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Occurrence and molecular characterization of *Bartonella* spp. and hemoplasmas in neotropical primates from Brazilian Amazon

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ABSTRACT

Little is known about the prevalence and genetic diversity of *Bartonella* spp. and hemoplasmas in nonhuman primates (NHP). The present study aimed to investigate the occurrence of and assess the phylogenetic position of *Bartonella* spp. and hemoplasma species infecting neotropical NHP from Brazilian Amazon. From 2009 to 2013, a total of 98 blood samples from NHP belonging to the Family Cebidae were collected in the island of São Luís, state of Maranhão, of which 87 NHP were from Wild Animal Screening Center (CETAS) in the municipality of São Luís, and 11 (9 *Sapajus* sp. and 2 *Saimiri sciureus*) were from NHP caught in the Sítio Aguahy Private Reserve. DNA samples were screened by previously described PCR protocols for amplifying *Bartonella* spp. and *Mycoplasma* spp. based on *nuoG*, *gltA* and 16S rRNA genes, respectively. Purified amplicons were submitted to sequencing and phylogenetic analysis. Bacteremia with one or more *Bartonella* spp. was not detected in NHP. Conversely, 35 NHP were PCR positive to *Mycoplasma* spp. The Blastn analysis of seven positive randomly selected sequencing products showed percentage of identity ranging from 95% to 99% with other primates hemoplasmas. The Maximum Likelihood phylogenetic analysis based on a 1510 bp of 16S rRNA gene showed the presence of two distinct clusters, positioned within *Mycoplasma haemofelis* and *Mycoplasma suis* groups. The phylogenetic assessment suggests the presence of a novel hemoplasma species in NHP from the Brazilian Amazon.

1. Introduction

Arthropod-borne pathogens are important cause of morbidity and mortality in a wide spectrum of animal species, including humans [1,2]. Because 75% of emerging pathogens cause disease in humans and animals, a *One Health* approach to zoonotic infections is needed to correctly address animal and human health [1]. In this context, hemotropic mycoplasmas (also known as hemoplasmas) and *Bartonella* spp. emerge as important arthropod-borne pathogens that impact humans and animals' health [1,3]. While hemoplasmas are obligate bacteria that attach to red cells' surface, *Bartonella* spp. are facultative intracellular bacteria that also cause prolonged intraerythrocytic bacteremia in a wide variety of wild and domestic animals, including humans [4–6].

There is a limited number of reports on the occurrence of *Bartonella* and hemoplasmas on nonhuman primates (NHP). *Bartonella quintana* has been isolated from blood culture or PCR detected in a cynomolgus monkey (*Macaca fascicularis*) imported by the USA from the South East Asia [7], and from captive Rhesus and cynomolgus monkeys in China [8,9].

Among NHP, hemotropic mycoplasma infection has been reported in experimental and breeding colony monkeys, such as squirrel (*Saimiri sciureus*), cynomolgus (*M. fascicularis*) and owl (*Aotus trivirgatus*) monkeys [10–13] in French Guiana, the United States of America and the United Kingdom, respectively. In two cases, molecular studies allowed the description of new hemoplasma species namely '*Candidatus* Mycoplasma kahanei' [14] and '*Candidatus* Mycoplasma aoti' [13]. In Brazil, *Mycoplasma* spp. closely related to '*Candidatus* Mycoplasma kahanei' was detected in an adult female free-ranging black howler monkey (*Alouatta caraya*) in the state of Parana, Brazil [15].

The present study aimed to investigate the occurrence of and assess the phylogenetic relationship of *Bartonella* spp. and hemoplasma species infecting neotropical primates from the island of São Luís, state of Maranhão, northeastern Brazil.

