Genotype Detection of Circovirus Type 2 (PCV2) in Swine Clinical Samples from Brazil

Rovian Miotto  
Federal Institute of Education, Science and Technology of Santa Catarina State

Caroline Pissetti  
Animal Health Diagnostic Center (CEDISA)

Luiz Carlos Bordin  
Embrapa Swine and Poultry

Janice Reis Ciacci Zanella (janice.zanella@embrapa.br)  
Embrapa Swine and Poultry

Research Article

Keywords: Genotyping, Circovirus, PCV2a, PCV2b, PCV2d

Posted Date: August 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3276107/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Porcine circovirus disease (PCVD) is one of the most impactful diseases in commercial pig production. It is caused by the infection of a small, circular DNA virus, porcine circovirus type 2 (PCV2). Currently, four types of PCV have been identified: PCV1, PCV2, PCV3, and PCV4. PCV2 is classified into 9 different genotypes (a-i). Although there is widespread use of commercial vaccines for PCV2 and PCVD is not a new disease, its presentation has changed. In addition, PCV2 has also evolved genetically, requiring monitoring of vaccinated herds for clinical and genotypic aspects. The objective of this study was to identify genotypes of PCV2, namely, PCV2a, PCV2b, and PCV2d. Additionally, the study aimed to identify coinfections of different PCV2 genotypes and PCV3 coinfections. Herein, 333 PCVD clinical samples of pigs that tested positive for PCV2 from various tissues, states in Brazil, and production phases were analyzed. Of those, 266 were genotyped, and PCV2b was the most frequent genotype found in 56.77% of positive samples, mainly in nursery samples. However, PCV2d was present in 33.10% of the samples, mainly from finishing pigs and breeding sows. The PCR used in this study was compared to a commercial kit and was effective in PCV2 genotyping. This study shows that it is important to genotype PCV2, and conventional PCR demonstrated a good option for this. PCV2b remains the most prevalent in pig farms in Brazil and continues to cause disease. It is necessary to state that in Brazil, the most commonly used commercial vaccines are for PCV2a. Even though PCV2a vaccines provide heterologous protection for other genotypes, the adequate diagnosis and study of the need for updating vaccines for the genotypes currently circulating must be considered.

1 Background

Porcine circovirus type 2 (PCV2) is the etiological agent of porcine circovirus-associated disease (PCVD). PCV2 was identified in 1998 in tissues of pigs suffering from multisystem postweaning wasting syndrome (PMWS) [1], [2], a syndrome initially recognized in pigs in high-health Canadian herds during 1995[1]. Since then, PCV2 has been associated with several clinical manifestations, including systemic, enteric, respiratory, and reproductive diseases and porcine dermatitis and nephropathy syndrome, which are now known as PCV2-associated disease, PCVAD or PCVD [3]. PMWS is the most prevalent and severe clinical manifestation of PCV2 infection [4], being characterized by loss and severe lesions in lymphoid tissue, classified as systemic or subclinical [5]. Other clinical conditions associated with PCV2 infection include reproductive failure, dermatitis and nephropathy, and respiratory and enteric diseases. Despite the high worldwide prevalence and economic impact, it can be controlled by correcting risk factors and using vaccines [5].

PCV2 belongs to the family Circoviridae, genus Circovirus [1], and it is the smallest single-stranded DNA virus with autonomous replication and nonenveloped and circular symmetry [6]. Its genome has 1767 to 1768 nucleotides and contains two main ORFs located on opposite strands, ORF-1 and ORF-2, which encode proteins related to replication and capsid, respectively [9]. Due to the high genomic variability identified in PCV2, isolates have been divided into nine genotypes – PCV2a to PCV2i – based on ORF-2 or complete genome sequences [10] [11]–[15].
The evolution of the PCV2 genotype in recent years was marked by severe outbreaks of PCVD, reported in North America during 2004–2006, and the genomic composition of PCV2 isolates present in affected herds and of previously found isolates were compared [3], [16], [17]. Using the restriction fragment length polymorphism pattern (RFLP) technique, a new RFLP 321 pattern was identified in newer PCV2 isolates compared to the previously dominant RFLP 422 viruses [16]. Later, in North America, China and many swine-producing countries, a new PCV2 variant was detected in PCV2-vaccinated herds [18]–[20]. This evolution was associated with PCV2 vaccine failures. In Brazil, a PCV2 mutant associated with vaccine failures was first identified in 2012, and the viral strain was almost identical to that in China between 2004 and 2008 [21]. The virus was isolated from pigs vaccinated against PCV2 that showed typical clinical signs of PCVD and positive laboratory diagnosis for PCV2. Genomic analyses indicated additions or substitutions of nucleotides generating modifications in the genome of the virus, called mPCV2, which in turn led to changes in amino acids located in epitopes responsible for the activation of the immune system. The variant presented three extra amino acids at the end of ORF2 (viral capsid protein) [22].

