SEMINA
Ciências Agrárias

DOI: 10.5433/1679-0359.2022v43n1p431

Hemotropic mycoplasmas in bats from forest fragments, state of Paraná, southern Brazil

Detecção e caracterização de micoplasmas hemotrópicos em morcegos de fragmentos florestais, Paraná, Sul do Brasil

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Highlights

Bats as reservoir of several pathogens of concern for human and animal health.
Human's 'Candidatus Mycoplasma haematohominis' probably has its natural host in bats.
Hemotropic Mycoplasma spp. has been poorly reported in bats.

Abstract

The order Chiroptera is the second largest group of mammals with bats being identified as reservoir of several viral zoonoses, although, little is known about their role in other groups of pathogens, including hemotropic *Mycoplasma* spp. To date, hemoplasma species have been found infecting several species of bats with high genetic diversity between 16S rRNA gene sequences. On this study, we aimed to identify the occurrence and characterize 16S and 23S rRNA genes of hemoplasma species in four bats species (*Artibeus lituratus, Carollia perspicillata, Sturnira lilium* and *Sturnira tildae*) from forest fragments in Paraná State, southern Brazil, using PCR-based assays. Spleen tissue samples were collected, DNA extracted

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Received: Aug. 31, 2021 - Approved: Nov. 09, 2021

and further screened by a pan-hemoplasma PCR assay. All samples consistently amplified the mammal endogenous *gapdh* gene. One out of 15 (6.66%; 95% CI: 0.2-31%) bats tested positive for hemotropic *Mycoplasma* sp. by the PCR assay targeting the 16S rRNA gene. Sequencing of the 16S rRNA gene fragment from the hemoplasma-positive bat showed 99.14% identity with hemotropic *Mycoplasma* sp. detected in *Sturnira parvidens* from Belize. Sequencing of the 23S rRNA gene fragment from the hemoplasma-positive bat showed 86.17% identity with 'Candidatus Mycoplasma haemosphiggurus' detected in orange-spined hairy dwarf porcupines (*Sphiggurus villosus*) from Southern Brazil.
Key words: Bats. Hemotropic mycoplasmas. Hemoplasmas. *Mycoplasma* sp.

Resumo

A ordem Chiroptera é considerada a segunda maior ordem de mamíferos do mundo, sendo os morcegos identificados como reservatórios de diversas zoonoses de origem viral, contudo, pouco se sabe sobre seu papel em outros grupos de patógenos, incluindo *Mycoplasma* spp. Até o momento, *Mycoplasma* sp., foi encontrado infectando várias espécies de morcegos ao redor do mundo, com alta diversidade genética entre sequências de genes 16S rRNA. O objetivo do presente estudo foi detectar a infecção por hemoplasmas em quinze morcegos insetívoros de quatro diferentes espécies (*Artibeus lituratus, Carollia perspicillata, Sturnira lilium* and *Sturnira tildae*) provenientes de fragmentos florestais dos municípios de Mandaguaçu, Maringá e Paiçandu, no Estado do Paraná, sul do Brasil. Amostras de tecido foram coletadas e o DNA extraído, para posterior análise por PCR para detecção de hemoplasmas. Todas as amostras amplificaram o gene *gapdh.* Um morcego, do total de 15 (6.66%; 95% CI: 0.2-31%), foi positivo para *Mycoplasma* sp. na análise do gene16S rRNA. O sequenciamento deste fragmento genético mostrou 99,14% de identidade com *Mycoplasma* sp. detectado em *Sturnira parvidens* em Belize. O sequenciamento do fragmento do gene 23S rRNA do morcego positivo mostrou 86,17% de identidade com '*Candidatus* Mycoplasma haemosphiggurus' detectado em ouriço-cacheiro (Sphiggurus villosus) no sul do Brasil.
Palavras-chave*:* Morcegos. Micoplasmas hemotrópicos. Hemoplasmas. *Mycoplasma sp.*

Introduction

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic Gram-negative bacteria lacking a cell wall that are obligate parasites of red blood cells in a wide range of mammals worldwide. They may cause immune-mediated hemolytic anemia (Millán, Di Cataldo, Volokhov, & Becker, 2020). In Brazil, hemotropic *Mycoplasma* spp. have been reported in several wild mammals, including neotropical bats (Ikeda et al., 2017; Santos et al., 2020).

