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Short Communication

Detection of Hemotropic *Mycoplasma* sp. in white-eared opossums (*Didelphis albiventris*) from Southern Brazil

Detecção de *Mycoplasma* sp. hemotrópico em gambás-de-orelha-branca (*Didelphis albiventris*) no Sul do Brasil

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Abstract

Opossums are marsupials from the New World of the genus *Didelphis* and known as synanthropic animals due to their proximity with human beings. To date, '*Candidatus* Mycoplasma haemodidelphis' has been solely found infecting the North American opossum (*Didelphis virginiana*). Accordingly, the aim of this study was to screen eight white-eared opossums (*Didelphis albiventris*) from a public park in Maringa city, Paraná State, southern Brazil, for hemoplasma infection. Blood samples were taken from caudal venipuncture, and DNA was extracted and further screened by a pan-hemoplasma PCR assay. Seven out of eight (87.50%; CI 95%: 47.35-99.68%) white-eared opossums were positive for *Mycoplasma* spp. Sequencing of the 16S rRNA fragment showed 98,97% identity with '*Ca.* M. haemodidelphis' detected in the USA. Three out of eight (37.50%; CI 95%: 8.52-75.51%) white-eared opossums were infested by *Amblyomma dubitatum* ticks. This is the first report on detection of a potentially novel hemotropic *Mycoplasma* sp. infecting opossums from South America.

Keywords: Marsupials, hemotropic mycoplasmas, hemoplasmas, *Mycoplasma* sp.

Resumo

Gambás são marsupiais do Novo Mundo, pertencentes ao gênero *Didelphis*, e considerados animais sinantrópicos devido à sua proximidade com seres humanos. Atualmente, a espécie '*Candidatus* Mycoplasma haemodidelphis' só foi encontrada infectando gambá norte americano (*Didelphis virginiana*). O objetivo do presente estudo foi detectar a infecção por hemoplasmas em oito gambás-de-orelha-branca (*Didelphis albiventris*) capturados em um parque público da cidade de Maringá, no Estado do Paraná, sul do Brasil. Amostras de sangue foram coletadas por venopunção caudal para a extração do DNA e posterior análise pela PCR para espécies de hemoplasmas. Sete de oito animais (87,50%; CI 95%: 47,35-99,68%) foram considerados positivos para *Mycoplasma* spp. O sequenciamento do fragmento do gene 16S rRNA obtido apresentou 98.97% de similaridade com sequências de '*Ca.* M. haemodidelphis' detectadas nos Estados Unidos. Três gambás (37,50%; CI 95%: 8,52-75,51%) estavam infestados por carrapatos da espécie *Amblyomma dubitatum*. Esse é o primeiro relato de detecção de uma potencial nova espécie de *Mycoplasma* hemotrópico infectando gambás na América do Sul.

Palavras-chave: Marsupiais, micoplasmas hemotrópicos, hemoplasmas, *Mycoplasma* sp.

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Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic bacteria that may cause immune-mediated hemolytic anemia in animals and human beings (MESSICK, 2004; SYKES et al., 2010). *Mycoplasma* sp. have been detected in wildlife worldwide (MESSICK, 2004; VOLOKHOV et al., 2017). In Brazil, hemoplasma species have been found in wildlife, such as lion (*Panthera leo*) (GUIMARAES et al., 2007), capybaras (*Hydrochaeris hydrochaeris*) (VIEIRA et al., 2009), small rodents (GONÇALVES et al., 2015; SOUSA et al., 2017), nonhuman primates (BONATO et al., 2015; CUBILLA et al., 2017b; RAMALHO et al., 2017; de MELO et al., 2019), coatis (*Nasua nasua*) (CUBILLA et al., 2017a; SOUSA et al., 2017), wild canids (ANDRÉ et al., 2011; de SOUSA et al., 2017), free-ranging and/or captive jaguars (*Panthera onca*) (ANDRÉ et al., 2011; FURTADO et al., 2018), pumas (*Puma concolor*), jaguarundis (*Puma yagouaroundi*), little spotted cats (*Leopardus tigrinus*) (ANDRÉ et al., 2011), ocelots (*Leopardus pardalis*) (ANDRÉ et al., 2011; SOUSA et al., 2017), bats (IKEDA et al., 2017) and wild boars (DIAS et al., 2019).

