

Prevalence of Porcine Circovirus Type 2 (PCV-2) in Smallholder Pig Farms in Thung-Yai, Nakhon Si Thammarat, Thailand

Vassakorn KHOPHLOIKLANG^{1,#}, Ladawan SARIYA²,
Pornsak NARA-ARJ³, Manta POOMIKASEMSAK¹,
Kosit AREEKIT¹, Nilobol KAMYUN⁴, Suttida SANGPOOM⁵,
Aekkapot CHAMKASEM^{1,6,#} and Dusit LAOHASINNARONG^{7,*}

¹Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat 80240, Thailand

²The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Nakhonpathom 73170, Thailand

³Nakhon Si Thammarat Provincial Livestock Office (Thung Yai), Department of Livestock Development, Nakhon Si Thammarat 80000, Thailand

⁴Department of Mathematics, School of Science, University of Phayao, Phayao 56000, Thailand

⁵Division of Mathematics and Statistics, School of Science, Walailak University, Nakhon Si Thammarat 80160, Thailand

⁶Akkraratchakumari Veterinary College, Walailak University, Nakhon Si Thammarat 80160, Thailand

⁷Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhonpathom 73170, Thailand

(* Corresponding author's e-mail: dusit.lao@mahidol.ac.th)

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Abstract

Porcine circovirus type 2 (PCV2), which is a member of the Circovirus genus in the family of Circoviridae, is a small non-enveloped, closed-circular ssDNA. The PCV2-associated disease is one of the most important infectious agents on pig productivity worldwide, including China, India, Malaysia, and Thailand. It caused 2 major syndromes; postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). In addition, PCV2 may cause complexity with a pathogenic agent into porcine circovirus associated diseases (PCVADs). This study aimed to determine the prevalence of PCV2 in smallholder farms in Thung Yai district, Nakhon Si Thammarat, Thailand. A cross-sectional study was performed; 100 blood samples were collected from 13 smallholder pig farms. The samples were classified into 4 groups based on the pig's age; gilts, 6 sows, nursery-to-starter, and from growing to finishing pigs. Blood samples were carried out for DNA +extraction and nested-PCR. The epidemiological study showed 9 % positive by genetic detection.

The result suggested that growing-to-finishing pigs had significant PCV2 infection, followed by nursery-to-starter pigs and sow groups. In addition, multiple farms showed a high positive and significant correlation ($Cr \approx 0.245$). These results reveal a low prevalence of PCV2 in endemic regions in southern Thailand, which may help in the local control evaluation and eradication programs. Furthermore, the phylogenetic study of local strain should be investigated for the occurrence of PCV2 genetic evolution in Thailand and neighboring countries.

Keywords: Porcine circovirus type 2, PCV2, Nakhon Si Thammarat, PCR, ORF2, Capsid

[#]Equal contribution

Introduction

Circovirus is the smallest virus in Circovirus genus members in the family of Circoviridae, a small non-enveloped, closed-circular ssDNA genome of approximately 1.7 kb in length. The genomic material consists of 3 major open reading frames; (ORFs); ORF1 (Rep), ORF2 (Cap), and ORF3 (Protein associated with apoptosis) [1-4]. Circoviruses were mainly from avian species. Great members of the family Circoviridae have a wide host range in fish, insects, and mammals [5-8]. From the previous report, 2 types of circovirus were isolated from the domestic pig. Porcine circovirus 1 (PCV1) was first isolated in cell culture contamination and considered as non-pathogenic [9-11]. On the contrary, PCV2 has been associated with several clinical signs in domestic pigs and caused economic loss directly as Post Weaning Multisystemic Wasting Syndrome (PMWS) and Porcine Dermatitis and Nephropathy Syndrome (PDNS) [7,12,13].

