UNIVERSIDADE ESTADUAL PAULISTA - UNESP CÂMPUS DE JABOTICABAL

MOLECULAR DETECTION AND CHARACTERIZATION OF HEMOPLASMAS AND *Bartonella* spp. IN SMALL MAMMALS, LARGE RUMINANTS AND ASSOCIATED ECTOPARASITES

Luiz Ricardo Gonçalves

Biólogo

2020

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Microbiologia Agropecuária

2020

G635m

Gonçalves, Luiz Ricardo
Molecular detection and characterization of hemoplasmas and Bartonella spp. in small mammals, large ruminants and associated ectoparasites / Luiz Ricardo Gonçalves. -- Jaboticabal, 2020
149 p. : il., tabs., fotos, mapas

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Marcos Rogério André
Coorientadora: Rosangela Zacarias Machado

1. Bartonella. 2. Hemoplasmas. 3. Diversidade genética. 4. Pequenos mamíferos. 5. Grandes ruminantes. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

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TÍTULO DA TESE: MOLECULAR DETECTION AND CHARACTERIZATION OF HEMOPLASMAS AND *Bartonella* spp. IN SMALL MAMMALS, LARGE RUMINANTS AND ASSOCIATED ECTOPARASITES

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Jaboticabal, 31 de julho de 2020

DADOS CURRICULARES DO AUTOR

Luiz Ricardo Gonçalves – nascido na cidade de Jaboticabal, São Paulo, em 4 de novembro de 1985. Formado em Ciências Biológicas (Bacharelado) pela Universidade Estadual Paulista “Júlio de Mesquita Filho” – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Jaboticabal, São Paulo, no ano de 2013. Durante a graduação, foi bolsista CNPQ e FAPESP (2010-2010: CNPQ - Apoio Técnico/ Bolsa: 370406/2010–9; 2010–2011: FAPESP - 2010/12873–0; 2012–2014: CNPQ - Apoio Técnico/ Bolsa: 350104/2012–3), trabalhando com pesquisas na área de Parasitologia sob a orientação dos Professores: Profa. Dra. Rosangela Z. Machado e Prof. Dr. Marcos R. André. Em junho de 2016, concluiu o Curso de Mestrado em Microbiologia Agropecuária na FCAV/UNESP, com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Em agosto de 2016, ingressou no Curso de Doutorado no mesmo programa de pós-graduação e instituição sob a orientação dos Professores: Prof. Dr. Marcos R. André e Profa. Dra. Rosangela Z. Machado. No segundo semestre de 2016, ministrou as aulas de protozoologia na disciplina de Parasitologia para o Curso de Ciências Biológicas da FCAV/UNESP, sob a supervisão do Prof. Dr. Marcos R. André. Entre novembro de 2018 e abril de 2019, realizou Estágio de Doutorado Sanduíche na Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment – The Hebrew University of Jerusalem – Rehovot, Israel, sob orientação do Prof. Dr. Shimon Harrus, com bolsa da CAPES. Durante o primeiro semestre de 2020, atuou como docente na disciplina de Parasitologia Geral e Humana para o curso de Ciências Biológicas da FCAV/UNESP sob a supervisão do Prof. Dr. Marcos R. André e Profa. Dra. Rosangela Z. Machado.

Epígrafe

Retribui-se mal a um mestre continuando a ser apenas um aluno.

"Ecce homo"

Friedrich Nietzsche

DEDICATÓRIA

Aos meus queridos pais, Luzia C. Gonçalves e Luiz C. Gonçalves,

pelo amor, carinho e apoio em todas

as decisões da minha vida.

Aos meus irmãos, Kátia, Maiko e Luana e minhas sobrinhas, Aghata e Yasmin,

pelo carinho e apoio incondicional.

À minha namorada Rafaela, pelo amor, parceria,

compreensão e companherismo diário.

AGRADECIMENTOS

A Deus, por tudo aquilo que tenho experienciado.

À UNESP e ao Programa de Pós-graduação em Microbiologia Agropecuária pela oportunidade e pelo crescimento acadêmico e científico que me proporcionaram durante minha formação doutoral.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelas bolsas de estudos.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo Auxílio-pesquisa (Processo 2018/02753-0) concedido para a realização deste trabalho.

Ao Professor Heitor Miraglia Herrera, pelo respeito, confiança e por todo suporte durante as coletas. Esse auxílio foi imprescindível para o desenvolvemento deste projeto.

Ao Professor Shimon Harrus, pela oportunidade, confiança e ensinamentos durante o estágio de Doutorado Sanduíche. Sempre serei grato.

Aos meus parceiros de coleta de amostras, Wesley, Filipe, Grasiela, Wanessa, Gabriel, William, João, Thiago e Inalda.

Ao Dr. Ricardo Gutiérrez, pelos ensinamentos compartilhados e amizade durante meu estágio de Doutorado Sanduíche.

À Professora Darci Moraes Barros-Battesti, pelo auxílio na identificação dos ectoparasitas amostrados.

À Dra. Keyla C. M. de Sousa, pelo companherismo e todo auxílio em Israel.

Aos funcionários do Deparatamento de Patologia, Reprodução e Saúde Única, em especial à Mabel Mastro Custódio.

Aos amigos que passaram ou que ainda estão no Laboratório de Imunoparasitologia: Arvelino, Carlos, Otávio, Paulo, Márcia, Carla, Thiago, Natalia, Victória, Ana Cláudia, Matheus, Lucas, Paulo, Inalda, Simone, Kayo, Renan, Jyan, Priscila, Maria, Lucas, Lívia, Laryssa, Ana Júlia, Jhessye, Leidiane, Jaqueline e Ana Carolina. Venho aprendendo com vocês a cada dia.

AGRADECIMENTOS ESPECIAIS

Minha imensa gratidão ao Professor Dr. Marcos R. André e à Professora Dra. Rosangela Z. Machado. Orientadores e motivadores incansáveis que, com entusiasmo, sabedoria e competência, sempre me apoiaram, mesmo nos momentos turbulentos. Sempre os terei como um exemplo a ser seguido.

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unesp "JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Isolamento e caracterização molecular de *Bartonella* spp. em bovinos e roedores sinantrópicos"**, protocolo nº 01952/18, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 08 de março de 2018.

Vigência do Projeto	01/08/2016 a 01/07/2020
Espécie / Linhagem	Ordem Rodentia e Bovideo
Nº de animais	-
Peso / Idade	Variável
Sexo	Variável
Origem	Captura de vida livre e amostragem de bovídeos em áreas rurais

Jaboticabal, 08 de março de 2018.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
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DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPLASMAS E *Bartonella* spp. EM PEQUENOS MAMÍFEROS, GRANDES RUMINANTES E ECTOPARASITOS ASSOCIADOS

RESUMO – Hemoplasmas e *Bartonella* spp. compreendem dois importantes grupos bacterianos amplamente distribuídos e que infectam animais e humanos. Hemoplasmas são bactérias epicelulares, não cultiváveis, sem parede celular, e que se ligam à superfície dos glóbulos vermelhos de mamíferos. Por outro lado, *Bartonella* spp. são bactérias Gram-negativas que infectam principalmente eritrócitos e células endoteliais de várias espécies de mamíferos. Com o objetivo de avaliar a ocorrência e a diversidade genética desses grupos bacterianos, pequenos mamíferos e seus ectoparasitos foram amostrados em Campo Grande, Mato Grosso do Sul (MS) e Três Barras, Santa Catarina (SC), Brasil. Além disso, ruminantes e ectoparasitas associados foram amostrados em Campo Grande, MS, e Passos, Minas Gerais (MG), Brasil. Por fim, amostras de sangue de búfalos selvagens submetidos a um processo de translocação entre dois locais em Moçambique, África, também foram analisadas. Ensaios de PCR convencional, em tempo real quantitativo com sonda de hidrólise e com intercalante de DNA seguido de dissociação em alta resolução, análises filogenéticas – Máxima verossimilhança, Neighbor-joining e Inferência Bayesiana – e de bioinformática – Neighbor-Net e Templeton Crandall e Sing (TSC) network, foram utilizados para avaliar a ocorrência e diversidade genética de espécies de hemoplasmas e *Bartonella* spp. O presente estudo mostrou que 31,4% (33/105) dos pequenos mamíferos (sete [50%] capivaras; 14 [32,5%] gambás; 12 [30,7%] ratos) e 4,3% (5/116) dos ectoparasitas (três [2,6%] *Amblyomma* spp.; dois [1,7%] *Polypax spinulosa*) amostrados em Campo Grande foram positivos para hemoplasmas baseado no gene 16S rRNA. As análises filogenéticas e de distância mostraram que os hemoplasmas identificados parecem ser espécie-específicos. Em contraste, nenhum dos roedores e marsupiais, assim como seus ectoparasitas, mostraram-se positivos para *Bartonella* sp. nos ensaios moleculares e/ou na cultura. Por outro lado, ensaios moleculares dirigidos para os genes *nuoG* e 16S e para região ITS mostraram a presença de DNA de *Bartonella coopersplainsensis* em dois (3,6%) dos 55 *Rattus rattus* amostrados em Três Barras, SC. Este é o primeiro relato de *B. coopersplainsensis* na América do Sul. Além disso, DNA de *Bartonella bovis* foi encontrado em 21 das 75 (28%) amostras de sangue de bovinos e em 13 dos 126 (10,3%) carrapatos (*Rhipicephalus microplus*) coletados em Campo Grande. Da mesma forma, um (1%) dos 101 búfalos e quatro (4,2%) das 95 amostras de piolhos (*Haematopinus tuberculatus*) obtidas em Passos, MG, foram positivas para *Bartonella* spp. Enquanto o DNA de *B. bovis* foi identificado em uma amostra de sangue de búfalo, o DNA da espécie de *Bartonella* sp. amplificado nos piolhos foi geneticamente relacionado à *Bartonella* sp. previamente detectada em *Haematopinus quadripertusus* em Israel. Em contraste com as sequências idênticas detectadas nos piolhos, vários genótipos de *B. bovis* foram identificados dentre as amostras de sangue de bovinos. Por fim, entre os 97 búfalos africanos, DNA de *Bartonella* spp. e hemoplasmas foi detectado em 4,1% e 15,4% dos animais, respectivamente. A análise pelo BLAST mostrou a ocorrência de três agentes associados a bovinos: *Bartonella bovis, Mycoplasma wenyonii* e '*Candidatus* M. haemobos'. Tais achados contribuem para o entendimento sobre a

distribuição e diversidade genética de hemoplasmas e *Bartonella* spp. que circulam em roedores, gambás, bovinos, búfalos e ectoparasitos no Brasil e Moçambique.

Palavras-chave: Bovinos, Brazil, búfalos, capivaras, gambás, Moçambique, ratos

MOLECULAR DETECTION AND CHARACTERIZATION OF HEMOPLASMAS AND *Bartonella* spp. IN SMALL MAMMALS, LARGE RUMINANTS AND ASSOCIATED ECTOPARASITES

ABSTRACT – Hemoplasmas and *Bartonella* spp. comprise two important and widely distributed bacterial groups that can infect humans and animals. While hemoplasmas are cell wall-less uncultivated epicellular bacteria that attach to mammals red blood cells' surface, *Bartonella* is a successful group of Gram-negative bacteria that infect mainly erythrocytes and endothelial cells from a wide range of mammals. Aiming to assess the occurrence and the genetic diversity of these bacterial groups, small mammals and associated ectoparasites were trapped in Campo Grande, Mato Grosso do Sul (MS) and Três Barras, Santa Catarina (SC), Brazil. Also, large ruminants and associated ectoparasites were sampled in Campo Grande, MS, and Passos, Minas Gerais (MG), Brazil. Finally, blood samples from wild buffaloes submitted to a translocation process between two sites in Mozambique, Africa, were also analyzed. PCR assays, namely conventional, real-time quantitative using hydrolysis probe and DNA intercalant followed by High Resolution Melt, phylogenetic analyses – Maximum likelihood, Neighbor-joining and Bayesian inference – and bioinformatics – Neighbor-Net and Templeton Crandall and Sing (TSC) network – approaches were used to assess the occurrence and the genetic diversity of these bacterial species. The present study showed that 31.4% (33/105) small mammals (seven [50%] capybaras; 14 [32.5%] opossums; 12 [30.7%] rats) and 4.3% (5/116) ectoparasites (three [2.6%] *Amblyomma* spp.; two [1.7%] *Polypax spinulosa*) sampled in Campo Grande were positive for hemoplasmas targeting the 16S rRNA gene. The phylogenetic and distance analyses of showed that the amplified hemoplasmas DNA seem to be quite species-specific regarding mammalian hosts. In contrast, none of these small mammals and ectoparasites showed positivity for *Bartonella* sp. based on molecular assays and/or in the culture. On the other hand, *Bartonella coopersplainsensis nuoG* and 16S gene and as well as the ITS region were amplified in two (3.6%) out of 55 *Rattus rattus* sampled in Três Barras, SC. This is the first report of this rat-associated *Bartonella* species in South America. Also, *Bartonella bovis* DNA was found in 21 of 75 (28%) cattle blood samples and 13 of 126 (10.3%) associated ticks (*Rhipicephalus microplus*) collected in Campo Grande. Likewise, one (1%) out of 101 buffaloes and four (4.2%) of 95 lice (*Haematopinus tuberculatus*) DNA samples obtained in Passos, MG, were positive for *Bartonella* spp. While *B. bovis* was identified in a buffalo blood sample, the *Bartonella* sp. amplified in lice was closely related to a *Bartonella* sp. previously found in cattle-lice from Israel. In contrast to identical sequences detected in lice, several *B. bovis* genotypes were found in the cattle blood samples DNA. Lastly, among the 97 wild African buffaloes, *Bartonella* spp. and hemoplasmas DNA were detected in 4.1% and 15.4% of the animals, respectively. The BLASTn analysis disclosed at least three bovine associated pathogens, namely *B. bovis*, *Mycoplasma wenyonii* and '*Candidatus* M. haemobos'. These findings shed light on the distribution and genetic diversity of hemoplasmas and *Bartonella* spp. circulating in rodents, opossums, cattle, buffaloes and associated ectoparasites in Brazil and Mozambique.

Keywords: buffaloes, Brazil, capybara, cattle, Mozambique, opossums, rats

LIST OF ABBREVIATIONS

BAPGM: *Bartonella* Alpha Proteobacteria Growth Medium
cPCR: Convetional PCR (PCR: polymerase chain reaction)
ELISA: Enzyme-Linked Immunosorbent Assay
FISH: Fluorescence *in situ* hybridization
ftsZ: Cell division protein ftsZ
gapdh: Glyceraldehyde-3-Phosphate Dehydrogenas
gltA: Citrate synthase
HRM: High Resolution Melt
IFAT: Immunofluorescence antibody test
ITS: Intergenic transcribed spacer
IUCN: International Union for Conservation of Nature
LPSN: List of Prokaryotic names with Standing in Nomenclature
MLST: Multilocus sequence typing
nuoG: NADH Dehydrogenase Gamma Subunit
qPCR: Quantitative PCR
ribC: Riboflavin synthase
rpoB: Beta-subunit of RNA polymerase
WGS: Whole genome sequencing

CHAPTER 1 – General consideration

1. INTRODUCTION

Over the last decades, the Earth has witnessed intense changes in their ecosystems. Infectious diseases have emerged or re-emerged in several geographical regions causing global health and economic challenges. Changes in the climate, landscape, and ecosystems associated with other factors such as illegal wildlife trade, water storage, and globalization have affected the ecology and epidemiology of vector-borne diseases around the world (Harrus and Baneth, 2005; Otranto et al., 2015). These events, together with other factors such as environment encroachment and livestock intensification – caused by anthropogenic pressures – may affect the transmission dynamics of pathogens and the spillover of humans and domestic animals' pathogens to wildlife and vice-versa (Chomel et al., 2007; André, 2018; Price et al., 2019).

It is estimated that between 60% and 80% of newly emerging diseases are zoonotic in origin, and therefore dependent on an animal host for survival. Of these emerging diseases, nearly 70% are associated with wildlife, with cross-species spread transmission and showing an inherent reaction to the evolutionary pressures of the pathogen ecology. Even though both wildlife and domesticated animal hosts can be considered important sources of emerging diseases, the anthropogenic influence on ecological systems is essential in zoonotic disease emergence (Smolinsky et al., 2003; Jones et al., 2008; Hassell et al., 2016).

Several studies have assessed the ecological role – e.g., prey, predator, decomposer, preserver and pollinator – of wild animals (Dorst, 1991; Curtin et al., 2002; McCreless et al., 2016; Malhi et al., 2016; Hempson et al., 2017). Despite wildlife animal's contribution to ecological balance, they are notorious reservoirs for many important zoonotic pathogens (Gilbert, 2018; Jansen et al., 2018; Ye et al., 2020). Urban-adapted (referred here as synanthropic) wildlife, which are abundant in cities, have also been shown to carry or act as reservoir hosts for many zoonotic pathogens (Lowry et al., 2012). Anthropogenic changes associated with urbanization can also bring animals – e.g., rats, birds, and bats – into closer contact with

domesticated animals – e.g., pets and livestock – and humans, favoring an increase in the risk of pathogens spilling over to humans or livestock and vice-versa (Deplazes et al., 2004; Pulliam et al., 2012; Himsworth et al., 2013).

Livestock, a large land-use sector on Earth, is experiencing deep changes over the few last decades as a result of anthropogenic pressures. The increasing human population coupled with changes in livestock product consumption patterns has led to an intensification on livestock systems. This certainly should raise questions about the impact of the intensification on the evolution and emergence of zoonotic diseases, a facet often overlooked (Herrero and Thornton, 2013; Hassell et al., 2016).

In this scenario, epidemiological studies associated with genetics and phylogenetic approaches are elementary to know the distribution, the genetic variation that occurs within and between different pathogen populations, to establish the evolutionary relationships as well as to reconstruct most likely species origin and transmission pathways (Hassell et al., 2016).

Thus, the present study aimed to assess the occurrence, genetic diversity, and phylogenetic relationships of two important bacterial group, namely *Bartonella* and hemotropic mycoplasmas (hemoplasmas), that infects human and animals worldwide. Despite the countless number of studies carried out targeting these bacteria in humans or animals, few studies targeting wild animals were performed in Brazil. Therefore, the elucidation of these bacteria distribution, evolutionary relationships, genetic diversity, identification of potential hosts, and vectors in a particular ecotope shows great importance.

2. LITERATURE REVIEW

2.1. Infection by hemotropic mycoplasmas (hemoplasmas)

2.1.1. Taxonomy

The hemotropic mycoplasmas (hemoplasmas) belong to Mollicutes Class, Mycoplasmatales Order, and Mycoplasmataceae Family (Tully et al., 1993). Previously, rodent-associated hemoplasmas species, namely *Mycoplasma haemomuris* and *Mycoplasma coccoides*, were positioned into different genera and classified as *Haemobartonella muris* and *Eperythrozoon coccoides*, respectively. Besides, these species were considered members of Anaplasmataceae Family, within the Rickettsiales Order. Likewise, the cattle-associated hemoplasma species *Eperythrozoon wenyonii* – now *Mycoplasma wenyonii* – was also classified as rickettsiae (Rickettsiales Order). However, based on 16S rRNA gene sequences analysis, Neimark et al. (2001; 2005) transferred these three species to the genus *Mycoplasma*.

Thereafter, new *Candidatus* to hemoplasmas species were described, including but not limited to '*Candidatus* Mycoplasma haemodidelphis' reported in *Didelphis virginiana* in the USA (Messick et al., 2002) and '*Candidatus* Mycoplasma haemobos', the second cattle-associated hemoplasma species detected in Japan (Tagawa et al., 2008). Also, the subdivision of *M. haemomuris* into two subspecies, namely '*Candidatus* Mycoplasma haemomuris subsp. musculi' and '*Candidatus* Mycoplasma haemomuris subsp. ratti ', has been suggested (Harasawa et al., 2015).

Currently, different hemoplasma genotypes have been reported in rodents and marsupials based on genetic and phylogenetic analysis of 16S rRNA (Vieira et al., 2009; Sashida et al., 2013; Gonçalves et al., 2015; Hornok et al., 2015; Massini et al., 2019). Thus, these findings highlight the possibility that new hemoplasmas species may be described in the future.

2.1.2. Etiologic agents

Hemotropic mycoplasmas are uncultivable cell wall-less bacteria that attach to red blood cells surface of a broad variety of animals, including humans. Some

hemoplasmas species commonly occur as ‘ring forms’, while in other species these forms are rare or absent. Sometimes the hemoplasmas can be found free in plasma, however, they are usually found in an indentation or deformation of the erythrocyte membrane. Electron microscopy has shown that these bacteria present a coccoid shape, and usually with a diameter of less than 0.9 µm (Neimark et al., 2001). Studies regarding genome sequences of these species showed that although hemoplasmas have reduced genomic sizes (Brown et al., 2011), resulting in the loss of many of their biosynthetic abilities, they retain crucial genes for their life cycle (Santos et al., 2011; do Nascimento et al., 2013).

Among all hemoplasmas described, *M. haemomuris* and *M. coccoides* are associated with rodents. Besides, other studies have suggested the occurrence of possible new species circulating in wild and synanthropic rodents from Brazil (Vieira et al., 2009; Gonçalves et al., 2015), Japan (Harasawa et al., 2015) and Hungary (Hornok et al., 2015).

Among marsupials, ‘*Candidatus* Mycoplasma haemodidelphis’ was reported in (*D. virginiana*) from USA (Messick et al., 2002), and a closely related genotype has been recently detected in (*Didelphis albiventris*) from Brazil (Massini et al., 2019).

Regarding bovids-related hemoplasmas species, only *M. wenyonii* (Neimark et al., 2001) and ‘*Candidatus* Mycoplasma haemobos’ (Tagawa et al., 2008) have been reported so far.

2.1.3. Transmission

The epieritrocytic hemoplasmas lifestyle suggests a blood-sucking arthropods – e.g., ticks, fleas or lice – transmission. However, experimental studies aiming to investigate the vectorial competence of these arthropods are scarce (reviewed by Biondo et al., 2009) and the exact routes for transmission are still unknown.

Concerning the rodent-associated species, a study demonstred the mechanical transmission of *M. coccoides* by *Polyplax serrata* and *Polyplax spinulosa* lice in mice (Berkenkamp and Wescott, 1988). Recently, Cohen et al. (2018) suggested that *M. haemomuris*-like is mainly transmitted by rodent-rodent contact during aggressive interactions. Besides, the authors found no evidence of vertical

transmission of *M. haemomuris*-like in *Gerbillus andersoni* neither horizontally by fleas (*Synosternus cleopatrae*).

Bovids-related hemoplasmas DNA have been detected in ticks, lice, flies and mosquitoes (Hornok et al., 2011; Song et al., 2012; Hasan et al., 2017). Although the potential role of these blood-sucking arthropods in the transmission has been reported (Hofmann-Lehmann et al., 2004; Hornok et al., 2011), to the best of authors' knowledge, no study has shown the vectorial competence of these bacterial species so far. On the other hand, there is molecular evidence of trans placental transmission of *M. wenyonii* and '*Candidatus* M. haemobos' in beef and dairy cattle (Hornok et al., 2011; Girotto-Soares et al., 2016; Niethammer et al., 2018).

2.1.4. Diagnosis

Historically, the diagnosis of this group of bacteria has relied on the demonstration of hemoplasmas on the surface of parasitized erythrocytes of Romanowsky stained blood smears, especially in the acute phase of infection (Sykes, 2010). However, false-positive results may occur due to refractory artifacts, dye precipitates and Howell-Jolly bodies (Tasker and Lapin, 2002; Willi et al., 2007). Bacterial concentrations in the blood of humans or animals may also fluctuate during the course of the infection, and the low hemoplasma bacteremia could not be detected by microscopy due to the low sensitivity of this methodology, especially in immunocompetent chronically hemoplasma-infected patients (Volokhov et al., 2017).

Since hemoplasmas have not been cultured *in vitro*, the molecular detection of bacterial DNA, either by conventional (c) or real-time (q) PCR assays, is the main technique used for the diagnosis of infections caused by these agents (Willi et al., 2007; Sykes, 2010). Several PCR protocols targeting different gene fragments (16S rRNA, 23S rRNA, *RNase P* [Ribonuclease P], *gyrB* [DNA gyrase subunit B gene], *dnaK* [chaperone protein Dnak] and *rnpB* [β subunit of RNA polymerase]) were developed and have been facilitating the diagnosis of hemoplasmosis (Birkenheuer et al., 2002; Maggi et al., 2013a; Satoh et al., 2016; Volokhov et al., 2017; Mongruel et al., 2020).

Besides, the amplification of full-length 16S rRNA gene sequences followed by sequencing and phylogenetic analysis are specially necessary to distinguish between

closely related hemoplasmas species (Volokhov et al., 2017). In addition, qPCR assays have brought greater speed and sensitivity, coupled with a lower risk of contamination during DNA amplification, facilitating diagnosis, especially when low bacteremia is present (Willi et al., 2009).

2.1.5. Epizootiology of hemoplasmas in rodents, marsupials and associated ectoparasites

Even though the occurrence of hemoplasmas in domesticated animals has been well documented throughout the world, (do Nascimento et al., 2012; de Mello et al., 2019; Shi et a., 2019; Barker, 2019), few studies have been carried out targeting hemoplasmas in rodents, marsupials and associated ectoparasites.

During a rodent control performed in an animal hospital in Morioka, Japan, nine *Rattus norvegicus* were trapped and one showed positivity to hemoplasmas. The 16S rRNA gene and 16S-23S intergenic spacer region sequences analyses revealed the presence of a hemoplasma closely related to *M. haemomuris*, albeit not previously described in rodents (Sashida et al., 2013).

Analyzing spleen DNA samples of *R. norvegicus* (n = 14), *Mus musculus* (n = 37) and *Micromys minutus* (n = one) as well as their ectoparasites (*Leptopsylla segnis* – flea, *Laelaps algericus* – mite and *P. spinulosa* – louse) collected on the North-Eastern and Southern border of Hungary, Hornok et al. (2015) reported the molecular detection of novel hemoplasma species. Among these, 92% (13/14) *R. norvegicus*, 56.7% (21/37) *M. musculus* and 100% (1/1) *P. spinulosa* samples were positive.

In Brazil, the first molecular evidence of hemoplasmas in rodents (*Hydrochaeris hydrochaeris*) was reported in Paraná state. Out of 21 free-ranging and ten captive capybaras, the 16S rRNA hemoplasma-DNA was amplified in 17 (80%) and three (30%) capybaras, respectively (Vieira et al., 2009). The phylogenetic results also suggested a possible new hemoplasma species circulating in capybaras in Brazil.

In the same year, Conrado et al. (2015) and Gonçalves et al. (2015), showed the occurrence of hemoplasmas DNA in synanthropic rodents (*R. norvegicus* and *Rattus rattus*) in different sampling sites.

Among the 43 free-ranging *R. norvegicus* and 20 laboratory rats sampled in Curitiba city, Paraná state, 31 (72%) and nine (45%) were positive, respectively, for the PCR screening for hemoplasmas. Twenty-five out of the 26 obtained 16S rRNA sequences showed identity ranging from 98% to 100% with *M. haemomuris*. Besides, one sequence shared 99% of identity with a new hemoplasma species previously detected in *R. norvegicus* from Hungary. The authors did not find statistically significant alterations in hematologic parameters between positive and negative rats for *M. haemomuris* (Conrado et al., 2015). In addition to hemoplasmas detection in *R. rattus* (48.2%; 14/29), Gonçalves et al. 2015 also reported distinct *Mycoplasma* genotypes in wild rodents trapped in five Brazilian biomes. The authors reported an overall prevalence of 21.9% (100/457), and highlighted the statistical difference in the hemoplasmas occurrence between the sampling sites. Lastly, the authors raised the hypothesis that synanthropic rodents-related hemoplasmas were introduced in Brazil during the European colonization (Gonçalves et al., 2015).

By sampling wild rodents from Brazilian Pantanal biome, de Sousa et al. (2017) showed the lowest hemoplasma prevalence among wild rodents in Brazil up to now (0,9%; 1/110). The amplified 16S rRNA sequence was phylogenetically positioned near to *Mycoplasma* sp. previously detected in *Trichomys fosteri* by Gonçalves et al. (2015) in the same region. The authors reported that none of the ectoparasites (*Amblyomma* spp. larvae or nymphys [n = 176] and *Polygenis bohlsi bohlsi* [n = 75]) analyzed showed positivity to *Mycoplasma* spp.

To the best of authors' knowledge, only three studies were performed aiming to assess the molecular prevalence of hemoplasmas in marsupials despite their wide distribution around the world.

‘*Candidatus* M. haemodidelphis’ was initially detected in an anemic North American opossum (*D. virginiana*) (Messick et al., 2002).

The first molecular evidence of hemoplasmas in marsupials from Brazil was reported recently by Massini et al. (2019) in white-eared opossums (*D. albiventris*) from a public park in Maringa city, Paraná state, Southern Brazil. Seven out of eight opossums sampled were positive for hemoplasmas. The 16S rRNA sequence shared 98.97% identity with ‘‘*Candidatus* M. haemodidelphis’ previously detected in the USA. The authors also reported that three out of eight positive opossums were

infested by *Amblyomma dubitatum* ticks; however, these arthropods were not screened for hemoplasmas.

Finally, among 30 marsupials – 14 *Thylamys macrurus,* 11 *Gracilinanus agilis,* four *Monodelphis domestica* and one *D. albiventris* – and associate ectoparasites (18 *Amblyomma parvum* nymphs and five *Polygenis bohlsi bohlsi* fleas) sampled in Pantanal biome, Mato Grosso do Sul state, none showed positivity for hemoplasmas in PCR assays based on 16S rRNA (de Sousa et al., 2017).

2.1.6. Epizootiology of hemoplasmas in bovids

The occurrence of *M. wenyonii* and '*Candidatus* M. haemobos' in bovids (cattle and buffaloes) has been well documented worldwide. Several studies were carried out aiming to assess the prevalence of bovids-associated hemoplasmas species (**Figure 1)**. The prevalence of hemoplasmas in bovids is generally high (up to 97%), albeit it varies widely across different studies, and among distinct populations in the same country (Meli et al., 2010; Giroto et al. ,2012; de Mello et al., 2019).

