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Genetic characterization of porcine circovirus-2 field isolates from PMWS pigs

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ABSTRACT

PMWS is a new emerging disease in swine herds worldwide. Field isolates of PCV-2, a putative major causative agent of PMWS, were isolated and genetically characterized. Viral genome of two field isolates (PC201DJ and PC201SS) from pigs showing typical PMWS was sequenced. The nucleotide sequence homology with other PCV-2 isolates was ranging from 95% to 99% in complete viral genomic sequence. The highly conserved nonanucleotide motif of replication origin was identical to that of other PCV-2 isolates. To determine the genetic heterogeneity of PCV-2 isolates, the phylogenetic tree based on the complete genome of PCV-2 isolates were constructed. Two PCV-2 field isolates were closely related to Canadian isolates of PCV-2. PCV-2 isolated from field may have an origin of North America and is possibly originated from importation of breeding stocks. The result indicates that although the genome of PCV-2 is relatively stable in general, minor genetic variations exist among PCV-2 isolates from the different geographic locations. These differences of viral genome might have an important implication for genetic characteristics of PCV-2 infection. Three major immunorelevant epitopes of capsid protein showed variations in amino acid sequences. Also, the variance of amino acid sequence in antigenic epitope existed between two Korean PCV-2 isolates.

Key words: porcine circovirus, replication origin, sequence homology, phylogenetic tree, epitope

Introduction

Porcine Circovirus (PCV) is a small non-enveloped virus containing a single-stranded circular DNA genome. The PCV belongs to the family *Circoviridae* that has two types such as PCV-1 and PCV-2 [1, 5, 6, 12]. The two other animal circoviruses in this family are chicken anemia virus (CAV), psittacine beak and feather disease virus (PBFDV). The three plant circoviruses are known as banana bunchy top virus, coconut foliar decay virus, and subterranean clover stunt virus. Recently, a human circovirus, TT virus (TTV), was identified from patients with post-transfusion hepatitis. The human TTV has similarities to CAV in its genomic organization [1, 9, 10, 35, 43]. Besides, circoviruses show similarities to the family *Geminiviridae* with characteristics of single stranded circular form of DNA genome and using the rolling circle replication (RCR) strategy in its replication [7, 15]. PCV-1 consisting of 1,759 nucleotides shows neither cytopathic effects in tissue culture cells nor any specific diseases [12, 32, 42]. In contrast, PCV-2 has 1,768 nucleotides of viral genome and is speculated as a major agent causing post weaning multisystemic wasting syndrome (PMWS) in pigs [4, 6, 11, 26, 18, 19, 20, 24, 25, 34, 36, 38, 40, 41].

PMWS, a newly emerging disease in pigs, usually occurs in swine herds with good health condition and causes a low rate of morbidity in Canada, the United States, Asia, and many European countries. However, it affects weaners and finishers from 5 to 12 weeks old with relatively high mortality. PMWS pigs show clinical signs like dyspnea, anemia, visibly enlarged lymph nodes, diarrhea, pallor, progressive weight loss and jaundice. Histologically, main lesions associated with PMWS are lymphadenopathy, granulomatous interstitial pneumonia, hepatitis, and nephritis. Also, they include macrophage and lymphocytes infiltration in affected organs.

PCV is regarded as not only a crucial agent causing economical losses in swineherds, which is associated with PMWS, but also potential hazard in human health when xenotransplantation is addressed. Pig is a strong candidate to be developed as future donors of tissues and organs for those who need transplantation to replace impaired tissues and organs.

Though PCV-2 seems to be a quite important pathogenic agent, PCV-2 is not yet characterized in Korea. Presumably, this characterization of Korean PCV-2 isolates is valuable for developing diagnostic tools and vaccines. Consequently, in this study, we purposed to isolate PCV-2 from PMWS pigs in Korea and characterize PCV-2 isolates genetically by nucleotide sequence analysis. And we determined the origin

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of Korean PCV-2 isolates and genetic similarity by homology comparison and phylogenetic tree analysis.