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2. Material and methods

2.1. Study areas and blood samples from NHP

The Legal Amazon Region comprises the Brazilian states of Acre, Amapá, Amazonas, Pará, Rondônia, Roraima, Tocantins, Mato Grosso and Maranhão. The present work was carried out in two municipalities which are part of the island of São Luís, state of Maranhão, northeastern Brazil (Fig. 1 – Capture sites – Map of Maranhão State showing the Island of São Luís where NHP blood samples were collected (MapInfo Professional 7.5 SCP). From 2009 to 2013, a total of 112 blood samples from NHP belonging to the Family Cebidae [16] were collected, of which 98 NHP were from Wild Animal Screening Center (CETAS) (2°56′80″ S, 44°21′01″ W), which is located in the urban area of the municipality of São Luís, and 14 (11 *Sapajus* sp. and 3 *S. sciureus*) were from NHP caught in the Sítio Aguahy Private Reserve (2°38′76″ S, 44°08′22″ W), which is located in the rural area of the municipality of São José de Ribamar, an area of environmental preservation named Guarapiranga, that has less than 300 inhabitants [17]. The activities were approved by the Animals Ethics Committee of FCAV-UNESP Jaboticabal (Protocol No. 020091/09) and by the Chico Mendes Biodiversity Institute (ICMBio) (license No. 23078-1).

Blood sampling was performed through venipuncture of the jugular or femoral veins of the NHP, which had previously been anesthetized using a combination of zolazepam hydrochloride and tiletamine hydrochloride, according to each species. The samples collected were placed in Vacutainer® tubes containing EDTA [17].

2.2. PCR assays

2.2.1. DNA extraction

DNA was extracted from 200 μL of each NHP whole blood sample using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California, USA), according to manufacturer's instructions. DNA concentration and absorbance ratio (260/280) nm were measured using a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA). Microtubes containing ultra-pure sterile water were used as negative extraction controls, intercalated between each series of twenty NHP blood samples and submitted to DNA extraction.

2.2.2. Internal control PCR

In order to discard the presence of PCR inhibitors, each sample of blood extracted DNA was used as a template in an internal control PCR targeting the mammal GAPDH gene [18]. Five microliters of DNA were used as a template in 25 μL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.6 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA), and 0.5 μM of GAPDH-F (5′-CCTTCATTGACCTCAACTACAT-3′) and GAPDH-R (5′-CCAAAGTTGTCATGGATGACC-3′) primers. PCR amplifications were performed at 94 °C for 5 min followed by 35 repetitive cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Positive samples to above described internal control PCR protocol were subsequently submitted to quantitative real time PCR (qPCR targeting *nuoG* gene) and conventional PCR (cPCR targeting *gltA* gene) for *Bartonella* spp. and cPCR assays for *Mycoplasma* spp. targeting 16S rRNA and RNaseP genes as described below.

2.2.3. qPCR and cPCR assays for Bartonella spp

qPCR reactions for *Bartonella* spp. targeting *nuoG* gene were performed using the 10 μL PCR mixtures contained 5 μL of Go Taq® Probe qPCR Master Mix, dTTP (Promega) with a final concentration of 1.2 μM of each primer (F-Bart [5′-CAATCTTCTTTTGCTTCACC-3′] and R-Bart [5′-TCAGGGCTTTATGTGAATAC-3′] and hydrolysis probe (TexasRed-5′-TTYGTCATTTGAACACG-3′[BHQ2a-Q]3′) and

