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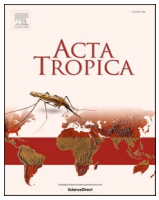

Hemoplasmas in wild rodents and marsupials from the Caatinga Biome, Brazil

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ABSTRACT

A total of 231 blood samples from wild mammals belonging to the orders Rodentia ($n = 142$) and Didelphimorphia ($n = 89$) were screened by real-time PCR assay (qPCR), being six *Rhipidomys* sp., 118 *Thrichomys laurentius*, nine *Rattus rattus*, four *Kerodon rupestris*, five *Necromys lasiurus*, 42 *Didelphis albiventris* and 47 *Monodelphis domestica*. Results using qPCR showed that 32 of the total 231 (13.85 %) samples were positive for hemoplasma sequences of the 16S rRNA gene. Sequences from two *D. albiventris* showed 99.77–99.89 % identity with '*Candidatus* Mycoplasma haemoalbiventris' and 99.09 % with '*Candidatus* Mycoplasma haemodidelphidis', respectively. Furthermore, one *M. domestica* and five *T. laurentius* showed 99.72–99.77 % identity with *Mycoplasma* sp., and one *K. rupestris* showed 98.13 % identity with '*Candidatus* Mycoplasma haematohydrochaerus'; and from two *Rattus rattus* showed 99.65–99.89 % identity with *Mycoplasma* sp. and '*Candidatus* Mycoplasma haemomuris'. The 23S rRNA gene sequences obtained from the two *D. albiventris* showed 100 % identity with '*Ca.* M. haemoalbiventris' whereas the sequences from the *R. rattus* showed only 85.31 % identity with '*Candidatus* Mycoplasma haematohydrochaerus'. Two *T. laurentius* and one *K. rupestris* showed 84.66–92.97 % identity with '*Candidatus* Mycoplasma haemosphiggurus'. Based on phylogenetic and Neighbor-Net network analyses of the 16S and 23S rRNA genes, potential novel species are described. In addition, '*Ca.* M. haemoalbiventris' was detected in *Didelphis albiventris*, and *Mycoplasma* sp. was detected in *Rattus* sp. rodents from the Caatinga biome, Brazil.

1. Introduction

Hemotropic mycoplasmas (HM, hemoplasmas) are Gram-negative bacteria that attach to erythrocyte surfaces and may cause hemolytic anemia in several mammalian species, including humans (Messick, 2004; Maggi, 2013; Hattori et al., 2020). HM species have also been reported in Japan, Hungary, Israel, Brazil, Chile, Türkiye and Kyrgyzstan (Neimark et al., 2001; Neimark et al., 2005; Vieira et al., 2009; Barker et al., 2010; Steer et al., 2011; Sashida et al., 2013; Hornok et al., 2015; Harasawa et al., 2015; De Sousa et al., 2017; Gonçalves et al., 2019; Erol et al., 2023; Altay et al., 2023). *Mycoplasma coccoides* (Neimark et al., 2005) and '*Ca.* Mycoplasma haemomuris' (Neimark et al., 2001) can infect both laboratory and wild rodents. Two novel HM species, '*Ca.* M. haemosphiggurus' (Valente et al., 2020) and '*Ca.* M. haematohydrochaerus' (Vieira et al., 2021) have been characterized in hairy dwarf porcupines (*Sphiggurus villosus*) and capybaras

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(*Hydrochoerus hydrochaeris*). Additionally, potentially novel HM species have been reported to infect wild rodents in Brazil and Chile (Gonçalves et al., 2015; De Sousa et al., 2017; Gonçalves et al., 2019).

In marsupials, '*Ca.* M. haemodidelphis' and '*Ca.* M. haemoalbiventris' were reported to infect the Virginia opossum (*Didelphis virginiana*) in the USA (Messick et al., 2002), and the white-eared (*D. albiventris*) (Massini et al., 2019; Gonçalves et al., 2019; Pontarolo et al., 2021), and black-eared opossum (*D. aurita*) in Brazil (Oliveira et al., 2023).

Rodents and marsupials are considered synanthropic animals because of their ability to adapt to urban environments due to urbanization pressures (Hornok et al., 2015). Some wild rodents are endemic in the Caatinga in Brazil, which is considered the world's most biodiverse biome (MMA, 2022). The Caatinga is an exclusive and understudied biome in a semi-arid region with high environmental and species richness (Barbosa and Filho, 2022). The area contains approximately 183 mammal species, of which 41 belong to the order Rodentia and 13 to the order Didelphimorphia (Silva et al., 2017). The interactions between people and nature in the Caatinga are complex, with the rural population sometimes depending on native vegetation for their subsistence. Although the region is predominantly urban and is home to approximately 28.6 million people, it has low human development indicators in Brazil. Human activities and urbanization have modified the region's ecosystems (Silva et al., 2018). Furthermore, this region is one of the most vulnerable to climate change.

Expanding urban areas into forested areas increases human-wildlife interactions and may promote the spillover of zoonotic pathogens (Santos et al., 2022). Regarding hemoplasma infection, the first report of a human hemoplasma infection was in 2008, in an HIV-positive patient co-infected with *Mycoplasma haemofelis* and *Bartonella henselae*, in Brazil (dos Santos et al., 2008). Later, a study in China of 65 veterinarians and farm workers found Mycoplasma suis infection in 49 % of patients (Yuan et al., 2009), and another study detected *Mycoplasma ovis* (Sykes et al., 2010) and *Mycoplasma haematoparvum* (Maggi et al., 2013) infection in veterinarians from USA. Later, another study in Brazil detected *Mycoplasma* sp. infection in a human who lived in contact with dogs and horses in a rural settlement (Vieira et al., 2015). Moreover, it has been hypothesized that '*Candidatus* Mycoplasma haematohominis', that causes disease in humans, probably has its natural host in bats (Millan et al., 2020). Thus, constant contact with domestic, synanthropic and even wild animals is of great concern for public health.

