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

Suphattra Jittimanee  Suparlark Nuntawan Na Ayudhya  Roongtham Kedkovid  Komkrich Teankum  Roongroje Thanawongnuwech*

Abstract

Porcine circovirus type 2 (PCV2) was first recognized as a causative agent of post weaning multisystemic syndrome or PMWS in Canada in 1991. Subsequently, it has been reported in almost all intensive pig production countries, and currently all associated diseases linked to PCV2 are included under the term porcine circovirus associated diseases or PCVAD. PCV2 can be divided into 2 major genotypes referred to genotype 1 (PCV2b) and genotype 2 (PCV2a). In Thailand, PMWS was first reported in 1998. However, only one isolate of Thai PCV2 from our group has been submitted to GenBank (AY864814). Therefore, the objective of this study was to determine the genetic characterizations of 12 ORF2 of PCV2 isolates from Thai pigs with PCVAD during 2007-2010. Twelve Thai PCV2 sequences were analyzed together with 19 representative ORF2 sequences. Interestingly, all 12 recent Thai PCV2 sequences belong to genotype 1 consisting of subgroup 1A/B (10/12, 83.33%) and 1C (2/12, 16.67%). In summary, based on genetic characterization and phylogenetic analysis, a few specific substitution patterns were observed in amino acid positions and genotype 1, subgroup 1A/B was predominated in Thai pigs with PCVAD in this study. More samples are needed to investigate the existence of genotype 2.

Keywords: genetic characterization, phylogenetic analysis, pigs, porcine circovirus type 2, Thailand

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บทคัดย่อ
ลักษณะทางพันธุกรรมและการวิเคราะห์วงศ์วานวิวัฒนาการของเชื้อเซอร์โคไวรัสชนิดที่สองที่แยกได้จากสุกรของประเทศไทยซึ่งป่วยด้วยกลุ่มอาการพีซีวีเอดีในระหว่างปี ค.ศ. 2007-2010

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Introduction
Porcine circovirus type 2 (PCV2) was first recognized as a causative agent of postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease in swine in Canada in 1991 (Harding and Clark, 1997). Subsequently, it has been reported in almost all intensive pig production countries worldwide (Allan and Ellis, 2000; Chae, 2005). PCV2 causes several clinical and pathological conditions in pigs including porcine respiratory disease complex (PRDC), reproductive failures, porcine dermatitis and nephropathy syndrome (PDNS), proliferative and necrotizing pneumonia and congenital tremor (Darwich et al., 2004; Chae, 2005). Currently, these associated diseases and conditions linked to PCV2 are called porcine circovirus associated diseases (PCVAD).

PCV2 belonging to the family Circoviridae, is a smallest mammalian, non-enveloped, single-stranded DNA virus enclosing a circular genome about 1.76 kb (Mankertz et al., 1997). The genome of PCV2 contains 3 major open reading frames (ORFs): ORF1, ORF2 and ORF3. The Cap protein is the main structural and major immunogenic of PCV2, which is encoded by ORF2. As a result, ORF2 is commonly used for reconstruction of the phylogenetic tree similar to the whole PCV2 genome study (Olvera et al., 2007).

Several studies suggested that PCV2 could be divided into 2 major genotypes (Carman et al., 2006; Cheung et al., 2007; Ma et al., 2007; Takahagi et al., 2008; Kim et al., 2009). Recently, both genotypes were proposed and referred to PCV2a (PCV2-genotype 2) and PCV2b (PCV2-genotype 1). However, PCV2c genotype has been described, but only found in Denmark (Segales et al., 2008). Interestingly, the virulence of PCV2a and PCV2b isolates was similar in the conventional SPF pig model, but the virulence of the isolates within the same cluster differed (Opriessnig et al., 2008). Alternatively, PCV2 can be classified into 8 subgroups 1A to 1C and 2A to 2E (Olvera et al., 2007), but those were not associated with the disease conditions or geographic areas. Recently, a new type of PCV referred to PCV1/2a was reported and later found to be a chimeric virus containing ORF1 of PCV1 and ORF2 of PCV2a in Canada in 2009 (Gagnon et al., 2010).

