Title: New insights about the genetic diversity of *Porcine circovirus 3* strains in Brazil

Running head: Genetic diversity of PCV3 strains

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Summary

Porcine circovirus 3 (PCV3) is a newly emerged circovirus discovered in 2016, and since then is threatening the swine industry worldwide. In this study, we evaluated the presence of different PCV3 strains in swine herds from Brazil. PCV3 was detected by qPCR in different samples from different life stages. Sequencing was performed with seventeen positive samples. This study reported the coinfection of different PCV3 strains in one animal. This study provides insights into the genetic diversity of PCV3 strains circulating in the Brazilian swine herds.

Keywords: Porcine circovirus 3, genetic diversity, swine.

1. Introduction

Porcine circovirus 3 (PCV3) has drawn the attention of the swine industry since it was identified through metagenomics approach in swine with clinical signs of porcine dermatitis and nephropathy syndrome, reproductive failure, and cardiac and multi-systemic inflammation (Palinski et al., 2016; Phan et al., 2016).

Until now, four species of circovirus are known to infect swine. *Porcine circovirus 1* (PCV1) was discovered in 1974 as a cell culture contamination and has not been associated with clinical disease. *Porcine circovirus 2* (PCV2) was discovered in 90 decades and it is considered an economically significant pathogen, that has been associated with a diverse range of clinical diseases as like porcine circovirus-associated diseases, postweaning multisystemic wasting syndrome, respiratory and enteric disease, porcine dermatitis and nephropathy syndrome, and reproductive failure (Palinski et al., 2016; Ouyang et al., 2019). PCV3 was discovered in 2016 and has been found in swine samples with several clinical syndromes (Palinski et al., 2016; Phan et al., 2016). And PCV4 was discovered in 2019 and it was identified in swine with several clinical diseases, including respiratory signs, enteric signs, and PDNS (Zhang et al., 2019).

As a new member of the *Circovirus* genus PCV3 has the common genomic organization, however, PCV3 is distantly related to other known circoviruses (Chen et al., 2019). Since the first report, researchers from many countries around the world have been reporting the detection of PCV3. Retrospective studies detected PCV3 DNA in swine samples from 1993 and 1996, indicating that PCV3 was circulating and has been present in swine populations worldwide for a long time (Klaumann et al., 2018c; Ye et al., 2018; Mora-Díaz et al., 2020). In Brazil, a retrospective study detected PCV3 DNA in swine samples from 1967 and is the oldest PCV3 partial capsid sequence described (Rodrigues et al., 2020). Moreover, PCV3 had been detected in animals with different clinical symptoms and even in asymptomatic animals. The detection of PCV3 in apparently healthy animals could indicate a subclinical infection (Klaumann et al., 2018b) which leads some researchers to the question if PCV3 has clinical relevance in the field (Mora-Díaz et al., 2020). However, considering the economic importance of PCV2 and

the effects that it caused to the swine industry worldwide, PCV3 as a new member of the same family should not be neglected (Klaumann et al., 2018a).

This work aimed to detect PCV3 in several Brazilian farms using qPCR and sequencing to elucidate some questions about PCV3: (I) How much genetic diversity is there among Brazilian PCV3 strains? (II) Is there the same PCV3 strain circulating predominantly among different Brazilian swine herds? (III) Is PCV3 more frequently in fetuses from reproductive failure cases compared to other life stages?

2. Methods

2.1. Sampling

We analyzed 261 swine samples (serum, vaginal swab, umbilical cord, intestine, spleen, liver, heart, lung, cerebrum, and lymph nodes) from sows (92), weaning (17), growing (65) and stillbirth/mummified fetuses (87). These samples were collected in 2019 from 19 swine farms located in Minas Gerais State, which is an important swine producer in Brazil.

