV1, PCV2, PCV3, and PCV4 (Opriessnig et al., 2020). The genome size of PCV1 varies between 1758 and 1760 nucleotides (nt) (Meehan et al., 1997; Fenaux et al., 2003). In contrast, PCV2 and

PCV3 have genome sizes in the approximate range of 1766-1769 and 1999-2001 nt, respectively (Guo et al., 2010; Palinski et al., 2017). While a novel PCV4 has a genome size of 1770 nt (Zhang et al., 2020; Nguyen et al., 2021).

In general, PCV genomes typically exhibit an ambisense organization, encoding two major open reading frames (ORFs): ORF1 (rep gene) and ORF2 (cap gene). The rep gene is located on the positive strand of the genome and is involved in viral replication. On the other hand, the cap gene is located on the negative strand of the genome and is responsible for encoding the capsid protein of the virion and (Zhao et al., 2019). Moreover, cap protein is hypervariable region and possesses immunogenic properties (Fenaux et al., 2000; Nawagitgul et al., 2000; Fenaux et al., 2003).

The relationship among PCVs was assessed through phylogenetic analysis of the cap gene. The amino acid similarities were as follows: 67% between PCV1 and PCV2 (Meehan et al., 1998), 24% between PCV1 and PCV3 (Phan et al., 2016), and 26-37% between PCV2 and PCV3 (Phan et al., 2016; Palinski et al., 2017). In the case of PCV4, it shared amino acid similarities of 43.1%, 45%, and 24.5% when compared to PCV1, PCV2, and PCV3, respectively (Zhang et al., 2020).

History of porcine circoviruses (PCVs)

Porcine circovirus 1 (PCV1)

In 1974, an unidentified virus was found as a contaminant in a porcine kidney cell line (PK-15, ATCC CCL-33) without causing any cytopathic effect (Tischer et al., 1974). The source of contamination was suspected to be the serum and swine tissue used in the cell culture (Dulac and Afshar, 1989). Apart from PK-15 cells, PCV1 has the capability to infect Vero cells (Allan et al., 1994).

Regarding pathogenicity, PCV1 can infect several tissues, including lymph nodes, thymus, spleen, lung, liver, and intestine, without inducing microscopic lesions (Tischer et al., 1986; Allan et al., 1995). Infected pigs generally remain clinically healthy but able to elicit specific antibodies to PCV1 (Tischer et al., 1986; Allan et al., 1995). In contrast, previous reports showed that PCV1 cultured in cells has the ability to infect porcine fetuses at 55 days of gestation, leading to the induction of microscopic lung lesions with severe hemorrhage (Saha et al., 2011) and is associated with congenital tremors (Allan et al., 1995; Stevenson et al., 2001). However, PCV1 is widely recognized as non-pathogenic to pigs.

In general, PCV1 are prevalent worldwide (Allan et al., 1998; Fenaux et al., 2000). The seroprevalence of PCV1 at herd level ranges from 10% (Puvanendiran et al., 2011) to 100% (Labarque et al., 2000).

Porcine circovirus 2 (PCV2)

During the 1990s, Canada experienced a puzzling viral disease outbreak that led to nursery mortality rates ranging from 12% to 15% (Ellis, 2014). This outbreak was characterized by several clinical signs, including jaundice, diarrhea, and respiratory disease. In the mid-1990s, the virus was identified in typical lesions within the lymph nodes of affected pigs using an immunohistochemistry assay. This discovery led to the proposal of the name "postweaning multisystemic wasting syndrome (PMWS)" for this apparently new disease (Clark, 1996; Harding, 1996; Nayar et al., 1997; Allan et al., 1998; Ellis et al., 1998; Harding et al., 1998), which was designated as PCV2 (Meehan et al., 1998). Interestingly, the retrospective study revealed that PCV2 infection could be detected in pigs as early as 1962, and associated lesions like porcine dermatitis and nephropathy syndrome (PDNS) and PMWS were already observed in 1985 (Jacobsen et al., 2009).

Geographic distribution of PCV2

PCV2 is considered as a major cause of production losses in the swine industry (Madec et al., 2008; Ramamoorthy and Meng, 2009; Segales et al., 2013). Since its initial identification, the virus quickly became widespread in swine populations across all continents (Firth et al., 2009; Patterson and Opriessnig, 2010; Grau-Roma et al., 2011; Xiao et al., 2015; Afolabi et al., 2017; Franzo and Segales, 2018; Franzo et al., 2022). The worldwide distribution of PCV2-positive countries has

been reported based on publications and genomic sequences submitted in NCBI genomes repository (Figure 1).

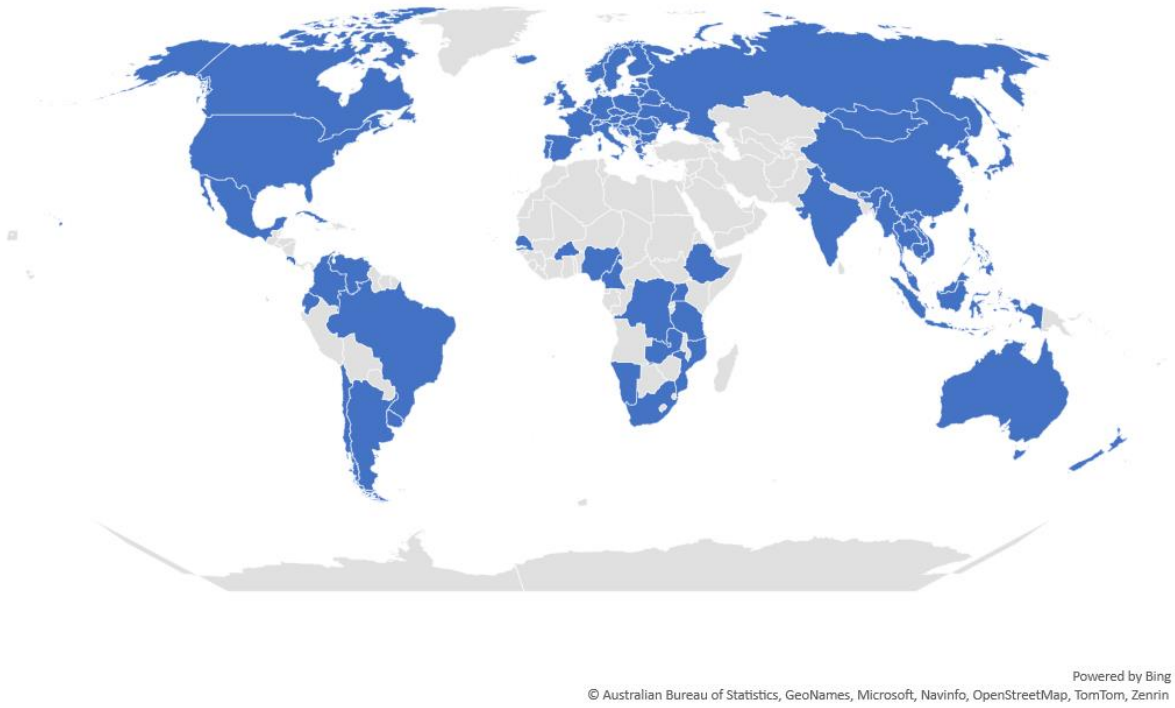

Figure 1 PCV2-infected countries are indicated in blue-colored.

Clinical manifestations associated to PCV2 infection

Infection with PCV2 can trigger various clinical presentations that are referred to as porcine circovirus diseases (PCVDs) (Segales et al., 2005; Segales and Sibila, 2022) or porcine circovirus-associated diseases (PCVADs) (Opriessnig et al., 2007) depending on geographic origin. In this review, PCVDs will be used. Currently, PCVDs are classified into 4 major groups: PCV2 systemic disease (PCV2-SD), PCV2 reproductive disease (PCV2-RD), PCV2 subclinical disease (PCV2-SI), and porcine dermatitis and nephropathy syndrome (PDNS) (Segales, 2012; Segales and Sibila, 2022). The clinical manifestations of PCVDs have been extensively documented and reviewed elsewhere (Segales and Sibila, 2022).

First, PCV2-SD is characterized by wasting, weight loss, respiratory and gastrointestinal involvement. This condition is now referred to as PCV2-SD instead of PMWS. It's worth noting that PCV2-SD can sporadically affect vaccinated pigs, underlining the complex nature of the disease's causation (Segales et al., 2005). Secondly, PCV2-RD is the condition related to reproductive problems including abortions or mummifications and return to estrus. Thirdly, PCV2-SI refers to subclinical infections where pigs do not display apparent clinical signs but may still carry the virus. This often leads to decreased growth rates and increased vulnerability to other diseases, leading to widespread use of vaccines worldwide, regardless of the clinical status of the pigs. Finally, PDNS is a condition characterized by skin lesions, primarily in the hind limbs and perianal area. Additionally, it can lead to microscopic

kidney lesions, resulting in necrotizing and fibrinous glomerulonephritis. The clinical manifestations resulting from PCVDS were summarized in Table 1.

Table 1 *Summary of clinical manifestations of PCVDs (adapted from Segales, 2022)*

PCVDs	Clinical signs and diagnostic criteria
PCV2-SD	● Multisystemic involvement: respiratory and gastrointestinal disorders ● Wasting, decreased rate of weight gain ● Severe lymphoid depletion with high amount of PCV2 in lymphoid or affected tissues
PCV2-RD	● Abortions or mummifications ● Regular return to estrus ● SMEDI conditions: stillbirth, mummification, embryonic death, and infertility ● Myocarditis and high amount of PCV2 in heart ● PCV2 seroconversion and/or PCV2 positivity around return to estrus occurrence
PCV2-SI	● Absence of clinical signs ● Decreased average daily gain ● No or minimal microscopic lesions in lymphoid tissues ● Low amount of PCV2 in lymphoid tissues
PDNS	● Hemorrhagic and necrotizing skin lesions, mainly in hind limbs and perineal area ● Swollen and pale kidneys with microscopic lesions as systemic necrotizing vasculitis, necrotizing and fibrinous glomerulonephritis

Genotypes of PCV2

The rapid global emergence of PCV2 may be attributed to its high mutation rate around 1.2×10^{-3} substitutions/site/year, which aligns its evolutionary dynamics more closely with single-stranded RNA viruses (Firth et al., 2009). As of now, the classification of genotypes is firmly established and relies on three main criteria: a maximum intra-genotype p-distance of 13% for the ORF2 gene, a minimum bootstrap support of 70% at the relevant internal node, and the availability of at least 15 sequences for analysis (Franzo and Segales, 2018). Following the proposed criteria, nine genotypes have been identified, ranging from PCV-2a to PCV-2i (Franzo and Segales, 2018; Wang et al., 2020). Moreover, PCV2 genotype classification has become more difficult due to its evolution facilitated by genetic recombination. Interestingly, several reports confirmed the existence of PCV2 recombinants by intra- and inter-genotypic recombination in several countries (Ma et al., 2007; Hesse et al., 2008; Kim et al., 2009b; Cai et al., 2011; Huang et al., 2013; Ramos et al., 2013; Neira et al., 2017; Rajkhowa et al., 2021). Thus, the evidence and pathogenicity of the PCV2 recombinants need further investigation.

Since its identification of PCV2, phylodynamic studies have unveiled two major genetic shifts in PCV2: one from PCV2a to PCV2b, occurring around 2003, and another from PCV2b to PCV2d, observed around 2012 (Patterson and Opriessnig, 2010; Guo et al., 2012; Xiao et al., 2015; Franzo and Segales, 2018). Notably, PCV2d has now surpassed PCV2b to become the dominant genotype worldwide (Xiao et al.,

2015). Intriguingly, the virus has evolved to enhance its virulence compared to existing PCV2a and PCV2b strains (Guo et al., 2012). Therefore, the presence of PCV2d can be attributed to the virus itself exerting strong selection pressure or being influenced by vaccine-driven selection pressure due to widespread vaccination efforts across the globe.

PCV2 vaccination

Commercial vaccine was introduced to swine industry around 2004-2006 to combat PCVDs. At present, the two main types of commercial vaccines are inactivated vaccines and subunit vaccines, both of which are based on the cap gene (Guo et al., 2022). It appears that the current vaccines, which are based on PCV2a strains, have the capability to effectively manage the major circulating strains of PCV2 worldwide based on clinical, virological, immunological and pathological evaluation (Chae, 2012; Opriessnig et al., 2014). Nevertheless, it is important to note that all commercial vaccines do not grant sterile immunity. Therefore, additional research and investigation are warranted. A list of commercial PCV2 vaccines available worldwide is presented in Table 2.

Table 2 *Commercially available PCV2 vaccines used in swine industry.*
(adapted from Mukherjee, 2018)

Vaccine	**Antigen**	**Dosage**	**Licensed for**	**Geographical availability**
Circovac	Inactivated PCV2a	2 ml 0.5 ml	Sows/Gilts Pigs	Worldwide, except USA Worldwide
Ingelvac CircoFlex	PCV2a Cap protein	1 ml	Pigs	Worldwide
Porcilis PCV	PCV2a Cap protein	2 ml	Pigs	Europe, several south-and central American and Asian countries
Circumvent™ PCV	PCV2a Cap protein	2 ml	Pigs	North America, several south-and central American and Asian countries
Circumvent G2 PCV	PCV2a Cap protein	1 or 2 ml	Pigs	USA and Canada
Suvaxyn PCV	Inactivated recombinant PCV1 expressing the PCV2a Cap protein	2 ml	Pigs	Europe, several Asian countries
Fostera™ PCV	Inactivated recombinant PCV1 expressing the PCV2a Cap protein	1 or 2 ml	Pigs	North America, south Africa, several Asian countries

Porcine circovirus 3 (PCV3)

In 2015, PCV3 was initially discovered in the USA through next-generation sequencing (NGS) techniques, and it was associated with porcine dermatitis and nephropathy syndrome (PDNS), reproductive problems, and multisystemic and cardiac inflammation (Phan et al., 2016; Palinski et al., 2017). The complete genome of PCV3 was 2000 nucleotides in length and includes three ORFS: ORF1, ORF2, and ORF3 (Phan et al., 2016). PCV3 antigens were predominantly detected through *in situ* hybridization in the myocardium, leiomyocytes of tunica media, and inflammatory cells (Phan et al., 2016). Interestingly, retrospective studies have revealed PCV3-positive samples in various countries, including Brazil since 1967 (Rodrigues et al., 2020), Sweden since 1993 (Ye et al., 2018), and Spain and China since 1996 (Klaumann et al., 2018b; Sun et al., 2018). However, there was skepticism about the economic impact on the swine industry in the initial phase following the identification of PCV3.

Geographic distribution of PCV3

In the decade following the initial identification of PCV3, numerous studies globally uncovered the existence of PCV3 in different countries, showcasing a range of clinical manifestations (Faccini et al., 2017; Ku et al., 2017; Zhai et al., 2017; Kedkovid et al., 2018; Kim et al., 2018; Klaumann et al., 2018b; Saraiva et al., 2018; Arruda et al., 2019; Franzo et al., 2019; Ouyang et al., 2019; Dei Giudici et al., 2020; Opriessnig et al., 2020; Saporiti et al., 2020; Molini et al., 2021; Turlewicz-Podbielska

et al., 2022; Vargas-Bermudez et al., 2022). The worldwide distribution of PCV3-positive countries has been reported based on publications and genomic sequences submitted in NCBI genomes repository (Figure 2).

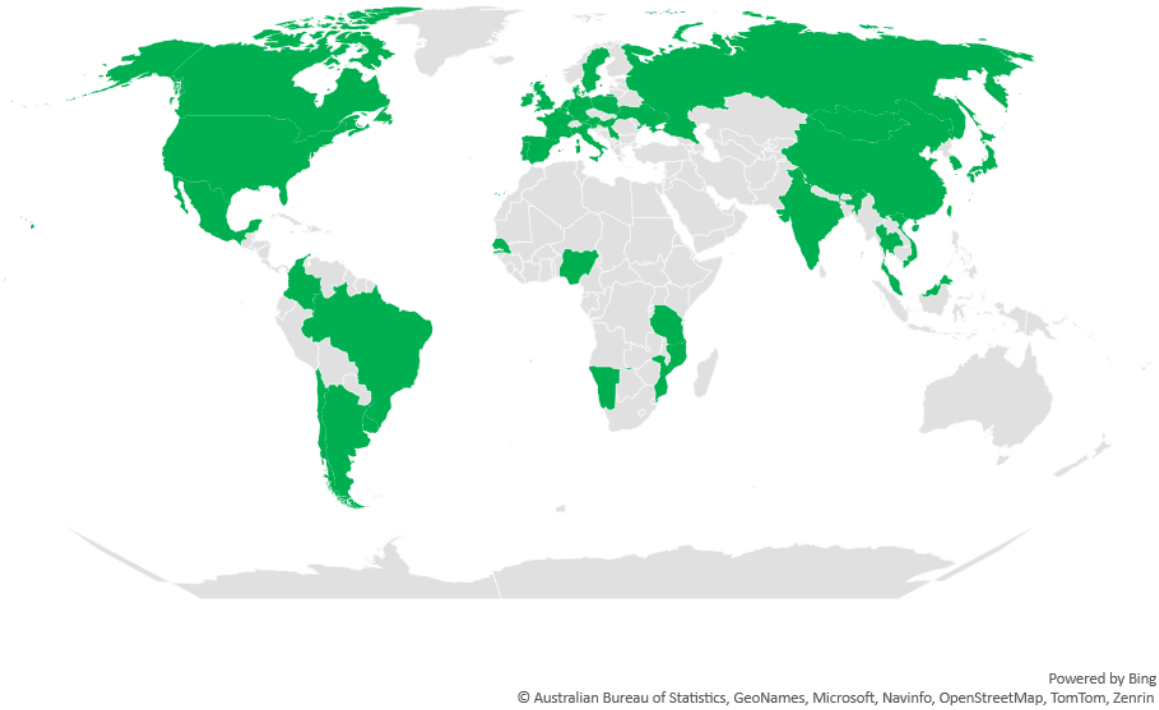

Figure 2 *PCV3-infected countries are indicated in green-colored.*

Clinical manifestations associated to PCV3 infection

At present, PCV3 has been detected in pigs worldwide, showing clinical manifestations, though it can also be detected in apparently healthy pigs. PCV3 infection has been associated with a multitude of clinical and pathological presentations, including reproductive disorders (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Bera et al., 2020), respiratory disorders (Zhai et al., 2017; Kedkovid et al., 2018; Kim et al., 2018), gastrointestinal disorders (Zhai et al., 2017), multisystemic inflammation (Phan et al., 2016; Arruda et al., 2019), neurological signs (Chen et al., 2017), and PDNS (Palinski et al., 2017; Bera et al., 2020). However, PCV3 can be detected in healthy pigs (Zhai et al., 2017; Ye et al., 2018; Saporiti et al., 2020). Moreover, PCV3 was found in pigs suffering from porcine respiratory disease complex (PRDC) than clinical healthy pigs (Kedkovid et al., 2018).

PCV2-associated disease (PCV2-AD) diagnostic criteria is well-established relying on available data that offer clinical, pathological, and virological assessments of PCV-2 infection cases (Segales and Sibila, 2022). Interestingly, PCV-2 and PCV-3 exhibit several parallel clinical presentations and a case definition for PCV3-associated diseases has been established (Saporiti et al., 2021). The case definitions for PCV3-associated diseases consisted of PCV-3 reproductive disease (PCV3-RD) and PCV-3 systemic disease (PCV3-SD) (Table 3.)

Table 3 *Proposed diagnostic criteria have been outlined for the case definition of PCV-3 associated diseases (PCV-3-AD)*
(adapted from Saporiti, 2021).

PCV-3-AD	Clinical signs and diagnostic criteria
PCV3-SD	● Mummified fetuses, stillborn fetuses, weak born piglets ● Late abortion ● Multisystemic lymphoplasmacytic to lymphohistiocytic perivascular inflammation ● Moderate to high amount of PCV3 antigen in affected tissues
PCV3-RD	● Wasting, weight loss ● Neurological disorders ● Multisystemic lymphoplasmacytic to lymphohistiocytic perivascular inflammation ● Moderate to high amount of PCV3 antigen in affected tissues

Genotypes of PCV3

To date, PCV3 has been circulated worldwide. The phylodynamic approach revealed the evolutionary rate of PCV3 is 10^{-5}-10^{-6} substitutions/site/year that lower than the PCV2 (Franzo et al., 2019). As of now, PCV3-genotyping proposal is established based on a maximum within-genotype genetic distance of 3% at the complete genome and 6% at the cap gene, bootstrap support higher than 90% (Franzo et al., 2020a). The result indicated that PCV3 has only one genotype (PCV3a). Nonetheless, it is possible for a novel genotype to emerge and propagate in the future, driven by genetic mutations.

PCV3 vaccination

Viral isolation and propagation are critical steps for vaccine development. Unfortunately, the isolation of PCV3 is often unsuccessful. The studies revealed that porcine kidney (PK-15) and swine testis (ST) cells can be used for PCV3 isolation (Ha et al., 2018). However, one study suggested that PK-15 can be used for PCV3 isolation. It is possible that variations within different subpopulations of PK-15 may influence the success of virus isolation. Besides PK-15, primary porcine kidney cells have been reported as suitable for virus isolation and propagation (Oh and Chae, 2020). Currently, there is a scarcity of commercial PCV3 vaccines. Only one PCV3 vaccine, which is based on RNA particles, has been developed. Further investigation into the vaccine's efficacy is needed.

Porcine circovirus 4 (PCV4)

A novel porcine circovirus, designated PCV4, was documented in China in 2019, exhibiting several clinical manifestations such as respiratory signs, diarrhea, and PDNS-like lesions (Zhang et al., 2020). However, healthy pigs were also found positive to PCV4. Retrospective study have shown that PCV4 has been circulating in China since 2012 (Hou et al., 2022) and in Inner Mongolia since 2016 (Ha et al., 2021). Furthermore, several studies have demonstrated a gradual increase in the PCV4 positive rate after its initial identification (Tian et al., 2021; Hou et al., 2022; Xu et al., 2022a). However, PCV4 tested negative in various types of swine samples in both Italy and Spain (Franzo et al., 2020b). Currently, PCV4 was detected in approximately 3.28% of positive rates among sick and healthy pigs in South Korea in 2021 (Nguyen et al., 2021). Interestingly, our preliminary results showed that PCV4 has existed in Thailand since 2019. This suggests that PCV4 has relatively restricted geographical transmission within the Asian continent. Further research is required to investigate its pathogenesis and assess its potential economic impact on the swine industry.

Geographic distribution of PCV4

Currently, PCV4 has been reported in both clinically affected pigs and healthy pigs in China, South Korea, and Thailand. PCV4-positive countries have been reported based on publications and genomic sequences submitted in NCBI genomes repository (Figure 3).

Figure 3 *PCV4-infected countries are indicated in red-colored.*

Clinical manifestations associated to PCV4 infection

Infection with PCV2 and PCV3 presents parallel clinical manifestations that are clearly described as mentioned before. Interestingly, clinical signs in PCV4-infected pigs do not differ significantly from those seen in PCV2 and PCV3 infections. However, the proposed diagnostic criteria of PCV4 have not yet been established. The clinical manifestations based on field observation consisted of respiratory disorders (Zhang et al., 2020; Sun et al., 2021; Tian et al., 2021; Hou et al., 2022), reproductive disorders (Hou et al., 2022), gastrointestinal disorders (Zhang et al., 2020; Tian et al., 2021), neurological disorders (Tian et al., 2021; Hou et al., 2022), and PDNS (Zhang et al., 2020; Sun et al., 2021; Hou et al., 2022). Interestingly, only one animal experiment demonstrated that PCV4 infectious clone was pathogenic when administered to pigs (Niu et al., 2022b). However, these challenged pigs remained clinically healthy. Therefore, it is possible that clinical manifestations resulting from PCV4 infection may be influenced by multiple factors, which warrant further investigation.

Genotypes of PCV4

Unlike PCV2 and PCV3, genotyping methodology of PCV4 has not yet been established. Several studies proposed different criteria to classify PCV4 genotypes for 2-group classification (PCV4a and PCV4b) and 3-group classification (PCV4a, PCV4b, and PCV4c) (Wang et al., 2022; Wu et al., 2022; Xu et al., 2022a; Xu et al., 2022b). Therefore, the use of differing nomenclatures can lead to ambiguity and the potential for misinterpretation of results. Additional research is needed to develop

standardized criteria for genotyping PCV4, especially considering the limited availability of sequence information at present.

PCV4 vaccination

Similar to PCV3, isolation of PCV4 is often challenging. Efforts to isolate PCV4 using porcine kidney (PK-15) and swine testis (ST) cells have been attempted, but these attempts have failed to result in virus isolation (Zhang et al., 2020). Currently, there are no commercial PCV4 vaccines available on the market, and further research is required in this area.

Detection of PCVs

Generally, diagnostic tools for PCVD primarily target the presence of PCV2 antigen or nucleic acid within affected tissues (Opriessnig et al., 2007). The gold standard methods for detecting PCVs are immunohistochemistry (IHC) and *in situ* hybridization (ISH). However, these techniques are known to be time-consuming and labor-intensive. ISH and IHC are widely used within tissues and cells to visualize viral nucleic acid and protein antigens, respectively. In general, PCV2-specific ISH and IHC assays were well established (Rosell et al., 1999; Sorden et al., 1999). For PCV3, RNAscope®-based ISH assay was frequently performed on various tissues of infected pigs, including heart, lung, kidney, and lymphoid tissue (Phan et al., 2016; Arruda et al., 2019; Mora-Diaz et al., 2020). Concurrently, specific IHC assays for PCV3 and PCV4 were developed for understanding the viral tissue distribution and cellular tropism

(Palinski et al., 2017; Li et al., 2018b; Niu et al., 2022a). As a result, the advancement of ISH and IHC has aided in the study of PCV pathogenesis.

