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Genetic diversity and hematological and biochemical alterations in *Alouatta* primates naturally infected with hemoplasmas in Brazil

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ABSTRACT

Mycoplasma spp. and *Bartonella* spp. are Gram-negative bacteria transmitted by arthropod vectors that infect red blood cells of several mammal species. This study investigated the occurrence and genetic diversity of hemoplasmas and *Bartonella* spp. in 68 howler monkeys kept in captivity in São Paulo, a southeastern state in Brazil. In addition, possible hematological, biochemical and electrophoretic changes of serum proteins associated with the occurrence of hemoplasmas and *Bartonella* spp. in captive primates were also investigated. The cPCR results showed that all sampled howler monkeys were negative for *Bartonella* spp. based on the *gltA* gene. The cPCR results indicated that 18 (26.47%) non-human primates (NHP) were positive for hemoplasmas based on the 16S rRNA gene. Monocyte and lymphocyte counts were higher in hemoplasma-positive howlers ($P < 0.05$). Platelet counts decreased in nonhuman primates (NHP) positive for hemoplasmas ($P < 0.05$). The results from the blood serum proteinogram and biochemistry analyses were not significantly different between NHPs positive and negative for hemotrophic mycoplasmas. Phylogenetic analysis using Bayesian Inference (BI) based on the 16S rRNA gene positioned the obtained sequences close to '*Candidatus* Mycoplasma kahanei'. The analysis of sequence diversity of the 16S rRNA gene showed that 5 different genotypes are circulating in NHP in Brazil and in the world; besides, a clear separation between the sequences of hemoplasmas that infect NHP of the *Sapajus* and *Alouatta* genus in Brazil was found, probably corresponding to two different species. The pathogenic potential of this hemoplasma species in NHP should be further investigated.

1. Introduction

Alouatta primates are large arboreal animals that have the highest survival ability in the remaining of degraded forest areas due to their folivorous feeding habit [1]. Checking the health of wild animals kept in captivity allows diagnosing diseases that endanger the life or well being of these animals.

Checking the health of wild animals kept in captivity allows diagnosing diseases that endanger the life or well being of these animals. *Mycoplasma* spp. (hemoplasmas or hemotrophic mycoplasmas) includes arthropod-borne bacteria that attach to the erythrocyte surface of various animal species, including humans [2]. Such microorganisms lack cell wall and have the lowest known non-viral genome, ranging from 580 kb to approximately 2000 kb, with ribosomes and double-stranded circular DNA. They have been implicated as causing infectious hemolytic anemia in several mammal species [3,4]. Animals parasitized by hemoplasmas may present extravascular hemolysis, especially in the spleen, liver, lung and bone marrow. In addition, parasitism by such agents may be associated with intravascular hemolysis due to the fragility of erythrocytes parasitized by such bacteria [5].

Hemoplasmas have been detected in non-human primates (NHPs) in captivity, such as *'Candidatus* Mycoplasma aoti' in *Aotus trivigatus* in the United States [6], *'Candidatus* M. haemominutum' in *Saimiri sciureus* in Guyana [7], *'Candidatus* Mycoplasma haemomacaque' in *Macaca fuscata* in Japan [8], and *'Candidatus* Mycoplasma haemomacaque' in *Macaca fascicularis* in the United States [3]. *'Candidatus* Mycoplasma kahanei' in *Saimiri sciureus* was first described by [6], at the Pasteur Institute in the French Guiana. In Brazil, hemoplasmas have been detected in primates

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in the wild (*Sapajus* sp., *Alouatta caraya*, *Sapajus nigritus*) and in captivity (*Callithrix jacchus, Saguinus midas niger, Saimiri sciureus*) in the states of Paraná, Maranhão and Paraíba [9–11].

The genus *Bartonella* spp. includes Gram-negative, facultative aerobic and fastidious bacteria of medical and veterinary importance that infect a wide variety of hosts, with several mammals as reservoirs [12]. *Bartonella quintana* was detected in a free-ranging *Macaca fascicularis* in the USA, *Macaca mulatta* and *Macaca fascicularis* maintained in captivity in China, in captive *Gorilla gorilla* in West Africa, and in captive *Macaca fuscata* in Japan [13–16]. In Brazil, to date, *Bartonella* spp. has not yet been reported in NHP.

In view of this, this study investigated the occurrence and genetic diversity of hemoplasmas and *Bartonella* spp. in howler monkeys maintained in captivity in São Paulo, a southeastern state in Brazil. In addition, this work researched the possible hematological, biochemical and electrophoretic alterations of serum proteins associated with positivity to hemoplasmas and *Bartonella* spp. in captive primates

2. Material and methods

2.1. Research approval

This research was approved by the Biodiversity Authorization and Information System (SISBIO, protocol n ° 51662-2) and the Ethics Committee on Animal Use (CEUA) of the Sao Paulo State University "Júlio de Mesquita Filho" (protocol no. 5595/16).

2.2. Animals

Between April 2016 and January 2017, blood samples were collected from 68 *Alouatta* monkeys kept in captivity in nine cities in the state of São Paulo: Ilha Solteira Zoo; Municipal Zoo in Missina; Palmeira Zancaner in Catanduva; Joaquim Garcia Franco Municipal Zoo in Guaíra; Thermas dos Laranjais Zoo in Olímpia; Fábio Barreto Municipal Forest in Ribeirão Preto; Guarulhos Municipal Zoo; Quinzinho de Barros Municipal Park in Sorocaba; Center for Medicine and Research on Wild Animals (CEMPAS) in Botucatu, and still, in the Mucky Project, in Itu (Fig. 1). The animals were anesthetized intramuscularly (IM) with 6 mg/kg of tiletamine hydrochloride combined with zolazepam hydrochloride (Virbac Brasil®).