Materials and Methods

Clinical samples

Pigs showing PMWS signs were submitted from swine farms Korean nation-wide to the Immunopathology laboratory Konkuk University. Tissue samples used for our research include lung, lymph nodes, spleen, tonsil, kidney, and liver.

Polymerase chain reaction (PCR) and cloning

Primer sets were designed on the basis of the sequence of PCV-1 (GenBank accession no. U49186) and PCV-2 (GenBank accession no. AF027217). DNA extraction was performed by the commercial DNA extraction kit, DNAzol (GIBCO BRL) according to manufactures instruction. Raw materials for the DNA extraction include 100mg mixture of lung, spleen, liver, kidney, inguinal lymph node, mesenteric lymph node and tonsil of PMWS pigs. Five hundred$\mu\ell$ cell lysates of PK-15 cells as a control were used. Oligonucleotide sequences the primers used for the amplification were shown in Table 1 [26]. PCR product with 886 bp in length specific for both PCV-1 and PCV-2 was amplified using primers F1 and R1. Primers F2 and R1 specific for PCV-2 was used to amplify 469 bp of DNA fragments from the samples collected in PMWS pigs. Full length PCV-2 genome was amplified using specific primers F1 and 1768R. For the sequencing of the complete genomic DNA, overlapping viral gene from 433 bp to 1695 bp was amplified using internal primers (Table 1). The direction of the amplification was opposite to that of the first round full-length genomic DNA amplification step. Annealing temperature for PCR was 52℃. The amplified linear forms of PCR products were purified by GENECLEAN II Kit (Bio 101, Inc., USA) and cloned into pGEM T-easy vector (Promega, U.S.A.). Plasmid constructs containing viral gene were pGEM DJ1768, pGEM DJ506 from PC201DJ and pGEM SS1768, pGEM SS506 from PC201SS, respectively. Plasmid DNA with insertion of the PCV viral genes were prepared for the sequencing by midi-prep using QIA filter Plasmid Midi Kit (QIAGEN).

Isolation of porcine circovirus associated with PMWS

PCV-2 positive samples such as inguinal lymph node, lung, tonsil, spleen, and kidney by PCR were frozen in liquid nitrogen, and homogenized in mortar with autoclaved sea sands. The inocula composed of homogenized tissues and minimum essential media (MEM, GIBCO BRL) containing 10% antibiotics were centrifuged and filtered through 0.22 ㎛ filter to eliminate bacterial contaminant. The virus isolation was performed in PK-15 cell line free from PCV-1 and PCV-2. Dr. Nayar G.P.S (University Crescent, Canada) kindly provided PCV free PK-15 cells. The semi-confluent PK-15 cells were inoculated with 3 ml of inoculum and placed in a incubator for 90 minutes at 37℃ with 5 % CO_2. Then, fresh MEM containing 2 % fetal bovine serum, 1% antibiotics and antimycotics, 2.5 % HEPES, 1 % non essential amino acid and 1 % Na pyruvate was replaced. At twenty-four hours post-inoculation, cells were washed with Hanks balanced salt solution (HBSS, GIBCO BRL), treated with 300mM D-glucosamine for 30minutes and washed once with HBSS only [35, 44]. Cells were incubated for 48 hours to allow virus replication prior to further passage for cell culture adaptation. Samples were passed three times with D-glucosamine treatment and the presence of the PCV was tested by PCR using PCV specific primers.

Sequencing and genetic analysis

The pGEM DJ1768, pGEM DJ506 and pGEM SS1768, pGEM SS506 were sequenced by Sangers methods (Bionex, Seoul Korea) using automated nucleotide sequencer. The sequence of PCV-2 isolates, PC201SS and PC201DJ, were analyzed with computer programs Clustal X 1.81 and GeneDoc to construct phylogenetic tree for comparing with those of other known PCV isolates. Sequence homology was searched by BLAST from NCBI Genbank database.