Consequently, the detection of PCV nucleic acids through polymerase chain reaction (PCR) offers rapid results with high specificity and sensitivity. Notably, quantitative real-time PCR (qPCR) possesses the capability to quantify the number of genes of interest at each cycle. This suggests that qPCR could potentially serve as a tool for distinguishing PCV2 infections with or without PCVD. In this scenario, PCV2 DNA copy numbers were utilized to classify PCVD as follows: negative ($<10^6$ copies), suspect (10^6 copies), and positive ($>10^6$ copies) (Opriessnig et al., 2007). Unfortunately, criteria for identifying PCV3-associated diseases and PCV4-associated diseases based on genomic copy numbers have not yet been established. As previously noted, PCVs infections exhibiting similar clinical presentations can complicate the interpretation of the primary cause of diseases, especially co-infection. Several duplex qPCR for PCV2 and PCV3 has been established with various limitation of detection (LOD) (Li et al., 2018c; Wang et al., 2019). For PCV3 and PCV4, SYBR green I-based duplex qPCR assay was designed with 51.7 copies and 67.7 copies LOD for PCV3 and PCV4, respectively (Hou et al., 2021). The evidence demonstrates that co-infections involving PCVs were observed in all PCV4-positive countries, including China, Korea, and Thailand (Ha et al., 2021; Nguyen et al., 2021; Hou et al., 2022). To address this issue, a limited number of multiplex real-time PCR was developed to simultaneous detection of all PCVs (Chen et al., 2021; Kim et al., 2022;

Xu et al., 2022a) and a standardized protocol has not yet been established. Thus, a multiplex qPCR assay could be used in epidemiological studies, including a diagnostic tool for monitoring the co-infection that may help in the management strategies.

Situation of PCVs in Thailand

In Thailand, PCV2 was first reported in PMWS-affected pigs in 1998 (Tantilertcharoen et al., 1999). However, retrospective research discovered the virus in formalin-fixed and formalin-fixed paraffin-embedded tissues from PMWS-affected pigs dating back to 1993 (Kiatipattanasakul-Banlunara et al., 2002). During 2007-2008, the prevalence of PCV2 was found at 37% positive rate in Thai swine farms with PCV2b predominant (Jantafong et al., 2011), and then increased PCV2 positive farms were up to 80% between 2009-2015 (Thangthamniyom et al., 2017). Interestingly, the PCV2d genotype has replaced PCV2b as the dominant genotype during 2013-2014 (Thangthamniyom et al., 2017), which seems to be occurring worldwide (Xiao et al., 2015; Franzo et al., 2016). However, since then, the molecular epidemiology and prevalence of PCV2 have not been studied in Thailand. Hence, the current situation of PCV2 is of interest and urgently needed.

In 2018, the seminal research on PCV3 in Thailand reported a higher prevalence of PCV3 in PRDC-affected pigs than in clinically healthy pigs, suggesting PCV3 might be involved in PRDC (Kedkovid et al., 2018). However, the retrospective analysis revealed that PCV3 had been widely distributed among Thai pigs since 2006 (Sukmak et al., 2019). It indicated that Thai swine farms are struggling with PCV2 and

PCV3 infections. Co-infection of PCV3 with PCV2 and PRRSV can be seen in clinical cases of PRDC and PCVAD in Thai swine farms (Sukmak et al., 2019; Visuthsak et al., 2021). However, there was no link between PCVD-related clinical symptoms and PCV3 infection (Visuthsak et al., 2021). Thus, the underlying causes for clinical manifestations of PCV3 required further investigation. The phylogenetic analysis exhibited that Thai PCV3 were mostly clustered within PCV3a with a high genetic identity (Visuthsak et al., 2021). In the preliminary results, PCV4 was detected in Thailand in clinically affected pigs. However, retrospective studies are needed to gain information on the epidemiology of PCV4 to monitor this virus for disease prevention and control.

To gain a better understanding of the prevalence of all PCVs in Thailand, this study conducted a retrospective analysis to determine the prevalence of PCVs in Thailand from 2019 to 2020. The outcomes of this study can help address the following questions: 1) What is the prevalence of porcine circoviruses in Thailand during 2019-2020? 2) Is PCV4 present in Thailand? 3) What are the genetic diversity and characteristics of PCV2, PCV3, and PCV4 in Thailand? 4) Can a novel multiplex qPCR method for detecting PCV2, PCV3, and PCV4 be utilized?

This study aims to provide insights into the prevalence and characteristics of PCVs in Thailand during the specified period.

CHAPTER 3

EMERGENCE OF NOVEL PORCINE CIRCOVIRUS 2D STRAINS IN THAILAND, 2019-2020

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Abstract

Porcine circovirus 2 (PCV2) has been recognized as a causative agent of porcine circovirus diseases (PCVDs) affecting the global swine industry. In this study, the genetic diversity of PCV2 strains circulating in Thailand between 2019 – 2020 was investigated using 742 swine clinical samples from 145 farms. The results showed PCV2-positive rates of 54.2% (402/742) and 81.4% (118/145) at the sample and farm levels, respectively. Genetic analysis of 51 Thai PCV2 genomic sequences showed that 84.3% (43/51) was PCV2d, 13.7% (7/51) was PCV2b and 1.9% (1/51) was PCV2b/2d recombinant virus. Surprisingly, the majority of the Thai PCV2d sequences from this study (69.77%, 30/43) formed a novel cluster on a phylogenetic tree and contained a unique ^{133}HDAM^{136} on the ORF2 deduced amino acid sequence, which is in one of the previously identified immunoreactive domains strongly involved in virus neutralization. The PCV2b/2d recombinant virus also carried ^{133}HDAM^{136}. The emergence of the novel PCV2d strains predominating in Thailand was discussed. This study highlights the need for further investigations on the spreading of these PCV2d strains in other regions and the efficacy of current commercial vaccines.

Keyword: porcine circovirus 2, PCV2d, mutation, recombination, pigs, Thailand

Introduction

Porcine circovirus (PCV) 2, the causative agent of porcine circovirus diseases (PCVDs) affecting the global swine industry, is a non-enveloped single-stranded DNA virus containing a circular genome of 1766-1768 nucleotides (nt) (Guo et al., 2010) containing three main open reading frames (ORFs). Replicase protein encoded by ORF1 (Rep gene) is essential for viral replication (Mankertz et al., 1998). Capsid protein encoded by ORF2 (Cap gene) is a viral structural protein playing a significant role in the immunogenicity, virulence, and characteristics of the virus genotypes (Nawagitgul et al., 2000; Olvera et al., 2007). Finally, an ORF3 protein could induce apoptosis (Olvera et al., 2007). Since its discovery, PCV2 has been recognized as a viral pathogen with a significant economic impact on the pig industry in various regions, particularly in North America, Europe, and Asia (Grau-Roma et al., 2011; Afolabi et al., 2017; Franzo and Segales, 2018).

To date, PCV2 is classified into eight genotypes, PCV2a – h, based on the ORF2 nucleotide sequence (Franzo and Segales, 2018). PCV2d is currently the predominant genotype worldwide (Xiao et al., 2015), possibly due to selection pressure from the global PCV2 vaccination or the previously circulating PCV2 strains. In general, mutation at neutralizing epitopes might render the mutant virus less susceptible to the pre-existing antibodies (from vaccination or previously circulating viruses) (Huang et al., 2020). Hence, a further genetic shift from the current PCV2d strains was not unexpected. In this study, novel variants of PCV2d with a unique

mutation at a previously recognized immunoreactive domain on the ORF2 were identified and found to rapidly dominate in Thailand. This finding may raise awareness for further investigations on the spreading of these viruses in other regions and the cross-protection with current commercial vaccines.

Materials and methods

Clinical samples

A set of 742 swine clinical samples, each collected from a different pig, were retrieved from the sample repository of Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL) and Diagnostic Laboratory of Large Animal Hospital and Students Training Center (DLSTC). The samples were originally submitted to CU-VDL and DLSTC as part of routine diagnosis from January 2019 to December 2020. These samples were obtained from 145 swine farms located in 18 provinces across different geographical regions of Thailand, primarily in the high pig-density areas in the Western, Central, and Eastern parts. The corresponding data of these samples were also obtained, including sample types, sample collection dates, age groups or statuses of the pigs, clinical signs, and farm locations.

PCV2 detection and DNA sequencing

Viral DNA was extracted from the clinical samples by using the IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) on the automated extraction platform. The obtained DNA was stored at -80°C until used.

For PCV2 detection, a real-time PCR assay was done using Luna® Universal Probe qPCR master mix (NEB, MA, USA) with previously described protocol(Wang et al., 2019). The PCV2-PCR positive samples were further systematically selected for genetic characterization.

The sample selection process aimed to fulfill three criteria; 1) obtaining at least one PCV2 sequence from each of the six geographical regions of Thailand (the Northern, Northeastern, Central, Western, Eastern, and Southern regions), 2) including PCV2 sequences from both 2019 and 2020, and 3) acquiring a maximum of one PCV2 sequence from each individual pig. Whenever possible, samples with ct values lower than 30 during the PCV2 detection process were selected, to increase the likelihood of successful whole genome sequencing.

For genome sequencing, PCR amplicons were prepared and then submitted to the third-party sequencing company. The PCR assay was performed as previously described (Fenaux et al., 2000; An et al., 2007). The PCR reactions were done using Onetaq® 2x Master Mix (NEB, MA, USA). The PCR products were examined by 1% agarose gels and purified using NucleoSpin™ Gel and PCR Clean-up (MACHEREY-NAGEL, Germany). The PCR products were then submitted to Celemics, Inc. (Seoul, Korea) for barcode-tagged sequencing. The obtained nucleotide sequences were assembled and validated with SeqMan and EditSeq software v.5.03 (DNASTAR Inc., Madison, Wisconsin, USA) and submitted to GenBank.

Sequence analysis

Classification of the Thai PCV2 sequences was done using a previously proposed phylogeny-based method (Franzo and Segales, 2018). The Thai PCV2 sequences (n = 51) were aligned with a set of PCV2a-h reference sequences (Franzo and Segales, 2018). In total, 317 sequences were used for phylogenetic analysis. Phylogenetic trees were constructed based on the Neighbor-Joining (NJ) algorithm using p-distance data. The classification was separately done using the complete sequences of genomic, ORF1, and ORF2 data. The tree was also reconstructed using the Maximum Likelihood method with a sequence of PCV1 (GenBank accession number: KJ408798) as an outgroup to confirm the topology.

For the initial PCV2d sequence analysis, a phylogenetic tree of complete ORF2 sequences of Thai PCV2d from 2010 – 2020 was built (n = 124). The data from 2010 – 2015 were retrieved from GenBank and the data from 2019 – 2020 (n = 51) were from this study. Nucleotide sequences were aligned, and the phylogenetic tree was then constructed based on the NJ algorithm with the Maximum Composite Likelihood model (NJ-MCL method).

NCBI BLAST function (https://blast.ncbi.nlm.nih.gov) was performed using 19RBR58 ORF2 as a query sequence (6th February 2023) to retrieve sequences with high similarity from GenBank database for further PCV2d ORF2 analysis. The dataset was named 19RBR58/BLAST. The non-redundant version of 19RBR58/BLAST

(19RBR58/BLAST/NR, n = 158) was used in phylogenetic analysis. A phylogenetic tree was constructed using NJ-MCL method.

Otherwise stated, all sequence alignment was done using the Clustal W algorithm (Thompson et al., 1994) of BioEdit 7.2.5 (Hall, 1999). Phylogenetic tree construction was done using MEGA version 10.2.6 (Tamura et al., 2013) with bootstrap analysis of 1000 replications.

Recombination analysis was carried out using Recombination Detection Program (RDP, version 4.22) (Martin et al., 2010). Seven recombination detection methods were used; i.e., RDP, GENECOV, Bootscan, MaxChi, Chimaera, SiScan, and 3Seq. A recombination event was accepted when it was detected by at least five methods with the p-values < 0.01. Bonferroni correction was applied. In the final step, the identified recombinant virus was re-analyzed with SIMPLOT software v. 3.5 by Bootscan methods (Martin et al., 2010) and a direct PCR sequencing covering the recombination breakpoint.

Results

PCV2d is the major genotype in Thailand

PCV2 screening by real-time PCR was done on 742 pigs from 145 farms. The results are shown in Table 4. Overall, animal-level and farm-level positivity were 54.2% (402/742) and 81.4% (118/145), respectively. Fifty-one PCV2-positive samples (from 51 pigs) from 48 farms were genetically characterized. The nucleotide

sequences were deposited in the NCBI GenBank database under accession no. OL677572 – OL677622. Phylogeny-based genotyping of the ORF2 data showed that the Thai strains were PCV2b and PCV2d (Figure 4), found at 13.73% (7/51) and 84.31% (43/51), respectively (similar results were observed when ORF1 or genome data were used). However, one strain, 19NPT29, was not grouped within any genotype clusters. At the farm level, PCV2b and PCV2d were found at 14.58% (7/48) and 87.50% (42/48), respectively.

Novel PCV2d variants were identified and dominated among the PCV2d strains

Due to the high detection rate of PCV2d in this study, a phylogenetic tree was constructed to examine the genetic relationship between the current Thai PCV2d sequences (2019 – 2020) and the previously identified Thai PCV2d sequences (2010 – 2015). A cluster of PCV2d strains exclusively from 2019 – 2020 with a high bootstrap support was identified (data not shown). This cluster was named 19RBR58-like cluster, which accounted for 69.77% (30/43) of the PCV2d strains or 58.82% (30/51) of the PCV2 in this study. Percent nucleotide sequence identity of the 19RBR58-like cluster was as follows; genomic: 99.60 – 100, ORF2: 99.29 -100, and ORF1: 99.58 – 100. Amino acid sequence identity was as follows; capsid: 99.15 – 100, and replicase: 99.36 – 100.

ORF2 nucleotide and amino acid sequence alignment were examined to identify a distinctive genetic characteristic of the 19RBR58-like cluster. A unique

^{133}HDAM^{136} and ^{232}K were found in all amino acid sequences from the 19RBR58-like cluster. Other Thai PCV2 sequences (PCV2a, b, d, and h) were ^{133}ANAL^{136} or ^{133}ATAL^{136}. To our knowledge, PCV2 strains with ^{133}HDAM^{136} have not been reported previously.

NCBI BLAST function using 19RBR58 ORF2 (6th February 2023) (https://blast.ncbi.nlm.nih.gov) was used to retrieve sequences with high similarity from GenBank database for further analysis. From the dataset, PCV2d strains with ^{133}HDAM^{136} from Japan and Taiwan during the period of 2018 - 2020 were found. However, those sequences were direct submissions. Moreover, PCV2d with amino acid sequences other than HDAM, ANAL, and ATAL at the position 133 – 136 were also identified such as HAAM and HNAM. Clustering of the 19RBR58-like viruses was shown in Figure 5.

The novel PCV2d variants was a parental strain of a PCV2b/2d recombinant virus

Recombination analysis using seven different methods provided strong statistical support (average p-value = 3.84×10^{-9}) confirming that 19NPT29 is an intergenotypic recombinant virus of PCV2b and PCV2d. The analysis indicated that PCV2b strains (such as South Korea/2016/KU-1605-like viruses) and PCV2d strains (such as Thailand/2019/19RBR10-like viruses) were the potential parental strains involved in the recombination event. Notably, the parental PCV2d strain was also found within the 19RBR58-like cluster, and the presence of ^{133}HDAM^{136} and ^{232}K in the capsid protein was observed in 19NPT29. The putative recombination breakpoints

were identified at nucleotide positions 508 (ORF1) and 1356 (ORF2) (Figure 6). The detection rate of the recombinant strain at the animal and farm levels was found to be 1.96% (1/51) and 2.08% (1/48), respectively.

Table 4 *The prevalence of PCV2 in all tested samples during 2019-2020*

		Types of samples							Prevalence at sample level	Prevalence at farm level
Periods	Group of pigs†	Internal organs	Serum	Feces	Semen	Oral fluids	Colostrum	Umbilical cords	Positive rate	Positive rate
Jan-Dec 2019	Suckling	16/21	0/7	4/7	-	-	-	-	194/373 (52.0%)	62/81 (76.5%)
	Nursery	66/75	24/98	0/1	-	-	-	-		
	Growers	28/28	15/38	0/1	-	-	-	-		
	Breeders	2/4	14/48	1/1	1/10	-	-	-		
	Fetuses	23/34	-	-	-	-	-	-		
Jan-Dec 2020	Suckling	3/8	2/13	0/1	-	-	-	-	208/369 (56.4%)	66/74 (89.2%)
	Nursery	38/43	23/53	1/4	-	0/2	-	2/2		
	Growers	14/15	90/132	-	-	-	-	-		
	Breeders	0/2	10/38	-	1/12	-	2/2	-		
	Fetuses	22/42	-	-	-	-	-	-		
Jan 2019-Dec 2020		212/272 (77.9%)	178/427 (41.7%)	6/15 (40.0%)	2/22 (9.1%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	402/742 (54.2%)	118/145 (81.4%)††

† Suckling: < 4 weeks; Nursery: 5 - 8 weeks; Growers: 9 - 20 weeks; Breeders: boars, gilts, and sows

†† The PCV2 positive farms were calculated during Jan 2019 – Dec 2020.

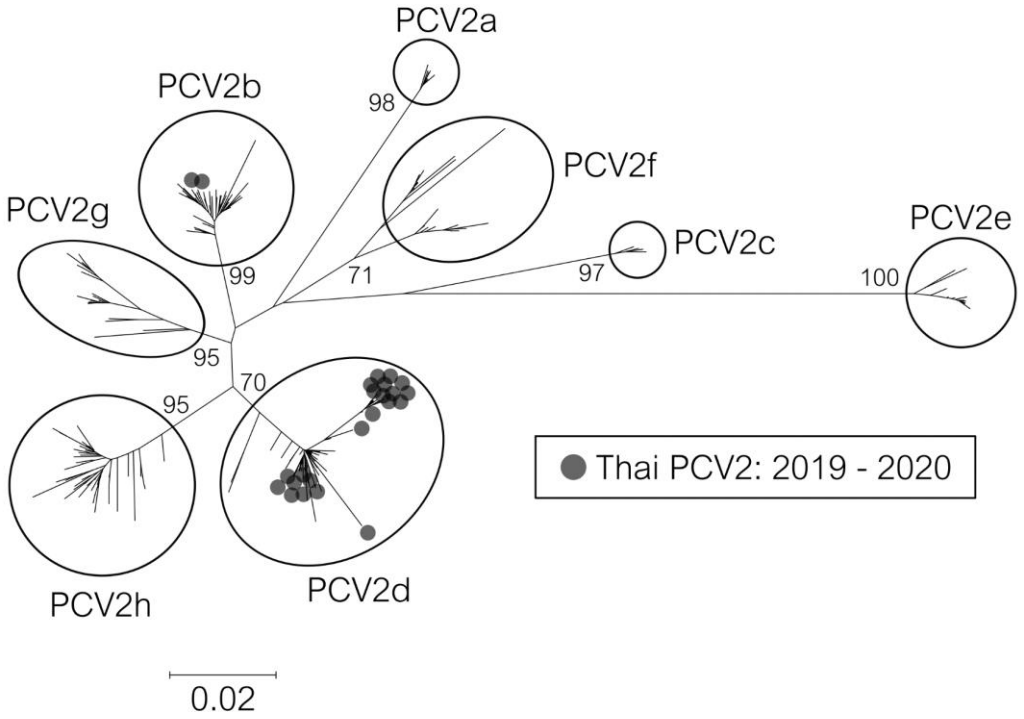

Figure 4 *Phylogenetic tree of PCV2 ORF2 sequences from Thailand. The 317 complete ORF2 sequences were Thai PCV2 sequences (2019–2020) in this study and reference sequences from an available database (Franzo and Segales, 2018). The tree was constructed using the Neighbor-Joining method with a p-distance model and bootstrapping at 1,000 replicates. Node labels indicate bootstrap values. The taxon position markers were adjusted to enhance readability.*

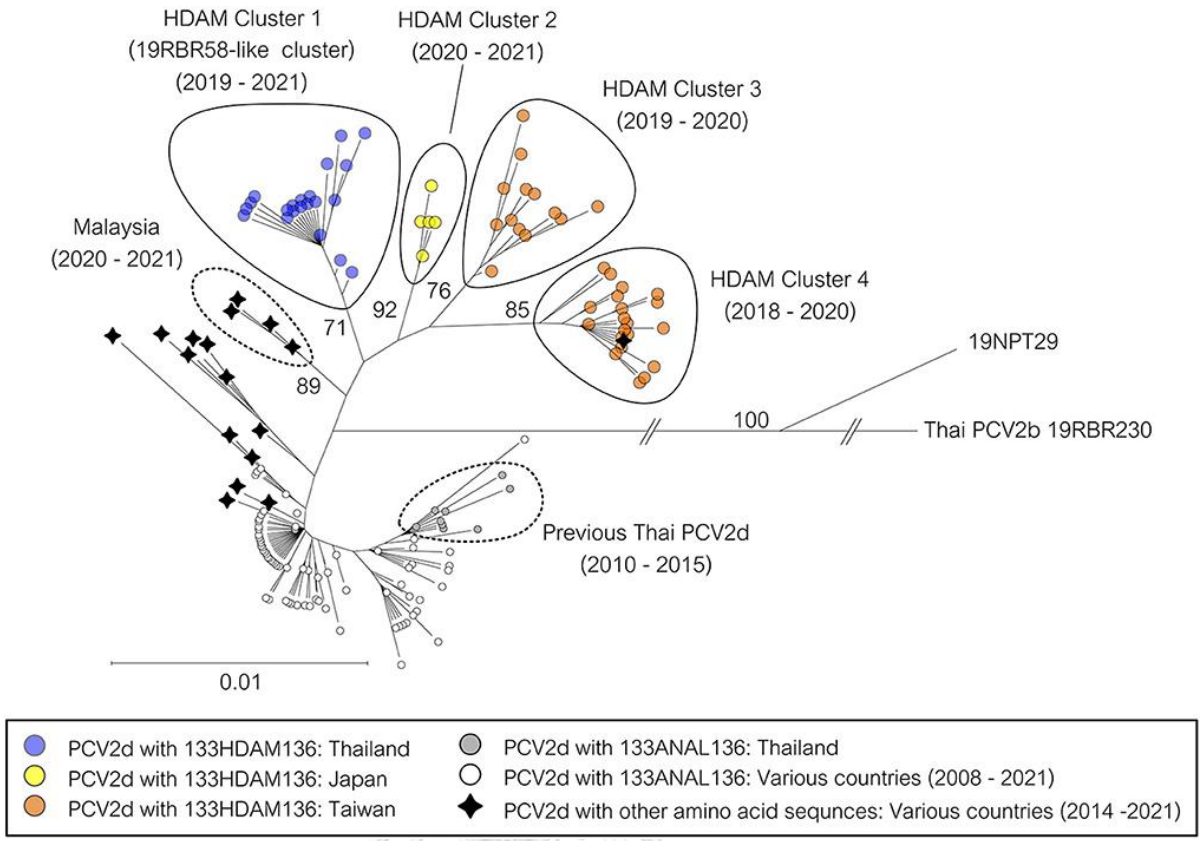

Figure 5 *Phylogenetic tree of PCV2d ORF2 sequences from Thailand. The 158 complete ORF2 sequences were Thai PCV2d sequences in this study and the related PCV2d sequences from NCBI BLAST. A sequence of Thai PCV2b was used as an outgroup. The tree was constructed using the Neighbor-Joining method with the Maximum Composite Likelihood model and bootstrapping at 1,000 replicates. Node labels indicate bootstrap values. Specific branches were shortened, and the taxon position markers were adjusted to improve readability.*

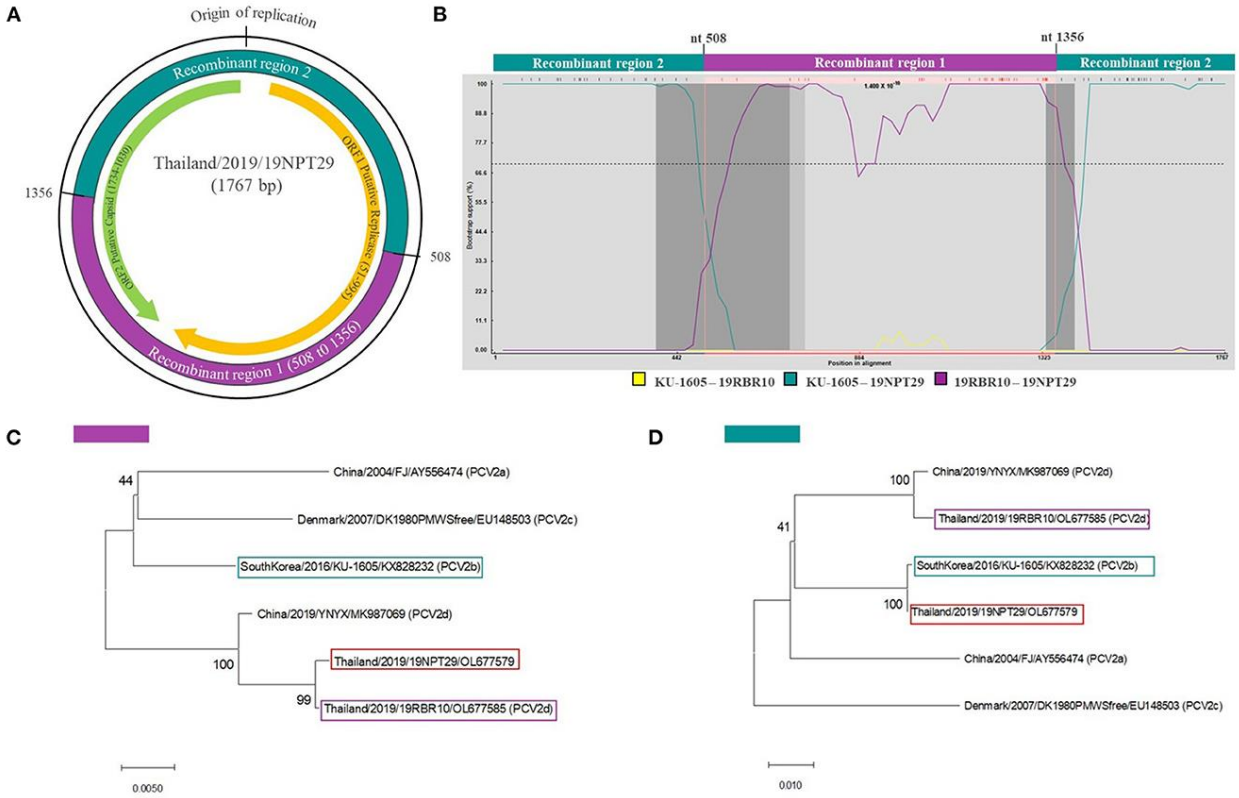

Figure 6 *Recombination analysis of 19NPT29. Recombination analysis was done using RDP software. Recombination breakpoints were identified in ORF1 and ORF2 regions of the 19NPT29 genome, resulting in recombinant region 1 (purple shaded) and 2 (dark green shaded) (A). Bootscanning analysis shows KU-1605 (South Korea) and 19RBR10 (Thailand, in this study) as parental strains (B). The phylogenetic trees were constructed based on recombinant region 1 (C) and 2 (D) to confirm recombination event.*

Discussion

PCV2 is a major swine virus causing economic losses. Although vaccines have been widely used, vaccine failures and immune escaping mutation of PCV2 has been proposed (Xiao et al., 2015). In this study, novel variants of PCV2d were identified providing a clue on the PCV2 evolution and epidemiology.