Although wild animals may act as sentinels for zoonotic diseases, they can also harbor pathogens previously found only in domestic animals, which can cause damage to or even endanger wild species (Hornok et al., 2015), enhancing the importance of HM studies in wild mammals. Especially since infection by Mycoplasma-type organisms has recently been considered a zoonosis after humans contracted the infection when they came into contact with bats (Descoulux et al., 2021). Thus, this study aimed to investigate HM occurrence and genetic diversity in rodents and marsupials from the Caatinga biome in Northeastern Brazil.

2. Material and methods

2.1. Study area

The study was conducted in 14 municipalities in four states located in the Caatinga biome of Northeastern Brazil (Fig. 1). The Caatinga biome occupies an area of 844,453 km^2, approximately 9.9 % of the Brazilian territory, comprising all nine states in northeastern Brazil (Alagoas, Bahia, Ceará, Maranhão, Pernambuco, Paraíba, Rio Grande do Norte, Piauí, Sergipe) and the north portion of the Minas Gerais state (IBGE, 2004). This region is characterized by a semi-arid climate, low humidity and rainfall, high temperatures, and long, dry periods (Mendonça and Danni-Oliveira, 2007). It is considered the most diverse

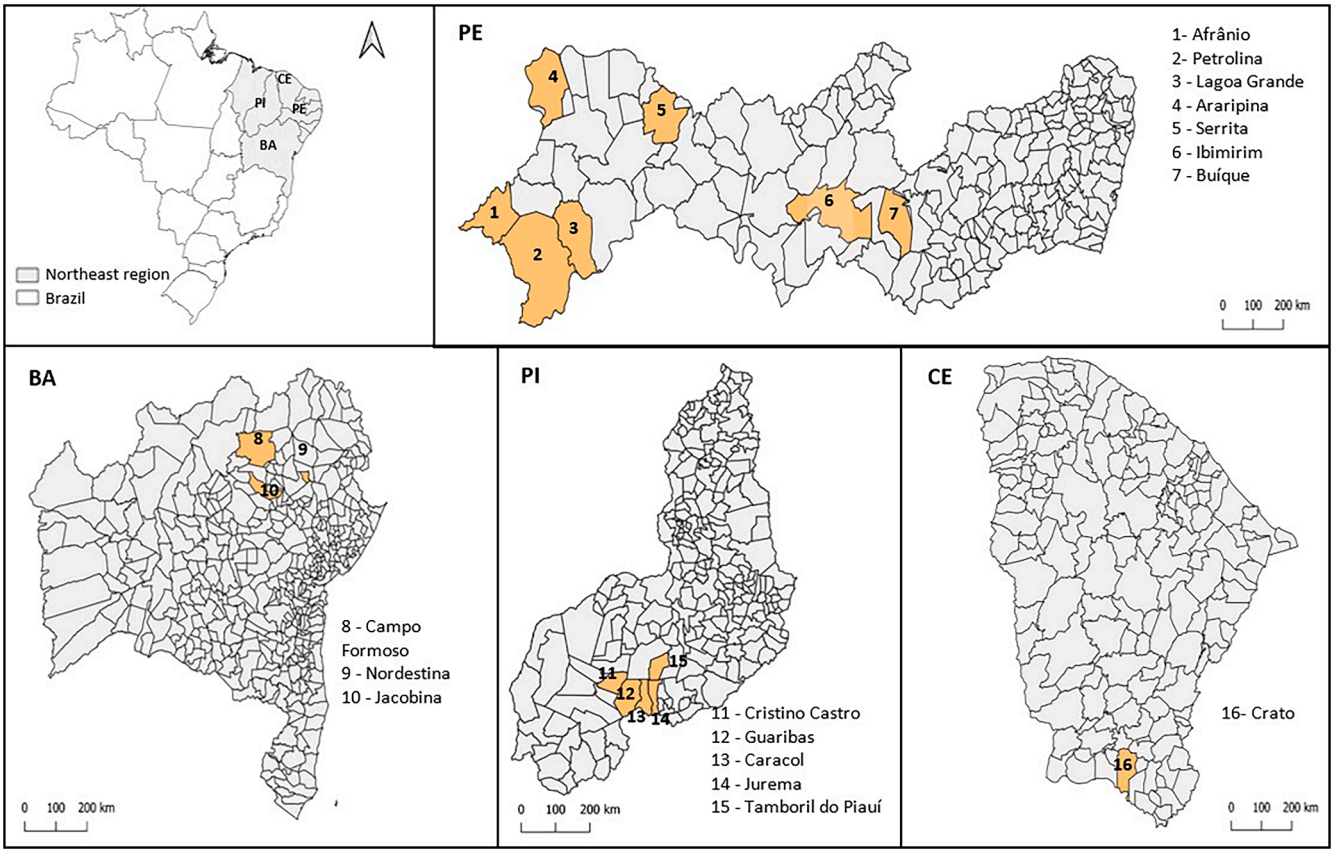

Fig. 1. Sampling sites of wild rodents and marsupials in municipalities from northeast Brazil. PE = Pernambuco, PI = Piaui, BA = Bahia and CE = Ceará.

ecosystem in the world, with a great multiplicity of animal species and vegetation (MMA, 2022).

2.2. Blood samples

A total of 231 blood samples from wild mammals in the order Rodentia ($n = 142$) and Didelphimorphia ($n = 89$) were used in this study, being six *Rhipidomys* sp., 118 *Thrichomys laurentius*, nine *Rattus rattus*, four *Kerodon rupestris*, five *Necromys lasiurus*, 42 *Ddidelphis albiventris* and 47 *Monodelphis domestica*. Blood collection was performed through the caudal vein and/or intra-cardiac puncture. Animal blood samples were centrifuged (3000 × *g*, 15 min), and the sera obtained were aliquoted into 1.5 mL tubes and stored at −20 °C (samples previously screened for other pathogens) (Horta et al., 2018; Santos et al., 2021). Samples were stored at −20 °C until molecular procedures were performed.

