

Survey of porcine circovirus type 2 and parvovirus in swine breeding herds of Colombia

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Abstract

Background: One of the consequences of the presentation of reproductive failures in sows is the economic losses in production because it alters the estimated values of the volume of production, decreasing the productivity of the farm. Porcine circovirus by porcine circovirus 2 (PCV2) has been associated with reproductive disorders, and porcine parvovirus (PVP) is one of the pathological agents most related to the presentation of reproductive failure in pigs. In Colombia, there are reports of the presence of PCV2 through molecular techniques, and PVP through serum tests; however, in the department of Tolima, the prevalence of these two viruses is unknown.

Objective: In this study, the aim was to establish a report of the prevalence of viruses in five municipalities of the department of Tolima-Colombia.

Methods: Blood samples from 150 breeding sows of five municipalities in Tolima, Colombia, were obtained. Quantitative polymerase chain reaction (qPCR) was used to detect the PCV2 and PVP virus in the blood samples followed by PCR and sequencing of 16 PCR products of the amplification of the *cap* gene of PCV2. A phylogenetic tree was constructed to identify the genotype of the PCV2 virus.

Results: The presence of PCV2d in sows was detected in 135 samples (90%), as well as the identification of PVP in 2.6% of the samples. In addition, the phylogenetic analysis showed that 16 isolates were the PCV2d2 genotype.

Conclusion: PCV2d and PVP were found to coinfect the females, and the identification of variability in regions in the predicted amino acid sequence of the PCV2 capsid may be associated with virus pathogenicity.

KEYWORDS

molecular characterization, reproductive failures in sows, PCVAD, PCV2d

1 | INTRODUCTION

Porcine circovirus is a worldwide disease caused by porcine circovirus 2 (PCV2), a DNA virus of the Circoviridae family (Allan & Ellis,

2000), which affects both young and adult pigs, resulting in a strong economic impact on swine production (Alarcon et al., 2013; Zaveckas et al., 2015). Porcine circovirus by PCV2 has been associated with the majority of clinical cases in sick pigs, within this signology are

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reproductive disorders (Olvera et al., 2007; Zhao et al., 2014), and the variety of clinical presentations are collectively referred to as porcine circovirus-associated diseases (PCVAD).

Porcine parvovirus (PVP) is one of the pathological agents most related to the presentation of reproductive failure in sows around the world (Streck et al., 2015). When the virus enters a nursery-stage production, it can have devastating effects. Some manifestations of the disease are described by the acronym SMEDI (stillbirth, mummification, embryonic death, infertility) (Foerster et al., 2016).

One of the consequences of the presentation of reproductive failures, mainly abortion, is the economic losses in the productions, due to the fact that it alters the estimated values of the volume of production, increasing the non-productive days of the sows and decreasing the productivity of the exploitation (Salogni et al., 2016).

Despite cataloguing PCV2 as one of the main viruses that causes reproductive problems in pigs (O'Connor et al., 2001; Ruiz-Fons et al., 2006), the mechanism that generates this pathology is still unknown, postulating the coinfection with other agents such as PVP, as a possible mechanism (Meng, 2013; Mészáros et al., 2017).

Works oriented to the diagnosis of causal agents of reproductive problems in sows describe PCV2 and PVP as the main viruses that lead to abortions, return to heat, stillbirths, and mummies (Chae, 2005; Tzika et al., 2012; Ransburgh, 2015). Field studies and laboratory tests have shown coinfection of PCV2 and PVP, which potentiates the effect of PCV2 on the development of PCVAD (Gillespie et al., 2009). Age, management factors, immune status, and time of infection can significantly modify the outcome of coinfection (Mészáros et al., 2017).

It has been described that PVP could facilitate PCV2 infection either indirectly, by reducing immunoprotection and/or stimulating virus replication by activating the host cell, or directly, by promoting viral DNA replication by infecting the same cells. Additionally, PVP has been associated with transient immunosuppressive effects (Mészáros et al., 2017).

In Colombia, there are reports of the presence of PCV2 using molecular techniques (Rincón-Monroy et al., 2014), and of PVP through serum tests (Rico et al., 2003); however, in the department of Tolima, the prevalence of these two viruses is unknown. This study seeks to establish a report on the prevalence of viruses in five municipalities of the department of Tolima.