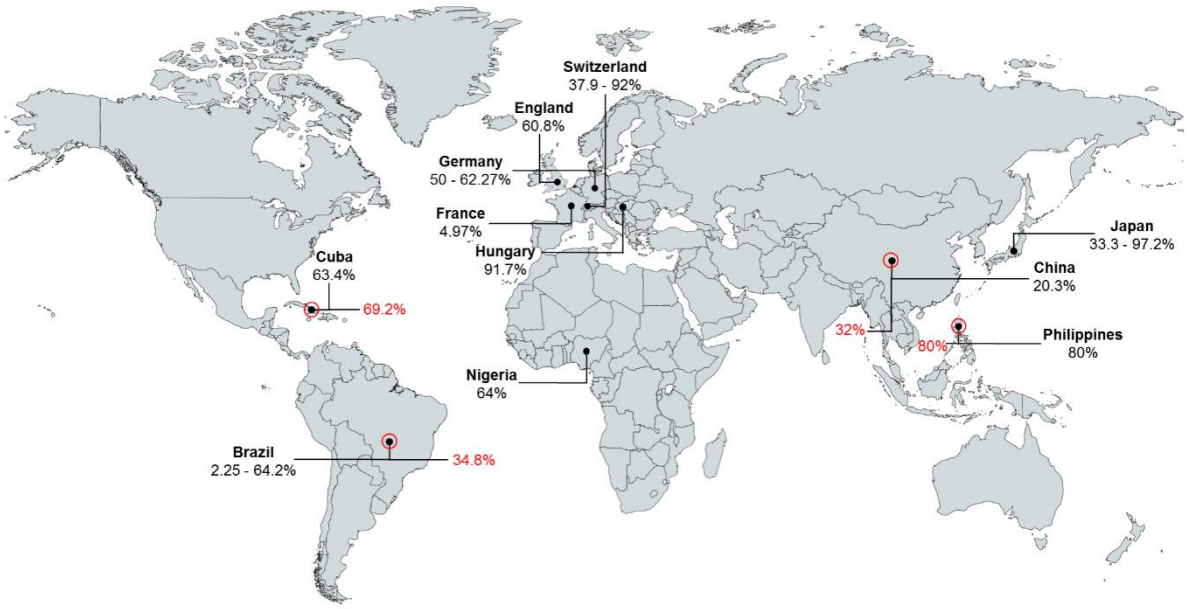

Figure 1. Molecular prevalence of bovids-related hemoplasmas species. The red numbers and circles refer to prevalence rates reported in buffaloes.

In Brazil, *M. wenyonii* and/or '*Candidatus* M. haemobos' were detected in bovids DNA blood samples from five states. The first molecular detection was performed by Giroto et al. (2012) in Londrina, Paraná state. The authors found an overall prevalence of 61% (164/433). The phylogenetic analyses based on a fragment of 16S rRNA (368 bp) revealed the presence of '*Candidatus* M. haemobos'.

Giroto-Soares et al. (2016) reported the occurrence of *'Candidatus* M. haemaobos' DNA in 40.9% (9/22) the dairy cows and 18.2% (4/22) aborted fetuses sampled in Presidente Getulio, Santa Catarina state. The authors suggested a possible hemoplasma transplacental transmission.

Witter et al. (2017), analyzing blood-DNA samples from cattle sampled in 64 farms localized in Ji-Paraná municipality, Rondônia state, found a prevalence of 64.2% (207/320). Besides, the age (>24–month–old cows) was also correlated with '*Candidatus* M. haemobos' infection rates.

Santos et al. (2018) carried out the first molecular detection of hemoplasmas in water buffaloes (*Bubalus bubalis*) from Brazil. A total of 34.8% (101/290) buffaloes sampled in four rural dairy farms in Maranhão state were positive for *Mycoplasma* spp.

Finally, when sampling beef cattle from Brazilian Pantanal, an area endemic for bovine trypanosomiasis, de Mello et al. (2019) reported a low hemoplasma prevalence rate (2.25%; 9/400). The authors also described the occurrence of five genotypes of *M. wenyonii* and one of '*Candidatus* M. haemobos' among the nine sequenced amplicons.

Additionally, the impact of these two hemoplasmas species in hematological parameters (Tagawa et al., 2010), their effect on cattle productivity (Tagawa et al., 2013a), their behavior during coinfection with other arthropod-borne pathogens - *Anaplasma* spp. and *Theileria* spp. (Hofmann–Lehmann et al., 2004; Hornok et al., 2012; Tagawa et al., 2013b), as well as the trans placental transmission (Hornok et al., 2011; Girotto-Soares et al., 2016; Niethammer et al., 2018) have been assessed.

2.1.7. Human infection with hemoplasmas species

The human infection with hemoplasmas is rare, and there have been few studies on the molecular characterization of hemoplasmas in humans (Alcorn et al.,

2020; Hattori et al., 2020). However, increasing contact with animals may favor the transmission of pathogens between humans and animals.

For instance, *Mycoplasma haemofelis*-like (dos Santos et al., 2008), *Mycoplasma suis*-like (Yuan et al., 2009), *Mycoplasma ovis* (Sykes, 2010; Maggi et al., 2013a), '*Candidatus* Mycoplasma haemohominis' (Steer et al., 2011) and '*Candidatus* Mycoplasma haematoparvum' (Maggi et al., 2013a,b) were detected in humans.

The hemoplasmosis in humans is not well established, possibly because hemoplasmas are unculturable and due to the fact these bacteria are usually overlooked by physicians (Maggi et al., 2013a; Hattori et al., 2020). However, recent studies have improved our knowledge about this hemoplasmas infecting humans.

Recently, the infection with '*Candidatus* M. haemohominis' was reported in a Japanese 42–year–old man (physician) and the clinical features of the infection were clarified (Hattori et al., 2020). During the hemoplasma infection, the authors reported various life-threatening symptoms in the patient, such as hemophagocytic syndrome, liver damage, and bleeding. Also, the patient showed hypoglycemia, pyrexia, anemia, whole-body erythema and pruritus. Electron microscopy, in–situ hybridization and draft genome analyses were performed in order to confirm the hemoplasma infection. The hemoplasma infection level was monitored using a real–time PCR assay based on 16S rRNA gene after the treatment. The hemoplasma load from the patient's serum samples decreased below the detection limit (<10 copies/reaction) 14 days after the patient treatment with levofloxacin, but then increased again. The hemoplasma genetic analysis showed that only 2–nt mutations were identified, including a non-synonymous mutation in the quinolone resistance–determining regions (QRDR) of the DNA gyrase subunit A GyrA. After a combination therapy with moxifloxacin and minomycin, the bacterial load decreased (Hattori et al., 2020).

A second case, in a 69–years–old Australian woman that worked as a wildlife reserve assistant was reported. The patient was admitted to a hospital with a four–day history of fever and myalgia, with a preceding history of four-months of weight loss and fatigue. Initial examination revealed fever, neutropenia, thrombocytopenia and the computerized tomography revealed splenomegaly. Later, the patient showed fever, weakness, abdominal pain, severe nonhemolytic anemia (hemoglobin 89g/L)

and marked hypoalbuminemia. After successive and unsuccessful treatments with piperacillintezobactom, doxycycline and prednisolone, given the extensive potential for zoonotic infection, itraconazole (later amphotericin), piperacillin/tazobactam and azithromycin were commenced. However, this approach also failed to improve symptoms. At that moment, a blood smear examination revealed small ring-like inclusions inclusions. Thereafter, the hemoplasma identification was performed using WGS, followed by 16S rRNA partial sequence amplification and fluorescence in–situ hybridization (FISH). The results showed the presence of '*Candidatus* M. haemohominis'. After the final diagnosis was achieved, a new treatment approach was started using minocycline (instead of doxycycline) and moxifloxacin. The patient received four of the planned six months of antibiotics, and remained well two months after treatment cessation (Alcorn et al., 2020).

Despite the fact that the two above-mentioned studies were case reports, the analyses performed to connect the clinical signs to *'Candidatus*. M. haemohominis' infection were essential to a definite diagnosis. Although these studies may have limitations, the results provide useful knowledge about human hemoplasma infection.

2.2. Infection by *Bartonella* spp.

2.2.1. Taxonomy

Bartonella genus belongs to the Order Rhizobiales, and Family Bartonellaceae (Birtles et al., 1999). *Bartonella* species are genetically close to *Brucella*, *Agrobacterium*, *Ochrobactrium*, and *Rhizobium* species within the subdivision of Alfa-Proteobacteria (Kosoy et al., 2012). The *Bartonella* genus was proposed more than a century ago when Alberto Barton identified the etiologic agent of Carrión's disease analyzing intracellular bacteria in blood smears from Oroya fever patients in 1905. Thereafter, this bacterium was named as *Bartonella bacilliformis* in his honor (Strong et al., 1915; Minnick et al., 2014).

Curiously, the genus *Bartonella* was represented by only *B. bacilliformis* for more than 80 years. This genus was substantially expanded after Brenner et al. (1993) suggested to unify *Bartonella* and *Rochalimaea* genera. The authors also proposed four new species, namely *Bartonella quintana*, *Bartonella*

vinsonii, *Bartonella henselae*, and *Bartonella elizabethae* based on DNA–DNA hybridization and comparison of 16S rRNA sequences (Brenner et al., 1993; reviewed by Kosoy et al., 2012).

Likewise, Birtles et al. (1995) proposed to include the *Grahamella* genus – a previously described hemotropic bacterial group – in the *Bartonella* genus.

During the last 25 years, the number of studies related to *Bartonella* genus has significantly expanded, as well as the number of species described and our knowledge about this important bacterial group (Kosoy et al., 2012; Breitschwerdt, 2017; Kosoy et al., 2018).

2.2.2. Etiologic agents

Bartonella species are Gram-negative facultative intracellular bacteria that promote prolonged intraerythrocytic bacteremia in mammalian hosts across the world (Birtles et al., 1999; Harms and Dehio, 2012). One of the most fascinating aspects of *Bartonella* spp. infection is the erythrocyte tropism, which is an intriguing and central event in the pathogenesis of this group of bacteria (Minnick and Battisti, 2009). This trait provides a partial immune system evasion in addition to enabling continuous transmission by blood-sucking arthropods (Harms and Dehio, 2012).

Currently, 36 *Bartonella* species and three subspecies validly published are reported in the List of Prokaryotic names with Standing in Nomenclature (LPSN – accessed on May 13, 2020: http://www.bacterio.net/bartonella.html). These species were found infecting mainly rodents, felids, canids, ruminants, and humans around the world **(Table 1)**.

Table 1. *Bartonella* species described, distribution, hosts and human infection

***Bartonella* species**	**Description**	**Distribution**	**Main reservoir**	**Human infection**
B. acomydis	Sato et al., 2013	Japan and Israel	Rodents	-
B. alsatica	Heller et al., 1999	Europe and North America	Rabbits	Yes
B. ancashensis	Mullins et al., 2015	Peru	Human	Yes
B. apis	Kešnerová et al., 2016	Europe, USA and China	Honey bee	-
B. bacilliformis	Strong et al., 1915	Colombia, Ecuador and Peru	Human	Yes
B. birtlesii	Bermond et al., 2000	Europe	Rodents	-
B. bovis	Bermond et al., 2002	Worldwide	Ruminants	-
B. callosciuri	Sato et al., 2013	Japan	Rodents	-
B. capreoli	Bermond et al., 2002	Europe, USA and Japan	Ruminants	-
B. chomelii	Maillard et al., 2004	Europe, China, Algeria and Palestine	Ruminants	-
B. clarridgeiae	Lawson and Collins, 1996	Worldwide	Felids	Yes
B. coopersplainsensis	Gundi et al., 2009	Asia, Australia and Europe	Rodents	-
B. doshiae	Birtles et al., 1995	Europe, Afghanistan, Brazil and China	Rodents	Yes
B. elizabethae	Brenner et al., 1993	Worldwide	Rodents	Yes
B. florencae	Mediannikov et al., 2014	France	Shrew	-
B. fuyuanensis	Li et al., 2016	China	Rodents	-
B. grahamii	Birtles et al.,1995	North Hemisphere	Rodents	Yes
B. heixiaziensis	Li et al., 2016	China	Rodents	-
B. henselae	Brenner et al., 1993	Worldwide	Felids	Yes
B. jaculi	Sato et al., 2013	Japan	Rodents	-
B. japonica	Inoue et al., 2010	Japan and China	Rodents	-
B. koehlerae	Droz et al., 2000	Worldwide	Felids	Yes
B. kosoyi	Gutiérrez et al., 2020	Israel	Rodents	Yes
B. krasnovii	Gutiérrez et al., 2020	Israel	Rodents	-
B. pachyuromydis	Sato et al., 2013	Japan	Rodents	-
B. peromysci	Birtles et al., 1995	United Kingtom	Rodents	-
B. queenslandensis	Gundi et al., 2009	Asia, Australia, Africa and Canary Islands	Rodents	-
B. quintana	Brenner et al., 1993	Worldwide	Human	Yes
B. rattaustraliani	Gundi et al., 2009	Australia	Rodents	-
B. rochalimae	Eremeeva et al., 2007	North Hemisphere and South America	Canids	Yes
B. schoenbuchensis	Dehio et al., 2001	China, Europe, South Korea and USA	Ruminants	Yes
B. senegalensis	Mediannikov et al., 2014	Senegal	Unknown	-
B. silvatica	Inoue et al., 2010	Japan	Rodents	-
B. talpae	Birtles et al., 1995	United Kingtom	Mole	-
B. taylorii	Birtles et al., 1995	Asia and Europe	Rodents	-
B. tribocorum	Heller et al., 1998	Worldwide	Rodents	Yes
B. vinsonii subsp. *arupensis*	Welch et al., 1999	Brazil, France, Greece, Italy, North America and Thailand	Rodents	Yes
B. vinsonii subsp. *berkhoffii*	Kordick et l., 1996	America, Europe, North Africa and Israel	Canids	Yes
B. vinsonii subsp. *vinsonii*	Kordick et l., 1996	North America	Rodents	-

(-) Not reported. Rodents-related *Bartonella* species are highlighted in gray

Additionally, several *Candidatus* to new species as well as different genotypes have been also identified in several regions (Mckee et al., 2016; Dahmani et al. 2017; Gutiérrez et al., 2018a).

Morphologically, these species are bacilli or coccobacilli with or without pili and flagella. Their length and genome sizes range from approximately 1 to 2 µm and 1.45 to 2.90 Mb, respectively (Bermond et al., 2002; Li et al., 2015; Mullins et al., 2015; Gutiérrez et al., 2020).

Rodents and bats harbor the largest *Bartonella* genetic diversity described so far (Mckee et al., 2016; Gutiérrez et al., 2018a). It has been estimated that these bacterial species began infecting mammals 62 million years ago near the Cretaceous-Paleogene boundary. In addition, bats have been incriminated as the ancestral hosts of all mammals-related *Bartonella* and appear to be responsible for the early geographic expansion of the genus (Mckee et al., 2020).

Out of all *Bartonella* species/subspecies described, rodents are considered as the main reservoirs for 22 species. Also, three *Bartonella* species have been reported in bovids, and none validly published *Bartonella* species has been identified in opossums so far **(Table 1)**.

2.2.3. Transmission

Due to their hemotropic lifestyle, *Bartonella* species are mainly transmitted by blood-sucking arthropods within mammalian communities. Several arthropods, such as fleas, lice, flies and ticks, are associated with *Bartonella* sp. transmission (Billeter et al., 2008; Chomel et al., 2009a). However, other transmission routes – scratch, bite and transplacental – have been reported (Chomel et al., 2010; Gutiérrez et al., 2015).

Fleas are considered key players in *Bartonella* sp. life cycle. In addition to efficiency in the *Bartonella* sp. transmission, these ectoparasites harbor a high genetic diversity of *Bartonella* spp. and also represent additional reservoirs for this bacterial group (Birtles, 2005; Morick et al., 2013; Gutiérrez et al., 2015). Additionally, experimental studies have demonstrated vector competence of sand flies (*Lutzomyia* spp.) for *B. bacilliformis* (Battistini,1931; Battistini et al., 2015), human body lice (*Pediculus humanus humanus*) for *B. quintana* (Bruce, 1921; Kim et al., 2017), and

Ixodes ricinus ticks for *B. henselae* and *B. birtlesii* (Cotté et al. 2008; Reis et al., 2011).

Several experimental studies have demonstrated the capability of fleas to acquire and transmit rodent-associated *Bartonella*. In a pioneer study, Krampitz (1962) showed that *Xenopsylla cheopis* fleas were competent vectors of an unidentified *Bartonella* sp. found in *Myodes glareolus* rodents. Similary, Bown et al. (2004) demonstrated that *Ctenophthalmus nobilis* fleas were able to transmit *B. grahamii* and *B. taylorii* to *M. glareolus.* Also, *Xenopsylla ramesis* has been shown to be competent to transmit *Bartonella* sp. OE 1-1 – now *B. krasnovii* – to *Meriones crassus* (Morick et al., 2013). In that study, the authors highlighted that about 69%–100% of the fleas acquired the bacteria within a period of 72 h, and a similar time was enough for the fleas to infect rodents. More recently, in an experiment involving artificial feeding of *X. cheopis* with rat blood inoculated with *B. elizabethae*, McKee et al. (2018) demonstrated that these fleas were able to acquire, and may maintain and excrete viable *B. elizabethae* for a minimum of 13 days post-infection.

In addition to vectorial transmission, the vertical *Bartonella* transmission from infected female rodents to their offspring was reported (Kosoy et al., 1998; Boulouis et al., 2001). Initially, Kosoy et al. (1998) isolated *Bartonella* spp. from the placental tissues of pregnant females, embryos, and neonatal pups of wild *Sigmodon hispidus* and *Peromyscus leucopus* rodents. Also, exploring the vertical transmission of *B. birtlesii* in BALB/c mice, Boulouis et al. (2001) reported similar results regarding transplacental transmission of *Bartonella* sp. in bacteremic pregnant mice. Besides, *Bartonella* OE 1-1 DNA was detected in one of 15 pups born to experimental infected *M. crassus* female rodents (Morick et al., 2013). Finally, the horizontal transmission of *Bartonella* sp. between male and female rodents in an arthropod-free system was also evaluated. The authors reported the absence of *Bartonella* sp. transmission from the experimentally infected to the naïve rodents (Bown et al. 2004).

Among cattle-associated ectoparasites, to the best of the author's knowledge, none study has been carried out so far in order to assess the role of these arthropods on *Bartonella* transmission. Despite transplacental *Bartonella* sp. transmission has been reported for rodents, Chastant-Maillard et al. (2015) did not find molecular evidence of this mechanism for *B. bovis* in cattle. The authors reported that none of

the 29 (52%) bacteremic cows gave birth to bacteremic calves, and that all calves analyzed were seronegative at birth. In addition, neither placentitis nor vasculitis was observed in all sampled cotyledons (Chastant-Maillard et al., 2015).

2.2.4. Diagnosis

The definitive diagnosis of bartonellosis in humans and animals remains a clinical, laboratory and microbiological challenge. There is no accurate technique for which a negative result that certifies the absence of *Bartonella* sp. infection (Álvarez-Fernández et al., 2018).

Currently, the most used approaches for the diagnosis of *Bartonella* sp. infection are DNA amplification using PCR, microbiological culture, and serology (Breitschwerdt, 2017; Álvarez-Fernández et al., 2018). However, all the mentioned approaches have limitations. Ideally, the *Bartonella* diagnosis should be confirmed by culturing the bacteria from aseptically obtained tissues (Breitschwerdt, 2017).

A chemically modified, insect-based liquid culture medium (BAPGM) was developed to support the growth of *Bartonella* species (Duncan et al., 2007). The enrichment culture of the biological sample in the liquid growth medium followed by a highly sensitive and broad-range PCR assay associated to solid culture has facilitated the *Bartonella* spp. detection and isolation (Breitschwerdt, 2017). Because *Bartonella* species are fastidious, it is important to highlight that a negative culture after a long incubation time does not exclude infection by this group of bacteria. Furthermore, the investigator must consider that humans and animals can be intermittently bacteremic (Kordick et al., 1999; Pultorak et al., 2013).

In addition, despite the improvements in *Bartonella* spp. culture methods, these approaches are still laborious, time-consuming, and their sensitivity to detect *Bartonella*-positive samples from wild animals is considerably low (Gutiérrez et al., 2017). On the other hand, the DNA amplification methods – cPCR and qPCR assays – offer a more rapid, specific, and sensitive tool to identify the *Bartonella* infections. Also, using the appropriate PCR targets followed by sequencing and bioinformatics analysis, the obtained DNA sequences can give more discriminatory power for *Bartonella* spp. identification (Gutiérrez et al., 2017). Serological tests, mainly IFAT and ELISA, can also be used to confirm prior or ongoing *Bartonella* spp. exposure.

However, due to poor associations between seroreactivity and bacteremia, and potentially cross-reaction with closely related bacteria, these assays show limited value for predicting *Bartonella* spp. infection. Meanwhile, these approaches have been widely used in seroepidemiologic studies (Lashnits et al., 2018).

In agreement with previously published studies (Gutiérrez et al., 2017; Kosoy et al., 2018), we also encourage attempting at *Bartonella* spp. isolation followed by a broader molecular characterization to properly identify this important group of bacteria. Thus, we suggested a work-flow for *Bartonella* spp. detection in human and animal samples **(Figure 2)**.

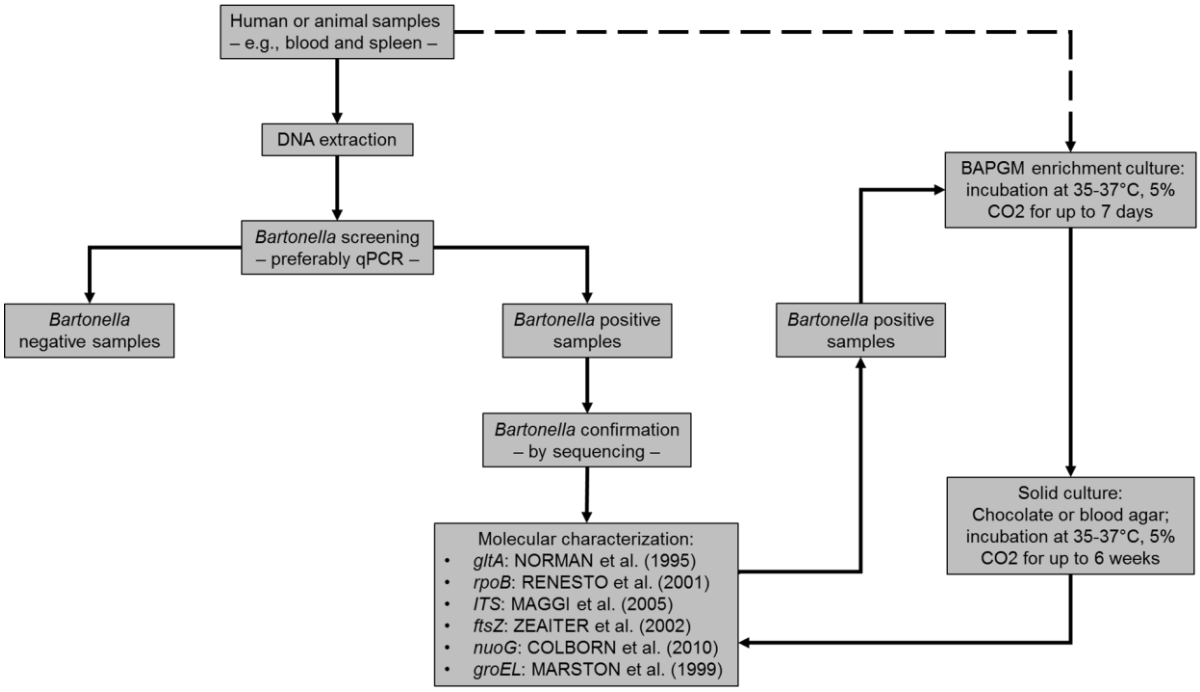

Figure 2. Suggested work-flow for detection and isolation of *Bartonella* spp. in human and animal samples. The “dashed step” may be conducted simultaneously with the DNA extraction followed by the qPCR screening. Adapted from Gutiérrez et al. (2017) and Kosoy et al. (2018).

2.2.5. Epizootiology of *Bartonella* spp. in rodents, marsupials and associated ectoparasites

Out of all *Bartonella* species listed in LPSN to date, 22 have rodents as main reservoirs. Among them, six species were reported in human infections causing different disease manifestations (Iralu et al., 2006; Breitschwerdt, 2017). Additionally,

other bartonellae have already been detected in rodents, such as the zoonotic *B. rochalimae* (Marciano et al., 2016) and *B. henselae* species (Helan et al., 2018).

The *Bartonella* occurrence, genetic diversity, transmission as well as other biological issues have been extensively analyzed in rodents communities. In order to have a idea of the representativeness of these works, a search using the words "*Bartonella* and rodents" in the PubMed database, retrieved 465 published studies **(Figure 3)**.

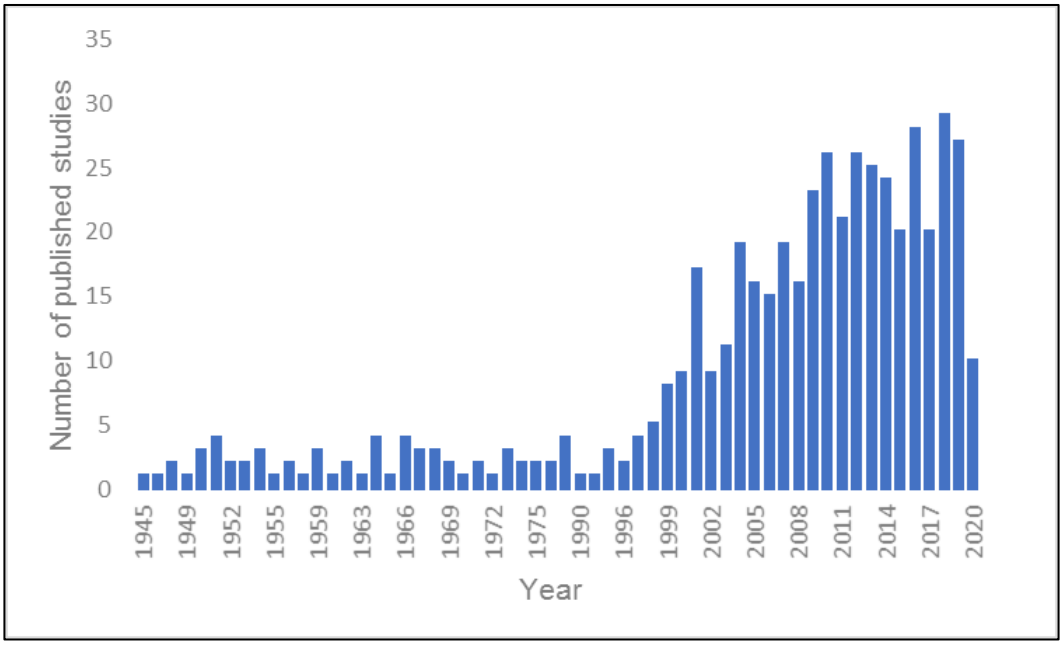

Figure 3. Number of studies reported in GenBank database – PubMed – using the combination of the words "*Bartonella* and rodents". PubMed – accessed on May 29, 2020. https://pubmed.ncbi.nlm.nih.gov/?term=Bartonella+and+rodents.

Bartonella spp. have been virtually reported in wild and synanthropic rodent communities worldwide (Buffet et al., 2013; Gutiérrez et al., 2015; Kosoy and Bai, 2019). The prevalence of *Bartonella* spp. in rodent communities is generally high, albeit it varies widely across different studies, ranging from apparent uninfected animals (Fontalvo et al., 2017) up to 90% (Bai et al., 2007). Several ecological factors, including but not limited to size and structure of rodent populations and behavior, temperature and sampling sites have been associated to *Bartonella* spp. prevalence rates in rodent communities (Bai et al., 2007; Kosoy and Bai, 2019).

Despite fleas being considered key players in the transmission of *Bartonella* spp. to rodents, rodent-associated *Bartonella* species have also been detected in lice (*Polyplax* spp. and *Hoplopleura* spp.), mites (*Leptotrombidium* spp., *Ascoschoengasti*a spp. and *Haemolaelaps* spp., *Blankarrtia* spp.) and ticks (*Haemaphysalis* spp., *Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor reticulatus*) (Kamani et al., 2013; Klangthong et al., 2015; Silaghi et al., 2016). However, it is worth mentioning that molecular detection does not imply vectorial capacity.

Phylogenetic analyses have shown that all rodent-related *Bartonella* species but except *B. rochalimae* and *B. tamiae* – species not listed in LPSN – belong to the same lineage (Buffet et al., 2013). Interestingly, the *Bartonella* genetic diversity verified in rodents apparently contributes to their high prevalence rates. Thus, this high diversity allows the bacteria to avoid the host immune responses against a specific genotype (Buffet et al., 2013). This idea is sustained by long-term studies carried out in Europe and North America with mark–recapture approaches, showing that these small mammals remain infected during long periods of their life, with different *Bartonella* genotypes continually replacing one another (Kosoy et al., 2004; Paziewska et al., 2012).

It is currently accepted that *Bartonella* spp. diversification mainly occurs by homologous recombination (Buffet et al., 2013). Rodent-related *Bartonella* spp. have a larger amount of imported genes than other *Bartonella* species. Moreover, genomic comparative studies of different *Bartonella* species have evidenced large genomic variations, such as rearrangements and deletions. These studies have proposed that phage-related elements (e.g., prophages and gene transfer agents [GTAs] associated with a run-off replication origin) may contribute largely to the diversity as well as evolution of some *Bartonella* species (Berglund et al., 2009; Gutiérrez et al., 2018b). In contrast, single-nucleotide mutational divergence proved to be the main driving force of diversity in other lineages, such as those comprising *B. bacilliformis* and *B. apis* (Paul et al., 2016; Segers et al., 2017).

In Brazil, all studies related to *Bartonella* spp. in rodents have been performed aiming the bacterial molecular detection and characterization. In this country,

Bartonella species were have been detected in wild and synanthropic rodents distributed in 13 out of 26 Brazilian states **(Figure 4)**.