2.3. Hematological analyses

Blood samples were drawn from the cephalic or jugular veins and stored in tubes containing 7.2 mg of ethylenediaminetetraacetic acid dipotassium (K_2EDTA).

Becton Dickinson (BD). Subsequently, after homogenization, the counts of leukocytes, platelets and red blood cell (RBCs), hemoglobin concentration (Hgb), hematocrit value (HT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were performed using an automatic cell counter (HORIBA MEDICAL Analyzer, model ABS Micros ESV 60). For that purpose, an automatic cell counter that allows the evaluation of hematological parameters of 10 animal species, including monkeys, was employed, using specific cards (HORIBA MEDICAL Analyzer, model ABS Micros ESV 60).

The differential count of leukocytes evaluated the percentage of basophils, eosinophils, rod neutrophils, segmented neutrophils, lymphocytes, and monocytes, was performed in Giemsa-stained blood smears (Sigma-Aldrich) using a light microscope (1000X).

2.4. Biochemical blood tests

Blood samples were also collected in clot-activating tubes, centrifuged to separate blood serum that was stored in liquid nitrogen (-196 °C) until further biochemical and serum analyses to evaluate renal and hepatic integrity. The evaluations were conducted using commercial kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) to measure urea (enzymatic method UV) and creatinine (enzymatic trinder), as well as alanine aminotransferase (ALT) (kinetic method UV) and alkaline phosphatase (FA) (method kinetic fixed time). The hematological and biochemical analyses were performed at the Laboratory of Veterinary Clinical Pathology in the Veterinary Hospital of FCAV, UNESP, Jaboticabal, SP, Brazil.

2.5. Serum protein electrophoresis

Blood samples were also collected in tubes containing clot activator to perform serum protein electrophoresis. Initially, total proteins were measured by the Biuret Method, using a set of Labtest Diagnostics (Santa Lago, MG, Brazil) reagents, while reading was performed using a semiautomatic spectrophotometer (Labquest/Labtest, Lagoa Santa, MG, Brazil) in the Laboratory of Veterinary Clinical Pathology of the Veterinary Hospital of FCAV, UNESP, in Jaboticabal, SP, Brazil. Subsequently, electrophoresis was performed in the Support Laboratory of the Department of Clinics and Veterinary Surgery of the FCAV, UNESP. The technique consisted of identifying and quantifying serum proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a vertical system (PROTEAN II XI VERTICAL ELECTROPHORESIS CELLS®-BIO-RAD), 4% stacking gel and 10% separation gel as described by [18]. The electrophoretic runs were performed at pre-fixed 20 and 25 mA for the stacking and separation gels, respectively, lasting 5 h and 30 min on average. Subsequently, the gel was stained with Coomassie brilliant blue while excess dye was removed with solution containing 250 mL methanol, 100 mL glacial acetic acid, and 1000 mL distilled water. Gel reading was performed in a computerized densitometer (SHIMADZU CS-9301-Japan).

2.6. Polymerase chain reaction (PCR)

2.6.1. DNA extraction

The blood samples from 68 howler monkeys were collected into Vacutainer tubes (BD Vacutainer with K_2EDTA), transported into styrofoam boxes containing ice until the laboratory, where they were maintained at - 70 °C until DNA extraction. The genomic DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA), following the manufacturer recommendations. DNA concentration and absorbance (260/280) nm were measured using a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA). The extracted DNA samples were stored at -20 °C until submitted to a conventional PCR assay (cPCR) based on the *gapdh* gene (endogenous control) [19].

2.6.2. Conventional PCR for hemoplasma and Bartonella spp

The samples positive for the *gapdh* gene were subjected to additional cPCR assays directed to the 16S rRNA and RNase P genes of *Mycoplasma* spp. and citrate synthase (*gltA*) gene fragment of *Bartonella* spp.

The *Mycoplasma* spp. 16S rRNA gene fragments were identified using two sets of oligonucleotide primers: HemMycop16S-41 s (5′-GYATGCMTAAYACATGCAAGTCGARCG-3′), HemMyco16S-938as (5′-CTCCACCACTTGTTCAGGTCCCCGTC-3′) (for amplifying an 800 bp fragment) and HemMycop 16S-322 s (5′-GCCCATATTCCTACGGGAAG CAGCAGT-3′), HemMycop16S-1420as (5′-GTTTGACGGGCGGTGTGTA CAAGACC-3′) (for amplifying an 800 bp fragment). The amplification was performed as follows: 95 °C for 2 min, followed by 55 cycles of 94 °C for 15 s, 68 °C for 15 s, and 72 °C for 18 s, followed by a final extension at 72 °C for 1 min [2]. Additionally, samples positive for *Mycoplasma* spp. in cPCR assays based on the 16S rRNA gene were submitted to cPCR based on the RNase P gene, using the primers HemoMyco RNaseP30 s (5′- GATKGTGYGAGYATATAAAAAATAAARCTC-RAC-3′) and HemoMyco RNaseP200as (5′-GMGGRGTTTACCGCGTTT-CAC-3′), for amplifying a 165 bp fragment of *Mycoplasma* spp. The