2 | MATERIALS AND METHODS

2.1 | Location

The study was carried out in five municipalities of the department of Tolima, Colombia, known for having farrowing to finishing farms. The selected municipalities were Ibagué (1250 m.a.s.l. and an average temperature of 24 and 28°C), Falan (990 m.a.s.l. and an average temperature of 32°C), Chaparral (854 m.a.s.l. and an average temperature of 24°C), Purificación (329 m.a.s.l. and an average temperature of 26°C), and Cajamarca (1814 m.a.s.l. and an average temperature of 24°C).



FIGURE 1 Abortion in the last third of pregnancy in sows of the sampled population

2.2 | Sample collection

The sample size was estimated through the formula described by Thrusfield (2018) where 150 samples were calculated taking into account a prevalence of 11.4% of PCV2 (Rincón-Monroy et al., 2014). On the other hand, the prevalence is unknown for PVP, due to this the sampling was distributed proportionally in the five municipalities depending on the size of the productions. The sampling was carried out in breeding sows older than 240 days, with a history of reproductive failures (abortions, return to heat, vaginal discharges, stillbirths, mummifications, and macerations) (Figure 1); the sampled productions were going to initiate a vaccination plan against PCV after the sampling. Note that 5 ml of blood was obtained by puncture of the jugular vein, using purple-top Vacutainer® tubes with EDTA anticoagulant and stored at -20°C until use. For the extraction of total DNA, 300 μl of blood was taken and tested using the Wizard Genomic DNA kit (Promega, USA) following the procedure recommended by the manufacturer for the extraction of genomic DNA from whole blood.

2.3 | Molecular detection of PCV2 and PVP by real-time PCR

The real-time PCR (qPCR) reaction, a master mix with a final volume of 20 μl was used, consisting of 7 μl of distilled-deionized water, 10 μl of Luna® Universal qPCR Master Mix (NEB, USA), 0.5 μl of each primer (forward and reverse) 10 pmol/ μl each, and 2 μl of the genomic DNA sample (100 ng/ μl). The amplification was performed in a QuantStudio 3 Real-Time PCR Systems thermocycler (Thermo Fisher Scientific, United States), with the following programming: predenaturation at 95°C for 1 min, 40 denaturation cycles at 95°C for 15 s, and extension at 72°C for 30 s. The melting curve was then run from 60 to 95°C at melting rates of 0.15°C/s; the melting temperature was defined as the

TABLE 1 Sequence of primers used for identification and characterization of the nucleotide sequences of the viruses

	Sequence	Amplicon	T _m °C	%G-C	Accession number
PCV2 full ORF2	GCCAGTTCGTCACCCTTTC	824 bp	58.8	57.9	MN196673.1
	CAGCGCACTTCTTTCGTT		56.4	50	
PCV2 qPCR	GGTCCACGGCTGTATTCT	99 bp	60.1	55	
	GGTTATGGTATGGCGGGAGG		59.7	60	
Identification PVP*	TACTTCTCAGAGCAAAGCG	998 bp	55.8	45	MN970191.1
	CCTGTGGAGAATTCATCTCC		55.2	50	
PVP qPCR	CCCAGGAACACTACCAGCAG	98 bp	60	60	
	CCTGAGCTGGCTAATTGCT		60.1	55	

Abbreviations: bp, base pairs; ORF2, open reading frame 2; PCV2, porcine circovirus 2; PVP, porcine parvovirus; qPCR, quantitative polymerase chain reaction; T_m, melting temperature.

*Zimmermann et al. (2006).

peak of the curve, with the intention of verifying the specific binding of the primer.

2.4 | Sequencing analysis

For the confirmation of the presence of the viruses in the positive samples and for the construction of the phylogenetic analysis, primers for PCV2 were designed to amplify the complete open reading frame 2 (ORF2), which encodes the viral capsid protein (Nawagitgul et al., 2000), using the primers described in Table 1; in the same way for PVP amplifying the *vp2* gene.