Reducing the dissemination and severity of the disease, vaccination and the use of risk control measures are the most effective actions to control circovirus in pigs [23]. There are several commercial vaccines currently available, most of which are based on genotype “a” (PCV2a) of porcine circovirus [24]. However, with the increase in circulating genotypic diversity, the need for more effective vaccines with updates and/or inclusion of genotypes in vaccines is being questioned [25].

Considering the losses caused by PCV2 infection to pig production and the resulting economic impact, as well as the continued detection of PCV2 in vaccinated herds, the objective of this work was to characterize PCV2 genotypes to estimate the presence of PCV2a, PCV2b, and PCV2d in the field, the presence of coinfections among genotypes, and which genotype was most present in coinfections with PCV3 as a quick and effective diagnostic measure presenting a low cost.

2 Results

PCV2a, PCV2b, or PCV2d were detected in 266 out of the 333 analyzed samples that were submitted to genotyping, and genotyping was not possible in 67 samples, as shown in Table 1. Of all the genotyped samples, 151 were positive for PCV2b (51.7%), 88 tested positive for PCV2d (30.1%), and 27 samples presented coinfection of two genotypes (PCV2b and PCV2d) (9.2%) (Table 1). No sample tested positive for PCV2a.

Table 1. Percentage and type of PCV2 genotyped samples
<table>
<thead>
<tr>
<th>Sample or tissue</th>
<th>Number of analyzed samples</th>
<th>Positive for PCV2b</th>
<th>Positive for PCV2d</th>
<th>Positive for PCV2b and PCV2d (coinfection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organs (pool)</td>
<td>52</td>
<td>41 (78.85%)</td>
<td>9 (17.30%)</td>
<td>2 (3.85%)</td>
</tr>
<tr>
<td>Brain</td>
<td>2</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Fetuses</td>
<td>40</td>
<td>29 (72.5%)</td>
<td>10 (25%)</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>Feces</td>
<td>3</td>
<td>3 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Intestine</td>
<td>29</td>
<td>13 (44.82%)</td>
<td>13 (44.82%)</td>
<td>3 (10.34%)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>6</td>
<td>6 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Lungs</td>
<td>44</td>
<td>28 (63.63%)</td>
<td>13 (29.54%)</td>
<td>3 (6.81%)</td>
</tr>
<tr>
<td>Kidney</td>
<td>3</td>
<td>2 (66.67%)</td>
<td>0 (0%)</td>
<td>1 (33.33%)</td>
</tr>
<tr>
<td>Serum</td>
<td>81</td>
<td>23 (28.4%)</td>
<td>43 (53.1%)</td>
<td>15 (18.51%)</td>
</tr>
<tr>
<td>Rectal swab</td>
<td>3</td>
<td>1 (33.33%)</td>
<td>0 (0%)</td>
<td>2 (66.67%)</td>
</tr>
<tr>
<td>Uterus</td>
<td>3</td>
<td>3 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 1. Percentage and type of PCV2 genotyped samples

PCV2b was detected most frequently in brain samples (2/2), lymph nodes (6/6), feces (3/3), and uterus (3/3) among the different received samples. On the other hand, serum samples were the most frequently positive for PCV2d, with 43 positive samples out of 81 total positive samples. Therefore, it can be observed that PCV2b is distributed in all types of received samples. Rectal swab samples had the highest incidence of coinfection between PCV2b and PCV2d genotypes.