Results

Isolation of porcine circovirus associated with PMWS

The PCV-2 virus PC201DJ and PC201SS were isolated from pig tissue samples in PCV free PK-15 cells. But cytopathic effect was not clearly detected in field virus after inoculation.

PCR and cloning

Vero cells and PCV free PK-15 cells were used for negative control in PCR. PK-15 cells (ATCC CCL-33)

Table 1. Sequence of oligomers used for confirmation of the viral presence in field samples, cloning and sequencing

Primer	Sequence(5′ – 3′)	Size	Position in viral strand	Position in complementary strand
F1	ACCAGCGCACTTCGGCAG	18nt	1~18	
F2	TGAGTACCTTGTTGGAGAGC	20nt	418~437	
R1	GTAATCCTCCGATAGAGAGC	20nt		867~886
1768R	AATACTTACAGCGCACTTCTTTCG	24nt		1745~1768
1696F	GGTGTCTTCTTCTGCGGTAACG	22nt	1696~1717	
433R	TCCAACAAGGTACTCACAGCAG	22nt		412~433

showed PCV-1 positive in PCR with 886 bp DNA product. Two PCV-2 isolates, PC201DJ and PC201SS, showed both DNA bands of 886 bp and 469 bp in 1.5% electrophoresis gel (Fig.1). PCR products of 1768 bp and 506 bp using F1/1768R and 1686F/433R were inserted into pGEM T-easy vector to construct each of pGEM DJ1768, pGEM DJ506, pGEM SS1768, pGEM SS506 hybrid plasmid. Restriction endonuclease *Not I* was used to confirm insertion of the PCV DNAs from hybrid plasmid since there is no restriction endonuclease *Not I* in PCV-2 genomic sequence (Fig. 2).

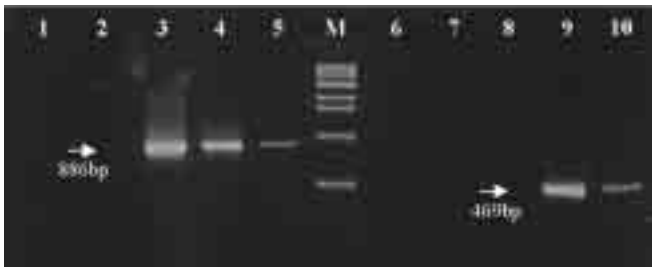

Fig. 1. PCR amplification for differentiation and identification of PCV-1 and PCV-2 using primer set common to both PCV-1 and PCV-2, and primers specific for the PCV-2. PCR products with 886 bp for PCV-1 and PCV-2 were amplified with F1 and R1, and F2 and R1 primer set amplified 469 bp only in PCV-2. M: 1Kb DNA marker (Bioneer, Seoul, Korea), lane 1 and lane 6: Vero cells, lane 2 and lane 7: PK-15 cells free from PCV-1, lane 3 and lane 8: PK-15 cells (ATCC CCL-33), lane 4 and lane 9: PC201DJ of PCV-2 isolate, lane 5 and lane 10: PC201SS of PCV-2 isolate.

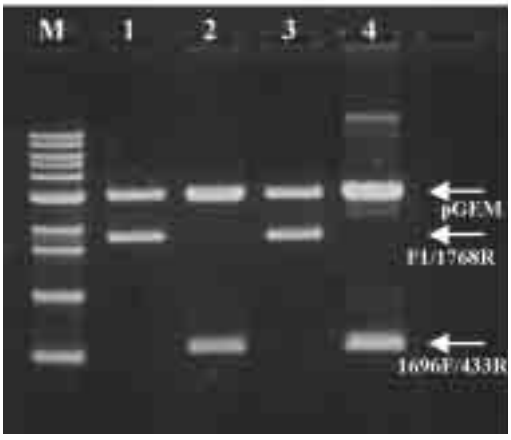

Fig. 2. Digestion of restriction endonuclease *Not I* for hybrid plasmid containing PCV DNA. PCR products of 1768 bp and 506 bp amplified by each primer sets of F1/1768R and 1696F/433R were inserted into pGEM T-easy vector. Restriction endonuclease *Not I* digested pGEM DJ1768 (lane 1), pGEM DJ506 (lane 2), pGEM SS1768 (lane 3) and pGEM SS506 (lane 4) released corresponding size of the insert. M: 1Kb DNA size marker (Bioneer, Seoul, Korea).