These amplifications were performed by conventional PCR using a T-100 thermocycler (Bio-Rad, USA), with a final reaction volume of 25 µl composed of 14.87 µl of deionized distilled water, 5 µl of 5x GoTaq® Colorless Flexi Buffer, 1 µl dNTPs (1.5 mM), 1 µl each primer (forward and reverse) 10 pmol/µl each, 1 µl MgCl₂ (25 mM), 0.125 µl GoTaq® Flexi DNA polymerase (Promega, USA), and 1 µl of the genomic DNA sample (100 ng/µl). The amplification consisted of an initial denaturation cycle at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and the last extension step at 72°C for 5 min.

Amplicons were revealed by horizontal 1% agarose gel electrophoresis, stained with Hydragreen® (ACTGene, USA) and visualized under ultraviolet light using an ENDURO GDS™ gel documenter (Lab-Net Intl, USA). The amplicons were sequenced using the Sanger method (Macrogen Inc, Seoul, Korea), subjected to bioinformatic analysis using Geneious prime software (Biomatters Ltd.) (Kearse et al., 2012) and compared with those reported in the GenBank of the National Center for Biotechnology Information, USA.

2.5 | Phylogenetic analysis

Multiple sequence alignments were carried out using Geneious prime software (Biomatters Ltd.). To determine the genotype of PCV2 iso-

lates, 18 reference porcine circovirus genome sequences were downloaded from the GenBank database (PCV2a: EF394775, FJ905471; PCV2b: FN398025, DQ220728, FN398024, DQ220736, DQ629115; PCV2c: EU148503, EU148505; PCV2d: HM038030, MG833033, EF524539, AY682991, AY682994, AY682996; PCV2e: EF524526, EF524533 and PCV1: FJ475129). Phylogenetic trees were constructed with the cap gene sequence of the 16 isolates and 18 reference sequences with the Geneious software using the neighbour-joining (NJ) method, with the Jukes-Cantor model as the nucleotide substitution model. The reliability of the generated trees was determined with 1000 replicates of the data set. The PCV2 sequences obtained in this study were submitted to GenBank, through the BankIt platform for registration and accession number MW718670-MW718685.

3 | RESULTS

3.1 | PCV2 and PVP detection

All blood samples collected were diagnosed by real-time PCR. Using this technique, a PCV2 prevalence of 90% (135/150) was obtained. In the case of PVP, a prevalence of 2.6% was found (4/150); the samples were run in triplicate to average the values of cycle threshold (Ct) obtained (Figure 2). PCV2 positive samples were found in all municipalities (Figure 3). The positive samples for PVP were only found in the municipality of Falan (Figure 3).

3.2 | Phylogenetic analysis and identification of genotypes

For the sequencing of PCV2, 16 representative samples were chosen for the municipalities (two from Ibagué, four from Falan, three from Chaparral, three from Purificación, and four from Cajamarca) (Figure 4), which were used to confirm the diagnosis and perform the bioinformatic analysis. These were compared with 18 sequences