2.2. DNA extraction and qPCR detection of PCV3

Total DNA was extracted from samples using Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions. For detecting and quantifying the PCV3 viral load in analyzed samples, a quantitative real-time PCR (qPCR) was standardized using the probe 5'–6-carboxyfluorescein–ACC CCA TGG–Zen–CTC AAC ACA TAT GAC C–Iowa Black–3', forward primer 5'-AGT GCT CCC CAT TGA ACG-3', and reverse primer 5'-ACA CAG CCG TTA CTT CAC-3' previously described by Palinski et al., (2016). As endogenous control, primers that amplified a region of 107 base pairs of the 18S ribosomal gene of swine were designed: 5′-GCCTCGAAAGAGTCCTGTATTG-3′ and 5′-CTGAGAAACGGCTACCACATC-3'. One PCV3 positive sample, confirmed by Sanger sequencing was used for obtaining

a standard curve. The amplicon of this positive sample was ligated into a cloning vector, Clone JET PCR Cloning kit (Thermo Fisher), and transformed into *Escherichia coli* DH5α competent cells. The plasmid DNA was recovered using Fast-n-Easy Plasmid Mini-prep (Cellco) and quantified using QuantiFluor ONE dsDNA System (Promega) and Qubit (Thermo Fisher). The standard curve was generated using ten-fold dilutions of the plasmid DNA, with a detection range of 1.4 to $1.4x10^9$ molecules. Samples with threshold cycles (Ct) values ≤ 38 and with a typical amplification curve were considered positive. The values of the quantification cycle (Cq) were 7.56 to 38.17 cycles with a linear relationship (R) of 0.98 and reaction efficiency (E) of 90%. In this study, $1.4x10^1$ molecules could be detected by qPCR. Data were statistically analyzed. A chi-square test was used to evaluate the association between positive samples in different life stages. Anova and Tukey's multiple comparisons test were used to compared viral load among different samples, using p-value < 0.05.

2.3. Sequencing and analysis of ORF2 of PCV3

We sequenced 17 strains in this work. Partial ORF2 sequences were obtained from positive samples (Supplementary Material, Table S1) that were amplified by Nested PCR, using a combination of primers (Ku et al., 2017), the amplicons were gel purified and sequenced.

The Sanger sequencing data were analyzed using CLC Genomics Workbench version 8.5.4 (Qiagen). Sequences were trimmed for removing poorly sequenced regions (quality score limit: 0.01; ambiguous nucleotide residues: 0) and assembled into contigs using the Assemble Sequences tool (Supplementary Material, Table S1). Sequences were further clustered using the cd-hit-est tool of CD-HIT version 4.7 (Li and Godzik, 2006) to remove redundancy, i.e. identical sequences identified in different samples (Supplementary Material, Table S2).

A dataset containing 17 ORF2 sequences of Brazilian PCV3 strains and 83 ORF2 sequences of reference PCV3 strains (Franzo et al., 2020) from different countries was downloaded from GenBank (Supplementary Material, Table S3) to establish a better framework to discuss the molecular epidemiology of Brazilian strains analyzed in this study. The ORF2 sequences were aligned by MAFFT version 7.307 (Katoh and Standley, 2013), using a local pairwise alignment with 100,000 iterations of refinement parameters to improve the alignment. The polymorphisms identified were screened using MEGA version 10.1.6 (Kumar et al., 2018) (Supplementary Material, table S4).

To expedite the construction of the phylogenetic tree, the model of nucleotide substitution HKY+G was chosen from the full alignment (including positions with gaps and excluding stop codons) using jModelTest version 2.1.10 (Darriba et al., 2012). The phylogenetic tree was calculated using the Bayesian Markov Chain Monte Carlo (MCMC) method using MrBayes 3.2.7a (Ronquist et al., 2012), in two runs with 5,000,000 generations and a sample frequency of 1,000 and diagnostic frequency of 10,000. At the end of runs, 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 burned to produce the consensus tree, which was annotated using Iroki (https://www.iroki.net/) (Moore et al., 2020).