In addition, PCV2-associated diseases have shown genetic diversity; the nucleotide sequence identity between the PCV1 and PCV2 is approximately 86 % in the replication gene and 66.4 % in the capsid gene [7,14,15]. The PCV2-associated disease is one of the most important infectious agents on pig productivity worldwide, including China, India, Malaysia, and Thailand. PCV2 leads to a morbidity range from 4 - 30 %, occasionally 50 - 60 %, and mortality ranges from 4 - 20 %. PMWS-associated PCV has a high economic impact on productivity, causing chronic wasting syndromes at 5 - 12 weeks of age with clinical signs, such as growth retardation, weight loss, dyspnea, and anemia jaundice, and lymph nodes enlargement. The pathological lesions have shown granulomatous interstitial pneumonia, lymphadenopathy, multifocal lymphoplasmacytic hepatitis, lymphohistiocytic interstitial nephritis, and pancreatitis. However, the virulent of PMWS-outbreak depend on the herd's immunity and vaccination programs [2,5,16-21]. In contrast, PDNS-associated PCV was first reported in 1990 and has been affected in grower and finishing pigs. The infected pig demonstrated the typical lesion on the skin as multifocal red-purple blotches/scabs and petechial hemorrhage with renal enlargement on gross. The microscopic lesion was marked the necrotizing vasculitis (skin, kidney, mesentery, and spleen), fibrinonecrotizing glomerulitis with nonpurulent interstitial nephritis, and lymphoid-depletion. In addition, PCV2 plays a significant role in complex disease. It is called Porcine Circovirus Associated Diseases (PCVADs) as it undergoes a process of co-infection with bacteria (e.g., *Haemophilus parasuis*, *Streptococcus suis*, *Mycoplasma hyopneumoniae*) and virus (e.g., Swine influenza virus, Porcine Reproductive and Respiratory Syndrome virus). This co-infection leads to high clinical signs and farm production problems [5,8,21-24].

A field study of PCV2 was performed in some countries of North America, Europe, and Asia. Mukherjee *et al.* [25] reported the prevalence of PCV2 in India's North-Eastern region, approximately 62.25 % in blood samples and 18.94 % in tissue via PCR detection. This suggested that the rural area without vaccination have a high prevalence with PCV2 positive, whereas the prevalence was decreased dramatically with vaccination. Moreover, PCV2a DNA was only detected in non-vaccinated pigs [25-28]. PCV2 was detected the viral shedding in boar semen showing 18.2 % but no difference with antibody detection. Borkenhagen study (2018) demonstrated PCV2 was detected in oral secretion proximately 87.8 % and 23.1 % in farm bioaerosol samples. These studies suggested the PCV2 can be a recurrence in the farm environment [30,31]. In Thailand, PCV2 has been reported since 1993; and PWMS in 1998 PCV2 has been spread widely in the Central and Eastern part of Thailand with high pig population density during 2009 - 2015. The results showed a high positive about 44.09 % (n = 306/694) from 80 % (56/70) in farms. However, Jantafong (2018) reported that the prevalence of PCV2 infection in Thailand's central region was approximately 10 %. Furthermore, PCV2 detection was not significantly different between blood and fecal sample; however, it was related to age-dependent clearly [2,3,28,30-33].

Although Nakhon Si Thammarat province has the highest number of farms in the southern part and the rank of 14th in production in Thailand, the problem becomes more complicated by limited information on PCV2 prevalence and suitable control strategies. This indicates that the investigation of PCV2 prevalence in the southern part is essential to optimize control for smallholder pig farms. Therefore, this project investigates the prevalence of PCV2 in a smallholder pig farm in Southern Thailand, which would

help understand the epidemiology and improve the effective biosecurity system in smallholders and farms in tropical zone conditions.

Materials and methods

Sample collection

A total of 100 blood samples with ethylenediamine-tetra-acetic (EDTA) via marginal ear veins of pigs for adult pigs and jugular vein for nursery pigs from 14 smallholder pig farms in Thung Yai district, Nakhon Si Thammarat Province, Thailand (**Figure 1**) was used in the study. The samples were divided into 4 groups based on the pigs' age, gilt, sow, nursery to starter, and grower to finisher. All blood samples were stored at 4 °C during transportation and -80 °C for subsequent analysis of DNA extraction and polymerase chain reaction (PCR) for PCV2-specific nucleic acids. This experimentation was performed in accordance with DR256126012010. The permission of animal care and use protocol was conducted under RMUTSV IAC-03-13-61.