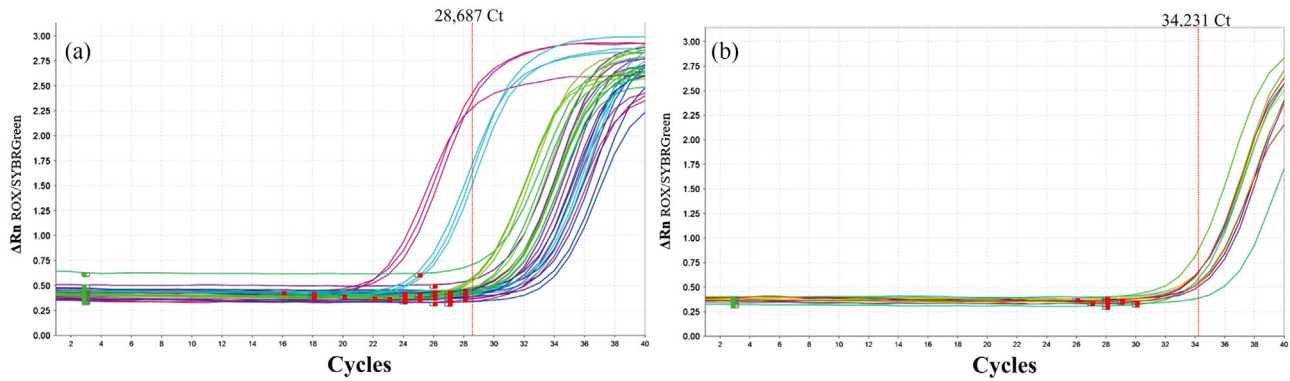


FIGURE 2 Amplification curves of blood samples positive for porcine circovirus 2 (PCV2) and porcine parvovirus (PVP): (a) PCV2-positive blood samples; (b) PVP-positive blood samples

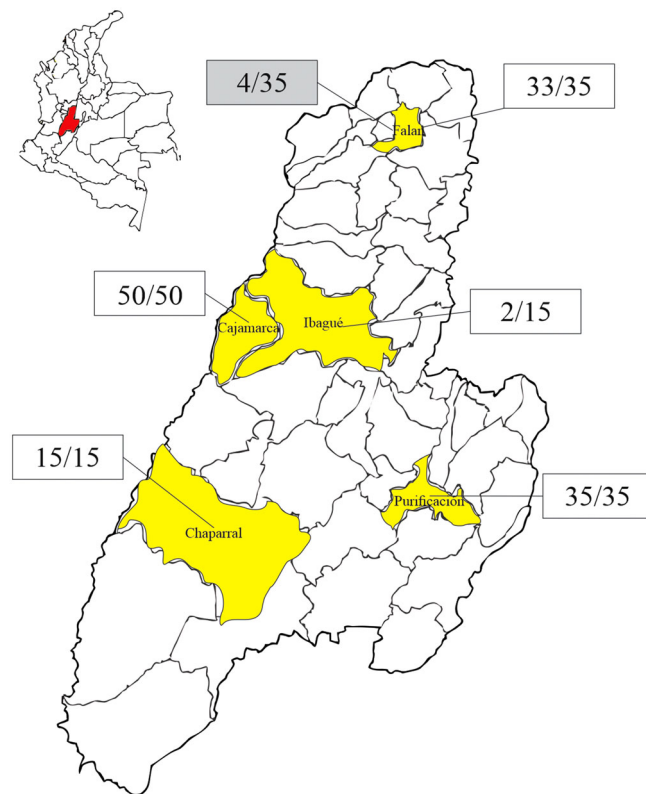


FIGURE 3 Distribution map of positive samples by municipality. White boxes indicate number of porcine circovirus 2 (PCV2) positive samples, whereas grey box indicate positive samples for porcine parvovirus (PVP).

reference deposited in the GenBank database, representing PCV genotypes from Colombia and other countries. The phylogenetic tree included the most reported PCV2 genotypes and a PCV1 sequence as an outgroup, showing that all 16 isolates cluster were the PCV2d genotype, specifically the PCV2d2 genotype. In the case of PVP, it was not possible to obtain a sequence with sufficient length, which would allow us to carry out a bioinformatic analysis (Figure 5).

3.3 | Molecular characterization

The predicted amino acid sequences of the PCV2 capsid proteins were analyzed and aligned using the BLOSUM62 array algorithm in Geneious prime software (Figure 6), to identify point changes in the amino acid sequence. Changes were identified in seven sequences, at positions 78 (Asp-Glu), 79 (Phe-Leu), 123 (Val-Leu), 130 (Val-Ala), 134 (Asn-Ile), and 207 (Tyr-Cys).

4 | DISCUSSION

In this study, PCV2 was detected in 135/150 blood samples from sows with reproductive failure in production in the department of Tolima, with a prevalence of 90%. In addition, a prevalence of 2.6% was found due the detection of four out of 150 samples positive for PVP, presenting a coinfection of the viruses in these samples. The real-time PCR technique was used because it is described as highly sensitive and specific for the detection of nucleotides of the PCV2 *cap* gene and the PVP *vp2* gene (Oliver-Ferrando et al., 2018; Wilhelm et al., 2006; Yang et al., 2016).