3. Results

3.1. qPCR detection of PCV3

At the farm level, 78.94% (15/19) of the farms were PCV3 positive. The PCV3 positivity among the farms ranged from 16.67% to 95.24%. At the animal level, 39.85% (104/261) of the animals tested were PCV3 positive. When we analyzed the life stages, the sows had 28.26% (26/92), weaning pigs had 35.29% (6/17), stillbirth/mummified fetuses had 51.72% (45/87), and growing animals had 41.54% (27/65) of PCV3 positivity

(Figure 1A). Among the tested samples, we detected PCV3 DNA in all the ten different types of samples, including vaginal swabs (3/5).

Based on the PCV3 positivity rate in the different life stages, we observe a different frequency of PCV3 positivity among different life stages (Figure 1A). A statistic difference was observed between sows and stillbirth/mummified fetuses, therefore we decided to investigate the viral load and samples from them. However, it was not possible to observe significant differences that indicated a higher viral load in any type of sample (Figure 1B). The variation in viral load occurred in each animal individual.

3.2.Sequencing and phylogenetic analysis of ORF2 of PCV3

Among the PCV3 positive samples, 17 samples had the ORF2 of their viral strains sequenced. The sequences of ORF2 were clustered into four non-redundant sequences that were named as UFV01/BR/MG/2019, UFV02/BR/MG/2019, UFV03/BR/MG/2019 and UFV04/BR/MG/2019 (Supplementary Material, Table S2), and that were deposited in GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MT497513, MT497514, MT497515, and MT497516, respectively.

It is interesting to observe that we identified four different strains in samples collected from the same farm [UFV/01/MG/BR/2019 (MT497513), UFV/02/MG/BR/2019 (MT497514), UFV/03/MG/BR/2019 (MT497515), and UFV/04/MG/BR/2019 (MT497516)] suggesting that different PCV3 strains can be circulating in the same herd. And two strains [UFV/01/MG/BR/2019 (MT497513) and UFV/02/MG/BR/2019 (MT497514)] were obtained from different tissue samples (lymph node and intestine) of the same animal, which presented clinical signs of wasting. This result confirms that more than one PCV3 strain can be infecting the same animal. This is the first time that different PCV3 strains were detected in different tissue samples from

the same animal. PCV3 co-infection with different strains could increase the chances of viral recombination occurs.

Analysis of polymorphisms confirmed high conservation among the ORF2 sequences of PCV3 strains (Supplementary Material, table 4). UFV01/BR/MG/2019 (MT497513) sequence is identical to the PCK3-1701 strain (GenBank accession MF611876.1), which was identified in South Korea (2016), and the PCV3-CN-JL22-2018 (MK178309.1), originated from China (2018). UFV02/BR/MG/2019 (MT497514) differs by one synonymous substitution from two PCV3 strains from Brazil (MK645718.1 and MK645719.1; 2018), three strains from China (MK645718.1, MK645719.1, and MK178321.1; 2018), one strain from Italy (MF162298.1; 2017) and one strain from South Korea (MK503331.1; 2018). UFV03/BR/MG/2019 (MT497515) differs by two synonymous and one non-synonymous substitution from UFV02/BR/MG/2019 (MT497514). UFV04/BR/MG/2019 (MT497516) differs by one synonymous from UFV01/BR/MG/2019 (MT497513).

Taken into consideration only the sequences of Brazilian PCV3 strains (Figure 2), most of the substitutions are located at third-codons positions of the ORF2. The overall mean number of synonymous substitutions (dS) is equal to 2.96 and the number of non-synonymous (dN) is equal to 1.35, with a dN/dS rate of 0.46 among the Brazilian strains. Nine amino acid residues showed to be polymorphic among sequences of the Cap protein, and three of them (V24A, K27R, and S77T|G) are polymorphic in at least four of all nineteen strains.

In the phylogenetic tree (Figure 3), all Brazilian strains were classified in the monophyletic clade of the PCV3a genotype, according to the most recent genotyping proposal for PCV3 (Franzo et al., 2020).