2.3. DNA extraction

DNA from 200 μL of whole blood was extracted using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, USA), following the manufacturer's instructions. Ultrapure water was used in parallel as a negative control to monitor cross-contamination. To monitor DNA extraction, a conventional PCR assay targeting a fragment of the mammalian endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was performed on all samples (Rottman et al., 1996).

2.4. Real-time polymerase chain reaction (qPCR)

All DNA samples were initially screened using a universal SYBR Green real-time PCR (qPCR) assay targeting the 16S rRNA gene of HMs, as previously described (Willi et al., 2009). A standard curve was generated using a ten-fold serial dilution of gBlock™ (Integrated DNA Technologies, Coralville, IA, USA). The expected melt-temperature mean for the reaction was 77.398. All parameters were analyzed according to the standards established by the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) (Bustin et al., 2009). Samples with a median cycle quantification (Cq) value < 32 were considered positive (Vieira et al., 2015).

2.5. PCR assays and DNA sequencing

All HM-positive samples by the qPCR assay were subjected to PCR targeting using a fragment (~ 950 bp) of the 16S rRNA HM gene as previously described (Hoelzle et al., 2011; Machado et al., 2017). DNA-positive samples detected in the PCR assay using the 16S rRNA gene were subjected to PCR targeting a fragment (~800 bp) of the 23S rRNA gene from HMs (Mongruel et al., 2020) (Table 1). Nuclease-free water and *Mycoplasma ovis* DNA obtained from naturally infected goats (*Capra aegagrus hircus*) (Machado et al., 2017) were used as negative and positive controls, respectively, for both PCR assays.

2.6. Phylogenetic, genotype diversity, and distance analysis

The assembled consensus sequences were generated using Geneious Prime v. 2019.2.3, subjected to BLASTn analysis (Altschul et al., 1990), and aligned with the sequences available in the GenBank® database using MAFFT (Katoh and Standley, 2013). The best-fit evolutionary models were estimated as GTR+G+I for 16S rRNA and GTR+G for 23S rRNA genes based on the Akaike information criterion (AIC) by using jModeltest version 2.1.10 (Darriba et al., 2012). Phylogenetic analysis used Bayesian inference with three independent runs of 100 million generations of Monte Carlo Markov Chain (MCMC) with one sampling/10,000 generations and a 10 % burn-in on the CIPRES Science Gateway (Miller et al., 2010). The reconstruction was visualized using FigTree 1.4.0 software.

Table 1
List of primers, target gene, number of amplicon size (pb), respective references, of the PCR tests.

Test	Primers	Target genes	Amplicon Size (pb)	Reference
PCR	(Forw) CCT TCA TTG ACC TCA ACT ACA T	gapdh	-	Birkenheuer et al. (2003)
PCR	(Rev) CCA AAG TTG TCA TGG ATG ACC	gapdh		
qPCR	SYBR HAEMO (Forw) AGC AAT RCC ATG TGA ACG ATG AA	16S rRNA	155	Willi et al. (2009)
qPCR	SYBR HAEMO (R1) TGG CAC ATA GTT TGC TGT CAC TT	16S rRNA		
qPCR	SYBR HAEMO (R2) GCT GGC ACA TAG TTA GCT GTC ACT	16S rRNA		
PCR	16S HAEMO (Forw) GGC CCA TAT TCC TRC GGG AAG	16S rRNA	950	Hoelzle et al. (2011); Machado et al. (2017)
PCR	16S HAEMO (Rev) ACR GGA TTA CTA GTG ATT CCA	16S rRNA		
PCR	23S HAEMO (Forw) TGA GGG AAA GAG CCC AGA C	23S rRNA	800	Mongruel et al. (2020)
PCR	23S HAEMO (Rev) GGA CAG AAT TTA CCT GAC AAG G	23S rRNA		

The genotypic diversity among the 16S rRNA and 23S rRNA gene sequences detected in this study and closely related to HMs was determined and aligned as described above and submitted for analysis using DnaSP6 software (Librado and Rozas, 2009). Inference and graphic representations were made using the TCS Network method in PopART software (Leigh and Bryant, 2015). Furthermore, distance analysis based on a split-network was performed using SplitStree v. 4.14.6 software (Huson and Bryant, 2006) by applying the Neighbor-Net method with the same alignment.

Herein, sequences of hemoplasma-positive samples were deposited in the GenBank® database under the accession numbers for 16S rRNA: OP271909, OP271917, OP271915, OP271910, OP271914, OP271916, OP271918, OP271919, OP271911, OP271912, OP271913, and 23S rRNA: OP271903, OP271905, OP271907, OP271908, OP271904, OP271906). Analysis was performed using hemoplasma sequences deposited in the Genbank® database (accession numbers for the 16S rRNA: MN423258, MN423260, MW703800, MT170012, AF178676, KT215626, KT215626, MN423261, K T215635, KM258432, AB820289, 23255, FJ667773, MW616881; MW617219, MW617212, MN692881).

3. Results

The *gapdh* gene was consistently amplified in all the samples. A total of 32/231 (13.85 %; 95 %CI: 9.99–18.90 %) animals were positive for HM species by qPCR. The HM-positive animals belonged to the Rodentia and Didelphimorphia families, and 22 (68.75 %) were collected in

Pernambuco, seven (21.8 %) were from Piaui, two (6.2 %) were from Bahia, and one (3.1 %) was from Ceará State. The efficiency of qPCR for HMs was 97.976 %, the r^2 was 0.998, the slope was -3.371, and the y-intercept was 34.024. The gBlock melt-curve mean was 77.369°C, and the melting temperature of positive samples varied from 77.108–77.631. Regarding hemoplasma infection rate in each species, it was 10.52 % (8/42) in *D. albiventris*, 2.13 % (1/47) in *M. domestica*, 50 % (3/6) in *Rhipidomys* sp., 12.71 % (15/118) in *T. laurentius*, 22.22 % (2/9) in *R. rattus*, 25 % (1/4) *K. rupestris* and 40 % (2/5) in *N. laurentius*. Mycoplasma-positive samples, GenBank® accession numbers, percentage sequence and identities are shown in Table 2.