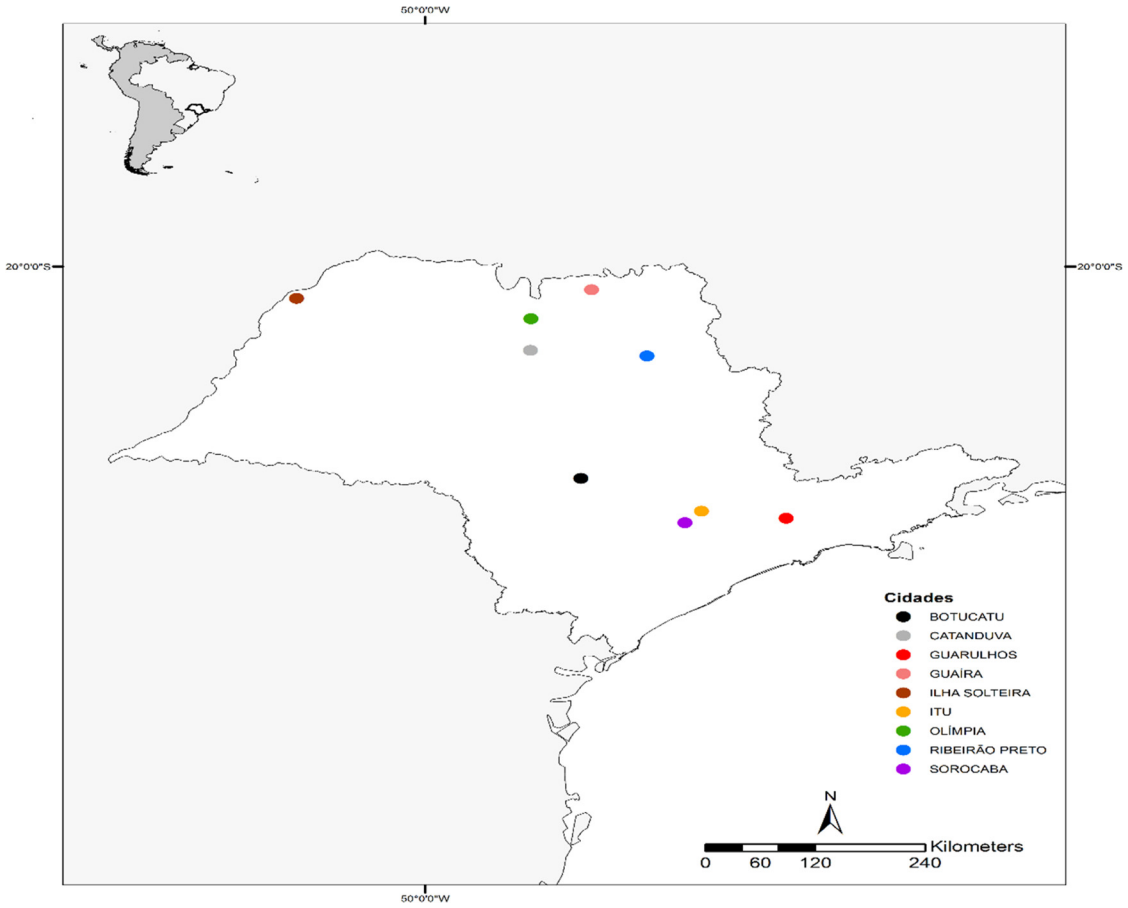

Fig. 1. Map showing the cities where blood samples from howler monkeys were collected in São Paulo, Brazil, 2018.

cycling conditions were: 95 °C for 2 min, followed by 55 cycles of 94 °C for 15 s, 59 °C for 15 s, and 72 °C for 18 s, followed by a final extension at 72 °C for 1 min [2]. Amplification reactions were performed using 25 µL final volume reactions containing 0.2 mM of each deoxynucleotide (Life Technologies®), 0.5 µM of each primer oligonucleotide (Integrated DNA Technologies® - IDT, Coralville, Iowa, USA), 1.5 mM Magnesium Chloride (Life Technologies®), 1.25U Taq DNA Polymerase (Life Technologies®), 10X PCR buffer (Life Technologies®), and sterile ultra-pure water q.s.p. 25 µL. *Mycoplasma* spp. DNA previously detected in NHPs [9] was used as a positive control in the PCR reactions. Sterilized ultra-pure water (Nuclease-Free Water, Promega, Madison, Wisconsin, USA) was used as a negative control in all cPCR assays.

The samples used for the *Bartonella* spp. molecular analyses were submitted to a cPCR based on a gene fragment (350 bp) of the citrate synthase gene (*gltA*). The amplification reaction contained 0.5 µM of each primer (BhCS.1137 (AATGCAAAAAGAACAGTAAACA) and CSH1f (GCGAATGAAGCGTGCCTAAA), 5 µL DNA, 25 µL buffer, 1.0 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate (dNTPs), 1.0 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA). The *Bartonella henselae* DNA obtained from a naturally infected feline [20] and sterilized ultra-pure water were used as positive and negative controls, respectively. The cycling conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min [21,22].