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

**Materials and Methods**

**Field samples:** Clinical samples (serum, tonsil or lymph nodes) from different farms in high pig density provinces of Thailand submitted to Chulalongkorn University-Veterinary Diagnostic Laboratory (CVDL) and Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University during 2007-2010 were included in this study. These samples were kept in -80°C until performing DNA extraction and PCR. Viral DNA was extracted from lymphoid tissue homogenates and serum samples using NucleoSpin Extract Viral DNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

**PCR amplification of ORF 2 gene:** A full-length ORF2 gene of PCV2 was amplified by PCR with forward primer, PCV2-f1 (5’-CCA TGC CCT GAA TTT CCA TA-3’) and reverse primer PCV2-r1 (5’-ACA GCG CAC TTC TTT CGT TT-3’) published by Takahagi et al. (2008), in a 50 µl reaction mixture. The amplification reaction was performed with an initial step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The PCV2 positive samples of 702 nt were used for DNA sequencing.

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

### Table 1 Classification of 12 Thai PCV2 isolates compared with 8 PCV2 reference strains in the same group by nucleotide and deduced amino-acid sequences of ORF2

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Nucleotide sequence (262-267)</th>
<th>Amino acid sequence (86-87)</th>
<th>Genotype</th>
<th>subgroup</th>
<th>Country/area</th>
<th>% NT identity²</th>
</tr>
</thead>
<tbody>
<tr>
<td>FJ905468</td>
<td>CCCCGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Korea</td>
<td>100</td>
</tr>
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<td>AY484409</td>
<td>CCCCGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Netherlands</td>
<td>100</td>
</tr>
<tr>
<td>THA_07NP88</td>
<td>CCCCGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Nakohn Pathom</td>
<td>98.9/98.6</td>
</tr>
<tr>
<td>THA_07NP144</td>
<td>CCCGGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Nakohn Pathom</td>
<td>99.6/99.6</td>
</tr>
<tr>
<td>THA_08NP113</td>
<td>CCCGGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Nakohn Pathom</td>
<td>99.8/99.8</td>
</tr>
<tr>
<td>THA_08NP266</td>
<td>CCCGGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Nakohn Pathom</td>
<td>99.8/99.8</td>
</tr>
<tr>
<td>THA_10SB01</td>
<td>CCCGGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Saraburi</td>
<td>99.6/99.6</td>
</tr>
<tr>
<td>THA_10SB02</td>
<td>CCCGGC</td>
<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Saraburi</td>
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</tr>
<tr>
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<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Saraburi</td>
<td>99.4/99.4</td>
</tr>
<tr>
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<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Saraburi</td>
<td>99.1/99.1</td>
</tr>
<tr>
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<td>PCV2b</td>
<td>1A/B</td>
<td>Nakohn Pathom</td>
<td>99.5/99.5</td>
</tr>
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<td>PR</td>
<td>PCV2b</td>
<td>1A/B</td>
<td>Ayudhya</td>
<td>99.3/99.3</td>
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<tr>
<td>AY864814 (01NP1)</td>
<td>CCCCTC</td>
<td>PL</td>
<td>PCV2b</td>
<td>1C</td>
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</tr>
<tr>
<td>THA_09NP290</td>
<td>CCCCTC</td>
<td>PL</td>
<td>PCV2b</td>
<td>1C</td>
<td>Nakohn Pathom</td>
<td>97.7/98.1</td>
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<td>PCV2a</td>
<td>2C</td>
<td>Hungary</td>
<td>100</td>
</tr>
<tr>
<td>AY322004</td>
<td>AAAATC</td>
<td>KI</td>
<td>PCV2a</td>
<td>2D</td>
<td>France</td>
<td>100</td>
</tr>
<tr>
<td>EU148503</td>
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<td>PL</td>
<td>PCV2c</td>
<td>-</td>
<td>Denmark</td>
<td>100</td>
</tr>
<tr>
<td>AY193712</td>
<td>ATCTTC</td>
<td>IF</td>
<td>PCV1</td>
<td>-</td>
<td>China</td>
<td>100</td>
</tr>
</tbody>
</table>

