Coincidental detection of genomes of porcine parvoviruses and porcine circovirus type 2 infecting pigs in Japan

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ABSTRACT. The infection status of 15 viruses in 120 pigs aged about 6 months was investigated based on tonsil specimens collected from a slaughterhouse. Only 5 species of porcine parvoviruses and porcine circovirus type 2 (PCV2) were detected at high frequencies; 67% for porcine parvovirus (PPV) (PPV-Kr or -NADL2 as the new abbreviation), 58% for PPV2 (CnP-PARV4), 39% for PPV3 (P-PARV4), 33% for PPV4 (PPV4), 55% for PBo-like V (PBoV7) and 80% for PCV2. A phylogenetic analysis of PPV3 suggested that Japanese PPV3s showed a slight variation, and possibly, there were farms harboring homogeneous or heterogeneous PPV3s. Statistical analyses indicated that the detection of PCV2 was significantly coincidental with each detection of PPV, PPV2 and PPV3, and PPV and PPV4 were also coincidentally detected. The concurrent infection with PCV2 and porcine parvoviruses in the subclinically infected pigs may resemble the infection status of pigs with the clinical manifestations of porcine circovirus associated disease which occurs in 3–5 months old pigs and is thought to be primarily caused by the PCV2 infection.

KEYWORDS: coinfection, porcine circovirus associated disease, porcine circovirus type 2, porcine parvoviruses, prevalence


A number of new parvoviruses have been identified during the past 15 years and given various names, and thereby, the updated taxonomy of the family Parvoviridae was proposed in 2014 [6]. The classical porcine parvovirus (PPV), which was first identified in the 1960s [4] and now present worldwide, causes embryonic death, stillbirths and mummification when embryos or fetuses in seronegative dams are infected. The newly identified porcine parvoviruses have been detected in various areas of the world, but its relationship with any diseases remains unclear. PPV is thought to be one of the cofactors for porcine circovirus associated disease (PCVAD) whose main etiologic agent is porcine circovirus type 2 (PCV2) [1, 10, 13]. The PCV2 infection alone does not cause a clinical disease, but concurrent viral or bacterial infections may augment the severity of PCVAD possibly through stimulating the PCV2 replication or suppressing the PCV2 clearance by altered cytokine regulation [8, 9, 18].

During our screening for known viral genomes and newly identified porcine parvovirus genomes in specimens of apparently healthy pigs, we found and now report that the genomes of PCV2 and the classical and new porcine parvoviruses were coincidentally detected. The 5 porcine parvoviruses we studied include PPV [4], PPV2 [11], PPV3 [14], PPV4 [5] and porcine bocavirus-like virus (PBo-like V) [2]. According to the proposed taxonomy of the family Parvoviridae [6], most of the virus names have been changed as indicated in Table 1. However, we use the previous abbreviations in this paper to avoid confusion.

MATERIALS AND METHODS

Sample collection and viral nucleic acid purification: Tonsil specimens from 120 pigs were collected from a slaughterhouse in 2010 when most of the pigs were probably not injected with the inactivated PCV2 vaccine in Japan. The pigs were about 6 months old and obtained from 22 farms with 1–10 samples per farm. The procedures for the viral DNA and RNA isolation were previously described [23]. Briefly, the tonsil homogenates were prepared using a Micro Smash machine (Tomy Seiko, Tokyo, Japan), and after centrifugation at 15,000 g for 15 min, aliquots of the supernatant were stored at −80°C. The viral DNA and RNA were isolated by a DNA/RNA purification machine, Magtration System 6GC (Precision System Science, Chiba, Japan) and a solution kit, GC series Magtration-MagaZorb RNA Common Kit (Precision System Science). The isolated nucleic acids were reverse-transcribed by Superscript II reverse transcriptase and primers of random hexamers according to the manufacturer’s instructions (In-
vitrogen, Carlsbad, CA, U.S.A.) and used as templates for
the various PCRs detecting the genomes of both the DNA
and RNA viruses. For the validation of reverse transcription-
PCR assay, control RNA and PCR primers of the kit were
used in each experiment.

