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MICROBIOLOGY

Molecular detection and phylogenetic analysis of porcine circovirus 3 in wild boars from the State of São Paulo, Brazil

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Detecção molecular e análise filogenética do circovírus suíno 3 em javalis selvagens do Estado de São Paulo, Brasil

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Molecular detection and phylogenetic analysis of porcine circovirus 3 in wild boars from the State of São Paulo, Brazil

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ABSTRACT: Porcine circovirus 3 (PCV3) belongs to the *Circoviridae* family and has been identified in domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*). Accordingly, this study aimed to investigate the molecular detection of PCV3 in wild boars and to analyze their phylogenetic relationships. To conduct the investigation, samples were collected from 25 wild boars culled during regulated hunting and population control activities in Brazil. During necropsy, tissue fragments from the spleen, liver, lungs, and lymph nodes were collected and subjected to polymerase chain reaction (PCR) to detect PCV3. The results showed that, of the 25 wild boars analyzed, nine (36%) tested positive for PCV3 in at least one tissue. Lymph nodes, spleens, and lungs yielded the highest number of positive samples. Additionally, the complete sequence of the open reading frame 2 (ORF2) region of the PCV3 genome was determined for three positive samples using the Sanger sequencing method; 100% nucleotide identity was observed among these three sequences. The sequences identified in this study clustered with PCV3 sequences previously reported in Brazil and other countries, reflecting the limited geographic structuring currently observed for PCV3. Furthermore, nucleotide identity with ORF2 sequences previously reported in Brazilian wild boars ranged from 98.76% to 99.38%, while identity with sequences from Brazilian domestic pigs also previously reported ranged from 98.45% to 99.07%. Therefore, the results corroborate the high genetic conservation reported for PCV3 worldwide. To the best of our knowledge, this study represents the first report of PCV3 detection in wild boars in the state of São Paulo, Brazil.
Key words: porcine circovirus, viral detection, molecular surveillance.

Detecção molecular e análise filogenética do circovírus suíno 3 em javalis selvagens do Estado de São Paulo, Brasil

RESUMO: O circovírus suíno 3 (PCV3) pertence à família Circoviridae e foi identificado em suínos domésticos (*Sus scrofa domesticus*) e javalis (*Sus scrofa)*. Neste sentido, este estudo teve como objetivo investigar a detecção molecular do PCV3 em javalis selvagens e suas relações filogenéticas. Para realizar a investigação, amostras foram coletadas de 25 javalis abatidos durante atividades de caça regulamentada e controle populacional no Brasil. Na necropsia, fragmentos de baço, fígado, pulmões e linfonodos foram coletados e submetidos à reação em cadeia da polimerase (PCR) para detecção do PCV3 nesses tecidos. Os resultados da coleta das amostram revelou que dos 25 javalis analisados, nove (36%) apresentaram resultado positivo para PCV3 em pelo menos um tecido. Além disso, os tecidos de linfonodo, baço e pulmão apresentaram o maior número de amostras positivas. Do mesmo modo, a sequência completa da região de leitura aberta 2 (ORF2) do genoma do PCV3 foi sequenciada em três amostras positivas utilizando o método de sequenciamento de Sanger. Em que foi observado, 100% de identidade de nucleotídeos entre essas três sequências. As sequências identificadas neste estudo agruparam-se com sequências de PCV3 previamente relatadas no Brasil e em outros países, refletindo a limitada estruturação geográfica atualmente observada para o PCV3. Ademais, a identidade de nucleotídeos com sequências de ORF2 de PCV3 previamente relatadas em javalis brasileiros variou de 98,76% a 99,38%, e para as sequências de suínos domésticos brasileiros, também previamente relatadas, a variação foi de 98,45% a 99,07%. Portanto, os resultados corroboram a alta conservação genética relatada para o PCV3 em todo o mundo. Até onde sabemos, este estudo representa o primeiro relato de detecção de PCV3 em javalis selvagens no estado de São Paulo, Brasil.
Palavras-chave: circovírus suíno, detecção viral, vigilância molecular.