The cPCR-amplified products were subjected to horizontal electrophoresis in 1.0% agarose gel stained with ethidium bromide (0.5 µL/mL) in TEB run buffer pH 8.0 (44.58 M Tris- base, 0.44 M boric acid, 12.49 mM EDTA). Electrophoresis was performed at 90 V/150 mA for 60 min. The products were determined using a 100 base pair molecular weight marker (Life Technologies®). The cPCR results for *Mycoplasma* spp. and *Bartonella* spp. were visualized and analyzed using an ultraviolet light transilluminator (ChemiDoc MP Imaging System, Bio Rad®).

The amplified products of the reactions were purified with the "Silica Bead DNA Gel Extraction" Kit, following the manufacturer recommendations. The samples were sent for sequencing at the Center for Biological Resources and Genomic Biology (CREBIO) of the FCAV, UNESP, in Jaboticabal, SP, Brazil. The amplified products were sequenced by an automated technique based on the dideoxynucleotide chain termination method [23].

2.7. Phylogenetic analyses

For phylogenetic analysis, the sequences obtained from positive samples were submitted to screening tests using the Phred-Phrap software, version 23 [24], to evaluate the quality of the electropherogram and to obtain consensus sequences. The BLAST program [25] was used to analyze the nucleotide sequences (BLASTn) aiming at comparing the obtained sequences with those deposited in the GenBank database [26]. Sequences were aligned with other GenBank sequences available using the MAFFT software version 7 [27].

The phylogenetic analysis was based on Bayesian Inference (BI) performed in the MrBayes 3.1.2 software [28]. We run 10^9 generations of Monte Carlo Markov chain (MCMC) with one sampling per 100 generations and a 25% burn-in. The best evolutionary model was selected by the ModelTest 2 program (version 2.1.6) in XSEDE [29] based on the Akaike Information Criterion (AIC) [30]. All phylogenetic analyses were performed using the CIPRES Science Gateway platform [31]. The trees were edited in the Treegraph 2.0.56-381 beta software [32].

The alignment of the 16S rRNA gene sequences of amplified hemoplasmas in the present study and sequences of hemotrophic mycoplasmas detected in NHPs in other regions of Brazil and the world were used to calculate the number of variable sites, polymorphism level (genotype diversity - [dh] and number of genotypes (h) using the DnaSP

v5 software [33]. Additionally, these same sequences were submitted to the Median-Joining network [34] and distance-based Split-network analyses, which were inferred using the Population Analysis with Reticulate Trees (popART) and SplitsTree 4 [35,36], respectively.

The *Mycoplasma* sp. sequences generated in this study are deposited in the Genbank database under the accession numbers MH734374 to MH734381.

2.8. Statistical analysis

For the statistical analysis, the sampled animals were separated into two groups, one positive and one negative for hemoplasmas. Subsequently, statistical analysis was performed to determine the means and standard deviation of the parameters evaluated in the hemogram, biochemistry, and protein electrophoresis. Subsequently, means were compared by Tukey test at 5%. Statistical analyses were processed in the Statistica software version 7.0.

3. Results

All 68 howler monkeys evaluated were positive for the cPCR assay based on the endogenous gene *gapdh*, of which 18 (26.47%) were positive for *Mycoplasma* spp. based on the 16S rRNA gene, and only 1 (5.55%) was positive for hemoplasmas based on the RNAse P gene. On the other hand, the cPCR based on the *gltA* gene showed that all animals were negative for *Bartonella* spp. Of the 18 samples positive for hemoplasmas in cPCR based on the 16S rRNA gene, 8 amplicons were sent for sequencing, considering the intensity of bands in agarose gel electrophoresis. Phylogenetic analysis based on Bayesian Inference (Fig. 2) resulted in two large clades supported by 100% posterior probability. Clade #1 consisted of sequences belonging to the *Mycoplasma haemofelis* group. The sequences obtained in this study were positioned in clade #2 (*Mycoplasma suis* group), along with *Mycoplasma* spp. (KT314166 and KT314165), and *'Candidatus* Mycoplasma kahanei' (AF338269) obtained from NHP *Saimiri sciureus* sampled in Maranhão, northeastern Brazil, and French Guiana, respectively, corroborating the BLASTn results. The sequences detected by BLAST in this study showed identity and Query coverage varying between 97–100% and 99–100%, respectively, with the same sequences detected in NHP clustered in the *M. suis* group in the phylogeny.

Among the 19 primate-derived *Mycoplasma* spp. 16S rRNA gene sequences analysed, including sequences retrieved from GenBank (n = 11) and those obtained in the present study (n = 8), 4 different genotypes were identified ($\pi = 0.13768$, Hd = 0.825 and K = 24.09357) (Table 1).

All genotypes were composed of more than one sequence. The distribution of the remaining sequences in their respective genotypes, as well as their spatial arrangement, obtained by the analysis of median-joining network, can be observed in Table 2 and Fig. 3A, respectively. The genotype network showed clear separation between them, evidencing several mutational events between the genotype groups (#1, #2 and #3) and (#4 and #5). The sequences detected in NHP in this study integrated genotypes #2 and #3 while genotype #3 is composed of this study sequences only.

The non-rooted distance obtained from the Splits Tree program showed a clear separation between the genotype sequences (#1, #2, and #3) and (#4 and# 5), corroborating the current results (Fig. 3B).