The order Chiroptera is considered to be the second largest group of mammals in the world, with more than 1,200 species present on all continents except Antarctica (Schipper et al., 2008). Bats have been widely studied as reservoir hosts for zoonotic pathogens (Volokhov et al., 2017; Millán, López-Roig, Delicado, Serra-Cobo, & Esperón, 2015), although whether bats are equally competent hosts of non-viral pathogens, such as bacteria, remains an open and unanswered question (Volokhov et al., 2017; Brook & Dobson, 2014). In this regard, the numbers of studies on hemoplasmas infecting bats have been increasing over the past decade. Previous studies showed that hemoplasmas were common in little brown bats (*Myotis lucifugus*) in eastern and northeastern USA (Mascarelli et al., 2014). Additionally, the prevalence of

hemotropic *Mycoplasma* spp. was found to be 97% in Schreibers's bats (*Miniopterus schreibersii*) in Spain (Millán et al., 2015), 67% in vampire bats (*Desmodus rotundus*) in Peru and Belize (Volokhov et al., 2017), 83.33% in D. *rotundus* in Brazil (Santos et al., 2020), 26.66% in molossids (*Molossus molossus* and *M. nigricans*) in Brazil (Ikeda et al., 2017) and 60% in fruit bats (*Eidolon* sp.) in Nigeria (Di Cataldo, Kamani, Cevidanes, Msheliza, & Millán, 2020).

Several 16S rRNA gene sequences from hemotropic Mycoplasma spp. that are phylogenetically related to known hemoplasma species have been identified in bats. Sequences with similarity to *Mycoplasma coccoides* in molossids in Brazil (Ikeda et al., 2017), '*Candidatus* Mycoplasma haematohominis' in *Miniopterus schreibersii* in Spain (Millán et al., 2015) and *Pteropus* spp. in New Caledonia (Descloux et al., 2020), along with other potentially novel hemoplasma genotypes, have been described in bats (Millán et al., 2020). However, due to the high genetic diversity between 16S rRNA gene sequences from hemoplasmas found in bats, studies involving other genes of this group of bacteria are needed in order to better characterize the species. Accordingly, the aim of the present study was to identify and characterize 16S and 23S rRNA genes of hemoplasma species occurring in four bat species (*Artibeus lituratus, Carollia perspicillata, Sturnira lilium* and *Sturnira tildae*) from forest fragments in the state of Paraná, southern Brazil, using PCR-based assays.

Materials and Methods

Ethical approval

This study was approved by the Ethics Committee for Animal Experimentation and Animal Welfare of the Federal University of Paraná, Brazil (protocol number 053/2018). The animal and laboratory procedures were approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol number 63433).

Study area and sampling

The study was carried out in an urban public park in the city of Maringá: Ingá Park (23° 25′ 48.4″ S 51° 55′ 43.2″ W) and rural areas of the municipalities of Mandaguaçu (23° 19′ 4.6″ S 52° 4′ 21.3″ W;) and Paiçandu (23° 29′ 55.3″ S 52° 8′ 16.1″ W), in Paraná. The areas studied are located in the northwestern region of Paraná, which is characterized by semideciduous Atlantic Forest fragments. This region has a subtropical climate with an average temperature of 21.7 °C. The areas studied have diverse fauna that commonly includes populations of capybaras (*Hydrochoerus hydrochaeris*), non-human primates, reptiles, Didelphis spp., *Nasua nasua* and bats, as well as a wide variety of birds and fishes.

