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<Short Communication>

Genetic variations in open reading frame 2 gene of porcine circovirus type 2 isolated in Korea during 2016-2017

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Abstract: The capsid protein of porcine circovirus type 2 (PCV2) encoded by open reading frame 2 (ORF2) is important for neutralizing activity against PCV2 infection. This study investigated the heterogeneity of the ORF2 gene of PCV2 isolated in Korea during 2016–2017. The results revealed that PCV2d is currently the predominant genotype. Moreover, comparison of ORF2 from 17 PCV2 isolates revealed 88.3–100% homology at the nucleotide (deduced amino acid 86.3–100%) level. Interestingly, 61.5% (8/13) of the PCV2d isolates had glycine at position 210. These data provide a useful information for PCV2 epidemiology in Korea.

Keywords: capsid proteins, genetic variation, phylogenetic analysis, porcine circovirus type 2

Porcine circovirus type 2 (PCV2) is a small, nonenveloped, circular single-stranded DNA virus belonging to the genus *Circovirus* of the family *Circoviridae* [16]. PCV1 is nonpathogenic but pathogenic PCV2 is the primary etiological agent of PCV2-associated diseases (PCVAD) that include post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome, which both cause major economic losses in the swine industry worldwide. PCV3 was recently reported as a novel porcine circovirus and its pathogenesis is still unknown [6].

Table 1. Porcine circovirus type 2 (PCV2) isolates analyzed in this study

PCV2 strain	Geographic origin	Year of the isolation	Genome size (nt)	ORF2 size (nt)	Genotype	GenBank accession number
HID5697	Nonsan	2016	1768	702	PCV2b	KY810319
HID5701	Pocheon	2016	1767	705	PCV2d	KY810320
HID5703	Sangju	2016	1768	702	PCV2a	KY810321
HID5705	Yeoju	2016	1768	702	PCV2a	KY810322
HID5707	Sejong	2016	1767	705	PCV2d	KY810323
HID5709	Sejong	2016	1767	705	PCV2d	KY810324
HID5715	Jeju	2016	1767	705	PCV2d	KY810325
HID5733	Cheongyang	2016	NA	705	PCV2d	MG715490
HID5734	Icheon	2016	NA	702	PCV2b	MG715491
HID5735	Jeju	2016	NA	705	PCV2d	MG715492
HID5736	Nonsan	2017	NA	705	PCV2d	MG715493
HID5737	Nonsan	2017	NA	705	PCV2d	MG715494
HID5738	Nonsan	2017	NA	705	PCV2d	MG715495
HID5739	Nonsan	2017	NA	705	PCV2d	MG715496
HID5740	Nonsan	2017	NA	705	PCV2d	MG715497
HID5741	Nonsan	2017	NA	705	PCV2d	MG715498
HID5742	Nonsan	2017	NA	705	PCV2d	MG715499

ORF, open reading frame; nt, nucleotide; NA, not available.

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PCV2 has a genome of 1766–1768 nucleotides in length that contains at least four major open reading frames (ORFs). The largest ORF1 encodes proteins (Rep and Rep') essential for viral replication in host cells. ORF2 encodes a structural capsid (Cap) protein, which is the immunodominant region inducing neutralizing antibody in hosts. ORF3 and ORF4 encode nonstructural proteins associated with apoptotic and anti-apoptotic activity in PCV2-infected cells, respectively.

PCV2 can be classified into five genotypes (PCV2a, b, c, d, and e) based on the ORF2 sequence. The first identified PCV2a was the most prevalent genotype in pigs from 1996 to the early 2000s [1]. Since 2005, PCV2b has been reported to be the dominant genotype associated with PCVAD in pig-producing countries [12]. The newly emerging PCV2d (formerly mutant PCV2b) has been recognized in the USA, Korea, China, Germany, and Brazil [13]. In Korea, PCV2 was first identified in pigs showing PMWS [2]. PCV2a circulated in Korean pig farms until a genotype shift occurred from PCV2a to PCV2b in 2002 [7]. PCV2d is currently the predominant genotype although major PCV2 genotypes (PCV2a and PCV2b) are still being detected in the Korean pig population [8].

PCV2 Cap protein is the major immunogenic protein that induces protective immunity against PCV2 infection and plays an important role in binding to a receptor, heparan sulfate [10]. The ORF2 gene of PCV2a has a high level of nucleotide similarity with that of PCV2b, but is significantly different from that of PCV2d, which spread rapidly worldwide [11]. To allow molecular diagnosis and vaccine development, further studies are required to understand the genetic variation of the ORF2 gene associated with production of neutralizing antibodies against PCV2 genotypes. In this study, we sequenced on either full-length or ORF2 gene of PCV2 isolates in Korea from 2016 to 2017, and investigated the heterogeneity in the ORF2 gene of the PCV2 isolates.