A blood count comparison of 18 healthy howler monkeys and 18 howler monkeys positive for *Mycoplasma* spp. from all localities of the study is shown in Table 3, and showed significant difference in the lymphocyte (P = 0.033), monocyte (P = 0.004) and platelet counts (P = 0.003) of the positive and negative animals. In this study, lymphocyte values were above the reference limits in 3/18 (16.6%) *Alouatta* positive for hemoplasmas and in 4/18 (22.2%) negative NHP, showed significant difference (P = 0.033). The platelet counts decreased in 4/18 howler monkeys (22.22%) positive for *Mycoplasma* spp. Monocyte counts were above the reference limits in 3/18 (16.6%) *Alouatta* positive and 1/18 (5.5%) *Alouatta* negative for hemoplasmas.

Of the 18 *Mycoplasma* positive animals, 16 blood serum samples were submitted to the evaluation of renal function (urea and creatinine) and liver integrity (alkaline phosphatase and alanine aminotransferase), and the results were compared to those found in 16 howler monkeys negative for hemoplasmas. The results of the biochemical blood tests were not significantly different between the positive and negative howler monkeys for hemoplasmas.

A total of 11 blood serum samples from captive howler monkeys positive (11) and negative (11) for *Mycoplasma* spp. were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Table 4).

The NHPs serum samples showed nine proteins with the following molecular weight, immunoglobulin A (139 kD), ceruloplasmin (125 kD), transferrin (79 kD), albumin (69 kD), heavy chain immunoglobulin G (58 kD), haptoglobin (45 kD), α 1 antitrypsin (42 kD), light chain immunoglobulin G (32 kD), and non-identified (22 kD). The SDS-PAGE results were not significantly different between the NHP blood serum samples positive and negative for hemoplasmas.

4. Discussion

The cPCR results showed that all blood samples of the howler monkeys were negative for *Bartonella* spp. Likewise, [10], sampled primates in captivity in Maranhão, Brazil, and reported negative results using conventional and real-time PCR assays based on the *gltA* and *nuoG* genes, respectively.

Also in Brazil, a previous study conducted with samples of human blood donors reported that 3% of the individuals were bacteremic to *Bartonella* spp. when tested by a culture enrichment liquid medium prior to PCR [38]. The liquid culture followed by agar plate seeding, and subsequent DNA amplification by PCR, increases *Bartonella* detection sensitivity in biological samples with low bacteremia [39]. It should be also noted that a very low-grade bacteremia would be expected when *Bartonella* spp. is present in blood samples from NHP and, probably, in levels below the detection threshold of the molecular techniques used in this study. Therefore, this study would benefit from using pre-culture in liquid enrichment medium prior to inoculation in solid media (blood or chocolate agar), followed by detection using molecular techniques [40–42].

In the United States, *Bartonella quintan*a was isolated from *Macaca fascicularis* blood samples using both bovine blood and chocolate agar and PCR assay [13]. In Japan, *B. quintana* was isolated in sheep blood agar from 2/36 blood samples collected from *Macaca fascicularis* [14]. Still in Japan, *B. quintana* was detected in 6/45 *Macaca fuscata* blood samples by isolation in chocolate agar followed by PCR [17]. These data show the importance of *Bartonella* spp. culture prior to PCR as to increase detection sensitivity [16]

Hemoplasma diameter varies between 0.3 and 0.8 μL, and appear as basophilic, spherical structures under light microscopy in blood extensions stained with Giemsa and Romanowsky-based dyes [3]. In this study, hemoplasmas were not observed in the blood smears of howler monkeys, emphasizing the low sensitivity of this technique for this purpose [9]. On the other hand, hemoplasmas were found in the blood-smear of 8/40 (20%) sampled primates in southern Brazil; 10/40 (25%) of these primates were positive for *Mycoplasma* spp. using cPCR, including the eight samples positive for the blood smear [11].

This study reports the occurrence of hemoplasmas infecting NHP in captivity for the first time in the state of São Paulo, southeastern Brazil. The 16S rRNA sequences of *Mycoplasma* spp. used for phylogeny were grouped into two large clades: *Mycoplasma haemofelis* and *Mycoplasma suis* clusters. All the sequences detected in this study agree with results previously reported in *Saimiri sciureus* in Maranhão [10] and *Alouatta caraya* in Foz do Iguaçu [11], northeastern and southern Brazil, respectively, which were positioned in the *M. suis* cluster close to