The state with the highest positivity rate was Mato Grosso do Sul, where genotyping was possible in 100% of the samples (Fig. 1). Most of the samples were received from the three southern states (Paraná, Rio Grande do Sul, and Santa Catarina), which represent the largest swine production in the country. Regarding genotype distribution by state, PCV2b was most frequently detected in samples from Paraná state (61.99%), while PCV2d was more frequent in samples from São Paulo state. However, it was in Mato Grosso do Sul where the highest rates of coinfection between the two genotypes were found.

Figure 1. PCV2 genotype detection per sample by each Brazilian state, showing the most prevalent genotype in each sample.

Regarding the production phase, the largest number of genotyped samples was for the finishing phase, in which PCV2d was the most frequently detected genotype in 62 samples, while PCV2b was detected in 41 samples. In the nursery phase, the most prevalent genotype was PCV2b, present in 51 samples, while
PCV2d was detected in 11 samples. The highest rates of coinfection of PCV2b and PCV2d were found in the finishing phase samples, a total of 20 samples. The highest rates of PCV2b were found in samples that were not informed about the production phase they came from, and PCV2d was more prevalent in sow samples, as shown in Table 2.

Table 2. PCV2 genotype detection per production phase, with the most positive genotypes and the total number of positive samples.

<table>
<thead>
<tr>
<th>Production Phases</th>
<th>Number of analyzed samples</th>
<th>Positive for PCV2b</th>
<th>Positive for PCV2d</th>
<th>Positive for PCV2b and PCV2d (coinfection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replacement pigs</td>
<td>12</td>
<td>8 (66.67%)</td>
<td>1 (8.33%)</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Sows</td>
<td>9</td>
<td>3 (33.33%)</td>
<td>5 (55.56%)</td>
<td>1 (11.11%)</td>
</tr>
<tr>
<td>Gestation sows</td>
<td>4</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Farrowing sows</td>
<td>24</td>
<td>17 (70.83%)</td>
<td>6 (25%)</td>
<td>1 (4.16%)</td>
</tr>
<tr>
<td>Nursery pigs</td>
<td>64</td>
<td>51 (79.68%)</td>
<td>11 (17.18%)</td>
<td>2 (3.12%)</td>
</tr>
<tr>
<td>Finishing pigs</td>
<td>119</td>
<td>42 (35.3%)</td>
<td>57 (47.9%)</td>
<td>20 (16.8%)</td>
</tr>
<tr>
<td>Uninformed</td>
<td>34</td>
<td>28 (82.35%)</td>
<td>6 (17.65%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 2. PCV2 genotype detection per production phase, with the most positive genotypes and the total number of positive samples.

2.1 Occurrence of PCV2 genotypes and/or PCV3 Coinfections

Twenty-seven samples showed coinfection between PCV2b and PCV2d genotypes, representing 9.2%. However, no other coinfection between PCV2 genotypes was found, and most of the coinfections were found in samples obtained from swabs, representing 66.67% of positive samples. Moreover, coinfections with PCV3 were shown in 26 samples for both PCV2 and PCV3, which represent 8.9%. Samples from fetuses showed the highest positivity for coinfection between PCV2 and PCV3. In addition, a higher presence of "b" genotype samples was observed in these coinfections, with 20 positive samples for PCV2b and 5 positive samples for PCV2d. Only one sample showed positivity for coinfection between PCV2b, PCV2d, and PCV3.

2.2 Assessment of the agreement between the two tests for PCV2 genotyping
A total of 18 samples were tested with the commercial kit (Kylt® PCV-2 Typing kit) and conventional PCR, and the results showed that most of the samples presented coinfection between the genotypes (Table 3).

**Table 3.** Comparison between the two PCV2 genotyping tests

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conventional PCR</th>
<th>Commercial Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCV2b</td>
<td>PCV2b</td>
</tr>
<tr>
<td>2</td>
<td>PCV2b</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>PCV2b and PCV2d</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>7</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>9</td>
<td>PCV2b and PCV2d</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>11</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>12</td>
<td>PCV2b</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>PCV2b</td>
<td>Negative</td>
</tr>
<tr>
<td>14</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>15</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>16</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>17</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
<tr>
<td>18</td>
<td>PCV2b and PCV2d</td>
<td>PCV2b and PCV2d</td>
</tr>
</tbody>
</table>

The results were submitted to the Kappa test, with the objective of evaluating the concordance between the tests, and the test results are exemplified in Table 4.