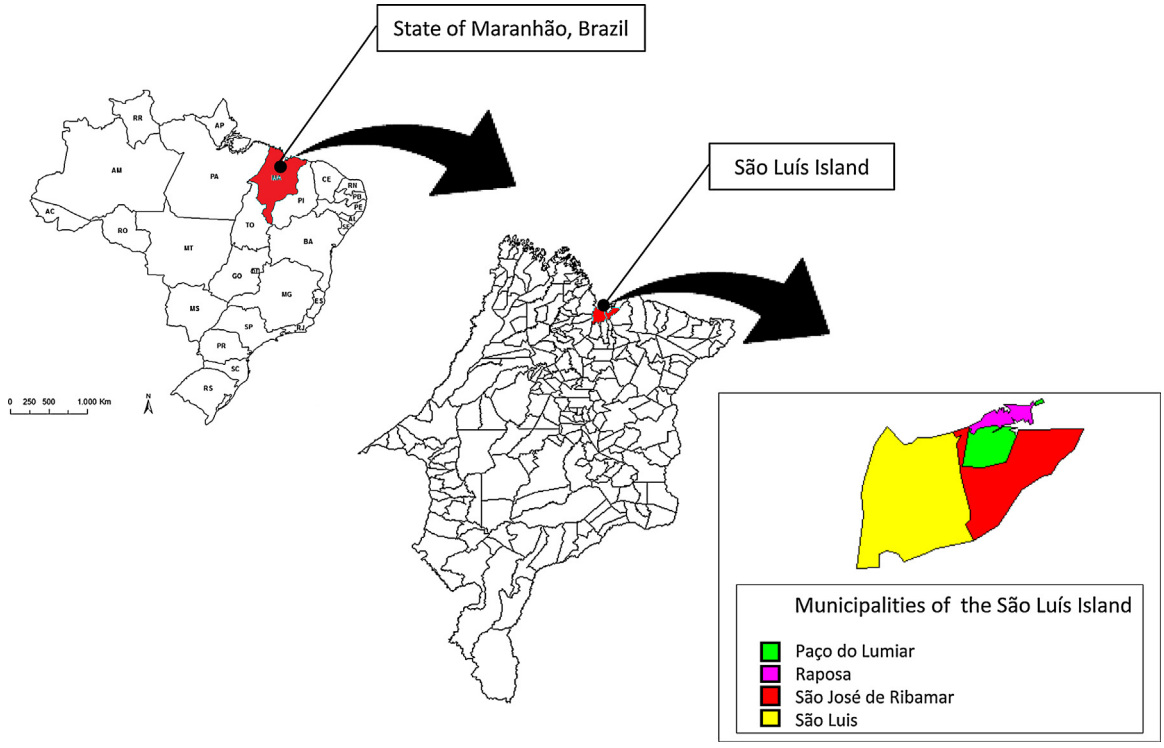

Fig. 1. Capture sites. Map of Maranhão State showing the Island of São Luís where nonhuman primates blood samples were collected (MapInfo Professional 7.5 SCP).

1 μL of DNA sample [19]. PCR amplifications were conducted in Low-Profile Multiplate™ Unskirted PCR Plates (BioRad, Hercules, CA, USA) using CFX96 Thermal Cycler (BioRad, Hercules, CA, USA). The amplification conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 10 min and 52.8 °C for 30 s. The qPCR was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [20]. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/\text{slope}}$). Copy numbers were estimated using 10-fold serial dilutions of pIDTSMART plasmids (Integrated DNA Technologies, Coralville, IA, USA) encoding *nuoG B. henselae* sequence (insert containing 83 bp). The number of plasmid copies was determined according to the formula (Xg/μL DNA/[plasmid length in bp × 660]) × 6.022 × 10^{23} × plasmid copies/μL. Plasmids (Integrated DNA Technologies, Coralville, IA, USA) encoding *nuoG B. henselae* sequence were used as positive controls.

Additionally, a previously described PCR protocol based on *gltA* gene was utilized to amplify *Bartonella* spp. DNA, using the combined primers namely BhCS.1137 (AATGCAAAAAGAACAGTAAACA) [21] and CSH1f (GCGAATGAAGCGTGCCTAAA) [22] (fragment of ~350 bp). Five microliters of DNA were used as a template in 25 μL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.0 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and 0.5 μM of each primer. *B. henselae* DNA (GenBank accession number KC331015) obtained from a naturally infected cat [23] was used as positive control. Ultra-pure sterile water was used as negative control in all PCR assays described above. PCR amplifications were performed by an initial cycle of 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, and an extension final by 72 °C for 5 min.

2.2.4. cPCR assays for Mycoplasma spp.