Phylogenetic analyzes of the 16S rRNA gene of the HMs detected in the two *D. albiventris* (OP271909 and OP271917) in the present study were grouped with '*Ca.* M. haemoalbiventris', previously detected in *D. aurita* (OP279616 and OP279617) and *D. albiventris* (MN423260, MN423257, MT170016, MT170014, MH158515, MN423256, MT170015, MN423259, MT170012, MT170013, MN423258, MH158514, MW703800, MW703802 and MW703801) in Brazil, and *D. marsupialis* (AF178676) in the USA (Fig. 2). The sequence obtained of one *K. rupestris* (OP271913) of the current study was positioned in a separate clade to '*Ca.* M. haematohydrochoerus' detected in capybaras from Brazil (MN423254, MN423255, FJ667773, MW616882, and MW616880) with a posterior probability of > 99 % (Fig. 2). The sequences obtained of the five *T. laurentius* (OP271910, OP271914, OP271916, OP271918 and OP271919) and one *M. domestica* (OP271915) of the present study were grouped as *Mycoplasma* hemotrope sp. of sequences previously detected in *Rhipidomys macrurus* (KT215626) from northwestern Brazil, and are closely related to HM species detected in wild rodents from Brazil (KT215621, KT215624, KT215626, KT215627 and KT215630) and an HM species detected in *Neovison mink* from Chile (MT446244 and MT462251) (Fig. 2). The sequences from *R. rattus* (OP271911 and OP271912) obtained in this study were grouped with sequences previously detected in *R. rattus* (MN423261 and KT215635) and *R. novergicus* (KM258432) from Brazil, and *R. tanezumi* (ON733033) from South Africa, with posterior probabilities >99 % for the closely related '*Ca.* M. haemomuris subsp. ratti' that was described in *R. rattus* from Japan (AB758439) (Fig. 2).

In agreement with the Baye-sian inference, the neighbor-net network analysis (Fig. 3) showed a clear phylogenetic separation between HMs found in rodents and marsupials and those previously detected in other mammalian species. In Hap_1, the HM sequences from *T. laurentius* and *M. domestica* were grouped close to the sequences previously detected in wild rodents from Brazil. In Hap_2, the sequences obtained from *R. rattus* grouped with those from rodents from Brazil and South Africa. In Hap_3, the sequences obtained from *D. albiventris* grouped with those previously described in opossums from Brazil. Hap_4 comprised only the HM sequence detected in *K. rupestris.* Additionally, genotype network analysis was performed using HM species sequences of the 16S rRNA gene detected in mammals in previous studies and showed geographic separation between the detected haplotypes (Fig. 4).

The 23S rRNA gene sequences obtained from *D. albiventris* (OP271903 and OP271905) were closely positioned to '*Ca* M. haematoalbiventris' detected in *D. albiventris* from Brazil (MN442085) (Fig. 5). The *23S rRNA* gene sequence obtained from *K. rupestris* positioned in a separate clade to '*Ca.* M. haematohydrochoerus' (MW617210 and MW617212), with posterior probabilities > 97 % (Fig. 5). The 23S rRNA gene sequences obtained from *T. laurentius* (OP271907 and OP271908) were positioned in separate clades with sequences obtained from *R. rattus* (OP271904), with posterior probabilities > 99 % (Fig. 5).

4. Discussion

In the present study, we indicated cross-species transmission of HMs among small rodents and marsupials, differing from a previous study that showed a lack of cross-species transmission between small rodents and opossums (Gonçalves et al., 2019). Herein, 10.9 % of *D. albiventris* samples from Petrolina (Pernambuco, northeastern Brazil) were positive for hemotropic *Mycoplasma* sp. Previous studies have found similar HM prevalence rates in *D. albiventris*: 32.5 % in central-western Brazil (Gonçalves et al., 2019) and 40 % and 23.08 % in southern Brazil (; Antonangelo et al., 2021). However, in another study in southern Brazil, hemoplasma *Mycoplasma* sp. infection was not detected in the *D. albiventris* (Antonangelo et al., 2021). Different biomes, climates, and vegetation influence the habits and behavior of opossums, and thus, their exposure to HMs, which may explain the discrepancy in results from different studies in other regions of Brazil (Fig. 4). To date, all studies on the detection of HMs in Brazilian opossums have used conventional PCR assays as a diagnostic method (Massini et al., 2019; Gonçalves et al., 2019; Pontarolo et al., 2021; Antonangelo et al., 2021). Consequently, to the best of our knowledge this is the first study to use qPCR to detect HM species in opossums from Brazil.

Phylogenetic analysis of the 16S rRNA (Fig. 2) and 23S rRNA (Fig. 5) gene sequences from the two *D. albiventris* clustered together with those previously described in the *Mycoplasma suis* group and revealed 99.89 % and 100 % identity, respectively, with other HM sequences previously detected in opossums from Brazil. The haplotype networks support these results, confirming that these animals were infected with *'Ca.* Mycoplasma haematoalbiventris' (Fig. 3).

In contrast, HMs infecting *M. domestica* clustered together with those in the *Mycoplasma haemofelis* group from rodents (*T. laurentius*) in the phylogenetic analysis (Fig. 2), as evidenced by genotype analysis in Hap_1, demonstrating that they were infected by the same HM species (Fig. 3). Additionally, different HM genotypes were circulating in *K. rupestris, R. rattus, T. laurentius*, and *M. domestica*, compared to other genotypes described in wild rodents from Brazil.

In qPCR experiments, 16.2 % of the rodent samples were positive for HM species. This is a lower prevalence rate when compared to that in other studies of small wild and synanthropic rodents, with positive rates in animals varying between 21.9–25 % for HM species in central-western Brazil (Gonçalves et al., 2015; Gonçalves et al., 2019). However, the rate was higher than that found in Japan, 11.1 % (Sashida et al., 2013). Many wild rodent species, mainly from Brazil, have been investigated for HMs (Millán et al., 2020). An HM prevalence above 50 % has been detected in synanthropic rodents (*R. rattus* and *Mus musculus*) from Brazil, Japan, Switzerland, and Hungary (Hornok et al., 2015; Gonçalves et al., 2015; Oliveira Conrado et al., 2015), and a recent study in Chile reported 8 % positivity (Alabí et al., 2020).