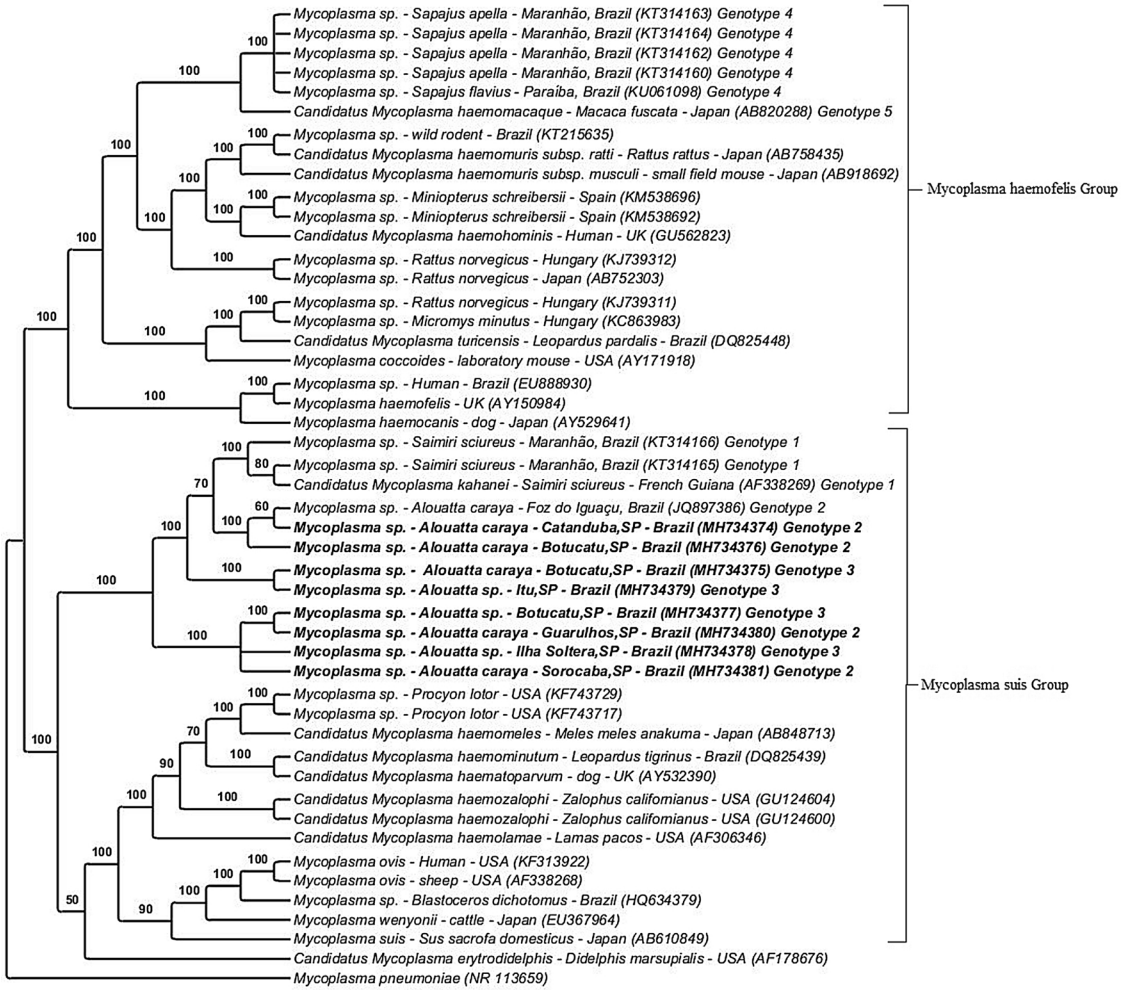

Fig. 2. Phylogenetic relationship within the *Mycoplasma* genus based on approximately 800 bp fragments of the 16S rRNA gene. The inferred phylogenetic tree resulting from Bayesian analysis and the GTR + I + G evolution model. The sequences of this study are highlighted in bold. Distribution of *Mycoplasma* spp. 16S rRNA sequences detected in NHP in Brazil and the world.

Table 1
Polymorphism and genetic diversity between 16S rRNA sequences of *Mycoplasma* spp. detected in NHP in Brazil and in the world.

Gene	(pb)	N	VS	GC%	h	Hd (mean ± DP)	π (mean ± DP)	K
16S rRNA	800pb	19	51	45.4	5	0.825 ± 0.040	0.13768 ± 0.01483	24.09357

N, number of analyzed sequences; VS, number of variable sites; GC, G + C content; h, number of genotypes; Hd, genotype diversity; SD, standard deviation; π, nucleotide diversity (per site); K, nucleotide differences number.

Table 2
Distribution of *Mycoplasma* spp. 16S rRNA sequences detected in NHP in Brazil and the world in the five genotypes found in this study.

Genotype #1	Genotype #2	Genotype #3	Genotype #4	Genotype #5
KT314165 (Maranhão)	**Sorocaba***	**Ilha Solteira***	KT314160 (Maranhão)	KC512401 (USA)
KT314166	**Botucatu 24.0***	**Itu***	KT314164 (Maranhão)	AB820288 (Japan)
(Maranhão)	**Guarulhos***	**Botucatu 22.0***	KT314162 (Maranhão)	
AF338269 (French Guyana)	**Catanduva***	**Botucatu 27.0***	KT314163 (Maranhão)	
	JQ897386		KU061098 (Paraíba)	
	(Foz do Iguaçu)			

Sequences detected in this study in NHPs in São Paulo state, southeastern Brazil.

Mycoplasma sp., forming a monophyletic group with the '*Candidatus* Mycoplasma kahanei' sequence.

On the contrary, the sequences detected in *Sapajus apella* and *Sapajus flavius* specimens sampled in Maranhão and Paraíba [10,42], northeastern Brazil, respectively, differed from our results, and were clustered in the *M. haemofelis* group together with the '*Candidatus* Mycoplasma haemomacaque', similarly to the results of the sequences detected in *Macaca fuscata* in Japan [8].

