Characterization of Porcine Circovirus Type 2 in Taiwan

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ABSTRACT. In an effort to understand the genetic diversity of porcine circovirus type 2 (PCV2) and the prevalence of PCV2 infection in Taiwanese herds, we have sequenced the complete genomes from PCV2-infected specimens and individually measured the antibody titre against PCV2 from pigs reared in Taiwan between the years 2000 and 2002. A total of 623 specimens originating from pigs displaying varied clinical signs were screened with the polymerase chain reaction (PCR). Results showed that 309 pigs (49.6%) tested positive for PCV2. Eight of the positive specimens were used for the amplification of the complete viral genome. Sequence comparison of the complete genomes indicated that the 8 Taiwanese PCV2 isolates shared 95-99% similarity. Phylogenetic analysis of all 40 PCV2 isolates from North America, Europe, Asia and Taiwan revealed that those isolates were grouped together in one large group containing two minor subgroups. The Taiwanese PCV2 isolates were classified into the two minor subgroups. The prevalence of serum antibodies to PCV2 in pigs was investigated, and results showed that approximately 83.5% of the pigs in Taiwan were seropositive. Finishing pigs possess the highest titers of antibodies, while 9-week-old pigs contained the lowest titers for specific antibodies. Our results suggest that PCV2 infections have become common in Taiwanese pig farms.

KEY WORDS: phylogenetic analysis, polymerase chain reaction, porcine circovirus type 2, seropositive.

FULL PAPER

Porcine circovirus (PCV) is a non-enveloped virus with a size of 17 nm and contains a single-stranded circular DNA genome [9, 21]. PCV was classified as a member of Circoviridae family [3]. The other six animal members in Circoviridae are chicken anemia virus [21], psittacine beak-and-feather disease virus [21], human transfusion-transmitted virus [18], goose circovirus [30], pigeon circovirus [30] and canary circovirus [22]. Porcine circovirus type 1 (PCV1) and type 2 (PCV2) have two major open reading frames (ORF1 and ORF2). ORF1 is believed to code for the replication-associated protein [16], while ORF2 encodes a major structural capsid protein [20]. Sequence differences between PCV1 and PCV2 are mainly located in ORF2 [5, 9, 16]. PCV2 can be distinguished from PCV1 on the basis of genome sequence and antigenicity [2, 5, 9, 14].

PCV1 has been isolated from a contaminant in the PK-15 cell line [6]. This virus is not considered to be pathogenic to pigs [3, 29]. PCV2 infection has been strongly correlated as the causative agent of postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis nephropathy syndrome (PDNS), congenital tremors, and reproductive failure in pigs [2, 8, 17, 25]. PMWS was first reported in western Canada [7]. PMWS is now known to occur in pig farms in Europe, North America and Asia [2, 11, 19, 26]. However, PCV2 has been identified in pigs even when typical clinical and histological lesions were absent [12]. Serological surveys employing indirect immunofluorescence assays (IFA), indirect immunoperoxidase monolayer assays (IPMA) and enzyme linked immunosorbent assays (ELISA) revealed that antibodies to PCV1 were common in North American and European swine populations [6, 28, 29]. Other studies have shown that cross-reaction exists between PCV1 and PCV2 [13, 14, 23], and that PCV2 appears to be the main PCV type circulating amongst pig populations [13].

PMWS has been reported in Taiwanese pigs [4]. The affected pigs display weight loss, dyspnea and jaundice. Gross lesions include lymphadenopathy, interstitial pneumonia, interstitial hepatitis and nephritis. Histopathological lesions include macrophage infiltration, depletion of lymphoid cells, variable numbers of syncytial cell formation in the lymphoid tissues, and characteristic basophilic intracytoplasmic inclusions are present in macrophage cells. The purpose of this study was to detect PCV2 in various clinical cases using polymerase chain reaction (PCR) assays, compare the complete genomes of Taiwanese PCV2 isolates and realize the seroprevalence of antibodies to PCV2 in pig populations.