Initially, two species were described in rodents, *Mycoplasma coccoides* and '*Ca.* M. haemomuris', both belong to the haemofelis group and infects both laboratory and wild mice (Thurston, 1954; Kreier and Hall, 1968). Recently, two new HM species were described in rodents, '*Ca.* M. haemosphiggurus' in hairy dwarf porcupines (*Sphiggurus villosus*) and '*Ca.* M. haematohydrochaerus' in capybaras (*Hydrochoerus hydrochaeris*) from southern Brazil (Valente et al., 2020; Vieira et al., 2021). The 16S rRNA sequences obtained in this study from *K. rupestris, M. domestica, T. laurentius,* and *R. rattus* showed between 84.66 and 100 % identity with other HM species sequences detected in rodents from different regions of Brazil (Fig. 2). Corroborating these findings, the Neighbor-Net network analysis highlighted the genetic distinction between the HM species identified in the present study and other species of mammals in Brazil (Fig. 3).

Phylogenetic analyses of the sequence of *Kerodon rupestris* of 16S rRNA (Fig. 2) and 23S rRNA (Fig. 5) genes of the HMs detected in this study demonstrated a close relationship to '*Ca.* M. haematohydrochoerus' described in capybaras from Brazil, but in separate clades supported by high posterior probabilities. The results were corroborated by the haplotype networks (Fig. 3). Our sequence was assigned to the *M. haemofelis* group as an isolated genotype, with a high number of mutations in the hypothetical haplotypes that link these sequences with the sequences of other species. Based on the 16S rRNA and 23S rRNA gene sequences amplified in the present study, the name '*Candidatus* Mycoplasma haematorupestris' was proposed for this novel organism.

Table 2
Hemoplasmas-positive samples (n = 32) and GenBank® accession numbers of 16S and 23S rRNA genes from wild animals, Northeast Brazil.

Species	Hemoplasma-positive	GenBank® accession number		Identidade GenBank®							
				16S rRNA				23S rRNA			
	N° and (%)	16S rRNA	23S rRNA	(%) Identity	Agent identity	Animal species/ origin	GenBank® accession number	(%) Identity	Agent identity	Animal species/ origin	GenBank® accession number
Didelphis albiventris	8/42 (19 %)	OP271909	OP271903	99.77 - 99.89	*Ca.* M. haemoalbiventris'	*Didelphis albiventris* / Brasil	MN423258, MN423260, MW703800 and MT170012	100	*Ca.* M. haemoalbiventris	*Didelphis albiventris* / Brasil	MW694786
		OP271917	OP271905	99.09	*Ca.* M. haemodidelphidis	*Didelphis virginiana* / EUA	AF178676				
Monodelphis domestica	1/47 (2.1 %)	OP271915	-	99.72 - 99.77	Hemotropic *Mycoplasma* sp.	*Rhipidomys macrurus* / Brasil	KT215626	-	-	-	-
Rhipidomys sp.	3/6 (50 %)	-	-	-	-	-	-	-	-	-	-
Thrichomys laurentius	15/118 (12.7 %)	OP271910 OP271914 OP271916 OP271918 OP271919	OP271907 OP271908	99.72 - 99.77	Hemotropic *Mycoplasma* sp.	*Rhipidomys macrurus* / Brasil	KT215626	84.66 -92.97	*Ca.* M. haemosphiggurus	*Sphiggurus villosus* / Brasil	MN692881
Rattus rattus	2/9 (22.2 %)	OP271911	OP271904	99.65 - 100	Hemotropic *Mycoplasma* sp.	*Rattus rattus* / Brasil	MN423261 and KT215635	85.31	*Ca.* M. haematohydrochaerus	*Hydrochoerus hydrochaeris* / Brasil	MW617219 e MW617212
		OP271912				*Rattus norvegicus* / Brasil	KM258432				
					Ca. M. haemomuris	*Rattus rattus* / Japão	AB820289				
Kerodon rupestris	1/4 (25 %)	OP271913	OP271906	98.13	*Ca.* M. haematohydrochaerus	Capivaras / Brasil	MN423255, FJ667773 and MW616881	84.66-92.97	*Ca.* M. haemosphiggurus	*Sphiggurus villosus* / Brasil	MN692881
Necromys lasiurus	2/5 (40 %)	-	-	-	-	-	-	-	-	-	-