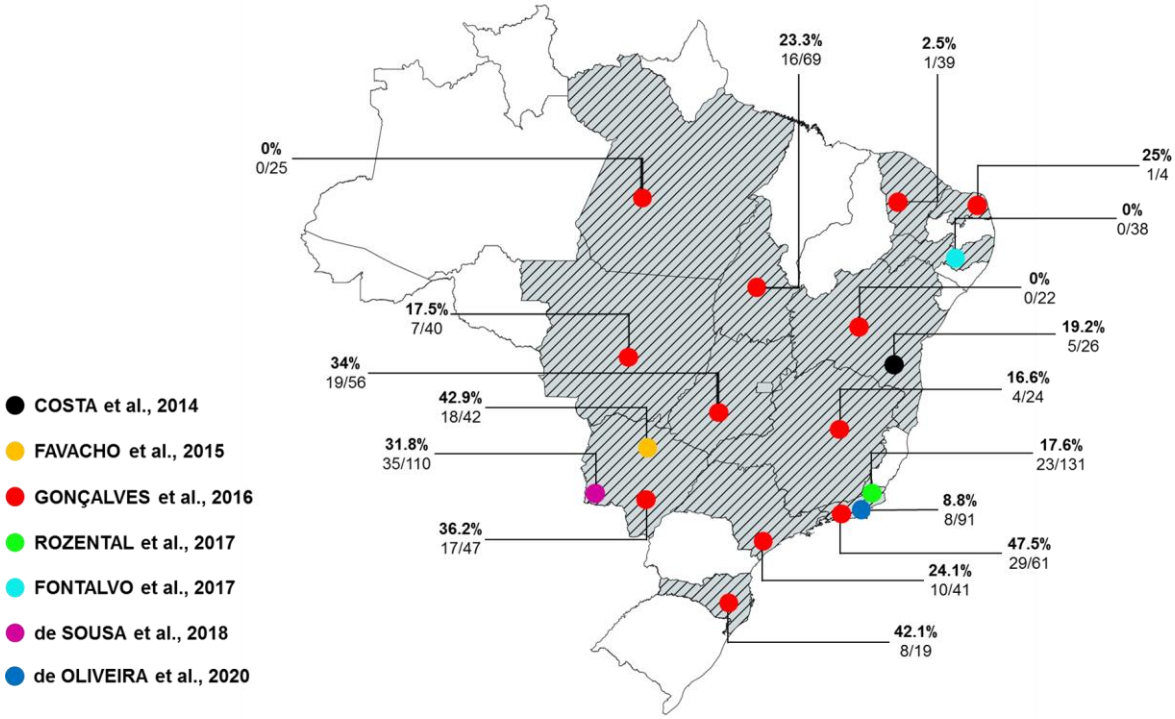

Figure 4. Prevalence and distribution of rodent-associated *Bartonella* species in Brazil.

The first detection of *Bartonella* species in rodents was reported in slum areas of Salvador, Bahia (Costa et al 2015). The authors isolated *Bartonella* spp. in five out of 26 *R. norvegicus* (19.2%). Isolates from four of the five positive synanthropic rats were identified as *B. queenslandensis* and the another one as *B. tribocorum*.

Later, sampling wild rodents captured from Dois Irmãos do Buriti and Sidrolândia municipalities, Mato Grosso do Sul – MS, Favacho et al. (2015) revealed the presence of *Bartonella* DNA similar to *B. vinsonii* subsp. *arupensis* in 42.9% (18/42) of sampled animals. Despite the authors stated that the amplified *gltA* and ITS sequences shared 98% identity with *B. vinsonii* subsp. *arupensis*, the identified sequences, to the best of author's knowledge, have never been submitted to GenBank data, precluding comparative analyses.

In a broad study, Gonçalves et al. (2016) screened *Bartonella* in rodent-spleen DNA samples belonging to 52 species distributed in thirteen Brazilian states. An overall prevalence of 25.6% (117/457) was reported. The molecular analysis based on three molecular markers, demonstrated that the amplified sequences were

phylogenetically related to *B. vinsonni* complex sequences detected in rodents and their fleas in North America.

Among the 131 rodents belonging to 18 species captured in eight municipalities in Rio de Janeiro state, Rozental et al. (2017) amplified *Bartonella* DNA in 23 animals (17.6%). The phylogenetic inference based on the *gltA* gene partial sequences revealed two different bacterial species. Out of 23 amplified sequences, while 22 were closely related to *B. vinsonni* complex sequences, one obtained sequence was phylogenetically positioned near to *B. doshiae*.

In contrast to the above mentioned studies, Fontalvo et al. (2017) reported absence of *Bartonella* sp. DNA in all 38 blood-DNA samples from wild rodents caught in Petrolina and Lagoa Grande cities, Pernambuco. However, among the ticks and lice found parazitying the small mammals, *Bartonella* sp. DNA was amplified in one *Polyplax* sp. louse. Unfortunatelly, the amplicon was not sequenced.

Recently, analyzing 110 wild rodent-spleen DNA samples trapped at the Nhumirim ranch, Corumbá – MS, de Sousa et al. (2018) detected *Bartonella* DNA in 31.8% of animals. After the screening, the positive DNA samples were submitted to cPCR targeting five other protein coding genes. The phylogenetic analysis performed using the *gltA* and *ftsZ* sequences (n = 4) obtained from *Oecomys mamorae*, clustered with *B. vinsonni* complex sequences. On the other hand, the eight *nuoG* and *ftsZ* sequences amplified from *Thrichomys fosteri* were phylogenetically positioned between *B. alsatica* and *B. pachyuromydis*. Also, three (7.8%) *Polygenis bohlsi bohlsi* flea pools showed positivity in the *Bartonella* screening. The *gltA* and *ftsZ* fragments obtained from those fleas (n = 2) were grouped with *B. vinsonni* complex sequences.

Out of 91 wild rodents sampled in Rio de Janeiro state, de Oliveira et al. (2020) reported that eigh DNA samples (8.8%) were positive for *Bartonella* spp. Except the *ftsZ* and *groEL* regions amplified from a *Akodon cursor* that were positoned near to B. rochalimae, all other detected sequences (gltA, ftsZ and groEL) were clustered with *Bartonella vinsonii* complex sequences previoulsly detected in Brazil.

In contrast to a high number of published studies regarding detection and characterization of bartonellae in rodents, few studies have been performed aiming to assess the occurrence of *Bartonella* spp. in marsupials.

Founier et al. (2007) proposed a new *Bartonella* species isolated from five kangaroos (*Macropus giganteus*) from Australia. Despite the authours have suggested *Bartonella australis* as new species based on five genes results, the nomenclatural status has been not validly published.

Analyzing *Ixodes tasmani* (n = 41) and *Ixodes trichosuri* (n = 1) ticks collected from wild koalas (*Phascolarctos cinereus*) at the Koala Convention Centre, Australia, Vilcins et al. (2009) detected *Bartonella* DNA in four out of 19 pooled tick samples. Phylogenetic analyses based on *gltA* gene grouped the amplified sequences with *Bartonella* sp. sequences previously detected in *Ixodes scapularis* ticks from Maryland, USA.

Similar to the above mentioned studies, Kaewmongkol et al. (2011a) reported the identification of a novel *Bartonella* species in fleas (*Acanthopsylla jordani*) and ticks (*Ixodes antechini*) collected from a small carnivorous marsupial (*Antechinus flavipes)* in Australia. After the molecular characterization targeting five different regions, the authors proposed the name '*Candidatus* Bartonella antechini n. sp.' for the recently characterized organism. The phylogenetic analysis of the amplified sequences showed the close relationship between this new *Candidatus* to *Bartonella* species and *B. australis*.

Likewise, Kaewmongkol et al. (2011b), based on PCR assays targeting 16S, *gltA*, *rpoB*, *ftsZ* genes and ITS intergenic region, reported two novel *Candidatus* to *Bartonella* species detected in marsupials. While '*Candidatus* Bartonella woyliei n. sp.' was detected in both fleas (*Pygiopsylla hilli*) and ticks (*Ixodes australiensis*) collected from woylies (*Bettongia penicillata*), '*Candidatus* Bartonella bandicootii n. sp.' was detected in fleas (*Pygiopsylla tunneyi*) collected from western barred bandicoots (*Perameles bougainville)*.

In the USA, cat-related *Bartonella* spp. have been molecularly detected in *Ctenocephalides felis* from North America opossums (*D. virginiana*) (Reeves et al., 2005; Nelder et al., 2009).

In Brazil, analyzing DNA-blood samples from *D. albiventris* (n = 27) and *M. domestica* (n = 10) trapped in Pernambuco state, Northeastern Brazil, Fontalvo et al. (2017) reported absence of *Bartonella* DNA in all analyzed DNA samples. Likewise, all *Amblyomma* spp. (n = 21) and Argasidae larvae (n = 6) ticks and *C. felis* (n = 3) fleas removed from the sampled marsupials showed negative results in a PCR assay based on *gltA* and *ribC* genes.

Among the 30 marsupials (*T. macrurus* [n = 14], *G. agilis* [n = 11), *M. domestica* [n = 4], and *D. albiventris* [n = 1]) specimens trapped in Corumbá, MS, none of them showed positivity in a qPCR assay based on *nuoG* gene. Besides, all 18 *Amblyomma* spp. ticks and five *P. bohlsi* fleas sampled from the marsupials were negative during the *Bartonella* DNA screening (de Sousa et al., 2018).

Lastly, out of 38 marsupials (*Didelphis aurita* [n = 22], *Gracilinanus microtarsus* [n = 1], *Marmosops incanus* [n = 8], *Marmosa paraguayana* [n = 2], *Monodelphis americana* [n = 3] and *Philander frenatus* [n = 2]) captured in Rio de Janeiro state, all DNA samples were negative for *Bartonella* sp. in PCR assay targeting three different genes (*gltA*, *ftsZ* and *groEL*) (de Oliveira e al., 2020).

2.2.6. Epizootiology of *Bartonella* spp. in bovids

Bartonella spp. infection in cattle has been extensively reported across the globe (Chang et al., 2000; Bermond et al., 2002; Bai et al., 2013; Boularias et al., 2020). On the contrary, only one study assessed the occurrence of *Bartonella* in buffaloes (6.8% (7/103) so far (Bai et al., 2013) **(Figure 5)**. Despite the fact that Brazil has one the largest cattle herds in the world (Gilbert et al., 2018), none study has been performed aiming to assess the prevalence of *Bartonella* in bovids in this country to date.

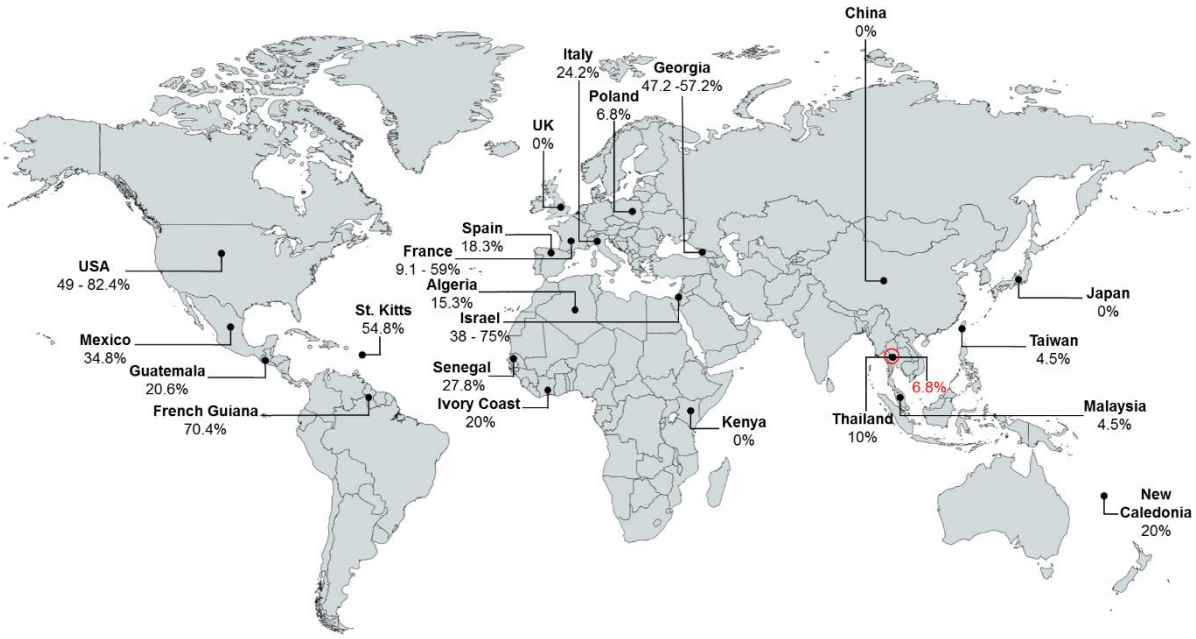

Figure 5. Molecular prevalence of bovid-related *Bartonella* species. The red numbers and circles refer to prevalence rates reported in buffaloes.

Different *Bartonella* species – *B. schoenbuchensis*, *B. bovis* and *Bartonella chomelii* – including "*Candidatus*" to new species and different genotypes have been detected in bovids (Bai et al., 2013; Dahamani et al., 2016; Boularias et al., 2020). *Bartonella bovis* was the most common species identified in these animals (Bai et al., 2013), except in few studies where *B. chomelii* was the most frequent (Antequera-Gomes et al., 2015) or the only species found (Mediannikov et al., 2011).

The global distribution of cattle-related *Bartonella* species has shown a great inconsistency in the prevalence rates across and within geographical sites (Bai et al., 2013; Gutiérrez et al., 2014) **(Figure 5)**. Analyzing cattle from five countries, Bai et al. (2013) reported prevalence rates ranging from 0% (0/221) in Kenya to 57.2% (91/159) in Georgia. Also, the authors reported a wide variation (0% [0/39%] to 90% [18/20]) within the same country. Recently, Boularias et al. (2020) reported *Bartonella* occurrence rates ranging from 2.6% (1/38) to 23% (6/26) among dairy cattle sampled in different areas from Algeria. Since *Bartonella* ssp. are mainly vector-transmitted, it is speculated that the distribution and abundance of specific vectors play a major role

in the *Bartonella* spp. prevalence (Bai et al., 2013). Nonetheless, there is no study showing the vectorial competence of bovid-related *Bartonella* species to date.

Even though the actual role of the blood-sucking arthropods in the bovid-related *Bartonella* spp. transmission is still unknown, *Bartonella* sp. DNA has been amplified in several bovid-associated ectoparasites. For instance, *Bartonella* sp. DNA was detected in 15.7% (40/254) of the *R. microplus* ticks collected from cattle in Taiwan (Tsai et al., 2011). Also, *B. bovis* was detected in eight out of 200 (4%) *H. bispinosa* ticks and none of the 70 *R. microplus* collected from cattle in Peninsular Malaysia (Kho et al., 2015). Recently, *Bartonella* sp. DNA was identified in 19 out of 277 (6.8%) ticks – *Rhipicephalus* (n = 8), *Hyalomma* (n = 8) and *Ixodes* (n = 3) – collected from cattle in Algeria. Of these, *B. bovis* and *B. chomelli* DNA was detected in 17 and two tick samples, respectively (Boularias et al., 2020).

Likewise, *Bartonella* sp. DNA was detected in 84.6% (11/13) of the louse (*Haematopinus quadripertusus*) pool samples collected from cattle in Israel (Gutiérrez et al., 2014). In addition, *Bartonella* sp. DNA closely related to *Bartonella* sequences previously reported in lice from Israel was amplified in 27.3% (3/11) eggs and 22.4% (22/98) *Haematopinus* spp. adult lice collected from cattle in Thailand (Promrangsee et al., 2019).

Additionally, a low *Bartonella* sp. prevalence was reported in *Stomoxys* spp. (6.6% [4/60]) and *Haematobia* spp. (2.2% [1/45]) flies collected from beef cattle from California, USA. *Bartonella bovis* was the bacterial species identified in the stable flies, whereas *B. henselae* was reported in the horn fly (Chung et al., 2004). Besides, all (100% - 12/12) *Hippobosca equina* collected from three cows from France showed positivity based on PCR assay. The amplified fragment was sequenced and the sequence was 100% similar to *B. chomelii* (Halos et al., 2004). Lastly, *B. chomelli* was detected in 78.8% (26/33) of the Hippoboscidae flies collected from cattle in Algeria (Boularias et al., 2020).

In contrast to rodent-associated *Bartonella* spp., the genetic diversity of *Bartonella* spp. in bovids has been poorly assessed. In an extensive study that sampled bovids from Thailand (n = 40), Japan (n = 305), Georgia (n = 159), Guatemala (n = 389) and Kenya (n = 221), Bai et al. (2013) detected three closely related but distinct lineages of *B. bovis* – suggesting a clonal population structure.

Besides, the authors speculated that two *Bartonella* lineages (i.e., I and II) could be associated with the “taurine” (*Bos taurus taurus*) and “zebu” (*Bos taurus indicus*) cattle lineages, respectively. Finally, a third lineage (i.e. III) was correlated with the water buffalo (Bai et al., 2013). Based on MLST results, the authors distinguished 22 sequence types (STs) among the 28 *B. bovis* strains. Also, they showed that *B. bovis* STs might retain geographical particularity (Bai et al., 2013). Similar results were found in Malaysia based on MLST analysis targeting eight loci. Among the nine *B. bovis* isolated from cattle blood samples, six new genotypes were reported (Kho et al., 2015).

Even though *Bartonella* spp. has been recognized to cause endocarditis in humans and animals (Chomel et al., 2009b), cattle infection with *Bartonella* sp. is usually asymptomatic (Welc-Faleciak and Grono, 2013). In a retrospective study aiming to determine the role of *Bartonella* as an endocarditis agent in cattle, tissues and serum samples from 22 animals were analyzed. Among the vegetative valves screened for *Bartonella* DNA, *B. bovis* was reported in two (10%) tissues – aortic and mitral valves – samples. Moreover, using the indirect fluorescent antibody test (IFAT), four additional samples showed antibody titers (>40 and 640<) against *B. bovis, B. chomelii* and/or *B. schoenbuchensis* (Maillard et al., 2007). Furthermore, *B. bovis* was isolated in a cow with endocarditis found dead in the field. The histopathological examination revealed that the aortic valve cusp was expanded by an acidophilic coagulum containing multifocal mineral deposition, bacteria, neutrophils, and red blood cells. Additionally, the adjacent valve cusp was expanded by moderate fibrous connective tissue with multifocal mild plasma cells, lymphocytes, macrophages, and neutrophils in the subendocardium (Erol et al., 2013).

Despite a considerable number of studies performed, mainly those associated with molecular detection of *Bartonella* sp. in cattle, the impact of bartonellosis in livestock, as well as the transmission routes of these bacteria among these animals is poorly understood. Therefore, the elucidation of these intriguing issues should be addressed in future studies.

2.2.7. Human infection with *Bartonella* species

Even though several *Bartonella* species have been linked to human infection **(Table 1)**, the most common species involved in human diseases are *B. bacilliformis*, *B. quintana*, and *B. henselae*, which are the etiological agents of Carrion's disease, trench fever and cat-scratch disease (CSD), respectively (Breitschwerdt, 2017). These infections may cause a variety of clinical manifestations, ranging from mild symptoms (fever, headache, and malaise) to more serious disease expressions (hallucinations and acute hemolytic anemia) (Minnick et al., 2014; Cheslock and Embers, 2019). In addition to the above-mentioned disorders, human diseases caused by *Bartonella* spp. infections may include endocarditis, myocarditis, bacilliary angiomatosis, hepatic peliosis, vasculitis, uveitis, retinitis, erythema, urticaria, neuritis, chronic lymphadenopathy, weight loss, muscle fatigue, partial paralysis and aneurysm (Breitschwerdt, 2017; Cheslock and Embers, 2019; Lins et al., 2019).

In contrast to *B. bacilliformis* and *B. quintana* species, which have humans as the main hosts, domestic cats are the main reservoirs for *B. henselae* (Chomel and Kasten, 2010). Additionally, *B. henselae* appears to be the most medically important *Bartonella* zoonotic species involved in human infection (Breitschwerdt, 2017).

Besides, different rodent-associated *Bartonella* species have been also implicated as the causative agents of human diseases **(Table 2)**. Indeed, rodents are widely dispersed around the globe and some rodent species are well adapted to human dwellings. These characteristics reveal the need for control measures and epidemiological studies related to rodents as well as their ectoparasites aiming to prevent or decrease the risk of human exposure to *Bartonella* sp. infection.

Table 2. Clinical manifestations in human infections potentially associated with rodent-related *Bartonella* species

***Bartonella* species**	**Clinical manifestations**	**Reference**
B. elizabethae	Endocarditis	(Daly et al., 1993)
B. vinsonii subsp. *arupensis*	Fever, endocarditis and neurologic disorders	(Welch et al., 1999) and (Fenollar et al., 2005)
B. grahamii	Intraocular neuroretinitis and lymphadenopathy	(Kerkhoff et al., 1999) and (Oksi et al., 2013)
*B. washoensis**	Fever, myocarditis and meningitis	(Kosoy et al., 2003) and (Probert et al., 2009)
*B. tamiae**	Fever	(Kosoy et al., 2013)
Bartonella sp. close related to *B. elizabethae* complex	Fever	(Kosoy et al., 2013)
B. tribocorum	Fatigue, muscle pain and headache	(Vayssier-Taussat et al., 2016)
B. doshiae	Fatigue, blurred vision and arthralgia	(Vayssier-Taussat et al., 2016)
B. kosoyi	Fever, lymphadenopathy, weakness and malaise	(Kandelaki et al., 2016)

* These *Bartonella* species are not listed in LPSN

Bat-related *Bartonella* species have been also associated with human infection. Lin et al. (2010) isolated *Bartonella* sp. from aortic valve tissues of a 59-year-old man from the USA. The man was initially hospitalized showing progressive shortness of breath, weight loss, fatigue, and altered mental status. The physical examination and the echocardiogram showed cardiac disorders. After *Bartonella* sp. isolation from aortic valve tissue and molecular characterization targeting five genes, the authors proposed the name '*Candidatus* Bartonella mayotimonensis' for the new isolate. Although the reservoir hosts in nature also remained elusive, the above described *Bartonella* species was later reported in bats from Finland (Veikkolainen et al., 2014).

In a study aiming to investigate whether *Bartonella* spp. circulating in bats and associated flies also occurred in human beings from the same areas, Bai et al.

(2018) sampled 50 bat-flies (*Eucampsipoda africana*), 177 bats (*Rousettus aegyptiacus*) and 204 individuals from Nigeria. Based on the MLST of the *Bartonella* strain found in bats, the authors proposed a new species named *Bartonella rousetti*. Also, antibodies against *B. rousetti* were detected in 8 (3.9%) patient serum samples without cross-reactivity to other *Bartonella* species, suggesting that bat-associated *Bartonella* strains might be capable of infecting humans (Bai et al., 2018). It's interesting to point out that five out of the eight seropositive humans had a history of contact with bats, either by catching or eating them.

Bartonella schoenbuchensis, a ruminant-associated bartonellae*,* was isolated from a patient showing fatigue, muscle pain and fever after a tick bite. The French patient exhibited high bacteremia – 850 CFU/mL. It was the first report of human infection with cattle-associated *Bartonella* (Vayssier-Taussat et al., 2016).

In Brazil, the most commom *Bartonella* species found in humans are *B. henselae* and *B. quintana* (Magalhães et al., 2010; Favacho et al., 2010; Siciliano et al., 2015).

During a serosurvey for arthropod-borne bacteria, serum samples of 437 healthy people from a rural community in Minas Gerais state, Brazil, were analyzed. The authors reported antibodies against *B. henselae* and *B. quintana* in 13.7% (n = 60) and 12.8% (n = 56) of the samples, respectively (Costa et al., 2005).

Lamas et al. (2010) reported 38.4% seroprevalence rate to *B. henselae* in among 125 HIV patients in Rio de Janeiro state.

Analyzing 500 blood donors at Campinas, São Paulo state, Brazil, antibodies against *B. quintana* and *B. henselae* were detected in 32.0% and 16.2% of the donors, respectively. Besides, the authors detected *B. henselae* (3%) and *B. clarridgeiae* (0.2%) DNA in the blood samples subjected to liquid culture (Pitassi et al., 2015). Later, aiming to determine the risk factors associated with *Bartonella* species infection in the same blood donors, the authors performed a multivariate logistic regression. The results revealed that infection with *B. henselae* or *B. clarridgeiae* was associated with cat contact or history of tick bite (Diniz et al., 2016).

These findings reinforce the need for studies on epidemiological surveillance targeting *Bartonella* spp. in domestic, wild and synanthropic animal populations.

These studies may shed light on *Bartonella* species distribution and assist the authorities in developing control measures related to emerging diseases.

3. GENERAL OBJECTIVES

The current study aimed to assess the occurrence and genetic diversity of hemoplasmas and *Bartonella* species in small mammals, large ruminants and associated ectoparasites.

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CHAPTER 2 – Genetic diversity, and lack of molecular evidence for hemoplasma cross-species transmission between wild and synanthropic mammals from Central-Western Brazil[1]

Abstract

Globally, hemotropic mycoplasmas (hemoplasmas) comprise an emerging or remerging bacteria group that attaches to red blood cells of several mammal's species and in some cases, causing hemolytic anemia. Herein, we assessed the occurrence, genetic diversity, the factors coupled to mammals infection, and the phylogeographic distribution of hemoplasmas in sylvatic and synanthropic mammals and their associated ectoparasites from Brazil. We collected spleen and/or blood samples from synanthropic rodents (*Rattus rattus* [N=39] and *Mus musculus* [N=9]), sylvatic rodents (*Hydrochoerus hydrochaeris* [N=14]) and opossums (*Didelphis albiventris* [N=43]). In addition, ticks (*Amblyomma* spp. [N=270] and lice (*Polyplax spinulosa* [N=6]) specimens were also sampled. Using a PCR targeting the 16S rRNA region, out of 48 small rodents, 14 capybaras and 43 opossums DNA samples, hemoplasma DNA was found in 25%, 50%, and 32.5% animals, respectively. Besides, we reported hemoplasma DNA in *Amblyomma* sp. (22.2% [2/9]) and lice (100% [2/2]) pools samples from rats, and one female *A. sculptum* DNA sample (3% [1/33]) obtained from a capybara. Additionally, and in agreement with ML analysis, the network analyses showed a clear phylogenetic separation among the hemoplasmas genotypes found in the different host species sampled, thus, suggesting the absence of cross-species hemoplasmas transmission between the mammals trapped. Finally, using the TCS network analysis, we reported the same 16S rRNA *Mycoplasma* genotype circulating in *Rattus* sampled in Brazil, Hungary, and Japan.

Keywords: capybaras, hemotropic mycoplasmas, lice, opossum, phylogenetic analysis, *Rattus rattus* and ticks

1. Introduction

Interactions among sympatric vertebrate hosts, vectors and pathogens shape infectious diseases occurrence, infection load, the timing of outbreaks and invasiveness of pathogens (Hoberg and Brooks, 2015; Cohen et al., 2018). Recently, the occurrence of arthropod-borne diseases in humans and animals have increased due to distinct factors, such as climate change (higher temperatures increased pathogen propagation and disease incidence), affecting the species behavior and

[1] Este capítulo corresponde ao artigo publicado na revista Acta Tropica: 105303, 2020

immunity; urbanization (causing biotic homogenization); deforestation and natural environment encroachment (predisposing a higher contact among wildlife, humans and domestic animals); and globalization (e.g. wildlife and livestock trades). As a consequence, these factors may affect the transmission dynamics of pathogens, the emergence and reemergence of infectious diseases and the spillover of humans and domestic animals' pathogens to wildlife and vice-versa (Chomel et al., 2007; Dar and Reshi, 2014; Otranto et al., 2015; Price et al., 2019).

Rodentia and Didelphimorphia comprise two important mammal groups, playing an crucial role in the maintenance of the ecological balance. Whereas rodents (order Rodentia) are widely distributed in different habitats and represent the largest order of mammals (over 2200 species), the family Didelphidae (order Didelphimorphia) is found only throughout the nearctic and neotropical zones and comprises 98 species (Wilson and Reeder, 2005; IUCN – https://www.iucnredlist.org – Accessed on August, 2019).

Among the different ecological roles, these animals are important reservoirs for many pathogens (e.g. *Trypanosoma* spp., *Leishmania* spp., *Rickettsia* spp., *Anaplasma phagocytophilum*), and play a crucial role to immature tick stages development (Labruna, 2009; Stuen et al., 2013; Roque et al., 2014; Rodrigues et a., 2019).

In the current scenario, the hemotropic mycoplasmas (also known as hemoplasmas) emerge as pathogens that could impact on humans and animals' health (Maggi et al., 2013a). The hemoplasmas are uncultivated cell wall-less bacteria that attach to red blood cells surface of a wide range of animals. Besides, the hemoplasmas are mainly transmitted via direct contact with blood - e.g., aggressiveness among animals -, and produce a primary acute infection that is followed by a persistent latent infection (Neimark et al., 2005; Cohen et al., 2018). Once the host is infected, hemoplasmas could induce acute hemolysis, and the disease is characterized by anorexia, lethargy, dehydration, weight loss, and occasionally death (Willi et al., 2007).

In Brazil, hemoplasmas DNA has been detected in domestic animals, such as cats (Miceli et al., 2013; André et al., 2014), dogs (Ramos et al., 2010; Valle et al., 2014), goats (Machado et al., 2017), cattle (Girotto et al., 2012; de Mello et al.,

2019) and sheep (Souza et al., 2019). Concerning the wildlife, these agents have been molecularly detected in free-ranging and captive wild felids (Willi et al., 2007; André et al., 2011; de Souza et al., 2017; Furtado et al., 2019), wild and captive canids (André et al., 2011; de Souza et al., 2017), bats (Ikeda et al., 2017), non-human primates (Bonato et al., 2015; de Melo et al., 2019), wild and synanthropic rodents (Vieira et al., 2009; Gonçalves et al., 2015), procyonids (de Souza et al., 2017; Cubilla et al., 2017), deer (Grazziotin et al., 2011), wild boars (Dias et al., 2019) and opossums (Massini et al., 2019). On the other hand, hemoplasmas DNA has not been detected in ectoparasites in Brazil so far (de Souza et al., 2017).

Although extensive surveys have been conducted in Brazil, several biological aspects of hemoplasmas epidemiology remain poorly assessed. Thus, the elucidation of these bacterial cycles in nature, including the genetic diversity, identification of hosts, vectors and the species distribution in a particular ecotope shows great importance.

Thereby, the present study aimed to investigate the occurrence and the genetic diversity of hemoplasmas infecting marsupials, wild and synanthropic rodents and associated ectoparasites in urban areas and forest fragments in Central-Western Brazil.