MATERIALS AND METHODS

Samples: Various tissue samples (including tonsil, lymph nodes, spleen, liver, lung and brain) were collected from 623 pigs possessing a variety of clinical conditions from 80 pig farms between the years 2000 and 2002. These included 237 pigs with nonspecific clinical signs (excluding PMWS, PDNS, reproductive failure, nervous signs and sudden death), 162 pigs with PMWS, 95 pigs with sow abortions, 68 pigs with nervous signs, 39 pigs with sudden death and 22 pigs with PDNS. Tissue samples were stored at –80°C until used. A total of 1,320 pig sera were randomly collected from 12 pig farms and 5 abattoirs covering an area from northern to southern Taiwan. Serum samples were
collected from 200 sows and 960 were collected from 3- to 20-week-old pigs. Additionally, 160 samples were collected from finishing pigs in 5 abattoirs. Serum samples were submitted to the National Institute for Animal Health, Council of Agriculture from May 2000 to March 2002.

**Primer and PCR:** Total DNA was extracted from pig tissue samples using a QIAamp Tissue Kit (QIAGEN). Two specific oligonucleotide primers, F66 and B67 (Table 1), were used as previously described [15]. A 50 μl reaction mixture consisted of the following: 5 μl of 10X buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 1.25 μM of dNTPs, 20 μM of each primer, 0.5 μl POWER TAQ (5U/μl; POWER TAQ), and 6 μl of DNA sample. PCR was performed at 95°C, 12 min, followed by 42 cycles that consisted of denaturation at 95°C for 20 s, annealing at 57°C for 45 s. Samples were finally subjected to a terminal extension step at 72°C for 7 min.

**Nucleotide sequencing and phylogenetic analysis:** Different sets of PCV2 specific primers, based on the sequences of PCV2 isolates from Taiwan (GenBank accession number AF154679), were used to amplify the complete PCV2 genome. Primers sets are given in Table 1. A 100 μl reaction mixture consisted of the following: 10 μl of 10X buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 1.25 μM of dNTPs, 20 μM of each primer, 1.0 μl POWER TAQ (5U/μl; POWER TAQ), and 12 μl of DNA sample. PCR was performed at 95°C for 5 min, followed by 35 cycles that consisted of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Samples were finally subjected to a terminal extension step at 72°C for 10 min.

**Statistical analysis:** Duncan’s new multiple range test was applied in an effort to compare the differences in the means of antibody titers amongst the different age groups. Comparisons of the seropositive rates in different age groups were made by Chi-square analysis. Values of \( P<0.05 \) were considered to indicate statistical significance.

**RESULTS**

**PCR analysis:** Each pig was tested for PCV2. Two primers (F66 and B67) designed for PCV2 were used to amplify the 416 bp DNA fragment. Results indicated that 309 pigs (49.6%, 309/623) were positive for PCV2. Among the 309 pigs, the most common occurrence of PCV2 infection was associated with nonspecific clinical signs (131 pigs, 42.4%). Other PCV2 infections were associated with a variety of clinical signs that included 116 pigs with PMWS (37.6%, 116/309), 7.4% pigs (23/309) resulting from sow abortions, 5.8% pigs (18/309) with nervous signs, 4.9% pigs (15/309) with PDNS cases and 1.9% pigs (6/309) resulting from sudden death. Additionally, the PCV2 positive rate for each clinical signs were 71.6% pigs (116/162) with PMWS, 42.4% pigs (131/309) with nonspecific clinical signs, 23.0% pigs (74/315) with PDNS cases and 2.2% pigs (7/315) resulting from sudden death.
68.1% pigs (15/22) with PDNS, 55.2% pigs (131/237) with nonspecific clinical signs, 26.4% pigs (18/68) with nervous signs, 24.2% pigs (23/95) with aborted fetuses and 15.3% pigs with sudden death (6/39).

Sequence analysis: In this study, the complete genome of 8 Taiwanese PCV2 isolates were amplified and sequenced, including 4 PCV2 isolates from pigs with PMWS (Chia-Yi/TWN, SC/TWN, GH/TWN, and KS/TWN), 2 PCV2 isolates with PDNS (PF/TWN and YL/TWN), 1 PCV2 isolate with nervous signs (PT-1/TWN) and 1 PCV2 isolate resulting from sow abortion (TD/TWN). Sequence analysis of the complete genomes of 8 Taiwanese PCV2 isolates from this study showed that they all contained 1,768 bp. Sequence information for 2 PCV2 isolates has been submitted to GenBank (accession number AF364094 (Chia-Yi/TWN) and AF456211 (SC/TWN)).