**Table 4.** Kappa statistical test showing the accuracy of the test.
Table 4. Kappa statistical test showing the accuracy of the test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>KAPPA</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2b</td>
<td>0.478 (0.094 – 0.863)</td>
<td>77.8</td>
</tr>
<tr>
<td>PCV2d</td>
<td>0.658 (0.308 – 1.000)</td>
<td>83.33</td>
</tr>
<tr>
<td>PCV2</td>
<td>0.478 (0.094 – 0.863)</td>
<td>77.8</td>
</tr>
</tbody>
</table>

Conventional PCR was more sensitive for some samples; therefore, 4 samples were genotyped, while in the commercial kit, it was not possible to characterize the genotypes in the analyzed samples. In only one sample (sample 2) was there discrepancy between the commercial kit and conventional PCR, where the commercial kit identified a coinfection (PCV2b and PCV2d) and the PCR test only found PCV2b. It was possible to analyze the data between the genotypes and the two tests. The PCV2b test and the general PCV2 (conventional PCR and commercial kit) must be observed and showed the same accuracy (77.8). In the PCV2d test, the accuracy was higher, at 83.33. The kappa test showed a confidence interval greater than 0; thus, it shows us that the two tests are capable of identifying the genotypes and show agreement between them. However, the largest concordance was found in the identification of PCV2d, as it presented an accuracy and a larger confidence interval.

3 Discussion

PCV2 infection has a significant impact on the swine industry, leading to various losses for producers, with subclinical disease being of paramount importance [36]. There are nine recognized PCV2 genotypes: PCV2a, PCV2b, PCV2c, PCV2d, PCV2e, PCV2f, PCV2g, PCV2h and PCV2i [11], [44], [45]. Among these, PCV2a, PCV2b, and PCV2d are currently considered of greater importance and worldwide distribution, with PCV2d being the current predominant genotype [7], [46]. The other groups, including PCV2c, PCV2e, PCV2f, PCV2g, and PCV2h, largely represent small clusters (few isolates) of probably minor significance [47]. PCV2e, in particular, its viral structure contains 12 or 15 extra nucleotides at ORF2 sequences compared to those of PCV2a, PCV2b, and PCV2d. Due to the presence of these extra nucleotides at the 3' end of ORF2, PCV2e was considered a progenitor of PCV2a, 2b, and 2d. This distinct genetic characterization may affect the efficiency of PCV2e in vivo replication and deserves further investigation [47].

Herein, we analyzed clinical PCVD diagnostic samples that previously tested positive for PCV2 at the CEDISA laboratory. In our study, 79.88% (266/333) of the samples were genotyped for PCV2. The results demonstrated that PCV2b was the most prevalent, detected in 151 out of 266 analyzed samples, which is in agreement with several studies worldwide that demonstrate that PCV2b and PCV2d are currently the most prevalent genotypes of PCV2 infection in pigs [18], [37], [41]. Among them, a study conducted in Australia where this virus was detected in 7 out of 17 analyzed samples [41] and another study conducted in Austria in 2020 by [42] showed a higher prevalence of PCV2b in a retrospective report from 2002 to 2017, where PCV2b was found in all studied years, as well as in the retrospective study from
2015 to 2018 by [37] in China. Furthermore, our study showed that PCV2b and PCV2d genotypes were found in analyzed samples from all Brazilian states studied. This finding is similar to a study conducted by [43], where these two genotypes were found in all Chinese provinces analyzed. Previous studies have shown a shift in the most prevalent genotypes in pig production over the years [17] from the PCV2a genotype to PCV2b in 2002 [48], [49], and in 2012, a shift occurred from PCV2b to PCV2d [34], [50]. Since then, PCV2d has become the most prevalent in various regions of the world, such as China [37], South Korea [51], Austria [42], the United States [13], Colombia [52], and the Dominican Republic [53].