Sequence analysis

Complete viral genomic sequence was generated from sequence data obtained with overlapping sequencing analysis using internal primer sets. The schematic diagram of overlapping sequence is shown in Fig. 3. Complete viral genomic sequences of PC201DJ and PC201SS were aligned with PCV-2 (AF027217) and PCV-1 (U49186) as depicted in Fig. 4. PCV-2 isolates showed identical genetic characteristics known prototype PCV-2 such as overlapped putative eleven ORFs as shown in Fig. 3. The largest ORF1 and ORF2 code for *Rep* protein and viral capsid protein showed opposite orientation. Nonanucleotide motif of replication origin is observed in the same position with other PCV-2 strains, which is an essential element for the rolling circle replication (Fig. 3 and Fig. 4) [21, 28, 30, 37]. The nonanucleotide motif of 5-**AAGTATTAC**-3, which is different from PCV-1s of 5-TAGTATTAC-3, was conserved in both PCV-2 field isolates.

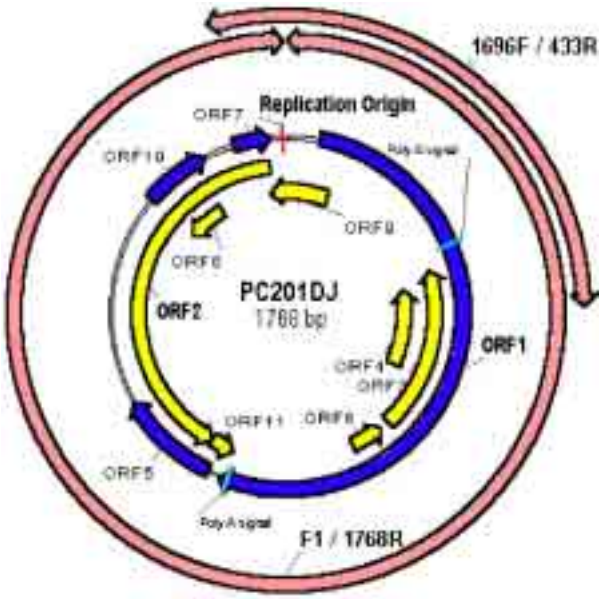

Fig. 3. Schematic diagram of complete viral genome of PC201DJ. Overlapped putative eleven ORFs were found in circular viral genome. Replication origin composed of nonanucleotide motif was located between ORFs 1 and 7. Two primer sets for sequencing were displayed as overlapped sequencing.

Homology analysis

Sequence homology was compared with other known PCV isolates in Table 2. Sequences of other known fifteen PCV-2 isolates and three PCV-1 isolates were downloaded from GenBank [17, 25, 31, 34]. The geographic locations of PCV isolates were varied as USA, Canada, France and Ireland. Two Korean isolates, PC201DJ and PC201SS, have 97 % of complete viral genomic sequence homology. Both PC201DJ and PC201SS showed 95~99 % sequence homology of complete viral genome in PCV-2 but 76~77 % in PCV-1. Especially, PC201DJ shows 99 % complete sequence homology