The prevalence of PCV2 in Thailand remained consistently high during the period of 2019 – 2020 compared to the period of 2009 – 2015. The prevalence at the animal level from 2009 – 2015 was 44.09% (Thangthamniyom et al., 2017). In the current study, the prevalence increased to 54.2%. At the farm level, the prevalence from 2009 – 2015 was 80% (Thangthamniyom et al., 2017), and in 2019 – 2020, it reached 81.4%. These findings suggests that PCV2 was still circulating, despite the implementation of PCV2 vaccines in Thailand. Unfortunately, the PCV2 vaccination status of each farm was not available in this study. Therefore, no conclusion can be made regarding the effect of the PCV2 vaccination and the overall PCV2 prevalence in Thailand.

In recent years, a genotype shift towards PCV2d can be observed in various countries, particularly in Asia. These countries include China (Nan et al., 2022), South Korea (Kwon et al., 2017), Vietnam (Doan et al., 2022), Malaysia (Tan et al., 2022), and Thailand (Thangthamniyom et al., 2017). In Thailand, the prevalence of PCV2d has been increasing since 2010, with only PCV2d detected by 2015 (Thangthamniyom et

al., 2017). However, in this study, a novel strain of PCV2d, which accounted for 69.77% of all the current Thai PCV2d, was identified. Therefore, this novel strain of PCV2d plays a crucial role in the prevailing PCV2d strain in Thailand during 2019 – 2020. Moreover, this finding suggests that it may serve as the starting point for the next genetic shift within PCV2d.

This study identified the dominance of novel PCV2d strains, the 19RBR58-like cluster, over the previously circulating PCV2 strains in Thailand. At position 133 – 136 of the capsid protein, the 19RBR58-like cluster was ^{133}HDAM^{136} while other Thai PCV2 strains were ^{133}ANAL^{136} or ^{133}ATAL^{136}. Notably, this region of amino acids resides in one of the antibody recognition domains (domain B) previously described (Mahe et al., 2000; Lekcharoensuk et al., 2004), i.e., domain A (aa 51-84), B (aa 113-139), C (aa 161-207), and D (aa 228-233). A single mutation at position 134, 135 or 136 has been shown to strongly reduce the neutralization activity (Huang et al., 2020). Therefore, the capsid protein with ^{133}HDAM^{136} might render the 19RBR58-like cluster less susceptible to the antibodies from the previously circulating strains and the vaccines. In fact, PCV2 vaccination is widely implemented in Thailand (personal communication). The observed immune escaping mechanism is further supported by the rapid increase of the 19RBR58-like cluster. Prior to 2015, the 19RBR58-like cluster was not detected in Thailand, and there is a lack of sequence data from 2016 to

2018. Thus, it is possible that the emergence of the 19RBR58-like cluster occurred during the period of 2016 – 2018.

In addition to Thailand, this study also identified PCV2d sequences with ^{133}HDAM^{136} from Japan and Taiwan (direct submission in GenBank). Interestingly, the strains carrying ^{133}HDAM^{136} from each region formed a distinct cluster on the phylogenetic tree. This suggests that the current situation of these viruses may not be attributed to recent spreading between regions. Phylogenetic analysis further revealed that all the clusters harboring ^{133}HDAM^{136} (Thailand, Japan, and Taiwan) likely share a common ancestor with PCV2d strains from Malaysia. At present, the prevalence of the ^{133}HDAM^{136} PCV2d variants in Japan and Taiwan is unknown. Further investigations are needed to determine whether the prevalence of these PCV2d variants is high, similar to that observed in Thailand.

Recombinant viruses derived from PCV2d strains have been reported in various countries, including China, India, and South Korea (Wei et al., 2019; Jang et al., 2021; S et al., 2022). In this study, a recombinant PCV2d/PCV2b strain, named 19NPT29, was identified. Interestingly, ^{133}HDAM^{136} was also found in the capsid protein of 19NPT29. It would be valuable to conduct further studies to investigate whether the presence of ^{133}HDAM^{136} provides any advantages to the recombinant PCV2 strain, particularly in the case of inter-genotypic recombinants. Unfortunately,

conducting further epidemiological studies on the 19NPT29-like viruses from the source farm is not possible as the farm is no longer operational.

The main limitation of this study was the absence of information regarding the vaccination status of the farms, along with the passive surveillance nature of the study. The observed mutation in the antibody recognition domain of the capsid protein within the 19RBR58-like cluster is suspected to have played a role in its emergence. Therefore, the information regarding the vaccination status would have been invaluable for interpreting the data and generating hypotheses for further studies on cross-protection. Furthermore, it is important to note that the samples used in this study were obtained from two diagnostic laboratories (CU-VDL and DLSTC), which may have led to potential underrepresentation of certain geographical regions. However, it is worth mentioning that this study managed to collect samples from all the high-pig-density regions in the country. Conducting active surveillance in the future may provide a more precise assessment of the prevalence of PCV2 and the PCV2d status.

In conclusion, this study reveals the presence of a novel PCV2d strain with ^{133}HDAM^{136} in the capsid protein as the predominant PCV2 strain in Thailand. Additionally, a recombinant virus between PCV2b and the novel PCV2d was identified. The emergence of these novel PCV2d strains might have been influenced by both vaccination and the previously circulating viruses. Conducting active

surveillance can provide a comprehensive understanding of PCV2 evolution and facilitate the implementation of early interventions against the emergence of novel strains.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors helped in designing the research. CS, TC and TJ collected the samples and did the PCR and sequencing. CS, RK and RT analyzed the data and interpreted the results. CS, RK and RT prepared the manuscript.

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Data Availability Statement

Datasets are available on request.

CHAPTER 4

PREVALENCE AND GENETIC CHARATERIZATION OF PORCINE CIRCOVIRUS 3 IN THAI SWINE FARMS DURING 2019-2020

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Abstract

To date, porcine circovirus 3 (PCV3) has been reported worldwide with various clinical manifestations. The virus has been confirmed in Thai swine farms. Nevertheless, prevalence and genetic characteristics of PCV3 across a wide range of Thai swine farms in high density regions has not yet been firmly established. We investigated PCV3 in 734 samples from 145 Thai farms in 2019 and 2020. Results revealed a significant presence, with 28.88% of samples and 46.90% of farms positive. Prevalence in 2019 and 2020 was 25.5% and 32.52% at the sample level and 46.91% and 48.65% at the farm level. Genetic analysis of 33 Thai PCV3 strains showed high nucleotide identity (97.4% to 100%) among them, compared to the reference strains (87.5% to 99.6%). Only PCV3a was found in this study. In conclusion, this study highlights a substantial prevalence of PCV3 in Thai swine farms and provides insights into the genetic diversity of the virus. The findings emphasize the importance of ongoing surveillance and management efforts for PCV3 in swine populations, considering its potential for cross-border transmission.

Keyword: porcine circovirus 3, prevalence, genetic analysis, swine, Thailand

Introduction

In 2015, porcine circovirus 3 (PCV3) was first identified in the US pig population with various clinical manifestations, including multi-systemic inflammation, reproductive failure, and porcine dermatitis and nephropathy syndrome (Phan et al., 2016; Palinski et al., 2017). Since its discovery, PCV3 has been reported worldwide in almost all intensive pig production countries (Faccini et al., 2017; Ku et al., 2017; Kedkovid et al., 2018; Kim et al., 2018; Klaumann et al., 2018b; Saraiva et al., 2018; Saporiti et al., 2020; Molini et al., 2021; Turlewicz-Podbielska et al., 2022; Vargas-Bermudez et al., 2022). Currently, PCV3-associated diseases were classified into PCV3-reproductive disease (PCV3-RD) and PCV3-systemic disease (PCV3-SD) (Saporiti et al., 2021). Furthermore, the virus was also detected in healthy pigs, raising questions about its pathogenicity. This suggests that clinical presentations induced by PCV3 could be influenced by unidentified factors, warranting further investigations.

The worldwide distribution of PCV3 has led to an increase in PCV3 molecular epidemiology studies, resulting in a variety and amount of genetic and molecular data that have produced confusion regarding the classification of viruses among different research groups. Therefore, new unified classification criteria for PCV3 were recently proposed using a maximum within-genotype genetic distance of 3% at the complete genome and 6% at the ORF2 levels, with bootstrap support higher than

90% (Franzo et al., 2020a). Hence, PCV3a was proposed and regarded as the only one genotype based on based on these characteristics.

In Thailand, there is a scarcity of comprehensive PCV3 genetic epidemiology data across a wide range of the Thai swine farms. Therefore, the objective of this study aimed to investigate the prevalence and genetic diversity of PCV3 based on ORF2 gene in high density areas of Thai swine farms. Consequently, the insights gained from this research will also contribute to a deeper understanding of the prevalence and genetic epidemiology of PCV3 in Thailand.

Materials and methods

Sample collection

A total 734 of swine samples gathered from different farms in high-density pig zones in Western, Central, and Eastern Thailand for diagnostic proposes during January 2019- December 2020 were included in this study. These samples were kindly provided by Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL), and Chulalongkorn University Diagnostic Laboratory of the Large Animal Hospital and Students Training Center.

Viral DNA extraction and PCV3 detection

DNA extraction was performed by IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) according to the manufacturer's instructions. The extracted DNA samples were subsequently kept at -80°C until used.

To detect PCV3, a real-time PCR assay (qPCR) was performed using Luna™ Universal Probe qPCR master mix (NEB, MA, USA) following a previously established protocol (Wang et al., 2019) (Table 5). PCR reactions were conducted in a 20 µl reaction volume, comprising 0.4 µM of both forward and reverse primers, 0.2 µM of probes, 10 µl of Luna® Universal Probe qPCR master mix (NEB, MA, USA), and 4 µl of the extracted DNA. The PCR begins with an initial denaturation step at 95°C for 60 seconds, followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. This amplification was performed using the QuantStudio 5 real-time system (Applied Biosystems, USA).

PCV3 sequencing and phylogenetic analysis

Samples that tested positive with a qPCR cycle threshold (ct) value lower than 30 were subsequently selected for amplification and sequencing**.** The complete ORF2 gene of PCV3 was carried out following a previously established protocol (Ku et al., 2017) (Table 5). Briefly, the PCR reactions were conducted in 25 ul reaction volume, consisting of 0.2 uM of both forward and reverse primers, 12.5 ul of OneTaq® Quick-Load 2X master mix (NEB, MA, USA), and 5 ul of the extracted DNA. The thermocycling conditions consisted of an initial denaturation step at 94°C for 30 seconds, followed by 30 cycles of denaturation at 94°C for 15 seconds and annealing at 48°C for 30 seconds and extension at 68°C for 45 seconds and final extension step at 68°C for 5 minutes. The PCR products were purified using the NucleoSpin™ Gel

and PCR clean-up kit (MACHEREY-NAGEL, Germany) and then sent for sequencing through a barcode-tagged sequencing platform (Celemic, Seoul, Korea).

The obtained nucleotide sequences were further analyzed and assembled with SeqMan, and Editseq software v.5.03 (DNASTAR Inc., Madison, Wisconsin, USA). To perform pairwise comparisons and genetically characterize PCV3, the ORF2 gene sequences were aligned using the Clustal W algorithm within BioEdit 7.2.5 (https://bioedit.software.informer.com/), alongside reference PCV3 strains obtained from the GenBank database. Phylogenetic trees were constructed using MEGA version 10.2.6, employing the maximum likelihood method based on the Hasegawa-Kishino-Yano model+G along with the bootstrap analysis of 1000 replications for robustness. (Tamura et al., 2013).

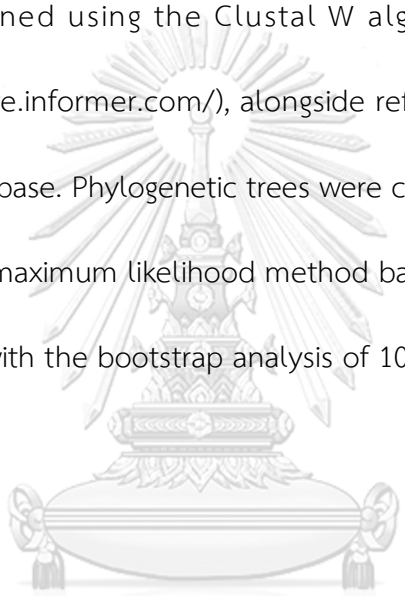

Table 5 *List of primers and probe used in this study.*

Primers/probe	Nucleotide sequence (5'-3')	Target gene	Purpose	Reference
PCV3F-qPCR	GGTGAAGTAACGGCTGTGTTTT	ORF2 (86 bp)	Detection	(Wang et al., 2019)
PCV3R-qPCR	ACACTTGGCTCCARGACGAC			
PCV3-Probe	FAM-ATGCGGAAAGTTCCACTCGK-BHQ1			
PCV3-F	TTACTTAGAGAACGGACTTGTAACG	ORF2 (649 bp)	Genome sequencing	(Ku et al., 2017)
PCV3-R	AAATGAGACACAGAGCTATATTCAG			

Results

Prevalence of PCV3 in Thai swine farms during 2019-2020

In this study, a total 734 swine samples from 145 different farms in high density areas across Western, Central, and Eastern Thailand were tested with qPCR. The results showed that the positive rates of PCV3 were 28.88% (212/734) and 46.90% (68/145) for sample level and farm level, respectively. Data were analyzed by year, the prevalence of PCV3 in the samples was as follows: 25.5% (92/365) in 2019 and 32.52% (120/369) in 2020 at the sample level. At the farm level, the prevalence was 46.91% (38/81) in 2019 and 48.65% (36/74) in 2020. Figure 7 illustrates the prevalence of PCV3 in Thai swine farms from 2019 to 2020.

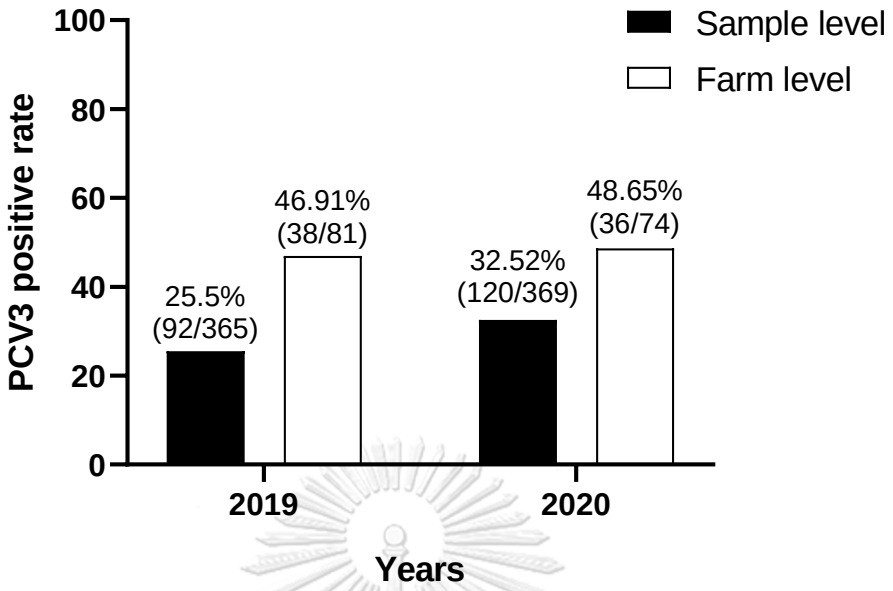

Figure 7 *The prevalence of PCV3 in Thai swine farms during 2019-2020. Percentages of PCV3-positive rate are shown. Number of positive samples and farms are shown in parenthesis.*

Genetic characteristics of PCV3

The obtained cap gene sequences of 33 Thai PCV3 strains were 636 nucleotides, indicative of partial ORF2 sequences. In this study, phylogenetic analysis based on partial cap gene sequences was aligned against those reference strains. The sequence analysis revealed that the 33 Thai PCV3 strains shared a nucleotide identity ranging from 97.4% to 100% among themselves. Moreover, when compared to the reference strains, they exhibited nucleotide identities spanning from 87.5% to 99.6%. Additionally, they displayed a nucleotide identity range of 97.9% to 100% with the initial PCV3 sequence reported in Thailand (PCV3/PB01/MG310152).

The phylogenetic tree demonstrated that all Thai PCV3 strains were highly similar and formed a closely clustered group. According to PCV3 genotyping criteria outlined by Franzo et al. (Franzo et al., 2020a), the finding indicated that all 33 Thai PCV3 strains were clustered as PCV3a (Figure 8). The representative PCV3 strain (PCV3/P119/2020) was employed for comparison with the reference strains. The phylogenetic analysis additionally demonstrated a remarkably close relationship with PCV3 strains from Italy (99.6%, MF162298), Korea (99.6%, KY996341), China (99.5%, MF405272), Spain (99.5%, MW167068), Malaysia (99.5%, MN725080), and Brazil (99.5%, MF079254) based on partial ORF2 nucleotide identity. Interestingly, the PCV3 strain (PCV3/328/2019), which was in a distinct branch from the Thai PCV3 strains, exhibited a 98.7% nucleotide identity with a PCV3 strain from Sweden.

At the amino acid (aa) level, the Thai PCV3 strains in this study shared amino acid identities ranging from 98.1% to 100% with each other. In comparison to the PCV3 reference strains, the similarities between them ranged from 90.5% to 100%. Among the 33 Thai PCV3 strains, 29 of them exhibited 24V and 27K variations in the cap gene, while 3 Thai PCV3 strains displayed 24A and 27R substitutions in the cap gene. Notably, one PCV3 strain (PCV3/248/2019) exhibited a single amino acid mutation (R25K) within the antibody recognition domains (23–35aa and 119–131aa) of the PCV3 cap gene, which has been reported previously (Wang et al., 2020). Furthermore, Thai PCV3 strain (PCV3/328/2019) exhibited two amino acid mutation (N56D and Q98R) in predicted B-cell epitopes as previously reported (Li et al., 2018a).

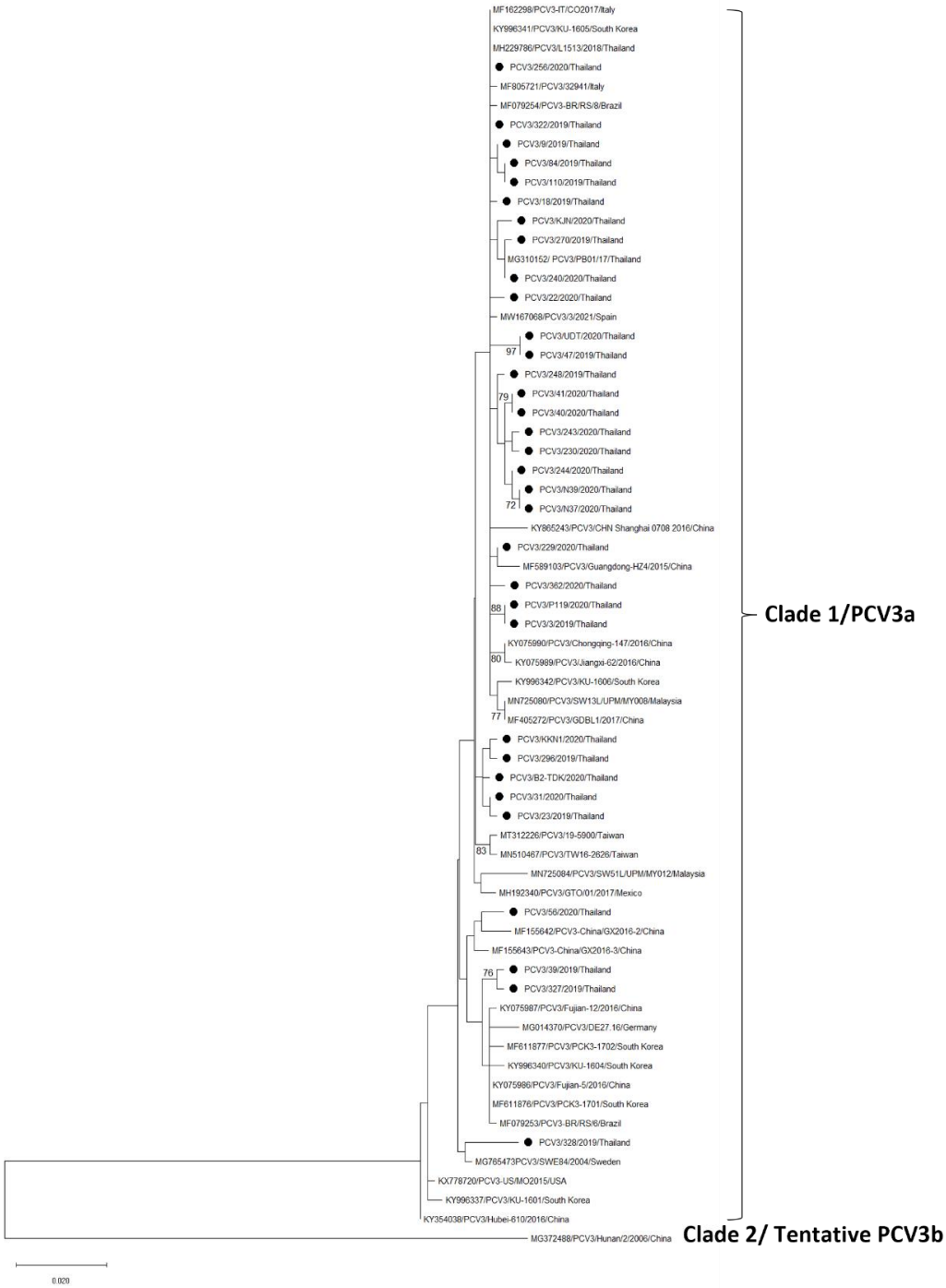

Figure 8 *A phylogenetic tree was constructed based on the partial capsid gene using the Maximum Likelihood method and the Hasegawa-Kishino-Yano model with 1000 bootstrap values. The Thai PCV3 strains in this study were denoted by black solid circles.*

Discussion

In this study, a total of 734 swine samples were collected from 145 distinct farms located in high-density pig farming regions throughout Western, Central, and Eastern Thailand between 2019 and 2020. These samples were subjected to qPCR screening to assess the prevalence of PCV3. The overall positive rates of PCV3 were determined to be 28.88%, which is lower than the rates previously reported in Thailand (Visuthsak et al., 2021). However, when analyzed by year, the PCV3 prevalence exhibited a slight increase, rising from 25.5% in 2019 to 32.52% in 2020 at the sample level and increased from 46.91% in 2019 to 48.65% at the farm levels. This suggests that the virus has been active in the Thai swine farms, but clinical manifestations induced by PCV3 remain controversial. Nevertheless, the prevalence of PCV3 in Thai swine farms in this study was higher than the prevalence reported in Malaysia and Vietnam (Tan et al., 2020; Dinh et al., 2023), implying a higher transmission rate within Thai swine farms than that of the neighboring countries. However, the study in China demonstrated the high level of PCV3 infection, spanning from 7.41-70% (Chen et al., 2022).

In comparison to other PCVs, a previous study revealed that PCV2 was the most prevalent in Thailand during 2019-2020, with rates of 54.2% at the sample level and 81.4% at the farm level (Sirisereewan et al., 2023a). It indicated that co-circulation of PCV2 and PCV3 in Thai swine farms may more or less cause the economic consequences for the swine industry. In general, both PCV2 and PCV3

exhibit similar clinical manifestations, making diagnosis based solely on clinical signs challenging. Hence, PCV3 detection is crucial for understanding its prevalence, evolution, and devising effective disease control strategies.

For genetic analysis, all Thai PCV3 strains were classified as PCV3a based on the genotyping classification purposed by Franzo et al (Franzo et al., 2020a). For cap gene sequences, it indicated that Thai PCV3 strains had a high nucleotide identity (97.4-100%) for each other. In this study, the representative PCV3 strain (PCV3/P119/2020) exhibited nucleotide identity of over 99% with numerous PCV3 strains worldwide, including those from Italy, Korea, China, Spain, Malaysia, and Brazil. Thus, these findings align with previous studies, suggesting that PCV3 has generally remained stable over time, lacking significant molecular evolution specific to specific regions of the world (Klaumann et al., 2018a; Yang et al., 2022). This could be attributed to the relatively low genetic variability and the evolutionary rate of PCV3, estimated at 10^{-5} substitutions per site per year (Franzo et al., 2019). Notably, one PCV3 strain (PCV3/328/2019) was positioned in a separate branch from the Thai PCV3 strains and exhibited a 98.7% nucleotide identity with a PCV3 strain from Sweden dating back to 2004. The precise origin of this virus remains uncertain, but it is plausible that transmission may be significantly influenced by the importation of live animals and semen between the two countries.

Remarkably, nearly all Thai PCV3 strains displayed two amino acid mutations (A24V and R27K) when compared to the PCV3 prototype strain (US/MO2015, KX778720). Moreover, one PCV3 strain (PCV3/248/2019) exhibited a single amino acid mutation (R25K). It indicated that amino acids mutation fell within the antibody recognition domains (23–35aa and 119–131aa) of the PCV3 cap gene, which has been reported previously (Wang et al., 2020). This could potentially influence the immune evasion mechanism. Furthermore, the Thai PCV3 strain (PCV3/328/2019) displayed two amino acid mutations (N56D and Q98R) within predicted B-cell epitopes, consistent with previously reported (Li et al., 2018a) that may confer different immunogenicity.

In summary, this study investigated the prevalence of PCV3, revealing widespread infection in Thai swine farms, particularly in high-density regions, with an upward trend observed over the years. The most predominant strain of PCV3 in Thai swine farms was PCV3a with high genetic stability. While the economic impact of PCV3 infection on the Thai swine industry requires further investigation, conducting active surveillance for prevalence and genetic evolution will offer valuable insights for developing prevention and control strategies.

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CHAPTER 5

MOLECULAR DETECTION AND GENETIC CHARACTERIZATION OF PORCINE CIRCOVIRUS 4 (PCV4) IN THAILAND DURING 2019-2020

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Abstract

Porcine circovirus 4 (PCV4) is considered a novel PCV, firstly found in China in 2019 and later discovered in Korea. This present study investigated the prevalence and genetic characteristics of PCV4 from high pig-density areas in Thailand during 2019-2020. From 734 samples, three samples (0.4%) from aborted fetuses and porcine respiratory disease complex (PRDC) cases were found positive for PCV4, two of the PCV4-positive samples were coinfected with both PCV2 and PRRSV, and the other PCV4-positive sample was found coinfected with PCV2. *In situ* hybridization (ISH) revealed the presence of PCV4 in the bronchial epithelial cells and in lymphocytes and histiocyte-like cells in the lymphoid follicles of the PRDC-affected pig. The complete Thai PCV4 genome had over 98% nucleotide identity with other PCV4 strains and was closely related to the Korean and Chinese PCV4b strains. Importantly, the amino acid residue at position 212 of the Cap gene is recommended for differentiating PCV4a (^{212}L) from PCV4b (^{212}M) based on currently available PCV4 genome sequences. These findings provide important clues for the pathogenesis, epidemiology, and genetic characteristics of PCV4 in Thailand.