2. Materials and Methods

2.1. Study sites, mammal trapping and sample collection

Between May 2017 and August 2018, 105 mammals belonging to four species were sampled in different sites of Campo Grande municipality (-20º 42’ 30” S, -54º 61’ 60” W), state of Mato Grosso de Sul, Central-Western Brazil **(Table 1).**

Table 1. Number and animal species postitive to hemoplasmas targeting the 16S rRNA

Animal species	Sample type	Nº of sampled animals	Occurrence of hemoplasma % (Nº)	Ectoparasite species	Nº of sampled ectoparasites	Occurrence of hemoplasma % (Nº)
Mammals				**Arthropds**		
Rodentia				**Ixodida/Phthiraptera***		
R. rattus	DNA from spleen tissues	39	30.7% (12/39)	*Amblyomma* sp.[a]	62	22.2% (2/9) [b]
				*P. spinulosa**	6	100% (2/2) [b]
M. musculus	DNA from spleen tissues	9	0% (0/9)	-	-	-
H. hydrochaeris	DNA from whole blood	14	50% (7/14)	*A. dubitatum*	42	0% (0/42)
				A. sculptum	36	3.3% (1/33) [c]
				Amblyomma sp.[a]	2	0% (0/2)
Didelphimorphia						
D. albiventris	DNA from whole blood	43	32.5% (14/43)	*A. dubitatum*	70	0% (0/28) [b]

[a] *Amblyomma* sp. refers to larvae sampled – In these specimens only the genus was reported

[b] Ectoparasites-DNA pool samples

[c] Three out of 36 *Amblyomma*-DNA samples were negative to the endogenous control (16S rRNA)

Forty-eight small rodents were trapped in urban areas (four sites) and urban forest fragments (four sites). Additionally, fourteen capybaras (*Hydrochoerus hydrochaeris*) – the largest rodent in South America – were trapped in three urban forest fragments. Lastly, 43 marsupials were sampled in six urban forest fragments. The small mammals were caught using Tomahawk and Sherman live traps baited with a mix of bananas, *paçoca*, oat flakes and tinned sardines. Once captured, the small rodents were chemically immobilized using a combination of ketamine hydrochloride (100 mg/mL) and acepromazine (10 mg/mL) (1:9) intramuscularly. After that, spleen fragments were collected under sterile conditions, placed into DNase and RNase-free microtubes containing etanol (100%), and maintained at -20°C until DNA extraction. On the other hand, the marsupials (*Didelphis albiventris*) were anesthetized with a chemical association of Ketamine (20 mg/kg) and Xylazine (2 mg/kg) intramuscularly. Blood samples were collected from the marsupials' lateral caudal veins, placed to DNase and RNase-free anticoagulant ethylenediaminetetraacetic acid (EDTA)-contaning microtubes, and maintained at -20°C until DNA extraction. Finally, the capybaras were initially immobilized using an anesthetic dart containing TELAZOL® 100 (4mg/kg). Subsequently, approximately 2-5 mL of blood was collected from the femoral vein into EDTA-buffered vacutainer tubes. The samples were kept on ice until arrival in the laboratory and stored at -20°C until DNA extraction. The following data were recorded from each animal: the presence of ectoparasites, gender, weight, and sampling point.

All animals were checked for the presence of ectoparasites. Once collected, the ectoparasites were placed in microtubes containing absolute etanol (Merck) and maintained at -20°C until morphological identification and DNA extraction. The morphological identification was performed using previously described taxonomic keys (Onofrio et al., 2005; Martins et al., 2010).

All animal captures were in accordance with the licenses obtained from the Instituto Chico Mendes de Conservação da Biodiversidade (license number 56912-2), Imasul (license number 05/2017) and endorsed by the Ethics Committee of FCAV/UNESP University under the number:01952/18.

2.2. DNA extraction and molecular detection of hemoplasmas

DNA was extracted from 10 mg of each small rodent spleen tissue and 200 µL of blood samples from capybaras and marsupials, using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, California, USA), according to manufacturer's instructions. Furthermore, the collected ectoparasites were submitted to DNA extraction individually and/or in pools (the tick nymphs were pooled up to 3 individuals and the larvae up to 7 individuals from the same host - the lice were pooled up to 2 specimens from the same host), using the commercial kit above mentioned.

In order to discard the presence of PCR inhibitors, all extracted mammal DNA samples were used as a template in an internal control PCR targeting the mammal *gapdh* gene as previously described (Birkenheuer et al., 2003). Likewise, all arthropod DNA samples were submitted to internal control targeting the 16S rRNA as previously described (Black and Piesman, 1994). Internal control-PCR positive samples were subsequently submitted to a broad-range hemoplasma PCR assay targeting the 16S rRNA gene.

Previously described PCR protocols were utilized to amplify *Mycoplasma* spp. 16S rRNA gene, using two sets of primers, namely HemMycop16S-41s (5'-GYATGCMTAAYACATGCAAGTCGARCG-3') and HemMyco16S-938as (5'-CTCCACCACTTGTTCAGGTCCCCGTC-3') (fragment of 800 bp), and HemMycop16S- 322s (5'-GCCCATATTCCTACGGGAAGCAGCAGT-3') and HemMycop16S-1420as (5'-GTTTGACGGGCGGTGTGTACAAGACC-3') (fragment of 800 bp) as previously described (Maggi et al., 2013a). Sequences derived from each amplicon obtained from each primer set (with an overlap of 600 bp) were used to build a larger consensus sequence (approximately 1200 bp). '*Candidatus* Mycoplasma haemobos' DNA (MF992084) obtained from a naturally infected buffalo (Gonçalves et al., 2018) and ultra-pure sterile water were used as positive and negative controls, respectively.

2.3. Phylogenetic analysis

The amplicons obtained from 16S rRNA-based PCR assays were purified using the EXOSAP-IT® (Applied Biosystems). Purified amplified DNA fragments were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser – Applied Byosystem/ Perkin Elmer). Consensus sequences were

obtained through the analysis of electropherograms using the Phred-Phrap program (Ewing et al., 1998). The Phred quality score (peaks around each base call) was established at ≥20 (99% in accuracy of the base call). Hemoplasma-16S rRNA sequences were identified by BLASTn using the Megablast (following default parameters), aligned with sequences available in GenBank using Clustal/W (Thompson et al., 1994), and adjusted in Bioedit v. 7.0.5.3 (Hall, 1999). The phylogenetic analysis was performed using Maximum Likelihood (ML) method, inferred with RAxML-HPC BlackBox (7.6.3.) (Stamatakis et al., 2008) and performed in CIPRES Science Gateway (Miller et al., 2010). The Akaike Information Criterion (AIC) available on MEGA v. 5 software (Tamura et al., 2011) was applied to identify the most appropriate model of nucleotide substitution. GTR+ G + I model was chosen as the most appropriate for the phylogenetic analysis of the 16S rRNA alignment.

2.4. Identification and genetic relationship of identified hemoplasmas genotypes

The 16S rRNA aligned sequences amplified in the present study were utilized to identify the genotypes using the DnaSP v5.10 (Librado and Rozas, 2009). To investigate the genetic relationship among hemoplasmas genotypes detected in the present study and those previously detected in rodents and marsupials retrieved from GenBank, a Neighbor-Net network was constructed, using the pairwise genetic distances with SplitsTree v4.14.6 (Huson and Bryant, 2006). Additionally, the different genotypes identified were submitted to TCS network inferred using the Population Analysis with Reticulate Trees (popART v. 1.7) (Leigh and Bryant,2015).

3. Results

3.1. Ectoparasites and hemoplasma occurrence and BLAST analysis

Ectoparasites were found in 10.4% (5/48) of small rodents. Also, 71.4% (10/14) of trapped capybaras were infested by ticks. Lastly, ticks were observed in 32.5% (14/43) of the sampled opossums. All ectoparasites species sampled are shown in **(Table 1)**.

Except for three tick-DNA samples obtained from capybaras, all arthropod and mammal DNA samples were positive to 16S rRNA and *gapdh* endogenous control

PCR assays, respectively. The tick samples negative in arthropod-16S rRNA PCR assay were excluded from subsequent analyses.

Out of 48 small rodents, 14 capybaras and 43 opossums DNA samples, hemoplasma DNA was found in 25%, 50% and 32.5% animals, respectively. Besides, 16S rRNA hemoplasma was detected in 22.2% (2/9) of the *Amblyomma* sp. larvae and 100% (2/2) of the *Polyplax spinulosa* DNA samples collected from *R. rattus*. Additionally, one female *Amblyomma sculptum* DNA sample (3% [1/33]) obtained from one capybara was positive to hemoplasma. Nonetheless, all 28 *Amblyomma dubitatum* nymphs DNA samples were negative to *Mycoplasma* spp. **(Table 1)**.

The positive samples obtained in hemoplasmas-PCR showing high intensity amplicons in agarose gel electrophoresis were selected and submitted to sequencing. The BLASTn analysis showed that hemoplasmas-16S rRNA sequences detected in rodents and their associated ectoparasites (N=5) shared percentage of identity ranging from 99.5% to 100% with other murine hemoplasmas detected in Brazil (KT215635, KT215639, KT215642) and Japan (AB758439). In addition, the sequences detected in capybaras (N=3) were identical (100% of identity) to a *Mycoplasma* sp. sequence (FJ667773) previously reported in a capybara from Brazil. Finally, the sequences identified in opossums (N=5) were identical to *Mycoplasma* sp. sequence (MH158514) detected in an opossum (*D. albiventris*) from Brazil and shared 98.8% of identity with '*Candidatus* Mycoplasma haemodidelphis' (AF178676) detected in a marsupial (*Didelphis virginiana*) in the USA. All sequences amplified in the present study showed query coverage of 100%. The amplified 16S rRNA sequences were deposited in Genbank under accession numbers (MN423253-MN423265).

3.2. Phylogenetic and genotype analyses

In accordance with BLAST analysis, one sequence detected in *R. rattus* (MN423261) and another sequence amplified in *P. spinulosa* (MN423265) clustered together with '*Candidatus* M. haemomuris' sequences (KM258432, KT215635, AB758435) previously detected in synanthropic rodents (*R. novergicus* and *R. rattus*) from Brazil and Japan. Additionally, other two sequences detected in *R. rattus*

(MN423262 and MN423263) and another one identified in *Amblyomma* sp. larvae (MN423264) collected from *R. rattus* were positioned together with other sequences detected in synanthropic rodents from Brazil (KT215639), Japan (AB752303) and Hungary (KJ739312) **(Figure 1)**.

Regarding the sequences identified in capybaras (MN423253-MN423255), three sequences grouped with hemoplasma sequences (FJ667773 and FJ667774) previously detected in capybaras in Southern Brazil. Besides, these sequences closely positioned to a hemoplasma sequence (KY002651) detected in *Nasua nasua* trapped in Central-Western Brazil, albeit remaining in a separate branch **(Figure 1)**.

Regarding the hemoplasma sequences identified in opossums, five sequences (MN423256-MN423260) were phylogenetically related to *Mycoplasma* sp. sequences (MH158514 and MH158515) previously detected in *D. albiventris* in Southern Brazil, and to '*Candidatus* M. haemodidelphis' (AF178676), previously detected in *D. virginiana* from the USA. Interestingly, this cluster was closely related to *Mycoplasma* sp. sequences (KC920441 and KC920448) detected in *Procyon lotor* from the USA **(Figure 1)**. All clusters reported in the current study were supported by high bootstrap values ranging from 80% to 100%.

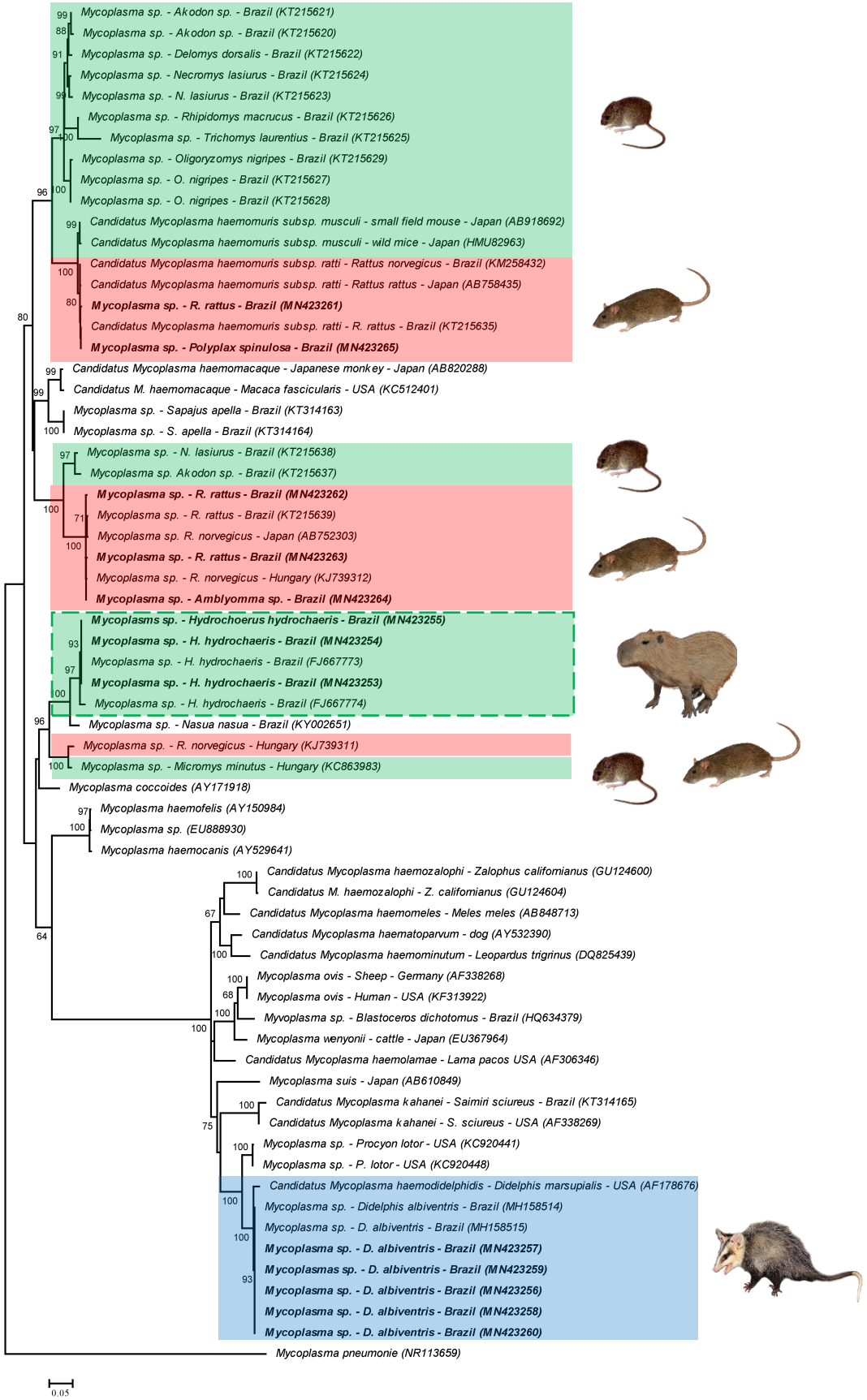
Mycoplasma sp. - Akodon sp. - Brazil (KT215621)
Mycoplasma sp. - Akodon sp. - Brazil (KT215620)
Mycoplasma sp. - Delomys dorsalis - Brazil (KT215622)
Mycoplasma sp. - Necromys lasiurus - Brazil (KT215624)
Mycoplasma sp. - N. lasiurus - Brazil (KT215623)
Mycoplasma sp. - Rhipidomys macrucus - Brazil (KT215626)
Mycoplasma sp. - Trichomys laurentius - Brazil (KT215625)
Mycoplasma sp. - Oligoryzomys nigripes - Brazil (KT215629)
Mycoplasma sp. - O. nigripes - Brazil (KT215627)
Mycoplasma sp. - O. nigripes - Brazil (KT215628)
Candidatus Mycoplasma haemomuris subsp. musculi - small field mouse - Japan (AB918692)
Candidatus Mycoplasma haemomuris subsp. musculi - wild mice - Japan (HMU82963)
Candidatus Mycoplasma haemomuris subsp. ratti - Rattus norvegicus - Brazil (KM258432)
Candidatus Mycoplasma haemomuris subsp. ratti - Rattus rattus - Japan (AB758435)
Mycoplasma sp. - R. rattus - Brazil (MN423261)
Candidatus Mycoplasma haemomuris subsp. ratti - R. rattus - Brazil (KT215635)
Mycoplasma sp. - Polyplax spinulosa - Brazil (MN423265)
Candidatus Mycoplasma haemomacaque - Japanese monkey - Japan (AB820288)
Candidatus M. haemomacaque - Macaca fascicularis - USA (KC512401)
Mycoplasma sp. - Sapajus apella - Brazil (KT314163)
Mycoplasma sp. - S. apella - Brazil (KT314164)
Mycoplasma sp. - N. lasiurus - Brazil (KT215638)
Mycoplasma sp. Akodon sp. - Brazil (KT215637)
Mycoplasma sp. - R. rattus - Brazil (MN423262)
Mycoplasma sp. - R. rattus - Brazil (KT215639)
Mycoplasma sp. R. norvegicus - Japan (AB752303)
Mycoplasma sp. - R. rattus - Brazil (MN423263)
Mycoplasma sp. - R. norvegicus - Hungary (KJ739312)
Mycoplasma sp. - Amblyomma sp. - Brazil (MN423264)
Mycoplasms sp. - Hydrochoerus hydrochaeris - Brazil (MN423255)
Mycoplasma sp. - H. hydrochaeris - Brazil (MN423254)
Mycoplasma sp. - H. hydrochaeris - Brazil (FJ667773)
Mycoplasma sp. - H. hydrochaeris - Brazil (MN423253)
Mycoplasma sp. - H. hydrochaeris - Brazil (FJ667774)
Mycoplasma sp. - Nasua nasua - Brazil (KY002651)
Mycoplasma sp. - R. norvegicus - Hungary (KJ739311)
Mycoplasma sp. - Micromys minutus - Hungary (KC863983)
Mycoplasma coccoides (AY171918)
Mycoplasma haemofelis (AY150984)
Mycoplasma sp. (EU888930)
Mycoplasma haemocanis (AY529641)
Candidatus Mycoplasma haemozalophi - Zalophus californianus (GU124600)
Candidatus M. haemozalophi - Z. californianus (GU124604)
Candidatus Mycoplasma haemomeles - Meles meles (AB848713)
Candidatus Mycoplasma haematoparvum - dog (AY532390)
Candidatus Mycoplasma haemominutum - Leopardus trigrinus (DQ825439)
Mycoplasma ovis - Sheep - Germany (AF338268)
Mycoplasma ovis - Human - USA (KF313922)
Myvoplasma sp. - Blastoceros dichotomus - Brazil (HQ634379)
Mycoplasma wenyonii - cattle - Japan (EU367964)
Candidatus Mycoplasma haemolamae - Lama pacos USA (AF306346)
Mycoplasma suis - Japan (AB610849)
Candidatus Mycoplasma kahanei - Saimiri sciureus - Brazil (KT314165)
Candidatus Mycoplasma kahanei - S. sciureus - USA (AF338269)
Mycoplasma sp. - Procyon lotor - USA (KC920441)
Mycoplasma sp. - P. lotor - USA (KC920448)
Candidatus Mycoplasma haemodidelphidis - Didelphis marsupialis - USA (AF178676)
Mycoplasma sp. - Didelphis albiventris - Brazil (MH158514)
Mycoplasma sp. - D. albiventris - Brazil (MH158515)
Mycoplasma sp. - D. albiventris - Brazil (MN423257)
Mycoplasmas sp. - D. albiventris - Brazil (MN423259)
Mycoplasma sp. - D. albiventris - Brazil (MN423256)
Mycoplasma sp. - D. albiventris - Brazil (MN423258)
Mycoplasma sp. - D. albiventris - Brazil (MN423260)
Mycoplasma pneumonie (NR113659)
0.05

Figure 1. Phylogenetic relationships within the *Mycoplasma* genus based on a fragment of 1500 bp 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 1000 replicates. *Mycoplasma pneumoniae* was used as outgroup.

In agreement with ML analysis, the Neighbor-Net network analysis showed a clear phylogenetic separation among the hemoplasmas found in the different host species sampled in the present study and highlighted the richness of *Mycoplasma* species infecting rodents. In addition, we did not find rodent-associated – including those reported in capybaras – genotypes circulating in opossums and vice-versa **(Figure 2)**. Likewise, the TCS network analysis, performed using the sequences amplified in the current study and others previously detected in rodents and opossums retrieved from GenBank data base, showed the presence of 21 different genotypes **(Figure 3)**. The capybaras sequences detected in the present study together with a sequence previously detected in a capybara (FJ667773) from Brazil were classified as genotype #1. Also, the opossum sequences were grouped in the genotype #2, including the sequences previously detected in an opossum (MH158514 and MH158515) from Brazil. In addition, the '*Candidatus* M. haemodidelphis' sequence was herein classified as genotype #19. Lastly, the synanthropic rodent-related sequences amplified in the current study were grouped as genotypes #3 and #4. The genotype #3 consisted of five sequences, one detected in *R. rattus* and another detected in *Amblyomma* sp. larvae. Other three sequences were previously reported in *Rattus* spp. from Brazil and Japan. Among the eight sequences comprising the genotype #4, five were previously reported in *Rattus* spp. trapped in Brazil, Japan and Hungary. Other three sequences were detected in *R. rattus* and *P. spinulosa* in the present study **(Figure 3)**.

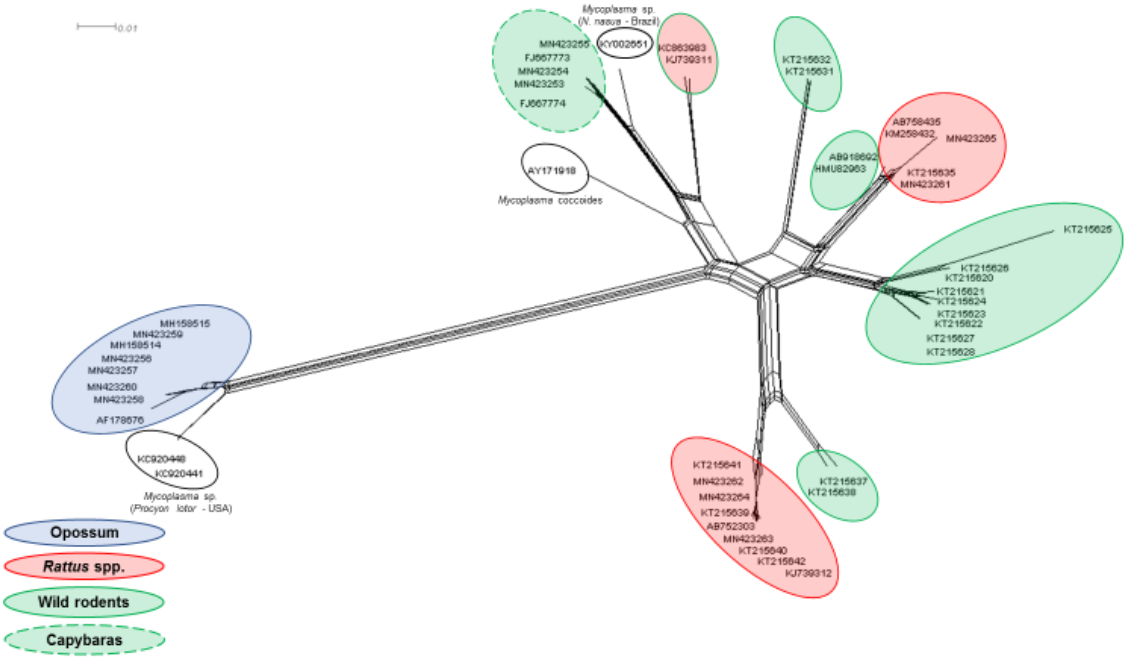

Figure 2. Neighbor-Net analysis of 16S rRNA sequences obtained from wild and synanthropic rodents, opossum sampled in the present study and compared to related hemoplasmas sequences previously deposited in GenBank.

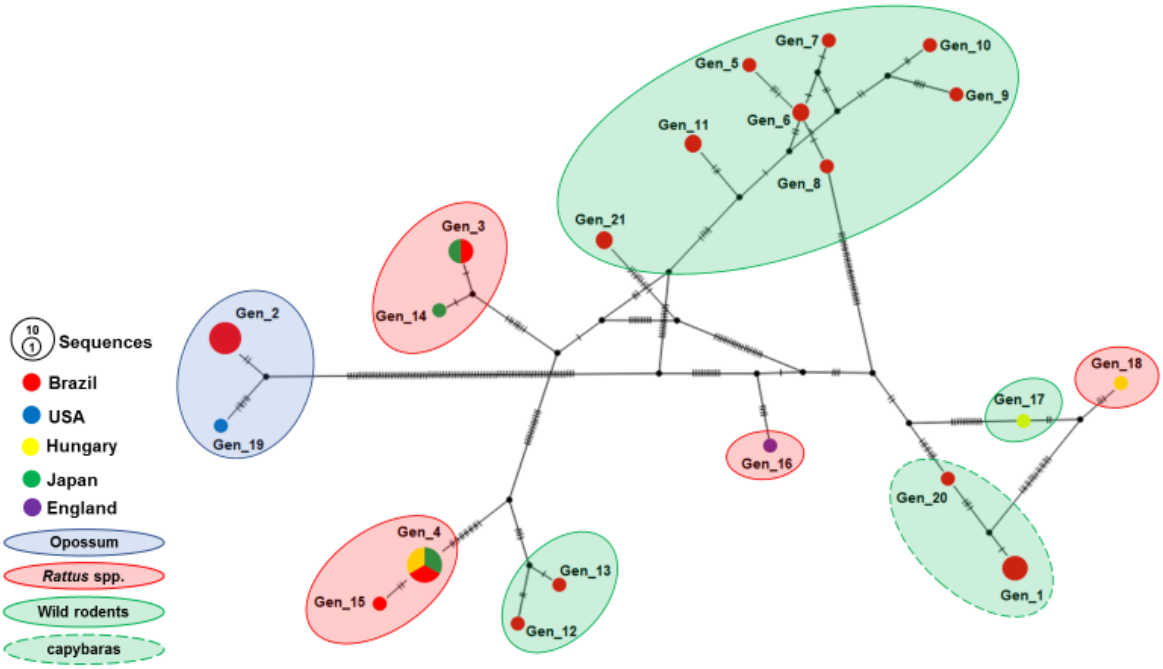

Figure 3. TCS network analysis of 16S rRNA genotypes detected in wild and synanthropic rodents, opossum and related ectoparasites

4. Discussion

In the present study, we reported the molecular occurrence and genetic diversity of hemoplasmas in rodents, marsupials and associated ectoparasites in Central-Western Brazil. Additionally, using a phylogeographic approach, the specificity of hemoplasma genotypes among different groups of mammals was assessed.

The prevalence of hemotropic mycoplasmas among different mammal species sampled around the world is widely variable, ranging between 0% to 78.8% (Willi et al., 2009; Vieira et al., 2015; de Souza et al., 2017; Volokhov et al., 2017; Millán et al., 2018; Souza et al., 2019). Herein, the occurrence of hemoplasmas was 25%, 50% and 32.5% among the small rodents, capybaras and opossuns samples, respectively. The difference observed in the occurrence of hemoplasmas has been assigned to distinct biological factors, such as group of analyzed animals (e,g., free-ranging vs captive animals) (Vieira et al., 2009), habitat types (e.g. undisturbed sites vs disturbed sites) (Volokhov et al., 2017), gregarious behavior of the hosts (de Souza et al., 2017), and age (Persichetti et al., 2018). Interestingly, we found highest hemoplasma occurrence in rodents trapped in forest fragments. These results are congruent to those previously reported in raccoons from Georgia, USA (Volokhov et al., 2017). The highest occurrence of hemoplasma infection in rodents trapped in forest fragments could be partially attributed to aggressive interactions among these animals due to lower food availability when compared to urban areas – since the main route of rodent-associated hemoplasma transmission seems to be direct (Cohen et al., 2018). On the other hand, a higher availability of food in urban areas may have led to higher tolerance by rodents, reducing the aggressive interaction and hence, the hemoplasma transmission through infected saliva or blood. Also, the higher availability of food might improve the immune response against hemoplasma infection (Becker et al., 2014). However, the low number of animals sampled in the present study prevent an accurate statistical analysis and, therefore any speculation about it should be treated with caution. Additionally, considering that the hemoplasma occurrence could be attributed to distinct biological factors, further studies aiming at determining the biological parameters (e.g., host interactions and

density, presence and richness of vectors species) that may play a role in hemoplasmas prevalence among different mammals are needed.

To the best of authors' knowledge, this was the first molecular detection of hemoplasmas in ticks and lice from Brazil. Although the vector competence of *P. spinulosa* and *P. serrata* to *M. coccoides* have been determined (Berkenkamp and Wescott, 1988), a recent study (Cohen et al., 2018) suggested that *M. haemomuris*-like is mainly transmitted by rodent-rodent contact. Additionally, the authors did not find evidence of horizontal transmission of hemoplasmas by fleas (*Synosternus cleopatrae*). Therefore, while the real role of ticks in the hemoplasma transmission cycles remains poorly assessed, the PCR positive results found in ectoparasites should be analyzed carefully, since it may represent reminiscent DNA from mammal blood samples. Indeed, the tick vector competence for hemoplasmas should be conducted in future studies.

The ML analysis corroborated the results obtained by BLASTn, since the *Rattus*-associated hemoplasma sequences grouped with other synanthropic rodents-associated *Mycoplasma* sequences previously detected (Gonçalves et al., 2015; Harasawa et al., 2015). Previously, studies proposed the split of *M. haemomuris* into two phylogenetic closely related subspecies based on different targets, such as 16S rRNA, ITS and *rnpB* (Sashida et al., 2013; Harasawa et al., 2015). Additionally, and in agreement with previous studies (Gonçalves et al., 2015; Sashida et al., 2013; Hornok et al., 2015), the ML analysis showed the occurrence of another hemoplasma species, which was strongly supported (bootstrap of 100%).

The phylogenetic analysis also showed that the capybaras sequences grouped with other sequences previously reported in this same rodent species and supported by high bootstrap value (97%). Finally, the opossum sequences that clustered with other sequences detected in an opossum from Southern Brazil (Massini et al., 2019) were positioned slightly separated from '*Candidatus* Mycoplasma haemodidelphis' reported in *D. virginiana* in the USA (Messick et al., 2002). Therefore, other studies sampling more animals and targeting different genes (23S rRNA, *rpoB gyrB*), should be performed to assess the phylogenetic positioning of these species.

Interestingly, the genotypes reported in the present study infecting the trapped animals showed to be host-specific, and thus, suggesting the absence of cross-species transmission of hemoplasmas among small rodents, capybaras and opossums – even sharing some tick species. Future experimental studies should confirm this hypothesis. Similar results were described between urban raccoons and sympatric feral cats in Georgia, USA (Volokhov et al., 2017). On the other hand, other study (Millán et al., 2018) reported that some genotypes were shared by different wild carnivores (between different mustelids specimens, and between mustelids and Viverridae species) sampled in Northern Spain. The spillover phenomenon – cross-species pathogens transmission – is promoted by a successive process that provides chances of an animal pathogen to establish the infection in another one (Plowright et al., 2017). The infection establishment is driven by synergism of distinct factors, such as host's distribution and behavior, pathogen prevalence, route of transmission, and genetic, physiological and immunological features of recipient hosts (Plowright et al., 2017). In this way, several biological features – not verified herein – can allow or not the cross-species transmission. Curiously, the cats and dogs-related hemoplasmas species/genotypes have been extensively reported in wild felids and canids, respectively (Willi et al., 2009; André et al., 2011; Harasawa et al., 2014; de Souza et al., 2017; Ghazisaeedi et al., 2017; Millán et al., 2018). Surely, these hemoplasmas species/genotypes carry specific factors that enable these pathogens to overcome every barrier and settle in another host. However, these factors seem to be absent among rodents. These findings are in accordance to a previous study (Gonçalves et al., 2015), who in a wide survey in Brazil sampling wild and synanthropic rodent species concluded that the hemoplasmas reported in wild rodents are restrict to these animals and seem not to infect *R. rattus* or *M. musculus*. However, further studies aiming at identifying the factors that are crucial to cross-species transmission are needed.