A total of 244 blood and saliva samples were randomly selected from 23 pig farms in Korea from 2016 to 2017. The geographic locations of the PCV2 isolates used in this study are summarized in Table 1. All samples were centrifuged for 10 min and the supernatants were collected and stored at −80ºC until further use. Viral DNA was extracted from 300 μL of samples using the Viral Gene-spin DNA/RNA Extraction Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. PCR was performed in total volume 20 μL reaction mixture containing 1 μL of viral DNA, 1 μL of 10 pM of each of two primers (ORF1: PCV2-F1, 5'-ACCAGCGCACTTCGGCAG-3'; PCV2-R976, 5'-GGAAATTCAGGGCATGGGGG-3' and ORF2: PCV2-F867, 5'-GCTCTCTATCGGAGGATTAC-3'; PCV2-R1768, 5'-AATACTTACAGCGCACTTCTTTCG-3'), TOPsimple DyeMIX-Tenuto (Enzynomics, Korea), and dH_2O. The PCR products were ligated into RBC TA Cloning Vector (RBC Bioscience, Taiwan) and transformed into competent *E. coli* DH5α cells. The correct clones having the cloned plasmid DNA were selected and the nucleotide sequences were determined with M13 forward and reverse primers (Cosmo Genetech, Korea).

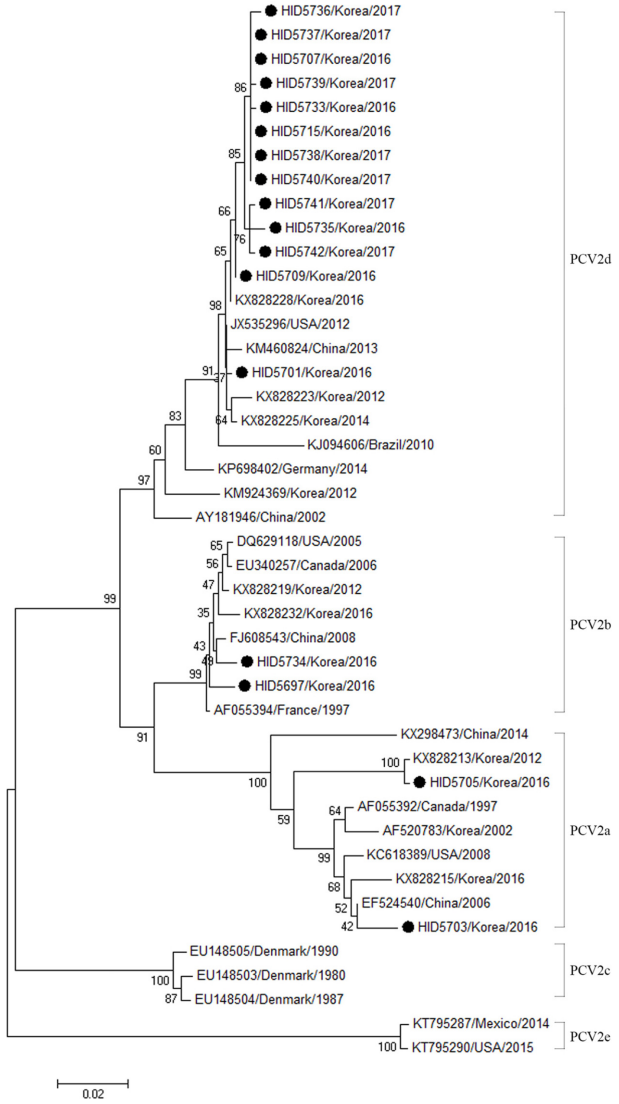

Fig. 1. Phylogenetic analysis of the Korean PCV2 isolates based on the nucleotide sequences of the ORF2 gene. Multiple-sequencing alignment was performed using ClustalW [9], and the phylogenetic tree was obtained using the neighbor-joining method in the MEGA 6.0 software [15]. The numbers at each branch represent bootstrap values greater than 50% of 1,000 replicates. The scale bar indicates 0.02 nucleotide substitutions per site.

