Genetic diversity of porcine circovirus 3 strains and the first detection of two different PCV3 strains coinfecting the same host in Minas Gerais, Brazil

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Abstract
Porcine circovirus 3 (PCV3) is a recently emerged circovirus discovered in 2016 that has drawn the attention of the swine industry worldwide. In this study, we evaluated the genetic diversity of PCV3 strains on pig farms. A total of 261 samples from sows, weaning pigs, growing pigs, and stillborn/mummified fetuses were analyzed by quantitative real-time PCR. The results revealed that at least two main lineages of PCV3 are circulating in Brazil. For the first time, it was possible to detect the presence of two different PCV3 strains in the same host.

Porcine circovirus 3 (PCV3) was discovered in 2016, in a survey using a metagenomics approach, in swine with clinical signs of porcine dermatitis and nephropathy syndrome, reproductive failure, and cardiac and multisystemic inflammation [1, 2]. Currently, four circoviruses are known to infect swine. PCV1 has not been associated with clinical disease. PCV2 is an economically significant pathogen associated with a diverse range of clinical diseases [3]. PCV3 and PCV4 emerged recently and have been detected in swine with several clinical diseases [1, 2, 4]. As a newly discovered member of the genus Circovirus, PCV3 has conserved elements in its genomic organization in common with members of other species; however, PCV3 is only distantly related to the other known circoviruses [5]. According to a previously proposed classification system, PCV3 strains can be divided into two clades [13]. Clade 1 (PCV3a) includes PCV3 sequences from different countries. Clade 2 included only two Chinese sequences, which could either represent recently emerged variants or the last descendant of previously circulating genotypes [13]. Researchers from many countries around the world have reported the detection of PCV3 in swine showing different clinical symptoms, and even in asymptomatic animals. The detection of PCV3 in apparently healthy swine could indicate subclinical infections [6], which has led to the question of whether PCV3 has clinical relevance in the field [7]. Considering the economic importance of PCV2 to the swine industry, PCV3, as an emergent pathogen and as a new member of the same family, should not be neglected.

This work aimed to detect PCV3 on several Brazilian farms using quantitative real-time PCR (qPCR) and sequencing to answer some questions about PCV3: (I) Is there genetic diversity among Brazilian PCV3 strains? (II) Is there a main PCV3 strain circulating among different Brazilian swine herds? (III) Is PCV3 found more frequently in fetuses from reproductive failure cases compared to other age groups?

We analyzed 261 swine samples (serum, vaginal swab, umbilical cord, intestine, spleen, liver, heart, lung, cerebrum, and lymph nodes). The samples were divided according to age group: sows (92), weaning pigs (17), growing pigs (65), and stillborn/mummified fetuses (87). These samples were
collected from healthy sows, weaning and slow-growing pigs, and fetuses in 2019 from 19 commercial farms located in Minas Gerais State, which is a crucial swine-producing state in Brazil.

Total DNA was extracted from samples using a Wizard SV Genomic DNA Purification System (Promega). To detect PCV3 and determine the viral load, we used qPCR primers and probes that have been described previously [1]. As an endogenous control, primers that amplified a region of 107 base pairs of the 18S ribosomal gene of swine were used [8]. One PCV3-positive sample, confirmed by Sanger sequencing, was used to obtain a standard curve. The amplicon of this positive sample was ligated into a cloning vector using a clone JET PCR Cloning Kit (Thermo Fisher). Samples with threshold cycle (Ct) values of ≤ 38 and with a typical amplification curve were considered positive. Data were analyzed statistically. A chi-squared test was used to evaluate the association between positive samples in different age groups, and ANOVA and Tukey’s multiple comparison tests were used to compare the viral load among different samples, using a p-value of < 0.05.

We sequenced 17 strains in this work. ORF2 sequences were obtained from positive samples (Supplementary Table S1) that were amplified by nested PCR using a combination of primers described previously [9]. The Sanger sequencing data were trimmed and assembled into contigs using CLC Genomics Workbench version 8.5.4 (QIAGEN) (Supplementary Table S1). Sequences were further clustered using the cd-hit-est tool of CD-HIT version 4.7 [10] to remove redundancy (Supplementary Table S2).

A dataset containing 17 ORF2 sequences of Brazilian PCV3 strains and 83 ORF2 sequences of reference PCV3 strains [11] from different countries was downloaded from GenBank (Supplementary Table S3). The ORF2 sequences were aligned using MAFFT version 7.307, and the polymorphisms identified were screened using MEGA version 10.1.6 (Supplementary Table S4).