The species belonging to the *Rattus* genus have been shown interesting models for ecological studies, mainly due to their dispersion around the globe (Aplin et al., 2011; Kosoy and Bai, 2019). Herein, using the Neighbor-Net and TCS network approaches, we demonstrated that the same genotypes circulate in synanthropic rodents sampled in Brazil, Japan and Hungary. Although only one partial gene was

herein analyzed, these results agree with the hypothesis previously raised that *Rattus*-related hemoplasma genotypes detected in Brazil may have been introduced in Brazilian territories during the European colonization period (Gonçalves et al., 2015). However, further studies targeting other genes as well as the genomes are needed to solve it. Despite the wide distribution of *Rattus* spp. in Brazil, coupled with the unknown zoonotic potential of these genotypes, future studies aiming to assess the impact of these hemoplasmas in human and animal health should be carried out.

5. Conclusion

The present study reported a high occurrence of hemoplasma in synanthropic rodents, capybaras, opossums and associated ectoparasites sampled in Central-Western Brazil. Also, the assessment of the network based on 16S rRNA disclosed the hemoplasmas genetic richness occurring in rodents and highlighted the presence of the same genotype infecting synanthropic rodents from different countries. Finally, we did not find molecular evidence suggesting hemoplasmas cross-species transmission among the wild and synanthropic rodents, capybaras and opossums.

Declaration of competing interest

None.

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CHAPTER 3 – Low occurrence of *Bartonella* in synanthropic mammals and associated ectoparasites in peri-urban areas from Central-Western and Southern Brazil[2]

Abstract

Worldwide, *Bartonella* species are known to infect a wide range of mammalian and arthropod hosts, including humans. The current study aimed to investigate the prevalence of *Bartonella* spp. in synanthropic mammals captured in peri-urban areas from Central-Western and Southern Brazil and their ectoparasites. For this aim, 160 mammals belonging to four species, and 218 associated arthropods were sampled. DNA was extracted and subjected to different *Bartonella* screening assays. Additionally, blood samples from 48 small rodents were submitted to liquid BAPGM culture followed by qPCR assay and solid culture. Two out of 55 *Rattus rattus* captured in Santa Catarina state were PCR-positive for *Bartonella* when targeting the *nuoG*, 16S, and ITS loci. Sequences showed high homology with *Bartonella coopersplainsensis*. Conversely, all 48 small rodents, 14 capybaras and 43 opossum DNA samples from animals trapped in Mato Grosso do Sul were *Bartonella* negative in the HRM real time PCR assays targeting the ITS locus and *gltA* gene. Additionally, all mammal-associated ectoparasites showed negativity results based on HRM real time PCR assays. The present study showed, for the first time, the occurrence of *B. coopersplainsensis* in Brazil, shedding some light on the distribution of rats-related *Bartonella* in South America. In addition, the majority of rodents and marsupials were negative for *Bartonella* spp. Since *B. coopersplainsensis* reservoirs - *Rattus* spp. - are widely dispersed around the globe, their zoonotic potential should be further investigated.

Keywords: *Bartonella coopersplainsensis*; BAPGM; culture; capybaras; fleas; lice; rodents; opossums; ticks

1. Introduction

Distinct biotic and abiotic features, such as habitat fragmentation, ectoparasites richness, host density and climate conditions, could affect pathogen transmission dynamics (Greer and Collins, 2007). Furthermore, the absence or the low prevalence of some pathogens in a specific population can be attributed to different factors, including but not limited to a small number of tested animals,

[2] Este capítulo corresponde ao artigo publicado na revista Acta Tropica: 105513, 2020.

refractory hosts, or, in particular cases when a population is established, by invasion/introduction of pathogen-free individuals in a new area (Kosoy and Bai, 2019).

Bartonella species are vector-borne Gram-negative intracellular facultative bacteria that infect a wide range of mammalian and ectoparasite hosts. These bacteria comprise a group showing distinct host specificity, distribution, pathogenesis and genetic diversity features (Arvand et al., 2010; Harms and Dehio, 2012; Mckee et al., 2016; Harms et al., 2017; Gutiérrez et al., 2018a).

The prevalence of *Bartonella* in rats (*Rattus* sp.), a rodent genus broadly dispersed around the globe, varies widely across different studies, and sampling sites and even among *Rattus* species in a same study (Kosoy et al., 2019). Ecological factors including landscape features, structure population, temperature, ectoparasites richness and sampling zone have been attributed to distinct rates of *Bartonella* infection in rats (Klangthong et al., 2015; Halliday et al., 2015; Peterson et al., 2017; Abreu-Yanes et al., 2018).

Unlike *Bartonella* species and genotypes isolated from *Rattus* genus that are usually highly *Rattus* specific (Buffet et al., 2013; Kosoy et al., 2019), wild rodents species harbor a great diversity of *Bartonella* species (Buffet et al., 2013; Gutiérrez et al., 2015).

As opposed to rodents, only a few studies have been performed aiming the molecular detection or isolation of *Bartonella* in marsupials. To date, *Bartonella* was only isolated from *Macropus giganteus* from Australia (Fournier et al., 2007). In addition, *Bartonella* DNA was detected in fleas and ticks collected from marsupials belonging to *Bettongia penicillata* and *Perameles bougainville* species from Australia, (Kaewmongkol et al., 2011). Moreover, cat-related *Bartonella* spp. have been molecularly detected in *Ctenocephalides felis* from an opossum (*Didelphis virginiana*) from the USA (Reeves et al., 2005; Nelder et al., 2009).

In Brazil, limited studies have been carried out addressing the detection and isolation of *Bartonella* in synanthropic small mammals. For instance, rat-related *Bartonella* spp. were isolated from five out of 26 (19%) *R. norvegicus* sampled in Salvador, Northeast Brazil (Costa et al., 2014). Additionally, *Bartonella* DNA was detected in two out of 29 *R. rattus* (6.8%) sampled from four Brazilian states

(Gonçalves et al., 2016). Concerning wild rodents, a *Bartonella* species phylogenetic related to the *Bartonella vinsonii* complex (Kosoy et al., 2012) has been detected in Cricetidae rodents from 12 Brazilian states (Favacho et al., 2015; Gonçalves et al., 2016; Rozental et al., 2017; de Sousa et al., 2018).

Only two studies have been performed aiming to detect *Bartonella* DNA in marsupials from Brazil. In both studies, none of the 68 marsupials' specimens from four different species were positive for Bartonella DNA (Fontalvo et al., 2017; de Sousa et al., 2018).

Since rodent-related *Bartonella* comprise some zoonotic species coupled with few studies targeting *Bartonella* in marsupials, we investigate here the presence of this bacterial group in synanthropic mammals and associated ectoparasites sampled in localities in Central-Western and Southern Brazil.

2. Materials and Methods

2.1. Sampling sites, mammals capture and biological samples collection

Between September 2016 and August 2018, 160 mammal specimens belonging to four different species were sampled in distinct sites of Campo Grande municipality (-20º 42' 30" S, -54º 61' 60" W), Mato Grosso do Sul state (MS), Central-Western Brazil, and Três Barras city (26° 8' 42" S , 50° 22' 53" W), Santa Catarina state (SC), Southern Brazil **(Table 1)**.

Table 1. Number and animal species positive to *Bartonella*.

Site	Animal species	Sample type	Nº of sampled animals	Occurrence of *Bartonella* % (Nº)	Ectoparasite species	Nº of sampled ectoparasites	Occurrence of *Bartonella* % (Nº)
		Mammals			**Arthropds**		
Santa Catarina	**Rodentia**						
	R. rattus	DNA from spleen tissues	55	3.6% (2/55)	*Tunga caecata* *Notoedres muris* *Myocoptes* sp.	2 1 1	Not tested Not tested Not tested
Campo Grande	**Rodentia**						
	R. rattus	DNA from spleen tissues and liquid culture	39	0% (0/39)	*Amblyomma* sp.[a] *Polyplax spinulosa*	62 6	0% (0/9) [b] 0% (0/2) [b]
	M. musculus	DNA from spleen tissues and liquid culture	9	0% (0/9)	-	-	-
	H. hydrochaeris	DNA from whole blood	14	0% (0/14)	*A. dubitatum* *A. sculptum* *Amblyomma* sp.[a]	42 36 2	0% (0/42) 0% (0/33) [c] 0% (0/2)
	Didelphimorphia						
	D. albiventris	DNA from whole blood	43	0% (0/43)	*A. dubitatum*	70	0% (0/28) [b]

[a] *Amblyomma* sp. refers to larvae sampled – In these specimens only the genus was reported

[b] Ectoparasites-DNA pool samples

[c] Three out of 36 *Amblyomma*-DNA samples were negative to the endogenous control (16S rRNA)

In Campo Grande municipality, 48 small rodents (*Rattus rattus* [n = 39] and *Mus musculus* [n =9]) were trapped in urban area (4 sites) and urban forestfragments (4 sites). Additionally, 14 capybaras (*Hydrochoerus hydrochaeris*), and 43 marsupials (*Didelphis albiventris*) were trapped in three and six urban forest fragments, respectively, in Campo Grande city. Additionally, 55 black rats (*R. rattus*) were captured in thirteen urban sites in Três Barras city, SC **(Figure 1)**.

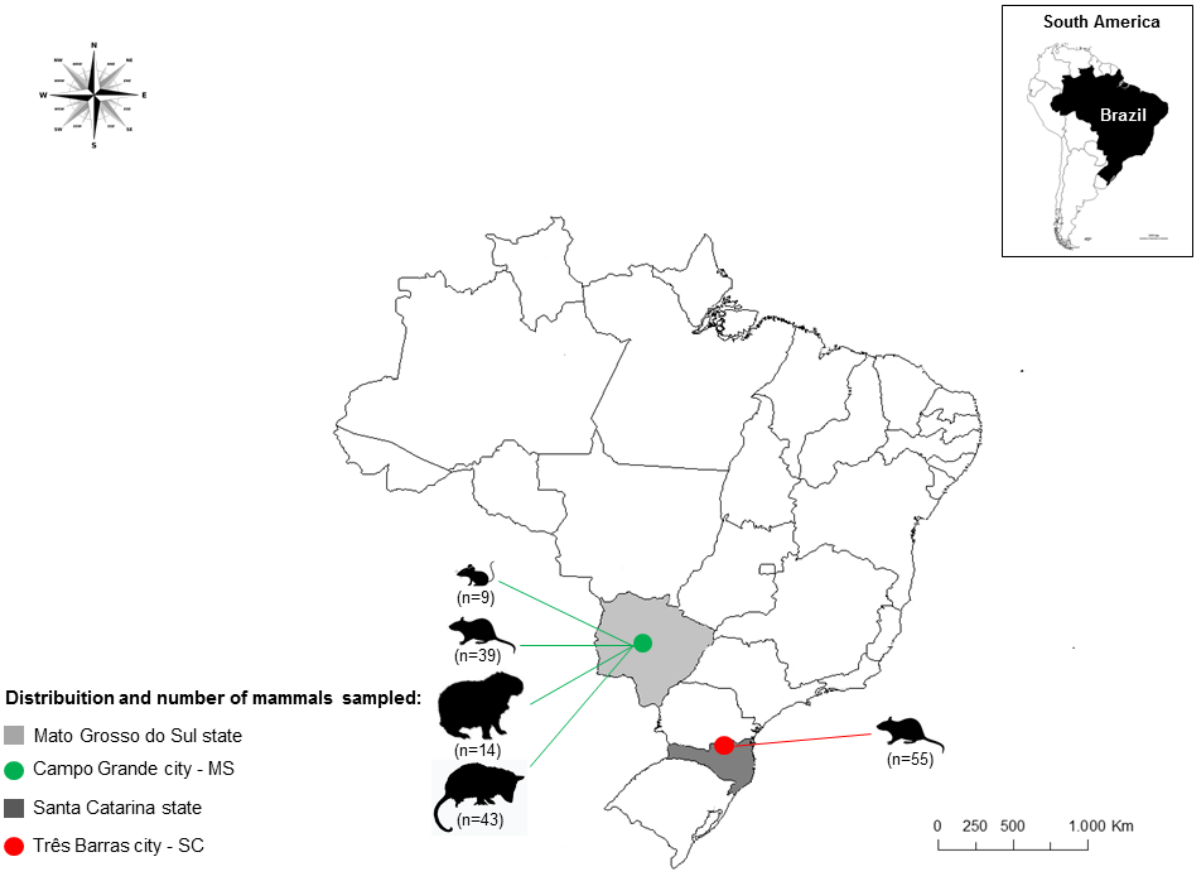

Figure 1. Sampling sites, number and distribution of mammals sampled in Campo Grande, MS and Três Barras, SC, Brazil.

All capture procedures were performed as previously described (Nantes et al., 2019; Gonçalves et al., 2020). Briefly, the small mammals were captured using Tomahawk (45 x 17,5 x 15 cm) and Sherman (42 x 11,5 x 14 cm) live traps baited with a mix of bananas, *paçoca*, oat flakes and tinned sardines. The small rodents were chemically immobilized using a combination of ketamine hydrochloride (100

mg/mL) and acepromazine (10 mg/mL) (1:9) intramuscularly. When the death of anesthetized small rodents did not occur after exsanguination, the euthanasia was performed through intracardiac injection of 19.1% potassium chloride (2 ml/kg). Thereafter blood and/or spleen fragments were collected under sterile conditions. On the other hand, marsupials were anesthetized with a chemical association of Ketamine (20 mg/kg) and Xylazine (2 mg/kg) intramuscularly. Blood samples were collected from the marsupials' lateral caudal veins and placed to DNase and RNase-free anticoagulant ethylenediaminetetraacetic acid (EDTA)-containing microtubes. Finally, after chemical immobilization using an anesthetic dart containing TELAZOL® 100 (4mg/kg), blood samples were collected from the capybaras' femoral vein into EDTA-buffered vacutainer tubes (Gonçalves et al., 2020). Except for the small rodents trapped in Campo Grande, for which blood samples were kept at -80°C for *Bartonella* culture, all other samples (blood or spleen) were kept on ice until arrival to the laboratory and stored at -20°C until DNA extraction. The following data were recorded from each animal: the presence of ectoparasites, gender, weight, and sampling point.

All sampled animals were checked for the presence of ectoparasites. Once collected, the ectoparasites were placed in microtubes containing absolute ethanol (Merck®) and maintained at -20°C until morphological identification and DNA extraction. The morphological identification was performed using previously described taxonomic keys (Onofrio et al., 2005; Martins et al., 2010; Linard et al., 2014; Anholt et al., 2014; Pereira et al., 2017).

All animal captures were in accordance with the licenses obtained from the Instituto Chico Mendes de Conservação da Biodiversidade (license number 56912-2), Imasul (license number 001/2017) and endorsed by the Ethics Committee of FCAV/UNESP and Contestado Universities under the numbers: 01952/18 and 15/16.

2.2. *Bartonella* isolation

The 48 blood samples obtained from the small rodents trapped in Campo Grande municipality were taken to the laboratory for bacterial culturing. Initially, the blood samples were subjected to a pre-enrichment culture as previously described (Maggi et al., 2005; Duncan et al., 2007). Briefly, the EDTA-anticoagulated blood

samples were thawed at room temperature and an aliquot of 200 µL was inoculated into filter cap cell culture flasks (Corning®) containing 2 mL of liquid *Bartonella* alphaproteobacterium growth medium (BAPGM – pH 6.2) supplemented with 10% of defibrinated sheep blood. Sheep blood samples were confirmed to be *Bartonella*-negative using a previously described qPCR assay (André et al., 2016). The flasks were incubated for 7 days at 37°C in 5% CO_2 in a water-saturated atmosphere and maintained under a constant shaking motion. A negative control flask (only liquid culture) was prepared and incubated simultaneously with all blood samples. After 7 days of incubation an aliquot of 500 µL was submitted to DNA extraction using a commercial kit (InstaGene Matrix - BIORAD®) followed by a *Bartonella* screening using the qPCR assay targeting the *nuoG* gene describe elsewhere (André et al., 2016). In addition, 300 µL aliquot was sub-inoculated onto an agar chocolate plate, which was maintained in incubation as described above during four weeks. The plates were examined twice a week for the presence of *Bartonella*-like colonies. If *Bartonella*-like colonies were observed, the colonies were submitted to DNA extraction, qPCR confirmation (André et al., 2016), conventional PCR targeting the *gltA* gene (Birtles and Raoult, 1996) and sequencing.

2.3. DNA extraction and quality

DNA was extracted from ten mg of each small rodent spleen tissue, and from 200 µL of each blood sample from capybaras and marsupials, using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, California, USA), according to manufacturer's instructions. Furthermore, the collected ectoparasites were submitted to DNA extraction individually and/or in pools (tick nymphs were pooled up to three individuals, larvae up to seven individuals) from the same host. The lice were pooled up to two specimens from the same host, using the commercial kit mentioned above.

In order to discard the presence of PCR inhibitors, all extracted mammal DNA samples were used as a template in an internal control PCR targeting the mammal *gapdh* gene as previously described (Birkenheuer et al., 2003). Likewise, all arthropod DNA samples were submitted to internal control targeting the 16S rRNA as previously described (Black and Piesman, 1994). Internal control-PCR positive samples were subsequently submitted to *Bartonella* screening High Resolution Melt

(HRM) real-time PCR assays targeting the ITS locus and *gltA gene.* Additionally, DNA samples from the rodents trapped in SC were screened using a qPCR assay targeting the *nuoG* gene.

2.4. Molecular detection of *Bartonella* DNA in mammals and associated ectoparasites from Campo Grande city, MS

Initially, DNA samples were screened for *Bartonella* DNA using an HRM real-time PCR assay targeting a fragment of approximately 200 bp of the 16S–23S internal transcribed spacer (ITS) locus, as previously described (Maggi and Breitschwerdt, 2005; Gutiérrez et al., 2013). To confirm the results, all DNA samples were submitted to an additional HRM real-time PCR assay targeting the *gltA* gene (approx. 350 bp), as previously described (Sofer et al., 2015). Briefly, the amplification reaction was performed using the StepOnePlus (Applied Biosystems) real-time system. The amplification protocol used was as follows: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 20 s at 65°C for both targets (data collection on HRM reporter), and 5 s at 72°C. The HRM stage was performed at the end of the cycling as follows: 15 s at 95°C, followed by a temperature increase from 70 to 95°C (data collection set in 0.3%, HRM reporter). PCR was carried out in 20 µL reaction volumes containing 0.5 µL of 10 mM of each primer, 0.6 µL of 50 µM solution of Syto9 (Invitrogen®, CA, US), 10 µL of Dream Taq Hot Start PCR Master Mix (Thermo Fisher Scientific®, San Jose, CA, USA), 6.4 µL ultrapure PCR water (Thermo Fisher Scientific®, San Jose, CA, USA), and 2 µL of DNA. DNA of *Bartonella krasnovii* (Gutiérrez et al., 2020) and ultra-pure water were used as positive and non-template controls, respectively, in all real-time PCR assays.

2.5. Molecular detection and characterization of *Bartonella* DNA in rodents from Três Barras, SC

The quantification and screening for the *Bartonella* DNA was performed using a qPCR assay targeting a fragment of 83 bp of the *nuoG* gene as described elsewhere (André et al., 2016). Briefly, the qPCR assay was 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 1 µL of DNA sample. The amplification conditions were used as follows: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 minutes and 52.8°C for 30 seconds) (André et al., 2016). Serial dilutions were performed with the aim of constructing standard curves with different concentrations of plasmid DNA (pIDT Smart; Integrated DNA Technologies) (2.0×10^7 to 2.0×10^0 copies/µl).The number of plasmid copies was determined in accordance with the formula (x grams per microliter of DNA/[plasmid size (base pairs) x 660]) x 6.022×10^{23} x plasmid copies per microliter. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the formula $E = 10^{-1/\text{slope}}$.

The positive DNA samples in the molecular screening for *Bartonella* were submitted to additional PCR assays targeting the *gltA* (Birtles and Raoult, 1996), *rpoB* (Renesto et al., 2001), *nuoG* (Colborn et al., 2010), *groEL* (Zeaiter et al., 2002 and Paziewska et al., 2011), 16S rRNA (Dauga et al., 1996), and ITS (Diniz et al., 2007). *Bartonella bovis* DNA (Gonçalves et al., 2020) and ultra-pure water were used as positive and non-template controls, respectively, in all PCR assays. Thereafter, the amplicons obtained were purified using the EXOSAP-IT® (Applied Biosystems). Purified amplified DNA fragments were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser – Applied Biosystem/ Perkin Elmer) (Sanger et al., 1977). Finally, consensus sequences were obtained through the analysis of electropherograms using the Phred-Phrap program with a Phred quality score (peaks around each base call) established at ≥20 (99% in the accuracy of the base call) (Ewing et al 1998).

3. Results

3.1. Ectoparasites and DNA extraction quality

Ectoparasites were found in 7.7% (8/103) of small rodents sampled in Campo Grande, MS and Três Barras, SC. Among them, 62 ticks (*Amblyomma* spp.), six lice (*Polyplax spinulosa*), two mites (one *Notoedres muris* and one *Myocoptes* sp.) and two fleas (*Tunga caecata* – including one specimen found in a *Bartonella*-positive rat - #27) were sampled. Also, 71.4% (10/14) of trapped capybaras in Campo Grande

were infested with ticks (*Amblyomma* spp.). Lastly, ticks (*Amblyomma* spp.) were observed in 32.5% (14/43) of the sampled opossums. All ectoparasite species sampled are shown in **Table 1**.

Except for three tick-DNA samples obtained from capybaras, all arthropod and mammal DNA samples were positive for 16S rRNA and *gapdh* internal control PCR assays, respectively. The tick samples that were found negative for arthropod-16S rRNA by PCR were excluded from subsequent analyses.

3.2. *Bartonella* isolation

None of 48 rodent blood samples submitted to the liquid pre-enrichment culture were positive by the *Bartonella* qPCR assay. Likewise, no *Bartonella*-like colonies were observed on the agar chocolate plates during a period of four weeks of incubation.

3.3. *Bartonella* prevalence and BLASTn results

Two (#22 and #27) out of 55 rats (3.6%) sampled in SC were positive by the qPCR assay for *Bartonella* based on the *nuoG gene*. The #22 and #27 DNA samples showed absolute quantification of 4.7 x 10^1 and 3.1 x 10^1 copies/µL, respectively. On the other hand, all mammal and arthropod specimens trapped in MS were negative by the qPCR assays for *Bartonella* targeting the ITS locus and *gltA* gene. The efficiency mean of the qPCR assays was *E*= 98.1% ([ranging from 91.4% to 103.9%]; slope = -3.371; r^2 = 0.997).

The two *nuoG*-qPCR positive samples were confirmed positive by conventional PCR assays targeting the *nuoG* and 16S rRNA genes and ITS locus. While the #22 and #27 DNA samples were positive in all above referred PCR assays, the #27 DNA sample showed weak band intensity in *nuoG* and 16S assays, precluding its sequencing. Both ITS sequences shared 99.6% identity to *Bartonella coopersplainsensis* (accession number EU111770) isolated from an Australian *Rattus leucopus*. The only 16S sequence obtained (#22) in the present study shared 100% identity to both *Bartonella* sp. (accession number AY993935) detected in *Rattus tanezumi* from China and *B. coopersplainsensis* (accession number NR_1116177) isolated from *R. leucopus* from Australia. Also, the obtained *nuoG*

sequence (#22) was identical (100% identity) to *Bartonella* sp. (JX131666) detected from *R. tanezumi* from South Africa. The *nuoG*, ITS and 16S sequences showed query coverages ranging from 81% to 100%. The sequences were deposited in GenBank database under accession numbers: *nuoG*: MT302378; ITS: MT271770-MT271771 and 16S: MT267730.

4. Discussion

In the current study, only two out of 55 rats trapped in SC were positive for *Bartonella* DNA. However, all DNA samples obtained from mammals' blood samples or ectoparasites sampled in MS were negative on the screening real-time PCR for *Bartonella* DNA targeting two different genomic regions. Considering that all DNA samples from MS were positive for the internal control (*gapdh*), confirming the presence of amplifiable DNA, the absence of *Bartonella* DNA suggests that all animals screened were not infected with *Bartonella* or that these animals presented an extremely low number of circulating *Bartonella* organisms, at least below the limit of detection of the HRM real-time PCR assays performed, which was previously estimated to be < 1 CFU per µl for the ITS locus (Gutiérrez et al., 2015b). In addition, we carried out the liquid BAPGM culture followed by qPCR approach aiming to improve the sensitivity of *Bartonella* diagnosis in the small rodent blood samples from MS. This approach has greatly facilitated the *Bartonella* detection from the blood samples of several animal species (Breitschwerdt, 2014). Nonetheless, none rodent blood sample subjected to BAPGM approach was positive, supporting the hypothesis that these animals were *Bartonella*-free.

The rat-associated *Bartonella* prevalence rates vary widely among the different studies and sampling zones within the same study (Kosoy and Bai, 2019). Several biotic and abiotic features have been attributed to prevalence variations. Peterson et al. (2017) reported that the *Bartonella* prevalence in rats ranged from 0 to 97% among the different sampling sites. In addition to environmental features, the authors reported that co-occurrence of rat species (*R. rattus* and *R. norvegicus*), flea infestation, and age class were significant predictors for *Bartonella* prevalence (Peterson et al., 2017). Likewise, during a study performed in Asembo and Kibera, Kenya, the *Bartonella* prevalence in *Rattus* varied from 0 to 60% according to the

trapping sites (Halliday et al.,2015). Different from Asembo sampling site that is more rural, the Kibera study site is a neighborhood within Nairobi - the Kenyan capital - few miles from the Nairobi center, thus is likely to have a higher international connectivity with a higher rodent movement through sea trade. Thereby, the higher prevalence observed at the Kibera might be explained by the repeated introduction of *Rattus*-associated *Bartonella* species to this site.

In the above scenario, Kosoy and Bay (2019) analyzing the prevalence of *Bartonella* in rats around the world, highlighted that *Bartonella* infection rates in rat populations are very high. However, the authors noticed that rats in some cities were not infected with *Bartonella*. One of the explanations raised by the authors is that the absence of *Bartonella* in some rat populations could be attributed to a kind of "island syndrome", in which at some point, certain parasites are absent mainly when a new rat population is formed by the introduction of a small number of individuals. Therefore, this could explain the absence or low prevalence of *Bartonella* in rats in some studies outside of Asia (Kosoy and Bay, 2019), as well as in our study.

Curiously, Ellis et al. (1999) reported that rats from US coastal cities (e.g. Baltimore, Miami and Los Angeles) were *Bartonella* positive, while rats sampled in non-coastal cities (Reno and Spencer) were *Bartonella*-free. Distinct from the first report of rat-associated *Bartonella* in Brazil, in which 19% *R. norvegicus* (n = 26) trapped in Salvador, a coastal city, were positive (Costa et al., 2014), the rats sampled in the present study in non-coastal cities of Campo Grande, MS, and Três Barras, SC, far approximately 1.000 Km and 170 Km, respectively, from the Brazilian coast, showed to be *Bartonella*-free and/or a low prevalence (3.6%) for this group of bacteria. Although a low number of rats have been analyzed (n = 39 in MS and n = 55 in SC), the molecular absence and the low *Bartonella*-DNA prevalence in rats could be attributed to possible isolation of the rat populations explored in this study.

Considering that rats were introduced to Brazilian territories during the European colonization (only about 500 years ago) (Hingston et al., 2016), we can speculate that these rat populations were established by *Bartonella*-free individuals or by only a few individuals harboring *Bartonella*. Likewise, the continuous arrival of new rats through seaports, as probably has happened in Salvador city, may have played a crucial role in the prevalence of rat-related *Bartonella* in coastal cities.

Finally, the low prevalence of rat fleas, that are considered key players in the *Bartonella* cycle (Gutiérrez et al., 2015a) during the ectoparasites survey, could have influenced the *Bartonella* prevalence found. However, new studies sampling a higher number of rats in different sites, coastal and non-coastal regions, are much necessary to solve this enigma.

In Brazil, the overall *Bartonella* prevalence in wild rodents varies from 0% to 42.9% (Favacho et al., 2015; Gonçalves et al., 2016; Rozental et al., 2017; Fontalvo et al., 2017; de Sousa et al., 2018). Similar to Fontalvo et al. (2017) that reported negative results for *Bartonella* DNA in all small wild rodents (n = 38) trapped in Pernambuco state, Northeastern Brazil, all capybaras (n = 14) analyzed in the current study showed negative results in the HRM real-time PCR assays. Despite the fact that several flea species have been reported in small rodents from Brazil (Carvalho et al., 2001), fleas were not observed on rodents at the time of sampling in the present study neither in the study performed by Fontalvo et al. (2017). Conversely, de Sousa et al. (2018) reported a *Bartonella* prevalence of 31.8% (35/110) among wild rodents trapped in the central region of Pantanal, municipality of Corumbá, MS – far approximately 350 km from our sampling sites in Campo Grande municipality, MS. Also, 75 fleas (*Polygenis bohlsi bohlsi*) were collected from 16 (14.5%) rodents. Finally, the authors reported *Bartonella* DNA in three (7.8%) flea pools, emphasizing the role of fleas in the rodent-associated *Bartonella* prevalence.

Capybaras have a broad distribution in South America. Usually, this rodent species is infested by ticks (Szabó et al., 2013), and only a few records of fleas in this rodent species were reported (Linardi and de Avelar, 2014). Although the rodent-associated *Bartonella birtlesii* transmission under experimental condition by *Ixodes ricinus* tick has been demonstrated (Reis et al., 2011), the role of ticks in rodent-related *Bartonella* transmission remains unclear (Harrison et al., 2012). Therefore, the absence of fleas may have influenced the *Bartonella* prevalence found in capybaras. Considering that a limited number of capybaras were screened, these results should be interpreted with caution. Thereby, future studies are needed in order to shed light on the role of capybaras as hosts for *Bartonella* in South America.

Except for the findings from Australia (Fournier et al., 2007*;* Kaewmongkol et al., 2011), no study has reported the detection of *Bartonella* DNA in marsupials from

America. Marsupials have a wide distribution in the American continents, but, only two studies reported the occurrence of *Bartonella* in ectoparasites from marsupials (Reeves et al., 2005; Nelder et al., 2009). Nonetheless, it is necessary to emphasize that the *Bartonella*-positive ectoparasites found in *D*. *virginiana* were the cat flea, *C. felis* - a common finding in the USA (Abramowicz et al., 2012; Blanton et al., 2016) - in which only cat-associated *Bartonella* DNA (*B. clarridgeiae* and *B. henselae*) were detected. The fact that the marsupials were not screened in both above mentioned studies, the role of these mammals in the *Bartonella* epidemiological cycles remains vague.