None of the previous studies conducted in Brazil named the *Mycoplasma* species found in the NHPs sampled since they could

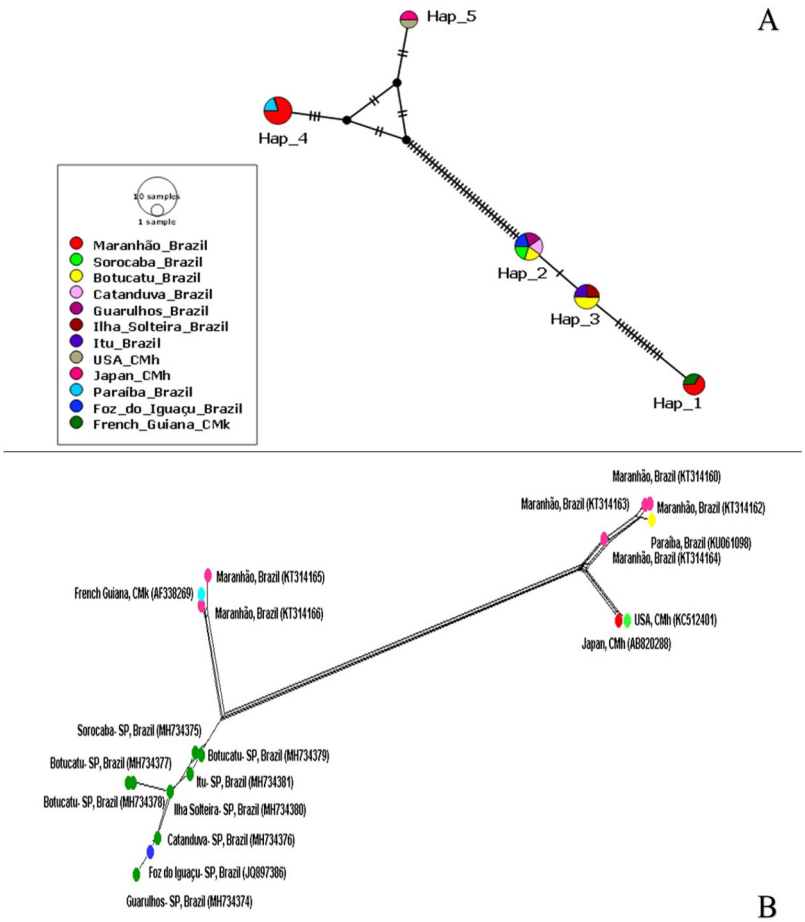

Fig. 3. A. Median-joining network of the genotypes of the *Mycoplasma* spp. 16S rRNA gene detected in monkeys sampled in Brazil and the world. CMh- '*Candidatus* Mycoplasma haemomacaque'; CMk- '*Candidatus* Mycoplasma kahanei'. B. Split-network based on the distance obtained from sequences of the *Mycoplasma* spp. 16S rRNA gene detected in NHP in Brazil and the world. CMh- '*Candidatus* Mycoplasma haemomacaque'; CMk- '*Candidatus* Mycoplasma kahanei'.

Table 3
Hemogram results of NHPs negative and positive for hemoplasmas in São Paulo state, Brazil, 2018.

Parameter	Group		*P Value*
	Positive (n = 18)	Negative (n = 18)	
RBCs (x10^6 cells/μL)	4.60 ± 0.55	4.59 ± 0.75	0.975
Hemoglobin (g/dL)	12.62 ± 1.55	13.17 ± 1.96	0.357
Hematocrit Value (%)	37.93 ± 4.61	39.35 ± 7.95	0.515
MCV(fL)	82.63 ± 7.28	85.24 ± 7.09	0.283
MCH (g/dL)	33.25 ± 2.31	33.83 ± 3.02	0.526
MCHC (g/dL)	27.46 ± 2.17	28.87 ± 3.38	0.145
Total leucocytes (x10^3/μL)	13.80 ± 4.27	12.56 ± 4.4	0.398
Basophils (x 10^3/μL)	2.7 ± 7.2	1.0 ± 4.2	0.324
Eosinophils (x 10^3/μL)	3.6 ± 4.2	2.6 ± 2.0	0.372
Band neutrophils (x 10^3/μL)	0.00 ± 0.00	3.4 ± 5.9	0.732
Segmented neutrophils (x 10^3/μL)	69.3 ± 2.6	79.7 ± 3.7	0.343
Lymphocytes (x 10^3/μL)	50.2 ± 1.40	36.2 ± 22.7	0.033*
Monocytes (x 10^3/μL)	10.5 ± 1.0	6.5 ± 4.5	0.004*
Platelets (x 10^3/μL)	223.44 ± 75.61	323.06 ± 109.27	0.003*

MCV: mean corpuscular volume. MCHC: mean corpuscular hemoglobin concentration, MCH: mean corpuscular hemoglobin *: P value < 0.05.

represent new species due to the low identity with the previously identified sequences [9,10,42]. We highlight, based on phylogenetic inference, a degree of genetic diversity among the *Mycoplasma* species infecting NHPs in Brazil. Therefore, this study revealed the occurrence of at least two species of hemoplasmas circulating among these hosts due to the clear phylogenetic separation between the sequences detected in *Sapajus* spp. and *Alouatta* spp. This genetic separation is also supported by genotype and distance network analyses, performed only with sequences of hemoplasmas detected in NHPs. The genotype network based in distance indicated that the hemoplasmas parasitizing NHPs are clearly divided into two clusters. Thus, the detected sequences were grouped together with two hemoplasma sequences: the first, *Candidatus* Mycoplasma kahanei detected in NHPs sampled in Maranhão, Brazil, and the second, *Mycoplasma* sp. detected in *Alouatta* spp. and *Saimiri sciureus* in the French Guiana and Foz do Iguaçu, respectively, corresponding to # 1, # 2 and # 3 genotypes. The other sequences detected in *Sapajus apella* and *S. flavius* in Maranhão and Paraíba, northeastern Brazil, were located at the other end of the network and the sequence of *Candidatus* Mycoplasma haemomacaque detected in *Macaca fuscata* in Japan corresponded to # 4 and # 5 genotypes.