Keywords: Porcine circovirus 4, pigs, complete genome, capsid, genetic characterization, Thailand

Introduction

To date, four species of porcine circoviruses (PCVs) have been identified, including PCV1, PCV2, PCV3, and PCV4 (Opriessnig et al., 2020). PCV1 is nonpathogenic in pigs. PCV2 causes various symptoms collectively called porcine circovirus-associated diseases (PCVADs) or porcine circovirus diseases (PCVDs) (Segales, 2012; Klaumann et al., 2018a; Opriessnig et al., 2020). PCV3 has been found in pigs with multiple clinical signs; however, the pathogenesis is still unknown (Ouyang et al., 2019; Opriessnig et al., 2020; Sirisereewan et al., 2022). Recently, a novel PCV4 has been identified in China and Korea both in clinically healthy and infected pigs with several clinical presentations, including respiratory and enteric signs and skin lesions suggestive of porcine dermatitis and nephropathy syndrome (PDNS) (Zhang et al., 2020; Ha et al., 2021; Nguyen et al., 2021; Tian et al., 2021; Hou et al., 2022; Xu et al., 2022a). Therefore, despite having limited information on PCV4, the virus should not be overlooked. Furthermore, due to the lesson learned from past experiences with other swine viruses in Asia (Kedkovid et al., 2020), many emerging viruses have spread among countries due to the consequences of globalization through international trade and travel, both officially and unofficially. Therefore, the recent findings of PCV4 in China and Korea would raise awareness of the virus to the Asian swine practitioners to investigate this novel pathogen in their areas as part of the focus area “prevent and detect” to understand the disease distribution and its

impact. In this study, we investigated from the samples obtained during 2019-2020 for the presence of PCV4 in Thailand and its genetic characterization.

Materials and methods

Sample collection and viral DNA extraction

Seven hundred thirty-four samples from 145 swine farms submitted for diagnostic purposes at the Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL), and Diagnostic Laboratory of Large Animal Hospital and Students Training Center during January 2019- December 2020 were used for this study. The samples consisted of serum (n = 426), tissue (n = 188), fetus (n = 75), semen (n = 25), feces (n = 16), colostrum (n = 2), and oral fluid (n = 2) that were mainly collected from the high pig density areas in the Western, Central, and Eastern parts of Thailand.

Total viral DNA was extracted using IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) following the manufacturer's instruction. The extracted DNA was stored at -80℃ until used.

Molecular detection of PCV4

TaqMan® real-time PCR was performed to detect PCV4 targeting the replicase gene (rep) of PCV4 using two newly designed primer pairs and probe (Appendix B). Briefly, PCR reactions were performed in a total 20 µl reaction containing 0.4 µM of forward and reverse primers, 0.2 µM of probes, 10 µl of Luna® Universal Probe qPCR master mix (NEB, MA, USA), and 3 µl of extracted DNA. The PCR condition consisted of initial denaturation at 95℃ for 60s followed by 45 cycles of 95℃ for 15s and 60℃

for 30s using Quantstudio5 real-time system (Applied Biosystems, USA). Positive control plasmid was synthesized by inserting a full length of PCV4 rep gene (891 bp) into the pUC18 vector by GenScript Company (Nanjing, China). Detection limit of the PCV4 TaqMan® real-time PCR was 200 copies/µl of standard plasmid DNA.

Complete genome amplification and sequencing

The complete genomes of PCV4-positive samples were amplified using two primer sets. The PCR reactions were performed in 25 µl reaction mixtures containing 3 µl of extracted DNA, 0.5 µM of forward and reverse primers, and 12.5 µl of Q5® High-Fidelity 2x master mix. The PCR thermal profile involved an initial denaturation of 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 72°C for 30 s, 72°C for 50 s, and final extension at 72°C for 2 min. The PCR products were purified using Nucleospin™ Gel and PCR clean-up (MACHEREY-NAGEL, Germany) and submitted for sequencing by a barcode-tagged sequencing platform (Celemic, Seoul, Korea). The obtained nucleotide sequences were further analyzed and assembled with SeqMan, and Editseq software v.5.03 (DNASTAR Inc., Madison, Wisconsin, USA), then deposited in GenBank under accession no. ON854861-ON854863.

Phylogenetic analysis

For pairwise comparison and genetic characterization of PCV4, the complete nucleotide sequences were aligned using the Clustal W algorithm of BioEdit 7.2.5 (https://bioedit.software.informer.com/) with the reference PCV4 strains from the GenBank database. Phylogenetic trees were reconstructed with MEGA version 10.2.6

using the neighbor-joining algorithm (NJ) with 1000 bootstrap replicates (Tamura et al., 2013).

Currently, guidelines for identifying PCV4 genotypes are not yet fully established, but they have been temporarily proposed and were not consistent. Generally, PCV4 strains have been classified into two main genotypes, PCV4a and PCV4b(Wang et al., 2022; Wu et al., 2022; Xu et al., 2022a). However, a single study has proposed that PCV4 might be classified into three genotypes, including PCV4c (Xu et al., 2022b). To simplify the analysis, in this present study, PCV4 strains were classified into two main genotypes, PCV4a and PCV4b. The phylogenetic analyses were carried out using the complete genome, Rep gene, and Cap gene, following the approach previously described (Xu et al., 2022a).

Detection of PCV4 in tissues using *in situ* hybridization

Of the three PCV4-positive samples, the formalin-fixed paraffin-embedded (FFPE) tissue samples were available for only one case, 19RBR247, and thus used for virus localization analysis. The FFPE tissues were stained with hematoxylin and eosin (HE) for histopathology study. To identify PCV4 tissue localization, *in situ* hybridization (ISH) was performed according to a previously described protocol with some modifications (Piewbang et al., 2022). Briefly, PCV4-specific probe targeting 110 bp of the Cap gene of PCV4 was constructed using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The sections were incubated overnight at 40°C in a moist chamber with PCV4-specific

probe. The PCV4-specific DIG probe was detected by using 1:200 anti-DIG AP Fab fragments (Roche Diagnostics, Basel, Switzerland). Hybridization signals were detected as pink to brilliant dark red colorimetric staining of Permanent Red (LPR) (Dako, Glostrup, Denmark) with 50% hematoxylin counterstaining. Slides incubated without the DIG probe were used as a negative control.

Results

Prevalence and geographical distribution of PCV4 during 2019-2020

To investigate the existence of PCV4, several types of swine samples (n =734) from different geographical regions mainly located in the high pig density areas of Thailand were used. At sample levels, the overall positive rates of PCV4 were 0.4% (3/734). Notably, three PCV4-positive samples from clinically infected pigs with abortion and respiratory signs were found coinfected with PCV2 (3/3) and PRRSV (2/3) (Table 6). Among the 145 pig farms in these 18 provinces, 2.07% (3/145) were PCV4-positive. The geographic distribution of PCV4-positive farms was shown in Figure 9.

Genetic characteristics of PCV4

In this study, all PCV4-positive samples were selected and sequenced for genetic characterization and phylogenetic analysis. The results showed that Thai PCV4 strains were 1770 nt in length, with 891 nt of ORF1 (Rep) and 687 nt of ORF2 (Cap). The complete genome sequences of the Thai PCV4 strains were aligned against those of reference viruses from China and Korea (Table 7). The results showed that the complete genome of the 3 Thai PCV4 strains shared 100%

nucleotide identity to each other and shared 98.3-99.0% nucleotide identity with other reference PCV4 strains (Table 7). An amino acid sequence analysis indicated that the Rep and Cap genes of the Thai PCV4 strains shared similarity of 98.6%-100% and 97.8%-99.1%, respectively, when compared to each other and the reference strains. The Thai PCV4 strains showed the highest similarity to PCV4 NM2 from China with 99% nucleotide identity (complete genome), 100% Rep amino acid identity, and 98.6% Cap amino acid identity. Based on the phylogenetic analysis of complete genome nucleotide sequences of 41 PCV4 strains, the viruses have undergone evolution, resulting in two main distinct branches (PCV4a and PCV4b) (Figure 10). The results showed that the Thai PCV4 strains belonged to PCV4b and clustered in the same branch with PCV4/KU-02010 strain found in Korea (Figure 10). In the present study, PCV4 strains identified in China belong to both PCV4a and PCV4b genotypes. Interestingly, in the Korean and Thai swine farms, only PCV4b strains were detected. Moreover, the phylogenetic trees based on Cap and Rep genes were constructed for assessing genetic relationships. The findings demonstrated that different genomic regions of PCV4 yielded similar outcomes.

Nucleotide sequence comparison and amino acid sequence analysis of PCV4

An analysis of nucleotide and amino acid sequences of the Cap and Rep genes against reference PCV4 strains demonstrated that Thai PCV4 genomes displayed nucleotide identities of 97.6%-98.9% amino acid and 98.4%-100% amino

acid in Cap gene and Rep genes, respectively compared to those of reference viruses. The Thai PCV4 strains shared amino acid identities of 97.8%-99.1% and 98.6%-100% in Cap gene and Rep gene compared to those of reference viruses. There were no amino acid deletions or insertions. Compared with prototype PCV4 strain HNU-AHG1-2019 (accession no. MK986820), Thai PCV4 genomes had 20 nucleotide substitutions (Table 8). For deduced amino acid analysis, one amino acid substitution (Q155K) was seen in Rep gene, while 3 amino acid substitutions (N27S, I80V, and I96V) were found in Cap gene (Figure 11). Among them, there was one unique amino acid substitution (I80V) in the Thai PCV4 strains of Cap gene compared with other PCV4 strains. Compared with the representative strains (Figure 11), the Cap gene of PCV4a contains specific amino acid patterns of 27S and 212L, while certain PCV4b strains have unique amino acid patterns of 27N and 212M. Interestingly, the sequences from the present study revealed that the Thai PCV4 strains were grouped in PCV4b, even though they showed amino acid 27S in the Cap gene, and possessed the same amino acid at position 212M, similar to the two PCV4 strains (KU-02010 and KU-02011) from Korea. The findings suggest that amino acid variation at position 212 of Cap gene could be used for differentiating PCV4a (^{212}L) from PCV4b (^{212}M) (Figure 11).

Detection of PCV4 by *in situ* hybridization

The PCV4-positive pig (19RBR247) with respiratory illness was submitted for necropsy. Prominently, lungs with enlarged tracheobronchial lymph nodes were diffusely mottled and failed to collapse with dark red to purple consolidation and diffuse fibrinous attachment in the pleura (Figure 12A). Microscopically, lungs revealed moderate to severe diffuse pulmonary and interlobular edema with mild multifocal hyperplasia of bronchus-associated lymphoid tissue, and moderate to severe diffuse broncho-interstitial pneumonia. Tracheobronchial lymph nodes showed moderate multifocal lymphoid depletion. To investigate the tissue localization of PCV4, lung and tracheobronchial lymph node were tested using *in situ* hybridization. The results showed that PCV4-ISH-positive signals were mainly observed in the cytoplasm of bronchial epithelium (Figure 12B). Moreover, a few positive signals were detected in the lymphocytes and histiocyte-like cells of the tracheobronchial lymph node (Figure 12C). No hybridization signals were seen in internal negative control of lung (Fig. 12D) and tracheobronchial lymph node tissue sections (Figure 12E).

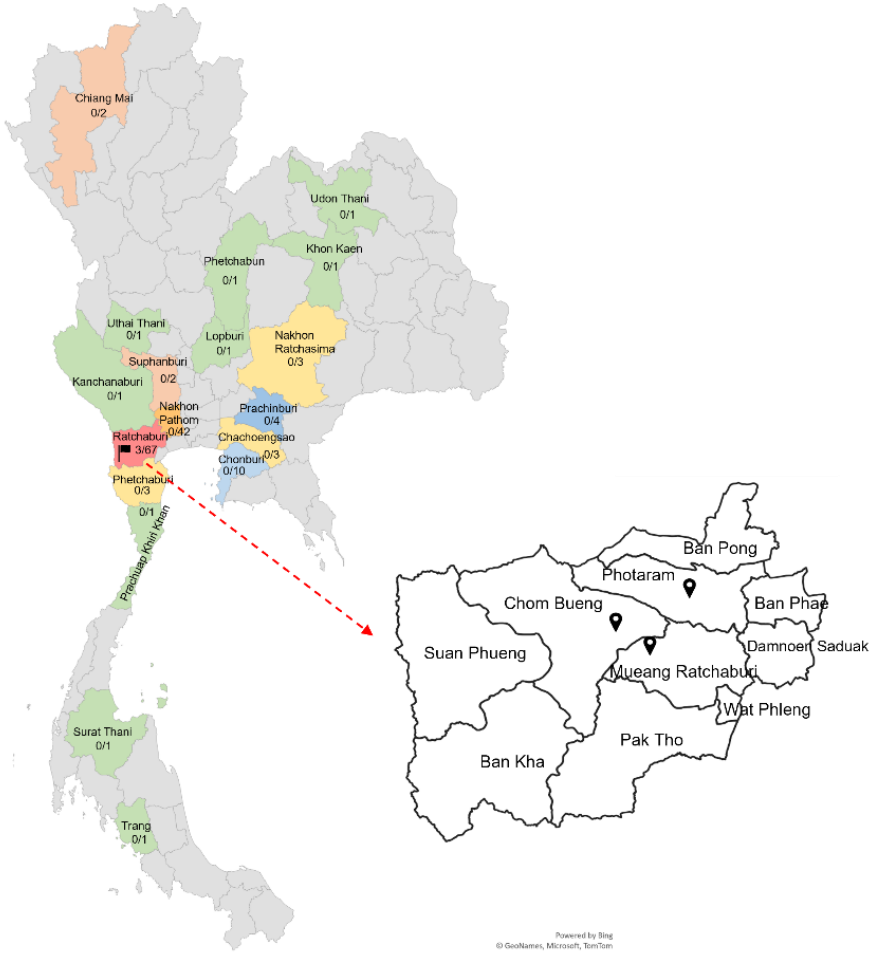

Figure 9 *Geographic distribution of PCV4-tested swine farms (n=145) in Thailand. The black flag indicates the Ratchaburi province where the positive samples were collected for complete genome sequencing. The locations of the 3 districts found PCV4-positive farms are indicated in the black pin.*

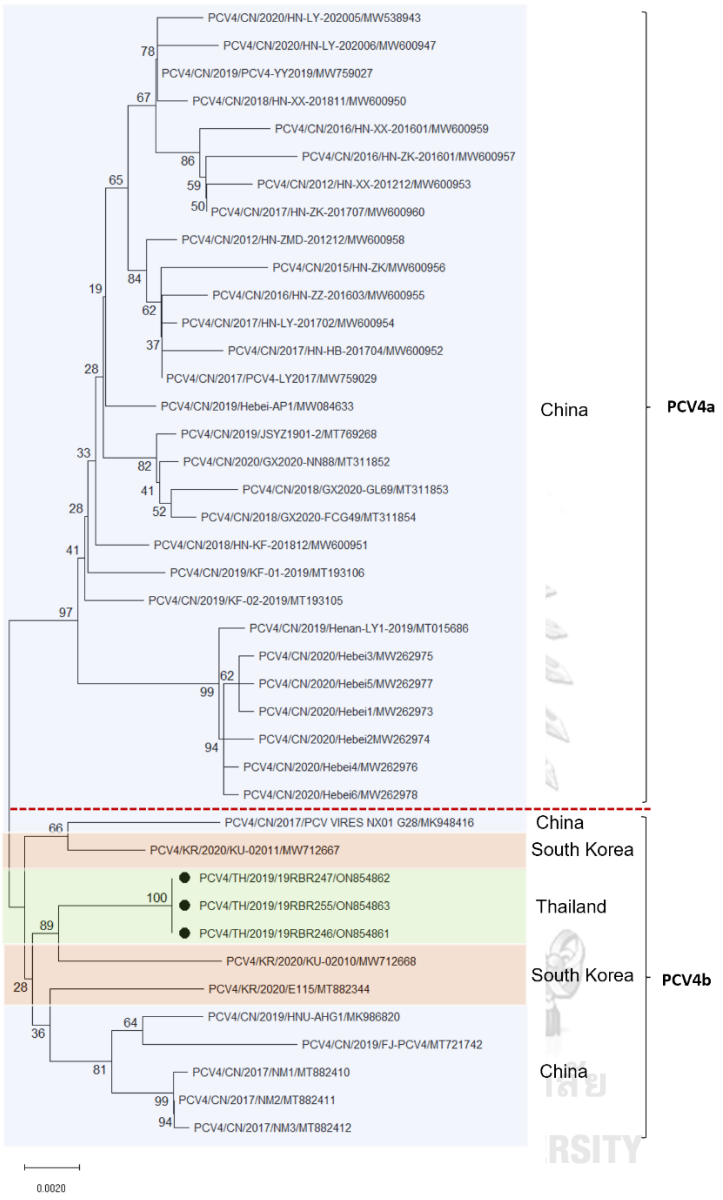

Figure 10 *Phylogenetic tree based on the complete genome sequences of 3 Thai PCV4 strains and other reference strains. The Thai PCV4 sequences obtained in this study were marked with solid black circles. The colored background represented the country of origin of the PCV4 viruses. The phylogenetic tree was constructed using the neighboring-joining method with a p-distance model and bootstrapping at 1000 replicates.*

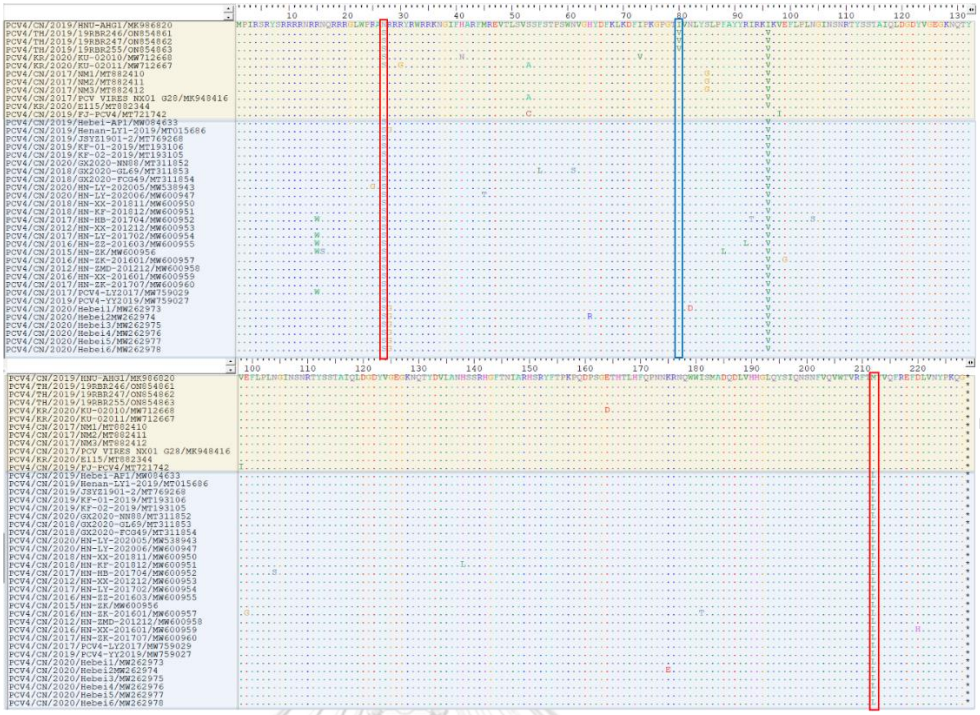

Figure 11 *Comparison of Cap amino acid sequences of PCV4 isolates in this study. Dots are used to denote the residues that are consistent with HNU-AHG1 (MK986820). The red boxes indicate the amino acid at positions 27 and 212, previously proposed for differentiation of PCV4a and PCV4b. Mutations at residues position 80 of Thai PCV4 strains are shown with the green box.*

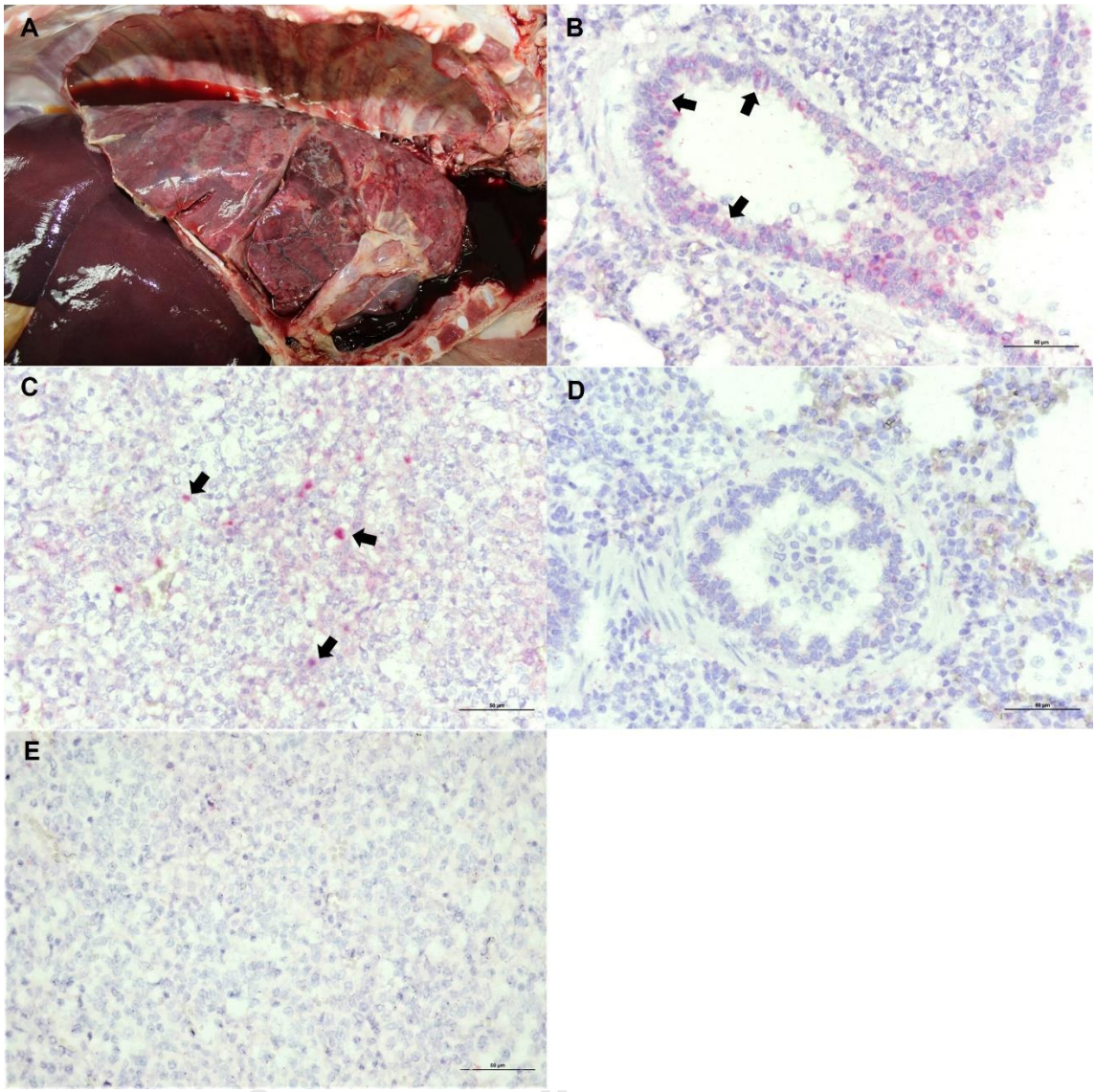

Figure 12 *Gross and microscopic lesions and in situ hybridization (ISH) using PCV4-specific probe targeting Cap gene of PCV4. Grossly, lung failed to collapse with dark red to purple consolidation and diffuse fibrinous attachment in the pleura (A). Lung: PCV4-positive cells were characterized by pink to brilliant red in the cytoplasm of bronchial epithelial cells (arrows) (B). Tracheobronchial lymph node: positive signals were observed in lymphocytes and histiocyte-like cells in the lymphoid follicle (arrows) (C). No hybridization signals were seen in the internal negative control of lung and tracheobronchial lymph node (D, E).*

Table 6 *PCV4-positive samples found in Thailand during 2019-2020*

							Coinfection			
Collection year	**Strain**	**Age of pigs**	**Sample**	**Location**	**Farm**	**History**	**PCV2**	**PCV3**	**PCV4**	**PRRSV**
Jan-2019	19RBR246	Fetus	Pooled organs	Ratchaburi	Farm A	Abortion	Yes	-	Yes	-
Jan-2019	19RBR247	Grower-finishing	Pooled organs	Ratchaburi	Farm B	Respiratory signs, sudden death	Yes	-	Yes	Yes
Feb-2019	19RBR255	Nursery	Pooled organs	Ratchaburi	Farm C	Respiratory signs	Yes	-	Yes	Yes

Table 7 *Percentage of nucleotide and amino acid identity (%) shared between Thai PCV4 strains and other reference strains.*