Previously described PCR protocols based on 16S rRNA gene were utilized to amplify *Mycoplasma* spp. DNA, using two sets of primers, namely HemMycop16S-41s (5′-GYATGCMTAAYACAT GCAAGTCGARCG-3′) and HemMyco16S-938as (5′-CTCCACCACTT GTTCAGGTCCCCGTC-3′) (fragment of ~800 bp), and HemMycop16S-322s (5′-GCCCATATTCCTACGGGAAGCAGCAGT-3′) and HemMycop16S-1420as (5′-GTTTGACGGGCGGTGTGTACAAGACC-3′) (fragment of ~800 bp) [24]. Five microliters of DNA were used as a template in 25 μL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and 0.3 μM of each primer. *Mycoplasma haemofelis* DNA (Genbank accession number KJ135315) obtained from a naturally infected cat [23] was used as positive control. Ultra-pure sterile water was used as negative control in all PCR assays described above. PCR amplifications were performed at 94 °C for 2 min followed by 55 repetitive 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. 16S rRNA *Mycoplasma* spp.-positive samples were additionally submitted to RNaseP gene-*Mycoplasma* spp. (165 bp) PCR assay using the oligonucleotides HemoMyco RNaseP30s (5′-GATKGTGYGAGYATATAAAAAATAAARCTCRAC-3′) and HemoMyco RNaseP200as (5′-GMGGRGTTTACCGCGTTTCAC-3′). The conditions of amplification were the same as described above, except for the annealing temperature (59 °C) [24]. In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms.

2.3. Phylogenetic analysis

The products derived from amplicons obtained from each primer set (~1300 bp for 16S rRNA of *Mycoplasma* spp., with an overlap of 600 bp to improve the identification of species detected) were purified using Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP, Brazil). Purified amplified DNA fragments from positive samples were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser – Applied Byosystem/Perkin Elmer, Waltham, MA, USA). Consensus sequences were obtained through the analysis of the sense and antisense sequences using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Primer sequences were trimmed from the consensus sequences prior Blastn analysis. Comparisons with sequences deposited in GenBank were done using the basic local alignment search tool (BLASTn) [25]. The sequences were aligned with sequences published in GenBank using Clustal/W [26] and manually adjusted in Bioedit v. 7.0.5.3 (Carlsbad, CA, USA) [27]. Phylogenetic inference based on maximum likelihood criterion (ML), was inferred with RAxML-HPC BlackBox 7.6.3 [28] through the CIPRES Science Gateway [29]. Akaike information criterion was used in Mega 5.05 to identify the most appropriate model of nucleotide substitution. GTRGAMMA model was chosen as the most appropriate for the Maximum Likelihood analysis of the 16S rDNA alignment.

3. Results

Out of 112 NHP blood samples analyzed, 98 were positive to internal control (GAPDH gene) (Median DNA concentration = 12.6 ng/uL; SD [standard deviation] ± 10.3; 260/280 ration = 2.6; SD ± 0.78). All negative samples to GAPDH gene-PCR were excluded from subsequent analysis. Bacteremia with one or more *Bartonella* spp. was not detected in NHP in both qPCR (*nuOG* gene; efficiency of qPCR reactions targeting *nuoG* gene: 90.9%, $r^2 = 0.999$; 91.3%, $r^2 = 0.998$; and 90.9%, $r^2 = 0.999$) and cPCR (*gltA* gene) assays. The analytical sensitivity per qPCR reaction was 10 copies/μL. Among 98 NHP blood samples analyzed, 35 (35.7%) were PCR positive to *Mycoplasma* spp. (Table 1), detected from the following host species: *Sapajus apella*, *S. sciureus* and *Saguinus midas niger*. Unfortunately, all positive samples to 16S rRNA gene that were tested by the RNaseP gene PCR yielded bands of weak intensity which precluded sequencing efforts of these fragments.