**Detection of viral genome by PCR**: Two multiplex PCRs
for 3 DNA viruses (porcine circovirus type 2 (PCV2), suid
herpesvirus 1 and porcine parvovirus (PPV)) and 6 RNA
viruses (porcine reproductive and respiratory syndrome
virus (PRRSV), Japanese encephalitis virus, porcine rotavi-
rus A (PoRV-A), porcine epidemic diarrhea virus (PEDV),
transmissible gastroenteritis virus (TGEV) and Getah virus)
were performed in separate tubes according to the published
method [17]. Other PCR primer pairs included; NP1200 and
NP1529 for swine influenza virus [15], HE5-1 and HE5-4m
for hepatitis E virus [26], Q1 F and Q2 R for porcine parvo-
irus 2 [11], PPV3 F and PPV3 R for porcine parvovirus 3
[25], PPV4 F and PPV4 R for porcine parvovirus 4 [25], and
SbocaF and SbocaR for PBo-likeV [32].

Viral genomes were amplified by PCR using Quick Taq
HS DyeMix (Toyobo, Osaka, Japan) including Taq poly-
merase. The PCR consisted of an initial enzyme activation
step at 94°C for 5 min, followed by 35 cycles of denaturation
at 94°C for 30 sec, annealing at 55°C for 30 sec, extension
at 72°C for 30 sec and a final extension at 72°C for 7 min.

**Phylogenetic analysis**: For the phylogenetic analysis of
PPV3, the 622 bp fragment of the VP gene were examined after ampli-
ifying the 713 bp fragment of the VP region (nucleotide posi-
tions from 3,359 to 4,072 of the strain AB916464) with the PCR
primers PPV3 P7F (3′-GGGGCACTCATTTCTCTGTAT-5′)
and PPV3 P7R (3′-CTGGCCTTTTCCACTTAGGA-5′) [25]
and sequencing with both primers and the two internal prim-
ers PPV3 7F2 (3′-GGAGAATAATGTTCTTCCTC-5′) and
PPV3 7R2 (3′-TCGTAATCATTAGAAGCCTG-5′).

The sequence data and phylogenetic tree were compiled
and analyzed using MEGA 5.1 [27] and Genetyx (Genetyx
Co., Tokyo, Japan). The phylogenetic trees were generated
by the maximum likelihood method.

The partial sequences of the PCR products of PPV3 have
been deposited in DDBJ under accession numbers from
LC011459 to LC011478.

**Statistical analysis**: Chi-square tests were used to evalu-
ate the statistical significance of co-isolation of two viral
genomes among PPV, PPV2, PPV3, PPV4, PBo-likeV and
PCV2 by dividing two categories, PCR-positive and PCR-
negative individuals for each virus. *P* values of <0.05 were
considered statistically significant.

**RESULTS**

**Prevalence of porcine parvoviruses and porcine circo-
virus 2 in 120 Japanese pigs**: We previously analyzed the
prevalence of the PPV2 genomes in the tonsil specimens
from 120 pigs [23]. With the same specimens, we extended
such a screening for 14 other viral genomes as listed in Table
1. Five of the 14 viral genomes were detected; four were
members of the family Parvoviridae and another one was
PCV2. The prevalences were 67% for PPV, 39% for PPV3,
33% for PPV4, 55% for PBo-likeV and 80% for PCV2 (Table
1), in addition to 58% for PPV2 [23]. Multiple viral genomes
were detected from the individual pigs, and thereby, as for
the 5 examined porcine parvoviruses, 3%, 23%, 53%, 78%
and 93% of the pigs were positive for more than 5, 4, 3, 2
and 1 virus (es), respectively. Only 9 pigs of various farms were
negative for the 5 parvovirus DNAs, and 4 of the 9 pigs were
negative for PCV2 DNA. Among the 8 farms with larger
sample numbers (8 to 10 samples per farm), 7 farms were
positive for all 5 parvoviruses, and one farm was negative
(0/10) for only one (PPV4) of the 5 parvoviruses. The results
suggested that a high proportion of the pigs in most farms
were co-infected with the five parvoviruses and PCV2.

We tested the possibility that the detections of these high-
ly prevalent viruses were random or coincidental. The chi
square analyses indicated that PCV2 was coincidentally de-