A total of 15 bats (five females and 10 males) were caught during September 2018 and March 2019 using mist nets. These were placed along natural or artificial trails and kept there for six-hour periods that straddled sunset time. The nets were checked every 30 min. The capture effort was 3.240 square hectometers. Four out of these 15 bats (26.7%) were caught in the rural area of the municipality of Mandaguaçu, six (40%) in Ingá Park and five (33.3%) in the rural area of the municipality of Paiçandu. These bats were placed in individual cloth bags, chemically restrained (xylazine 4.0 mg/kg and ketamine 20.0 mg/kg), euthanized using isoflurane in a carbon dioxide chamber and identified at the species level (Reis,

Peracchi, Batista, Lima, & Pereira, 2017). Four species were identified: *Artibeus lituratus* (7/15; 46.67%), *Carollia perspicillata* (2/15; 13.33%), *Sturnira lilium* (4/15; 26.67%) and *Sturnira tildae* (2/15; 13.33%). Spleen fragment samples were collected, labeled and stored at -80 °C until the time of molecular analyses.

DNA extraction and PCR assays

DNA was extracted from spleen fragment samples using a commercial kit (QIAamp™ DNA micro-kit, Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Ultrapure water was used as a negative control in parallel, to monitor for cross-contamination.

A conventional PCR to investigate the mammalian endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (Birkenheuer, Levy, & Breitschwerdt, 2013) was performed to ensure successful DNA extraction. Thereafter, all DNA samples were screened by means of a genus-specific PCR assay targeting a fragment (900 bp) of the 16S rRNA gene of hemoplasmas (Hoelzle et al., 2011; Machado et al., 2017). Bat DNA samples that were found to be positive in the screening PCR assay based on 16S rRNA were subjected to a genus-specific PCR assay targeting a fragment (800 bp) of the 23S rRNA gene of hemoplasmas (Mongruel et al., 2020). Nuclease-free water and *Mycoplasma ovis* DNA from a naturally infected goat blood sample were used as negative and positive controls, respectively, in both PCR assays. The amplified PCR products were subjected to electrophoresis on stained 1.5% agarose gel (SYBR® Safe DNA gel stain, Invitrogen) for 50 min at 100 V and then visualized.

Sequencing and phylogenetic analysis

Amplicons of the 16S rRNA and 23S rRNA genes obtained from one hemotropic *Mycoplasma* sp.-positive sample were sequenced in both directions using the Sanger method, with nucleotide sequences of the 16S rRNA and 23S rRNA genes of hemotropic *Mycoplasma* sp. submitted to the GenBank® database (accession numbers: MN170512 and MN172169).

The partial sequences of the 16S and 23S rRNA genes of hemotropic *Mycoplasma* spp. were aligned using MAFFT 7.110 (Katoh & Standley, 2013) on the Guidance 2 server (Drummond, Suchard, Xie, & Rambaut, 2012), for each gene. Phylogenetic analyses on the 16S and 23S genes were performed based on Bayesian inference (BI) using the Beast 1.8.0 package (Drummond et al, 2012). Three independent runs of 100,000,000 generations of Monte Carlo Markov Chain (MCMC) were performed, with one sampling per 10,000 generations and a 10% burn-in. The substitution models were estimated as GTR+G for the 16S rRNA gene and GTR+I+G for the 23S rRNA gene, based on the Akaike information criterion (AIC) using jModeltest 2.1.10 (Darriba, Taboada, Doallo, & Posada, 2012). Reconstructions were visualized using the FigTree 1.4.4 software (Sela, Ashkenazy, Katoh, & Pupko, 2015). The 16S rRNA gene and 23S rRNA gene trees were rooted with *Mycoplasma pneumoniae* (NZ_CP008895).