The presence of coinfections between genotypes (PCV2b and PCV2d) is becoming increasingly frequent, which was observed here in 27 samples. Previously published data [54] observed coinfections between the PCV2b and PCV2d genotypes in wild boars and domestic pigs in Ukraine. It was also possible to observe coinfection with PCV3 in our study in a total of 26 samples. Again, this result is related to other studies around the world, in which it was also possible to identify coinfection between PCV2 and PCV3 genotypes [33], [43], [55]. However, none of them relate to which PCV2 genotype was the most prevalent in coinfections with PCV3.

Lymph node samples provide a good indication of how PCV2 infection is in pig farms [22]. In our study, genotyping was possible for all lymph node samples received. In addition to careful diagnosis at the time of animal evaluation, the use of high-quality samples is of utmost importance. PCV2b was detected in all types of samples received, suggesting that this genotype causes systemic infection in animals. On the other hand, PCV2d was detected only in organ, lung, fetus, intestine, and blood serum samples. An important finding observed in this study was the identification of PCV2b in brain samples from finishing pigs. Brain lesions caused by PCV2 can be occasional [58]. In our study, it was not possible to correlate the clinical symptoms presented by the animals to the genotype of PCV2 detected.

PCV2d was the most common genotype in the finishing phase. This result is consistent with a study conducted in South Korea, in which [59] found a higher prevalence of PCV2d in pig lymph nodes at slaughter. On the other hand, in the nursery phase, PCV2b was the most common genotype. To our knowledge, this is the first study to find these data and seek a correlation between genotype and production phase. The finishing phase was the phase in which the most positive samples for PCV2 could be obtained and genotyped. This is probably due to the drop in vaccine immunity and maternal immunity, making pigs more susceptible to PCV2 infections [22].

Brazil is the fourth world pork producer, and PCV2 infection occurs broadly in Brazilian pig farms. Thus, PCVD is broadly controlled by commercial vaccines. Since the first detection of PCV2 infection associated with PCVD and reproduction of the disease, the evolution of the disease presentation and PCV2 genotype detection have been shown. A study of Brazilian swine herds analyzed clinical and PCV2-positive samples in 2019 from commercial pig farms from South, Southeast and Midwest Brazil [60]. The pig herds previously vaccinated against PCV2 presented clinical signs compatible with PCVD. A total of 75.47% was identified as PCV2d, 22.64% as PCV2b and 1.89% as coinfection of PCV2b and PCV2d [60]. There was also no detection of the PCV2a genotype. PCV2 clinical disease in vaccinated swine herds
leads to the question of virulence variation. Specially after the new genotype identification and
differences on disease presentation. However, only a single study comparing the three main PCV2
genotypes side by side found differences in virulence. Nonetheless, this was not repeatable by other
groups. Recently, a comparison of the virulence of PCV2a, PCV2b and PCV2d was made in a model of
PCV2 coinfection and porcine reproductive and respiratory syndrome virus (PRRSV). Interestingly, in pigs
coinfected with PRRSV, PCV2d appeared to be more virulent than PCV2a and PCV2b, which was evident
by an increased serum PCV2 load and a greater severity of lymph node depletion [61]. When the same
study was repeated with Mycoplasma hyopneumoniae and concomitant infection of PCV2a, PCV2b or
PCV2d, the results demonstrated once more that PCV2d was more virulent in the context of coinfection,
as measured by serum PCV2 load and severity of lymphoid lesions [62]. In pigs inoculated at 42 days of
age and then necropsied at 63 days of age, there were no significant differences between the groups of
genotypes evaluated. Pigs inoculated with PCV2a, PCV2b or PCV2d had significant levels of PCV2 loads
in the blood and lymph nodes compared to pigs inoculated with PCV2e. The results of this study
indicated that PCV2a, PCV2b, and PCV2d are more virulent than PCV2e based on PCV2 blood and
lymphoid viral load [63].

