Genotypic Change of Porcine Circovirus Type 2 on Japanese Pig Farms as Revealed by Restriction Fragment Length Polymorphism Analysis

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(Received 21 September 2007/Accepted 29 January 2008)

ABSTRACT. Porcine circovirus type 2 (PCV2) has been recognized as the causal agent of postweaning multisystemic wasting syndrome and can be divided into two major genotypic groups. We developed a method of restriction fragment length polymorphism (RFLP) analysis of PCV2 open reading frame 2 for easy discrimination between the two major groups. Genotyping of PCV2 isolates from 10 Japanese commercial pig farms was performed, and the analysis revealed that both PCV2 groups and at least five RFLP types of PCV2 are prevalent in Japan. On two farms, the genotypes of the PCV2 isolates in the spring of 2007 were different from those in the autumn of 2006. One genotype may have become dominant within only six months on these farms.

KEY WORDS: genotype, porcine circovirus type 2 (PCV2), restriction fragment length polymorphism (RFLP).

Porcine circovirus type 2 (PCV2) has been recognized as the causal agent of postweaning multisystemic wasting syndrome (PMWS), although it has not been proven as the sole factor responsible for the disease [1, 2, 11, 17]. PMWS was first described in Canada and has subsequently been reported by pig-rearing farms around the world. Pigs affected by PMWS exhibit respiratory clinical signs and wasting after weaning and develop multiple characteristic pathological changes, including lymphocyte depletion and granulomatous inflammation of the lymphoid tissues [1]. An outbreak of PMWS causes an increase in the number of slow-growing pigs and eventual loss of productivity on commercial pig farms.

PCV2 is an icosahedral, non-enveloped virion about 17 nm in diameter, which makes it the smallest currently known porcine virus. PCV2 has an approximately 1770-nucleotide genome of ambisense single-stranded circular DNA. Two major open reading frames (ORFs) are oriented in opposite directions. ORF1, the rep gene, and ORF2, the cap gene, encode replication-associated protein and capsid protein, respectively [4, 14]. ORF3 completely overlaps ORF1 on the PCV2 genome, but in the counter-direction. The apoptotic activity of the ORF3 protein has been reported to play an important role in viral pathogenicity [12].

The genotypes of PCV2 have not been considered to be important, since PCV2 isolates have been noted to share nucleotide identities of greater than 94.5% [8]. In 2004, Fenaux et al. reported that 2 amino acid mutations in ORF2 protein enhanced PCV2 replication in vitro [6]. Mutations in ORF3 have been reported to alter PCV2 pathogenicity in vivo [13]. These findings suggest that the pathogenicity of PCV2 might depend on its genotype.

Recently, Olvera et al. performed a comprehensive analysis of the PCV2 genotype [15]. PCV2 isolates were divided into two major groups: group 1, which was further subdivided into 3 clusters (1A to 1C), and group 2, which was subdivided into 5 clusters (2A to 2E; Olvera’s classification). PCV2 isolates belonging to groups 1 and 2 were originally detected in Europe and North America, respectively.

In Japan, PMWS was first described in 1997 [16], and a subsequent serological survey conducted in 1999 revealed the presence of PCV2 antibodies in 94.6% of clinically healthy pigs and 96.6% of randomly selected farms [10]. PCV2 DNA was detected in 86.2–100% of farms between 2000 and 2003 [9]. These studies indicate a high prevalence of PCV2 infection on pig farms in Japan. However, extensive genotyping of the PCV2 isolates from Japanese pig farms has not been performed.

In this study, we performed genotyping of PCV2 isolates from Japanese commercial pig farms based on restriction fragment length polymorphism (RFLP) analysis using PCV2 ORF2.

Sera were collected from fifteen 2- to 4-month old pigs (5 each of 2-, 3- and 4-month old pigs) at each of 10 farrow-to-finish pig farms in Japan with sow numbers of over 500 (farms A to J) in the autumn of 2006 and spring of 2007. Viral DNA was extracted from 100 µl of serum using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). One microliter of the extracted DNA was subjected to PCR. A fragment including ORF2 of PCV2 was amplified by PCR with 2 primers, PCV2-f1 (5’-CCA TGC ACT GAT ATT TAC TCA GTA-3’) and PCV2-r1 (5’-ACA GCG CAC TTC TTG CTT CTG-3’), in a 25-µl reaction mixture (Fig. 1a). PCR was carried out with an initial step at 94°C for 2 min; 40 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min; and a final extension step at 72°C for 7 min.
The PCR products were digested with *Ava*II, *Bam*HI and *Cla*I for 2 hr at 37°C. Figure 1b shows the cutting patterns of PCR-RFLP with each restriction enzyme. The RFLP types of the PCV2 isolates are indicated by numerical codes showing the cutting patterns of the restriction enzymes, namely, *Ava*II, *Bam*HI and *Cla*I, in that order. For example, RFLP type 321 gives patterns 3, 2 and 1 with *Ava*II, *Bam*HI and *Cla*I digestion, respectively.