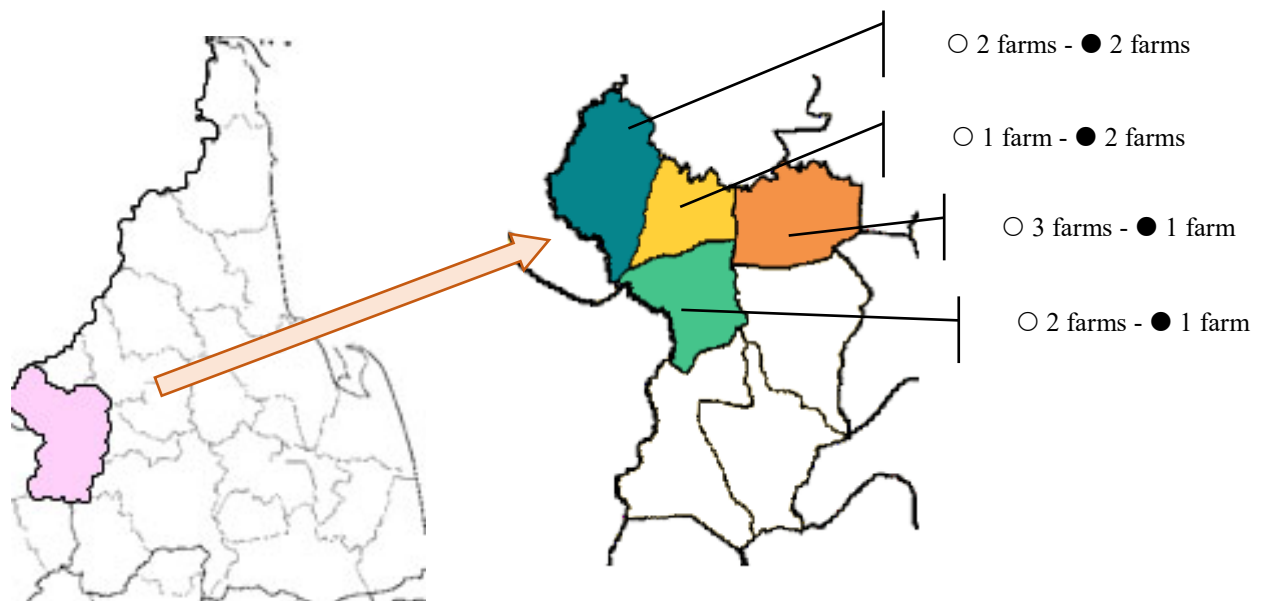


Figure 1 (A) Nakhon Si Thammarat province is in the white area, whereas Thung Yai district is in the shadow area. (B) Thung Yai district map (thick borderline). One hundred samples were collected from 14 farms in 4 sub-districts in Thung Yai district; 4 farms in Bang Rub, 3 farms in Thung Sang, 4 farms in Thung Yai, and 3 farms in Tha Yang sub-districts. All pig farms were divided by production type as 8 breeding farms (○) and 6 finishing farms (●).

DNA extraction and one-tube semi-nested PCR

DNA was extracted from 300 µL of blood using a commercial DNA isolation kit (Geneaid Biotech Ltd., Taiwan) by following the manufacturer's instructions. DNA samples were kept in -20 °C until used for PCR assays. One-tube semi-nested PCR was selected to amplify 226 bp of PCV2 ORF2 fragment by published primers [22,29]. The external primers are PCV-ORF2-290F (5'-TAG GTT AGG GCT GTG GCC TT-3') and PCV- ORF2-535R (5'- CCG CAC CTT CGG ATA TAC TG -3'). The inner primers are PCV-ORF2-290F and PCV-ORF2-516R (5'-ACT GTC AAR CGA ACC ACA GTC A-3'). The DNA sample was resuspended in 100 µL of DNA hydration buffer. One-tube semi-nested PCR was carried out in 25 µL of total reaction mixture that consisted of 1.25 U i-Taq DNA Polymerase (iNtRON