For phylogenetic analysis, the ORF2 genes of Korean PCV2 isolates were compared with 27 reference PCV2 sequences deposited in GenBank (National Center for Biotechnology Information, USA). A multiple sequence alignment was generated using the ClustalW program [9]. Phylogenetic tree was constructed by the neighbor-joining method and bootstrap analysis with 1,000 replicates using MEGA 6.0 software [15].

The full-length genomes ($n = 7$) or ORF2 genes ($n = 10$) of PCV2-positive samples from 8 pig farms were successfully sequenced. The ORF2 nucleotide sequences from the PCV2

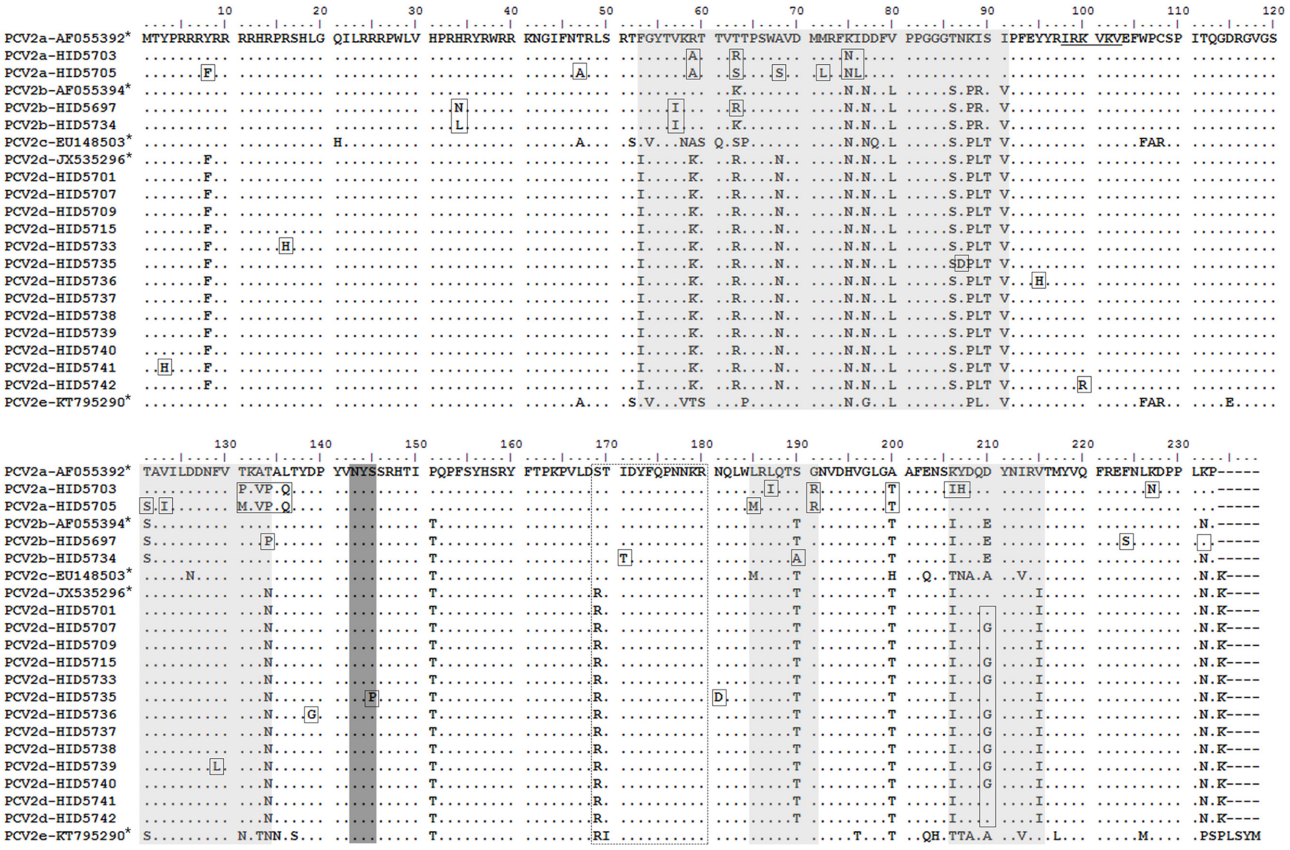

Fig. 2. Alignment of deduced amino acid sequences based on the ORF2 gene. Asterisks show the representative PCV2a, PCV2b, PCV2c, PCV2d and PCV2e genotypes. The open boxes indicate amino acid variations between Korean PCV2 isolates and representative PCV2 genotypes. Potential hypervariable domains are shown in light grey areas, and a dark grey area indicates potential sites of *N*-glycosylation [3]. The dashed line box represents the immunodominant decoy epitope [18]. The underline corresponds to the putative heparan sulfate-binding receptor domain described in Misinzo *et al*. [10].