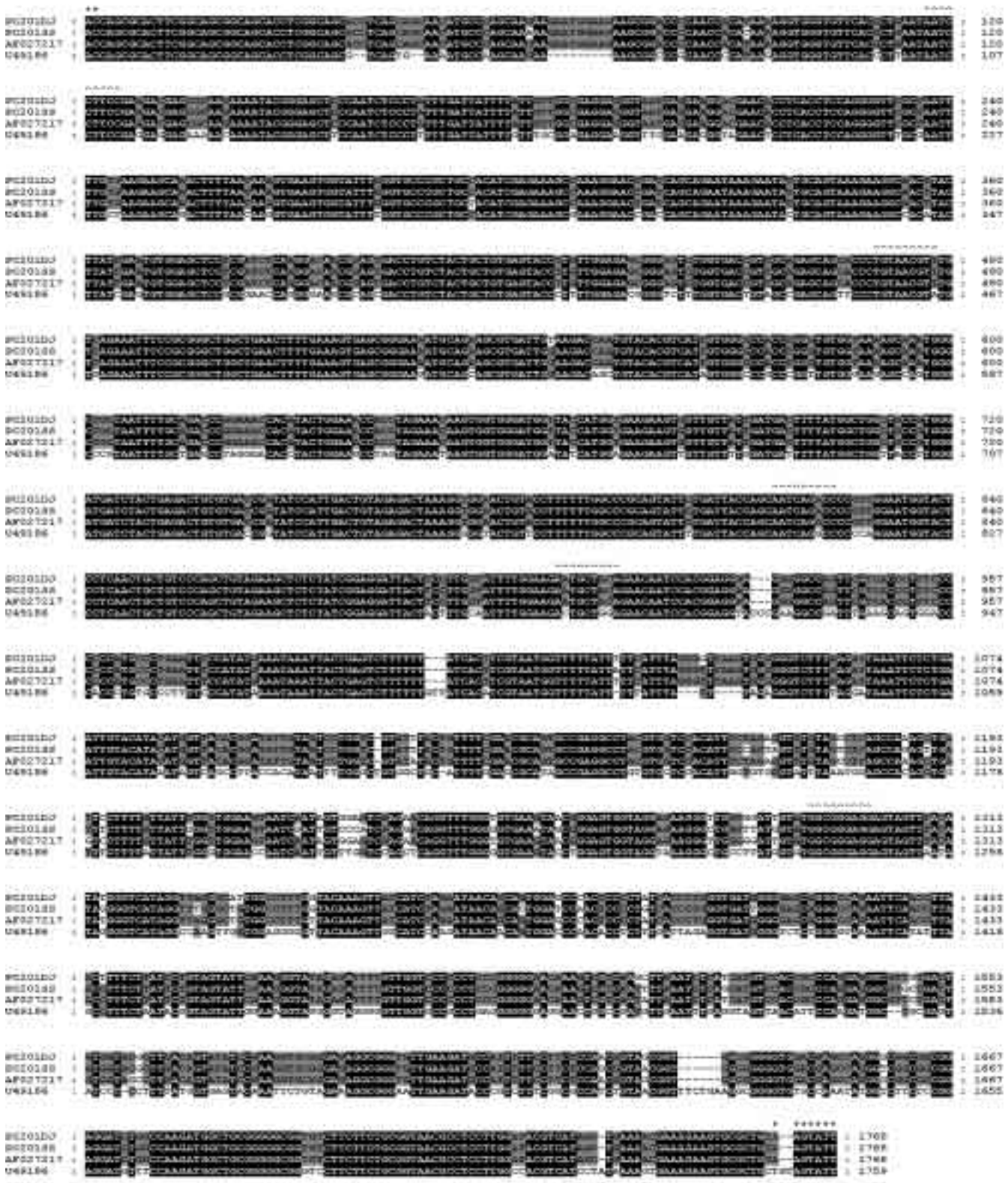

Fig. 4. Complete viral genomic sequence alignments of PC201DJ and PC201SS with PCV-2 (AF027217, USA) and PCV-1 (U49186, PK-15). Conserved sequences were shaded in three levels according to identity. Asterisks show nonanucleotide motif of PCV-2 as replication origin. Glycosylation sites were displayed by ^ marks.