4. **Discussion**

PCV3 infection is being related with several health problems as PDNS-like clinical signs and reproductive failure in sow (Palinski et al., 2016), cardiac and multi-systemic inflammation (Phan et al., 2016), the occurrence of stillbirths (Tochetto et al., 2018), digestive and respiratory disease (Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Savic et al., 2020). However, reproductive failure and multisystemic inflammation seem to be the most consistently reported across the current literature (Mora-Díaz et al., 2020). The association of PCV3 with several clinical presentations suggest that PCV3 could be a potential threat to the swine industry. This study aimed to contribute to the knowledge of the PCV3 strains that are circulating nowadays.

In the present study, different swine samples of different life stages from 19 farms were collected and subjected to qPCR to detect PCV3. We detected PCV3 DNA present in all different samples (serum, vaginal swab, lymph node, umbilical cord, intestine, liver, spleen, heart, lung, and cerebrum) and PCV3 was detected in all different life stages (stillbirth/mummified fetuses, weaning, growing swine, and sows). The detection of PCV3 DNA in 78.94% of the farms corroborates with the results of Saraiva et al., (2019) that PCV3 is disseminated among the Brazilian swineherd.

PCV3 positivity rate was homogeneous in the samples from weaning and growing swine, ranging from 35.29 to 41.54%. These results corroborate with the finds of Klaumann et al., (2018) that suggest that PCV3 has a homogeneous frequency of positivity in different life stages.

Our results demonstrated that stillbirth/mummified fetuses had a bigger PCV3 positivity rate (51.72%). PCV3 DNA was detected in internal organs (intestine, spleen, liver, heart, lung, and cerebrum) and umbilical cord samples from stillbirth/mummified fetuses. PCV3 was detected in 6 sows with reproductive failure and your respective stillbirth/mummified fetuses were also PCV3 positives. Also, we identified the strain

UFV01/MG/BR/2019 (MT497513) in samples from one sow with a reproductive problem and your respective three stillbirth fetuses. These results support the hypothesis that PCV3 could be vertical transmitted and reinforce this as a possible route of PCV3 transmission suggested by other researchers (Kedkovid et al., 2018; Deim et al., 2019; Sukmak et al., 2019).

PCV3 horizontal transmission from sows to weaning pigs could be possible according to Kedkovid et al., (2018) which studied sows viremia. Our results showed that PCV3 was detected in 70% (21/30) of the serums from sows clinical healthy. This positivity rate was higher than the 28% PCV3 positivity observed by Kedkovid et al., (2018) in sows. The average viral load in serum samples from sows clinical healthy was $4.12x10^3$ copies/µL. The low viral load of PCV3 could be caused by a subclinical infection, which justifies the fact that the animals were asymptomatic (Klaumann et al., 2018b; Feng et al., 2019). It is important to evaluate the impact of PCV3 subclinical infection because the health of the animals is important, especially the sow's health which is important for the success of the reproduction, and consequently to the swine production.

In this study, we also analyzed vaginal swab from clinical healthy sows, which had stillborn piglets, and it was collected immediately after parturition. We identify that 3/5 of vaginal swab were PCV3 positive. Until this date, this is the first report of PCV3 detection in a vaginal swab from clinical healthy sows after parturition. This positivity in vaginal swab reinforces the hypothesis that PCV3 can be related to reproductive failures. Also, the PCV3 DNA detection in vaginal swab could represent a risk of horizontal transmission, especially in farms that do natural insemination.

It is known that fetal death can be caused by diverse factors. PCV2 was associated with causing encephalitis (Corrěa et al., 2011) and myocarditis (Brunborg et al., 2007) resulting in fetal death. In this study, we detected PCV3 in cerebrum and heart of stillbirth

and mummified fetuses. We hypothesize that PCV3 could cause fetal death due to encephalitis and/or myocarditis, similar to PCV2. Also, in situ hybridization had identified PCV3 replication in heart and cerebrum samples (Arruda et al., 2019) which reinforce our hypothesis that PCV3 could be causing encephalitis and/or myocarditis, resulting in fetal death. However, further studies should be conducted in order to elucidate this hypothesis.

In this study, we did not observe differences in viral loads between the different samples collected. Our results demonstrate that PCV3 is present in a diverse range of samples but it is not possible to infer whether PCV3 has a tropism for any tissue.