Other virulence studies compared PCV2 loads in the blood and lymph nodes and in the severity of
lymphoid lesions. The isolated infected groups, PCV2a, PCV2b and PCV2d, resulted in similar virulence to
each other, and all were more virulent than the PCV2e groups. Within the doubly infected groups, the
combination of PCV2d and PRRSV was more virulent than the other PCV2 genotypes (2a, 2b, and 2e),
each in combination with PRRSV [14]. Further studies have shown that the higher virulence of PCV2d
compared to the other PCV2 genotypes (2a, 2b, and 2e) can be attributed to an extra amino acid (lysine
residue) found in PCV2d ORF2.

PRDC is considered a multifactorial disease caused by the interaction of noninfectious components
(environment, management, age, genetics, and nutrition) with viral and bacterial pathogens. Worldwide,
the most relevant pathogens in PRDC are influenza A virus (IAV), PRRSV, Mycoplasma hyopneumoniae
(Mhyo), PCV2 and pyogenic bacteria [29]. A study of respiratory agents in 74 lungs submitted for
diagnosis at CEDISA showed that all lung samples were positive for IAV. Coinfection of IAV and Mhyo
was seen in 31% of cases, and 14.9% of the lungs were positive for PCV [29]. In fact, Mhyo potentiated
the severity of lymphoid lesions associated with PCV2 and increased the amount of PCV2 loads in the
blood and lymph nodes, regardless of the PCV2 genotype [36].

Since there is no consensus on virulence variation among genotypes, PCV2 infection and disease in
vaccinated herds remains unknown. There are several research studies on PCV2 vaccination and cross-
protection. Most of the published knowledge of cross-protection between PCV2 genotypes results from
the use of available vaccines (commonly commercial PCV2a vaccines) in commercial herds with
continuous PCV2 field infections. Based on this, there is good cross-protection for many field isolates,
such as PCV2b or PCV2d. Severe problems of PCVD in large percentages of pigs in a given herd are rare
in vaccinated pig populations today. However, there is evidence that certain PCV2 genotypes, such as
PCV2d, can replicate and increase prevalence in vaccinated herds and may be associated with clinical
PCV2 in swine herds [17], [64]. Many studies using PCV2a vaccines and cross-protection presented generally satisfactory results; that is, vaccinated pigs have a clear advantage over unvaccinated pigs. Such studies used a challenge with PCV2a [65], [66], PCV2b [65], [67] or PCV2d [56], [68]. However, a study that investigated chimeric live PCV1-2a and PCV1-2b found better protection against PCV2b challenge by the homologous vaccine [19], [68], possibly suggesting that homologous protection may be better than heterologous protection. Similarly, a field investigation was conducted in 2012 in the U.S. after PCV2a vaccination was used for approximately 5 years in almost all growing pigs [57]. Interestingly, PCV2b (9.9%) and non-PCV2a were found in the serum of vaccinated healthy pigs, while PCV2a and PCV2b were found in the serum of unvaccinated pigs [57]. In a recent field study, a PCV2d vaccine had some advantages over a PCV2a vaccine in protecting pigs from the simultaneous challenge of PCV2d/PRRSV 1-7-4 and endemic IAV and bacterial coinfections. In unvaccinated pigs, severe PCVD was reproduced [69].

Interestingly, by genotyping PCV2-positive samples from clinical cases, PCV2a was not detected. It is suggested that this is due to the impact of PCV2 vaccines and vaccination pressure [10], [70] favoring the emergence and selection of new genotypes, mainly PCV2b and PCV2d [42], [59]. Obviously, not all vaccines are equal, and there are differences among them, such as adjuvant and production technology, administration, doses, and protection coverage. However, for PCV2 infection, all commercial vaccines show protection, but their ability to control the disease and their use may vary.

4 Conclusion

PCV2 genotyping is important to know which is the predominant genotype in each farm. For this, in the detected analyses, conventional PCR proved to be more effective than commercial kits, making it a lower cost alternative for rapid diagnosis. PCV2b remains highly present in farms, but it was also possible to observe an increase in the detection of PCV2d in the evaluated samples. It is important to note the existence of coinfections between genotypes, including coinfection with PCV3. Based on these data, attention should be given to monitoring the efficacy of vaccines and the emergence of clinical circovirus disease. Thus, it is important to study the evolution of PCV2 into different genotypes and evaluate the update of existing vaccines in the market, considering the high mutation rate presented by the virus. The control of circovirus disease should combine the appropriate use of vaccines with biosafety and sanitary management measures, seeking to improve the health of pigs.

