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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Brief Report

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Abstract

Porcine circovirus 3 (PCV3) is a recently emerged circovirus discovered in 2016, which since then has drawn the attention of the swine industry worldwide. In this study, we evaluated the genetic diversity of PCV3 strains in 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 PCV3 strains have at least two main lineages circulating in Brazil. For the first time, it was possible to detect the presence of two different PCV3 strains in the same host.

Background

Porcine circovirus 3 (PCV3) was discovered in 2016 through 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]. Until now, four species of circovirus were 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 had been detected in swine with several clinical diseases [1, 2, 4].

As a newly discovered member of the *Circovirus* genus, PCV3 has conserved elements in genomic organization in common with other species; however, PCV3 is only distantly related to the other known circoviruses [5]. The classification proposed divided PCV3 into two clades [13]. Clade 1 (PCV3a) included PCV3 sequences from different countries. Clade 2 included only two Chinese sequences, which could represent either 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 in several Brazilian farms using quantitative real-time PCR (qPCR) and sequencing to elucidate 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 health 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 the Wizard SV genomic DNA purification system (Promega). To detect and quantify the PCV3 viral load, we used qPCR primers and probes, as previously described [1]. As an endogenous control, primers that amplified a region of 10^7 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 (Clone JET PCR Cloning kit, ThermoFisher). 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 compared 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 Material, (SI)] that were amplified by nested PCR, using a combination of primers, as previously described [9]. The Sanger sequencing data were trimmed and assembled into contigs using CLC Genomics Workbench version 8.5.4 (Qiagen) [Supplementary Material, (SI)]. Sequences were further clustered using the cd-hit-est tool of CD-HIT version 4.7 [10] to remove redundancy [Supplementary Material, (SII)].

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 Material, (SIII)]. The ORF2 sequences were aligned by MAFFT version 7.307 and the polymorphisms identified were screened using MEGA version 10.1.6 [Supplementary Material, (SIV)].

To expedite the construction of the 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 using MrBayes 3.2.7a [13], in two runs with 5,000,000 generations. At the end, 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 [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, sows had 28.26% (26/92), weaning pigs 35.29% (6/17), stillborn/mummified fetuses 51.72% (45/87), and growing pigs 41.54% (27/65) PCV3 positivity (Fig. 1A). Among the tested samples, we detected PCV3 DNA in all 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 investigated the viral load of the samples from these groups. However, no significant differences were observed (Fig. 1B). No significant difference was found among different farms (data not shown).

Among the PCV3-positive samples, 17 had the ORF2 of their viral strains sequenced. The partial sequences of ORF2 were clustered into four non-redundant sequences, named UFV01/BR/MG/2019, UFV02/BR/MG/2019, UFV03/BR/MG/2019, and UFV04/BR/MG/2019 [Supplementary Material, (SII)] and deposited in the GenBank database under accession numbers MT497513, MT497514, MT497515, and MT497516, respectively.

It is interesting to observe that four different strains, i.e. MT497513, MT497514, MT497515, and MT497516, were present on the same farm, suggesting that different PCV3 strains can circulate in the same herd. Two strains, MT497513 and MT497514, were obtained from different tissue samples (lymph node and intestine, respectively) from the same animal, indicating co-infection.

Analysis of polymorphisms confirmed high conservation among the ORF2 sequences of PCV3 strains [Supplementary Material, (SIV)]. The MT497513 sequence is identical to that of the PCK3-1701 strain (MF611876.1), which was identified in South Korea (2016), and of PCV3-CN-JL22-2018 (MK178309.1), originating from China (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 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 are located at third-codon positions of 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 were shown to be polymorphic among sequences of the Cap protein, and three of them (V24A, K27R, and S77T|G) were polymorphic in at least four strains. In the phylogenetic tree (Fig. 3), all Brazilian strains were classified in the 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 aimed to contribute to the knowledge on 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 different samples and PCV3 was detected in all different age groups. The detection of PCV3 DNA in 78.94% of the farms corroborates the results of other researchers 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 corroborate with other researchers who suggested that PCV3 has a homogeneous frequency of positivity in different age groups [8].

Our results demonstrate that stillborn/mummified fetuses had a higher PCV3 positivity rate (51.72%). 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 strain 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.