Thus, the NHPs of the *Sapajus* genus in Brazil were infected by a hemoplasma species phylogenetically related to '*Candidatus* Mycoplasma haemomacaque, a species that infects *Macaca fuscata* in Japan. On the other hand, the species infecting the sampled *Alouatta* NHP was phylogenetically related to '*Candidatus* Mycoplasma kahanei', a parasite of *Saimiri sciureus* in the French Guiana [7].

Recently, the hemoplasma genotypes detected in *Alouatta caraya* sampled in Paraná, southern Brazil, were phylogenetically associated with '*Candidatus* Mycoplasma kahanei' [11]. However, specimens of *Sapajus flavius*, a species threatened with extinction, sampled in Paraíba, northeastern Brazil, were positive for a *Mycoplasma* sp. that was positioned within a monophyletic clade, composed of *Mycoplasma* sp. sequences detected in *Sapajus apella* from Maranhão, northeastern Brazil [42].

Although attempts to amplify a fragment of the RNase P gene have been performed, low-intensity bands were generated, probably due to the low bacteremia [2], preventing good quality sequencing. Future

Table 4
The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) results of 22 captive howler monkeys, 11 positive and 11 negative for hemoplasmas in São Paulo, Brazil, 2018.

		Negative (n = 11)		Positive (n = 11)		P Value
	Molecular weight (Daltons)	Mean	Standard Deviation	Mean	Standard Deviation	
TP	–	7836.36	2683.38	7872.73	1912.11	0.971
IgA	139 kD	108.18	62.74	94.82	48.43	0.582
Ceru	125 kD	64.09	61.14	56.36	69.44	0.784
Transf	79 kD	681.62	328.26	574.82	176.01	0.352
Alb	69 kD	5484.64	1250.77	4917.45	1772.54	0.396
Heavy IgG	58 kD	916.73	303.57	757.82	376.24	0.288
Hapt	45 kD	81.73	41.71	53.64	33.73	0.097
Alpha 1	42 kD	11.64	11.33	8.74	9.37	0.520
Light IgG	32 kD	259.36	127.02	251.30	131.93	0.885
Non identified	22 kD	137.45	61.54	129.36	49.46	0.737

TP: total proteins; IgA: immunoglobulin A; Ceru: ceruloplasmin; Transf: transferrin; Alb: albumin; IgG: Immunoglobulin G; Hapt: haptoglobin; Alpha 1: α 1 antitrypsin.

studies should be conducted to investigate the phylogenetic relationships between parasitic hemoplasmas of NHPs based on genes other than the 16S rRNA.

Hemoplasmas may induce the development of regenerative anemia [42,43]. In this work, the results of RBCs were not significantly different between howler monkeys positive and negative for *Mycoplasma* spp. Some primates that were positive for hemoplasmas had decreased RBCs, which was not associated with hemoplasma positivity. Generally, macrocytosis is observed in *Mycoplasma* positive animals as it has been described in domestic cats positive for *Mycoplasma haemofelis* [44]. Similarly, no statistically significant differences were observed in the hemogram of howler monkeys (*Alouatta caraya*) sampled in Paraná, positive and negative for hemoplasmas [10], corroborating the results of this work. Similarly, no evidence of hematological and biochemical abnormalities were observed associated with a '*Candidatus* Mycoplasma haemomacaque' infection in a *Macaca fascicularis* colony in the United States [2].

Although a significant association with hemoplasma infection was not detected in this study, increased monocyte counts were observed in 6/18 howler monkeys positive for hemoplasmas. Leukocytosis due to monocytosis in *Alouatta* usually occurs associated with capture stress in captivity and free-living [37,45–47].

In turn, increased lymphocyte values were observed in 18/18 howler monkeys and 14/18 negative for hemoplasmas. Previously, absolute lymphocyte count was higher in uninfected *Alouatta caraya* (2/3) compared to those infected (1/8) with '*Candidatus* Mycoplasma kahanei' [11]. Non-human primates kept in captivity usually show lymphocytosis due to stress [37].

The comparison of the biochemical analysis results between the *Mycoplasma* positive and negative animals shows no significant differences for urea, creatinine, ALT, and FA levels. These findings indicate that hemoplasma infection did not induce renal functional alterations or hepatocellular lesions in the studied population.

Regarding the serum proteinogram result, no statistical differences were observed between the *Alouatta* NHP positive and negative for hemoplasmas. Studies evaluating acute phase plasma proteins should be performed to detect the concentration of these proteins in inflammatory processes. Besides, statistically significant differences between the total protein dosages of howler monkeys infected and not infected with *Mycoplasma* spp. were not observed [11].

5. Conclusion

The present work showed the occurrence of *Mycoplasma* sp. phylogenetically related to '*Candidatus* Mycoplasma kahanei' in *Alouatta* primates kept in captivity in São Paulo, southeastern Brazil. Four different genotypes were detected in NHP in the present study, probably representing two different hemoplasma species. The serum biochemistry and serum proteinogram results of *Alouatta* infected or not with hemotrophic mycoplasmas were not significantly different. Thus, the actual pathogenic potential of this species of hemoplasma should be better investigated.

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