Only two studies have previously investigated the occurrence of *Bartonella* in marsupials from Brazil. None of the 68 marsupials sampled in Central-Western and Northeastern Brazil were positive for *Bartonella* (Fontalvo et al., 2017; de Sousa et al, 2018), as well as reported in the present study. Additionally, all ectoparasites including *Polygenis bohlsi bohlsi* fleas (n = 5) collected from *Monodelphys domestica* and *Thylamus macrurus* (de Sousa et al., 2018) and *C. felis* fleas (n = 3) collected from *D. albiventris* (Fontalvo et al. 2017) showed to be negative for *Bartonella* DNA. These findings raise interesting questions about the role of the American marsupials in the *Bartonella* life cycle. Are marsupials refractory hosts for *Bartonella* infection? Could the marsupial's immune system promote the clearance of *Bartonella* infection?

In the current study, although with a low prevalence, we showed, for the first time, the occurrence of *B. coopersplainsensis* in rats trapped in Brazil. This *Bartonella* species has not been reported in humans yet. However, since its description (Gundi et al., 2009), *B. coopersplainsensis* has been detected from *Rattus* collected in Southeastern Asia (Saisongkorh et al., 2009; Jiyipong et al., 2012; Tay et al., 2014), New Zealand (Helan et al., 2018), Spain and Italy (Obiegala et al., 2019), and wild rodents (*Apodemus agrarius*) from China (Li et al., 2015) and Lithuania (Mardosaitè-Busaitienè et al., 2019). The absence of positive results in conventional PCR assays based on *gltA* and *rpoB* genes might have been associated to the low number of *Bartonella* DNA copies present in the sampled rodent samples. Although the three amplified regions (16S rRNA, *nuoG* and ITS) shows a lower discriminatory power when compared with *gltA* and *rpoB* genes (La Scola et al., 2003), they provided sufficient discriminatory information for the

identification of *B. coopersplainsensis* infecting *R. rattus* in the current study. Additionally, we emphasized that most screened DNA samples were *Bartonella*-free. Therefore, ecological and biological factors (e.g. rodents' geographic isolation and absence of fleas) may also have influenced the prevalence observed. Finally, since *B. coopersplainsensis* reservoirs - *Rattus* spp. - are widely dispersed around the world, the zoonotic potential of this *Bartonella* species should be further investigated.

Declaration of competing interest

None.

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CHAPTER 4 – Molecular detection and genetic diversity of *Bartonella* species in large ruminants and associated ectoparasites from the Brazilian Cerrado[3]

Abstract

Currently, five *Bartonella* species and an expanding number of *Candidatus Bartonella* species have globally been reported in ruminants. Likewise, different *Bartonella* genotypes were identified. However, studies relating to ruminants-associated *Bartonella* in Brazil are scarce. The current study aimed to assess the prevalence and genetic diversity of *Bartonella* in cattle, buffaloes and associated ectoparasites in Brazil. For this purpose, EDTA-blood samples from 75 cattle and 101 buffaloes were sampled. Additionally, 128 *Rhipicephalus microplus* and one *Amblyomma sculptum* ticks collected from cattle, and 197 *R. microplus*, one *A. sculptum* and 170 lice (*Haematopinus tuberculatus*) collected from buffaloes were included. *Bartonella* DNA was initially screened through an HRM real-time PCR assay targeting the 16S-23S internal transcribed spacer (ITS), and the positive samples were submitted to an additional HRM assay targeting the *ssrA* gene. The HRM positive amplicons were sequenced, and the nucleotide identity was assessed by BLASTn. *Bartonella* spp.-positive DNA samples were analyzed by conventional PCR assays targeting the *gltA* and *rpoB* genes, and then the samples were cloned. Finally, the phylogenetic positioning and the genetic diversity of clones were assessed. Overall, 21 out of 75 (28%) cattle blood samples and 13 of 126 (10.3%) associated ticks were positive for *Bartonella bovis*. Out of 101 buffaloes, 95 lice and 188 ticks DNA samples, one (1%) buffalo and four (4.2%) lice were positive for *Bartonella* spp. Conversely, none of the ticks obtained from buffaloes were positive for *Bartonella*. The *Bartonella* sequences from buffalos showed identity ranging from 100% (ITS and *gltA*) to 94% (*ssrA*) with *B. bovis*. In contrast, the *Bartonella* DNA sequences from lice were identical (100%) to uncultured *Bartonella* sp. detected in cattle tail louse (*Haematopinus quadripertusus*) from Israel in all amplified genes. The present study demonstrates the prevalence of new *B. bovis* genotypes and a cattle lice-associated *Bartonella* species in large ruminants and their ectoparasites from Brazil. These findings shed light on the distribution and genetic diversity of ruminant and ectoparasites-related *Bartonella* in Brazil.

Keywords: bartonellosis, buffaloes, cattle, genetic diversity, *Haematopinus tuberculatus*, *Rhipicephalus microplus*

1. Introduction

The *Bartonella* genus comprises a large group of bacteria that infects mainly erythrocytes and endothelial cells from a wide range of animals including humans.

[3] Este capítulo corresponde ao artigo publicado na revista Transbound. Emerg. Dis.:00:1–10, 2020.

Bartonella species are Gram-negative and facultative intracellular vector-borne pathogens closely related to *Brucella*, *Agrobacterium*, *Ochrobactrium*, and *Rhizobium* within the alfa-2-proteobacteria subdivision (Birtles and Raoult, 1996; Birtles, 2005; Kosoy et al., 2012).

Currently, five *Bartonella* species named *Bartonella bovis*, *Bartonella chomelii*, *Bartonella schoenbuchensis*, *Bartonella capreoli* and *Bartonella melophagi* have been associated with ruminants. In addition, an expanding number of *Candidatus* to *Bartonella* species and different genotypes have been globally reported in ruminants (Dahmani et al. 2017; Gonçalves et al., 2018; Raya et al., 2018; Razanske et al., 2018). Interestingly, these species and recently detected genotypes are phylogenetically related and exclusively associated with these animals (Kosoy et al., 2016).

Ruminants have played a crucial role in agricultural systems throughout the world. The first ruminants evolved about 50 million years ago and were small (< 5 Kg) forest-dwelling omnivores. Nowadays, there are about two thousand living ruminant species, classified into six families (Antilocapridae, Bovidae, Cervidae, Giraffidae, Moschidae and Tragulidae), the majority of which are Bovidae and Cervidae (Hackmann and Spain, 2010). These animals are ecologically, agriculturally and economically important to humans as they are widely used for distinct purposes, such as ecological indicators, meat and dairy products and draught power (Hanley, 1996; Bai et al., 2013).

Although *Bartonella* infections have been reported in cattle worldwide (Bermond et al., 2002, Roilan et al., 2003, Tsai et al., 2011; Bai et al., 2013; Gutiérrez et al., 2014; Antequera-Gómez et al., 2015), only two studies reported *Bartonella* in buffaloes so far (Bai et al., 2013; Gonçalves et al., 2018). While the first study isolated *B. bovis* in Asian buffaloes (*Bubalus bubalis*) from Thailand, the second amplified *Bartonella* DNA in wild African buffaloes (*Syncerus caffer*) from Mozambique. Interestingly, the prevalence of *Bartonella* in cattle is generally high, albeit it varies widely across different studies, ranging from apparent uninfected animals to up to 80% (Cherry et al., 2009; Bai et al., 2013). As opposed to cattle, the buffaloes have shown a low prevalence of less than 7% (Bai et al., 2013).

Moreover, *Bartonella* DNA has been amplified in cattle-associated hematophagous arthropods, including *Haematobia* and *Stomoxys* spp. from California (Chung et al., 2004), *Hippobosca equi* from Europe (Halos et al., 2004), *Rhipicephalus microplus* from Taiwan (Tsai et al., 2011), *Haematopinus quadripertusus* from Israel (Gutiérrez et al., 2014), and *Haemaphysalis bispinosa* from Malaysia (Kho et al., 2015). However, the role of these arthropods in the transmission cycles of *Bartonella* is unknown and competence studies should be performed in order to elucidate their biological role. Additionally, the role of vampire bats in the transmission of ruminant-associated *Bartonella* has been discussed (Becker, Bergner, et al., 2018; Raya et al., 2018; André et al., 2019).

Although *B. bovis* has been associated with bovine endocarditis (Maillard et al., 2007; Erol et al., 2013), reports on biological aspects regarding pathogenicity, transmission and genetic diversity relating to ruminants-associated *Bartonella* are scarce. Likewise, to the best of our knowledge, no study has been performed in Brazil aiming to verify the prevalence of *Bartonella* in bovids to date. Thus, in the current study, the prevalence and genetic diversity of *Bartonella* in cattle and buffaloes as well as associated ectoparasites was investigated.

2. Material and Methods

2.1 Ruminants and ectoparasites sampling

All procedures were carried out according to the ethical guidelines for the use of animal samples permitted by the Institutional Animal Care and Use Committee (IACUC) of Universidade Estadual Paulista (FCAV/UNESP), Jaboticabal, São Paulo (Protocolo number: 01952/18).

Between August 2017 and March 2018, blood samples were collected, by convenience, from 75 cattle (64 Nelore cattle breed [*Bos taurus indicus*] and 11 mixed cattle breed) from Campo Grande city (-20º 42’ 30” S, -54º 61’ 60” W), Mato Grosso do Sul state, Central-Western Brazil (Figure 1). Additionally, in October 2017, 101 water buffaloes (*Bubalus bubalis*) were sampled from Passos municipality (-20° 71′ 60″ S, -46° 60′ 36″ W), Minas Gerais state, Southeast Brazil (Figure 1). Approximately 2-5 mL of whole blood were collected from the jugular vein into ethylenediaminetetraacetic acid (EDTA)-buffered vacutainer tubes. The blood

samples were kept on ice (maximum time of 1 hour) until arriving at the laboratory and subsequently stored at -20 ºC until DNA extraction. During the blood sample collection, ectoparasites were sampled from the animals and kept in absolute ethanol (Merck®) until the morphological identification and DNA extraction.

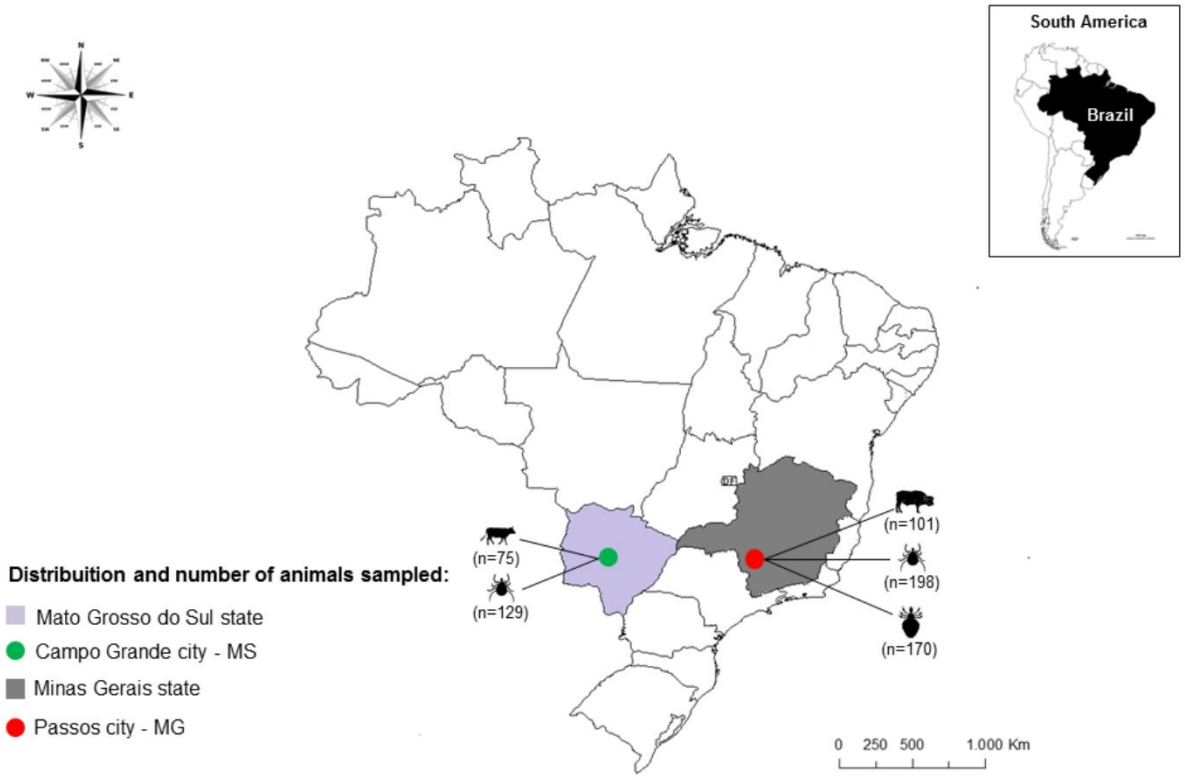

Figure 1. Sampling sites, number and distribution of cattle, buffaloes, ticks and lice sampled in the Brazilian Cerrado.

2.2 Morphological identification of the ectoparasites

Using a stereoscope and morphological keys, the collected ticks were identified, as described elsewhere (Onofrio et al., 2005). Likewise, the sampled lice were identified according to Meleny and Kin (1974). Out of 129 ectoparasites collected from cattle, 128 were identified as *Rhipicephalus microplus* tick species (113 adults [13 males and 100 females], 14 nymphs and one larva), and only one female specimen was classified as *Ambyomma sculptum*. The buffalo ectoparasites were identified as 197 *R. microplus* (148 adults [37 males and 111 females], 48 nymphs and one larva) and one female belonging to *A. sculptum* species. Also, 170

lice (163 adults [66 males and 97 females] and 7 nymphs) were identified as *Haematopinus tuberculatus* (Figure 1).

2.3 DNA extraction and endogenous control PCR

DNA was extracted from blood samples of each animal (300 µL) according to a protocol previously published (Kurumae-Izioka, 1997). Additionally, DNA from adult ticks was extracted individually. DNA from tick larvae and nymphs were extracted in pools consisting of 1-3 individuals collected from the same host. Likewise, DNA from lice was extracted individually or by pooling up to three individuals from the same host. The ectoparasites' DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen®, Valencia, CA, USA), according to the manufacturer's instructions. To confirm the presence of amplifiable DNA, a PCR assay targeting the mammals *gapdh* gene was performed (Birkenheuer et al., 2003). In addition, all ticks and lice DNA samples were submitted to an internal control targeting the 16S rRNA and *cox-1*, respectively, as previously described (Black and Piesman, 1994; Folmer et al., 1994). Endogenous gene-PCR positive samples were subsequently submitted to *Bartonella* screening HRM (High Resolution Melting) real-time PCR assays targeting the ITS locus and *ssrA* gene.

2.4 Molecular detection of *Bartonella* DNA from ruminants and associated ectoparasites

Firstly, DNA samples were screened for *Bartonella* DNA using an HRM real-time PCR assay targeting a fragment of approximately 200 bp of the 16S–23S internal transcribed spacer (ITS) locus, as previously described (Maggi and Breitschwerdt, 2005; Gutiérrez et al., 2013). Also, an additional real-time PCR assay targeting the transfer mRNA (*ssrA*) gene (approx. 300 bp) was performed on all ITS-HRM positive samples (Gutiérrez et al., 2013). Briefly, the amplification reaction was performed using the StepOnePlus (Applied Biosystems) real-time system. The amplification protocol used was as follows: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 20 s at 65°C (60°C for *ssrA*) (data collection on HRM reporter), and 5 s at 72°C. The HRM stage was performed at the end of the cycling as follows: 15 s at 95°C, followed by a temperature increase from 70 to 95°C (data collection set in

0.3%, HRM reporter). PCR was carried out in 20 µL reaction volumes containing 0.5 µL of 10 mM of each primer, 0.6 µL of 50 µM solution of Syto9 (Invitrogen®, CA, US), 10 µL of Dream Taq Hot Start PCR Master Mix (Thermo Fisher Scientific®, San Jose, CA, USA), 6.4 µL ultrapure PCR water (Thermo Fisher Scientific®, San Jose, CA, USA), and 2 µL of DNA. DNA of '*Candidatus* Bartonella krasnovii' (Gutiérrez et al., 2018a) and ultra-pure water were used as positive and non-template controls, respectively, in all real-time PCR assays.

2.5 Molecular characterization of *Bartonella* in ruminants and associated ectoparasites

The positive samples in the above described HRM assays were subjected to additional PCR assays targeting the *gltA* (750 bp) and *rpoB* (825 bp) genes as previously described (Birtles and Raoult, 1996; Renesto et al., 2001). Subsequently, the positive amplicons were submitted to pGEM-T Easy vector cloning (Promega® Madison, WI, USA), following the manufacturer's recommendations. Up to three clones from each positive sample were selected for sequencing, according to the blue/while colonies system. Firstly, the clones were subjected to plasmid DNA extraction using the Illustra® PlasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). Secondly, plasmid DNA extracted from the clones were subjected to a PCR assay using the primers M13 F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and M13 R (5′-GTCATAGCTGTTTCCTGTGTGA-3′) (Lau et al., 2010) that flank the multiple cloning site of the pGEM T-Easy plasmid and, therefore, including the inserts of the *gltA* and *rpoB* genes. Thereafter, the amplicons obtained were purified using the EXOSAP-IT® (Applied Biosystems). Purified amplified DNA fragments were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser – Applied Biosystem/ Perkin Elmer) (Sanger et al., 1977). Finally, consensus sequences were obtained through the analysis of electropherograms using the Phred-Phrap program with a Phred quality score (peaks around each base call) established at ≥20 (99% in the accuracy of the base call) (Ewing et al 1998).

2.6 *Bartonella* identification and phylogenetic analyses

The *Bartonella* species were identified by BLASTn analysis using the Megablast (following default parameters), aligned with sequences available in GenBank using Clustal/W (Thompson et al., 1994), and adjusted in Bioedit v. 7.0.5.3. (Hall, 1999). The phylogenetic analysis was performed using Maximum Likelihood (ML) method, inferred with RAxML-HPC BlackBox (7.6.3.) (Stamatakis et al., 2008) and performed in CIPRES Science Gateway (Miller et al., 2010). The Akaike Information Criterion (AIC) available on MEGA v.5 software (Tamura et al., 2011) was applied to identify the most appropriate model of nucleotide substitution. GTR+G+I model was chosen as the most appropriate for the phylogenetic analyses of the *gltA* and *rpoB* alignments.

2.7 Genetic diversity of detected *Bartonella* sequences

The *gltA* and *rpoB* aligned sequences amplified in the present study were applied to identify the genotypes to calculate the nucleotide diversity (□), the polymorphic level (genotype diversity [Gd]), the number of variable sites (v), and the average number of nucleotide differences (K), using the DnaSP v5.10 (Librado and Rozas 2009). Additionally, the different genotypes identified in the present study and other ruminant-associated *Bartonella* sequences (*B. bovis* and *Bartonella* sp. identified in cattle-lice) obtained from GenBank were submitted to TCS network (Templeton et al., 1992; Huson and Bryant, 2006) inferred using the Population Analysis with Reticulate Trees (popART v.1.7) (Leigh and Bryant, 2015). Only sequences of about 700 bp and 800 bp for *gltA* and *rpoB*, respectively, were used in the TCS network.

3. Results

All ruminants and arthropod DNA samples analyzed were positive to internal controls targeting the *gapdh*, 16S rRNA and/or *cox-1* genes, respectively.

Twenty-one (28%) and 13 (10.3%) cattle blood and *R. microplus*-DNA samples, respectively, were positive to *B. bovis* targeting the ITS *locus* (99-100% of identity) (Table 1). In addition, 57.1% (12/21) and 69.2% (9/13) cattle and *R. microplus*-ITS-positive DNA samples, respectively, were positive in the *ssrA* assay, sharing 99-100% of identity to *B. bovis*. *Bartonella* DNA was only detected in

engorged *R. microplus* female ticks collected from positive cattle. None of the tick-DNA samples obtained from *Bartonella*-negative cattle were positive in HRM assays. None of the larva (n=1), nymphs (n=11/pools) or male (n=13) tick-DNA samples were positive for *Bartonella*. Additionally, out of 101 buffaloes, 95 lice and 188 tick DNA samples, only one (1%) buffalo and four (4.2%) adult lice were positive for *Bartonella* targeting the ITS *locus*. Conversely, none of the ticks obtained from buffaloes were positive for *Bartonella* DNA.

Table 1. Number and animal species positive to *Bartonella* targeting the ITS *locus* and *ssrA*, *gltA* and *rpoB* genes.

Animals	**N**	% positive samples	**BLAST**	**Ectoparasite species**	**N**	% positive samples	**BLAST**
B. bubalis	101	1% (1/101)	(ITS)100% - *B. bovis*	*Rhipicephalus microplus*	187	0%	-
			(*ssrA*) 94% - *B. bovis*	*Amblyomma sculptum*	1	0%	-
			(*gltA*) 100% - *B. bovis*	*Haematopinus tuberculatus*	95	4.2% (4/95)	(ITS, *ssrA* and *gltA*) 100% - *Bartonella* sp. (clone Hq)
B. taurus	75	28% (21/75)	(ITS) 99-100% - *B. bovis*	*R. microplus*	125	10.4% (13/125)	(ITS) 99-100% - *B. bovis*
			(*ssrA*) 99-100% - *B. bovis*				(*ssrA*) 99-100% - *B. bovis*
			(*gltA*) 99-100% - *B. bovis*				(*gltA*)100% - *B. bovis*
			(*rpoB*) 99-100% - *B. bovis*	*A. sculptum*	1	0%	-
Total	**176**	**12.5% (22/176)**			**401**	**4.2% (17/401)**	

The *Bartonella* sequences identified in the only positive buffalo blood sample showed identities of 100% (ITS) and 94% (*ssrA*) with *B. bovis*. All *Bartonella* DNA sequences detected in lice were identical (100%) to an uncultured *Bartonella* sp. detected in cattle tail lice (*H. quadripertusus*) from Israel (Gutiérrez et al., 2014) (Table 1).

The *gltA* (n=14 [10 from cattle, two from ticks and two from buffaloes]) and *rpoB* (n=15 [all from cattle]) sequences showed identity ranging from 97.86% to 100% to *B. bovis* identified in cattle from different countries (KF199895 – France; KF199897 – Guatemala; KJ909808 – Israel and KR733192 – Malaysia). All amplified sequences showed query coverage of 100%. The *gltA* and *rpoB* sequences were deposited in GenBank under accession numbers: *rpoB*: MN615904-MN615918; *gltA*: MN615919-MN615937.

In agreement with BLASTn analysis, the *Bartonella* sequences detected from cattle, buffaloes, ticks and lice in the present study clustered with other ruminant-associated *Bartonella* species in both target genes (Figure 2).

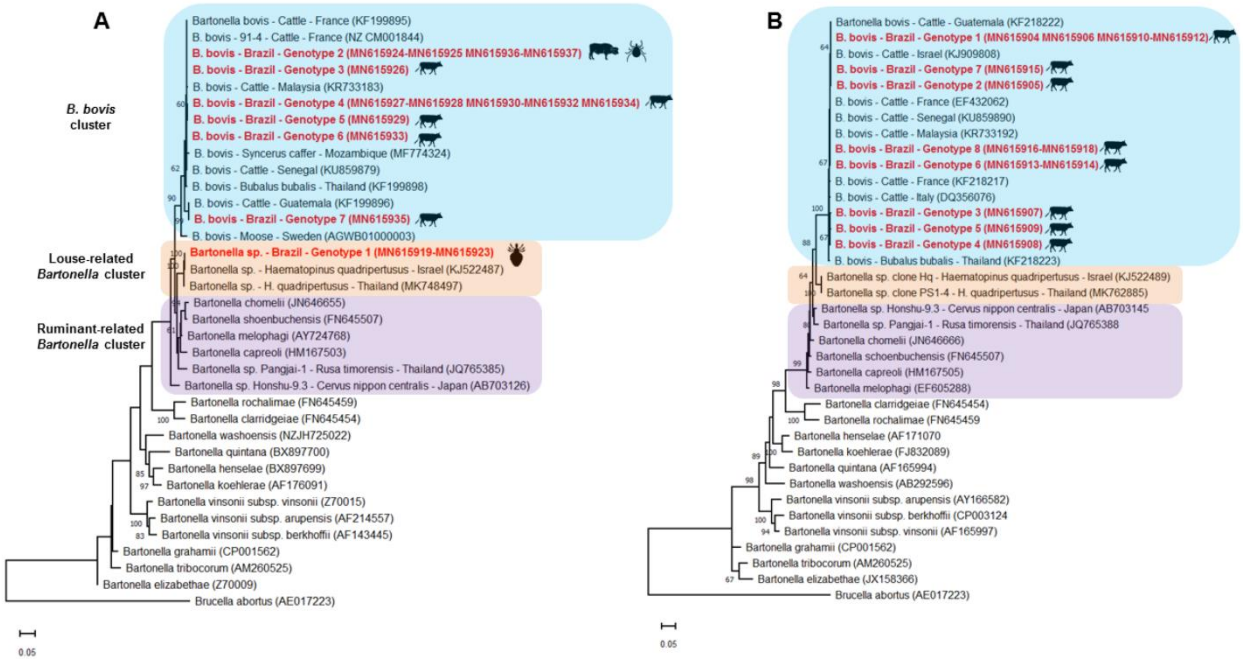

Figure 2. Phylogenetic relationships within the *Bartonella* genus based on the *gltA* **(A)** *and rpoB* **(B)** genes. The tree was inferred by using the maximum likelihood (ML) method with the GTR+G+I model. The sequences detected in the present study are highlighted in red. The numbers at the nodes correspond to bootstrap values higher than 60% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

Herein, three clones were obtained from 5 (4 cattle and 1 louse) out of 13 positive samples for *Bartonella* spp. targeting the *gltA* and *rpoB* genes. Among them, while 2 samples (1 cattle and 1 louse) showed three clones identical to each other, the other three samples (all from cattle) showed, at least, one and up to three different sequences.

The amplified sequences (*gltA*) and other related *Bartonella* species retrieved from GenBank (n=12) were distributed into 14 genotypes in the TCS network analysis (Figure 3). Three *B. bovis* genotypes identified in cattle in this study (Gen_3, Gen_5 and Gen_6) were distinct from any other genotypes analyzed to date. Besides, the other three *B. bovis* genotypes (Gen_2, Gen_4 and Gen_7) were previously identified in different countries. The Gen_2 included six sequences, four detected in buffalo and *R. microplus* ticks from the present study and the other two sequences identified in cattle from France (KF199895 and NZ_CM001844). Interestingly, two sequences identified in a tick (#tick27) were different from that reported in their associated host (#cattle27 - Gen_7). Seven sequences were classified as Gen_4, including six from cattle from the present study and one amplified from cattle from Guatemala (KF199897). Also, the Gen_7 comprised two sequences reported in cattle, one from the current study and another one (KF199896) from Guatemala (Figure 3). Finally, the Gen_1 comprises six sequences, five of them were identified in *H. tuberculatus* in the present study and another one was previously reported in *H. quadripertusus* (KJ522487) from Israel (Figure 3).

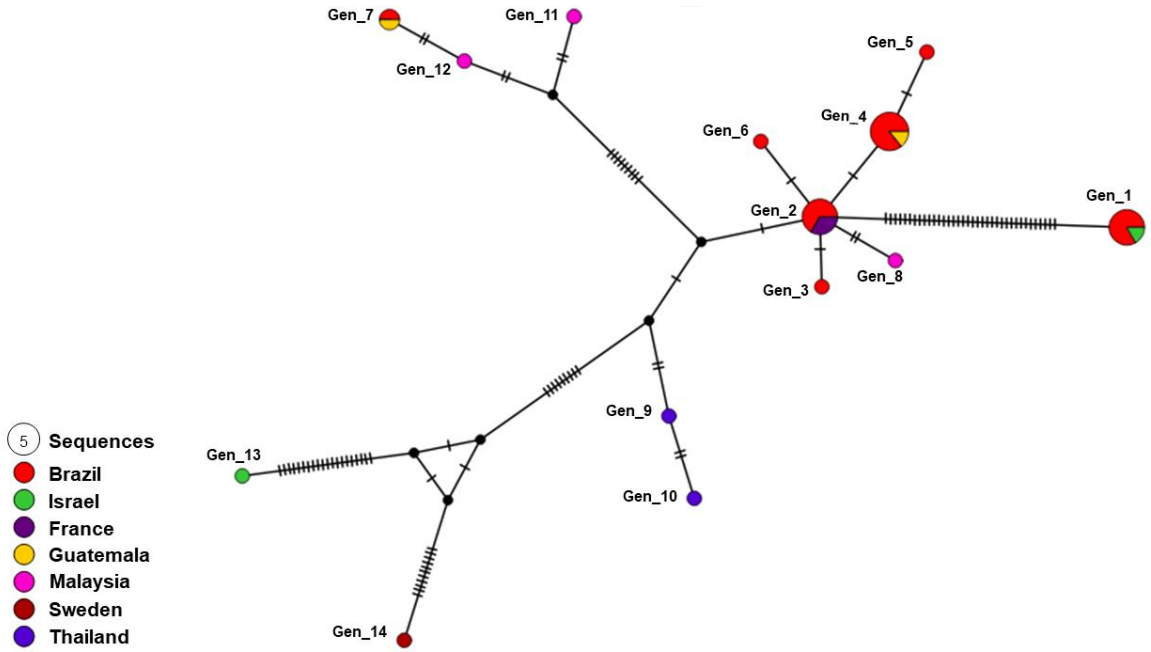

Figure 3. TCS network analysis of *gltA Bartonella* genotypes detected in cattle, buffalo, ticks and lice. Gen_= genotype. The traces refer to the nucleotide modification.

Likewise, the amplified *B. bovis* (*rpoB*) sequences and those retrieved from GenBank (n=19) were grouped into 16 genotypes (Figure 4). Five *B. bovis* genotypes detected in cattle from Brazil (Gen_2, Gen_3, Gen_5, Gen_6 and Gen_7) were distinct from any other genotypes analyzed (Figure 4). In addition, the other three genotypes (Gen_1, Gen_4 and Gen_8) were previously reported in cattle in different countries. Ten sequences were classified as Gen_1, including five *B. bovis* sequences identified in the current study, three (KJ909807-KJ909809) from Israel, one (KF218222) from Guatemala, and another one (AY166581) from France. In addition, the Gen_4 comprised two sequences, one sequence from cattle sampled in the current study, and another one (KF218221) from Guatemala. Finally, eight sequences were classified as Gen_8, comprising three from the present study, two (EF432061 and EF432062) from France, two (KU859890 and KU859891) from Senegal, and another one (KR733192) from Malaysia (Figure 4).