²Boldface letters are reference strains

Results

Genetic characterization: All 12 Thai ORF2 of PCV2 sequences in this study had a genome length of 702 nt and revealed nucleotide identities ranged between 97.7-99.8% (Table 1), indicating no significant differences between PCV2 genotype. However, the nucleotide substitutions in the ORF2 gene among the 12 Thai PCV2 sequences compare to the former Thai
PCV2 sequences in 2004 (THA_01NP1, AY864814) were observed. These substitutions led to amino acid changes located between amino acid positions 46-90, 121-131 and 190-191 (Fig. 1).

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

![Figure 1](image)

**Figure 1** Alignment of amino acids predicted from the ORF2 nucleotide sequences of the 12 Thai PCV2 sequences during 2007-2010 compared with the former Thai sequences in 2004 (01NP1) revealing amino acid changes located between amino acid positions 46-90, 121-131 and 190-191. Regions reported by Grau-Roma et al. (2008) as more heterogenic are highlighted in black lines.
Discussion

Porcine circovirus type 2 (PCV2) is divided into 2 major genotypes based on sequencing analysis. However, several nomenclature have been used such as group I and II, pattern 321 and 422, SG3 and SG1/SG2, group 1 and 2, and Group A and B (Olvera et al., 2007; Segales et al., 2008; Timmusk et al., 2008).

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

Previous studies revealed that both genotypes were associated with PCVAD-affected and non-affected herds (Cheung et al., 2007; Carman et al., 2008; Grau-Roma et al., 2008; Horlen et al., 2008; Wiederkehr et al., 2009). However PCV2b is currently prevailing in naturally occurring infections (Takahagi et al., 2008; Timmusk et al., 2008; Cortey et al., 2010). Although the virulence of PCV2a and PCV2b isolates was similar in the SPF pig model, the virulence of isolates within the same cluster was different (Opriessnig et al., 2008). On the other hand, many recent publications have reported a shift from PCV2a to PCV2b genotype that might be related to the occurrence of PMWS outbreaks in Canada (Gagnon et al., 2007), Sweden (Timmusk et al., 2008), Switzerland (Wiederkehr et al., 2009) and Spain (Cortey et al., 2010), indicating that PCV2b may be more virulent than PCV2a.

All 12 Thai PCV2 isolates in this study were classified within only one PCV2 genotype (genotype 1: 1A and 1B) similar to several countries such as Cuba (genotype 1: 1A) and Indonesia (genotype 1: 1C). Interestingly 10 Thai PCV2 sequences were located within subgroup A/B (10/12, 83.33%) together with the other PCV2 sequences from Korea (FJ905468), the Netherlands (AY484409) and USA (EU340258). These PCV2 isolates subgroup A/B were found in PCVAD pigs from Nakhon Pathom (2007, 2008 and 2010), Saraburi (2010) and Ayudhya province (2010). The other 2 Thai PCV2 sequences (THA_09NP165 and THA_09NP290) from Nakhon Pathom province were classified within subgroup 1C (2/12, 16.67%) similar to the previous Thai PCV2 isolate (THA_01NP1, AY864814), a Chinese isolate (AY035820) and 2 Indonesian isolates (EU340258) together with the other PCV2 sequences from Korea (CU cluster Emerging H-15-75-53). These PCV2 isolates subgroup A/B were found in Thailand. A few specific substitution patterns in each subgroup were observed in amino acid positions. This report revealed that PCV2 genotype 1 was predominated in Thai pigs with PCVAD. This finding provides some useful information about PCV2molecular epidemiology in Thailand with at least 2 introductions into the country.

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