PCV2 DNA was detected in 2 to 15 of the 15 pigs tested from each farm. Five RFLP types (111, 122, 222, 311 and 321) of PCV2 were detected at the 10 Japanese pig farms (Table 1), and a single RFLP type was observed at one individual farm at the same time. At farms B and E, RFLP types 311 and 111 were detected in the autumn of 2006 and spring of 2007, respectively.

The ORF2 nucleotide sequences of the PCR products were determined using two primers, PCV2-sf2 (5'-TAC CGG GAG TGG TAG GAG AA-3') and PCV2-sr2 (5'-CTC TCC CGC ACC TTC G-3'), and were analyzed with GENETYX (Genetyx, Tokyo, Japan). The phylogenetic tree of PCV2 ORF2 was constructed by the neighbor-joining method using ClustalW [18] and was visualized with Treeview.

The ORF2 nucleotide sequence identities between the

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**Table 1.** RFLP types and accession numbers of PCV2 isolates and nucleotide identities between PCV2 isolates in the autumn of 2006 and spring of 2007 for each individual farm

<table>
<thead>
<tr>
<th>Farm</th>
<th>Autumn of 2006</th>
<th>Spring of 2007</th>
<th>Nucleotide identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A0111 111 AB361566</td>
<td>A2439 111 AB361567</td>
<td>99.8</td>
</tr>
<tr>
<td>B</td>
<td>B0044 311 AB361568</td>
<td>B1201 111 AB361569</td>
<td>92.3</td>
</tr>
<tr>
<td>C</td>
<td>C0040 311 AB361570</td>
<td>C1313 311 AB361571</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>D1742 311 AB361572</td>
<td>D3645 311 AB361573</td>
<td>99.5</td>
</tr>
<tr>
<td>E</td>
<td>E1249 311 AB361574</td>
<td>E2637 111 AB361575</td>
<td>92.3</td>
</tr>
<tr>
<td>F</td>
<td>F1440 321 AB361576</td>
<td>F3241 321 AB361577</td>
<td>99.4</td>
</tr>
<tr>
<td>G</td>
<td>G2342 222 AB361578</td>
<td>G3544 222 AB361579</td>
<td>99.8</td>
</tr>
<tr>
<td>H</td>
<td>H2337 122 AB361580</td>
<td>H3544 122 AB361581</td>
<td>99.7</td>
</tr>
<tr>
<td>I</td>
<td>I1328 311 AB361582</td>
<td>I3943 311 AB361583</td>
<td>99.8</td>
</tr>
<tr>
<td>J</td>
<td>J4446 111 AB361584</td>
<td>J9242 111 AB361585</td>
<td>99.7</td>
</tr>
</tbody>
</table>
PCV2 isolates in 2006 and those in 2007 were 92.3–100% for each farm (Table 1). The PCV2 isolates detected at the same time shared 99.5–100% nucleotide sequence identities for ORF2 at each individual farm.

Phylogenetic analysis based on PCV2 ORF2 was performed using the isolates detected in this study and reference sequences available on a public database (38 PCV2 and one PCV1). The PCV2 isolates from this study are highlighted in black. The reference sequences are shown using accession numbers. The bootstrap values (1000 replicates) for each cluster are shown. The names of the clusters are given according to Olvera’s classification [15]. The RFLP types are shown in boldface and on the right side of the clusters, respectively.

Fig. 2. Phylogenetic analysis of PCV2 based on ORF2 nucleotide sequences. An unrooted phylogenetic tree was constructed by the neighbor-joining method with 20 PCV2 ORF2 sequences determined in this study and reference sequences available on a public database (38 PCV2 and one PCV1). The PCV2 isolates from this study are highlighted in black. The reference sequences are shown using accession numbers. The bootstrap values (1000 replicates) for each cluster are shown. The names of the clusters are given according to Olvera’s classification [15]. The RFLP types are shown in boldface and on the right side of the clusters, respectively.