Analysis of the complete genomes of our 8 PCV2 isolates showed that these shared 95–99% similarity. The 8 Taiwanese PCV2 complete genomes reported in this study were compared to 32 PCV2 isolates obtained from GenBank (Fig. 2). Phylogenetic tree analysis classified these isolates into 1 large group containing two minor subgroups (Fig. 2).

Phylogenetic analysis: In an effort to understand the interrelationships of the viral isolates, the complete genome sequences of the 8 Taiwanese PCV2 isolates from this study and 32 PCV2 isolates obtained from GenBank (Fig. 2) database were aligned and analyzed. Phylogenetic tree analysis classified these isolates into 1 large group containing two minor subgroups (Fig. 2). The first subgroup contained 7 Canadian isolates (9741/CAN, IAF614/CAN, IAF2897/CAN, Imp-999/US, 9741/CAN, SPA3, GER2, KSY-2/KOREA and PMWS-No.35/JAPAN), 5 American isolates (Imp-999/US, AF147751/US, ISU31/US, 26606/US and 26607/US), 2 Korean isolates (JHP/KOREA and KSY-2/KOREA) from Spain (SPA3), 1 isolate from Germany (GER2), 1 isolate from Korea (KSY-2/KOREA) and 1 isolate from Japan (PMWS-No.35/JAPAN). A comparison of the complete genomes revealed that all 14 PCV2 isolates were closely related, possessing 92–99% similarity. The deduced amino acid sequences of ORF2 from the 8 Taiwanese PCV2 isolates were aligned with the Imp-999/US, 9741/CAN, SPA3, GER2, KSY-2/KOREA and PMWS-No.35/JAPAN PCV2 isolates (Fig. 1), showing high degrees of similarity ranging from 91–99%.

Phylogenetic analysis: In an effort to understand the interrelationships of the viral isolates, the complete genome sequences of the 8 Taiwanese PCV2 isolates from this study and 32 PCV2 isolates obtained from GenBank (Fig. 2) database were aligned and analyzed. Phylogenetic tree analysis classified these isolates into 1 large group containing two minor subgroups (Fig. 2). The first subgroup contained 7 Canadian isolates (9741/CAN, IAF614/CAN, IAF2897/CAN, Imp-999/US, 9741/CAN, SPA3, GER2, KSY-2/KOREA and PMWS-No.35/JAPAN), 5 American isolates (Imp-999/US, AF147751/US, ISU31/US, 26606/US and 26607/US), 2 Korean isolates (JHP/KOREA and KSY-2/KOREA), 1 isolate from Spain (SPA3), 1 isolate from Germany (GER2), 1 isolate from Korea (KSY-2/KOREA) and 1 isolate from Japan (PMWS-No.35/JAPAN). A comparison of the complete genomes revealed that all 14 PCV2 isolates were closely related, possessing 92–99% similarity. The deduced amino acid sequences of ORF2 from the 8 Taiwanese PCV2 isolates were aligned with the Imp-999/US, 9741/CAN, SPA3, GER2, KSY-2/KOREA and PMWS-No.35/JAPAN PCV2 isolates (Fig. 1), showing high degrees of similarity ranging from 91–99%.
2/KOREA), 1 Chinese isolate (BX/CHINA), 1 Japanese isolate (PMWS-NO.33/JAPAN) and 1 Taiwanese isolate (SC/TWN). The second subgroup contained 2 Chinese isolates (BF/CHINA and HR/CHINA), 2 Japanese isolates (PMWS-NO.26/JAPAN and PMWS-NO.35/JAPAN), 2 German isolates (GER1 and GER2), 3 Spanish isolates (SPA1, SPA2, SPA3), 1 Canadian isolate (PCV-2D/CAN), 3 French isolates (FRA1, FRA2, FRA3), 7 isolates from this study (PT-1/TWN, KS/TWN, YT/TWN, TD/TWN, PF/TWN, Chia-Yi/TWN and GH/TWN) and 3 Taiwanese isolates (PT-2/TWN, V00-52-2/TWN and MLTW-98/TWN).