-: Samples not sequence

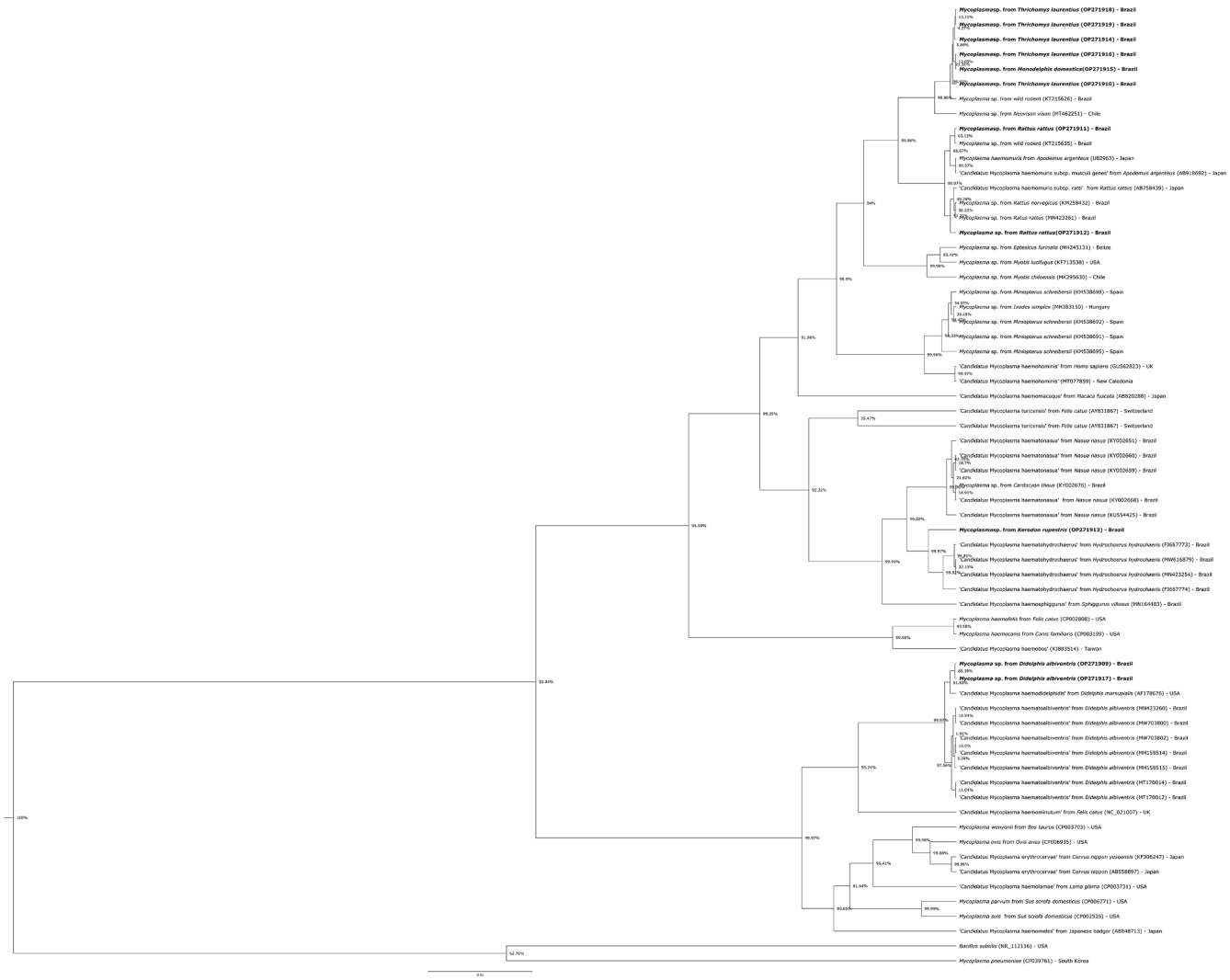

Fig. 2. Phylogenetic tree based on partial 16S rRNA gene showing the relationship of sequences detected in wild rodents and marsupials from this study (in bold) to the other hemoplasmas species. Tree was constructed by Bayesian Inference and a sequence of *Bacillus subtilis* (NR_112116) and *Mycoplasma pneumoniae* (CP039761) were used as outgroup.

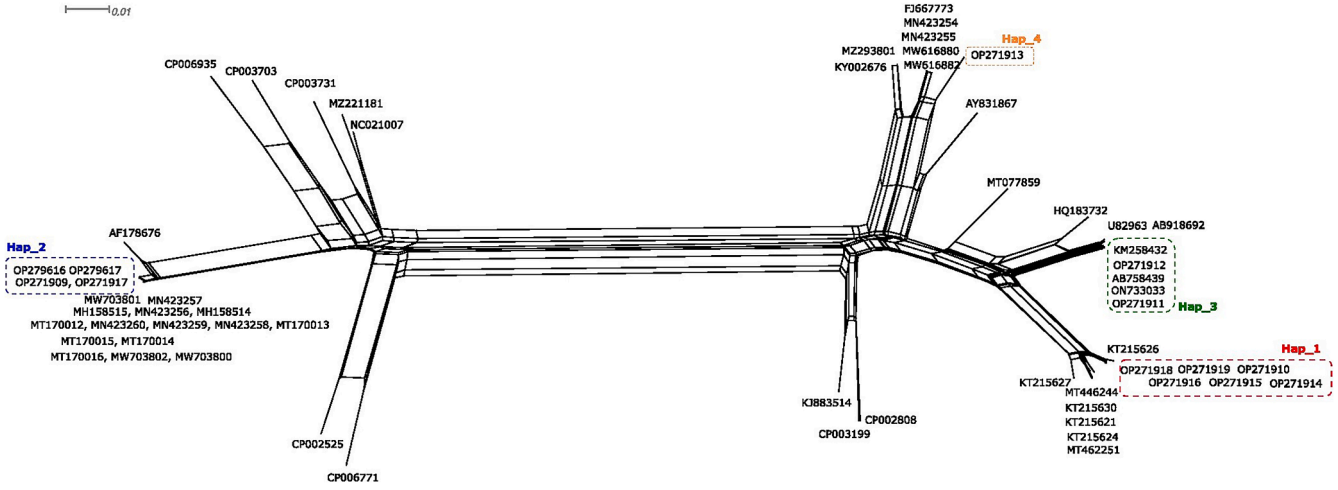

Fig. 3. Distance analysis of partial 16S rRNA gene obtained from wild mammals sampled in the present study with sequences from related hemoplasmas previously deposited in GenBank.