				% Nucleotide identity (%amino acid identity)		
Strain	**Year**	**Accession no**	**Country**	**Complete genome**	**Cap gene**	**Rep gene**
19RBR246	2019	ON854861	Thailand	100	100 (100)	100 (100)
19RBR247	2019	ON854862	Thailand	100	100 (100)	100 (100)
19RBR255	2019	ON854863	Thailand	100	100 (100)	100 (100)
HNU-AHG1-2019	2019	MK986820	China	98.8	98.9 (98.6)	98.6 (99.6)
KU-02010	2020	MW712668	Korea	98.9	98.6 (98.2)	99.1 (100)
KU-02011	2020	MW712667	Korea	98.9	98.6 (98.6)	99.2 (100)
NM1	2017	MT882410	China	99	98.8 (98.6)	99.1 (100)
NM2	2017	MT882411	China	99	98.8 (98.6)	99.2 (100)
NM3	2017	MT882412	China	99	98.8 (98.6)	99.1 (99.6)
E115	2020	MT882344	Korea	98.8	98.9 (99.1)	98.8 (100)
PCV_VIRES_NX01_G28	2017	MK948416	China	98.7	98.3 (98.6)	99.3 (99.6)
FJ-PCV4	2019	MT721742	China	98.4	98.2 (97.8)	98.4 (98.6)
Hebei-AP1	2019	MW084633	China	98.9	98.8 (99.1)	99.2 (100)
Henan-LY1-2019	2019	MT015686	China	98.5	98.1 (98.6)	98.9 (99.6)
JSYZ1901-2	2019	MT769268	China	98.9	98.6 (99.1)	99.1 (99.6)
KF-01-2019	2019	MT193106	China	98.8	98.6 (99.1)	98.8 (99.3)
KF-02-2019	2019	MT193105	China	98.8	98.8 (99.1)	99.1 (100)
GX2020/NN88	2020	MT311852	China	98.7	98.6 (99.1)	98.9 (99.3)
GX2020/GL69	2018	MT311853	China	98.6	98.5 (98.2)	98.8 (99.3)
GX2020/FCG49	2018	MT311854	China	98.8	98.8 (99.1)	98.9 (99.6)
HN-LY-202005	2020	MW538943	China	98.6	98.5 (98.6)	98.9 (99.6)
HN-LY-202006	2020	MW600947	China	98.5	98.6 (98.6)	98.6 (98.9)
HN-XX-201811	2018	MW600950	China	98.8	98.8 (99.1)	98.9 (99.3)
HN-KF-201812	2018	MW600951	China	98.8	98.6 (98.6)	99.1 (100)
HN-HB-201704	2017	MW600952	China	98.5	98.3 (97.8)	98.9 (99.6)
HN-XX-201212	2012	MW600953	China	98.4	98.6 (99.1)	98.7 (100)
HN-LY-201702	2017	MW600954	China	98.7	98.6 (98.6)	98.9 (100)
HN-ZZ-201603	2016	MW600955	China	98.6	98.3 (98.2)	99.1 (100)
HN-ZK	2015	MW600956	China	98.4	98.3 (97.8)	98.6 (98.9)
HN-ZK-201601	2016	MW600957	China	98.3	98.3 (98.2)	98.5 (98.6)
HN-ZMD-201212	2012	MW600958	China	98.7	98.6 (99.1)	98.9 (99.6)
HN-XX-201601	2016	MW600959	China	98.4	98.3 (98.6)	98.8 (98.9)
HN-ZK-201707	2017	MW600960	China	98.6	98.8 (99.1)	98.8 (99.3)
LY2017	2017	MW759029	China	98.8	98.6 (98.6)	99.1 (100)
YY2019	2019	MW759027	China	98.8	98.8 (99.1)	98.9 (99.6)
Hebei1	2020	MW262973	China	98.4	97.8 (98.2)	98.9 (99.6)
Hebei2	2020	MW262974	China	98.4	97.6 (97.8)	98.9 (99.6)
Hebei3	2020	MW262975	China	98.4	97.9 (98.6)	98.8 (99.6)
Hebei4	2020	MW262976	China	98.4	97.9 (98.6)	98.8 (99.3)
Hebei5	2020	MW262977	China	98.4	97.9 (98.6)	98.8 (99.3)
Hebei6	2020	MW262978	China	98.4	97.8 (98.6)	98.9 (99.6)

Table 8 *Nucleotide sequence comparison between Thai PCV4 strain (19RBR247) and the prototype PCV4 strain HNU-AHG1-2019*

Genome position	PCV4 HNU-AHG1-2019	PCV4 19RBR247
36	A	G
122	T	C
206	C	T
287	C	T
432	C	T
537	C	A
557	T	C
599	T	C
608	A	C
726	C	T
866	C	T
896	C	T
920	G	A
1251	T	G
1260	G	A
1448	T	C
1496	T	C
1527	C	T
1654	T	C
1680	G	T

Discussion

PCV2 and PCV3 are at least the two porcine circoviruses causing major threats to the global swine industry (Segales, 2012; Klaumann et al., 2018a; Opriessnig et al., 2020). The finding of PCV4 should not be neglected since Asia is the major world hub of pig production, and the virus itself may originate here and spread to other regions or vice versa. To date, PCV4 was discovered only in the Asian continent, China, Korea, and recently, Thailand, but not yet in others (Franzo et al., 2020b; Zhang et al., 2020; Ha et al., 2021; Nguyen et al., 2021; Tian et al., 2021; Hou et al., 2022; Vargas-Bermudez et al., 2022; Xu et al., 2022a)

In the present study, the results showed that only 3 of the 734 samples were tested positive for PCV4, with a positive rate of 0.4% (3/734) and 2.07% (3/145) of farm levels. Interestingly, extremely low prevalence of PCV4 was demonstrated in Thailand compared to previous reports (Zhang et al., 2020; Ha et al., 2021; Tian et al., 2021; Hou et al., 2022; Xu et al., 2022a) suggesting low transmission and infection rate within the pig herds. However, nearly 80% of the swine farms tested were mainly located in the high pig density areas of Thailand, which might not reflect the whole picture on PCV4 infection status of the country. Thus, in the future, large-scale field sampling of all regions could provide more information about PCV4 epidemiology in Thailand. Therefore, the threat of PCV4 to the Thai swine industry is skeptical and yet to be elucidated.

Recently, pigs inoculated with rescued PCV4 alone showed histopathological changes in several organs, but no obvious clinical signs found (Niu et al., 2022c). In the present study, we found that three PCV4-positive samples from clinically infected pigs were found coinfected with PCV2 (3/3) and PRRSV (2/3). These results apparently suggest that absence of other factors, such as coinfections, PCV4 infection might remain asymptomatic. However, the synergistic effect of PCV4 infection might exacerbate the disease severity and be associated with PRDC in clinically infected pigs as found in the present study. Therefore, co-infections and some unknown factors, yet to be elucidated, might involve PCV4 pathogenesis and its clinical outcomes.

According to *in situ* hybridization results, the virus presence was found in the bronchiolar epithelial cells and in lymphocytes and histiocyte-like cells in the lymphoid follicles consistently with the characteristics of PCV2 infection (Huang et al., 2008). Whether PCV4 infection in bronchiolar epithelial cells and tracheobronchial lymph node induces the observed broncho-interstitial pneumonia and the lymphoid depletion should be further studied. The contribution of PCV4 to the pathogenesis of PRDC and immune modulation should be of interest. These findings also suggest that a marked tropism of PCV4 for bronchial epithelial cells may impair the epithelial barrier function, thus predisposing the infected pigs to secondary infections and PRDC. The case history of the PCV4 positive cases found that the PCV4-infected pigs were found coinfected with PCV2, PRRSV, and/or *Streptococcus* spp. Additionally,

bronchial epithelial infection might contribute to viral shedding dynamics in the nasal secretion. Notably, it has been reported that the highest positive rates of PCV4 were detected in nasal swabs followed by serum samples (Zhang et al., 2020). Therefore, nasal swabs might be a better target specimen for PCV4 detection and surveillance. Further investigation is needed.

For genetic analysis, Thai PCV4 strains shared up to 98% nucleotide identity with other reference PCV4 strains. The high genetic stability of PCV4 is consistent with the previous reports (Ha et al., 2021; Hou et al., 2022). It is noted that the Thai PCV4 strains were closely related to the Chinese PCV4 sub-cluster. Although the virus had high genetic stability, some genetic variations could be observed among pig populations from different countries that probably specific to the geographic origin (Faustini et al., 2022). Additionally, the Thai PCV4 strains were highly related to each other, possibly, due to a single point introduction with low infection rate or restricted gene flow in the neighboring districts of Western Thailand, suggesting that the novel virus might be confined to these areas affecting the prevalence and genetic diversity.

Additionally, there are currently no fully established guidelines for classifying PCV4 genotypes, and the temporary proposals made so far have not been consistent. This is in contrast with PCV2 genotyping that a unified classification scheme based on Cap gene was proposed (Franzo and Segales, 2018). For PCV4 genotyping, there have been several studies proposing different criteria and markers

to differentiate PCV4 genotypes, such as 2-group classification (PCV4a and PCV4b) and 3-group classification (PCV4a, PCV4b, and PCV4c)(Wang et al., 2022; Wu et al., 2022; Xu et al., 2022a; Xu et al., 2022b). Among these studies, the residue at 27 and 212 of capsid gene were also proposed to be used in distinguishing PCV4a (27S and 212L) from PCV4b (27N and 212M) (Wang et al., 2022; Wu et al., 2022). Recently, PCV4c was proposed with specific amino acid pattern 27N, 28R, and 212M. However, in some genotyping criteria, PCV4c might be classified into PCV4b (Wu et al., 2022; Xu et al., 2022a). Therefore, the use of differing nomenclatures can create ambiguity and misinterpretation of results. Further investigation is required to establish the standardized criteria for genotyping PCV4, given the current paucity of sequence information. As mentioned above, previous reports showed that the amino acid at the position 27 of the Cap gene could be used as a marker to distinguish between PCV4a (27S) and PCV4b (27N) (Wang et al., 2022; Wu et al., 2022). However, the results in this study showed that the amino acid residue at position 27 cannot be used to differentiate between PCV4a and PCV4b since the Thai PCV4b strains and the Korean PCV4b strains (KU-02010 and KU-02011) showed N27S (Figure 11). The novel findings of this study suggest that amino acid variation at position 212 could be used for differentiating PCV4a (212L) from PCV4b (212M) for 2-group classification. Moreover, one unique amino acid substitution (I80V) was also found in the Thai PCV4 strains in Cap gene. The amino acid substitution occurred at residue 72-88 in B-cell epitopes on PCV4 capsid gene (Wu et al., 2022). It may alter antigenic properties of

the viruses and their immunogenic modifications caused by genetic mutation. However, the effects of genetic mutation on pathogenicity need further investigation. In terms of the genetic variation of PCV4 and its genotypes, a study conducted in various regions in China found that PCV4a was the most common genotype followed by PCV4b (Hou et al., 2022; Wang et al., 2022; Wu et al., 2022; Xu et al., 2022a; Xu et al., 2022b). However, in pig populations from Korea and Thailand, only the PCV4b genotype was detected (Nguyen et al., 2021). It is plausible to consider that PCV4 strains isolated from various geographic regions or pig populations may potentially demonstrate genetic variations, which may imply the existence of geographical or host specificity in the virus.

To date, PCV4 was discovered only in the Asian continent, China, Korea, and recently Thailand (Franzo et al., 2020b; Zhang et al., 2020; Ha et al., 2021; Nguyen et al., 2021; Tian et al., 2021; Hou et al., 2022; Vargas-Bermudez et al., 2022; Xu et al., 2022a). This study described the presence of PCV4 in Thailand from the retrospective samples and firstly demonstrated the viral tropism in the bronchial epithelial cells. The three Thai PCV4 strains demonstrated their genetic similarity with the Korean and Chinese PCV4b strains. However, the prevalence of PCV4 in Thailand is extremely low, and clinical involvement of PCV4 remains unclear. Apparently, it should be noted that the amino acid residue at position 212 of the Cap gene should be used for differentiating PCV4a and PCV4b. Further studies are needed to determine the

role of PCV4 infection related to clinical signs and its impact on the Thai swine farms. The classification of PCV4 genotypes requires further investigation and clarification due to the limited available PCV4 sequences.

Data availability

The datasets generated and analysed during the current study are available in the NCBI genomes repository under the accession numbers ON854861-ON854863.

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Author contributions statement

CS and RT planned the experiments. CS, TC, CP, SJ and RK carried out the experiments. CS and RK analyzed and interpreted the results. CS, RK and RT drafted and revised the manuscript with input from all authors.

CHAPTER 6

PREVALENCE OF PORCINE CIRCOVIRUS 2, 3, AND 4 IN THAI SWINE FARMS FROM 2021-2023 USING A NOVEL TRIPLEX REAL-TIME PCR FOR SIMULTANEOUS DETECTION

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Abstract

Porcine circoviruses (PCVs) are currently classified into four species: PCV1, PCV2, PCV3, and PCV4. Pigs infected with PCV2, PCV3, and PCV4, either individually or in co-infections, exhibit comparable clinical manifestations, making difficulty in distinguishing them solely based on clinical observation. Therefore, the adoption of robust diagnostic methods, such as a multiplex qPCR, is urgently needed when having mixed PCVs infection. In this study, we have developed a triplex quantitative PCR (tqPCR) assay for the simultaneous detection and differentiation of PCV2, PCV3, and PCV4. This assay demonstrates high specificity and sensitivity, with a detection limit of 100 copies/μl for PCV2 and PCV4, and 200 copies/μl for PCV3. Additionally, 121 clinical swine samples collected during 2020-2023 were tested using the developed tqPCR assay. The positive rates for PCV2 and PCV3 were found to be 27.3% and 9.9%, respectively. The co-infection rate of PCV2 and PCV3 was 5.8%. However, PCV4 was not detected throughout this experiment. These findings indicate that PCV2 and PCV3 have been circulating in the Thai swine farms, and the novel tqPCR could provide an efficient diagnostic tool for facilitating the simultaneous detection and epidemiology investigation of PCV2, PCV3, and PCV4.

Keywords: PCV2, PCV3, PCV4, triplex-real time PCR, swine

Introduction

Over the past three decades, porcine circovirus (PCV) type 2 (PCV2) has emerged as a foremost economic pathogen in the global swine industry. PCV2 infection results in diverse clinical outcomes collectively referred to as porcine circovirus diseases (PCVDs), encompassing porcine circovirus systemic disease (PCV-SD), porcine circovirus reproductive disease (PCV-RD), porcine dermatitis and nephropathy syndrome (PDNS), and porcine circovirus subclinical infection (PCV-SI)(Segales, 2012; Segales and Sibila, 2022). Presently, at least four species of porcine circoviruses (PCVs) are recognized: PCV1, PCV2, PCV3, and PCV4 (Opriessnig et al., 2020). While PCV1 typically poses no significant threat to pigs, the remaining types have been documented to cause PCVDs (Opriessnig et al., 2020). Therefore, determining the origin of infection based on clinical outcomes proves to be intricate and might impact the development of mitigation and management strategies in the farm settings.

Currently, PCV2 and PCV3 are prevalent worldwide (Franzo and Segales, 2018; Turlewicz-Podbielska et al., 2022), while PCV4 is limited to the Asian continent, including China, Korea, and Thailand (Zhang et al., 2020; Chen et al., 2021; Ha et al., 2021; Nguyen et al., 2021; Tian et al., 2021; Hou et al., 2022; Kim et al., 2022; Wu et al., 2022; Xu et al., 2022a; Xu et al., 2022b; Zou et al., 2022; Sirisereewan et al., 2023b). However, the economic impact of PCV4 requires further investigation and verification. For detection, the gold standard for diagnosis involves the detection of

PCV antigen or nucleic acid, employing techniques like *in situ* hybridization (ISH), immunohistochemistry (IHC), and polymerase chain reaction (PCR) (Gillespie et al., 2009). Although both ISH and IHC offer greater sensitivity, the fact that these assays are time consuming and laborious is of concern. Commonly, real-time PCR has become the assay of choice for detecting PCV. As known, multiplex real-time PCR (mqPCR) has been demonstrated to accurately quantify multiple targets without compromising assay efficiency (Bustin et al., 2009). Therefore, in this study, a triplex real-time PCR (tqPCR) assay is developed for detecting PCV2, PCV3, and PCV4 in the same sample. The implementation of this developed technique will be valuable for disease surveillance and identification of PCVs in endemic countries.

Materials and methods

Nucleic acids of the viruses

All genomes (DNA and cDNA) used in this study were provided by Chulalongkorn University- Veterinary Diagnostic Laboratory (CU-VDL), including porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), classical swine fever virus (CSFV), swine influenza virus (SIV), pseudorabies virus (PRV), and porcine epidemic diarrhea virus (PEDV). Both DNA and cDNA were stored at -80 °C until used.

Primers and probes for tqPCR assay and plasmid standards

For the establishment of the tqPCR assay, a total of three primer and probe combinations were utilized to facilitate the distinct identification of PCV2, PCV3, and PCV4. The primers and probes designed to recognized genes of PCV2 and PCV3 were adopted from previously well-established qPCR assay (Wang et al., 2019). In this study, the conserved region for PCV4 was chosen for designing specific primers and probes, aligning their melting temperatures with the existing counterparts. Detailed primer and probe sequences are documented in Table 9.

Standard plasmid preparation

Positive control plasmids for PCV2, PCV3, and PCV4 were synthetized by Biobasic company (Ontario, Canada), incorporating specific target fragments into the pUC57 vector. Plasmid concentrations were assessed using a Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA, USA), and then translated into copies per microliter(μl). Plasmids were subsequently diluted with ddH_2O, resulting in a stock solution with 10^8 copies/μl. This solution was used to establish a standard curve through 10-fold dilutions (ranging from 10^2 to 10^7 copies/μl) of the standard plasmid.

Optimization of tqPCR assay

To optimize the concentrations of primers and probes, the standard plasmid was uniformly combined with PCV2, PCV3, and PCV4 plasmids, each containing $1x10^5$ copies/μl as templates. The tqPCR was conducted in 20 μl reactions, comprising

Luna® Universal Probe qPCR master mix (NEB, MA, USA), primers and probes for all three viruses, and templates. During the optimization phase, the primers and probes were varied within ranges of 0.1-0.9 μM and 0.1-0.7 μM, respectively, in the multiplex reaction setup. The annealing temperature ranged between 59-61°C. The tqPCR was performed using Quantstudio5 real-time system (Applied Biosystems, USA).

Validation of tqPCR assay

The tqPCR assay was utilized to examine the specificity of six viruses: PRRSV, PPV, CSFV, SIV, PRV, and PEDV. To assess sensitivity, 10-fold serial dilutions of each standard plasmids were conducted in triplicate, ranging from 10^7 copies/μl to 10^2 copies/μl. To validate the limit of detection (LOD) for the tqPCR assay, the lowest concentration corresponding to the expected detection threshold was evaluated using 23 replicates for each concentration, as previously described (Pan et al., 2020). The minimum concentration demonstrating a positive detection rate of 95% was determined as the established and credible LOD.

For tqPCR assay validation, intra-assay and inter-assay repeatability were evaluated in triplicate using standard plasmids at concentrations of 10^7, 10^5, and 10^3 copies/μl. Repeatability was quantified by calculating coefficients of variation (CV) from the analyses.

Interference test of tqPCR assay

To investigate how varying quantities of target pathogens impact the sensitivity of the tqPCR assay, we combined plasmid standards containing two or three target pathogens at equivalent and differing concentrations. A total of twelve combinations were subjected to triplicate testing using the tqPCR assay (Table 10).

Clinical sample detection

A total of 121 clinical swine samples, submitted to the CU-VDL for viral diagnostic detection between 2021-2023, were used to identify the positive rate of each pathogen by a newly established tqPCR assay. Total viral DNA extraction was performed utilizing the IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) following the manufacturer's instruction. The extracted DNA was stored at -80 ℃ until used.

Table 9 *Primers and probes used in this study*

Virus	Primer/Probe	Sequence (5'-3')	Position†	Amplicon	Reference
PCV2	PCV2F	GARACTAAAGGTGGAACTGTACC	762-784	118	(Wang et al., 2019)
	PCV2R	TCCGATARAGAGCTTCTACAGC	879-858		
	PCV2Pr	Hex-AGGAGTACCATTCCAACGGGG-BHQ1	823-843		
PCV3	PCV3F	GGTGAAGTAACGGCTGTGTTTT	1550-1571	86	(Wang et al., 2019)
	PCV3R	ACACTTGGCTCCARGACGAC	1635-1616		
	PCV3Pr	TAMRA-ATGCGGAAAGTTCCACTCGK-BHQ2	1592-1611		
PCV4	PCV4F	CTGGTATGATTATGTGGAGCTGAAA	833-857	81	This study
	PCV4R	TCCTCCCTCATGACCTGATAGG	913-892		
	PCV4Pr	FAM-AGCCCTGTACCGGCGCGTGAC-BHQ1	867-889		

† Genome position of primer- and probe-binding sites according to the complete genome sequence of PCV2 19RBR8 strain (OL677602), PCV3 US/MO2015 strain (KX778720), and PCV4 19RBR255 strain (ON854863).

Results

Optimization of the tqPCR assay

In this study, distinct probes were chosen for individual viruses, employing separate fluorescent molecules, including FAM, TAMRA, and HEX. Nevertheless, it is essential to consider potential interference between the fluorophores, as this could impact the sensitivity of the assay. Hence, the primary objective of reaction optimization was to attain optimal performance while minimizing potential interference among the fluorophores. The optimized tqPCR reaction was conducted in a 20 μl volume, comprising 10 μl of Luna® Universal Probe qPCR master mix (NEB, MA, USA), primers and probes for PCV2 and PCV3 at concentrations of 0.6 μM and 0.7 μM respectively, primers for PCV4 at 0.2 μM, and a 0.1 μM probe for PCV4. Additionally, 1 μl of template DNA was added, and the volume was made up to 20 μl with ddH_2O to ensure uniformity. The thermocycling conditions consisted of initial denaturation at 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Signal acquisition was automated at the conclusion of each cycle.

Establishment of standard curves

Standard curves were generated for each virus using 10-fold serial dilutions of linear plasmids from 10^7 to 10^2 copies/μl. These plasmids were equally mixed with plasmids of PCV2, PCV3, and PCV4. The findings indicated that the tqPCR method effectively detected all targeted genes from the three viruses. Each standard curve exhibited a desirable amplification efficiency with the correlation coefficients of PCV2

(R^2 =0.999; Eff%= 96.6), PCV3 (R^2 =0.996; Eff%= 91.5), and PCV4(R^2 =0.999; Eff%= 95.3), as shown in Figure 13.

Specificity of the tqPCR assay

The PCV2, PCV3, and PCV4 plasmids served as the positive control samples. Templates included DNA and cDNA sourced from the six selected viruses (PRRSV, PPV, CSFV, SIV, PRV, and PEDV), while ddH2O served as the negative control sample. The results showed successful detection of the target viruses, accompanied by negative results for the other viruses (Figure 14), indicating robust specificity.

Sensitivity of the tqPCR assay

In this study, the limit of detection (LOD) was established as the lowest concentration at which detection occurred in 95% of the replicates. Analyzing the standard curves across a concentration range of 10^7 to 10^2 copies/μl for each pathogen, the ability to identify positive samples at concentrations as low as 10^2 copies/μl was confirmed. Furthermore, the standard control plasmids underwent additional 10-fold dilutions from the last dilution, leading to concentrations spanning from 10^1 to 10^0 copies/μl. The obtained results indicated LOD of 100 copies/μl for PCV2 and PCV4, and 200 copies/μl for PCV3 (Table 11). The determination of the cutoff threshold was automatically executed by the QuantStudio 5 real-time system instrument.

This study employed Ct values near the LOD for each pathogen to establish criteria for differentiating positive and negative samples. Despite some samples displaying positivity below the LOD concentration, their Ct values were still used for analysis, even if the detection rate was not deemed qualified. Positivity thresholds were set at 35 for PCV2 and PCV3, and at 33 for PCV4, designating samples with Ct values at or below these thresholds as positive. However, samples with Ct values above 33 but below 35 for PCV4, and those exceeding 35 but not reaching 37 for PCV2 and PCV3, were classified as invalid. Thus, negative samples were determined by Ct values surpassing 35 for PCV4 and exceeding 37 for PCV2 and PCV3.

Repeatability and reproducibility of tqPCR

The study utilized standard plasmids at three different concentrations (10^7, 10^5, 10^3 copies/μl) to evaluate the repeatability and reproducibility of the tqPCR assay. As demonstrated in Table 12, the intra-assay and inter-assay coefficients of variation (CV) for Ct values in the tqPCR assay ranged from 0.03% to 0.63% and 0.13% to 1.76%, respectively. These findings highlight the high repeatability of the tqPCR assay.

Interference test of tqPCR assay

As demonstrated in Table 10, a comprehensive analysis encompassing twelve different concentration combinations was carried out. The results underscore the competence of the tqPCR assay in detecting target pathogens, whether in duplex or triplex simulations, indicating its applicability for clinical use.

Detection of clinical samples

A total of 121 swine samples collected between 2021 and 2023 were examined to assess the prevalence of PCV2, PCV3, and PCV4 (Table 13). Using the tqPCR assay, positive rates of 27.3% (33/121), 9.9% (12/121), and 0% (0/121) were observed for PCV2, PCV3, and PCV4. Additionally, seven out of 121 samples (7/121) exhibited co-infections of PCV2 and PCV3. Notably, PCV4 was not detected in this study.