PCV2 has been related to the presentation of late abortions, stillbirths, mummifications (Brunborg et al., 2007; Madson et al., 2009; Segalés, 2012), in addition to being related to return to heat, due to replication in the embryos causing their death (Mateusen et al., 2007). Pensaert et al. (2004) describe that the presentation of the reproductive disease caused by PCV2 is reported in low proportion in the field, which differs from what was found in this study.

The reproductive signology of PCV2 is similar to that presented in infections with PVP, which is the most common cause of reproductive failure of viral origin in pigs (Saekhow et al., 2015). Additionally, coinfection of these viruses has been reported in animals with reproductive problems (Sharma & Saikumar, 2010; Sun et al., 2015; Saekhow et al., 2015) such as the presentation of stillbirths and mummifications as well as viable seropositive born animals (Dias et al., 2013).

Co-infection with PVP has been reported to promote PCV2 infection by stimulating immune cells, providing target cells for virus

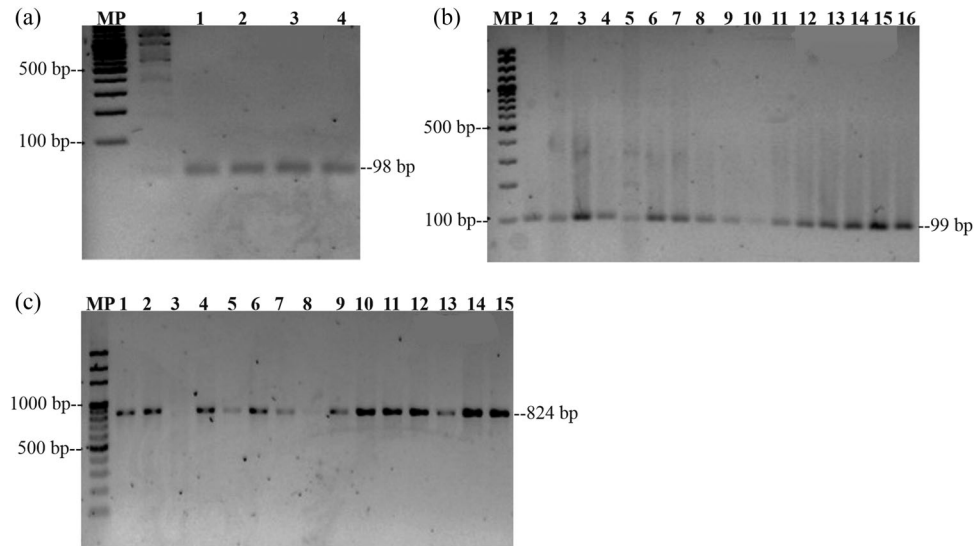


FIGURE 4 Electrophoresis gels with the amplifications obtained using the primers used. The images correspond to the electrophoresis gels of the endpoint PCR amplification products, using the primers described in Table 1. (a) Electrophoresis gel of the amplification products using the porcine parvovirus (PVP) quantitative polymerase chain reaction (qPCR) primers, where amplification of 98 base pairs (bp) is observed in wells 1–4. (b) Electrophoresis gel of the amplification products using the porcine circovirus 2 (PCV2) qPCR primers, where amplification of 99 bp is observed in wells 1–16. (c) Electrophoresis gel of the amplification products using the PCV2 Full ORF2 primers, where amplification of 824 bp is observed in wells 1–15. Abbreviation: Mp, molecular weight marker