<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>porcine parvovirus (porcine parvovirus)</td>
<td>PPV</td>
<td>67</td>
</tr>
<tr>
<td>porcine parvovirus 2 (porcine Cn virus)</td>
<td>PPV2 (CaP-PARV4)</td>
<td>58</td>
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<td>porcine parvovirus 3 (porcine hokovirus)</td>
<td>PPV3 (P-PARV4)</td>
<td>39</td>
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<td>porcine parvovirus 4 (porcine parvovirus 4)</td>
<td>PPV4 (PPV4)</td>
<td>33</td>
</tr>
<tr>
<td>porcine boca-like virus (porcine bocavirus 7)</td>
<td>PBo-likeV (PBoV7)</td>
<td>55</td>
</tr>
<tr>
<td>porcine circovirus 2</td>
<td>PCV2</td>
<td>80</td>
</tr>
<tr>
<td>suid herpesvirus 1</td>
<td>SuHV1</td>
<td>0</td>
</tr>
<tr>
<td>hepatitis E virus</td>
<td>HEV</td>
<td>0</td>
</tr>
<tr>
<td>swine influenza virus</td>
<td>SIV</td>
<td>0</td>
</tr>
<tr>
<td>porcine reproductive and respiratory syndrome virus</td>
<td>PRRSV</td>
<td>0</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>JEV</td>
<td>0</td>
</tr>
<tr>
<td>porcine epidemic diarrhea virus</td>
<td>PEDV</td>
<td>0</td>
</tr>
<tr>
<td>porcine rotavirus A</td>
<td>PoRV-A</td>
<td>0</td>
</tr>
<tr>
<td>transmissible gastroenteritis virus</td>
<td>TGEV</td>
<td>0</td>
</tr>
<tr>
<td>Getah virus</td>
<td>GETV</td>
<td>0</td>
</tr>
</tbody>
</table>

a) New names recently proposed for the family Parvoviridae [6]. The prevalence of PPV2 was previously described [23], but for convenience, the data were included in this table.
DETECTION OF PORCINE PARVOVIRUS GENOMES

As extracted, the text refers to the detection of porcine parvoviruses (PPV) and their prevalence among pigs. The study examines the tonsil specimens of 120 apparently healthy pigs for the screening of 15 viruses that can infect pigs. Only the five porcine parvoviruses, i.e., PPV, PPV2, PPV3, PPV4, and PBo-likeV, and PCV2 were detected, and their prevalences were quite high, ranging from 33% to 80% (Table 1) [23]. The high prevalences of the classical PPV and PCV2 at the age of about 6 months are common in most pig-producing countries, whereas the prevalences of PPV3, PPV4, and PBo-likeV are the first observations in Japanese pigs.

The PPV3 DNA was detected in 39% of the 120 Japanese pigs (Table 1). Since the first identification of PPV3 [14], the prevalence has been reported in several countries and appears to widely vary from lower frequencies (6–20%) in Hungary [7], China [21], the U.S.A. [29] and Germany [25] to higher frequencies (44–73%) in Hong Kong [14], China [16] and Thailand [22].

The prevalence of the PPV4 genome was 33% in this study which is comparable to the prevalence (44%) in Thailand [22], but higher than those of several other countries, that is, 1% in China [12], 6% in Hungary [7], 7% in Germany [25] and 4% in the U.S.A. [30].

PBo-likeV [2], which was also called PBoV (PBoV-SX) [31] or PBoV1 [24, 33], is one of several porcine bocaviruses which have recently been discovered [28]. The PBo-likeV infection was initially supposed to be associated with respiratory tract diseases in pigs due to the remarkable difference in the prevalences between sick (39% (74/191)) and healthy (7% (3/41)) pigs [32]. The prevalence of PBo-likeV was 55% in our study (Table 1), in contrast to 18% in Thailand [22], 7% in China [32], 63% in different areas of China [24], 2% in Romania [7] and 13% in the wild boars of Romania [3].

Although the prevalences of PPV3, PPV4 and PBo-likeV show some variation among countries, the available data suggest that these newly identified parvoviruses have already spread worldwide.

The phylogenetic analysis of PPV3 suggested that, compared to the variation of 87 sequences deposited from around the world, the 20 Japanese sequences were less variable and belonged to limited branches (Fig. 1). In the 4 farms we analyzed, 2 farms appeared to have heterogeneous PPV3s (Table 3 and Fig. 2). Although the variations within the farms were