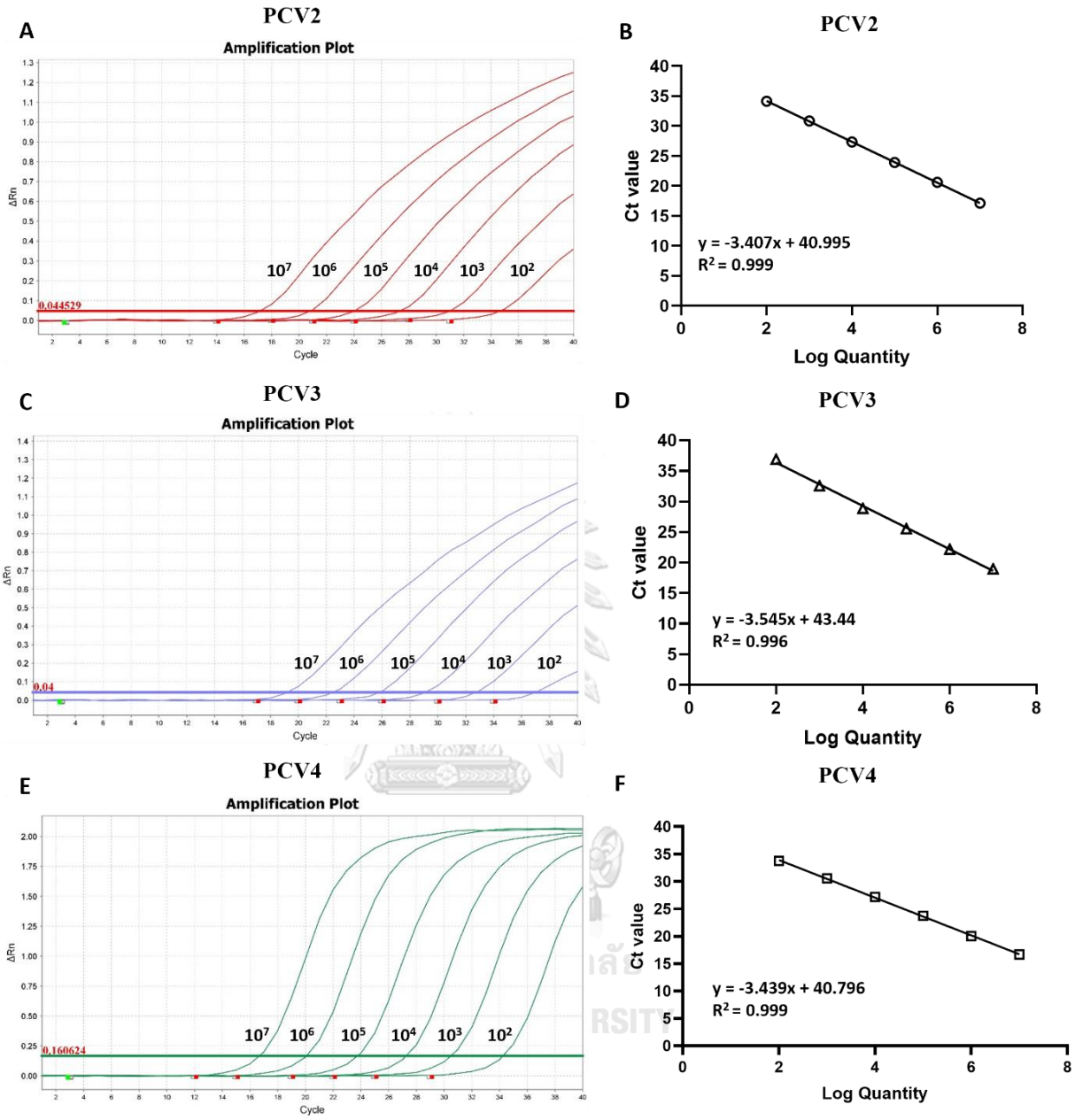

Figure 13 *Amplification and standard curves of the tqPCR assay were obtained by utilizing serially diluted plasmids mixed at equal concentrations, ranging from 10^7 to 10^2 copies/**μ**l, as templates. The amplification curves of the triplex assay for detecting PCV2 (A), PCV3 (C), and PCV4 (E) are displayed. The standard curves for PCV2 (B), PCV3 (D), and PCV4 (F) were constructed by plotting the Ct values (Y-axis) against the logarithm of plasmid copy numbers (X-axis).*

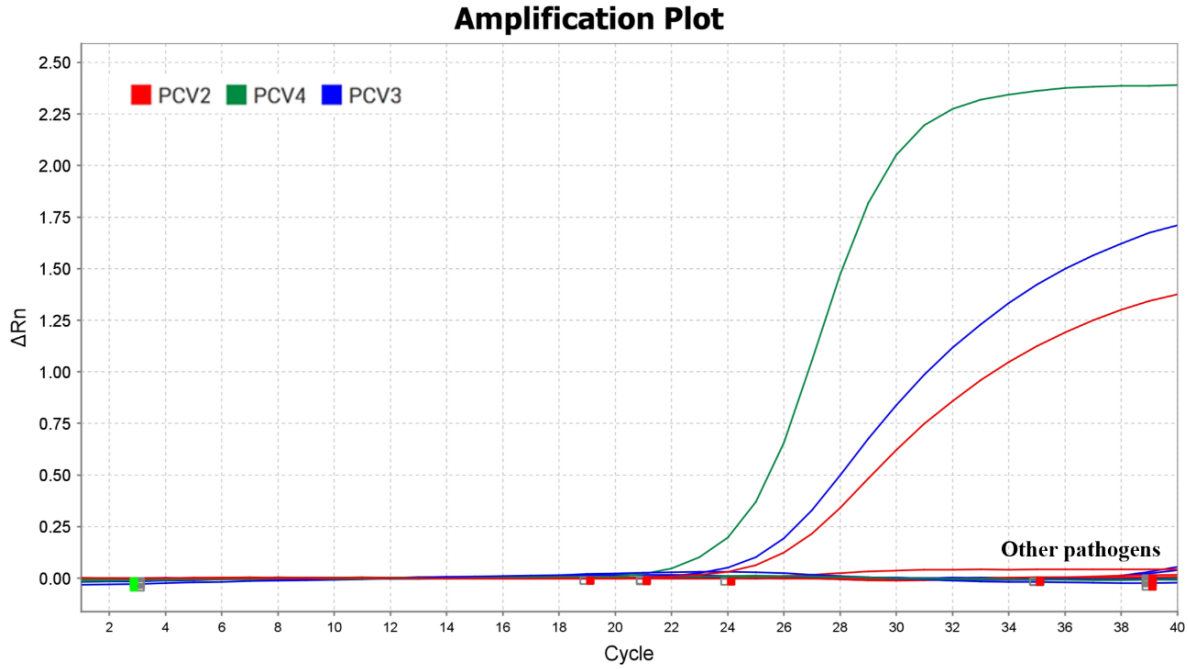

Figure 14 *Specificity of the tqPCR assay, PCV2, PCV3, PCV4, and other major swine viruses (including PRRSV, PPV, CSFV, SIV, PRV, and PEDV) were tested using tqPCR assay. The results indicated positive detection exclusively for PCV2, PCV3, and PCV4.*

Table 10 *Interference test by tqPCR assay*

Co-infection proportion	Number of DNA copies (copies/ul)			Co-infection real-time PCR Ct value (mean ± SD)		
	PCV2	PCV3	PCV4	PCV2	PCV3	PCV4
PCV2:PCV3: PCV4 = 10:1:1	1×10^7	1×10^6	1×10^6	18.62±0.04	23.14±0.22	20.23±0.04
PCV2:PCV3: PCV4 = 10:5:1	1×10^7	5×10^6	1×10^6	18.92±0.04	20.41±0.15	19.56±0.03
PCV2:PCV3: PCV4 = 1:5:10	1×10^6	5×10^6	1×10^7	21.72±0.15	19.91±0.09	16.78±0.04
PCV2:PCV3 = 1:1	1×10^7	1×10^7	-	17.76±0.04	18.17±0.03	-
PCV2:PCV3 = 10:1	1×10^7	1×10^6	-	17.74±0.09	21.89±0.08	-
PCV2:PCV3 = 100:1	1×10^7	1×10^5	-	17.85±0.11	26.61±0.23	-
PCV2:PCV4 = 1:1	1×10^7	-	1×10^7	17.48±0.04	-	16.11±0.06
PCV2:PCV4 = 10:1	1×10^7	-	1×10^6	17.91±0.07	-	19.64±0.04
PCV2:PCV4 = 100:1	1×10^7	-	1×10^5	17.98±0.23	-	23.23±0.54
PCV3:PCV4 = 1:1	-	1×10^7	1×10^7	-	18.11±0.04	16.14±0.04
PCV3:PCV4 = 10:1	-	1×10^7	1×10^6	-	17.99±0.09	19.60±0.11
PCV3:PCV4 = 100:1	-	1×10^7	1×10^5	-	18.03±0.12	23.11±0.07

Table 11 *Sensitivity of the tqPCR assay*

Pathogens	Concentration	Repeat times	Positive	Detection rate	95% detection rate
PCV2	100 copies/ul	23	23	100%	>95%
	10 copies/ul	23	12	52.2%	<95%
	1 copies/ul	23	2	8.7%	<95%
PCV3	200 copies/ul	23	23	100%	>95%
	100 copies/ul	23	17	73.9%	<95%
	10 copies/ul	23	11	47.8%	<95%
	1 copies/ul	23	5	21.7%	<95%
PCV4	100 copies/ul	23	23	100%	>95%
	10 copies/ul	23	15	65.2%	<95%
	1 copies/ul	23	1	4.3%	<95%

Table 12 *Repeatability analysis of tqPCR assay*

Name	Number of DNA copies (copies/ul)	Intra-assay			Inter-assay		
		Mean	SD	CV (%)	Mean	SD	CV (%)
PCV2	1×10^7	17.50	0.11	0.63	17.81	0.10	0.55
	1×10^5	24.25	0.04	0.14	24.50	0.43	1.76
	1×10^3	31.35	0.01	0.03	31.37	0.04	0.13
PCV3	1×10^7	18.34	0.06	0.33	18.45	0.10	0.52
	1×10^5	25.18	0.11	0.42	25.72	0.27	1.05
	1×10^3	32.33	0.11	0.33	32.51	0.10	0.30
PCV4	1×10^7	16.33	0.02	0.12	16.63	0.18	1.09
	1×10^5	23.12	0.02	0.09	23.31	0.15	0.62
	1×10^3	30.06	0.16	0.54	30.36	0.19	0.62

Table 13 *The infection rates of clinical samples collected from 2021-2023 were detected by tqPCR assay.*

Pathogens	tqPCR assay	
	Positive	Rate (%)
PCV2	33/121	27.3
PCV3	12/121	9.9
PCV4	0/121	0
PCV2+PCV3	7/121	5.8
PCV2+PCV4	0/121	0
PCV3+PCV4	0/121	0
PCV2+PCV3+PCV4	0/121	0
Total	52/121	43

Discussion

Porcine circovirus diseases (PCVDs) are acknowledged for their multisystemic clinical manifestations caused by PCV2 infection, which poses a worldwide challenge to the swine industry (Segales, 2012; Opriessnig et al., 2020; Segales and Sibila, 2022). Remarkably, PCV2, PCV3, and PCV4 infections displayed similar clinical signs, including the possibility of co-infection or mixed infection, which can pose challenges in their differentiation, despite their contributions to clinical PCVDs. Hence, this underscores the critical importance of precise diagnostic techniques like a multiplex qPCR to accurately identify the specific PCVs found in the samples. In this study, a tqPCR assay utilizing TaqMan probes was developed for the simultaneous detection and identification of PCV2, PCV3, and PCV4. The tqPCR assay could detect those PCVs and shows no cross-reaction with other major swine diseases, affirming the high specificity of the primers and probes and making it suitable for the simultaneous detection of PCVs. The results from both the inter- and intra-assay analyses indicated that the experiment exhibited a high degree of repeatability. Moreover, the amplification efficiency and correlation coefficients (R^2) of the tqPCR exceeded 90% and 0.995, respectively, reflecting the robustness of the experimental conditions. With 23 replicates for each concentration, the developed tqPCR achieved a LOD as low as 100 copies/μl for PCV2 and PCV4, and 200 copies/μl for PCV3, with a confidence level exceeding 95%. It is pointed out that our tqPCR exhibited satisfactory sensitivity.

Notably, mixed infections involving the three PCVs have been observed in China, Korea, and Thailand (Zhang et al., 2020; Chen et al., 2021; Ha et al., 2021; Nguyen et al., 2021; Tian et al., 2021; Hou et al., 2022; Kim et al., 2022; Wu et al., 2022; Xu et al., 2022a; Xu et al., 2022b; Zou et al., 2022; Sirisereewan et al., 2023b). Therefore, it is possible that co-infections with varying quantities of these viruses could potentially impact tqPCR sensitivity due to inter-viral interference. In this study, co-infection simulations were conducted using varying concentrations of each virus. The results indicated that all combinations yielded positive results in the tqPCR assay. However, it should be noted that when one of the target viruses occurred at a high concentration or in combinations involving all three viruses, it could potentially introduce interference in detecting the other viruses at lower concentrations. It is consistent with the previous reports that a high concentration of one target gene has the potential to interfere with the amplification of the other target gene at a low concentration (Wang et al., 2007; Hyeon et al., 2010).

To investigate the prevalence of PCV2, PCV3, and PCV4, a total of 121 clinical samples collected from 2021 to 2023 were subjected to the developed tqPCR analysis. The results revealed that 27.3% (33/121) of the samples were positive for PCV2, 9.9% (12/121) tested positive for PCV3, and co-infections involving both PCV2 and PCV3 were observed in 5.8% (7/121) of cases. However, PCV4 was not detected in this study. It is possible that the detection of PCV4 may necessitate a larger

sample size due to its extremely low prevalence in Thailand, as previously described (Sirisereewan et al., 2023b). This suggests that the prevalence of PCV3 and PCV4 in Thailand was lower compared to the findings of the prevalence studies in China and Korea (Kim et al., 2022; Zou et al., 2022). Nevertheless, monitoring the prevalence and impact of PCV3 and PCV4 on the Thai swine farms is essential for the pig industry. Moreover, the present findings support the widespread presence of PCV2 in the Thai pig population. This is consistent with prior studies reporting PCV2 positivity in over 80% of Thai swine farms (Sirisereewan et al., 2023a).

In view of the high mutation and recombination rate of PCVs (Opriessnig et al., 2020), ongoing prevalence surveillance is a necessity. Currently, PCV2 has been identified in nine genotypes, namely PCV2a to PCV2i (Wang et al., 2020). However, it is essential to highlight that PCV2a, PCV2b, and PCV2d are the three active genotypes reported worldwide with evidence of their co-circulation (Correa-Fiz et al., 2018; Franzo and Segales, 2018). In Southeast Asian countries, PCV2d is the predominant genotype, aligning with a global trend observed in Thailand (Sirisereewan et al., 2023a), Malaysia (Tan et al., 2022), and Vietnam (Doan et al., 2022). In high endemic areas, multiplex qPCR assays offer the advantage of rapid identification but lack the capability to differentiate PCV2 genotypes. Consequently, PCV2 genotyping may be necessary to explore genetic diversity and characterization. Utilizing robust diagnostic approaches like the multiplex qPCR, along with genotyping, is pivotal for effective

monitoring. However, using multiplex qPCR to target multiple genes for genotyping is generally more complex and necessitates validation time.

In summary, this study offers a potent diagnostic tool for detecting related viruses and enhances the efficacy of surveillance efforts in the future. The advantages of this tqPCR assay include reduced time consumption, heightened sensitivity and specificity, and the ability to perform quantitative analyses for the clinical diagnostic and epidemiological analysis of PCV2, PCV3, and PCV4.

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CHAPTER 7

OVERALL DISCUSSION

Porcine circoviruses, particularly PCV2 and PCV3, have been identified globally. PCV4, on the other hand, has a limited geographical distribution only in the Asian continent. Various clinical presentations caused by these viruses can result in an economic impact on the swine industry, leading to both direct and indirect losses. The global distribution of these viruses has led to a worldwide effort in sequencing their genomes to better understand their phylogenetics and phylodynamics. Genome sequence analysis played a crucial role in tracking the global epidemiological and virological situation, providing valuable insights for disease prevention and control strategies. Remarkably, PCVs exhibit high genetic mutation rates similar to RNA viruses, despite being DNA viruses. Since the initial identification of PCV2 in the last three decades, phylodynamic studies have unveiled the emergence of periodic waves featuring various genotypes within the PCV2 population (Franzo et al., 2016). These waves have resulted in two significant genotype shifts: from PCV2a to PCV2b, and subsequently from PCV2b to PCV2d. Notably, the genotype shift may contribute to the development of overt clinical signs observed in PCV2d infections compared to PCV2a/b strains (Guo et al., 2012). In Thailand, PCV2 and PCV3 have been reported, however, PCV4 status was not yet revealed. Reports on the prevalence and genetic diversity of PCVs have not been published in the last five years. Therefore, it is

crucial to monitor these viruses for a better understanding of the epidemiological and virological situation in Thailand.

In this study, prevalence and genetic characteristics of PCVs in Thai swine farms during 2019-2020 were investigated. It should be noted that PCV4 had been identified in Thailand for the first time in this study. It indicated that Thai swine farms are exposed to various PCVs potentially leading to clinical manifestations. Firstly, the prevalence of PCV2 in Thai swine farms in this study remained consistently high (54.2%) during the period of 2019–2020, showing an increase percentage compared to the period of 2009–2015 (44.09%) (Thangthamniyom et al., 2017). Especially, the positive rates at the farm levels exceeded 80%. This suggests that PCV2 has continued circulating in Thai swine farms despite the widespread implementation of PCV2 vaccination. However, it is premature to conclude that PCV2 vaccination ineffectively controls PCV2 infection in Thailand. Various confounding factors may involve in PCV2 vaccination failure. Comparing to PCV2, the prevalence of PCV3 and PCV4 was 28.88% and 0.4% at the sample level and 46.9% and 2.07% at the farm level, respectively. It indicated that PCV2 is predominantly found, followed by PCV3 and PCV4. The co-circulation of those PCVs in Thai swine farms should raise awareness among all stakeholders. The findings highlight the importance of disease prevention and control strategies to minimize the economic impact caused by PCVs clinical manifestations.

Regarding PCV2 genotypes, in Thailand, PCV2d has also become the dominant strain since 2015 (Thangthamniyom et al., 2017), in line with the global trend. This study revealed PCV2d remaining the dominant strain in Thailand. However, the proportion of PCV2d in Thailand seems to be increasing, indicating that the predominant PCV2d was gradually taking over other genotypes in the country. Interestingly, in this study, the identification of a novel PCV2d cluster, called the 19RBR58-like cluster had not been previously detected in Thailand. This study showed that novel PCV2d strains were more prevalent than previously circulating PCV2d strains in Thailand. The virus has a unique amino acid sequence (^{133}HDAM^{136}) in the capsid protein, located within the antibody recognition domains. This raises the concerns about its potential to evade the immune system. Moreover, it may serve as the starting point for the next genetic shift within PCV2d. Additionally, inter-genotypic recombination of PCV2b and PCV2d was first identified in Thailand in this study. However, further research is needed to understand the pathogenicity. While this research provides valuable insights, it has limitations, including a lack of information on farm vaccination status and reliance on passive surveillance. Nevertheless, it underscores the importance of active surveillance for comprehending PCV2 evolution and early intervening against emerging strains.

In contrast to PCV2, both PCV3 and PCV4 exhibit high genetic stability. In this study, all Thai PCV3 strains were classified as PCV3a. While the prevalence of PCV3 at

both the sample and farm levels was lower than that of PCV2, there is an upward trend in positive rates observed over the years. There is a possibility that PCV3-associated diseases could potentially lead to economic impacts on Thai swine farms in the future. Although the pathogenesis of PCV3-associated diseases remains uncertain and requires further investigation, active surveillance for prevalence and genetic evolution is crucial for informing the development of effective prevention and control strategies.

Furthermore, the presence of PCV4 in Thai swine farms was firstly reported. However, the prevalence of PCV4 in Thai swine farms was significantly lower when compared to PCV4-positive countries such as China and Korea. We detected the virus in pigs with various clinical signs, including respiratory disorders and reproductive failure. Interestingly, co-infections with porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 were observed. Indeed, the pathogenesis and pathogenicity of PCV4 remain poorly understood, and further investigation is necessary to determine its impact on Thai swine farms. Phylogenetic analysis revealed Thai PCV4 strains were tentatively classified as PCV4b and are closely related to PCV4 strain from China. In this study, we first demonstrated the viral tropism in bronchial epithelial cells using ISH. Virus replication in the bronchial epithelial cells may impair the epithelial barrier function, thus predisposing the infected pigs to secondary infections and later, PRDC.

Intriguingly, the genetic analysis reveals that PCV2 in Thai swine farms is undergoing more significant genetic evolution compared to PCV3 and PCV4. This is evident from the detection of a novel PCV2d cluster that had not been previously identified. It is possible that PCV2 has a higher genetic evolution rate, estimated at about 10^{-3} substitutions/site/year (Opriessnig et al., 2020), compared to PCV3 and PCV4, which have genetic evolution rates estimated at about 10^{-4} to10^{-5} substitutions/site/year (Chen et al., 2019; Opriessnig et al., 2020; Faustini et al., 2022). Moreover, the selective pressure induced by widespread PCV2 vaccination may be accelerating the genetic evolution of PCV2 in Thai swine farms, which differs from PCV3 and PCV4 that lack commercially available vaccines on the market.

Our studies confirm the presence of PCV2, PCV3, and PCV4 in Thai swine farms, highlighting the challenges in clinical diagnosis due to their shared symptoms. To overcome the scenarios, a triplex real-time PCR assay (tqPCR) for simultaneous PCVs detection, was developed. Analysis of 121 clinical samples from 2021 to 2023 reveals PCV2's continuously to be the dominant virus, followed by PCV3. Interestingly, PCV4 was not detected in the studied samples, aligning with previous findings of its limited transmission. In summary, the tqPCR assay offers advantages, including reduced testing time and quantitative analysis, for clinical diagnostics and epidemiological studies of PCV2, PCV3, and PCV4.

CHAPTER 8

CONCLUSION

In conclusion, this study sheds light on the prevalence and genetic characteristics of porcine circoviruses (PCVs) in Thai swine farms during 2019-2020. The findings revealed the first identification of PCV4 in Thailand, indicating that Thai swine farms are exposed to various PCVs with potential clinical impacts. Our study demonstrated that PCV2 is the predominant species, followed by PCV3 and PCV4 in Thai swine farms. The study highlighted the dominance of a novel PCV2d cluster in Thai swine farms with unique features that raise concerns about immune evasion. The inter-genotypic recombinant PCV2b/2d was first detected in Thailand. PCV3a continues to be the predominant strain, with a noticeable upward trend in positive rates over the years. We first identified the presence of PCV4, although its prevalence is significantly lower compared to other countries. The tissue tropism of PCV4 in bronchiolar epithelial cells was first identified in our study. Moreover, the development of a triplex real-time PCR assay offers a promising approach for accurate and efficient clinical diagnostics and epidemiological studies of PCV2, PCV3, and PCV4. Overall, our studies provide valuable insights for better understanding of prevalence and genetic characteristics of PCVs in Thai swine farms.

APPENDIX A

EMERGENCE OF NOVEL PORCINE CIRCOVIRUS 2D STRAINS IN THAILAND, 2019-2020

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Emergence of novel porcine circovirus 2d strains in Thailand, 2019–2020

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Porcine circovirus 2 (PCV2) has been recognized as a causative agent of porcine circovirus diseases (PCVDs) affecting the global swine industry. In this study, the genetic diversity of PCV2 strains circulating in Thailand between 2019 and 2020 was investigated using 742 swine clinical samples from 145 farms. The results showed PCV2-positive rates of 54.2% (402/742) and 81.4% (118/145) at the sample and farm levels, respectively. Genetic analysis of 51 Thai PCV2 genomic sequences showed that 84.3% (43/51) was PCV2d, 13.7% (7/51) was PCV2b and 1.9% (1/51) was PCV2b/2d recombinant virus. Surprisingly, the majority of the Thai PCV2d sequences from this study (69.77%, 30/43) formed a novel cluster on a phylogenetic tree and contained a unique ^{133}HDAM^{136} on the ORF2 deduced amino acid sequence, which is in one of the previously identified immunoreactive domains strongly involved in virus neutralization. The PCV2b/2d recombinant virus also carried ^{133}HDAM^{136}. The emergence of the novel PCV2d strains predominating in Thailand was discussed. This study highlights the need for further investigations on the spreading of these PCV2d strains in other regions and the efficacy of current commercial vaccines.

KEYWORDS

porcine circovirus 2, PCV2d, mutation, recombination, pigs, Thailand

1. Introduction

Porcine circovirus (PCV) 2, the causative agent of porcine circovirus diseases (PCVDs) affecting the global swine industry, is a non-enveloped single-stranded DNA virus containing a circular genome of 1766–1768 nucleotides (nt) (1) containing three main open reading frames (ORFs). Replicase protein encoded by ORF1 (*Rep* gene) is essential for viral replication (2). Capsid protein encoded by ORF2 (*Cap* gene) is a viral structural protein playing a significant role in the immunogenicity, virulence, and characteristics of the virus genotypes (3, 4). Finally, an ORF3 protein could induce apoptosis (4). Since its discovery, PCV2 has been recognized as a viral pathogen with a significant economic impact on the pig industry in various regions, particularly in North America, Europe, and Asia (5–7).

To date, PCV2 is classified into eight genotypes, PCV2a–h, based on the ORF2 nucleotide sequence (6). PCV2d is currently the predominant genotype worldwide (8), possibly due to selection pressure from the global PCV2 vaccination or the previously circulating PCV2

TABLE 1 The prevalence of PCV2 in all tested samples during 2019–2020.

Periods	Group of pigs[†]	Types of samples							Prevalence at sample level	Prevalence at farm level
		Pooled tissues	Serum	Feces	Semen	Oral fluids	Colostrum	Umbilical cords	Positive rate	Positive rate
Jan-Dec 2019	Suckling	16/21	0/7	4/7	-	-	-	-	194/373 (52.0%)	62/81 (76.5%)
	Nursery	66/75	24/98	0/1	-	-	-	-		
	Growers	28/28	15/38	0/1	-	-	-	-		
	Breeders	2/4	14/48	1/1	1/10	-	-	-		
	Fetuses	23/34	-	-	-	-	-	-		
Jan-Dec 2020	Suckling	3/8	2/13	0/1	-	-	-	-	208/369 (56.4%)	66/74 (89.2%)
	Nursery	38/43	23/53	1/4	-	0/2	-	2/2		
	Growers	14/15	90/132	-	-	-	-	-		
	Breeders	0/2	10/38	-	1/12	-	2/2	-		
	Fetuses	22/42	-	-	-	-	-	-		
Jan 2019-Dec 2020		212/272 (77.9%)	178/427 (41.7%)	6/15 (40.0%)	2/22 (9.1%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	402/742 (54.2%)	118/145 (81.4%)[‡]

[†]Suckling: < 4 weeks; Nursery: 5–8 weeks; Growers: 9–20 weeks; Breeders: boars, gilts, and sows.
[‡]The PCV2 positive farms were calculated from Jan 2019 to Dec 2020.

(118/145), respectively. Fifty-one PCV2-positive samples (from 51 pigs) from 48 farms were genetically characterized. The nucleotide sequences were deposited in the NCBI GenBank database under accession no. OL677572–OL677622 (Supplementary Table 4). Phylogeny-based genotyping of the ORF2 data showed that the Thai strains were PCV2b and PCV2d (Figure 1), found at 13.73% (7/51) and 84.31% (43/51), respectively (similar results were observed when ORF1 or genome data were used). However, one strain, 19NPT29, was not grouped within any genotype clusters. At the farm level, PCV2b and PCV2d were found at 14.58% (7/48) and 87.50% (42/48), respectively.

3.2. Novel PCV2d variants were identified and dominated among the PCV2d strains

Due to the high detection rate of PCV2d in this study, a phylogenetic tree was constructed to examine the genetic relationship between the current Thai PCV2d sequences (2019–2020) and the previously identified Thai PCV2d sequences (2010–2015). A cluster of PCV2d strains exclusively from 2019 to 2020 with a high bootstrap support was identified (data not shown). This cluster was named 19RBR58-like cluster, which accounted for 69.77% (30/43) of the PCV2d strains or 58.82% (30/51) of the PCV2 in this study. Percent nucleotide sequence identity of the 19RBR58-like cluster were as follows; genomic: 99.60–100, ORF2: 99.29–100, and ORF1: 99.58–100. Amino acid sequence identity was as follows; capsid: 99.15–100, and replicase: 99.36–100.