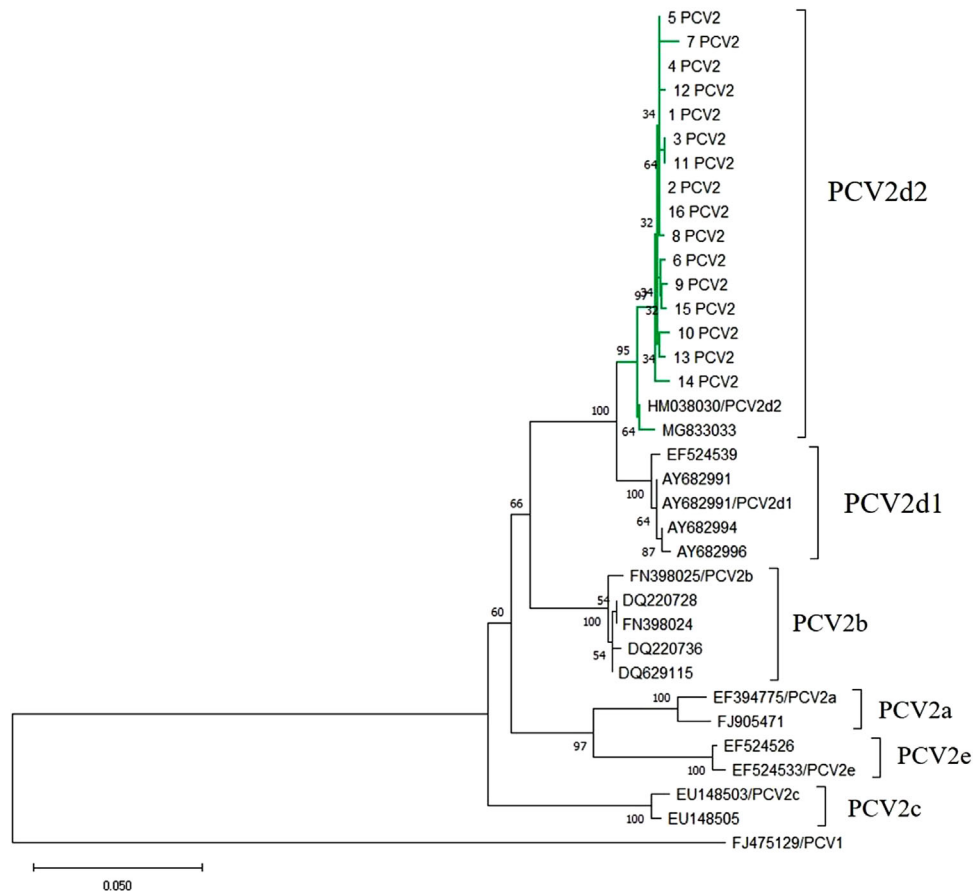


FIGURE 5 Phylogenetic tree based on the nucleotide sequence of porcine circovirus 2 (PCV2) open reading frame 2 (ORF2). Phylogenetic tree was constructed with the Geneious prime software using the neighbour-joining (NJ) method, with the Jukes–Cantor model as a nucleotide substitution model. The bar indicates the number of substitutions per site. The sequences marked in green correspond to the sequences obtained in the present study.