We were able to obtain 17 sequences of ORF2 of PCV3, which were clustered into four non-redundant sequences. Analysis of the genome of the sequences showed a high identity of nucleotides (98.98 to 100%) and amino acids (97.66 to 100%) among different PCV3 strains from different countries available into GenBank.

With the alignment of the ORF2 sequences, we observed some synonymous mutations that did not interfere in the cap sequence. However, the sequence UFV03/BR/MG2019 (MT497515) has one significant nonsynonymous mutation of lysine to glutamic acid in amino acid 101. This radical replacement changes the charge of the amino acid side chain. Nonsynonymous mutations in the cap protein could cause structure and function variations and consequently could cause pathogenic and antigenic variations.

In this study, we also observed amino acid mutations in PCV3 cap protein that suggests that different PCV3 strains are circulating in Brazilian swine farms. We performed a phylogenetic analysis based on the PCV3 cap sequence using the 17 PCV3 Brazilian strains identified in this study and 15 Brazilian strains previously deposited into GenBank. Our results demonstrate that PCV3 Brazilian strains can be arranged into four

different clusters considering ten amino acids of cap protein, which were previously described (Saraiva et al., 2018). This result reinforces the existence of genetic diversity among the PCV3 strains with at least two main lineages circulating in Brazil herds.

The phylogenetic tree showed that the strains sequenced in this study were grouped with reference strains of genotype PCV3a. The new classification based on the criteria proposed by Franzo et al., (2020) resulted in one major (PCV3a) with almost all ORF2 sequences of PCV3 from different countries, except for one Chinese sequence that is separate in another clade, which is the only reference representative of PCV3b genotype. The four Brazilian strains sequenced in this study were clustered into different subclades together with strains from Asia, Europe, and North America.

5. Conclusion

This study identified four PCV3 strains in samples collected in Brazilian farms demonstrating a genetic diversity among PCV3 strains, and the phylogenetic and polymorphism analyses reinforce two main lineages of PCV3 strains circulating in Brazilian herds. We detected two PCV3 strains coinfecting the same animal. We identify DNA of PCV3 in samples collected from pigs of all life stages, with more frequency of detection in fetuses from reproductive failure cases

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Conflict of interest statement

All authors have declared no conflict of interest.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review

committee approval has been received. The samples collected in this study carried out in strict accordance with the Animal Ethics Committee of the Federal University of Viçosa.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Figure 1. Detection and quantification of PCV3 in analyzed samples. A) Percentage of PCV3 positivity in different life stages (sows, stillbirth/mummified fetuses, weaning pigs, and growing pigs) using qPCR assay. A chi-square test was performed. *P-value < 0.05. **B)** PCV3 viral copies number using qPCR.

Figure 2. Polymorphisms identified in nucleotide sequences of ORF2 and amino acid sequences of Cap protein of Brazilian PCV3 strains. Each column corresponds to the positions of ORF2 and Cap that are variable.

Figure 3. Phylogenetic tree of ORF2 sequences of PCV3 strains. The midpoint rooted majority-rule consensus tree was obtained by Bayesian Inference (BI) analysis of 19 Brazilian strains of PCV3 and 83 reference PCV3 strains of other countries. The origin and collection year of each isolate are informed in the taxon labels, and the colors indicate the geographic region. The posterior probability (PP) values (expressed as percentages) are shown beside each node only for those with high support (PP>70). The sequences obtained in this study are highlighted in bold.

Supplementary Material

Table S1. Clustering of ORF2 sequences. Seventeen samples (Sample ID) had the ORF2 of their viral strains sequenced and these sequences were clustered (Cluster ID) into the non-redundant sequence.

Table S2. Representative sequences of ORF2 (nt) and Cap protein (aa) from the PCV-3 strains analyzed in this study.

Table S3. Sequence dataset of ORF2 sequences analyzed in this study.

Table S4. Counting of polymorphisms in ORF2 (nt) and Cap (aa) sequences among PCV-3 strains.