ORF2 nucleotide and amino acid sequence alignment were examined to identify a distinctive genetic characteristic of the 19RBR58-like cluster. A unique ^{133}HDAM^{136} and ^{232}K were found in all amino acid sequences from the 19RBR58-like cluster. Other Thai PCV2 sequences (PCV2a, b, d, and h) were ^{133}ANAL^{136} or ^{133}ATAL^{136}. To our knowledge, PCV2 strains with ^{133}HDAM^{136} have not been reported previously.

NCBI BLAST function using 19RBR58 ORF2 (6th February 2023) (https://blast.ncbi.nlm.nih.gov) was used to retrieve sequences with high similarity from GenBank database for further analysis (Supplementary Table 3). From the dataset, PCV2d strains with ^{133}HDAM^{136} from Japan and Taiwan during the period of 2018–2020 were found. However, those sequences were direct submissions. Moreover, PCV2d with amino acid sequences other than HDAM, ANAL, and ATAL at the position 133–136 were also identified (Supplementary Table 3) such as HAAM and HNAM. Clustering of the 19RBR58-like viruses was shown in Figure 2.

3.3. The novel PCV2d variants was a parental strain of a PCV2b/2d recombinant virus

Recombination analysis using seven different methods provided strong statistical support (average $p = 3.84 \times 10^{-9}$) confirming that 19NPT29 is an intergenotypic recombinant virus of PCV2b and PCV2d. The analysis indicated that PCV2b strains (such as South Korea/2016/KU-1605-like viruses) and PCV2d strains (such as Thailand/2019/19RBR10-like viruses) were the potential parental strains involved in the recombination event. Notably, the parental PCV2d strain was also found within the 19RBR58-like cluster, and the presence of ^{133}HDAM^{136} and ^{232}K in the capsid protein was observed in 19NPT29. The putative recombination breakpoints were identified at

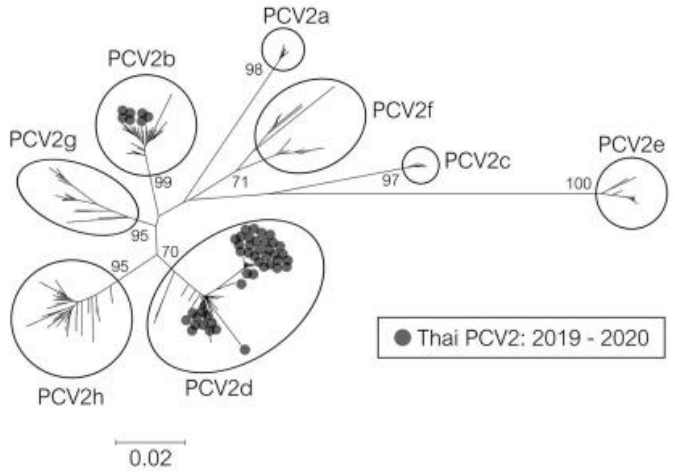

FIGURE 1
Phylogenetic tree of PCV2 ORF2 sequences from Thailand. The 317 complete ORF2 sequences were Thai PCV2 sequences (2019–2020) in this study and reference sequences from an available database (6). The tree was constructed using the Neighbor-Joining method with a p-distance model and bootstrapping at 1,000 replicates. Node labels indicate bootstrap values. The taxon position markers were adjusted to enhance readability.

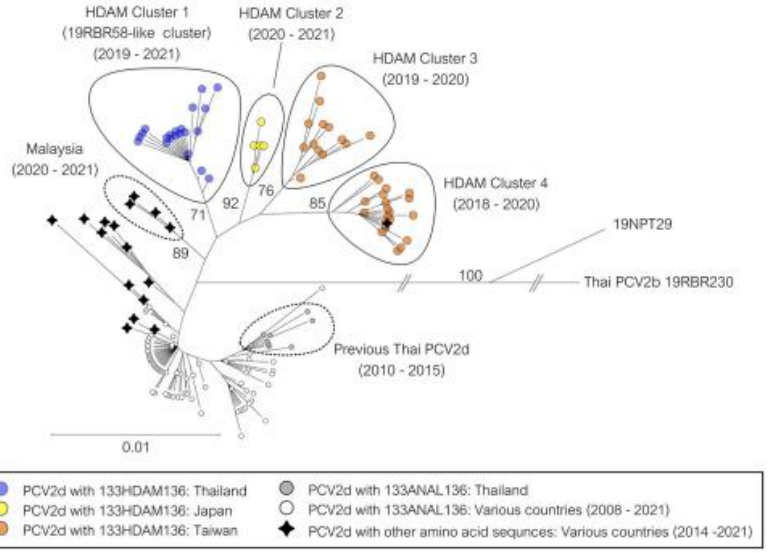

FIGURE 2
Phylogenetic tree of PCV2d ORF2 sequences from Thailand. The 158 complete ORF2 sequences were Thai PCV2d sequences in this study and the related PCV2d sequences from NCBI BLAST. A sequence of Thai PCV2b was used as an outgroup. The tree was constructed using the Neighbor-Joining method with the Maximum Composite Likelihood model and bootstrapping at 1,000 replicates. Node labels indicate bootstrap values. Specific branches were shortened, and the taxon position markers were adjusted to improve readability. A tree with each taxon label indicating the country of origin and the year of sample collection is available as Supplementary Figure 2.

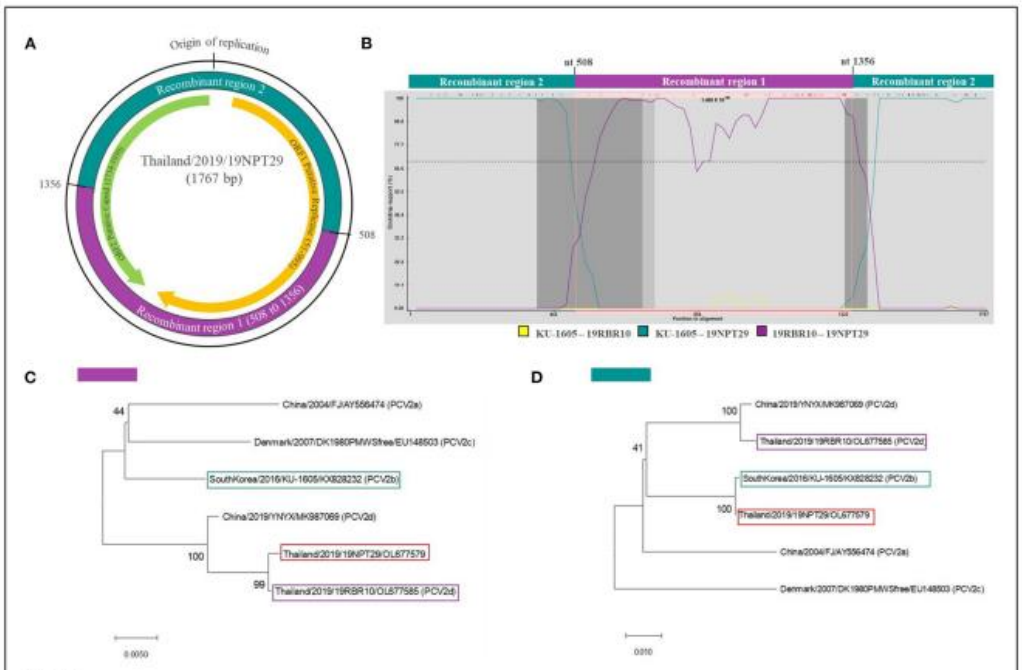

FIGURE 3
Recombination analysis of 19NPT29. Recombination analysis was done using RDP software. Recombination breakpoints were identified in ORF1 and ORF2 regions of the 19NPT29 genome, resulting in recombinant region 1 (purple shaded) and 2 (dark green shaded) **(A)**. Bootscanning analysis shows KU-1605 (South Korea) and 19RBR10 (Thailand, in this study) as parental strains **(B)**. The phylogenetic trees were constructed based on recombinant region 1 **(C)** and 2 **(D)** to confirm recombination event.

nucleotide positions 508 (ORF1) and 1356 (ORF2) (Figure 3). The detection rate of the recombinant strain at the animal and farm levels was found to be 1.96% (1/51) and 2.08% (1/48), respectively.

4. Discussion

PCV2 is a major swine virus causing economic losses. Although vaccines have been widely used, vaccine failures and immune escaping mutation of PCV2 has been proposed (8). In this study, novel variants of PCV2d were identified providing a clue on the PCV2 evolution and epidemiology.

The prevalence of PCV2 in Thailand remained consistently high during the period of 2019–2020 compared to the period of 2009–2015. The prevalence at the animal level from 2009 to 2015 was 44.09% (18). In the current study, the prevalence increased to 54.2%. At the farm level, the prevalence from 2009 to 2015 was 80% (18), and in 2019–2020, it reached 81.4%. These findings suggests that PCV2 was still circulating, despite the implementation of PCV2 vaccines in Thailand. Unfortunately, the PCV2 vaccination status of each farm was not available in this study. Therefore, no conclusion can be made regarding the effect of the PCV2 vaccination and the overall PCV2 prevalence in Thailand.

In recent years, a genotype shift toward PCV2d can be observed in various countries, particularly in Asia. These countries include China (19), South Korea (20), Vietnam (21), Malaysia (22), and Thailand (18). In Thailand, the prevalence of PCV2d has been increasing since 2010, with only PCV2d detected by 2015 (18). However, in this study, a novel strain of PCV2d, which accounted for 69.77% of all the current Thai PCV2d, was identified. Therefore, this novel strain of PCV2d plays a crucial role in the prevailing PCV2d strain in Thailand during 2019–2020. Moreover, this finding suggests that it may serve as the starting point for the next genetic shift within PCV2d.

This study identified the dominance of novel PCV2d strains, the 19RBR58-like cluster, over the previously circulating PCV2 strains in Thailand. At position 133–136 of the capsid protein, the 19RBR58-like cluster was ^{133}HDAM^{136} while other Thai PCV2 strains were ^{133}ANAL^{136} or ^{133}ATAL^{136}. Notably, this region of amino acids resides in one of the antibody recognition domains (domain B) previously described (23, 24), i.e., domain A (aa 51–84), B (aa 113–139), C (aa 161–207), and D (aa 228–233). A single mutation at position 134, 135 or 136 has been shown to strongly reduce the neutralization activity (9). Therefore, the capsid protein with ^{133}HDAM^{136} might render the 19RBR58-like cluster less susceptible to the antibodies from the previously circulating strains and the vaccines. In fact, PCV2 vaccination is widely implemented

in Thailand (personal communication). The observed immune escaping mechanism is further supported by the rapid increase of the 19RBR58-like cluster. Prior to 2015, the 19RBR58-like cluster was not detected in Thailand, and there is a lack of sequence data from 2016 to 2018. Thus, it is possible that the emergence of the 19RBR58-like cluster occurred during the period of 2016–2018.

In addition to Thailand, this study also identified PCV2d sequences with ^{133}HDAM^{136} from Japan and Taiwan (direct submission in GenBank). Interestingly, the strains carrying ^{133}HDAM^{136} from each region formed a distinct cluster on the phylogenetic tree. This suggests that the current situation of these viruses may not be attributed to recent spreading between regions. Phylogenetic analysis further revealed that all the clusters harboring ^{133}HDAM^{136} (Thailand, Japan, and Taiwan) likely share a common ancestor with PCV2d strains from Malaysia. At present, the prevalence of the ^{133}HDAM^{136} PCV2d variants in Japan and Taiwan is unknown. Further investigations are needed to determine whether the prevalence of these PCV2d variants is high, similar to that observed in Thailand.

Recombinant viruses derived from PCV2d strains have been reported in various countries, including China, India, and South Korea (25–27). In this study, a recombinant PCV2d/PCV2b strain, named 19NPT29, was identified. Interestingly, ^{133}HDAM^{136} was also found in the capsid protein of 19NPT29. It would be valuable to conduct further studies to investigate whether the presence of ^{133}HDAM^{136} provides any advantages to the recombinant PCV2 strain, particularly in the case of inter-genotypic recombinants. Unfortunately, conducting further epidemiological studies on the 19NPT29-like viruses from the source farm is not possible as the farm is no longer operational.

The main limitation of this study was the absence of information regarding the vaccination status of the farms, along with the passive surveillance nature of the study. The observed mutation in the antibody recognition domain of the capsid protein within the 19RBR58-like cluster is suspected to have played a role in its emergence. Therefore, the information regarding the vaccination status would have been invaluable for interpreting the data and generating hypotheses for further studies on cross-protection. Furthermore, it is important to note that the samples used in this study were obtained from two diagnostic laboratories (CU-VDL and DLSTC), which may have led to potential underrepresentation of certain geographical regions. However, it is worth mentioning that this study managed to collect samples from all the high-pig-density regions in the country. Conducting active surveillance in the future may provide a more precise assessment of the prevalence of PCV2 and the PCV2d status.

In conclusion, this study reveals the presence of a novel PCV2d strain with ^{133}HDAM^{136} in the capsid protein as the predominant PCV2 strain in Thailand. Additionally, a recombinant virus between PCV2b and the novel PCV2d was identified. The emergence of these novel PCV2d strains might have been influenced by both vaccination and the previously circulating viruses. Conducting active surveillance can provide a comprehensive understanding of PCV2 evolution and facilitate the implementation of early interventions against the emergence of novel strains.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

CS, TN, and TJ collected the samples and did the PCR and sequencing. CS, RK, and RT analyzed the data and interpreted the results and prepared the manuscript. All authors helped in designing the research and contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1170499/full#supplementary-material

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APPENDIX B

MOLECULAR DETECTION AND GENETIC CHARACTERIZATION OF PORCINE CIRCOVIRUS 4 (PCV4) IN THAILAND DURING 2019–2020

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Keywords: Porcine circovirus 4, pigs, complete genome, capsid, genetic characterization, Thailand

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Molecular detection and genetic characterization of porcine circovirus 4 (PCV4) in Thailand during 2019–2020

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Porcine circovirus 4 (PCV4) is considered a novel PCV, firstly found in China in 2019 and later discovered in Korea. This present study investigated the prevalence and genetic characteristics of PCV4 from high pig-density areas in Thailand during 2019–2020. From 734 samples, three samples (0.4%) from aborted fetuses and porcine respiratory disease complex (PRDC) cases were found positive for PCV4, two of the PCV4-positive samples were coinfected with both PCV2 and PRRSV, and the other PCV4-positive sample was found coinfected with PCV2. In situ hybridization (ISH) revealed the presence of PCV4 in the bronchial epithelial cells and in lymphocytes and histiocyte-like cells in the lymphoid follicles of the PRDC-affected pig. The complete Thai PCV4 genome had over 98% nucleotide identity with other PCV4 strains and was closely related to the Korean and Chinese PCV4b strains. Importantly, the amino acid residue at position 212 of the Cap gene is recommended for differentiating PCV4a (^{212}L) from PCV4b (^{212}M) based on currently available PCV4 genome sequences. These findings provide important clues for the pathogenesis, epidemiology, and genetic characteristics of PCV4 in Thailand.

To date, four species of porcine circoviruses (PCVs) have been identified, including PCV1, PCV2, PCV3, and PCV4[1]. PCV1 is nonpathogenic in pigs. PCV2 causes various symptoms collectively called porcine circovirus-associated diseases (PCVADs) or porcine circovirus diseases (PCVDs)[1–3]. PCV3 has been found in pigs with multiple clinical signs; however, the pathogenesis is still unknown[1,4,5]. Recently, a novel PCV4 has been identified in China and Korea both in clinically healthy and infected pigs with several clinical presentations, including respiratory and enteric signs and skin lesions suggestive of porcine dermatitis and nephropathy syndrome (PDNS)[6–11]. Therefore, despite having limited information on PCV4, the virus should not be overlooked. Furthermore, due to the lesson learned from past experiences with other swine viruses in Asia[12], many emerging viruses have spread among countries due to the consequences of globalization through international trade and travel, both officially and unofficially. Therefore, the recent findings of PCV4 in China and Korea would raise awareness of the virus to the Asian swine practitioners to investigate this novel pathogen in their areas as part of the focus area "prevent and detect" to understand the disease distribution and its impact. In this study, we investigated from the samples obtained during 2019–2020 for the presence of PCV4 in Thailand and its genetic characterization.

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Results

Prevalence and geographical distribution of PCV4 during 2019–2020. To investigate the existence of PCV4, several types of swine samples (n = 734) from different geographical regions mainly located in the high pig density areas of Thailand were used. At sample levels, the overall positive rates of PCV4 were 0.4% (3/734). Notably, three PCV4-positive samples from clinically infected pigs with abortion and respiratory signs were found coinfected with PCV2 (3/3) and PRRSV (2/3) (Table 1). Among the 145 pig farms in these 18 provinces, 2.07% (3/145) were PCV4-positive. The geographic distribution of PCV4-positive farms was shown in Fig. 1.

Genetic characteristics of PCV4. In this study, all PCV4-positive samples were selected and sequenced for genetic characterization and phylogenetic analysis. The results showed that Thai PCV4 strains were 1770 nt in length, with 891 nt of ORF1 (Rep) and 687 nt of ORF2 (Cap). The complete genome sequences of the Thai PCV4 strains were aligned against those of reference viruses from China and Korea (Table 2). The results showed that the complete genome of the 3 Thai PCV4 strains shared 100% nucleotide identity to each other and shared 98.3–99.0% nucleotide identity with other reference PCV4 strains (Table 2). An amino acid sequence analysis indicated that the Rep and Cap genes of the Thai PCV4 strains shared similarity of 98.6%-100% and 97.8%-99.1%, respectively, when compared to each other and the reference strains. The Thai PCV4 strains showed the highest similarity to PCV4 NM2 from China with 99% nucleotide identity (complete genome), 100% Rep amino acid identity, and 98.6% Cap amino acid identity. Based on the phylogenetic analysis of complete genome nucleotide sequences of 41 PCV4 strains, the viruses have undergone evolution, resulting in two main distinct branches (PCV4a and PCV4b) (Fig. 2). The results showed that the Thai PCV4 strains belonged to PCV4b and clustered in the same branch with PCV4/KU-02010 strain found in Korea (Fig. 2). In the present study, PCV4 strains identified in China belong to both PCV4a and PCV4b genotypes. Interestingly, in the Korean and Thai swine farms, only PCV4b strains were detected. Moreover, the phylogenetic trees based on Cap and Rep genes were constructed for assessing genetic relationships (Supplementary Figure 1). The findings demonstrated that different genomic regions of PCV4 yielded similar outcomes.

Nucleotide sequence comparison and amino acid sequence analysis of PCV4. An analysis of nucleotide and amino acid sequences of the Cap and Rep genes against reference PCV4 strains demonstrated that Thai PCV4 genomes displayed nucleotide identities of 97.6%-98.9% amino acid and 98.4%-100% amino acid in Cap gene and Rep genes, respectively compared to those of reference viruses. The Thai PCV4 strains shared amino acid identities of 97.8%-99.1% and 98.6%-100% in Cap gene and Rep gene compared to those of reference viruses. There were no amino acid deletions or insertions. Compared with prototype PCV4 strain HNU-AHG1-2019 (accession no. MK986820), Thai PCV4 genomes had 20 nucleotide substitutions (Table 3). For deduced amino acid analysis, one amino acid substitution (Q155K) was seen in Rep gene, while 3 amino acid substitutions (N27S, I80V, and I96V) were found in Cap gene (Fig. 3). Among them, there was one unique amino acid substitution (I80V) in the Thai PCV4 strains of Cap gene compared with other PCV4 strains. Compared with the representative strains (Fig. 3), the Cap gene of PCV4a contains specific amino acid patterns of 27S and 212L, while certain PCV4b strains have unique amino acid patterns of 27N and 212M. Interestingly, the sequences from the present study revealed that the Thai PCV4 strains were grouped in PCV4b, even though they showed amino acid 27S in the Cap gene, and possessed the same amino acid at position 212M, similar to the two PCV4 strains (KU-02010 and KU-02011) from Korea. The findings suggest that amino acid variation at position 212 of Cap gene could be used for differentiating PCV4a (^{212}L) from PCV4b (^{212}M) (Fig. 3).

Detection of PCV4 by in situ hybridization. The PCV4-positive pig (19RBR247) with respiratory illness was submitted for necropsy. Prominently, lungs with enlarged tracheobronchial lymph nodes were diffusely mottled and failed to collapse with dark red to purple consolidation and diffuse fibrinous attachment in the pleura (Fig. 4A). Microscopically, lungs revealed moderate to severe diffuse pulmonary and interlobular edema with mild multifocal hyperplasia of bronchus-associated lymphoid tissue, and moderate to severe diffuse broncho-interstitial pneumonia. Tracheobronchial lymph nodes showed moderate multifocal lymphoid depletion. To investigate the tissue localization of PCV4, lung and tracheobronchial lymph node were tested using in situ hybridization. The results showed that PCV4-ISH-positive signals were mainly observed in the cytoplasm of bronchial epithelium (Fig. 4B). Moreover, a few positive signals were detected in the lymphocytes and histiocyte-like cells of the tracheobronchial lymph node (Fig. 4C). No hybridization signals were seen in internal negative control of lung (Fig. 4D) and tracheobronchial lymph node tissue sections (Fig. 4E).

Collection year	Strain	Age of pigs	Sample	Location	Farm	History	Coinfection			
							PCV2	PCV3	PCV4	PRRSV
Jan-2019	19RBR246	Fetus	Pooled organs	Ratchaburi	Farm A	Abortion	Yes	–	Yes	–
Jan-2019	19RBR247	Grower-finishing	Pooled organs	Ratchaburi	Farm B	Respiratory signs, sudden death	Yes	–	Yes	Yes
Feb-2019	19RBR255	Nursery	Pooled organs	Ratchaburi	Farm C	Respiratory signs	Yes	–	Yes	Yes

Table 1. PCV4-positive samples found in Thailand during 2019–2020.

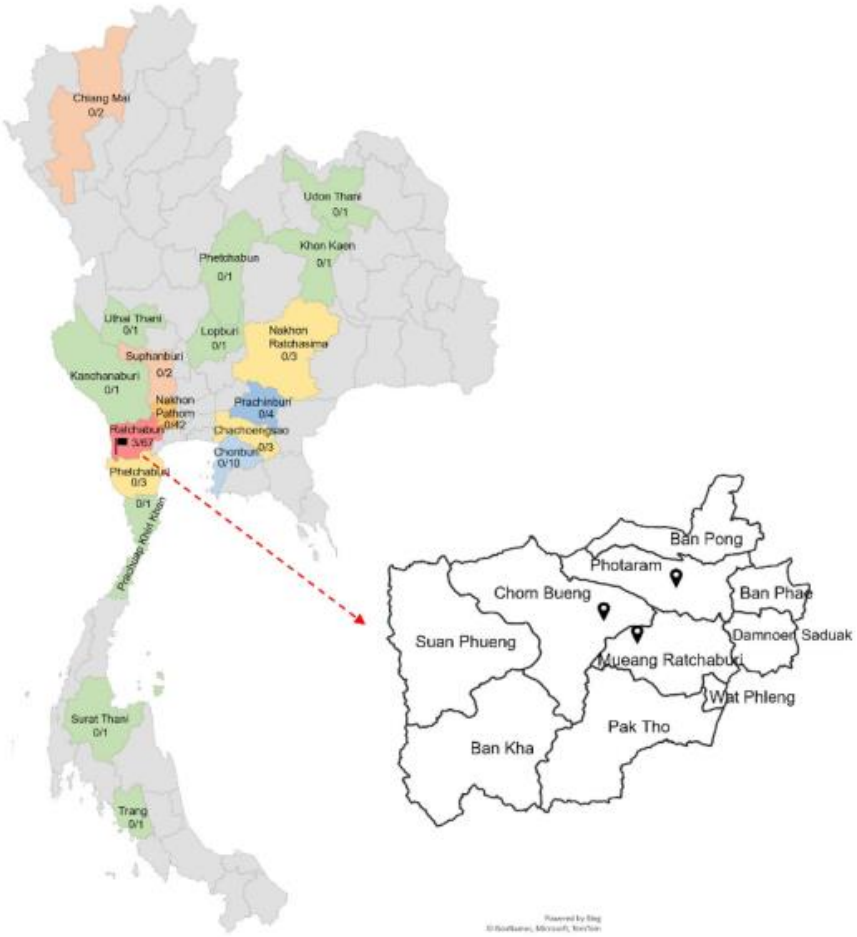

Figure 1. Geographic distribution of PCV4-tested swine farms (n = 145) in Thailand. The black flag indicates the Ratchaburi province where the positive samples were collected for complete genome sequencing. The locations of the 3 districts found PCV4-positive farms are indicated in the black pin.

Discussion

PCV2 and PCV3 are at least the two porcine circoviruses causing major threats to the global swine industry[1–3]. The finding of PCV4 should not be neglected since Asia is the major world hub of pig production, and the virus itself may originate here and spread to other regions or vice versa. To date, PCV4 was discovered only in the Asian continent, China, Korea, and recently, Thailand, but not yet in others[6–11,13,14].

In the present study, the results showed that only 3 of the 734 samples were tested positive for PCV4, with a positive rate of 0.4% (3/734) and 2.07% (3/145) of farm levels. Interestingly, extremely low prevalence of PCV4 was demonstrated in Thailand compared to previous reports[6,7,9–11] suggesting low transmission and infection rate within the pig herds. However, nearly 80% of the swine farms tested were mainly located in the high pig density areas of Thailand, which might not reflect the whole picture on PCV4 infection status of the country. Thus, in the future, large-scale field sampling of all regions could provide more information about PCV4 epidemiology in Thailand. Therefore, the threat of PCV4 to the Thai swine industry is skeptical and yet to be elucidated.