Table 2. Comparison of the complete viral genomic sequence, amino acid sequence of Rep protein, capsid protein, ORF3 and ORF4 of PCV-2 field isolates with other PCV strains

Isolates			% Sequence homology of complete viral genome		% Amino acid sequence homology of *Rep* protein		% Amino acid sequence homology of capsid protein		% Amino acid sequence homology of ORF3		% Amino acid sequence homology of ORF4	
GenBank accession no.	Strain name	Geographic location	PC201DJ	PC201SS	PC201DJ	PC201SS	PC201DJ	PC201SS	PC201DJ	PC201SS	PC201DJ	PC201SS
	PC201DJ	Korea	100	97	100	NT	100	NT	100	NT	100	NT
	PC201SS	Korea	97	100	NT	100	NT	100	NT	100	NT	100
AF027217	pmws PCV	USA	98	97	99	99	95	94	92	100	98	100
AF055391	Type II from USA	USA	98	97	99	100	95	94	94	100	98	100
AF055392	Type II from CAN	Canada	98	97	99	100	97	95	96	100	98	100
AF055393	Type II from FRA	France	95	95	99	99	92	92	95	96	98	100
AF055394	Type II from FRA	France	95	95	99	99	92	93	95	96	98	100
AF085695	412	Canada	98	96	96	97	97	95	94	95	93	94
AF086834	B9	Canada	98	96	96	97	97	94	NT	NT	NT	NT
AF086835	9741	Canada	98	96	96	96	97	96	NT	NT	NT	NT
AF086836	M226	Canada	97	96	96	96	97	96	NT	NT	NT	NT
AF109397	Bovine circo	France	98	97	99	100	95	94	96	100	98	100
AF109398	2-C	Canada	95	97	98	99	95	97	94	95	96	98
AF109399	2-E	Canada	96	96	99	99	94	94	99	99	98	100
AF112862	2-B	Canada	98	97	99	99	95	93	98	99	96	98
AF117753	2-D	Canada	95	97	99	99	92	95	93	94	96	96
AF118097	IAF-4370	Canada	99	97	NT	NT	100	94	NT	NT	NT	NT
AF071879	PCV-1	PK-15 cell	76	76	85	85	63	64	NT	NT	NT	NT
AF012107	PCV-1	France	76	76	85	86	NT	NT	NT	NT	NT	NT
U49186	PCV-1	Ireland	77	76	85	86	NT	NT	NT	NT	NT	NT

NT: Not tested

Table 3. Comparison of amino acid sequence in ORF2 immunorelevant epitopes of the PCV-2. Bold indicate variations between strains

Amino acid position of ORF2 of PCV−2	PCV−2 strains	Putative antigenic epitope of ORF2 of PCV−2
69 ~ 83	AF201311(France)	V D M **M** R F **N I N** D F **L** P P G
	AF027217(USA)	V D M **M** R F **N I D** D F **V** P P G
	PC201DJ(Korea)	V D M **M** R F **K L D** D F **V** P P G
	PC201SS(Korea)	V D M **L** R F **K I D** D F **V** P P G
117 ~ 131	AF201311(France)	G **C** G S **S** A V I L D D N F V **T**
	AF027217(USA)	G **V** G S **T** A V I L D D N F V **T**
	PC201DJ(Korea)	G **V** G S **T** A V I L D D N F V **P**
	PC201SS(Korea)	G **V** G S **S** A V I L D D N F V **P**
169 ~ 183	AF201311(France)	**F** T I D Y F Q P N N K **E** N Q L
	AF027217(USA)	**S** T I D Y F Q P N N K **R** T Q L
	PC201DJ(Korea)	**S** T I D Y F Q P N N K **R** N Q L
	PC201SS(Korea)	**G** T I D Y F Q P N N K **R** N Q L