INTRODUCTION

Porcine circovirus (PCV) has the smallest single-stranded circular DNA genome of the genus Circovirus, family Circoviridae (KIM et al., 2018). There are four identified species that infect swine: porcine circovirus 1 (PCV1), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), and porcine circovirus 4 (PCV4) (KROEGER et al., 2022).

PCV3 was first identified in 2015 in tissue samples from domestic swine, associated with dermatitis and nephropathy syndrome and

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reproductive pathologies (PALINSKI et al., 2017). Retrospective studies using PCR on tissue samples indicated the circulation of PCV3 long before its first detection, in pig samples in 1993 (YE et al., 2018) and in wild boar samples in 2004 (KLAUMANN et al., 2019).

PCV3 has been detected in wild boars in other countries around the world, such as Korea (DHANDAPANI et al., 2021), and Italy (FRANZO et al., 2018). In Brazil, PCV3 has been detected in free-living wild boars in Rio Grande do Sul (VARELA et al., 2021; SANTO et al., 2022) and Paraná (SOUZA et al., 2021). Wild boars can act as reservoirs for a wide range of infectious diseases that are important to domestic animals (MENGet al., 2009). Furthermore, they are susceptible to numerous pathogens with the potential for transmission to other animals and humans (DHANDAPANI et al., 2021).

In Brazil, wild boars were introduced in the 1960s, being imported from Uruguay, France, Germany, and Portugal (IBAMA, 2017). The population of free-living wild boars has grown significantly and is mainly concentrated in the Southeast region of the country (HEGEL et al., 2022).

In the state of São Paulo, Brazil, the wild boar population has increased considerably, yet no studies have investigated the occurrence of PCV3 in this species. Therefore, this study investigated the molecular detection of PCV3 in free-living wild boars from the state of São Paulo and characterized the virus phylogenetically in positive samples.

MATERIALS AND METHODS

Animals and sample collection

Twenty-five wild boars were culled by authorized Collectors, Sport Shooters, and Hunters (CACs) during official population control activities, in accordance with Brazilian Law No. 10,826 of December 22, 2003 (BRASIL, 2003). The animals were obtained during population control activities conducted in the municipalities of Braúna, Gabriel Monteiro, Birigui, and Guararapes, in the northwestern region of the state of São Paulo, Brazil. Samples were collected between April 2022 and August 2023.

A convenience sampling approach was used, without the application of biostatistical criteria for sample size determination. Due to the inherent difficulties associated with capturing and culling wild boars, the number of animals included in the study was limited.

Of the 25 wild boars evaluated, 16 were classified as adults (≥ 2 years of age) and nine as juveniles (< 2 years of age). Regarding sex, 14 were males and 11 were females. Gross examination of the carcasses and/or organs revealed no lesions suggestive of systemic disease.

The collection municipalities are located within the Atlantic Forest biome, in areas surrounded by agricultural crops, mainly peanuts, soybeans, sugarcane, corn, sorghum, and potatoes.

Although the samples were obtained from four different municipalities, these locations are geographically close to one another. Guararapes and Gabriel Monteiro share a common border, whereas Birigui and Braúna are separated by only one municipality.

These locations share similar environmental characteristics and land use patterns, with agriculture as the predominant activity. Thus, their geographic proximity and environmental similarity may have contributed to reduced heterogeneity within the sampled population and; consequently, influenced the observed frequency of PCV3-positive animals in this study.

After culling, the material received consisted of either whole carcasses or thoracic and abdominal viscera packaged in plastic bags. Gross examination of the whole carcasses revealed only lesions associated with firearm projectiles sustained during culling, with no gross lesions suggestive of systemic disease.

The material was sent refrigerated, in thermal boxes, to the Pathology Department, where it remained refrigerated until the time of necropsy, performed within 12 hours after slaughter. During the necropsy, fragments of spleen, liver, lung, and lymph nodes were collected, totaling 100 samples, 25 of each type of tissue. All collected fragments were kept at -80 °C until DNA extraction.