Horizontal transmission of PCV3 from sows to weaning pigs could be possible, according to Kedkovid et al. [25]. Our results show that PCV3 was detected in 70% (21/30) of the sera 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 be caused by a subclinical infection, which explains the swine being asymptomatic [6, 20]. It is crucial to evaluate the impact of subclinical PCV3 infection because animal health is important, especially that of sows, as it 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. We identified that 3/5 of the vaginal swabs were PCV3-positive. The PCV3 DNA detection in vaginal swabs could indicate a risk of horizontal transmission, especially in farms that carry out natural insemination.

Two strains were obtained from different tissue samples from the same animal, which presented clinical signs of wasting. This result 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. PCV3 co-infection 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 were clustered into four non-redundant sequences. Analysis of the genome sequences showed a high identity of nucleotides (98.98-100%) and amino acids (97.66-100%) among different PCV3 strains from different countries available on GenBank.

Considering the fact that Cap is the major structural protein and main antigen of PCV3 [21], it is important to analyze amino acid mutations in the Cap protein. The amino acid mutations observed in the PCV3 Cap protein suggest 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 the Brazilian PCV3 strains can be arranged into four different clusters by considering 10 amino acids 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. The four Brazilian strains sequenced in this study were clustered into

different subclades together with strains from Asia, Europe, and North America.

This study identified four PCV3 strains in samples collected from Brazilian pig farms in Minas Gerais State. The phylogenetic and polymorphism analyses indicate two main lineages of PCV3 strains circulating in Brazilian herds. This is the first description of two PCV3 strains coinfecting the same animal. We identified the DNA of PCV3 in samples collected from pigs of all age groups, and fetuses from reproductive failure cases, displaying a higher frequency of PCV3 detection.

Declarations

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Conflicts of interest: The authors have declared no conflicts of interest.

Ethics 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 Material of this article.

Author contributions: The study was conceived, designed, and critically revised by VSA, MRS, NCLR, GCB, JLRF, YFC, PMPV, and ASJ. Data analysis and the drafted 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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Figures