Opossums are marsupials comprised on the genus *Didelphis*, recognized as synanthropic animals due to human-environment interactions (MALTA & LUPPI, 2007). The occurrence of different vector-borne pathogens (VBPs) has been reported in *Didelphis* sp. (MESSICK et al., 2002; CASTELLAW et al., 2011; MELO et al., 2016; SILVA et al., 2017; FERREIRA et al., 2017; LONDOÑO et al., 2017; SOARES et al., 2017). '*Candidatus* Mycoplasma haemodidelphis' has been solely detected in the North American opossum (*Didelphis virginiana*) (MESSICK et al., 2000; MESSICK et al., 2002). In Brazil, although *Didelphis* sp. may be exposed to different VBPs, such as *Ehrlichia* spp., *Rickettsia* spp. and *Borrelia* spp. (MELO et al., 2016), to date only one opossum has been studied in this country and tested negative for hemotropic *Mycoplasma* spp. (SOUSA et al., 2017). Accordingly, the present study aimed to identify the occurrence of hemoplasma species in free-ranging opossums (*D. albiventris*) from Maringá city, Paraná State, southern Brazil, using PCR-based assays.

This study was approved by the Ethics Committee for Animal Experimentation and Animal Welfare at the Universidade Federal do Paraná, Brazil (protocol number 053/2018). Animal and laboratory procedures were approved and performed under regulations of the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol number 63433-2).

The study was carried out in an urban public park of Maringá city: Ingá park (51°55′59″ W, 23°25′28″ S). Maringá city is located in northwest region of Paraná State, which is characterized by semideciduous Atlantic Forest fragments and has a subtropical climate with an average temperature of 21.7 °C. The area has a diverse fauna, with populations of common marmoset (*Callithrix jacchus*), capybaras, and marsupials, as well as a wide variety of birds and fishes. *D. albiventris* is the only species of the genus *Didelphis* living in the park.

A total of eight adult (five females and three males) white-eared opossums (*D. albiventris*) were captured using wire mesh traps baited with fruit. Opossums were identified at species level based on morphological and phenotypical characteristics (LEMOS & CERQUEIRA, 2002). After chemical restraint (xylazine 4.0 mg/kg and ketamine 20 mg/kg), animals were individually microchipped with a subcutaneous implant (Animall TAG®, São Carlos, São Paulo, Brazil) and visually inspected for ectoparasites (ticks and fleas). Ticks were removed and stored in 70% ethanol-labeled tubes for further classification according to morphological taxonomic keys (ARAGÃO & FONSECA, 1961; GUIMARÃES et al., 2001; MARTINS et al., 2016). Thereafter, EDTA blood samples were collected by caudal venipuncture and stored at –20 °C until molecular analysis. After sampling, animals were monitored until total recovery from the chemical restraint and were then released at the park.

DNA was extracted from 200 µL blood using a commercially available kit (Illustra™ Blood Genomic Prep Mini Spin Kit, GE Healthcare Life Sciences, Little Chalfont, UK), according to the manufacturer's instructions. Ultrapure water was used as a negative control in parallel to monitor for cross-contamination.

To ensure successful DNA extraction, PCR for the opossum housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (BIRKENHEUER et al., 2003) was performed in all samples. Thereafter, samples were screened using conventional PCR with genus-specific primers targeting a portion of the 16S rRNA gene of *Mycoplasma* spp. (HOELZLE et al., 2011; MACHADO et al., 2017). The amplified PCR products were subjected to gel electrophoresis in 1.5% agarose gels for 1 h at 100 V, followed by SYBR safe staining (6 µg/ml; SYBR® Safe DNA Gel Stain, Invitrogen, CA, USA) and were viewed under a 312 nm UV light transilluminator.

Amplicons (871 bp) obtained from two *Mycoplasma* sp.-positive samples were purified from the agarose gel (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, EUA), evaluated by spectrophotometry for concentration and purity (NanodropTM 2000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, MA, USA), and sequenced in both directions by the Sanger method. The assembled partial sequences of the 16S rRNA gene were subjected to BLASTn analysis (ALTSCHUL et al., 1990) to determine the identity with sequences deposited in the GenBank database. Nucleotide sequences of the *Mycoplasma* sp. amplified herein were submitted to the GenBank database (accession numbers: MH158514 and MH158515).