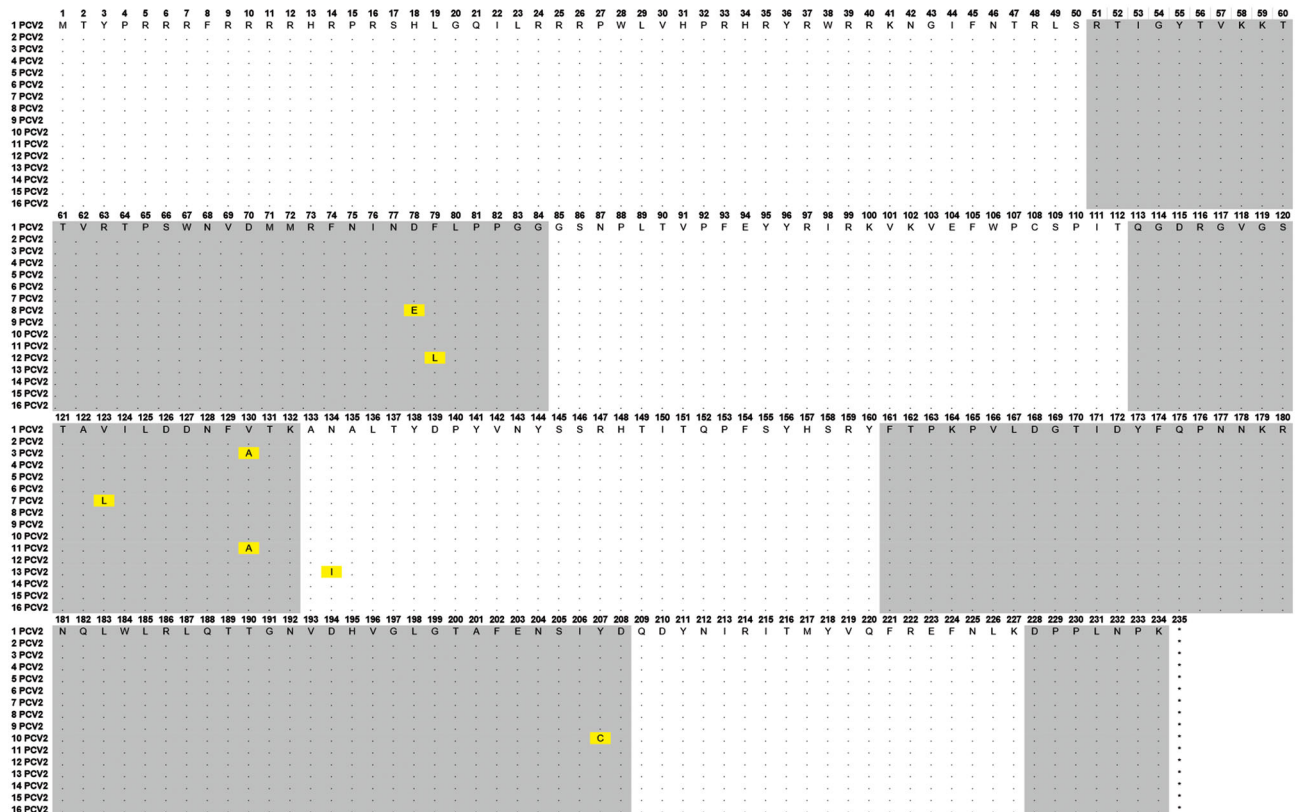


FIGURE 6 Comparison between the predicted capsid protein sequences of the obtained porcine circovirus 2 (PCV2) sequences. The grey areas correspond to the antibody recognition domains described in Tribble et al. (2011). The amino acids highlighted in yellow correspond to changes in the sequences.

replication or decreasing PCV2 neutralization by altering cytokine production and expression profiles (Allan et al., 1999; Opriessnig & Halbur, 2012). Both viruses replicate in circulating peripheral monocytes, contributing to cell-associated viremia and virus detection in lymphoid tissues (Kim et al., 2003). Although PCV2 + PVP coinfection in reproductive failure is common, in this study, it occurred in 2.6% of the samples (4/150).

All the females sampled presented reproductive problems such as abortions, return to heat, vaginal discharges, stillbirths, mummifications, and macerations (data not shown) that could be associated with the presence of PCV2 the same as reported before by Segalés et al. (2005). Animals without clinical signs are considered to have lower viral loads compared to animals with signs of the same production (Brunborg et al., 2004; Grau-Roma et al., 2009), suggesting a high prevalence to the presence of some risk factors that can contribute to the exacerbation of the infection and the presentation of signs (Rincón-Monroy et al., 2014).

The sequences obtained from the PCV2 positive samples revealed in the phylogenetic analysis that they belong to the PCV2d-2 subtype. In the case of PVP, it was not possible to obtain amplicons with the designed primers, despite the fact that in silico analyses showed efficiency in detection, an optimal length of the *vp2* gene was not achieved to characterize the sequences and perform phylogeny analysis. The

identification of this virus by molecular tests has not been reported in Colombia, only by serological tests (Rico et al., 2003).

PCV2 viruses, like other single-stranded DNA (ssDNA) viruses, are characterized by a high mutation rate (e.g., 1.2×10^{-3} substitution/site/year), within the typical range of RNA viruses, which has led to the appearance of an abundance of variants over time (Firth et al., 2009; Franzo et al., 2016).

Rincón-Monroy et al. (2014) reported the PCV2b subtype for the country and Almarío-Leiva et al. (2020) reported PCV2d in the Tolima and Huila regions; however, molecular characterization of this subtype had not been carried out, and the studies did not discriminate the isolates in production animals or those used for breeding stock.