Results and Discussion

All the samples consistently amplified the mammalian endogenous *gapdh* gene. One out of 15 bats (6.66%; 95% CI: 0.2-31%) tested positive for hemotropic *Mycoplasma*

sp. in the PCR assay targeting the 16S rRNA gene. Sequencing of the 16S rRNA gene fragment from the hemoplasma-positive bat showed 99.14% identity with hemotropic *Mycoplasma* sp. detected in a bat (*Sturnira parvidens*) (MH245137) in Belize, Central America, 98.90% identity with hemotropic *Mycoplasma* sp. (MH245136) detected in *Uroderma bilobatum* in Belize, and 98.29% identity with multiple hemotropic Mycoplasma sp. (KY932674, KY932675, KY932677, KY932678, KY932679, KY932680) detected in *D. rotundum* in Belize and Peru, with 100% query cover. Sequencing of the 23S rRNA gene fragment from the hemoplasma-positive bat showed 86.17% identity with '*Candidatus* Mycoplasma haemosphiggurus' detected in orange-spined hairy dwarf porcupines (*Sphiggurus villosus*) (MN692881 and MN164485) in southern Brazil and 80.46% identity with *Mycoplasma haemofelis* (CP002808, FR773153, NR_103993).

Phylogenetic analysis on the 16S rRNA and 23S rRNA genes of the bat hemoplasma detected in the present study clustered together with those of the *M. haemofelis* group (Figure 1 and Figure 2). Different branches were demonstrated in the phylogenetic analysis on 16S rRNA gene fragments from hemoplasmas previously detected in bats (Figure 1). The phylogenetic analysis showed that there was a close relationship between the *A. lituratus* hemoplasma detected here and the hemotropic *Mycoplasma* spp. detected in *Sturnira parvidens* and *Uroderma bilobatum* bats in Belize (Figure 1).