DNA extraction

DNA extraction was carried out using phenol: chloroform: isoamyl alcohol solution (GREEN & SAMBROOK, 2012). After extraction, the Nanodrop® instrument (Thermo Scientific) was used for DNA quantification and purity analysis of the extracted samples. The samples showed DNA concentrations ranging from 200 to 1,200 ng/µL, with purity values (A260/A280) between 1.8 and 2.0.

Real-Time Polymerase Chain Reaction

The following primers PCV3-A (5'-TGACGGAGACGTCGGGAAAT-3') and PCV3-1 (5'-CGGTTTACCCAACCCCATCA-3') were designed with a fragment size of 113 bp and were designed using Primer3 software (UNTERGASSER et al., 2012), based on similarity regions identified after alignment, performed with AliView and ClustalΩ

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software, of the PCV3 ORF1 sequences available in the public nucleotide database (NCBI-NIH).

PCR was performed on 100 samples (spleen, liver, lung, and lymph node) using the QuantiTect SYBR Green PCR kit (Qiagen) and the Mx3005P QPCR System real-time thermocycler (Agilent). The reaction solution (20 µL) consisted of: target DNA (100 ng), 10 µL of SYBR Green master mix, primers (500 nM), and nuclease-free water to complete the volume. Samples were subjected to an initial denaturation for 15 minutes at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 33 s and extension at 72 °C for 33 s. The dissociation curve was performed from 60 °C to 95 °C, with an increment of 0.5 °C every 5 seconds. Samples presenting Ct <35 together with a single melting peak at 80.5 °C were considered positive. Nuclease-free water was used as a negative control, and a lymph node tissue sample from a symptomatic pig positive for PCV3 was used as a positive control.

Among the positive samples, those with the lowest Ct values (n = 3) were selected for conventional PCR and sequencing of the complete ORF2 region of PCV3. These samples corresponded to two spleen samples (A40 and A41) and one lymph node sample (A42), from three different animals from the municipality of Guararapes. Due to budgetary limitations, it was not possible to sequence all positive samples. Therefore, samples with lower Ct values were prioritized, since they have a higher viral load and; consequently, a higher probability of generating high-quality sequences.

Sanger Sequencing of ORF2

Three PCV3 positive samples were submitted to Sanger sequencing. Primers used were PCR3-F (5'-CCCACATGCGAGGGCGTTTACC-3') and PCR3-R (5'-CGAGGCCGCTTCATCATCCACT-3'), which amplify an 895 base pair region that includes the complete ORF2 sequence (645 bps) of PCV3 (DAL SANTO et al., 2020).

Conventional PCR was performed using the Jumpstart® Taq ReadyMix kit (Sigma Aldrich) and a SimpliAmp® thermocycler (Thermo Fisher Scientific). Each reaction included a total volume of 50 µl, with JumpStart™ Taq ReadyMix (25 µl), target DNA (400 ng), primers (400 nM), and ultrapure water to complete the volume. Samples were subjected to an initial denaturation for 2 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, followed by a final cycle at 72 °C for 7 min.

Amplicon purification was performed using the Agencourt AMPure XP (Beckman Coulter, Inc.®) and quantification of the purified products was performed on the Qubit® 3.0 fluorometer (ThermoFisher Scientific). Sequencing was then performed using the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems), on the ABI3730xl® DNA Analyzer capillary electrophoresis instrument (Applied Biosystems). Electropherogram analysis was performed using the Chromas Lite and Geneious 4.8.3 software. It is important to highlight that we do not perform viral isolation prior to sequencing.

Phylogenetic analysis

The phylogenetic tree was constructed using nucleotide sequences produced by sequencing the ORF2 region of PCV3 on the FastTree platform (version 2.1.10), using the maximum likelihood (ML) method with the following parameters: NNI (Nearest-Neighbor Interchange), SPR (Subtree Prune and Regraft), and the GTR (General Time Reversible) evolutionary model with CAT (Category Approximation of Rate Heterogeneity) (PRICE et al., 2010). Pairwise nucleotide sequence divergence was estimated using the p-distance method implemented in MEGA 12 (KUMAR et al., 2024).