Biotechnology, Inc., Korea), 2.5 μ L of 10 \times PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 20 mM MgCl₂, and enhancer solution), 2.5 μ L of 10 mM each dNTP mix, 0.1 μ L of each primer, 5 μ L of template DNA, and nuclease-free water. One-tube semi-nested PCR was performed by standard thermal cycler (SimpliAmp®, Applied Biosystems) for 2 steps. First step, the conditions were initialized at 94 °C for 2 min, followed by 30 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, and final extension at 72 °C for 7 min. In the second step, the conditions were carried out as the first step at only 10 cycles, followed by changing annealing temperature to 60 °C for 25 cycles. The PCR products were electrophoresed in 2 % agarose gel and stained with GelRed® Nucleic Acid Gel Stain (Biotium, USA).

DNA sequences and genetic analysis

Three PCR-products with partial ORF2 were approximately 226 bp on 2 % agarose gel and purified with viral gene extraction. DNA elution was amplified and submitted to Macrogen sequencing with primers PCV-ORF2-290F, PCV-ORF2-535R, and PCV-ORF2-516R. Moreover, 8 ORF2 sequences were selected from Universal Protein Resource (UniProt) and GenBank® (NIH genetic sequence database) based on PCV2 subtypes. Then 3 partial PCR sequences and thirteen referred sequences of ORF2 were aligned using Clustal Omega and MEGA7 [30,31].

Statistical analysis

Data were analyzed by PASW Statistics for Windows, version 18 (SPSS Inc., Chicago). Data from each group were compared using variance analysis (ANOVA) with Least Significant Difference (LSD) to compare the incidence between groups. Statistical significance was considered at p -value ≤ 0.05 .

Results and discussion

PCV2 and its subtypes became the endemic disease in the swine industry around the world, including Thailand. PCV2 causes 2 syndromes and shows age-dependent susceptibility, namely Post-weaning Multisystemic Wasting Syndrome (PMWS) and Porcine Dermatitis and Nephropathy Syndrome (PDNS). Nowadays, the disease is named porcine circovirus disease (PCVD) or porcine circovirus associated disease (PCVAD) [32,33]. PCV2 comprises 4 subtypes based on ORF2 variation into PCV2a, PCV2b, PCV2c, and PCV2d, although the relation between clinicopathological features and PCV2 subtypes are still unclear. They should be better refined to study subtypes and clinical sign relation. Moreover, capsid protein plays an important role in the virus adhesion via heparan sulfate receptor on the target cell surface and antigenicity [34]. ORF2 was used in the viral detection for both parental and recombinant viruses by PCR detection and genetic determination [2,29].

One hundred blood samples from 14 farms in Thung Yai district, Nakhon Si Thammarat were collected and divided into 2 types based on pig production systems, farrowing-to-finishing, and nursery-to-finishing farms. β -actin gene was applied as an internal control to all DNA samples. Besides, the PCR product of ORF2 was approximately 226 bp in length on 2 % agarose gel electrophoresis (**Figure 2**). Nine out of 100 samples were positive to PCV2. No gilt was PCV2 positive; however, approximately 90 % of positive samples were finishing production, which composed of 67 % of growing-to-finishing pigs, 22 % of nursery-to-starter, and 11 % of sows (**Figure 3A**). The Pearson correlation shows the low level of correlation between the group of samples (replacement gilts, sows, nursery to starters, and growing to finishing pigs) and positive detection within $Cr \approx 0.245$. The growing to finishing pigs have the most positive result (67 % positive). Most growing to finishing pigs in smallholder farms in this study had no vaccination programs, which led to a high amount of positive results. For production type, the result showed slight correlation between breeding and finishing units ($Cr \approx 0.212$) (**Figure 3B**). The finishing farms rearing pigs from multiple sources are at greater risk than single-source pigs. This suggested that growing and finishing pigs should have a PCV2 vaccination. Moreover, the new groups of pigs should be vaccinated and evaluated for the immune status before entering the farm. However, it is not easy to do in smallholder farms. The most suitable methods are a pig-source selection from the negative farm and reliable sources [23,25,35].