To expedite the construction of a phylogenetic tree, the model HKY+G was chosen as the best-fit model of nucleotide substitution from the full alignment using jModelTest version 2.1.10 [12]. The phylogenetic tree was calculated using the Bayesian Markov chain Monte Carlo method in MrBayes 3.2.7a [13] in two runs with 5,000,000 generations. At the end of the procedure, the average standard deviation of the split frequencies was 0.008550. The chains reached a stationary distribution after 500,000 generations, and 10% of the trees generated were discarded as burn-in to produce the consensus tree, which was annotated using Iroki [14].

At the farm level, 78.94% (15/19) of the farms were PCV3 positive. At the animal level, 39.85% (104/261) of the swine tested were PCV3 positive. When we analyzed the age groups, 28.26% of sows (26/92), 35.29% of weaning pigs (6/17), 51.72% of stillborn/mummified fetuses (45/87), and 41.54% of growing pigs (27/65) were PCV3 positive (Fig. 1A). Among the samples tested, we detected PCV3 DNA in all of the 10 different types of samples, including vaginal swabs (3/5).

We observed that different age groups had a different frequency of PCV3 positivity (Fig. 1A). A statistically significant difference was observed between sows and stillborn/mummified fetuses. Therefore, we determined the viral load in the samples from these groups, but no significant differences were observed (Fig. 1B). Likewise, no significant differences were found among the different farms (data not shown).

The ORF2 region of the viral genome was sequenced, from 17 of the PCV3-positive samples, and four non-redundant partial sequences of ORF2 were identified, named UFV01/BR/MG/2019, UFV02/BR/MG/2019, UFV03/BR/MG/2019, and UFV04/BR/MG/2019 (Supplementary Table S2), and deposited in the GenBank database under accession numbers MT497513, MT497514, MT497515, and MT497516, respectively.

It is interesting to note that these four different variants were present on the same farm, suggesting that different PCV3 strains can circulate in the same herd. Two of the variants, MT497513 and MT497514, were obtained from different tissue samples (lymph node and intestine, respectively) from the same animal, indicating coinfection.
Analysis of polymorphisms confirmed high conservation among the ORF2 sequences of PCV3 strains (Supplementary Table S4). The MT497513 sequence is identical to that of the strain PCK3-1701 (MF611876.1), which was identified in South Korea in 2016, and of the strain PCV3-CN-JL22-2018 (MK178309.1), which was identified in China in 2018. MT497514 differs by one synonymous substitution from two PCV3 strains from Brazil (MK645718.1 and MK645719.1), three strains from China (MK645718.1, MK645719.1, and MK178321.1), one strain from Italy (MF162298.1), and one strain from South Korea (MK503331.1). MT497515 differs by two synonymous substitutions and one non-synonymous substitution from MT497514. MT497516 differs by one synonymous substitution from MT497513.

Taking into consideration only the sequences of Brazilian PCV3 strains (Fig. 2), most of the substitutions in ORF2 are located in third codon positions. The overall mean number of synonymous substitutions (dS) is 2.96, and the number of non-synonymous (dN) is 1.35, with a dN/dS rate of 0.46 among the Brazilian strains. Nine amino acid residues in the Cap protein were found to be polymorphic, and three of them (V24A, K27R, and S77T|G) were polymorphic in at least four strains. Phylogenetic analysis (Fig. 3) showed that all of the Brazilian strains belonged to a monophyletic clade of the PCV3a genotype according to the most recent genotyping proposal for PCV3 [11].

PCV3 infection is related to several health problems. However, reproductive failure and multisystemic inflammation seem to be the most consistently reported clinical signs [7]. The association of PCV3 with several clinical presentations suggests that PCV3 could be a potential threat to the swine industry. This study contributes new knowledge about PCV3 strains.

In this study, different swine samples of different age groups from 19 farms were collected and subjected to qPCR to detect PCV3. We detected PCV3 DNA in all of the sample types and age groups. The detection of PCV3 DNA in 78.94% of the farms corroborates the results of other researchers, who showed that PCV3 is disseminated throughout Brazil [15, 16].

The PCV3 positivity rate was homogeneous in samples from weaning and growing pigs, ranging from 35.29 to 41.54%. These results are in agreement with those of other researchers, who also showed that PCV3 has a homogeneous frequency of positivity in different age groups [8].