The Blastn analysis indicated that amplified sequences (GenBank accession numbers KT314160, KT314161, KT314162, KT314163, KT314164, KT314165, KT314166) demonstrated percentage of identity ranging from 95% to 99% with other NHP hemoplasmas ('*Candidatus* Mycoplasma haemomacaque', and '*Candidatus Mycoplasma kahanei*') sequences deposited in Genbank (Table 2). The sequences obtained were different from the positive control used in the PCR reactions (*M. haemofelis* – Genbank accession number KJ135315).

The ML phylogenetic analysis based on a fragment of 1510 bp (after the alignment by Bioedit) of 16S rRNA gene of seven positive

Table 1
Number of neotropical primates tested positive by hemoplasmas PCR, according to species, capture sites, and gender.

Scientific name	Capture sites	Gender	Number of animals positive for hemoplasma cPCR (16S rRNA)
Aotus infulatus	CETAS	1F	–
Sapajus sp.	CETAS and Sítio Aguahy	38M/27F	29
Callithrix jacchus	CETAS	6M/3F	–
Saimiri sciureus	CETAS and Sítio Aguahy	12M/4F	04
Saguinus midas niger	CETAS	3M/4F	02
Total		98	35

F = female; M = male.

Table 2
Closest identity percentage (Blastn) between NHP hemoplasma 16S RNA sequences detected in Brazil and other primate hemoplasma sequences previously submitted to GenBank from others regions of the world.

NHP sampled	Percentage of identity (%) by Blast analysis		
	'C. M. haemomacaque' (KC512401)	'C. M. haemomacaque' (AB820288)	'C. M. kahanei' (AF338269)
Sapajus apellaa[a]	95	95	–
Sapajus apella (C)	95	95	–
Sapajus apella (C)	95	95	–
Sapajus apella (C)	95	95	–
Sapajus apella (C)	96	97	–
Saimiri sciureus[a]	–	–	99
Saimiri sciureus[a]	–	–	99

C: CETAS.
[a] Free-ranging NHP.

randomly selected samples showed the presence of two distinct clusters (Fig. 2). The sequences belonging to cluster-1 were positioned within *M. haemofelis* group and showed a strong separation from '*Candidatus* Mycoplasma haemomacaque' detected in primates in USA and Japan supported by a high bootstrap value (99). On the other hand, the sequences belonging to cluster-2 were positioned within *Mycoplasma suis* group, clustering together (bootstrap value of 100) and showing a high percentage of identity (99%) with '*Candidatus* Mycoplasma kahanei', which was detected in *S. sciureus* in the USA.

4. Discussion

There is a limited number of reports on the occurrence and genetic diversity of *Bartonella* spp. and hemoplasmas in NHP around the world. Experimental inoculation of rhesus monkeys with *B. quintana* isolated from human patients resulted in chronic bacteremia [30,31] suggesting the possibility that NHP may be susceptible to the infection by *Bartonella* species acquired from humans or from other monkeys. Later, studies have shown the presence of different genotypes of *B. quintana* in NHP [7,8]. An occurrence of 30.7% for *B. quintana* has been reported in NHP (*Macaca mulatta* and *M. fascicularis*) maintained in Primatology Centers in China, using blood culture and/or PCR techniques [9]. In the present study, bacteremia with one or more *Bartonella* spp. was not detected in NHP. If *Bartonella* infection in NHP follows the same pathogenicity as in humans, one would expect that asymptomatic