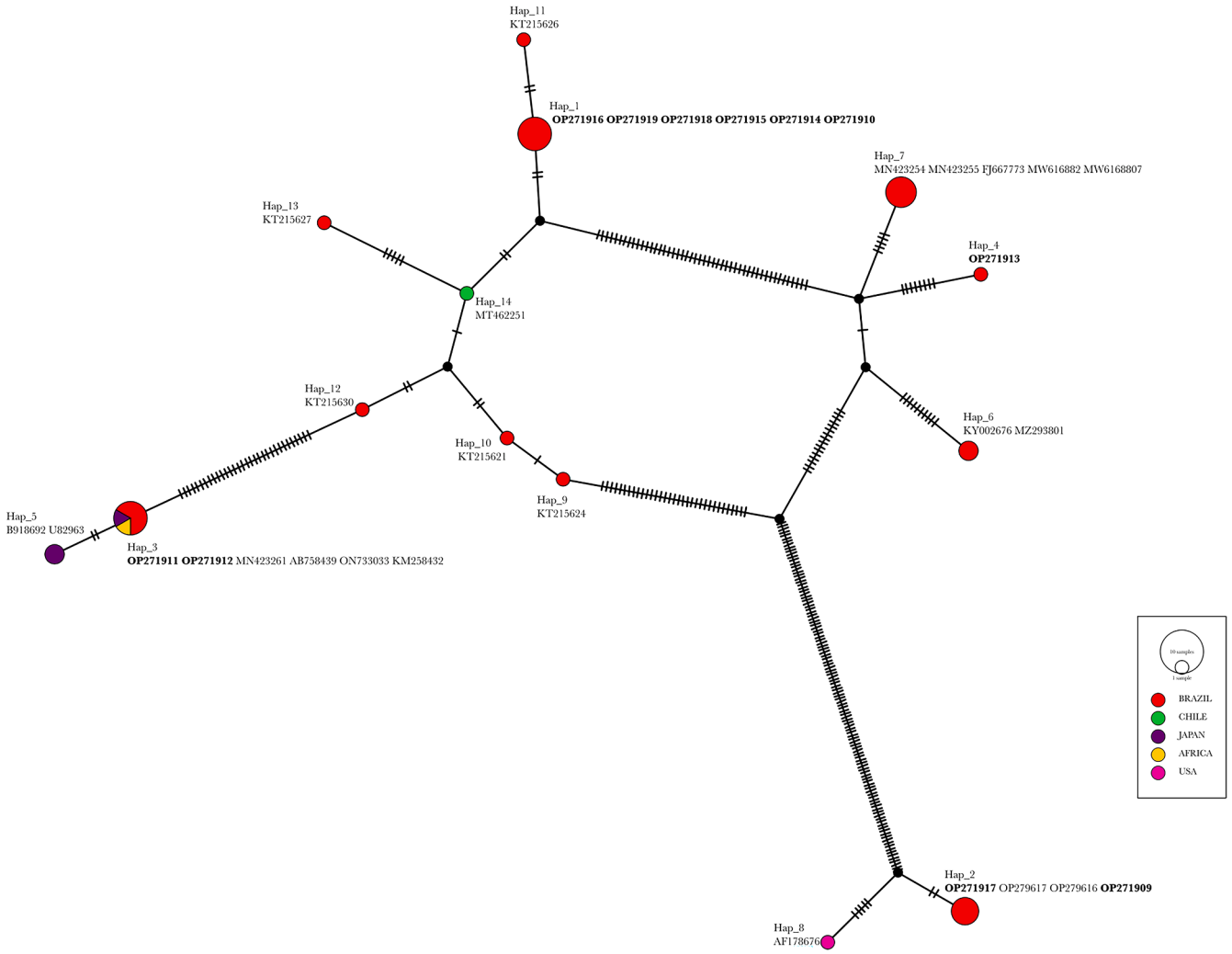

Fig. 4. Genotype diversity of hemotropic *Mycoplasma* species based on the 16S rRNA gene. Sequences detected herein are highlighted in bold.

According to Drancourt and Raoult (2005), if the similarity in the 16S rRNA gene between bacterial isolates were less than 97 %, they belonged to different species. Furthermore, it has been noted that relying on a single gene presents significant limitations because there may be an insufficient number of informative nucleotide sites and homologous recombination, making it difficult to distinguish very similar species (Hanage et al., 2006). On this pattern, we have also sequenced the 23S rRNA. This methodology has been globally adopted for novel hemoplasma species characterization (Pontarolo et al., 2021; Collere et al., 2021; Vieira et al., 2021).

Sequencing of 16S rRNA and 23S rRNA gene fragments from HM detected in *T. laurentius* and *M. domestica* revealed 99.72–99.77 % and 84.66–92.97 % identity with *Mycoplasma* sp. and 'Ca. M. haemosphiggurus', respectively (Table 2). Phylogenetic analyses of the 16S rRNA gene sequence of the HMs detected herein showed a close relationship to multiple hemotropic *Mycoplasma* sp. described in wild rodents from Brazil and Chile (Fig. 2), but in separate clades supported by high posterior probabilities, and in an isolated clade on the 23S rRNA gene phylogenetic tree (Fig. 5). The haplotype networks supported these results (Fig. 3). Our sequences were assigned to the *M. haemofelis* group as isolated genotypes, with a high number of mutations in the hypothetical haplotypes that linked these sequences with the sequences of other species. Accordingly, based on the 16S rRNA and 23S rRNA gene sequences amplified in the present study, the name '*Candidatus* Mycoplasma haematolaurentius' was proposed for this novel organism. Moreover, the *M. domestica* was infected with the same microorganism.

Similarly, sequencing of the 16S rRNA and 23S rRNA gene fragments from the two HM-positive *R. rattus* revealed 99.65–100 % identity with hemotropic *Mycoplasma* sp. and 99.89 % identity with '*Ca.* Mycoplasma haemomuris subsp. ratti' detected in *R. rattus* (Table 2). However, sequencing of the 23S rRNA gene fragments from HM-positive *R. rattus* showed only 85.31 % identity with '*Candidatus* Mycoplasma haematohydrochoerus' detected in capybaras (*Hydrochoerus hydrochaeris*) from Brazil (Table 2). Phylogenetic analyses showed that the 16S rRNA gene for HM from the two of *R. rattus* closely related to '*Ca.* Mycoplasma haemomuris subsp. ratti' from *R. rattus* (AB758439) detected in this study was previously described in *R. rattus* in Japan (Fig. 2). However, phylogenetic analyses of the 23S rRNA gene for HM isolated it in a separate clade, with only a few closely related 23S rRNA gene sequences available in the GenBank® database (Fig. 5). Corroborating this finding, the Neighbour-Net analysis demonstrated genetic similarity among the HM species circulating in the HM-positive *R. rattus* sequences obtained in this study, and other *Rattus* sp. species from Brazil, Japan and South Africa (Fig. 3).