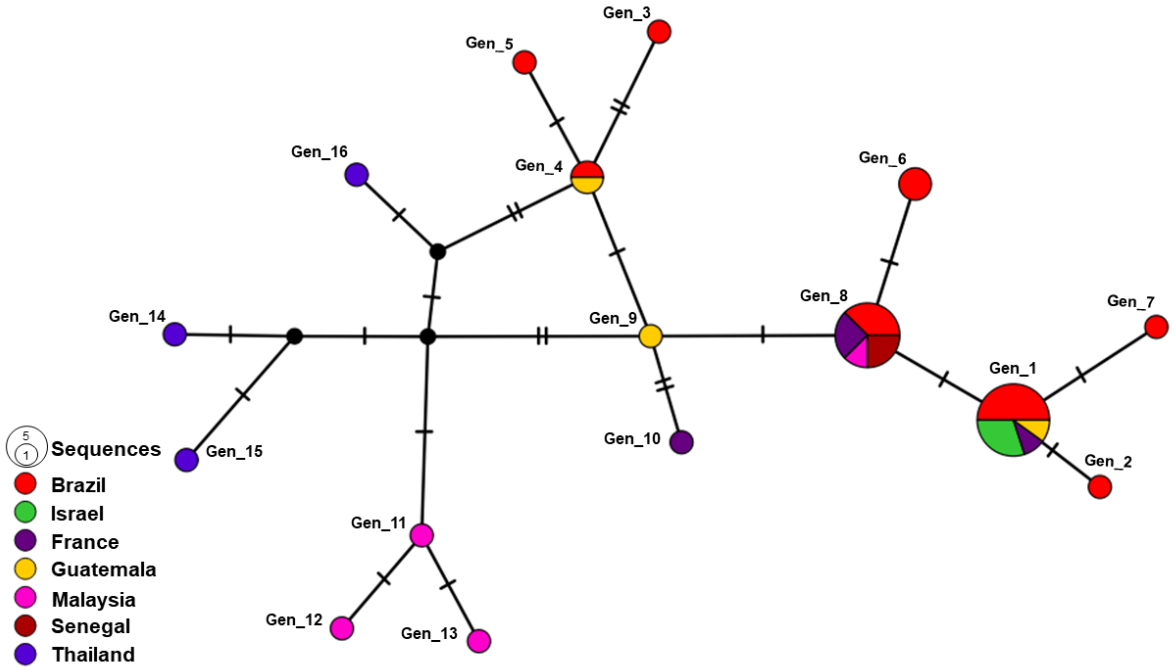

Figure 4. TCS network analysis of *rpoB Bartonella* genotypes detected in cattle. Gen_= genotype. The traces refer to the nucleotide modification.

The *gltA* and *rpoB B. bovis* sequences showed nucleotide diversity (π) of 0.003 and 0.002, respectively. The *Bartonella* sequences obtained from positive lice were identical to each other. The polymorphic level, number of variable sites and the average number of nucleotide differences for *B. bovis* sequences are shown in Table 2.

Table 2. Polymorphism and genetic diversity of *Bartonella* sequences detected in ruminants and associated ectoparasites from Brazil.

Species-Gene	(pb)	N	VS	GC %	h	Gd (mean ± SD)	π (mean ± SD)	k
B. bovis-gltA	750	14	6	38.8	7	0.769 ± 0.089	0.003 ± 0.002	2.96
B. bovis-rpoB	825	15	9	41.1	8	0.867 ± 0.067	0.002 ± 0.000	2.13
Bartonella from lice-*gltA*	750	5	0	39.3	1	0	0	0

N, number of sequences analyzed; VS, number of variable sites; GC, G+C content; h, number of haplotypes; Gd, genotype diversity; SD, standard deviation; π, nucleotide diversity (per site = PI); *K*, average number of nucleotide difference.

4. Discussion

In this study, we reported the prevalence and genetic diversity of *Bartonella* DNA sequences detected in large ruminants and associated arthropods sampled in Brazil. Although ruminant-associated *Bartonella* have been reported worldwide, to the best of our knowledge, this is the first report of ruminant-associated *Bartonella* in Brazil.

Bartonella bovis has been the most common species identified in cattle worldwide (Chang et al., 2000; Bai et al., 2013; Gutiérrez et al., 2014; Kho et al., 2015), except for Spain and New Caledonia, where *B. chomelii* was the most frequent (Antequera-Gomes et al., 2015) or the only species found (Mediannikov et al., 2011). Accordingly, in the present study, only *B. bovis* DNA was detected in cattle and associated ticks, and in the only positive buffalo. Herein, we found a relatively high prevalence of *Bartonella* DNA in cattle (28%), and a low prevalence in buffaloes (1%). Interestingly, *Bartonella* prevalence varied widely between different countries and between distinct regions in the same country (Bai et al., 2013). Since *Bartonella* species are mainly vector-transmitted, coupled with the lack of evidence for transplacental transmission of *B. bovis* (Chastant-Maillard et al., 2015), it has been speculated that the prevalence and abundance of specific arthropods play a crucial role in the *Bartonella* prevalence in these mammals (Bai et al., 2013). Although *R. microplus* has been suggested as a potential vector of *Bartonella* between cattle (Tsai et al., 2011), we did not find confirming evidence for this hypothesis in our study. Our results showed that the *B. bovis* DNA was present only in engorged *R. microplus* female ticks sampled from positive cattle, whereas all nymphs, larvae and male tick samples were negative. These results, coupled with the fact that *R. microplus* is a one-host tick, suggest that *R. microplus* probably do not play an important role in the transmission of *B. bovis* among cattle in Brazil. However, it is necessary to highlight that we reported the presence of different genotypes in a tick (Gen_2) and its respective host (Gen_7). A possible explanation for the latter finding could be attributed to the prevalence of both genotypes in cattle, which were not identified by our screening methods. Therefore, further studies are needed in order to elucidate the role of *R. microplus* as well as other blood-sucking arthropods (e.g. *Stomoxys calcitrans* and *Haematobia irritans*) in the *B. bovis* life cycle.

The *Bartonella* genotype found in buffalo lice was identical to those previously reported in *Haematopinus* lice from Israel (Gutiérrez et al., 2014). Furthermore, a closely related genotype was also recently identified in cattle tail lice from Thailand (Promrangsee et al., 2019). As opposed to the study performed in Thailand that sampled only lice, Gutiérrez et al. (2014) screened both cattle and lice samples and reported that *Bartonella* identified in either cattle blood and lice were different. Similarly, in this study, the genotype identified in lice was not detected in buffalo blood samples. Remarkably, this *Bartonella* variant shows low genetic diversity, and the sequences reported in *Haematopinus* lice from far-distant geographical sites are virtually identical. Previously, *B. melophagi* has been suggested as a potential endosymbiont of sheep keds (*Melophagus ovinus*) (Halos et al., 2004), and a similar evolutionary process between cattle tail louse and its detected *Bartonella* variant was suggested (Gutiérrez et al., 2014), thus we may face an equivalent event. As the relationship between this louse-associated *Bartonella* genotype and *Haematopinus* spp. is unknown, further studies elucidating this question are required.

While a previous study conducted in Mexico suggested a limited potential for transmission of *Bartonella* spp. by bites of vampire bats to their prey (Raya et al., 2018), recent studies conducted in Belize, Peru (Becker, Bergner, et al., 2018) and Brazil (André et al., 2019) highlighted the chance of *Bartonella* transmission by vampire bat' bites.

Even though we did not perform isolation of *Bartonella*, the cloning approach allowed a better resolution of the genetic diversity of the bacteria circulating in the sampled animals. Interestingly, while in 2 animals only one genotype was identified, up to three different genotypes were found in 3 animals. In an extensive study that sampled cattle from five countries and based on nine loci, Bai et al. (2013) demonstrated three closely related but distinct lineages of *B. bovis*, suggesting a clonal population structure for this species with a geographical particularity. Additionally, the authors hypothesized that two *Bartonella* lineages (i.e. I and II) could be associated with the "taurine" (*Bos taurus taurus*) and "zebu" (*Bos taurus indicus*) cattle lineages, respectively. Finally, a third lineage (i.e. III) was correlated with the water buffalo. In contrast, some sequences identified in zebu cattle in the present study shared the same genotype with sequences previously detected in taurine cattle

from France, Israel and Senegal. Since livestock trade play a central role in the cattle movements associated with the co-grazing of animals originating from different places and breeds, the association of *B. bovis* lineages to cattle breeds should be analyzed with caution. However, future studies are required to endorse these findings. Moreover, the *B. bovis* genotype identified in this study in a buffalo was not phylogenetically positioned near the *B. bovis* lineage (i.e. III) formerly reported in water buffaloes from Thailand (Bai et al., 2013). Instead, the buffalo sequences clustered together with other *B. bovis* sequences identified in cattle and cattle-ticks. A potential explanation for the latter finding may suggest an exchange of ruminant-associated *Bartonella* species between cattle and buffaloes in this geographic area, since the farm where the buffaloes were sampled had a close contact with cattle from the neighboring farms. As no experimental study has been performed aiming to identify whether the different *B. bovis* lineages have any specificity to different ruminants, further studies are required to confirm the hypothesis previously raised.

The genetic diversity is driven by distinct process, including but not restricted to mutation, recombination and demography. The genetic analysis performed in the current study suggests that the *B. bovis* genetic diversity is lower than those reported among rodent-associated *Bartonella* upon comparison with *gltA* sequences obtained in rodents from France (π = 0.077) (Buffet et al., 2013) and Brazil (π = 0.024) (Gonçalves et al., 2016) as previously described (Bai et al 2013). Despite the authors have been reported different *B. bovis* sequence types (STs) in ruminants from three countries, all STs were, in fact, very close to each other (Bai et al 2013). Even though *B. bovis* is known to be widely distributed in cattle worldwide, few studies have assessed the genetic diversity of this *Bartonella* species and the association of these different genotypes to pathogenicity.

In conclusion, this study demonstrated the prevalence of *Bartonella* in cattle, buffaloes and associated ectoparasites in Brazil, and that *B. bovis* was the most prevalent species reported to be circulating in the animals sampled. In addition, we reported distinct *B. bovis* genotypes in cattle. The genotypes identified in zebu cattle were identical to those previously reported in taurine cattle. Finally, our findings demonstrated the prevalence of a lice-related *Bartonella* in Brazil closely related to those reported in lice from Israel and Thailand. These findings shed light on the

distribution and genetic diversity of ruminant and ectoparasites-related *Bartonella* in Brazil.

Ethical statement: All procedures were carried out according to the ethical guidelines for the use of animal samples permited by the Institucional Care and Use Committee (IACUC) of Universidade Estadual Paulista (FCAV/UNESP), Jaboticabal, São Paulo - under protocol number: 01952/18.

Conflicts of Interest: The authors declare no conflict of interest.

Data Availability statement: The data that support the findings of this study are openly available in [National Center for Biotechnology Information] at [https://www.ncbi.nlm.nih.gov/], reference number [*rpoB*: MN615904-MN615918; *gltA*: MN615919-MN615937].

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CHAPTER 5 – Molecular detection of *Bartonella* species and hemoplasmas in wild African buffalo (*Syncerus caffer*) in Mozambique, Africa[4]

Abstract

The African buffalo (*Syncerus caffer*), a mammal species whose population in declining, can play a role as a reservoir or carrier of a wide number of arthropod-borne pathogens. Translocation procedures have been used as an alternative approach for species conservation. However, the veterinary aspects of this sort of procedures are extremely important to minimize the impact on animal health. In order to detect *Bartonella* and hemoplasmas, two important group of bacterial that have impact in both human and animal health, EDTA whole-blood samples were screened for the presence of these bacterial pathogens by molecular techniques. As a result, a molecular occurrence of 4.1% and 15.4% for *Bartonella* spp. and hemoplasmas, respectively, was reported among 97 wild buffaloes sampled during a translocation procedure from Marromeu to Gorongosa Reserve, Mozambique. Additionally, phylogenetic analyses of the obtained sequences were conducted. At least, three bovine-associated pathogens, namely *B. bovis*, *M. wenyonii* and '*Candidatus* M. haemobos', as well as a probably new *Bartonella* genotype/species were detected in *S. caffer.* Further studies are needed in order to determine whether these bacterial species may cause impact in buffaloes and other sympatric ruminant species living in the release site.

Keywords: African buffalo; *Bartonella*; Hemoplasmas; Phylogenetic assessment; *Syncerus caffer*

Introduction

The African buffalo (*Syncerus caffer*) plays a role as a reservoir or carrier of a wide number of arthropod-borne pathogens, such as *Theileria*, *Ehrlichia*, *Babesia* and *Anaplasma* species (Andrew and Norval, 1989; Allosopp *et al.* 2002; Eygelaar *et al*. 2015; Machado *et al*. 2016). Moreover, buffaloes are known to harbor other economically important infectious pathogens, such as *Mycobacterium tuberculosis*, foot-and-mouth disease virus and *Brucella abortus* (Godfroid, 2002; Michel *et al.* 2006; Van Schalkwyk *et al.* 2016). Although widely distributed throughout the sub-Saharan Africa, the African buffaloes are currently confined to protected areas. The species distribution and numbers have been strongly reduced by habitat loss and hunting (IUCN – Downloaded in 29 July 2016).

[4] Este capítulo corresponde ao artigo publicado na revista Parasitology Open. 4, e15, 2018.

Many native and exotic animal species have been selected for translocations procedures around the world (Seddon *et al*. 2014; Soorae, 2016). The translocation is defined as human-mediated movement of living organisms from one area to another (Woodford and Rossiter, 1993). Although the translocation of endangered species has become an important conservation approach, the project success depends to a large extent on the care with which wildlife biologists and veterinarians evaluate the suitability of the chosen release site (Woodford and Rossiter, 1993). Adequate managements are extremely important for the translocation procedures, since the introduction of pathogens into naive resident wildlife community or the translocation of animals from free-vectors and pathogens sites to enzootic areas can be catastrophic.

In this context, *Bartonella* species and hemotropic mycoplasmas (also known as hemoplasmas) emerge as important arthropod-borne pathogens that have impact in humans and animals' health (Maggi *et al*. 2013a; Breitschwerdt, 2014).

The *Bartonella* genus comprises a successful group of Gram-negative bacteria parasites (Birtles, 2005), which infects mainly erythrocytes and endothelial cells from a wide range of animal species, including humans (Breitschwerdt *et al*. 2010; Harms and Dehio, 2012). This success is characterized by the high prevalence of the infection and diversity of host species (Birtles, 2005; Kosoy *et al*. 2012). Currently, five (*B. bovis*, *B. chomelii*, *B. schoenbuchensis*, *B. capreoli* and *B. melophagi*) out of the 36 named *Bartonella* species/subspecies described have been associated with ruminants (Buffet *et al*. 2013; Breitschwerdt, 2017).

Contrariwise, hemoplasmas are cell wall-less uncultivated epicellular bacteria that attach to red blood cells surface of a wide range of animals, including humans (Neimark *et al*. 2001; Maggi *et al*. 2013b). Among the hemoplasmas, *Mycoplasma ovis*, *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haemobos' have been recognized as pathogens of domestic ruminants worldwide. In addition, an expanding number of *Candidatus* to new *Bartonella* and *Mycoplasma* species/genotypes have globally been reported in ruminants (Stoffregen *et al*. 2006; Watanabe *et al*. 2010; Sato *et al*. 2012; Maggi *et al*. 2013b; Dahmani *et al*. 2017).

Although the impact of bartonellae and hemoplasmas in livestock is still unknown, the gaps on the biology of these bacteria warrant further investigation.

Indeed, there are limited data on the occurrence, distribution, genetic diversity, pathogenicity and transmission of arthropod-borne agents among wild ruminants.

The elucidation of these bacterial cycles in nature, including the identification of hosts, vectors and the species distribution in a particular ecotope shows great importance (Gutiérrez *et al*. 2014). Additionally, the African buffalo plays a role as reservoirs for vector-borne pathogens and may represent serious threat to the livestock industry (Andrew and Norval, 1989; Allosopp *et al.* 1999; Eygelaar *et al*. 2015; Van Schalkwyk et al. 2016).

Therefore, the present study aimed to investigate the occurrence of *Bartonella* and hemoplasmas infection in wild buffaloes (*S. caffer*) submitted to translocation in Sofala province, Mozambique, Africa.

Material and methods

Blood collection of African buffaloes

The present study was carried out when Carlos L. Pereira was the Director of Conservation Gorongosa National Park, Mozambique. Animal management and welfare during the field work with Cape buffaloes were conducted in accordance with national legislation on the use of animals for research implemented by the National Administration for the Conservation Areas (ANAC) of Mozambique. In 2011, blood samples were collected from 97 wild African buffalo (*S. caffer*) in Marromeu Reserve, Mozambique. This reserve is a special buffalo protection area located in the Marromeu district (Sofala Province) **(Figure 1)**, with an area of 1.500 km² (www.jenmansafaris.com). Sampled animals were apparently healthy young male and female individuals. Approximately 10 mL of blood samples were collected of each animals before they were transferred from Marromeu Reserve (site where there was contact with cattle) to the Gorongosa National Park (site where there was no contact with cattle), distant around 300 kilometers from each other. The EDTA-blood were mixed (v/v) with ethanol, transported to the laboratory and posterior kept at -20°C until sent an aliquot (~1 mL) to Brazil (Machado *et al*. 2016; Rodrigues *et al.* 2017).

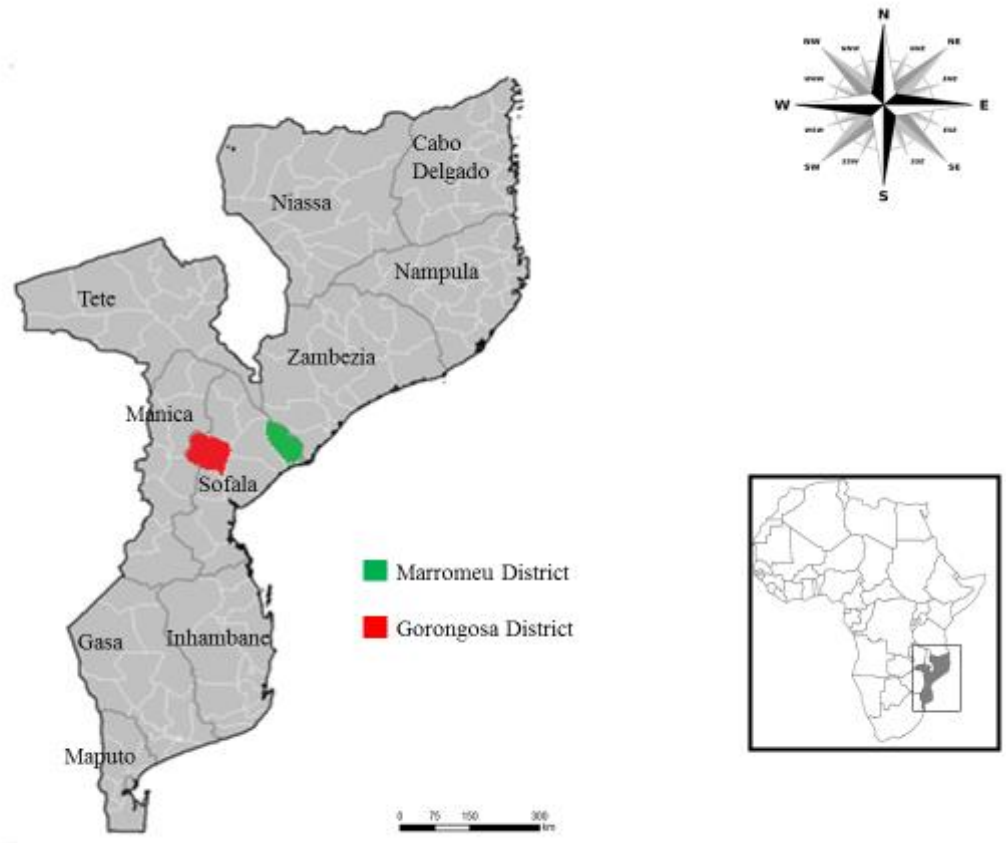

Figure 1. Map of Mozambique country highlighting the districts where the buffalo were translocated.

Blood samples and DNA extraction

EDTA-blood samples collected from buffaloes were mixed (v/v) with ethanol for further DNA extraction. In Brazil, the blood samples from these buffaloes were incubated in a lysis buffer (1% SDS, 100 mM EDTA at pH 8.0, 20 mM Tris-HCl at pH 8.0 and 350 mg/ml of proteinase K) at 37 °C for 18 h and centrifuged at 14.000 *g* for 5 min. The DNA was purified using Wizard Purification Systems (Promega). The concentration of each DNA sample was determined in a NanoDrop 2000c spectrophotometer (Thermo Scientific, San Jose, CA, USA) (Machado *et al.* 2016).

Molecular diagnosis of* Bartonella *and hemoplasmas species in African buffalo blood samples

A previously described broad range Taqman quantitative real-time PCR (qPCR) protocol based on *nuoG* gene was used aiming to detect *Bartonella* species

DNA as previously described (André *et al*. 2016). The qPCR amplifications were conducted in low-profile multiplate unskirted PCR plates (BioRad, CA, USA) using a CFX96 Thermal Cycler (BioRad, CA, USA). Serial dilutions were performed aiming to construct standard curves with different concentrations of plasmid DNA (pIDTSMART - Integrated DNA Technologies) ($2.0x10^7$ to $2.0x10^0$ copies/µL) encoding an 83 bp insert of *nuoG Bartonella henselae* (André *et al.* 2016). qPCR assays were performed including duplicates of each buffalo DNA sample. All the duplicates showing a difference in Cq values higher than were re-tested. Amplification efficiency (*E*) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/slope}$).

Additionally, two specific conventional PCR (cPCR) protocols based on 16S rRNA were used to amplify *M. wenyonii* (~530 bp) and "*Candidatus* M. haemobos" DNA (279 bp) as previously described (Nishizawa *et al*. 2010; Su *et al*. 2010). *Bartonella* (KX086714), "*Candidatus* M. haemobos" (KY328834) and *M. wenyonii* (KY328836) DNA samples previously obtained from naturally infected rodents and buffaloes (Gonçalves *et al*. 2016; Santos *et al*. 2018), respectively, were used as positive controls. Ultra-pure sterile water and blood samples from calves previously tested negative for both pathogens were used as negative controls in all PCR assays described above. In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms.

Molecular characterization of* Bartonella *and hemoplasma species

Samples from qPCR assays-positive buffalo were submitted to cPCR assays targeting four protein-coding genes with great potential to differentiation among related *Bartonella* species (La Scola *et al*. 2003; Kosoy *et al*. 2017), namely *gltA* (350 bp), *rpoB* (825 bp), *ftsZ* (515 bp) and *groEL* (752 bp) genes, as previously described (Norman *et al*. 1995; Birtles and Raoult, 1996; Renesto *et al*. 2001; Zeaiter *et al*. 2002; Paziewska *et al*. 2011). *Bartonella* DNA previously detected in rodents was used as positive control (Gonçalves *et al.* 2016). On the other hand, in order to better characterize the initial PCR assay results, hemoplasma-positive buffalo samples were additionally submitted to a cPCR assay targeting a 16S rRNA larger fragment (~800 bp), as previously described (Maggi *et al*. 2013b). *Mycoplasma haemofelis*

DNA previously detected in cats in Brazil was used as positive control (Santis *et al.* 2014).

All cPCR products were purified using Silica Bead DNA Gel Extraction Kit (Fermentas, SP, Brazil). Purified amplified DNA fragments were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser – Applied Byosystem/ Perkin Elmer) in both directions using the same primers used for PCR detection. In order to correctly determine the nucleotide composition, the electropherograms were submitted to PhredPhrap analysis (Ewing *et al*. 1998). The Phred quality score (peaks around each base call) was established higher than 20 (99% in accuracy of the base call). Subsequently, the sequences were submitted to BLASTn and phylogenetic analyses.

Phylogenetic analyses of gltA, ftsZ and 16S rDNA sequences

The sequences obtained from *gltA* and *ftsZ Bartonella* and 16S rRNA hemoplasmas cPCR assays were identified by BLASTn, using the Megablast (highly similar sequences – using default parameters). Subsequently, obtained sequences were aligned with those retrieved from GenBank database using Clustal/W (Thompson *et al*. 1994), adjusted in Bioedit v. 7.0.5.3 (Hall, 1999), and submitted to phylogenetic analysis. The Maximum Likelihood (ML) phylogenetic analysis was inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis *et al*. 2008). Also, in order to perform a robust phylogenetic analysis among the protein-coding gene sequences, the *Bartonella gltA* and *ftsZ* nucleotide sequences were submitted to other two methods. Phylogenetic analysis based on Bayesian inference (BI) was done using MrBayes on XSEDE (v. 3.2.6) (the *a posteriori* probability values higher than 50% were accessed with 10^6 replicates; the first 25% trees were discarded as burn-in). Finally, these sequences were analyzed by Neighbor-Joining (NJ) using the MEGA5.05 software. The Akaike Information Criterion (AIC) available on MEGA 5.05 software was applied to identify the most appropriate model of nucleotide substitution.

Results

Occurrence and molecular characterization of Bartonella *and hemoplasma species in African buffalo*

Out of 97 African buffalo blood samples submitted to *Bartonella*-qPCR, four (4.1%) were positive. The mean amplification efficiency was *E* = 90.7% ([ranging from 90.5% to 90.9%]; slope = -3.568; r^2 = 0.996). All blood samples showed low number of *Bartonella*-DNA copies/µL (#45 = 0.046 x 10^2; #50 = 0.041 x 10^2; #62 = 0.037 x 10^2; and #44 = 0.002 x 10^2 copies/µL). Among the four blood samples positive to *Bartonella* in qPCR assay, only the sample #62 showed to be positive in *gltA* and *ftsZ* cPCR assays. None blood samples showed positive results in cPCR assays targeting *rpoB* and *groEL* genes. The *gltA* nucleotide sequence amplified in the present study shared 99% identity with *B. bovis* (KF199897) detected in cattle from Guatemala (Bai *et al*. 2013). On the other hand, the obtained *ftsZ* nucleotide sequence shared 98% identity with *Bartonella* sp. (AB703117) previously detected in a Japanese Sika deer (*Cervus nippon centralis*) (Sato *et al*. 2012).

Additionally, 15.4% (15/97) samples were positive to hemoplasmas. Among them, 14.4% (14/97) and 4.1% (4/97) showed positive results to *M. wenyonii*, and '*Candidatus* M. haemobos', respectively. Four (4.1%) samples were simultaneously positive to *M. wenyonii* and '*Candidatus* M. haemobos'. The two hemoplasmas amplicons sequenced shared 99% and 99% identity with *M. wenyonii* (KX171205) and '*Candidatus* M. haemobos' (EF616468) nucleotide sequences detected in cattle from Mexico and Switzerland, respectively. None sample was simultaneously positive to *Bartonella* and hemoplasmas. The 16S rDNA sequences amplified in the present study showed query coverage ranging from 99 to 100%. All nucleotide sequences submitted to BLASTn and phylogenetic analyses were deposited in GenBank under the following access numbers: *Bartonella* (MF774324-MF774325) and hemoplasmas (MF981847 and MF992084).