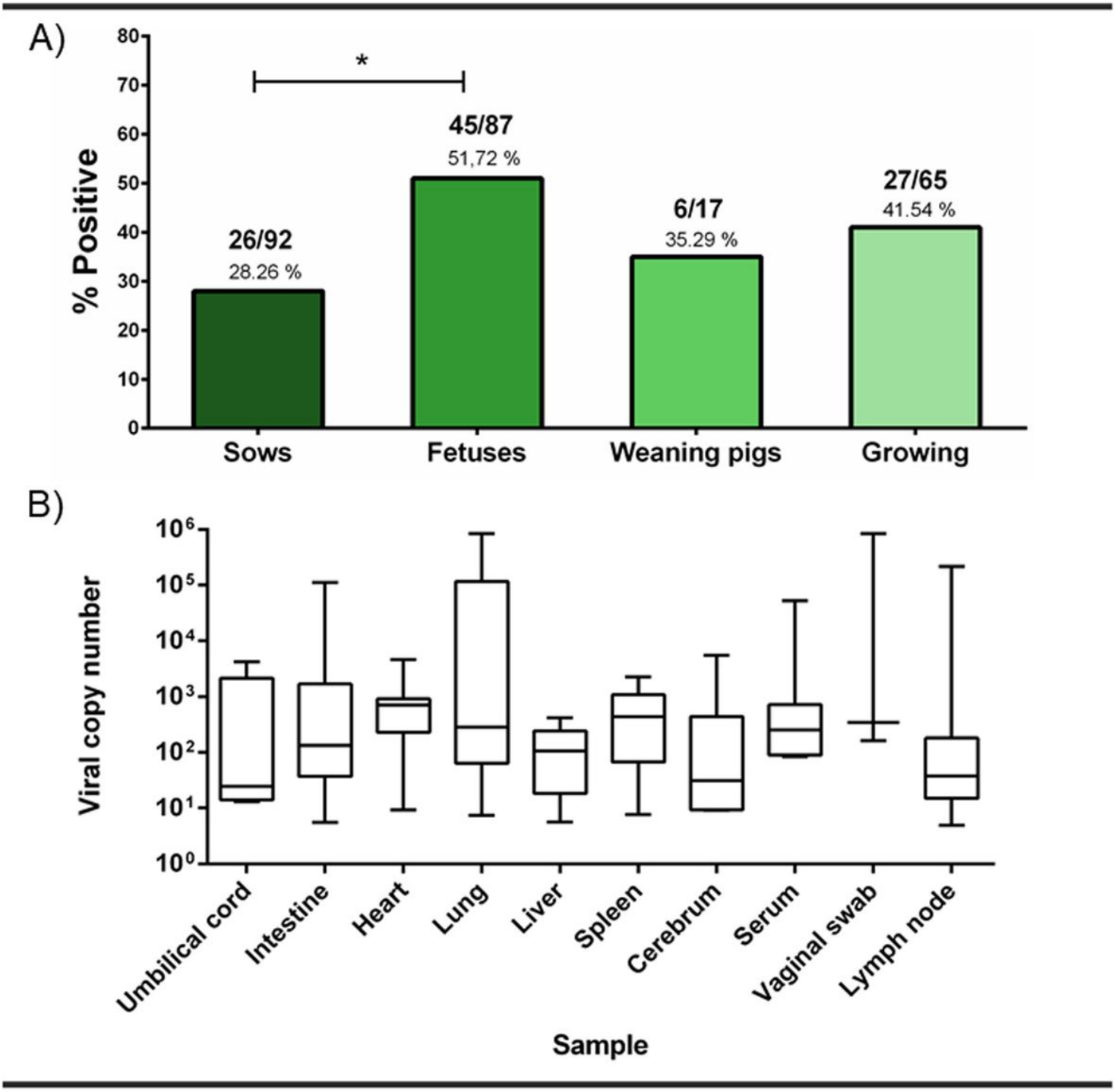

Figure 1

Detection and quantification of PCV3 using qPCR. A) Percentage of PCV3 positivity in different age groups (sows, stillborn/mummified fetuses, weaning pigs, and growing pigs). A chi-squared test was performed (*$p<0.05$). B) PCV3 viral copy numbers.