In our study, stillborn/mummified fetuses had a higher PCV3 positivity rate (51.72%) than the other groups. PCV3 DNA was detected in internal organs (intestine, spleen, liver, heart, lung, and cerebrum) and umbilical cord samples from fetuses, showing that PCV3 is present in a diverse range of tissues. However, no differences were observed in viral loads among different samples. PCV3 was detected in six sows with reproductive failure, and their respective stillborn/mummified fetuses were also PCV3 positive. Also, we identified the sequence MT497513 in samples from one sow with a reproductive problem and three of her stillborn fetuses. These results support the hypothesis that PCV3 can be transmitted vertically [17–19] and reinforce this as a possible route of PCV3 transmission.

The possibility of horizontal transmission of PCV3 from sows to weaning pigs has been reported by Kedkovid et al. [17]. In our study, PCV3 was detected in 70% (21/30) of the sera tested from clinically healthy sows. The average viral load in serum samples from clinically healthy sows was 4.12 × 10^3 copies/µL. The low viral load of PCV3 could
Fig. 3 Phylogenetic tree of ORF2 sequences of PCV3 strains. Analysis of 19 Brazilian strains of PCV3 and 83 reference PCV3 strains. The posterior probability (PP) values are shown beside each node only for those with high support (PP > 70). The sequences obtained in this study are highlighted in bold.
be caused by a subclinical infection, which would explain why the swine were asymptomatic [6, 20]. It is crucial to evaluate the impact of subclinical PCV3 infections, because animal health, especially that of sows, is vital for reproductive success.

In this study, we also analyzed vaginal swabs from healthy sows that had stillborn piglets. The swabs were collected immediately after parturition, and three out of five were PCV3 positive. The detection of PCV3 DNA in vaginal swabs could indicate a risk of horizontal transmission, especially on farms that carry out natural insemination.

Two strains were obtained from different tissue samples from the same animal, which showed clinical signs of wasting. This confirms that more than one PCV3 strain can infect the same animal. This is the first time that different PCV3 strains have been detected in different tissue samples from the same pig. Coinfection with different PCV3 strains could increase the chances of viral recombination within a single host.

We were able to obtain 17 sequences of ORF2 of PCV3, which included four non-redundant sequences. Sequence comparisons showed a high level of nucleotide (98.98-100%) and amino acid (97.66-100%) sequence identity among different PCV3 strains from different countries available in the GenBank database.

Considering the fact that Cap is the major structural protein and the main antigen of PCV3 [21], it is important to analyze amino acid mutations in the Cap protein. Our analysis of mutations observed in the PCV3 Cap protein sequences suggested that different PCV3 strains are circulating on Brazilian swine farms. We performed a phylogenetic analysis based on the PCV3 Cap protein sequences of the 17 PCV3 Brazilian strains identified in this study and those of 15 Brazilian strains previously deposited in the GenBank database. Our results demonstrate that the Brazilian PCV3 strains can be arranged into four different clusters based on differences in 10 amino acid positions of the Cap protein. This result reinforces the evidence of genetic diversity among PCV3 strains, with at least two main lineages circulating in Brazilian herds.

The phylogenetic tree showed that the strains sequenced in this study were grouped with reference strains of genotype PCV3a and clustered into different subclades together with strains from Asia, Europe, and North America.

In this study, we identified four PCV3 strains in samples collected from Brazilian pig farms in Minas Gerais State. Phylogenetic and polymorphism analysis indicated that two main lineages of PCV3 strains are circulating in Brazilian herds. This is the first description of two PCV3 strains infecting the same animal. We identified PCV3 DNA in samples collected from pigs of all age groups and fetuses from reproductive failure cases, in which a higher frequency of PCV3 infection was detected.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05032-y.

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**Author contributions** The study was conceived, designed, and critically revised by VSA, MRS, NCLR, GCB, JLRF, YFC, PMPV, and ASJ. Data analysis and drafting of the manuscript were carried out by VSA, MRS, PMPV, and ASJ. All authors have read and agreed to the published version of the manuscript.

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**Declarations**

**Conflict of interest** The authors have declared no conflicts of interest.

**Ethical approval** The authors confirm that the sample collection in this study was carried out in strict accordance with the Animal Ethics Committee of the Federal University of Viçosa.

**Availability of data and material** The data that support the findings of this study are available in the supplementary materials of this article.

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