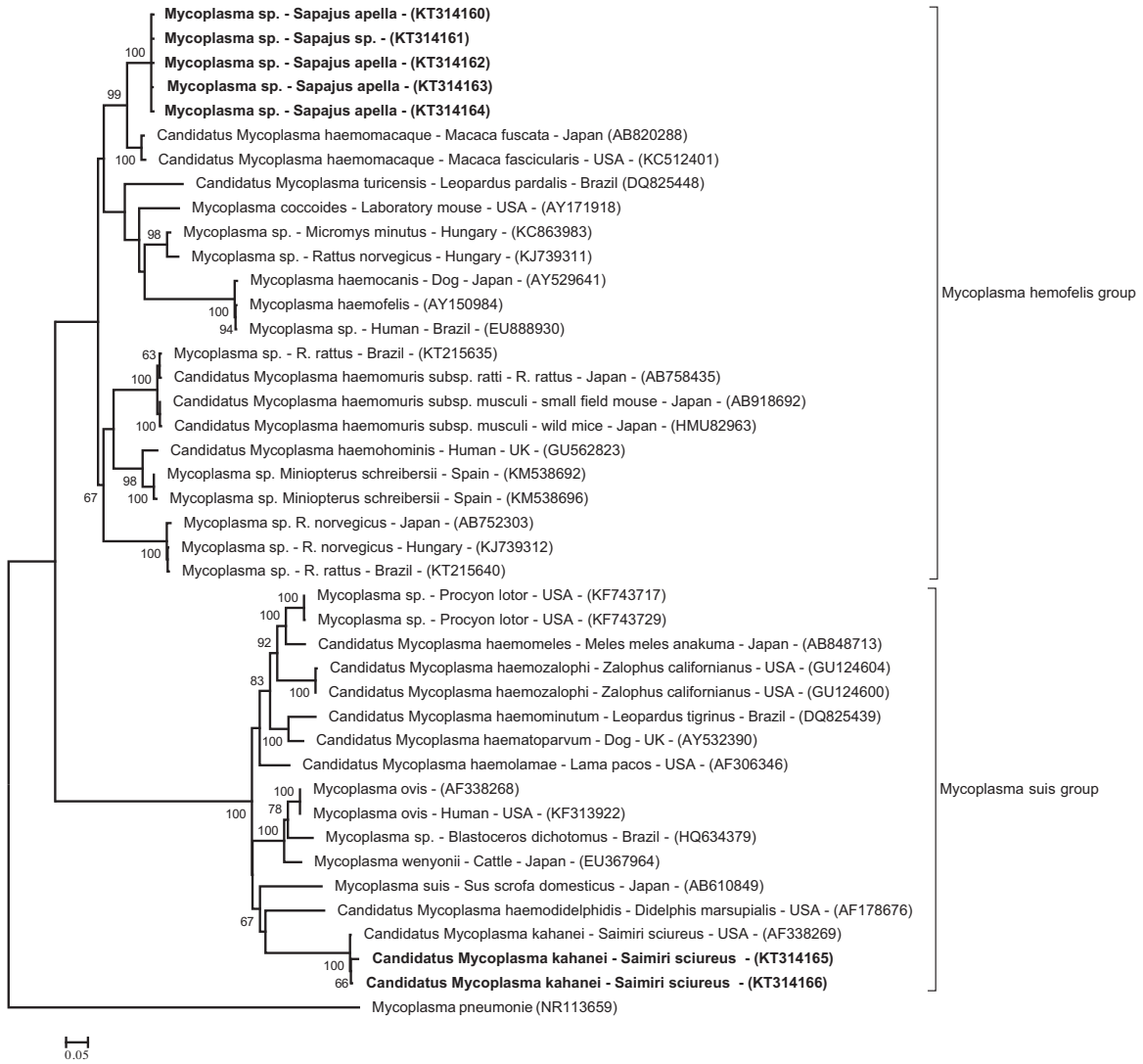

Fig. 2. Phylogenetic relationships within the *Mycoplasma* genus based on a 1510 bp fragment of the 16S rRNA gene. The phylogenetic tree was inferred by using the maximum likelihood method. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to boostrap values higher than 60% accessed with 1.000 replicates. *Mycoplasma pneumoniae* was used as outgroup.