5 Methods

5.1 Samples

Three hundred thirty-three samples of suspected PCVD clinical cases submitted to the Animal Health Diagnostic Center (CEDISA) between 2021 and 2022 from eight states (Goiás Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Rio Grande do Sul, Santa Catarina and São Paulo) from 2021 to 2022 were used in this study. Samples from different organs and materials were collected according to clinical
condition and compatible with PCVD lesions. The samples included a total of 105 serum samples, 10 sow uterus samples, 4 rectal swab samples, 3 kidney samples, 50 lung samples, 6 lymph node samples, 31 intestine samples, 61 fetus samples (which included stillbirths and mummified fetuses), 2 brain samples, and 58 organ pool samples (liver, kidney, lung, spleen, lymph nodes and heart tissue). The samples were obtained through a technical-scientific agreement among the Embrapa Swine and Poultry Research Center and CEDISA (Agreement 21000.18/0009 – 7).

All samples were submitted to detect PCV2 and PCV3 through qPCR. For this, samples were pretreated overnight with ATL (Qiagen) and proteinase K (Qiagen) at 56°C under agitation. Viral DNA was extracted using an IndiMag Pathogen Kit (Indical Bioscience) with an automated extraction system by IndiMag 48 s (Indical Bioscience). The qPCR for the diagnosis of PCV2 was performed targeting the amplification of a Cap gene region using specific primers PCV2_F (5’-CCAGGAGGGCGTTGTGACT-3’) and PCV2_R (5’-CGCTACCCTTGAGAAGGAA-3’) and the probe PCV2_S (5’-AATGGCATCTTCAACACCGCCTCT-3’), as previously described [31]. For the qPCR, 2.5 µL of extracted DNA was added to a GoTaq® Probe qPCR master mix (Promega), 1.25 µM and 0.5 µM of specific primers and probe, respectively. The fluorescence signal was performed at the end of each extension cycle phase in the QuantStudioTM Flex 6 thermocycler (Applied Biosystems). All samples with Ct < 38.00 were considered positive. For PCV3, the conserved rep gene was targeted with the primers PCV3_535_F (5’-TGA CGG AGA CGT CGG GAA AT-3’) and PCV3_465_R (5’-CGG TTT ACC CAA CCC CAT CA-3’) and the probe PCV3_535_F (5’-TGA CGG AGA CGT CGG GAA AT-3’) as described previously (Franzo et al., 2018). For qPCR, 2 µL of extracted DNA was added to a mix of GoTaq Probe qPCR master mix (Promega), 0.8 µM and 0.4 µM of specific primer and probe, respectively. The cycling parameters were 95°C for 2 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute in QuantStudio Flex 6 (Applied Biosystems). The fluorescence signal was acquired at the end of each cycle extension phase.

All samples were submitted to detect PCV2 and PCV3 through qPCR. For this, samples were pretreated overnight with ATL (Qiagen) and proteinase K (Qiagen) at 56°C under agitation. Viral DNA was extracted using an IndiMag Pathogen Kit (Indical Bioscience) with an automated extraction system by IndiMag 48 s (Indical Bioscience). The qPCR for the diagnosis of PCV2 was performed as previously described [31] and for PCV3 by Franzo et al. (2018) in QuantStudio Flex 6 (Applied Biosystems).

5.2 Positive Controls

To find positive controls for the reaction, 20 samples were randomly selected and subjected to sequencing. The sequencing reaction was performed with primers previously described [32], aiming to amplify the ORF-2 region of PCV2. Following the genomic sequencing analysis, it was possible to select positive control samples for PCV2b and PCV2d genotypes. For PCV2a, a previously sequenced reference sample from the “Collection of microorganisms of interest to swine and poultry farming” (CMISEA) Embrapa Swine and Poultry was used [21]. After that, the sequenced samples were tested to validate the PCR tests. The result of the positive control test is exemplified in Fig. 2.

