NOTE *Virology*

Genetic Diversity of Porcine Circovirus Type 2 in Korean Pigs with Postweaning Multisystemic Wasting Syndrome during 2005–2007

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ABSTRACT. The comparison of nucleotide and deduced amino acid sequence was conducted with 9 porcine circovirus type 2 (PCV2) strains isolated from PCV2-infected pigs with postweaning multisystemic wasting syndrome (PMWS) and 50 tissue samples obtained from PCV2-infected 50 pigs with PMWS during 2005–2007. At amino acid positions 88–89 of the ORF2 gene, 50 Korean PCV2 had amino acids PR/L consistent with group 1 PCV2, whereas 9 Korean PCV2 contained amino acids KI, characteristic of group 2 PCV2. Phylogenetically, 47, 3, 2 and 7 Korean PCV2 belonged to subgroups 1A/B (79.7%), 1C (5.1%), 2D (3.4%) and 2E (11.9%), respectively. Although the predominant Korean PCV2 was subgroup 1A/B, subgroups 1C and 2E were still circulating and subgroup 2D PCV2 were found to be newly emerged in Korea.

KEY WORDS: Korean pig, porcine circovirus type 2, prevalence.

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Porcine circovirus (PCV) belongs to the genus Circovirus of the Circoviridae family and is 17 nm in diameter, icosahedral and nonenveloped DNA virus [8, 19, 20, 28]. PCV has ambisense, single-stranded, closed circular genome that encodes proteins by the encapsidated viral DNA, and by the complementary DNA of the replicative intermediate synthesized in the host [9, 17, 27].

Two types of PCV have been identified as PCV type 1 (PCV1) and type 2 (PCV2). PCV1 viruses are naturally nonpathogenic and do not cause any pathological lesions in pigs [5, 29]. In contrast, PCV2 is accepted as the main causative agent involved in postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex, and reproductive disorders [2, 12, 14, 21, 22, 25, 26, 31]. Recently, the term "porcine circovirus diseases" has been proposed for the group of diseases and conditions linked to PCV2 [1].

Since PMWS was initially described in Western Canada, incidences of PMWS and PCV2 infection have been reported in many countries of Europe, America and Asia, and PMWS is nowadays considered a very significant disease in swine industry [3, 4, 7, 13, 18, 24, 30]. In Korea, first PCV2 infection was diagnosed in weaned pigs with PMWS by immunohistochemistry and PCR [10]. To date, PMWS is one of the most important diseases and causes considerable economic losses in pig producing industry in Korea [15]. A recent study reported a shift in PCV2 from group 2 to group 1 and the disappearance of the subgroups 1C and 2E in Korean pigs between 1999–2006 [6]. However, the precise current status of PCV2 infections is unclear because the previous study used only 16 PCV2-positive samples of pigs with PMWS during 2005–2006 [6]. Therefore, this study examined the groups of PCV2 recently circulating in Korean pigs with PMWS.

Tissue samples including the lung, lymph node, liver, spleen, and kidney were collected from 59 pigs with a clinical history of typical PMWS (wasting, unthriftiness, respiratory distress, jaundice and diarrhea) in 57 farms located in different geographic regions in Korea between 2005 and 2007 (Table 1). These samples were stored at –80°C after the necropsy until needed.

The total DNA was extracted from tissue samples or infected PK15 cells by the AccuPrep Genomic DNA Extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. The total DNA recovered was suspended in 100 μl of DNase free water and stored at –80°C until needed.

The PCR primers used to detect the PCV2 which were designed based on the ORF2 gene of reported PCV2 sequences (GenBank accession no. AY321990, AY181947, AY556474, AF109398, AY256459, AF264043 and AF544024) were as follows: Cap1, 5'-ATGACGTATCCAAGGAGGCG-3' (1735–1716); Cap2, 5'-GGGTTTAAGTGGGGGGTCT-3' (1037–1055). In order to sequence the entire genome [13], total four primer pairs were used as follows: CV1, 5'- AGGGCTGTGGCCTTTGTTAC-3' (1329–1348); CV2, 5'- TCTTCCAATCACGCTTCTGC-3' (549–530); CV3, 5'- TGGTGA CCGTTGCAGAGCAG-3' (445–464); CV4, 5'- TGGGCGGTGGACATGATGAG-3'(1537–1518); CV1–2, 5' AGGA CGAACACCTCACCTCCAG-3' (206–226); CV2–2, 5'-ACGTATCCAAGGAGGCGTTACC-3' (1732–1711); CV3–2, 5'- TTGTACATACATGGTTACACGG-3' (1076–1097); CV4–2, 5'- TGGTAATCAGAATACTGCGGGC-3'