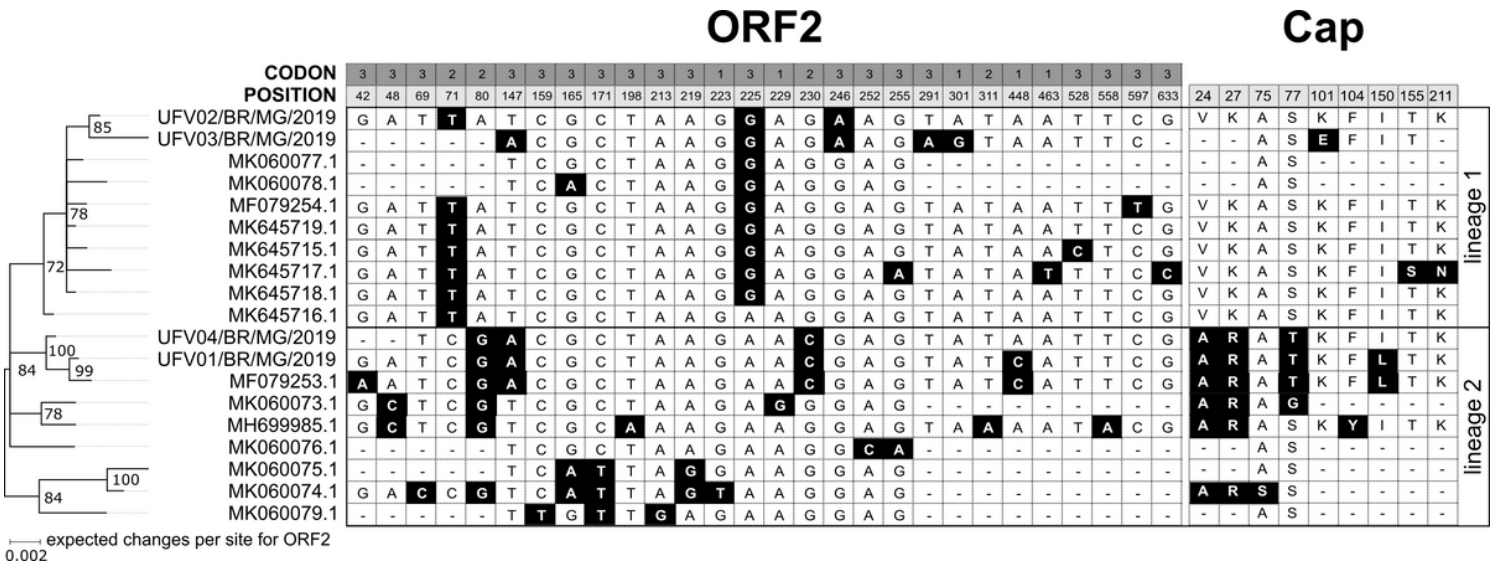

Figure 2

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

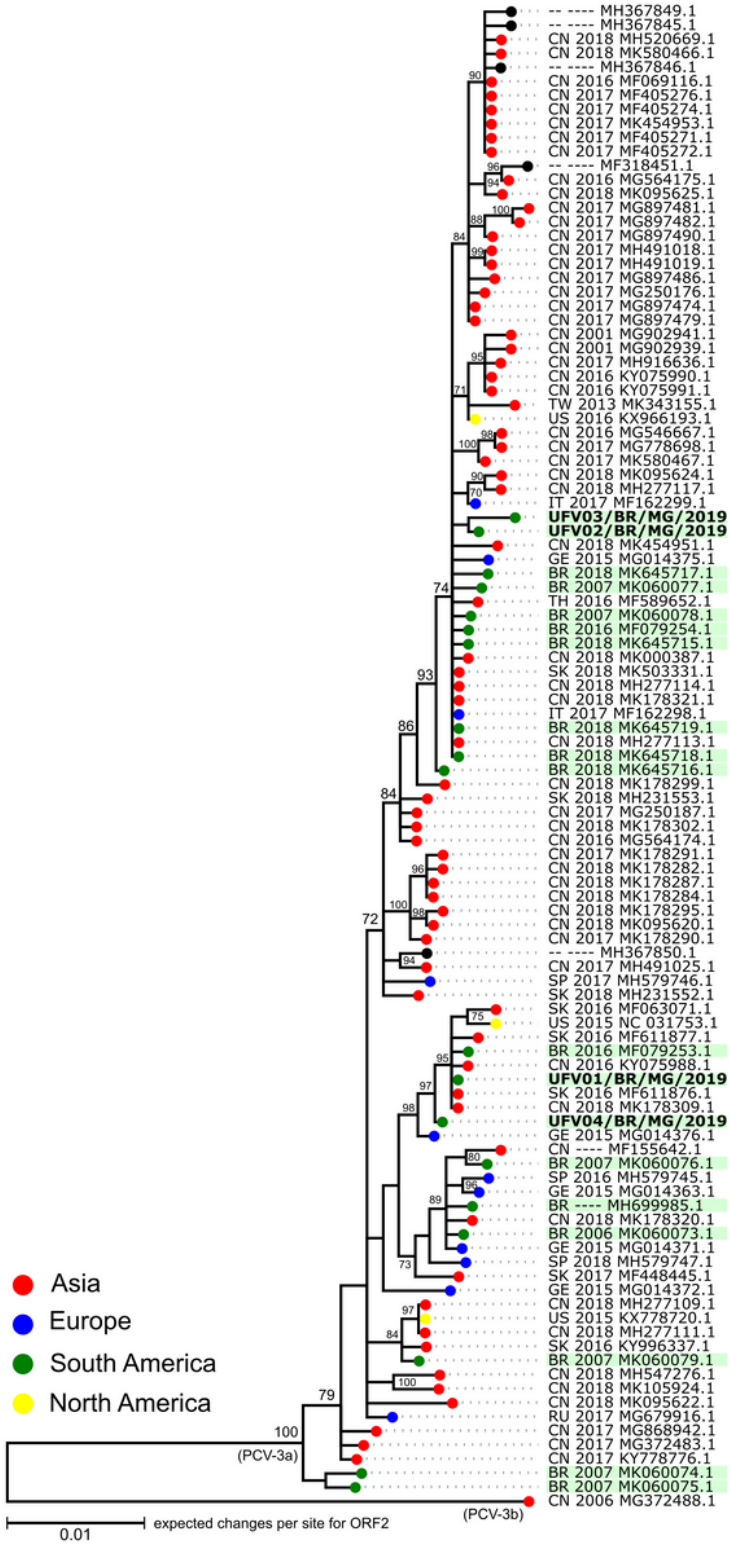

Figure 3

Phylogenetic tree of ORF2 sequences of PCV3 strains. 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.

Supplementary Files

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- SupplementaryMaterial.xlsx