At the time of sampling, ectoparasites (especially ticks) were found, but unfortunately, they were not collected. Ticks have been suggested as potential vectors of HMs and may be closely associated with the spread of the pathogen among wild animals, domestic animals and humans (Oliveira et al., 2021). However, further studies should confirm this hypothesis.

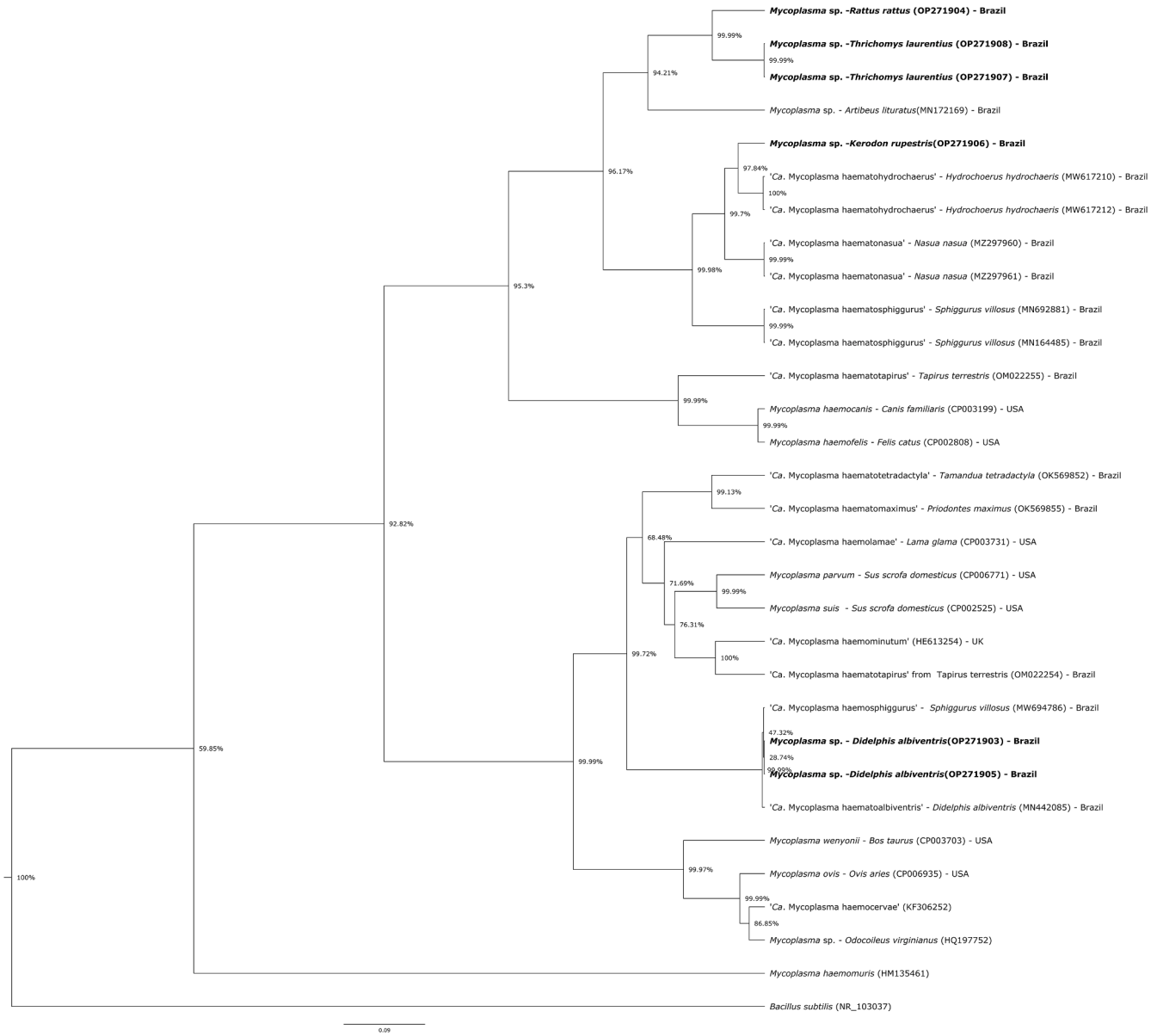

Fig. 5. Phylogenetic tree based on partial sequence of the 23S rRNA gene showing the relationship of sequences detected in wild rodents and marsupials from this study (in bold) to the other hemoplasmas species. Bayesian Inference constructed tree and a sequence of *Bacilus subtilis* (NR_103037) was used as outgroup.

5. Conclusion

This study showed that new HM species are highly prevalent in wild rodents from Brazil. Based on phylogenetic and Neighbor-Net network analysis of the 16S rRNA and 23S rRNA genes, two potentially novel species infecting *Kerodon rupestris, Trichomys laurentius* and *Monodelphis domestica* were described in the Caatinga biome, Brazil. The names '*Ca.* Mycoplasma haematorupestris' and '*Ca.* Mycoplasma haematolaurentius' are proposed for these novel organisms. Moreover, '*Ca.* Mycoplasma haemoalbiventris' was detected in *Didelphis albiventris,* and hemotropic *Mycoplasma* sp. was detected in *Rattus* sp. rodents from the Caatinga biome, Brazil.

Ethical approval

The authors confirm that have followed the ethical policies of the journal. This study was approved by the Ethics Committee on Animal Use of the Universidade Federal do Vale São Francisco (CEUA/ Univasf; protocol numbers 0004/070813 and 0010/021014); and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol numbers 36585-1, 45764-1, and 45764-2).

CRediT authorship contribution statement

Paula Talita Torres-Santos: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Anna Maria da Cruz Ferreira Evaristo:** Methodology. **Josenilton Rodrigues Santos:** Methodology. **Flávia Carolina Meira Collere:** Writing – review & editing, Validation, Methodology, Data curation. **Thállitha Samih Wischral Jayme Vieira:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Luiz Cezar Machado Pereira:** Methodology. **Patricia Avello Nicola:** Methodology. **Rafael Felipe da Costa Vieira:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Mauricio Claudio Horta:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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