RESULTS AND DISCUSSION

Real-time PCR and tissue positivity

In our study, nine (36%) of the 25 wild boars tested positive for PCV3. Of these, three were from the municipality of Gabriel Monteiro and six were from the municipality of Guararapes. Among the nine PCV3-positive wild boars, five (55.6%) were positive in all four tissues examined (liver, lung, spleen, and lymph node) (Table 1). Among the remaining four PCV3-positive wild boars, two were positive in the lymph node and spleen, one was positive only in the spleen, and one was positive only in the lung (Table 1). The Ct values of the PCV3-positive samples ranged from 21.33 to 34.88.

PCV3 replication has been documented in multiple porcine tissues (TEMEEYASEN et al., 2021); although a tropism for the lungs, spleen, and lymphoid tissues has been suggested (CHANG et al., 2024). Also in pigs, the highest viral load identified was in the thymus and lymph nodes (DEIM et al., 2019). In wild boars, PCV3 was most frequently detected in the spleen, liver, lungs, and tonsils (KLAUMANN et al., 2019). In another study, intermediate levels of PCV3 DNA were detected in

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Table 1 - Number of animals and percentage of tissues (spleen, liver, lung and lymph node) positive for porcine circovirus 3 (PCV3).

Positive tissues	Number of animals	Percentage
Liver, lung, spleen and lymph node	5	55.6%
Lung, spleen and lymph node	1	11.1%
Spleen and lymph node	1	11.1%
Spleen	1	11.1%
Lung	1	11.1%

serum samples, whereas high levels were detected in lung samples (SOUZA et al., 2021).

In the present study; although viral load was not quantified, PCV3 was detected exclusively in a single tissue (either the spleen or the lung) in 22% of the PCV3-positive wild boars. Notably, eight of the nine PCV3-positive wild boars tested positive in the spleen, consistent with previous reports and suggesting that the spleen is a suitable tissue for the molecular detection of PCV3.

In this dataset, the spleen, lung, and lymph node were the tissues with the highest frequency of PCV3 detection, consistent with the broad tissue distribution previously reported for this virus. These findings highlighted the importance of analyzing multiple tissues for the molecular detection of PCV3, as relying on a single tissue may reduce the likelihood of identifying infected animals.

Phylogenetic analysis and distribution of PCV3 in Brazil

For phylogenetic analysis, the complete PCV3 ORF2 sequence was used because this region, which encodes the capsid (Cap) protein, contains 17 lineage-specific amino acid substitutions distributed among 51 PCV3 lineages (QI et al., 2019) and has been widely used in previous phylogenetic studies (DUNDON et al., 2022; RESÉNDIZ-SANDOVAL et al., 2023; YANG et al., 2024).

For the phylogenetic analysis of the PCV3 ORF2 region, three PCR-positive samples (A40, A41, and A42) were sequenced. The resulting sequences were deposited in GenBank under accession numbers PZ577224, PZ577225, and PZ577226, respectively.

Through p-distance analysis, we verified that these three samples showed 100% nucleotide identity, indicating an absence of genetic variability among the analyzed sequences. However, it should be considered that the sampled wild boars came from the same geographic region, which may have limited the detection of greater genetic variability in the studied population.

According to the classification criteria proposed by FUX et al. (2018), the three sequences obtained in this study were assigned to the PCV3b1 group. However, this classification should be interpreted with caution, as the definition of PCV3 genotypes remains controversial and there is still no consensus regarding the robustness and applicability of the currently proposed classification systems (FRANZO et al., 2020; FRANZO & SÉGALES, 2023; COBOS et al., 2026).

P-distance analysis showed that the sequences obtained in this study shared 98.76% to 99.38% nucleotide identity with PCV3 sequences previously reported in Brazilian wild boars and 98.45% to 99.07% nucleotide identity with PCV3 sequences previously reported in Brazilian domestic pigs.