<table>
<thead>
<tr>
<th>Relationship between two viruses</th>
<th>Number of pigs</th>
<th>$\chi^2$ value</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPV / PPV2</td>
<td>$+/+$</td>
<td>47</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>PPV2</td>
<td>$+/+$</td>
<td>33</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>PPV3</td>
<td>$+/+$</td>
<td>32</td>
<td>7</td>
<td>48</td>
</tr>
<tr>
<td>PBo-likeV</td>
<td>$+/+$</td>
<td>46</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>PCV2</td>
<td>$+/+$</td>
<td>69</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>PPV2 / PPV3</td>
<td>$+/+$</td>
<td>28</td>
<td>19</td>
<td>41</td>
</tr>
<tr>
<td>PPV4</td>
<td>$+/+$</td>
<td>25</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>PBo-likeV</td>
<td>$+/+$</td>
<td>39</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>PCV2</td>
<td>$+/+$</td>
<td>60</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td>PPV3 / PPV4</td>
<td>$+/+$</td>
<td>15</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>PBo-likeV</td>
<td>$+/+$</td>
<td>28</td>
<td>38</td>
<td>19</td>
</tr>
<tr>
<td>PCV2</td>
<td>$+/+$</td>
<td>42</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>PPV4 / PBo-likeV</td>
<td>$+/+$</td>
<td>26</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>PCV2</td>
<td>$+/+$</td>
<td>32</td>
<td>64</td>
<td>7</td>
</tr>
</tbody>
</table>

a, *: significant (0.01<P<0.05). Others without asterisk mean not significant (P>0.05).

**DISCUSSION**

The present study, together with our previous study [23], examined the tonsil specimens of 120 apparently healthy pigs for the screening of 15 viruses which can infect pigs. Only the five porcine paroviruses, i.e., PPV, PPV2, PPV3, PPV4 and PBo-likeV, and PCV2 were detected, and their prevalences were quite high, ranging from 33% to 80% (Table 1) [23]. The high prevalences of the classical PPV and PCV2 at the age of about 6 months are common in most pig-producing countries, whereas the prevalences of PPV3, PPV4 and PBo-likeV are the first observations in Japanese pigs.

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not high, this raises the possibility that the observed variation within a farm resulted from multiple invasions of different strains rather than natural mutations within a farm after the invasion of one strain. The coexistence of different strains in a farm and coinfection of a pig with different strains must be risk factors for vaccine strategies and generation of a new recombinant virus strain.

The PCV2 genome was detected at a high frequency (80%) (Table 1) which is common worldwide. Interestingly, PCV2 was coincidentally detected along with PPV, PPV2 or PPV3, and PPV and PPV4 were also coincidentally detected (Table 2). These associations were weak, but statistically significant ($0.01 < P < 0.05$). Recently, similar associations were observed in pigs with PCVAD; the prevalences of the PPV and PPV2 DNAs were significantly higher in the

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Fig. 1. The phylogenetic tree was constructed, based on the 622 bases of the PPV3 VP gene, with the 20 Japanese PPV3s and 87 PPV3s currently deposited in the data bank. For the Japanese sequences, the 6 tentative sequence groups (Sequence groups 1–6) were defined by phylogenetic branch and % nucleotide difference, i.e., $<0.3\%$ (2/622) within each sequence group. The relationship between the farm and the sequence group of the detected PPV3 sequences is indicated in Table 3.
PCV2 is recognized as a causative agent of PCV AD. The clinical features of PCV AD or formerly called postweaning multisystemic wasting syndrome (PMWS) caused by PCV2 are systemic including enlargement of the lymph nodes, progressive loss of body weight or wasting combined with difficulty in breathing, diarrhea, pale skin and jaundice [9, 19]. The histopathologic changes in the affected lymphoid tissues are a severe lymphoid depletion, a diffuse infiltration of histiocytic cells and various inflammatory lesions. The pathogenesis of PCV AD or PCV2-induced diseases is complex, probably involving PCV2 infection and cofactors, such as other infections and altered cytokine or immune responses [8]. Particularly, the concurrent infection of PCV2-infected pigs by viruses (PPV, PRRSV, etc.), bacteria (*Mycoplasma hyopneumoniae*) or parasites may not be only a secondary infection after PCV2-induced depletion of lymphocytes, but could be important for the disease manifestation [18]. The experimental inoculation with PCV2 and PPV, but not PCV2 alone, could reproduce lesions similar to those of the field cases of PMWS [1, 10, 13]. The mechanism for the synergistic effect of coinfection was proposed that coinfection may promote the PCV2 infection by stimulating immune cells and providing target cells for the PCV2 replication or suppressing the PCV2 clearance by alteration of the cytokine production and profiles [1, 18].

The coincidental detections of PCV2 and PPVs in various combinations have been observed in both pigs with PCVAD at 3–5 months old and healthy pigs at about 6 months old. Therefore, the two stages may share a common mechanism for the proliferation of these viruses regardless of the presence or absence of PCVAD. Since circovirus and parvovirus are both DNA viruses, which require actively proliferating cells for efficient viral replication, lymphoproliferation or immunosuppression induced by infection with a virus could support the growth of other viruses.

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