The hemoplasma 16S rRNA sequences were aligned using MAFFT 7.110 (KATOH & STANDLEY, 2013). The best-fit evolutionary model was estimated as F81+G using jModeltest 2.1.4 (DARRIBA et al., 2012). The Bayesian information criterion (BIC) and maximum likelihood (ML) algorithms were used for phylogenetic inference. Reconstruction was visualized with FigTree 1.4.2 software.

All samples consistently amplified the opossum *gapdh* gene. Seven out of eight (87.50%; CI 95%: 47.35-99.68%) opossums were positive for *Mycoplasma* sp. by PCR. Sequencing of the 16S rRNA fragment showed 98.97% identity with '*Ca*. M. haemodidelphis' (GenBank® accession No: AF178676) detected in the USA. Phylogenetic 16S rRNA gene fragment analysis confirmed the close relationship of the white-eared opossum hemoplasma genotype with '*Ca*. M. haemodidelphis' detected in North American opossum. Moreover, the *Mycoplasma* sp. detected herein and the '*Ca.* M. haemodidelphis' detected in North American opossum formed a strongly supported branch with the hemotropic *Mycoplasma* sp. detected in raccoon (*Procyon lotor*) in the USA within the *Mycoplasma suis* group (Figure 1).

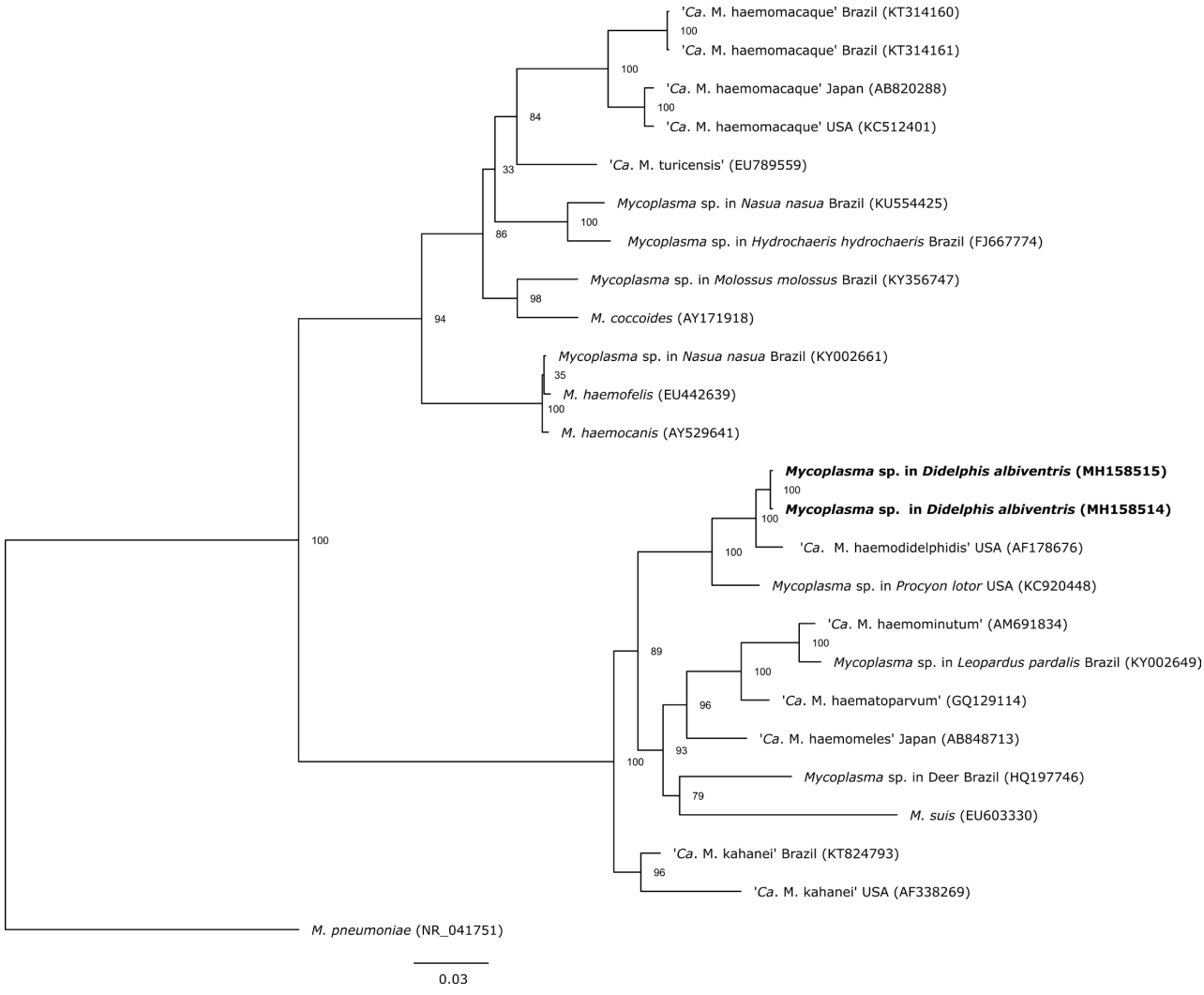

Figure 1. Phylogenetic tree based on partial sequences of the 16S rRNA gene, showing the relationship between the hemotropic *Mycoplasma* sp. detected in the white-eared opossums (*Didelphis albiventris*) from this study and other hemoplasmas. *Bacillus subtilis* was used as an outgroup. The GenBank accession number is in parentheses after the species name and origin of each bacterium. maximum likelihood analyses were carried out applying the F81+G model and 1000 bootstrap replicates for all analyses.

Three out of eight (37.50%; CI 95%: 8.52-75.51%) opossums were infested by ticks (6 nymphs and 14 larvae) identified as *Amblyomma dubitatum* nymphs and *Amblyomma* sp. larvae.

Herein, we report the molecular detection of hemotropic *Mycoplasma* sp. closely related to '*Ca*. M. haemodidelphis' in free-ranging opossums from Brazil. To the authors' knowledge, molecular detection of hemoplasma in opossums (*Didelphis* sp.) has only been described in the United States (MESSICK et al., 2000; MESSICK, 2004). In the Brazilian Pantanal region, a previous study screened 30 marsupials for hemoplasma infection, including one white-eared opossum, and all tested negative for *Mycoplasma* sp. by conventional PCR targeting the 16S rRNA of these bacteria (SOUSA et al., 2017).