The nucleotide identity analysis demonstrated low genetic variability between the sequences obtained in this study and those previously reported in Brazilian wild boars and domestic pigs, with a maximum nucleotide divergence of 0.6%.

These findings are consistent with the study conducted by SARAIVA et al. (2019), which suggested that the PCV3 Cap protein evolves under purifying selection pressure, not tolerating high levels of mutation in its amino acid sequence. Thus; although the ORF2 region is used in phylogenetic studies, performing analyses with the whole genome may provide better parameters for phylogenetic analysis.

The low nucleotide divergence (<3.80%) observed among all PCV3 sequences included in our analysis is consistent with the high genetic conservation reported for PCV3 and suggests the circulation of genetically similar sequences in wild boars and domestic pigs.

In addition, our sequences clustered with PCV3 sequences previously reported from other countries, and the low nucleotide divergence among all sequences analyzed further supports the low genetic diversity of PCV3 on a global scale (FRANZO et al., 2020; FRANZO& SÉGALES, 2023; KLAUMANN et al., 2018).

Considering the nucleotide divergence observed between the sequences identified in this study and PCV3 sequences previously reported in Brazilian wild boars and domestic pigs, together with the phylogenetic clustering of our sequences (Figure 1) and the low nucleotide divergence among all sequences analyzed (<3.80%), two hypotheses may be proposed. These findings may reflect either

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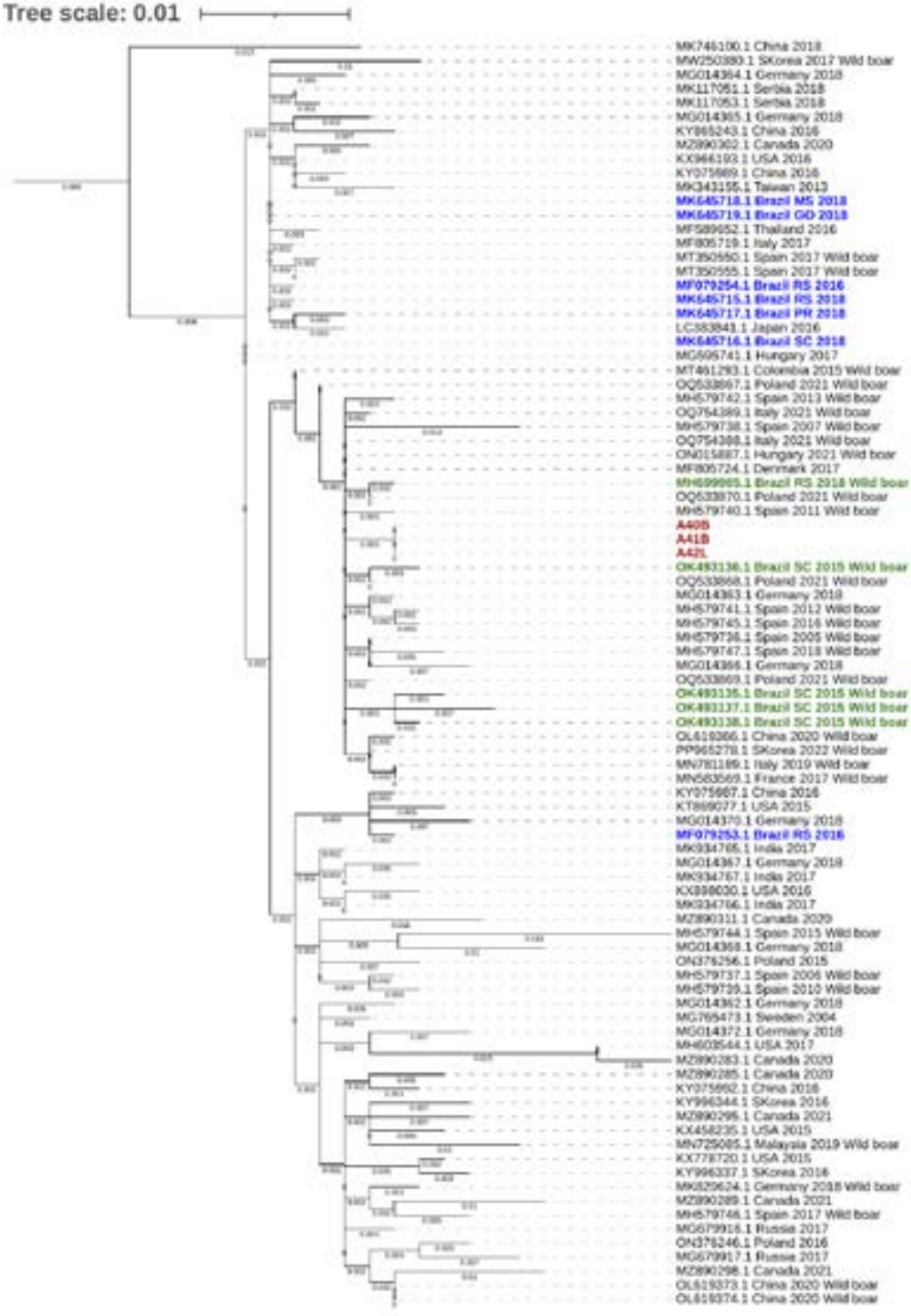