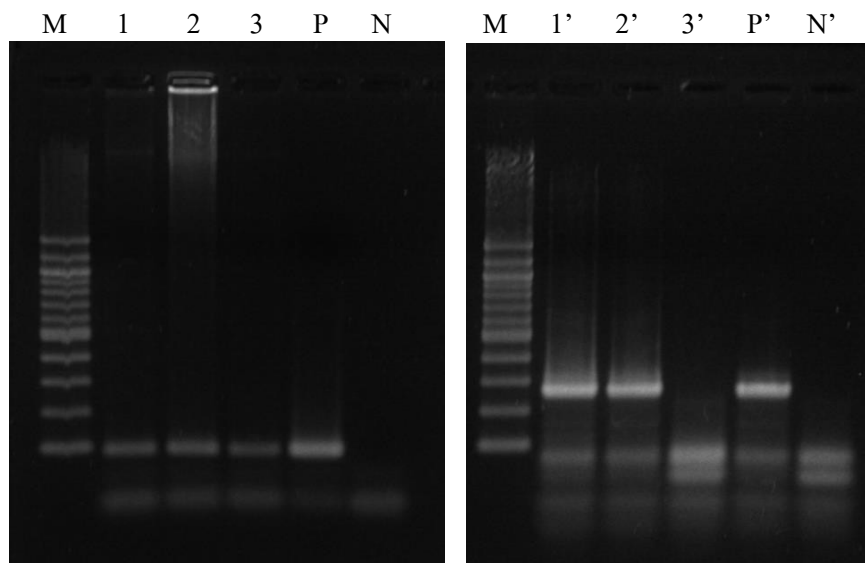


Figure 2 PCR products on 2 % Agarose gel electrophoresis. M: 100 bp DNA marker; Lane 1-3: β -actin detection around 94 bp in size; Lane 1'-3': PCV2 detection, and Lane P, N: positive and negative control for β -actin PCR, respectively; P', N': positive and negative control for ORF2 of PCV2 PCR.

PCV2 consists of 11 major genes but only 4 genes have been described, Replicase (ORF1), Capsid (ORF2), apoptosis associated PCV2 (ORF3), and caspase activity (ORF4) [36-40]. PCV2 has highly conserved nucleotides in ORF2. It has been classified on the basis of nucleotide sequencing evolution, which has 5 subtypes: PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e. Three positive samples were carried out for DNA sequencing and compared to 13 published sequences on UniProt® and GenBank® as nucleotide databases; PCV1: AY193712, AY184287; PCV2a: AF381177, AY699793; PCV2b: AY579893, DQ629117, KX831482; PCV2c: EU148504.1, EU148504; PCV2d: KX510085, MF169760; PCV3: KY418606, KX966193. The sequence alignment is shown in **Figure S1**. The 3 sequences were fully identical between sample group and over 95 % between published PCV2b. This was followed by PCV2c, PCV2a, PCV1, PCV2d, and PCV3 (**Table 1**). This suggests that these positive samples were related to other provinces of Thailand (THA_07NP88:Nakhon Pathom, THA_10SB01:Samut Prakan) and several countries, AY556477 (China, 2004), EU302140 (Indonesia, 2008), JX099786 (Vietnam, 2008), JF690923 (Malaysia, 2011), and KY985387 (Africa, 2017) [2,35,41,42].