NHP would have very low bacteremia [32], probably below the limit of detection of the PCR assay. *Bartonella* bacteremia is more readily documented in a primary reservoir species, such as cats or rodents [32] and might occur less frequently or to a much lower level in accidental hosts, such as humans or primates. In humans, the average bacterial levels in blood are 1–10 genome copies/μl, compared to the 10^5 to 10^6 copies/μl often found in cats [32]. In a recent study in blood donors in Brazil, only 3% of subjects were bacteremic for *Bartonella* spp. when tested by a combined enrichment liquid culture step prior PCR testing [33]. Further studies aiming to evaluating NHP from different Brazilian biomes are much needed, in order to better understand the possible role of monkeys as hosts or reservoirs for *Bartonella* species. Besides, the use of a combinational approach consisting of pre-enrichment culture of *Bartonella* species in BAPGM (insect-based liquid culture medium), sub-inoculation of the liquid culture onto agar plates, followed by DNA amplification using PCR is desirable, since may facilitate the growth and detection of specific wild types of *Bartonella*, providing enhanced sensitivity [34,35]. Culture of specimens in BAPGM pre-enrichment medium enhanced the PCR detection rate of *Bartonella* species over that of direct nucleic acid extraction from blood specimens from dogs, horses and humans [36–40]. It is of clinical and epidemiological relevance that failure to amplify *Bartonella* sp. gene targets, following extraction of DNA from NHP blood samples, does not rule out this bloodstream infection. It is estimated that *Bartonella* bacteremia in asymptomatic donors is approximately 10 CFU/mL of blood [32], which may be below the detection limit of most conventional or Real-Time PCR assays. According to Harms and Dehio [41], another reason for false-negative PCR or culture results is that *Bartonella* spp. typically cause a cyclic bacteremia. Obtaining three sequential specimens during a 1-week period appears to enhance detection of *Bartonella* bacteremia in human patients and should be considered as a diagnostic approach when bartonellosis is suspected [35]. The knowledge of epidemiology of human and animal bartonellosis in northeastern Brazil is scarce yet, with only one report on *B. henselae* and *B. clarridgeiae* in domesticated and stray cats in São Luis Island [42].

The development of molecular assays primarily targeting the 16S rRNA gene has resulted in the detection of a growing number of hemotropic mycoplasmas in animals and humans. These pathogens may have co-evolved with several animal species, including humans [5,24,43,44]. Some studies suggest that NHP may be susceptible to hemoplasma infection [12], showing mild to severe anemia [10,11].

Previously, *Mycoplasma* sp. was detected in a free-ranging *Alouatta caraya* sampled in Paraná, Brazil, which was presented with normochromic and macrocytic anemia. BLASTn and phylogenetic analyses showed a low percentage of identity (93.6) and a clear separation from other sequences of '*Candidatus* Mycoplasma kahanei' [15]. Herein, we reported for the first time the occurrence of hemoplasmas infecting neotropical primates from the Brazilian Amazon. The occurrence of *Mycoplasma* spp. in the evaluated NHP was lower [35.7% (35/98)] than previously described in a study performed in the USA among NHP maintained in captivity in a research colony [84.6% (44/52)] [3].

The phylogenetic analysis of 16S rRNA DNA sequences amplified from five *Sapajus apella* NHP's blood samples showed a strong separation from '*Candidatus* Mycoplasma haemomacaque'. This finding was supported by a percentage of identity ranging from 95% to 97% with other primates hemoplasmas by Blast analysis, indicating the designation of possible novel hemoplasmas in NHP in Brazil.

Conversely, the 16S rRNA DNA sequences detected in two free-ranging squirrel monkeys (*S. sciureus*) were positioned in the same clade as '*Candidatus* Mycoplasma kahanei', also detected in *S. sciureus* NHP in the USA [14]. This finding shows evidence that *S. sciureus* may play a role as hosts for '*Candidatus* M. kahanei'. However, future studies evaluating a larger number of animals are needed in order to better understanding the role of *S. sciureus* in the ecology of '*Candidatus* M. kahanei'. Additionally, our results show the presence of more than one hemoplasmas species in neotropical NHP in Brazil.

5. Conclusion

The results of this study demonstrate that neotropical primates in the Brazilian Amazon can be infected, and may serve as reservoirs, of hemoplasmas, including a potential novel hemoplasma species.

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