Figure 1 - Phylogenetic tree of porcine circovirus 3 (PCV3) constructed with FastTree (version 2.1.10) and the parameters NNI, SPR, GTR, and CAT. The analysis was based on the complete ORF2 region of PCV3, with three sequences obtained from this study, analyzed together with 87 other sequences from the ORF2 region of domestic pigs and wild boars available in the NCBI database. The sequences in green are from studies with free-living wild boars in Brazil, in blue, from domestic pigs in Brazil, and in red, the sequences from this study.

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multiple introductions of closely related PCV3 sequences into Brazil or the local evolution of a single introduced sequence that accumulated limited nucleotide changes while circulating among wild boars and domestic pigs.

Therefore, our study provided only preliminary phylogenetic conclusions. Future phylogeographic studies, including larger numbers of representative samples from different geographic regions and collection periods, complete genome sequencing, molecular clock analyses, spatiotemporal reconstruction of viral dispersal, and the integration of epidemiological, historical, and animal movement data, will be necessary to determine whether PCV3 was introduced into Brazil through wild boars, domestic pigs, or both.

Such studies could provide a comprehensive understanding of the geographic distribution of PCV3 and its dispersal patterns, as well as clarify the role of human activities and globalization in its spread.

CONCLUSION

This study represents the first report of PCV3 detection in free-living wild boars in the state of São Paulo, Brazil. Our findings highlighted the importance of analyzing multiple tissues to improve the molecular detection of PCV3. We also observed low genetic variability among the analyzed PCV3 sequences, consistent with the high genetic conservation reported for this virus worldwide. These findings reinforced the need for continued genomic surveillance to improve our understanding of the evolutionary dynamics and dispersal of PCV3 in Brazil.

DATA AVAILABILITY STATEMENT

The nucleotide sequences generated in this study are publicly available in the NCBI GenBank database under accession numbers PZ577224, PZ577225, and PZ577226, corresponding to samples A40, A41, and A42, respectively. The corresponding sequence data are also available from the authors upon reasonable request.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare that they have no financial, personal, or institutional interests that may have influenced the results and production of this scientific article.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was submitted to the Ethics Committee on the Use of Animals of the Faculdade de Medicina Veterinária de Araçatuba (FMVA) e Faculdade de Odontologia de Araçatuba (FOA), of the Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP). It was approved under Process FOA nº 321-2024 on June 26, 2024.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) exclusively for grammar and spelling corrections. The manuscript was written by the authors, and ChatGPT was not used to generate scientific content or to generate, analyze, or interpret the research data, results, or conclusions. The authors reviewed all AI-assisted changes and assume full responsibility for the final content of the manuscript.

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