In the present study, 87.5% of opossums were positive for hemotropic *Mycoplasma* sp. Previous studies have suggested high hemoplasma prevalence rates in tropical regions, which may favor transmission by arthropod vectors. Although previous studies have implicated ticks in the transmission of hemoplasmas, such as *Rhipicephalus sanguineus* sensu lato as a vector of *Mycoplasma haemocanis* in dogs (SENEVIRATNA et al., 1973) and *Haemaphysalis plumbeum* and *Rhipicephalus bursa* as vectors of *Mycoplasma ovis* in small ruminants (NEIMARK et al., 2004), to date there has not been adequate evidence to support the contention that hemoplasmas are truly vector-borne pathogens. Herein, although 37% of opossums were infested by *A. dubitatum* and *Amblyomma* sp. ticks, further studies are needed to elucidate the role of *A. dubitatum* ticks in the transmission of hemoplasmas.

Raccoons are considered adapted to urbanized and suburbanized environments (GEHRT et al., 2010), similar to opossums. A previous study has shown that raccoons from undisturbed habitats were more likely to be infected by hemoplasmas than animals from urban areas (VOLOKHOV et al., 2017). Herein, a high prevalence of hemotropic *Mycoplasma* sp. was found in opossums from public parks located in an urban area of Paraná State, southern Brazil.

Further studies are needed to elucidate differences in hemoplasma prevalence among environments.

Additionally, the analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting white-eared opossums from Southern Brazil. This data is supported since the inhabited areas for *D. virginiana* and *D. albiventris* do not overlap (COSTA et al., 2015; PÉREZ-HERNANDEZ et al., 2016), and thus, an infection of *D. albiventris* in Brazil with '*Ca.* M. haemodidelphis' from *D. virginiana* by direct transmission is less likely. Further studies evaluating other housekeeping genes (e.g. *rpoB*, 23S rRNA) are needed for characterization of this potentially novel hemotropic *Mycoplasma* species.

A potentially novel hemotropic *Mycoplasma* sp. is very prevalent in white-eared opossums from southern Brazil. This is the first report on detection of a hemotropic *Mycoplasma* sp. infecting opossums from South America.

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