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Table 1. Information of the geographic location, farm, age of pigs and outbreak year of Korean PCV2

Geographic location	Virus name[a)]	Accession no.	No. of Farm	Age (weeks)	Year	Geographic location	Virus name[a)]	Accession no.	No. of Farm	Age (weeks)	Year
Chungju, Chungbuk	C300	EU450595	1	8	2005	Damyang, Jeonnam	K48	EU450592	29	7	2006
Chungju, Chungbuk	C309	EU450596	2	7	2005	Damyang, Jeonnam	C51	EU450603	30	11	2006
Jincheon, Chungbuk	K398	EU450589	3	7	2005	Hampyeong, Jeonnam	C62	EU450608	31	8	2006
Boryeong, Chungnam	C374	EU450597	4	8	2005	Hampyeong, Jeonnam	C105	EU450612	32	5	2006
Cheongyang, Chungnam	C180	EU450622	5	10	2006	Hampyeong, Jeonnam	C141	EU450616	33	4	2006
Hongseong, Chungnam	C403	EU450598	6	7	2005	Hampyeong, Jeonnam	C206	EU450624	34	11	2006
Nonsan, Chungnam	K368–2	EU450585	7	7	2005	Hwasun, Jeonnam	C59	EU450605	35	8	2006
Nonsan, Chungnam	K368–3	EU450586	7	7	2005	Hwasun, Jeonnam	C146	EU450618	36	8	2006
Nonsan, Chungnam	K378–1	EU450587	8	6	2005	Hwasun, Jeonnam	C272	EU450632	37	9	2006
Nonsan, Chungnam	K378–2	EU450588	8	6	2005	Jangseong, Jeonnam	C167–4	EU450620	38	7	2006
Nonsan, Chungnam	C60	EU450606	9	10	2006	Muan, Jeonnam	C236	EU450628	39	9	2006
Nonsan, Chungnam	C103	EU450611	10	6	2006	Muan, Jeonnam	C276	EU450634	40	10	2006
Anseong, Gyeonggi	C80	EU450594	11	8	2005	Muan, Jeonnam	C277	EU450635	41	8	2006
Buan, Jeonbuk	C409	EU450599	12	4	2005	Muan, Jeonnam	C281	EU450636	42	11	2006
Gimje, Jeonbuk	C412	EU450600	13	3	2005	Muan, Jeonnam	C40	EU450642	43	14	2007
Gimje, Jeonbuk	K13	EU450590	14	6	2006	Naju, Jeonnam	K349	EU450584	44	8	2005
Gimje, Jeonbuk	C54	EU450604	15	10	2006	Naju, Jeonnam	C167–9	EU450621	45	5	2006
Gimje, Jeonbuk	C185	EU450623	16	10	2006	Sinan, Jeonnam	C144	EU450617	46	9	2006
Gimje, Jeonbuk	C241	EU450629	17	8	2006	Suncheon, Jeonnam	C61	EU450607	47	8	2006
Gochang, Jeonbuk	C73	EU450593	18	4	2005	Suncheon, Jeonnam	C207	EU450625	48	12	2006
Iksan, Jeonbuk	C125	EU450614	19	7	2006	Suncheon, Jeonnam	C234	EU450627	49	11	2006
Iksan, Jeonbuk	C155	EU450619	20	7	2006	Suncheon, Jeonnam	C24	EU450639	50	14	2007
Jeongeup, Jeonbuk	C413	EU450601	21	6	2005	Yeongam, Jeonnam	C640	EU450602	51	11	2006
Jeongeup, Jeonbuk	C255	EU450630	22	8	2006	Yeongam, Jeonnam	C94	EU450609	52	5	2006
Jeongeup, Jeonbuk	C268	EU450631	23	9	2006	Yeongam, Jeonnam	C101	EU450610	53	6	2006
Jeongeup, Jeonbuk	C275	EU450633	24	6	2006	Yeongam, Jeonnam	C224	EU450626	54	5	2006
Wanju, Jeonbuk	C111	EU450613	25	7	2006	Yeongam, Jeonnam	C6	EU450638	55	6	2007
Wanju, Jeonbuk	C128	EU450615	26	8	2006	Yeongam, Jeonnam	C25	EU450640	56	8	2007
Boseong, Jeonnam	K46	EU450591	27	10	2006	Seogwipo, Jeju	C282	EU450637	57	5	2006
Boseong, Jeonnam	C34	EU450641	28	9	2007						

a) The first letter of virus name, K and C, represents 9 Korean strains and 50 ORF2 gene sequences amplified directly from the PCV2-infected pigs, respectively.