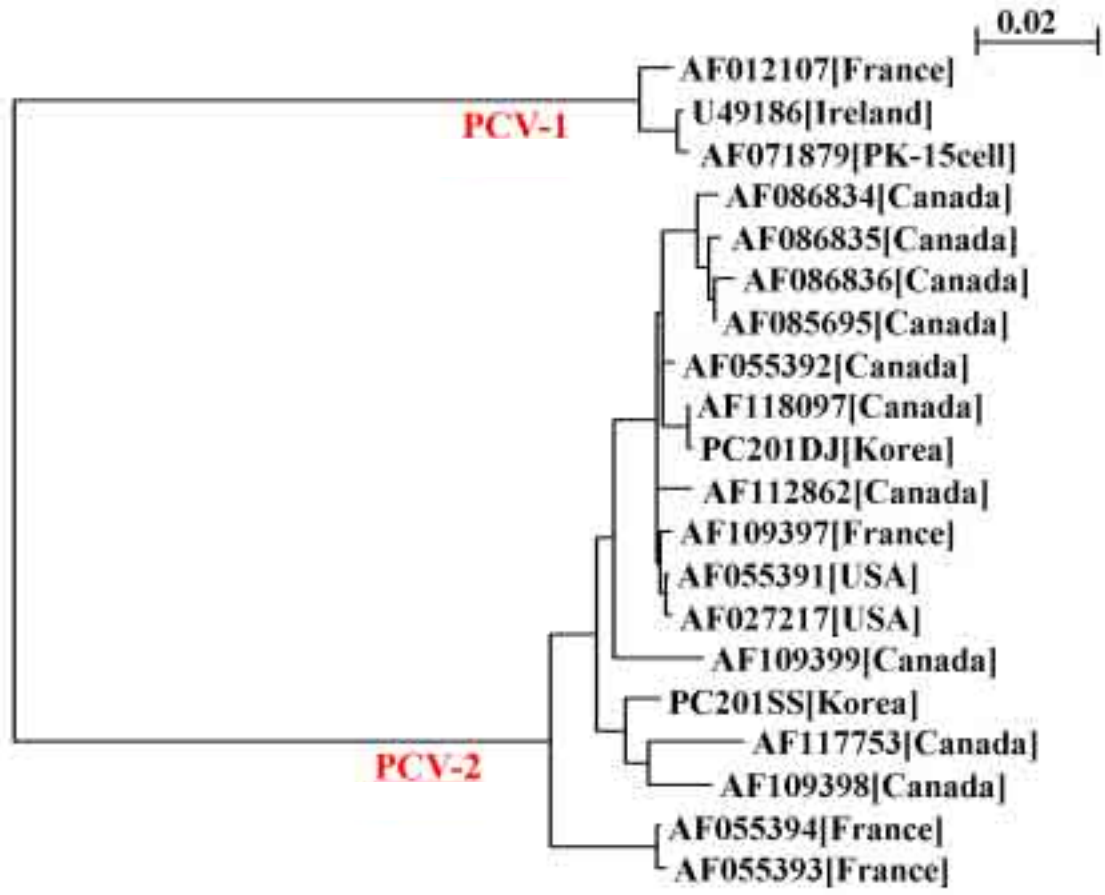

Fig. 5. Phylogenetic tree of the 20 PCV isolates including two Korean PCV-2s was constructed using computer analysis program Clustal X 1.81 on the basis of complete viral genome. Bootstrap neighbor joining method with the option of exclusion of positions with gaps was used. Branch lengths are proportional to the number of character-state changes. Scale bar: the number of character-state changes.

with AF118097. The amino acid sequence homology of both Korean isolates was compared in the point of ORF1, ORF2, ORF2 and ORF4. For both Korean isolates, the largest ORF1 of PCV-2, 314 amino acids encoding Rep protein, had over 98 % amino acid sequence homology in most case of PCV-2 except some Canadian isolates. 233 amino acids of ORF2, putative capsid protein, varied from 92 % to 97 % in amino acid homology of Korean isolates, but PC201DJ had exact consensus ORF2 with AF118097. The functions of 104 amino acids of ORF3 and 59 amino acids of ORF4 have been not clearly reported. The rate of homology in ORF3 and ORF4 of PCV-2 were varied from 92 % to 100%. Immunorelevant epitopes in the viral capsid protein showed considerable variations as shown in the Table 3. Especially first epitope located between amino acid 69 and 83 had higher mutation rate than others (Table 3). But the variation of putative immunorelevant epitopes was not consistent among strains.