isolates consisted of 702 or 705 bp. The ORF2 genes of the PCV2d isolates were three nucleotides (705 bp) longer than that of the PCV2a and PCV2b isolates. The degree of identity of ORF2 from the 17 PCV2 isolates ranged from 88.3% to 100% (86.3% to 100%) at the nucleotide (deduced amino acid) level. Among the 17 isolates, 2 (11.8%), 2 (11.8%), and 13 (76.4%) were determined as PCV2a, PCV2b, and PCV2d, respectively, based upon the DNA sequencing of ORF2 (Fig. 1). This results indicate that PCV2d is currently spreading rapidly in the Korean swine populations, which support that the genotype shift from PCV2a and 2b to PCV2d occurred on a nationwide scale reported by [8].

The similarity analysis of the 17 PCV2 isolates and representative PCV2 genotypes showed that the ORF1 gene was conserved, while the ORF2 gene was highly variable (data not shown). By multiple sequence alignment of deduced amino acid sequences, our results indicated that the ORF2 gene exhibited at least four major heterogenic regions at residues 53–91, 121–134, 185–191, and 206–215 (Fig. 2). Especially, these heterogenic regions were overlapped with the antibody recognition domains (residues 51–84, 113–132, 161–208, and 228–233) described by [17]. These results may reflect an effective strategy of PCV2 to escape from host immune responses.

There is considerable interest in the neutralizing epitope of the Cap protein that is a potential target for vaccine design. Recently, selection pressures of Korean PCV2 were identified in Cap protein (position 30, 59, 80, 131, 133, 190, and 232) at a significant level [8]. The alanine at amino acid position 59 of the PCV2a Cap protein is critical for neutralizing activity [4]. However, our results showed that PCV2b and PCV2d had single amino acid changes at position 59 to arginine and lysine, respectively. Also, it has also been reported that conformational epitopes (Cap 231–233) could be detected by neutralizing monoclonal antibodies to PCV2 [14]. In this study, PCV2a had amino acid residues L–K–P at positions 231–233, while the sequence is L–N/K–P in PCV2b strains. PCV2c and PCV2d had amino acid sequence L–N–P–K because of the addition of one amino acid (lysine, K) at position 234. In addition, compared with the representative PCV2 genotypes, amino acid substitutions were observed in the PCV2a isolates (Y8F, T47A, R59A, T63R/S, A68S, M72L, K75N, I76L, T121S, V123I, T131P/M, A133V, T134P, L136Q, L185M, L187I, G191R, A200T, K206I, Y207H and K227N),

PCV2b isolates (H34N/L, V57I, K63R, T134P, I171T, T190A, F224S and N232K), and PCV2d isolates (Y3H, R16H, N87D, Y95H, K100R, F129L, D139G, S145P and N181D). Interestingly, 61.5% (8/13) of PCV2d isolates had glycine at position 210, which has not been previously reported. The results of this study indicate that genetic evolution of Cap protein may allow virus to escape neutralizing antibodies induced by commercial vaccines based on PCV2a in pigs.

An immunological decoy exists in hypervariable regions such as glycoprotein 120 of human immunodeficiency virus and glycoprotein 5 of porcine reproductive and respiratory syndrome virus [19]. However, the PCV2 decoy epitope, 169-STIDYFQPNNKR-180, is highly conserved among almost all PCV2 [18]. Vaccination with Cap (169–180) monomer elicited strong antibody response to the decoy epitope, however, only a low level of virus-neutralizing activity was detected, resulting in viremia in response to PCV2 challenge similar to that in non-immunized pigs [19]. Also, the commercial PCV2 vaccine containing lower decoy epitope induced a significantly higher level of neutralizing antibody after immunization in guinea pigs [5]. In this study, we found a single amino acid substitution in the decoy epitope of a PCV2b isolate, isoleucine (I) to threonine (T) at position 171. This result may enhance antibody production to divert the host humoral immune response away from neutralizing activity against PCV2 infection.

In summary, our study revealed that major PCV2 genotypes (PCV2a, PCV2b, and PCV2d) co-circulate and that PCV2d is currently the predominant genotype in the Korean pig population. Furthermore, all PCV2 isolates are continually changing their Cap protein including multiple antibody recognition domains. Therefore, these data provide useful information for improving the epitope-based vaccine and molecular diagnosis against current circulating PCV2 genotype.

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