Recently, pigs inoculated with rescued PCV4 alone showed histopathological changes in several organs, but no obvious clinical signs found[15]. In the present study, we found that three PCV4-positive samples from clinically infected pigs were found coinfected with PCV2 (3/3) and PRRSV (2/3). These results apparently suggest that absence of other factors, such as coinfections, PCV4 infection might remain asymptomatic. However, the

Strain	Year	Accession no	Country	% Nucleotide identity (%amino acid identity)		
				Complete genome	Cap gene	Rep gene
19RBR246	2019	ON854861	Thailand	100	100 (100)	100 (100)
19RBR247	2019	ON854862	Thailand	100	100 (100)	100 (100)
19RBR255	2019	ON854863	Thailand	100	100 (100)	100 (100)
HNU-AHG1-2019	2019	MK986820	China	98.8	98.9 (98.6)	98.6 (99.6)
KU-02010	2020	MW712668	Korea	98.9	98.6 (98.2)	99.1 (100)
KU-02011	2020	MW712667	Korea	98.9	98.6 (98.6)	99.2 (100)
NM1	2017	MT882410	China	99	98.8 (98.6)	99.1 (100)
NM2	2017	MT882411	China	99	98.8 (98.6)	99.2 (100)
NM3	2017	MT882412	China	99	98.8 (98.6)	99.1 (99.6)
E115	2020	MT882344	Korea	98.8	98.9 (99.1)	98.8 (100)
PCV_VIRES_NX01_G28	2017	MK948416	China	98.7	98.3 (98.6)	99.3 (99.6)
FJ-PCV4	2019	MT721742	China	98.4	98.2 (97.8)	98.4 (98.6)
Hebei-AP1	2019	MW084633	China	98.9	98.8 (99.1)	99.2 (100)
Henan-LY1-2019	2019	MT015686	China	98.5	98.1 (98.6)	98.9 (99.6)
JSYZ1901-2	2019	MT769268	China	98.9	98.6 (99.1)	99.1 (99.6)
KF-01-2019	2019	MT193106	China	98.8	98.6 (99.1)	98.8 (99.3)
KF-02-2019	2019	MT193105	China	98.8	98.8 (99.1)	99.1 (100)
GX2020/NN88	2020	MT311852	China	98.7	98.6 (99.1)	98.9 (99.3)
GX2020/GL69	2018	MT311853	China	98.6	98.5 (98.2)	98.8 (99.3)
GX2020/FCG49	2018	MT311854	China	98.8	98.8 (99.1)	98.9 (99.6)
HN-LY-202005	2020	MW538943	China	98.6	98.5 (98.6)	98.9 (99.6)
HN-LY-202006	2020	MW600947	China	98.5	98.6 (98.6)	98.6 (98.9)
HN-XX-201811	2018	MW600950	China	98.8	98.8 (99.1)	98.9 (99.3)
HN-KF-201812	2018	MW600951	China	98.8	98.6 (98.6)	99.1 (100)
HN-HB-201704	2017	MW600952	China	98.5	98.3 (97.8)	98.9 (99.6)
HN-XX-201212	2012	MW600953	China	98.4	98.6 (99.1)	98.7 (100)
HN-LY-201702	2017	MW600954	China	98.7	98.6 (98.6)	98.9 (100)
HN-ZZ-201603	2016	MW600955	China	98.6	98.3 (98.2)	99.1 (100)
HN-ZK	2015	MW600956	China	98.4	98.3 (97.8)	98.6 (98.9)
HN-ZK-201601	2016	MW600957	China	98.3	98.3 (98.2)	98.5 (98.6)
HN-ZMD-201212	2012	MW600958	China	98.7	98.6 (99.1)	98.9 (99.6)
HN-XX-201601	2016	MW600959	China	98.4	98.3 (98.6)	98.8 (98.9)
HN-ZK-201707	2017	MW600960	China	98.6	98.8 (99.1)	98.8 (99.3)
LY2017	2017	MW759029	China	98.8	98.6 (98.6)	99.1 (100)
YY2019	2019	MW759027	China	98.8	98.8 (99.1)	98.9 (99.6)
Hebei1	2020	MW262973	China	98.4	97.8 (98.2)	98.9 (99.6)
Hebei2	2020	MW262974	China	98.4	97.6 (97.8)	98.9 (99.6)
Hebei3	2020	MW262975	China	98.4	97.9 (98.6)	98.8 (99.6)
Hebei4	2020	MW262976	China	98.4	97.9 (98.6)	98.8 (99.3)
Hebei5	2020	MW262977	China	98.4	97.9 (98.6)	98.8 (99.3)
Hebei6	2020	MW262978	China	98.4	97.8 (98.6)	98.9 (99.6)

Table 2. Percentage of nucleotide and amino acid identity (%) shared between Thai PCV4 strains and other reference strains.

synergistic effect of PCV4 infection might exacerbate the disease severity and be associated with PRDC in clinically infected pigs as found in the present study. Therefore, co-infections and some unknown factors, yet to be elucidated, might involve PCV4 pathogenesis and its clinical outcomes.

According to in situ hybridization results, the virus presence was found in the bronchiolar epithelial cells and in lymphocytes and histiocyte-like cells in the lymphoid follicles consistently with the characteristics of PCV2 infection[16]. Whether PCV4 infection in bronchiolar epithelial cells and tracheobronchial lymph node induces the observed broncho-interstitial pneumonia and the lymphoid depletion should be further studied. The contribution of PCV4 to the pathogenesis of PRDC and immune modulation should be of interest. These findings also suggest that a marked tropism of PCV4 for bronchial epithelial cells may impair the epithelial barrier function, thus predisposing the infected pigs to secondary infections and PRDC. The case history of the PCV4 positive cases found that the PCV4-infected pigs were found coinfected with PCV2, PRRSV, and/or *Streptococcus* spp. Additionally, bronchial epithelial infection might contribute to viral shedding dynamics in the nasal secretion.

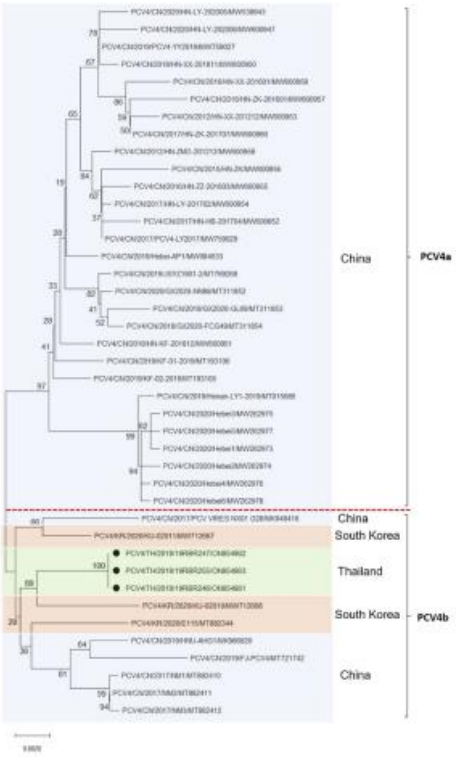

Figure 2. Phylogenetic tree based on the complete genome sequences of 3 Thai PCV4 strains and other reference strains. The Thai PCV4 sequences obtained in this study were marked with solid black circles. The colored background represented the country of origin of the PCV4 viruses. The phylogenetic tree was constructed using the neighboring-joining method with a *p*-distance model and bootstrapping at 1000 replicates.

Notably, it has been reported that the highest positive rates of PCV4 were detected in nasal swabs followed by serum samples[6]. Therefore, nasal swabs might be a better target specimen for PCV4 detection and surveillance. Further investigation is needed.

For genetic analysis, Thai PCV4 strains shared up to 98% nucleotide identity with other reference PCV4 strains. The high genetic stability of PCV4 is consistent with the previous reports[7,11]. It is noted that the Thai PCV4 strains were closely related to the Chinese PCV4 sub-cluster. Although the virus had high genetic stability, some genetic variations could be observed among pig populations from different countries that probably specific to the geographic origin[17]. Additionally, the Thai PCV4 strains were highly related to each other, possibly, due to a single point introduction with low infection rate or restricted gene flow in the neighboring districts of Western Thailand, suggesting that the novel virus might be confined to these areas affecting the prevalence and genetic diversity.

Additionally, there are currently no fully established guidelines for classifying PCV4 genotypes, and the temporary proposals made so far have not been consistent. This is in contrast with PCV2 genotyping that a unified classification scheme based on Cap gene was proposed[18]. For PCV4 genotyping, there have been several studies proposing different criteria and markers to differentiate PCV4 genotypes, such as 2-group classification (PCV4a and PCV4b) and 3-group classification (PCV4a, PCV4b, and PCV4c)[10,19–21]. Among these studies, the residue at 27 and 212 of capsid gene were also proposed to be used in distinguishing PCV4a (27S and 212L) from PCV4b (27N and 212M)[19,20]. Recently, PCV4c was proposed with specific amino acid pattern 27N, 28R, and 212M. However, in some genotyping criteria, PCV4c might be classified into PCV4b[10,20]. Therefore, the use of differing nomenclatures can create ambiguity and misinterpretation of results. Further investigation is required

Genome position	PCV4 HNU-AHG1-2019	PCV4 19RBR247
36	A	G
122	T	C
206	C	T
287	C	T
432	C	T
537	C	A
557	T	C
599	T	C
608	A	C
726	C	T
866	C	T
896	C	T
920	G	A
1251	T	G
1260	G	A
1448	T	C
1496	T	C
1527	C	T
1654	T	C
1680	G	T

Table 3. Nucleotide sequence comparison between Thai PCV4 strain (19RBR247) and the prototype PCV4 strain HNU-AHG1-2019.

to establish the standardized criteria for genotyping PCV4, given the current paucity of sequence information. As mentioned above, previous reports showed that the amino acid at the position 27 of the Cap gene could be used as a marker to distinguish between PCV4a (27S) and PCV4b (27N)[19,20]. However, the results in this study showed that the amino acid residue at position 27 cannot be used to differentiate between PCV4a and PCV4b since the Thai PCV4b strains and the Korean PCV4b strains (KU-02010 and KU-02011) showed N27S (Fig. 3). The novel findings of this study suggest that amino acid variation at position 212 could be used for differentiating PCV4a (212L) from PCV4b (212M) for 2-group classification. Moreover, one unique amino acid substitution (I80V) was also found in the Thai PCV4 strains in Cap gene. The amino acid substitution occurred at residue 72–88 in B-cell epitopes on PCV4 capsid gene[20]. It may alter antigenic properties of the viruses and their immunogenic modifications caused by genetic mutation. However, the effects of genetic mutation on pathogenicity need further investigation. In terms of the genetic variation of PCV4 and its genotypes, a study conducted in various regions in China found that PCV4a was the most common genotype followed by PCV4b[10,11,19–21]. However, in pig populations from Korea and Thailand, only the PCV4b genotype was detected[8]. It is plausible to consider that PCV4 strains isolated from various geographic regions or pig populations may potentially demonstrate genetic variations, which may imply the existence of geographical or host specificity in the virus.

To date, PCV4 was discovered only in the Asian continent, China, Korea, and recently Thailand[6–11,13,14]. This study described the presence of PCV4 in Thailand from the retrospective samples and firstly demonstrated the viral tropism in the bronchial epithelial cells. The three Thai PCV4 strains demonstrated their genetic similarity with the Korean and Chinese PCV4b strains. However, the prevalence of PCV4 in Thailand is extremely low, and clinical involvement of PCV4 remains unclear. Apparently, it should be noted that the amino acid residue at position 212 of the Cap gene should be used for differentiating PCV4a and PCV4b. Further studies are needed to determine the role of PCV4 infection related to clinical signs and its impact on the Thai swine farms. The classification of PCV4 genotypes requires further investigation and clarification due to the limited available PCV4 sequences.

Materials and methods

Sample collection and viral DNA extraction. Seven hundred thirty-four samples from 145 swine farms submitted for diagnostic purposes at the Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL), and Diagnostic Laboratory of Large Animal Hospital and Students Training Center during January 2019- December 2020 were used for this study. The samples consisted of serum (n = 426), tissue (n = 188), fetus (n = 75), semen (n = 25), feces (n = 16), colostrum (n = 2), and oral fluid (n = 2) that were mainly collected from the high pig density areas in the Western, Central, and Eastern parts of Thailand.

Total viral DNA was extracted using IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) following the manufacturer's instruction. The extracted DNA was stored at -80 °C until used.

Molecular detection of PCV4. TaqMan® real-time PCR was performed to detect PCV4 targeting the replicase gene (rep) of PCV4 using two newly designed primer pairs and probe (Supplementary Table S1). Briefly,

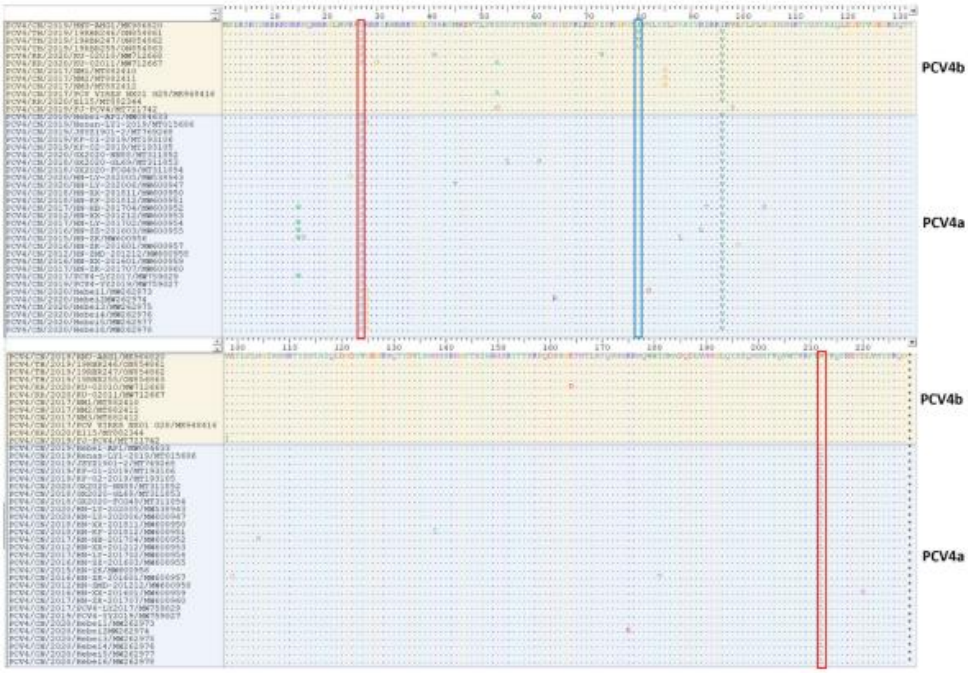

Figure 3. Comparison of Cap amino acid sequences of PCV4 isolates in this study. Dots are used to denote the residues that are consistent with HNU-AHG1 (MK986820). The red boxes indicate the amino acid at positions 27 and 212, previously proposed for differentiation of PCV4a and PCV4b. Mutations at residues position 80 of Thai PCV4 strains are shown with the green box.

PCR reactions were performed in a total 20 µl reaction containing 0.4 µM of forward and reverse primers, 0.2 µM of probes, 10 µl of Luna® Universal Probe qPCR master mix (NEB, MA, USA), and 3 µl of extracted DNA. The PCR condition consisted of initial denaturation at 95 °C for 60 s followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s using Quantstudio5 real-time system (Applied Biosystems, USA). Positive control plasmid was synthesized by inserting a full length of PCV4 rep gene (891 bp) into the pUC18 vector by GenScript Company (Nanjing, China). Detection limit of the PCV4 TaqMan® real-time PCR was 200 copies/µl of standard plasmid DNA.

Complete genome amplification and sequencing. The complete genomes of PCV4-positive samples were amplified using two primer sets (Supplementary Table S1). The PCR reactions were performed in 25 µl reaction mixtures containing 3 µl of extracted DNA, 0.5 µM of forward and reverse primers, and 12.5 µl of Q5® High-Fidelity 2 × master mix. The PCR thermal profile involved an initial denaturation of 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 72 °C for 30 s, 72 °C for 50 s, and final extension at 72 °C for 2 min. The PCR products were purified using Nucleospin™ Gel and PCR clean-up (MACHEREY–NAGEL, Germany) and submitted for sequencing by a barcode-tagged sequencing platform (Celemic, Seoul, Korea). The obtained nucleotide sequences were further analyzed and assembled with SeqMan, and Editseq software v.5.03 (DNASTAR Inc., Madison, Wisconsin, USA), then deposited in GenBank under accession no. ON854861-ON854863.

Phylogenetic analysis. For pairwise comparison and genetic characterization of PCV4, the complete nucleotide sequences were aligned using the Clustal W algorithm of BioEdit 7.2.5 (https://bioedit.software.informer.com/) with the reference PCV4 strains from the GenBank database. Phylogenetic trees were reconstructed with MEGA version 10.2.6 using the neighbor-joining algorithm (NJ) with 1000 bootstrap replicates[22].

Currently, guidelines for identifying PCV4 genotypes are not yet fully established, but they have been temporarily proposed and were not consistent. Generally, PCV4 strains have been classified into two main genotypes, PCV4a and PCV4b[10,19,20]. However, a single study has proposed that PCV4 might be classified into three genotypes, including PCV4c[21]. To simplify the analysis, in this present study, PCV4 strains were classified into two

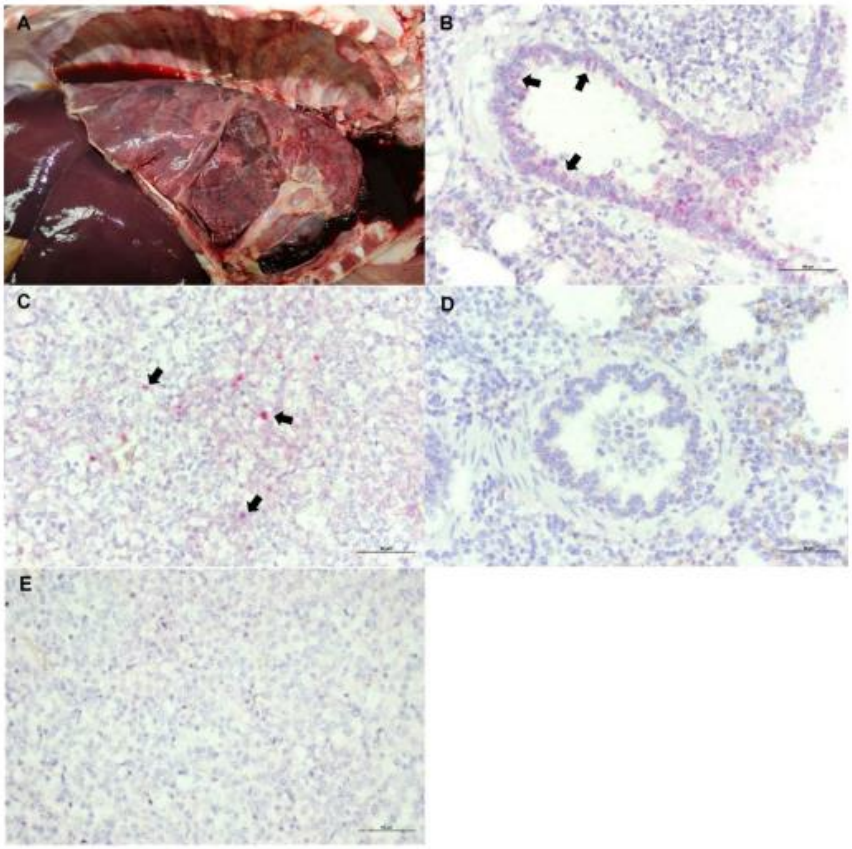

Figure 4. Gross and microscopic lesions and in situ hybridization (ISH) using PCV4-specific probe targeting Cap gene of PCV4. Grossly, lung failed to collapse with dark red to purple consolidation and diffuse fibrinous attachment in the pleura (**A**). Lung: PCV4-positive cells were characterized by pink to brilliant red in the cytoplasm of bronchial epithelial cells (arrows) (**B**). Tracheobronchial lymph node: positive signals were observed in lymphocytes and histiocyte-like cells in the lymphoid follicle (arrows) (**C**). No hybridization signals were seen in the internal negative control of lung and tracheobronchial lymph node (**D**,**E**).

main genotypes, PCV4a and PCV4b. The phylogenetic analyses were carried out using the complete genome, Rep gene, and Cap gene, following the approach previously described[10].

Detection of PCV4 in tissues using in situ hybridization. Of the three PCV4-positive samples, the formalin-fixed paraffin-embedded (FFPE) tissue samples were available for only one case, 19RBR247, and thus used for virus localization analysis. The FFPE tissues were stained with hematoxylin and eosin (HE) for histopathology study. To identify PCV4 tissue localization, in situ hybridization (ISH) was performed according to a previously described protocol with some modifications[23]. Briefly, PCV4-specific probe targeting 110 bp of the Cap gene of PCV4 was constructed using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The sections were incubated overnight at 40 °C in a moist chamber with PCV4-specific probe. The PCV4-specific DIG probe was detected by using 1:200 anti-DIG AP Fab fragments (Roche Diagnostics, Basel, Switzerland). Hybridization signals were detected as pink to brilliant dark red colorimetric staining of Permanent Red (LPR) (Dako, Glostrup, Denmark) with 50% hematoxylin counterstaining. Slides incubated without the DIG probe were used as a negative control.

Data availability

The datasets generated and analysed during the current study are available in the NCBI genomes repository under the accession numbers ON854861-ON854863.

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Author contributions

C.S. and R.T. planned the experiments. C.S., T.C., C.P., S.J. and R.K. carried out the experiments. C.S. and R.K. analyzed and interpreted the results. C.S., R.K. and R.T. drafted and revised the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary information

Molecular detection and genetic characterization of porcine circovirus 4 (PCV4) in Thailand during 2019-2020

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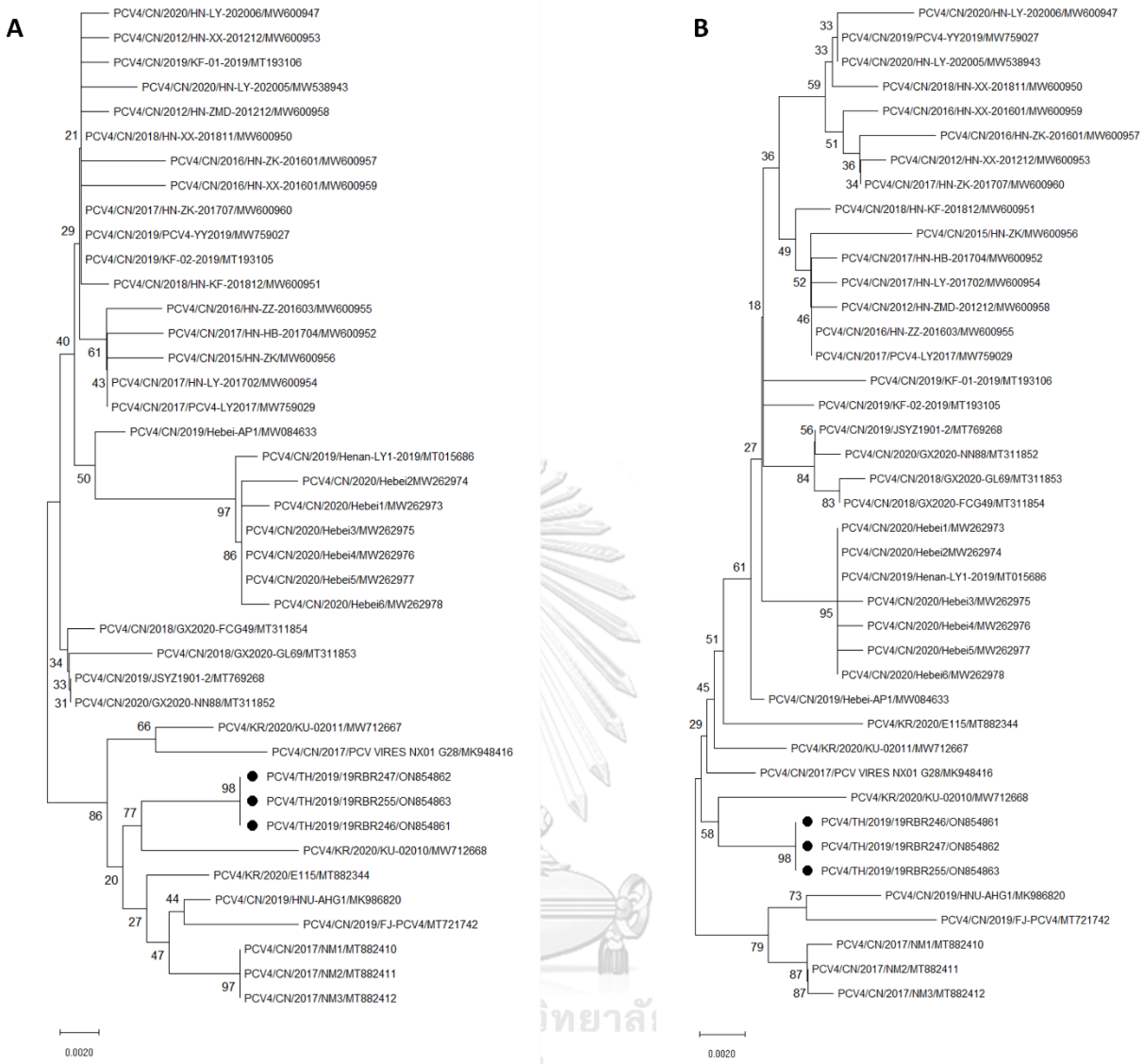

Supplementary figure 1. Phylogenetic tree based on full length of *Cap* gene (A) and *Rep* gene (B) using the neighboring-joining method with a *p*-distance model and bootstrapping at 1000 replicates. The Thai PCV4 sequences obtained in this study were marked with solid black circles.

Supplementary table S1. List of primers sequences used for detection and sequencing Porcine circovirus 4 (PCV4) in this study.

Primer	Sequences (5'-3')	Reference
PCV4-rF	AAAGCGCAGCGACCTTAAAG	For detection, this study.
PCV4-rR	CACGGGCCACTTCACTCATT	
PCV4-Probe	ROX-CTGTGGCCGCCCTGAATGCC-BHQ2	
PCV4-277F	GTGGCCTCCGGGACTACGTCAT	For genome sequencing. (Zhang et al., 2020)
PCV4-170R	GCACTGGGCTCTCCTACTTCCAG	
PCV4-88F	GCCACCCCGTGAAGAGATATT	For genome sequencing, this study.
PCV4-586F	CCGGGGATACCCACGATGAC	

Reference

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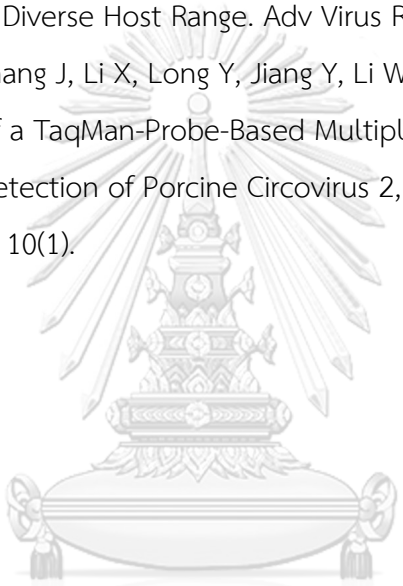

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