Phylogenetic tree analysis

As shown in Fig. 5, phylogenetic tree was constructed on basis of the complete viral genomic sequence of twenty PCV isolates worldwide using computer analysis program Clustal X 1.81. These sequences of eighteen PCV isolates available in GenBank and two Korean PCV-2 isolates PC201DJ and PC201SS were used for the analysis. Based on the phylogenetic analysis, two major genotypes representing PCV-1 and PCV-2 were distinct each other. Among PCV-1s, AF071879 isolated from PK-15 cell and other PCV-1s isolated in the European regions were also used for analysis. PCV-2 has two distinct branches according to the geographic regions. One major branch of PCV-2 genotype is found in Europe and another major branch is present in North America (USA and Canada) and Asia including Korea. Two Korean PCV-2 field isolates of PC201DJ from mid-western region of the Korean peninsula and PC201SS from Kyungki province were closely related to Canadian isolates but were clustered into different groups of PCV-2 genotypes. Bovine isolate of circovirus was most closely related to the USA isolates of PCV-2 [14].

Discussion

PMWS causes one of a major health problem in pig herds worldwide. This is supposed to be caused by complex of many different swine pathogens including porcine circovirus, swine influenza, swine parvovirus, PRRS virus, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *etc*. [1, 2, 3, 8, 13, 14, 22, 39]. There is no clear evidence supporting PCV as a culprit in PMSW. Recent research data showed a strong relationship between PCV-2 and PMWS [1, 4, 6, 16, 34, 36]. In this study, two Korean PCV-2 isolates was sequenced and genetic characteristics were analyzed. Two Korean PCV-2 isolates showed a high degree of sequence homology with other PCV-2 strains available. The nonanucleotide replication origin of PCV-2 was found as same conserved sequence and position as other strains of PCV-2. This sequence is critical in virus replication and the first nucleotide of the conserved nonamer was mutated from T in PCV-1 to A in PCV-2. When the first two nucleotide of the nonanucleotide were altered in PCV-1, a total loss of replication function was found [29, 31]. Stem-loop around nonanucleotide motif of replication origin has been reported also. Andthree repetitions of the 6-bp motif CGGCAG seem to be putative binding site for the Rep protein [28, 29]. ORF2 of PCV-2 corresponding to PCV-1s showed a homology of about 63 %, but no detectable cross-reactivity could be shown between ORF2 proteins of PCV-1 and PCV-2 [27]. ORF2 of Korean PCV-2 isolates showed over 98 % homology with PCV-2 isolates from USA, Canada and France. But Canadian isolates such as AF085695, AF086834, AF086835 and AF086836 showed slightly lower homology of 96 % with two Korean isolates. Mache *et al*. suggested that three peptides of ORF2 of PCV-2 could be related to antigenic epitopes on the base of the sequence of AF201311, which is French PCV-2 isolate [27, 29, 45]. Amino acid variability in antigenic epitope between Korean isolates and other PCV-2 strains may indicate that there is possible on going spontaneous genetic mutation, which could play an important role in antigenicity of the virus. But there is no antigenically distinct PCV-2 has been reported. An ORF3 showed slightly lower sequence homology than that of the ORF4 but it was not significant. Four glycosylation sites in ORF1 and one site in ORF2 existed in these two field isolates. By phylogenetic analysis based on the complete viral genomic sequence, it is assumed that two Korean PCV-2 isolates might be originated from North American continent. Not even live animals but other materials such as boar semen imported from North America for the reproduction would be a source of the virus transmission [23]. PC201DJ and PC201SS were closely related with Canadian isolates, but showed little divergence between the branch lengths. This study suggests that although the genome of PCV-2 is relatively conserved in general, but there are minor genetic variations exist among PCV-2 isolates from the different geographic locations.

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