5.3 Genotyping using conventional PCR (cPCR)
Each sample was individually tested for each of the three PCV2 genotypes of interest. PCR was based on the ORF-2 of PCV2, the region that presents higher diversity rates. The sequences of the primers used are shown in Table 5. The primers were diluted to 10 pmol, and the PCR used 2.5 µl of buffer, 1 µl with 25 mmol of MgCl2, 1 µl of dNTPs, 0.6 µl of each primer, and 2 µl of DNA from each sample. The PCR conditions used were as follows: predenaturation at 94°C for 1 minute, 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes [33]. The PCR products were subjected to 1% agarose gel electrophoresis and visualized under UV light after staining with 2% ethidium bromide.

To differentiate the PCV2a and PCV2b genotypes because they have the same amplicon size, each sample was submitted separately to PCR, thus making it possible to differentiate the genotypes.

Table 5. Primers used in the genotyping study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2ab - F</td>
<td>GGT TGG AAG TAA TCA ATA GTG GA</td>
<td>277pb</td>
<td>[34]</td>
</tr>
<tr>
<td>PCV2a - R</td>
<td>GGG GAA CCA ACA AAA TCT C</td>
<td></td>
<td>[33]</td>
</tr>
<tr>
<td>PCV2ab - F</td>
<td>GGT TGG AAG TAA TCA ATA GTG GA</td>
<td>277pb</td>
<td>[34]</td>
</tr>
<tr>
<td>PCV2b - R</td>
<td>GGG GCT CAA ACC CCC GCT C</td>
<td></td>
<td>[35]</td>
</tr>
<tr>
<td>PCV2d - F</td>
<td>GGT TGG AAG TAA TCG ATT GTC CT</td>
<td>343 pb</td>
<td>[34]</td>
</tr>
<tr>
<td>PCV2d - R</td>
<td>TCA GAA CGC CCT CCT GGA AT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Primers used in the genotyping study.

5.4 Genotyping using qPCR

Eighteen samples were submitted to genotype detection using a commercial qPCR kit (KyIt® PCV-2 Typing kit) and compared with cPCR results in relation to sensitivity and specificity. The results were submitted to the Kappa test.

Declarations

**Ethics approval:** All samples used in this study were obtained from pigs submitted for diagnostic testing at the Animal Health Diagnostic Center (CEDISA) laboratory in Concordia, Brazil. The study did not involve any human samples; therefore, the study was not presented to ethics committees.

**Consent to participate:** All the authors agree to participate in this research.

**Consent for publication:** All the authors agree to publish the findings of this research.
Availability of data and material: All the material was obtained under Agreement 21000.18/0009-7 between Embrapa Swine and Poultry and CEDISA. All the data published here are available.

Conflict of interest/Competing interests: The authors declare that they have no conflicts of interest. competing interests.

Funding: Research funded by Brazilian Agriculture Research Corporation – Embrapa, Projects no. 22.16.05.008.00.01.005, 20.21.10.006.00.03.001, and Agreement 21000.18/0009-7.

Authors’ contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Rovian Miotto, Caroline Pissetti, Luis Carlos Bordin and Janice Reis Ciacci Zanella. The first draft of the manuscript was written by Rovian Miotto, and all authors commented on previous versions of the manuscript. All the authors have read and approved the final manuscript.

Acknowledgments: The authors thank the expert collaboration of Neide Simon for the laboratory support, Arlei Coldebella for the statistical analyses and FAPESC - Foundation for Research and Innovation Support of Santa Catarina State for RM's scholarship.

References


47. M. Sibila et al., “Genotyping of Porcine Circovirus 2 (PCV-2) in Vaccinated Pigs Suffering from PCV-2-Systemic Disease between 2009 and 2020 in Spain”, *Pathogens*, vol. 10, n° 8, p. 1016, ago. 2021, doi:


60. H. I. D. NASCIMENTO, “Genetic variability of new brasilian PCV2d strains and their relation to commercial vaccines”, Master’s degree in Veterinary Science, Universidade Federal de Minas Gerais,


Figures

Figure 1

PCV2 genotype detection per sample by each Brazilian state, showing the most prevalent genotype in each sample.
**Figure 2**

Positive controls for each genotype. PCV2a (277bp); PCV2b (277bp) and PCV2d (343bp) using a 100bp marker.