(813–792). In addition, a pair of primers [SeqORF2F (5'-TTTTATCACTTCGTAATGGT-3', 1001–1020) and Seq ORF2R (5'-CGCACTTCTTTCGTTTTCAG-3', 1757–1738)] was also designed for cloning and sequencing of the ORF2 gene fragments.

The PCR was performed in 50 μl of a reaction mixture containing a final concentration of 1X Green GoTaq Reaction Buffer (Promege, U.S.A.), 200 μM dNTPs, 1 uM each primer, and 1.25 U of GoTaq^{TM} DNA polymerase (Promega, U.S.A.) with 5 μl of extracted DNA. The mixture was preheated for 5 min at 94°C, and subjected to 30 cycles of 1 min at 94°C, 1 min at the required temperature for each primer pair, 2 min at 72°C and a final 7 min incubation at 72°C. The amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by UV irradiation of ethidium bromide-stained samples.

Using the PCV1-free PK15 cells, PCV2 was isolated from PCV2-positive tissue samples showing a strong positive reaction for PCV2 by PCR [29]. The PCR products were purified using a QIAEX II gel extraction kit (QIAGEN Inc., U.S.A.) according to the manufacturer's instructions, and cloned into the pCR4-TOPO vector using a TA cloning kit, Version O (Invitrogen, U.S.A.). The constructs were transformed into TOP10 competent cells (Invitrogen, U.S.A.). The plasmids containing each PCR product were purified using a QIAGEN Plasmid mini kit (QIAGEN Inc., U.S.A.) according to the manufacturer's instructions. The DNA was sequenced using an automated DNA sequencer (ABI System 3700; Applied Biosystems, Inc., U.S.A.).

The nucleotide sequences of the whole genome of 9 Korean PCV2 strains and 59 ORF2 gene fragments (9 ORF2 fragments from 9 Korean PCV2 strains plus 50 ORF2 gene fragments directly amplified from 50 PCV2-infected pigs) were compared with the full genomes of 307 PCV2 and ORF2 gene sequences from the 433 PCV2 reported in GenBank until December 4, 2007 (data not shown) with each PCV1 (GenBank accession no. NC006266) and PCV2 group 3 ORF2 sequence (GenBank accession no. EU148503, EU148504 and EU148505) [11] using the DNA Basic module (DNAsis MAX; MiraiBio, Alameda, CA). Bootstrap test (1000 replicates) of phylogenetic analysis based on the nucleotide alignments without the primer sequences were constructed employing the Neighbor-Joining method and Kimura 2-parameter using the Molecular

Evolutionary Genetics Analysis (MEGA, version 3.1) [16]. Sequence homology analysis was carried out for the whole genome and ORF2 nucleotide and amino acid sequences of PCV2 using the LALIGN Query program of the GENESTREAM network server at the Institut de Génétque Humaine, Montpellier, France (http://www.eng.uiowa.edu/~tscheetz/sequence-analysis/examples/LALIGN/lalignguess.html).

From phylogenetic analysis of full-length genome without the primer sequences between the 9 Korean strains and other known viruses, seven Korean strains clustered with subgroup 1A, whereas the remaining 2 strains clustered with subgroups 1B and 2E PCV2 (data not shown). Phylogenetic analysis of the ORF2 gene without the primer sequences were conducted with 9 PCV2 strains isolated from PCV2-infected pigs with PMWS and 50 tissue samples obtained from PCV2-infected 50 pigs with PMWS. This analysis divided 59 Korean PCV2 into the following subgroups; 1A/B PCV2 (47/59, 79.7%), 1C PCV2 (3/59, 5.1%), 2D PCV2 (2/59, 3.4%) and 2E PCV2 (7/59, 11.9%), respectively. This indicates that the predominant Korean PCV2 is group 1 (84.8%), particularly subgroup 1A/B (Fig. 1). The comparison of nucleotide and amino acid sequences of the ORF2 gene between the Korean and other PCV2, which are known to be hypervariable and to be suitable phylogenetic marker [7, 13, 18, 19], supported immediately above results (data not shown). Our results are consistent with those reported in the literature [6, 11, 23], in which group 1 PCV2 have become the dominant strains particularly subgroup 1A/B. In addition, there has been a major shift from group 2 PCV2 to group 1 PCV2 in Korea and other countries. In contrast to the recent report on the epidemiology of PCV2 infection in Korean pigs from 2005 and 2006 [6], subgroups 1C and 2E PCV2 were also detected during 2005–2006 albeit in a small number of cases. This indicates that subgroups 1C and 2E PCV2 are still circulating in Korea. The most plausible explanation why subgroups 1C and 2E of PCV2 were not detected in previous study is that they used only 16 PCV2-positive samples during 2005–2006 [6], or that 1C and 2E PCV2 has reemerged from pigs imported from countries where these viruses circulate.