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Phylogenetic analysis based on PCV2 ORF2 was performed using the isolates detected in this study and reference sequences available on a public database. Figure 2 shows the phylogenetic tree, Olvera’s classification and the RFLP types. Olvera’s clusters 1A and 1B were classified into the same cluster by our analysis. RFLP types 111 and 112 were classified into group 1, while the other types were classified into group 2. RFLP type 311 belonged to Olvera’s clusters 2B, 2C and 2E. The analysis showed that RFLP types 122 and 222 were classified into the same cluster (cluster 2A of Olvera’s classification). RFLP types 111, 311 and 321 isolated in this study were classified into clus-
PCV2 was detected in some of the 2- to 4-month old pigs from all ten of the commercial pig farms examined in this study. The ages of the pigs infected with PCV2 differed among the farms. This difference may have been caused by many factors, including the housing and dietary conditions, head count of pigs, presence of other infectious agents and the levels of maternal PCV2 antibody.

As the PCR-RFLP method is a simpler, faster and less expensive than sequencing for genotyping, PCV2 isolates from a large number of pigs on farms can be analyzed regularly. The PCV2 dynamics in farms, such as entry of a new genotype of PCV2, can be detected immediately and precisely. ORF2 is a reliable phylogenetic marker of PCV2 isolates, and the phylogenetic tree derived from ORF2 nucleotide sequences has been shown to be similar to that derived from whole viral genome sequences [15]. Therefore, we performed PCR-RFLP analysis with PCV2 ORF2. RFLP-based PCV2 genotyping methods have been reported previously [3, 8, 19]. Nevertheless, using one of these methods, we could not discriminate between several group 2 and group 1 isolates [3], and five and eight restriction enzymes, respectively, needed to be used in two other methods [8, 19]. Thus, we developed an RFLP method requiring only three restriction enzymes (AvaiI, BamHI and CiaI) and by which Olvera’s group 1 isolates can be distinguished from group 2 isolates [3]. This finding confirmed that both groups, groups 1 (RFLP type 111) and 2 (RFLP types 122, 222, 311 and 321), and at least five RFLP types of PCV2 are prevalent in Japan.

We analyzed the PCV2 RFLP types in the autumn of 2006 and spring of 2007. On farms B and E, RFLP types 311 and 111 of PCV2 were detected in 2006 and 2007, respectively, implying that a genotypic change had occurred previously [3, 8, 19]. Nevertheless, using one of these methods, we could not discriminate between several group 2 and group 1 isolates [3], and five and eight restriction enzymes, respectively, needed to be used in two other methods [8, 19]. Thus, we developed an RFLP method requiring only three restriction enzymes (AvaiI, BamHI and CiaI) and by which Olvera’s group 1 isolates can be distinguished from group 2 isolates [3]. This finding confirmed that both groups, groups 1 (RFLP type 111) and 2 (RFLP types 122, 222, 311 and 321), and at least five RFLP types of PCV2 are prevalent in Japan.

We analyzed the PCV2 RFLP types in the autumn of 2006 and spring of 2007. On farms B and E, RFLP types 311 and 111 of PCV2 were detected in 2006 and 2007, respectively, implying that a genotypic change had occurred within only six months. For these farms, the ORF2 nucleotide sequence identities between the isolates in 2006 and those in 2007 were 92.3%, while the sequence identities were 99.4–100% for the other farms where the RFLP types had not changed. Thus, the PCV2 genotype difference observed on farms B and E might have been caused by entry of a new genotype of PCV2 from outside the farm. Such entry might be caused by introduction of new boars or sows infected with the new genotype of PCV2.

This study revealed that PCV2 groups 1 and 2 are prevalent on Japanese pig farms and that one genotype may have become dominant within only six months on two farms. Recently, the presence of PCV2 groups 1 and 2 in swine herds in the U.S.A. has been reported [5]. Only two of the diseased animals harbored PCV2-group 2 isolates, whereas PCV2-group 1 isolates were detected in all the diseased animals. Gagnon et al. claimed that the appearance of PCV2-group 1 isolates in Canada may explain the death rate increase related to PMWS in that country [7]. Further investigation is necessary to elucidate the differences in pathogenicity between the genotypes of PCV2. Further elucidation of the relationship between the genotypic changes in PCV2 and the increased death rate in relation to PMWS in Japanese pig farms is also required.

REFERENCES