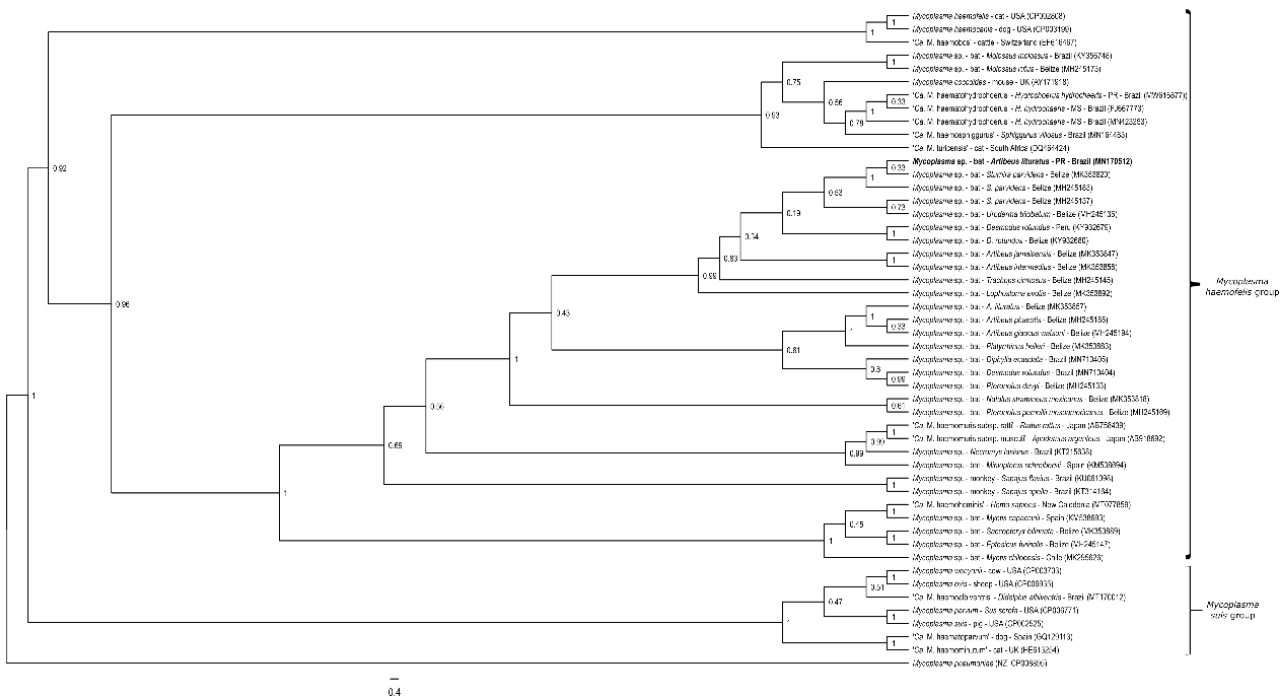

Figure 1. Phylogenetic tree based on partial sequences (~900 bp) of the 16S rRNA gene. The phylogenetic tree was inferred by using Bayesian inference (BI). The substitution models were estimated as GTR+G for the 16S rRNA gene, based on the Akaike information criterion (AIC) using jModeltest 2.1.10.. The sequence detected in the present study is highlighted in bold. *Mycoplasma pneumoniae* was used as an outgroup. Branch lengths represent units of substitutions per site.

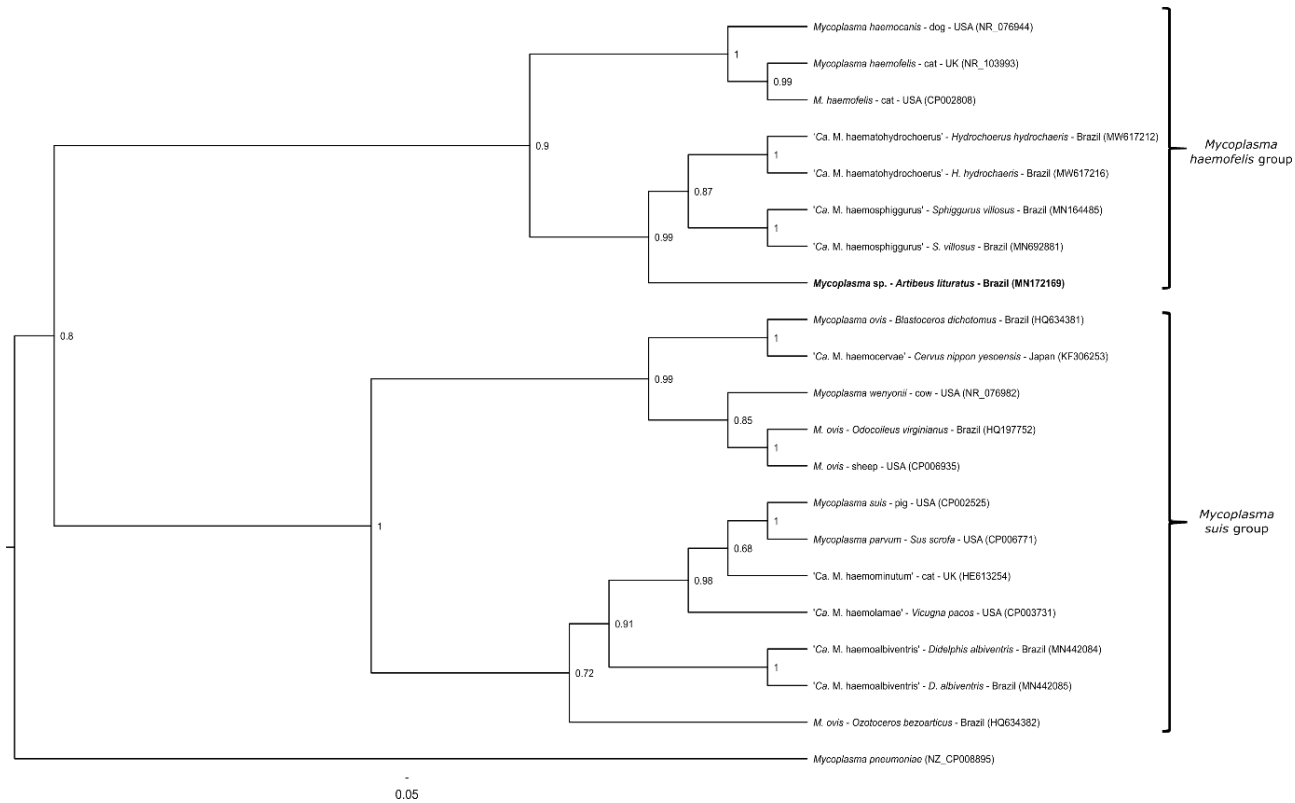

Figure 2. Phylogenetic tree based on partial sequences (~620 bp) of the 23S rRNA gene. The phylogenetic tree was inferred by using Bayesian inference (BI). The substitution models were estimated as GTR+I+G for the 23S rRNA gene, based on the Akaike information criterion (AIC) using jModeltest 2.1.10. The sequence detected in the present study is highlighted in bold. *Mycoplasma pneumoniae* was used as an outgroup. Branch lengths represent units of substitutions per site.