Data analysis suggests an ongoing genotype switch from PCV2b to PCV2d (Franzo & Segalés, 2020; Wei et al., 2019). PCV2d has been recognized as the predominant genotype worldwide (Xiao et al., 2015; Wei et al., 2019). Although PCV2a virus-based vaccines could provide protection against PCV2b and PCV2d infection, substantial genetic divergence between PCV2d strains has been observed over time, which could have changed the pathogenicity of the virus; this was evidenced in PCV2 strains collected from farms where the vaccine program failed (Wang et al., 2020). However, there is no relationship between PCV2 genotype and disease status or geographic area (Olvera et al., 2007; Timmusk et al., 2008).

The appearance of this subtype was reported in 1999, identifying two clades within this subtype PCV2d-1 and PCV2d-2 (Xiao et al., 2015). Later, it was proposed that PCV2d-1 was an ancestor of PCV2d-2 because it was only reported between 1999 and 2011. In our study, all strains are grouped with the PCV2d-2 subtype.

A comparison of the predicted amino acid sequences of the PCV2 capsid revealed 99% homology. Amino acid comparisons showed regions with changes related to antibody recognition regions (Trible et al., 2011). Changes in seven predicted sequences were identified as shown in Figure 6.

Some works demonstrated the antigenic characteristics of regions in the amino acid sequence of the capsid protein; amino acid residues at positions 145–162, 175–192 (Shang et al., 2009), and 165–200 (Lekcharoensuk et al., 2004) are recognized by neutralizing monoclonal antibodies, the region between positions 131 and 190 is important for virion neutralization (Saha et al., 2012). A mutation in the 163–180 region has been reported to generate false epitope recognition resulting in the production of non-neutralizing antibodies (Trible & Rowland, 2012; Tribble et al., 2011). However, the changes in the predicted sequences obtained in this study are not related to these described regions.

Work by Tribble et al. (2011) showed that the peptide generated from the region between 169–180 presented a greater response in the serum of experimentally infected animals and some with PCVAD, but in the same assay, the serum of vaccinated animals generated readings almost exclusively to the larger polypeptide (43–233) and showed much lower responses to the smaller polypeptides.

Since the PCV2 capsid protein is immunogenic, mutations in the capsid protein may explain the direction of PCV2 genotypic evolution and its pathogenesis (Wei et al., 2019). It should be considered that mutation in one or more amino acids of this protein can affect the virulence and pathogenicity of PCV2 (Huang et al., 2011; Saha et al., 2012).

5 | CONCLUSION

PCV2d and PVP in sows were detected by molecular techniques in the department of Tolima, and the two viruses were found coinfecting the females. Regions with variability in the predicted amino acid sequence of the PCV2 capsid may be associated with virus pathogenicity.

6 | RECOMMENDATIONS

Additional epidemiological studies are needed, including a retrospective analysis of the circulation of these viruses in Colombia with more continuous and up-to-date surveillance, to better understand their epidemic and evolutionary dynamics as well as the interactions in the presentation of coinfections.

AUTHOR CONTRIBUTIONS

Heinner F. Uribe-García was involved in conceptualization, and Ilang S. Rondón-Barragán was involved in the methodology. Heinner F. Uribe-

García, Rafael A. Suarez-Mesa, and Ilang S. Rondón-Barragán were involved in investigation. Validation and data curation were made by Heinner F. Uribe-García and Ilang S. Rondón-Barragán. Formal analysis was made by Heinner F. Uribe-García and Ilang S. Rondón-Barragán. Funding acquisition, resources, administration and supervision were provided by Ilang S. Rondón-Barragán and Rafael A. Suarez-Mesa. Heinner F. Uribe-García, Rafael A. Suarez-Mesa, and Ilang S. Rondón-Barragán were involved in writing of the original draft. Writing—review and editing were made by Ilang S. Rondón-Barragán. Finally, visualization of the paper draft was made by Heinner F. Uribe-García.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

All experimental procedures followed the Guidelines from the Bioethics Committee of the Central Office of Research from the University of Tolima based on Law 84/1989 and Resolution 8430/1993 and complied with the guidelines for animal care and use in research and teaching.

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PEER REVIEW

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