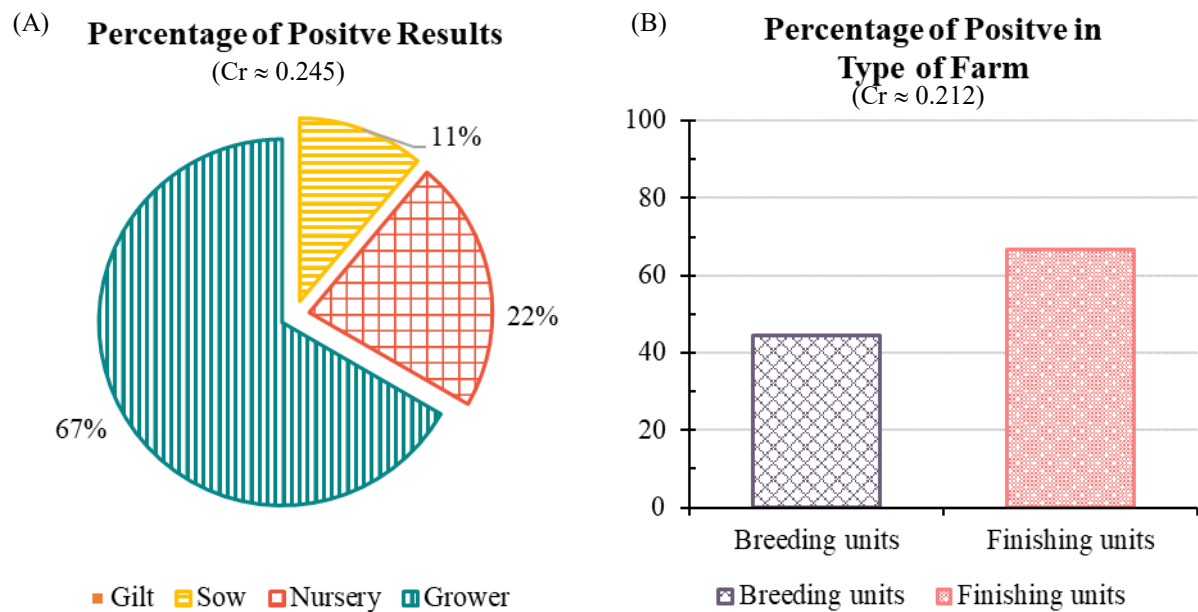


Figure 3 (A) The circular schematic diagram demonstrated the percentage of positive samples from blood samples (N=100). The growing to finishing group was labeled with green lines; nursery to starter (bright red), and sow (yellow). (B) The histogram showed the percentage of positive within production units. Both breeding units and finishing units had mild correlations value (Cr \approx 0.212) with a positive result (PCR detection) significantly at $p < 0.05$ using a Pearson correlation.

In addition, good management procedures of farms such as biosecurity systems and husbandry practices are the most important means. This study found that most of the finishing farms cannot perform the confined and acclimatized procedure, all in/all-out system, and effectively vaccination programs. This leads to retaining the PCV2 circulation in the farm environment and the effect of age dependent infection. The disinfectant selection is required to inactivate the viral load in the farm due to great disinfectant-resistance. For instance, potassium peroxymonosulfate (KHSO₅), sodium hypochlorite (NaOCl), sodium hydroxide (NaOH), quaternary ammonium, except the group of ethanol, chlorhexidine, and aldehydes are less active for PCV [43,44]. On the other hand, the breeding unit, both gilts, and sows have obtained intermittent vaccination programs due to the low level of PCV2 infection (11 % positive). The groups of nursery-to-starter and growing-to-finishing pigs are approximately 22 and 67 % positive, respectively. This conforms to the non-vaccination in nursery-to-finishing group. This suggests that both groups should be regularly vaccinated and monitored for viral detection sporadically. Furthermore, smallholder farmers should be educated on the effective biosecurity system and productive husbandry practices to improve pig production and reduce the risk of PCV2 infection.

Conclusions

This study examined the prevalence of PCV2 in pig smallholder farms in Thung Yai district, Nakhon Si Thammarat, Thailand. Findings suggest that the PCV2 is classified into PCV2 subtype B, similar to previous reports in Thailand and neighboring countries. Nevertheless, the complete PCV2 genome analysis and comparison of large holder farms (main pig source) and smallholder farms should be confirmed to practice the specific southern region's strategic control.

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Supplementary document

[illegible]

Figure S1 Nucleotides sequence alignment of partial ORF2 of 3 local sequences and 13 referred sequences from GenBank® (NIH genetic sequence database) of PCV2. The symbol (.) represents the fully conserved residue and (-) point to the gap between nucleotides.