High genetic diversity among 16S rRNA gene sequences from hemoplasmas detected in bats in 12 countries has been described (Millán et al., 2020). In rodents, high genetic diversity between hemoplasmas has also been found (Alabí et al., 2020). A previous study on bats in Brazil found hemotropic *Mycoplasma* sp. genotypes that were closely related to previously reported genotypes detected in rodents (Ikeda et al., 2017). From an evolutionary point of view, bats and rodents are large groups belonging to ancient orders of mammals that have shown high adaptation as hosts for different groups of pathogens. Here, the 16S rRNA gene sequence from the *A. lituratus* hemoplasma was seen to branch away from rodent hemoplasmas (Figure 1). Moreover, a hemotropic *Mycoplasma* sp. detected in *A. lituratus* in Belize was also seen to branch away from the hemoplasma sequence detected in this bat species in Brazil.

In order to better characterize the hemoplasma species infecting bats, we amplified and sequenced a fragment of the 23S rRNA gene. However, since there are no other closely related hemoplasma 23S rRNA

gene sequences available in the GenBank® database, the bat hemoplasma 23S rRNA gene detected herein showed only 86% identity with the 23S rRNA gene of 'Ca. M. haemosphiggurus' detected in orange-spined hairy dwarf porcupines (*Sphiggurus villosus*) in southern Brazil (Figure 2). Further studies targeting other genes (*RNaseP, rpoB, gyrB*) and/or whole genome sequencing should be performed in order to assess the phylogenetic positioning of bat hemoplasma species.

Previous studies found that hemoplasmas were highly frequent in Schreibers's bats (*Miniopterus schreibersii*) in Spain (Millán et al., 2015), vampire bats (*D. rotundus*) in Peru, Belize and Brazil (Volokhov et al., 2017; Santos et al., 2020), molossids (*Molossus molossus* and *M. nigricans*) in Belize and Brazil (Ikeda et al., 2017; Becker et al., 2020) and Eidolon fruit bats in Nigeria (Di Cataldo et al., 2020). However, in other studies, hemotropic *Mycoplasma* spp. was not found infecting *Pipistrellus pipistrellus* and *P. kuhlii* (Ikeda et al., 2017; Becker et al., 2020). In this regard, a recent study suggested that some bat species may be resistant to infection or have ecological characteristics that reduce their exposure to hemotropic *Mycoplasma* spp. (Millán et al., 2020). Here, only one out of 15 bats tested positive for hemoplasma. Therefore, future studies sampling higher numbers of bats are needed in order to elucidate that hypothesis.

Conclusion

Hemoplasmas are uncommon in bats from the forest fragment areas of the present study, in the state of Paraná, southern Brazil. Phylogenetic analysis on the 16S rRNA gene showed that the *A. lituratus* hemoplasma detected here had a close relationship with hemotropic *Mycoplasma* spp. detected in S. *parvidens* and *U. bilobatum* bats in Belize.

Acknowledgements

Flávia C. M. Collere was sponsored by a fellowship from the the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) at the time of research. The Brazilian National Council of Scientific and Technological Development (CNPq) provided a fellowship of research productivity (PQ) to Dr. Rafael F.C. Vieira (CNPq - 313161/2020-8).

Funding

No funding was used for this research

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

This study was approved by the Ethics Committee for Animal Experimentation and Animal Welfare of the Federal University of Paraná, Brazil (protocol number 053/2018). The animal and laboratory procedures were approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol number 63433).

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