Since the subgroups of PCV2 circulating in Korean pigs have been reported to be 1A/B, 1C and 2E [6], the above result suggests that subgroup 2D PCV2 has newly emerged in Korea (Fig. 1). The occurrence of subgroup 2D PCV2 has been reported in the countries including the Canada, U.S.A., Germany, Austria, France, Hungary, Sweden, Denmark, Brazil, China and Taiwan. Therefore, it is likely that subgroup 2D PCV2 appeared in Korea from imported live pigs, mainly from the U.S.A., Canada and France, where subgroup 2D PCV2 occurs. This means that subgroup 2D PCV2 in imported pigs might be spread to Korean pigs.

It was reported that PCV2 can be divided into two groups based on the ORF2 sequences at nucleotides 1486–1472 of group 1 PCV2 and 1487–1473 of group 2 PCV2 and amino acids 86–91 [7], and can be further subdivided into 1A/B (PR), 1C (PL) and group 2 (KI) based on the amino acid

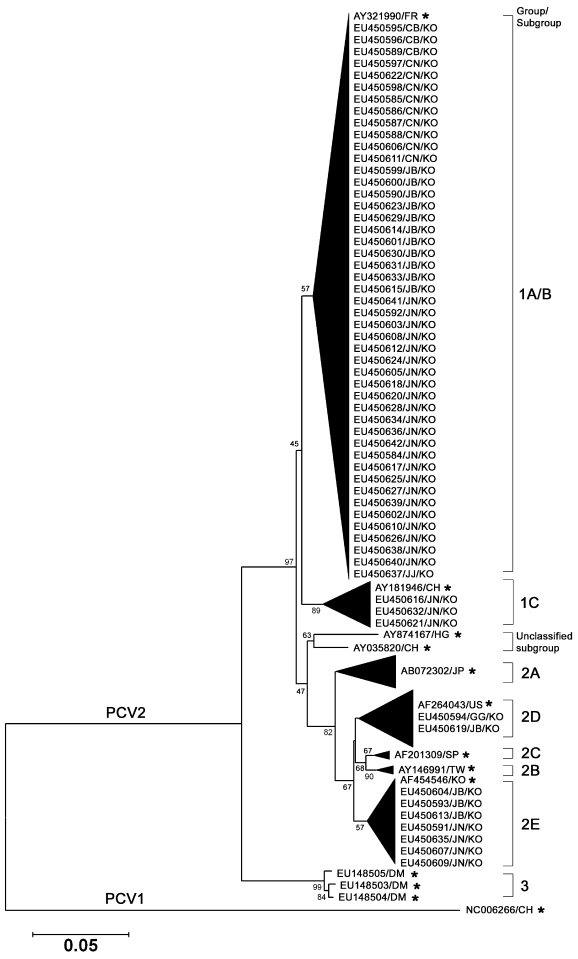

Fig. 1. Compressed phylogenetic tree based on the ORF2 gene sequences of 433 PCV2 reported in GenBank and 59 ORF2 gene sequences of Korean PCV2 from this study with each PCV1 (GenBank accession no. NC006266) and PCV2 group 3 (GenBank accession no. EU148503, EU148504 and EU148505) strain. Accession number and geographic origin of representative reference strains are indicated by asterisk (FR, France; CH, China; HG, Hungary; JP, Japan; US, U.S.A.; SP, Spain; TW, Taiwan; KO, Korea; DM, Denmark). The name of Korean strains belonging to each subgroup is listed in Table 1 (CB, Chungbuk; CN, Chungnam; GG, Gyeonggi; JB, Jeonbuk; JN, Jeonnam; JJ, Jeju).

positions 88–89 of ORF2 [6]. In this study, at amino acid positions 88–89 of ORF2 gene, 50 Korean PCV2 were found to belong phylogenetically to group 1 PCV2 with amino acids PR and PL, which is consistent with that of subgroups 1A/B and 1C PCV2. On the other hand, the remaining 9 Korean PCV2 were classified into group 2 PCV2 containing the amino acids, KI, which is characteristic of group 2 PCV2. These results also show that group 1 PCV2 is most common but there are sporadic occurrences of group 2 PCV2 in Korean pigs.

In conclusion, the most common PCV2 circulating in

Korean pigs with PMWS during 2005–2007 is group 1, particularly subgroup 1A/B. However, subgroups 1C and 2E are still circulating, and subgroup 2D PCV2 has newly emerged in Korea albeit in a small number of cases. These results will provide important epidemiological data for the control and establishment of a surveillance system for PMWS in Korea.

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