Surveillance of the PCV2 antibody: To investigate the prevalence of specific antibodies against PCV2 in pigs, a national survey of sera on pig populations was carried out. The results showed that approximately 83.5% (1103/1320) of pigs were seropositive (≥20) to PCV2 (Fig. 3). The sows and 3-week-old piglets had the higher level of antibody titers, the average titer was 3162.2 in the sows and 1819.7 in the 3-week-old piglets. The average titer for 6- and 9-week-old pigs was 134.8 and 20.8, respectively. However, the average titer for 12-week-old pigs was 31.6. In the case of 14- and 20-week-old pigs, the average titer was 1819.8 and 8511.3. The average titer for finishing pigs was 10232.9. The average titers for 20-week-old and finishing pigs were significantly higher than that for 9- and 12-week-old pigs (P<0.05). Additionally, the sows were 97.5 % seropositive. Six-week-old pigs were 77.5% seropositive while 9-week-old pigs were only 50% seropositive. Twenty-week-old and finishing pigs were each 100% seropositive. The seropositive rates for 20-week-old and finishing pigs were significantly higher than that for 9-week-old pigs (P<0.05).

DISCUSSION

In previous studies [2, 12, 19, 26], PCV2 was widely found in populations of diseased pigs that displayed a wide variety of clinical conditions and lesions. For these reasons, our laboratory has been routinely using the PCR assay to test pools of at least three tissues from each diseased pig since the year 2000. Our studies revealed that PCV2 was identi-
fied in 49.6% (309/623) of the pigs, highlighting the considerable predominance of the PCV2 viral pathogen in Taiwanese pig farms. PCV2 was not only identified in cases associated with PMWS (71.6%), but was also found associated with a variety of other clinical conditions (68.1% in PDNS, 26.4% in nervous cases, 24.2% in abortion cases and 15.3% in sudden death). These results suggest that PCV2 infections may occur in the absence of the typical clinical signs of PMWS.

The authors have evidence confirming the occurrence of various combinations of PCV2 with other viral pathogens (unpublished data), including PCV1, porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PrV), classical swine fever virus (CSFV), porcine enterovirus type 8 (PEV8), porcine teschovirus (PTV), porcine cytomegalovirus (PCMV) and other viral pathogens. However, PRRSV and porcine parvovirus (PPV) infection are internationally widespread, and include those areas where PMWS has been detected [11, 12]. Previous studies suggested that minor variations in ORF2 of PCV2 may be responsible for the variety of clinical signs observed [17]. However, the potential for PCV2 to cause these different clinical conditions requires further investigation [12]. Detailed studies are needed to determine the different factors associated with PCV2 pathogenicity in pigs.

Phylogenetic analyses of different PCV2 isolates have been carried out on PCV2 isolates identified from a variety of clinical cases [5, 12]. In this study, phylogenetic tree analysis suggested that 40 PCV2 isolates be grouped into two subgroups. Phylogenetic analyses also indicated that 10 Taiwanese PCV2 isolates were very similar, and that an association between groupings of isolates and clinical signs could not be found (Fig. 2). These results were in agreement with previous studies [5, 12]. Additionally, one Taiwanese PCV2 isolate (SC/TWN) was grouped into another minor subgroup. The complete genome of the SC/TWN isolate was similar to isolates from North America and Korea. This
suggests that the SC/TWN isolate may have originated from an invaded strain. These preliminary findings have proven useful in directing future studies of PCV2 in Taiwan.

Serological investigations in pig populations sampled from the entire age range and from the whole region of Taiwan have revealed that approximately 83.5% of pigs were seropositive to PCV2 (Fig. 3). Most of the sows contained PCV2 antibodies between 1:1,280 and ≥1:20,480. Investigations revealed that PCV2 antibodies increased with the onset of age, which may result from sustained PCV2 contact infection from the environment. Serological analysis of PCV2 revealed low antibody titers at 9-weeks of age that actively seroconverted during the growing period. This suggests that pigs 6- to 12-weeks old may be exposed to a high risk of PCV2 infection.

Additionally, serological investigations suggest that PCV2 infection in pigs is not accidental, but that the virus may persist in pigs and cause endemic infection in herds. Therefore, transmission via oral and respiratory routes may occur in herds. One possibility is that active viral particles from infected pigs present in the oronasal and fecal discharges could infect new hosts [27]. One interesting finding in our investigation was that PCV2 DNA in serum samples was able to be detected in 14- and 20-week-old pigs, and in finishing pigs (data not shown). The fact that PCV2 antibodies were not able to neutralize the virus and that production of neutralizing antibodies may have been severely delayed has been suggested [24]. To our knowledge, this is the first report detailing serological evidence in connection to PCV2 in Taiwan.

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