Phylogenetic analysis

The phylogenetic analyses performed among the protein-coding gene sequences (*gltA* and *ftsZ*), using different methods (ML, MI and NJ) yielded congruent tree topologies **(Figures 2-3 and S1-S4).**

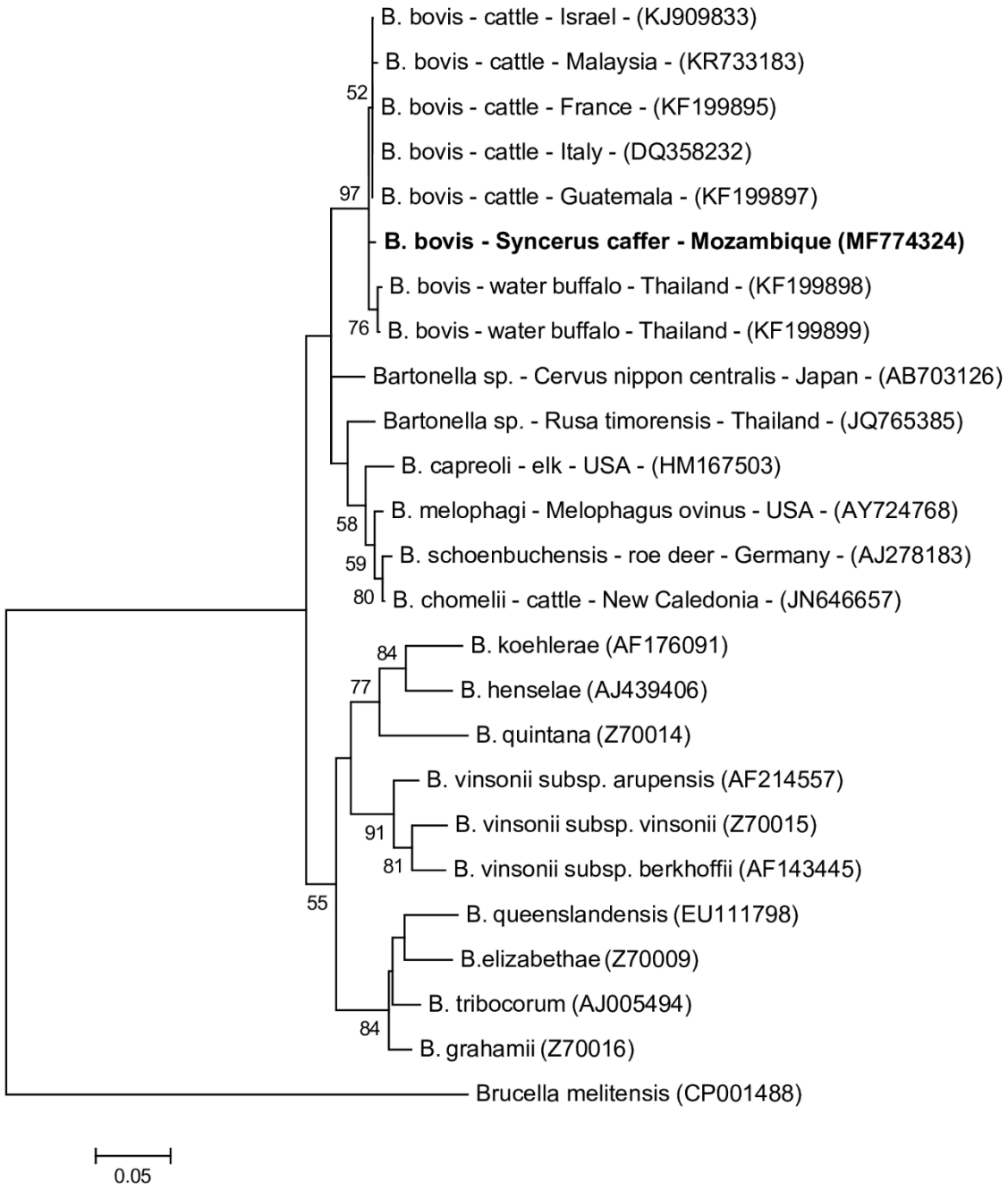

Figure 2. Phylogenetic relationships within the *Bartonella* genus based on the *gltA* gene. The tree was inferred by using the maximum likelihood (ML) method with the GTR+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

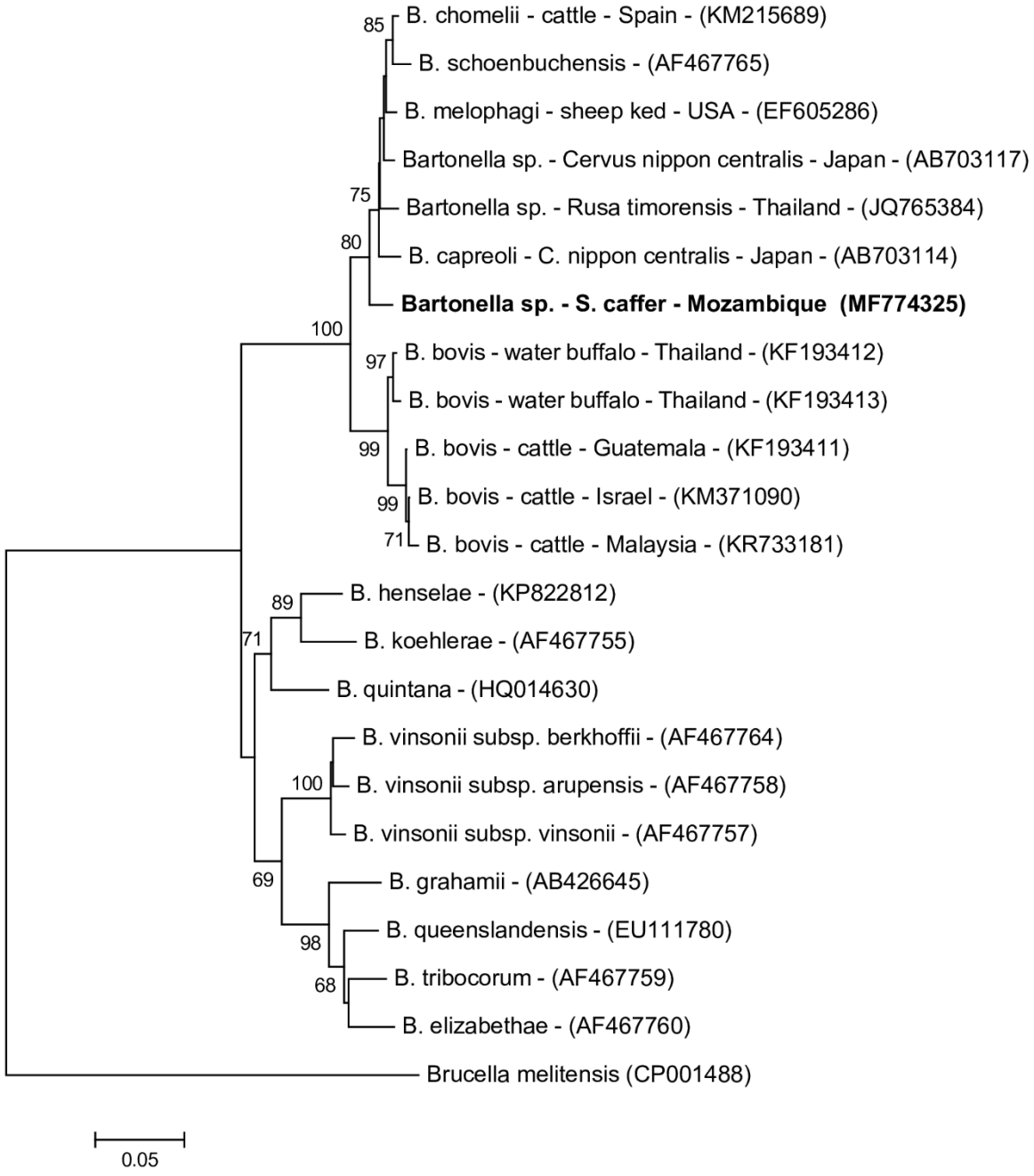

Figure 3. Phylogenetic relationships within the *Bartonella* genus based on the *ftsZ* gene. The tree was inferred by using the maximum likelihood (ML) method with the GTR+G model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

Additionally, according to BLASTn analysis, the *Bartonella gltA* and *ftsZ* sequences amplified from the same animal (#62) showed a distinct phylogenetic positioning in ML, BI and NJ analyses **(Figures 2-3 and S1-S4)**. The *gltA* sequence (MF774324), which shared 99% identity with *B. bovis*, clustered with other *B. bovis* sequences, including *B. bovis* sequences previously amplified in water buffalo from Thailand (Bai *et al*. 2013), showing high support index (ranging from 97% to 100%) (**Figure 2 and S1-S2)**. On the other hand, although the *ftsZ* sequence (MF774325) clustered with other ruminant-associated *Bartonella* sequences, it was not closely related to any of these sequences, remaining in a separate branch in all different methods and supported by a high support index (ranging from 80% to 100%) (**Figure 3 and S3-S4)**.

Also in agreement to BLASTn analysis, the 16S rRNA sequences (MF981847 and MF992084) belonging to *M. wenyonii* and to '*Candidatus* M. haemobos' when submitted to ML analysis were phylogenetic positioned near to other *M. wenyonii* and '*Candidatus* M. haemobos' sequences, respectively, detected around the world and supported by high bootstrap values (87 and 100%, respectively) **(Figure 4)**.

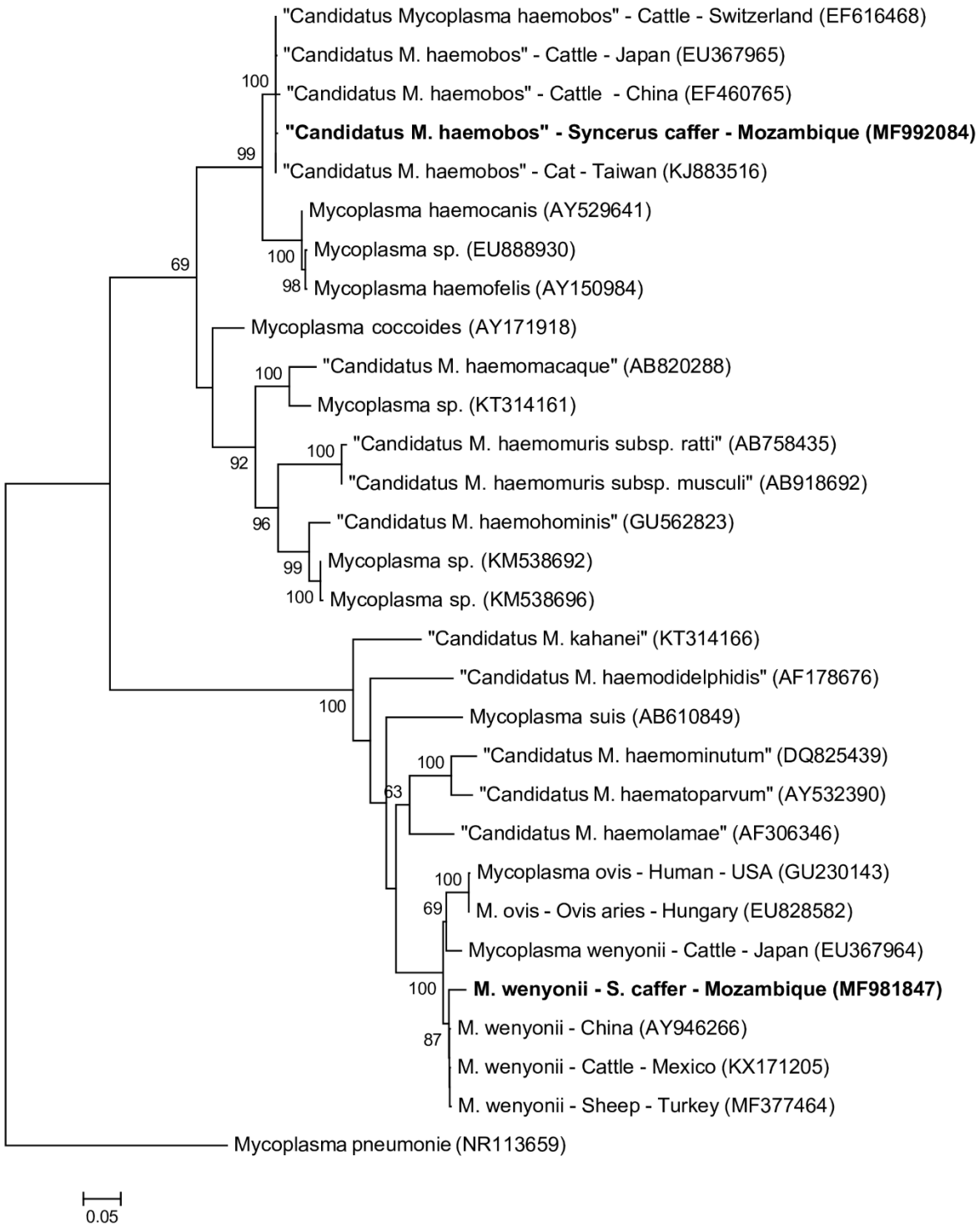

Figure 4. Phylogenetic relationships within the *Mycoplasma* genus based on a fragment of 800 bp of the 16S rRNA gene. The phylogenetic tree was inferred by using the maximum likelihood method with the GTR+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to boostrap values higher than 60% accessed with 1000 replicates. *Mycoplasma pneumoniae* was used as outgroup.

Discussion

In the present study, the occurrence of *Bartonella* and hemoplasmas species was assessed in African buffaloes translocated from Marromeu to Gorongosa National Park in Mozambique. Although *Bartonella* and/or hemoplasmas species have been previously reported in domestic animals (dogs, cats and cattle) (Gundi *et al*. 2004; Willi *et al*. 2006; Dahmani *et al*. 2017), wild animals (rodents, bats and cheetahs) (Kosoy *et al.* 2010; Kamani *et al*. 2013; Krengel *et al*. 2013) and hematophagous arthropods (soft ticks and bat flies) (Billeter *et al.* 2012; Mediannikov *et al*. 2014) from African continent, the present work presented, for the first time, the occurrence of these arthropod-bacteria species in *S. caffer*.

Similarly to the occurrence of *Bartonella* in water buffalo (*Bubalus bubalis* [6.8%; 7/103]) from Thailand (Bai *et al*. 2013), a low occurrence was observed among the animals analyzed in the present study (4.1%; 4/97). Additionally, the prevalence of hemoplasmas reported in the African buffaloes was lower than that previously reported in buffaloes (32%; 8/25) from China (Su *et al.* 2010). However, it is important to highlight that the animals selected in the latter study were showing different clinical signs, such as emaciation, anorexia, and decreased milk yields (Su *et al*. 2010). In addition to animal health status, the difference in occurrence of *Bartonella* and hemoplasmas observed in different countries have been attributed to different factors, such as distribution and abundance of arthropod vectors, and environment and landscape features, which could influence the exposure to these agents (Bai *et al*. 2013).

Syncerus caffer is frequently infested by species of *Hyalomma*, *Rhipicephalus,* and *Amblyomma* ticks (Carmichael, 1976; Anderson *et al*. 2012; Kariuki *et al*. 2012). These ticks are responsible for transmission of several pathogens frequently reported in African buffalo, such as *Theileria* spp., *Anaplasma* spp., *Ehrlichia* spp. and *Babesia* spp. (Andrew and Norval, 1989; Eygelaar *et al*. 2015; Machado *et al*. 2016). Therefore, it suggests that *Bartonella* and hemoplasma species detected in *S. caffer* in the present study may be potentially transmitted by these tick species. In addition to possible role of ticks in the transmission of *Bartonella*, several studies have reported the isolation or molecular detection of *Bartonella* species in other blood-sucking arthropods associated with wild and domestic ruminants (Halos *et al.* 2004;

Dehio *et al.* 2004; Chung *et al.* 2004; Duodu *et al*. 2013; Gutiérrez *et al.* 2014). Regarding the hemoplasma transmission, few studies have accessed the mechanism of transmission by arthropod vectors. Prullage *et al.* (1993) demonstrated the mechanical transmission of *Mycoplasma suis* by *Stomoxys calcitrans* and *Aedes aegyptii* among susceptible splenectomized pigs and Woods *et al.* (2005) showed that *Ctenocephalides felis* is a possible vector of *Mycoplasma haemofelis* and *'Candidatus* M. haemominutum' among cats. On the other hand, bovine-associated *Mycoplasma* species were molecularly detected in ticks belonging to *Dermacentor andersoni* (Neimark *et al.* 2001), *Rhipicephalus (B) microplus* and *Haemaphysalis bispinosa* species (Mohd Hasan *et al.* 2017) and among horn fly (*Haematobia irritans*), stable fly (*Stomoxys calcitrans*) and horse flies (*Tabanus* spp.) (Hornok *et al.* 2011), house fly (*Musca domestica*) and lice (*Haematopinus erysternus*) (Hofmann-Lehmann *et al.* 2004). These findings suggested that these arthropods may have an active role in the maintenance and transmission of ruminants-associated *Bartonella* and hemoplasma species. However, experimental studies aiming to analyze the vectorial competence of selected arthropod species are much needed.

In contrast to the phylogenetic analysis (ML) performed with hemoplasmas sequences amplified from *S. caffer* blood samples, which clustered with other *M. wenyonii* and '*Candidatus* M. haemobos' sequences detected around the world, the *Bartonella gltA* and *ftsZ* sequences detected in the same animal (#62) were positioned in different branches in all different methods analyzed (ML, BI and NJ). This uncertainty about the phylogenetic positioning could be explained by two hypotheses. Firstly, there would have been a coinfection with different *Bartonella* species/genotypes (*B. bovis* identified by *gltA* sequence, and a new *Bartonella* genotype closely related to other ruminant-associated *Bartonella* identified by *ftsZ* sequence). Alternatively, the amplified sequences might have represented an infection with a genotype that went through recombinant events. Indeed, the latter phenomena have been already reported in *Bartonella* species from wild rodents (Harrus *et al*. 2009; Paziewska *et al*. 2011), cattle (Guitiérrez *et al*. 2014) and bats (Bai *et al*. 2015). These distinct possibilities reinforce the great challenge on *Bartonella* identification based on direct molecular detection in blood, tissue or ectoparasite samples (Guitiérrez *et al*. 2014; Kosoy *et al.* 2017). Although the

multiple loci sequencing approach was attempted in the present study for a better understanding of sequences phylogenetic positioning and possible recombinant events and/or infection by multiple *Bartonella* species (Kosoy *et al*. 2017), the unique positive sample (#62) in cPCR assays was negative in PCR targeting additional genes (*rpoB* and *groEL*), thus precluding to solve this issue. Additional culturing of isolates would have benefited the differentiation of these genotypes/species (Kosoy *et al.* 2017).

Considering that buffaloes usually do not show clinical signs of tick-borne diseases, a surveillance on this animal species is much needed, since they act as carriers for arthropod-borne pathogens to the livestock (Andrew and Norval, 1989; Allosopp *et al.* 1999; Eygelaar *et al*. 2015; Van Schalkwyk et al. 2016). Furthermore, since these animals can migrate over large distances, they could spread different pathogens for susceptible wildlife. In the present study, at least three bovine-associated pathogens, namely *B. bovis*, *M. wenyonii* and '*Candidatus* M. haemobos' were reported in *S. caffer*. Regarding the pathogenic potential of the above mentioned agents, while *B. bovis* have been associated with bovine endocardits (Mailard *et al*. 2007; Erol *et al*. 2013), bovine hemoplasmas species have been associated with anemia, transient fever, decreased milk production, anorexia, weight loss and infertility (Smith *et al.* 1990; Su *et al*. 2010; Hoelzle *et al*. 2011).

In the Marromeu Reserve, site where animals were caught, buffaloes and cattle used to share the same area. Although little is known about the origin, evolution and dispersion of *Bartonella* and hemoplasmas species, the low prevalence detected in African buffaloes, mainly to *Bartonella*, coupled to a wide distribution and the constant detection of these bacterial in cattle around the world, suggest that these pathogens may have been transmitted via arthropod-vectors from cattle to buffaloes. On the other hand, there is no contact among cattle and wildlife in Gorongosa National Park, the site where the animals were released. Therefore, the sylvatic animals residing in this area, mainly other ruminant species may be exposed to *B. bovis, M. wenyonii*, '*Candidatus* M. haemobos' and *Anaplasma* spp., as previously reported (Machado *et al*. 2016). Although it is difficult to assess the impact of these infections in such population, this situation can be catastrophic to immunologically naïve wildlife (Woodford and Rossiter, 1993).

Even though translocation procedures have intended conservation benefits, this attempt may also impose risks to animal health (IUCN/SSC, 2013). During management procedures, it is important to consider that a translocated animal does not represent a single species, but is rather a biological package containing a selection of viruses, bacteria, protozoa, helminths and arthropods. Different reports have showed the complexity of the translocation procedures, either by diseases introduced through the animals or diseases contracted from the resident animals at the release site (reviewed by Woodford and Rossiter, 1993). Therefore, the veterinary aspects, prior, during and after translocation procedures are extremely important in order to minimize the impact on animal health. Although few studies have been conducted regarding arthropod-borne pathogens in the capture (Marromeu Reserve) and the release (Gorongosa National Park) sites, Rift Valley fever phlebovirus was detected on buffaloes and cattle sampled between 2013 and 2014 from both places (Moiane *et al.* 2017). Additionally, *Trypanosoma vivax* and *T. vivax*-like DNA was amplified in buffaloes and tsetse (*Glossina* spp.) captured between 2007 and 2014 from Gorongosa (Rodrigues *et al.* 2017). These findings reinforce the need for further studies to know the impact of the translocation process on resident fauna.

Although in the present study we were unable to solve the issue regarding the possible occurrence of co-infection or recombination events by distinct *Bartonella* species, future studies using cloning of amplicons prior to sequencing and the isolation of bartonellae in blood or chocolate agar may help solving this problem, improving the molecular identification and characterization of this important bacterial group in wild ruminants.

In summary, our study showed the molecular occurrence of *B. bovis*, a possible new *Bartonella* genotypes/species or a genotype resulted from recombination events, *M. wenyonii* and '*Candidatus* M. haemobos' in African buffaloes submitted to translocation from Marromeu to Gorongosa National Park. Additionally, African buffaloes and other sympatric ruminants living in the release site (Gorongosa National Park) should be evaluated in the future in order to assess the impact of these pathogens in the ecosystem.

Conflicts of interest: None.

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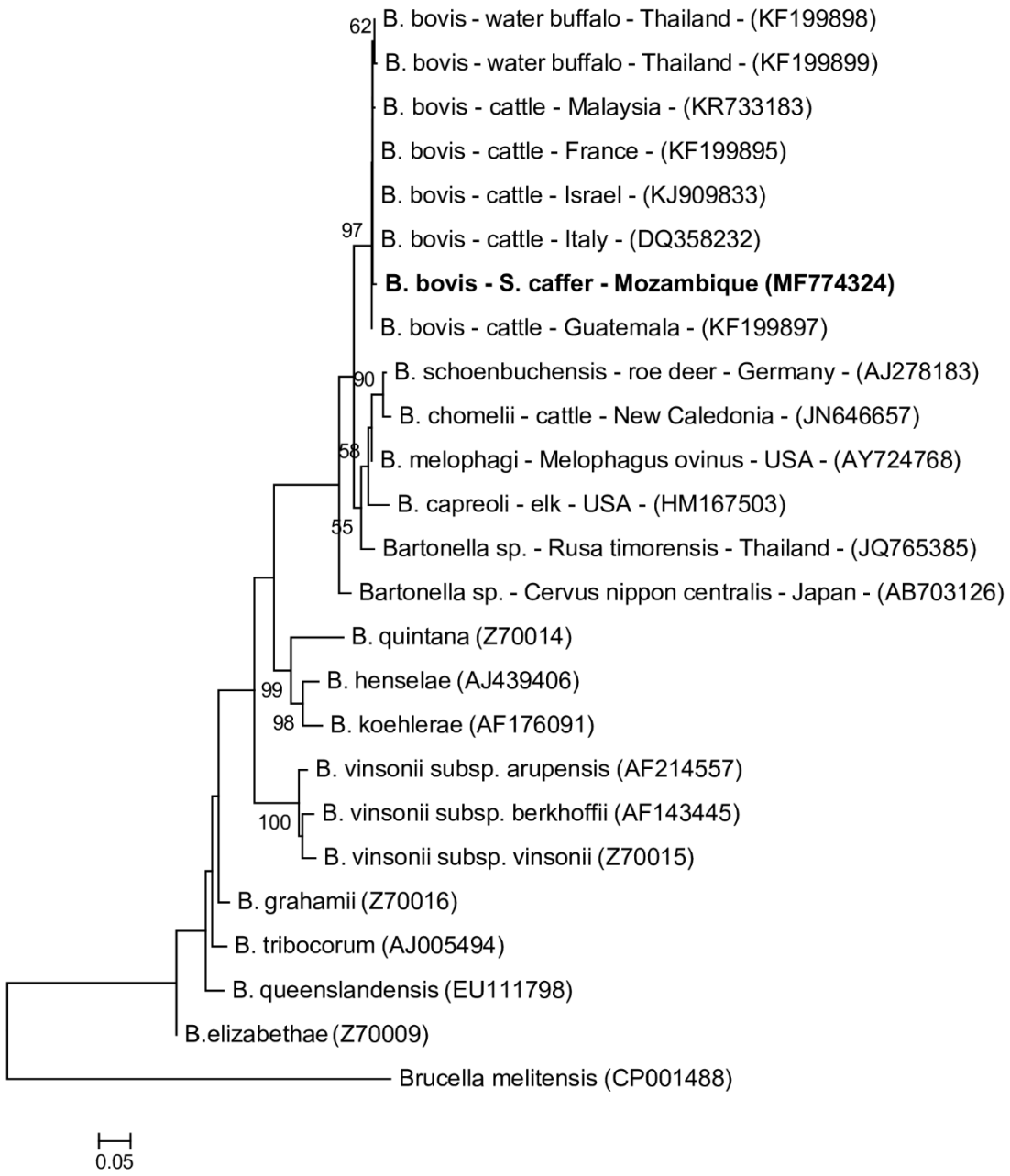

Figure S2. Relationships within the *Bartonella* genus based on the *gltA* gene. The tree was inferred by using the neighbor-joining (NJ) method with the GTR+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to posterior probability values higher than 50% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

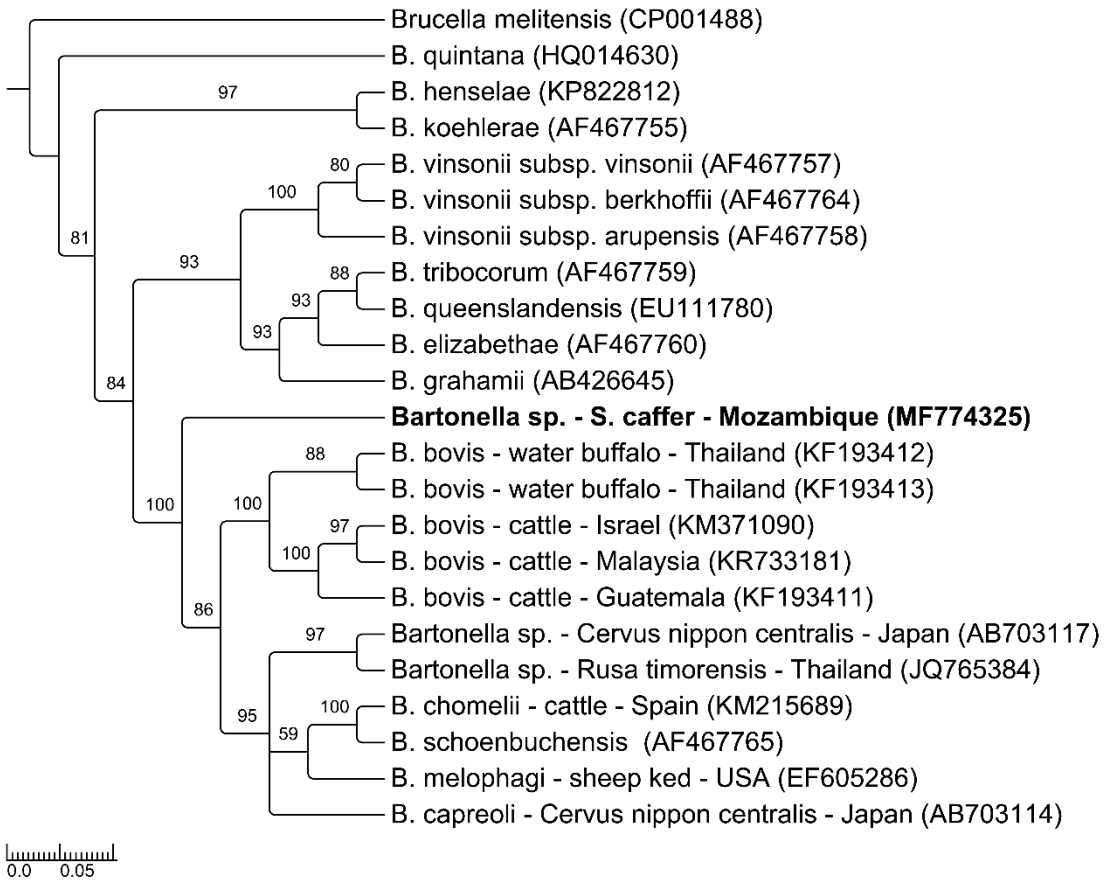

Figure S4. Relationships within the *Bartonella* genus based on the *ftsZ* gene. The tree was inferred by using the neighbor-joining (NJ) method with the GTR+G model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to posterior probability values higher than 50% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

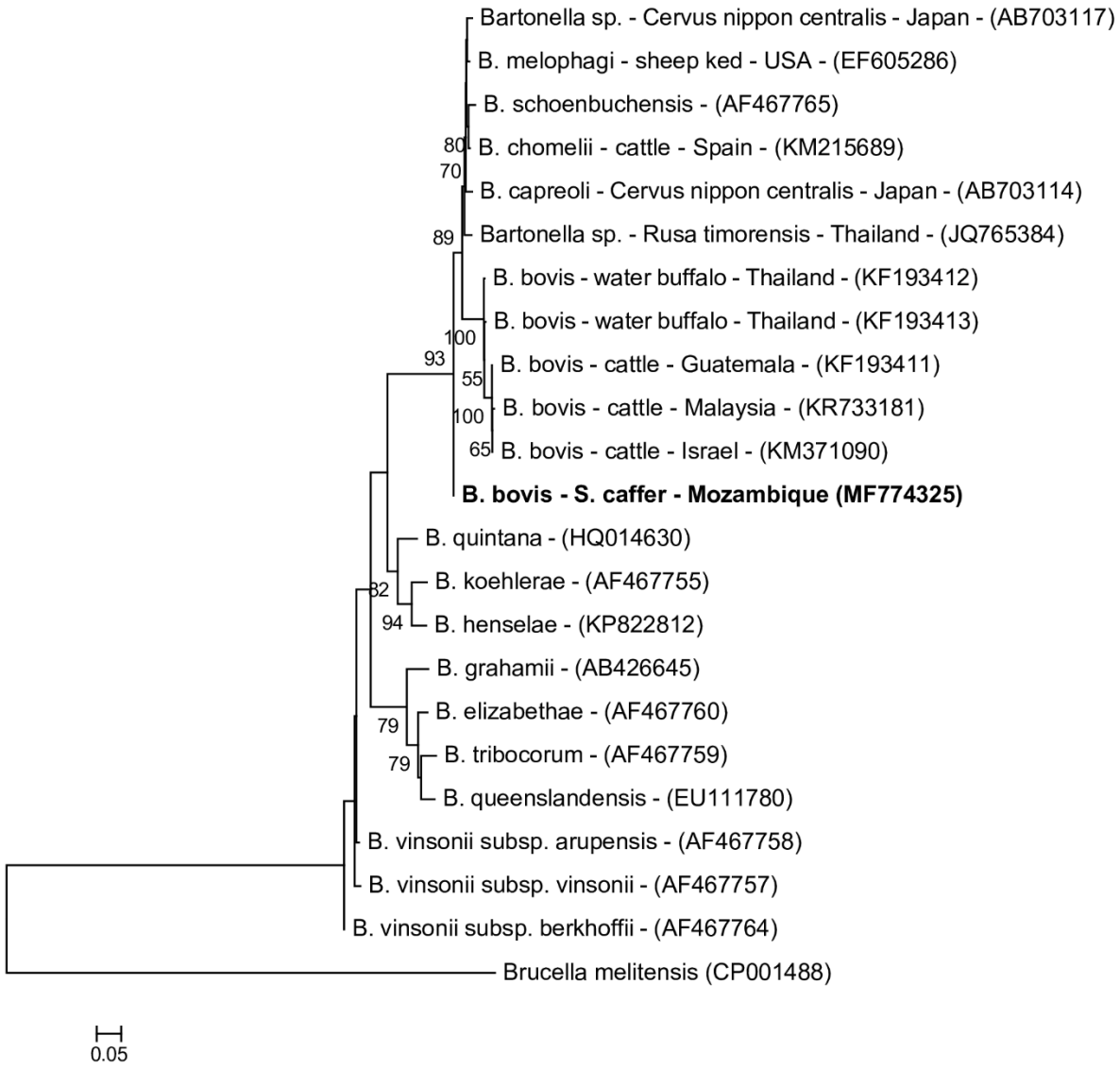

Figure S4. Relationships within the *Bartonella* genus based on the *ftsZ* gene. The tree was inferred by using the neighbor-joining (NJ) method with the GTR+G model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to posterior probability values higher than 50% accessed with 1000 replicates. *Brucella melitensis* was used as outgroup.

CHAPTER 6 – Final considerations

Hemoplasmas and *Bartonella* spp. are two interesting bacterial groups. The evolutionary success of these groups is irrefutable and may be expressed by their wide geographic distribution associated with the richness of hosts.

Over the years, several researchers around the world have improved our knowledge about these bacterial groups. Despite the undeniable advances achieved, other biological issues – e.g., transmission routes and pathogenicity – remain poorly understood. Using molecular, phylogenetic and bioinformatic tools, the current study aimed to increase our knowledge about the occurrence and genetic diversity of hemoplasmas and *Bartonella* spp. infecting small mammals, large ruminants and associated arthropods.

Herein, we reported the occurrence and genetic diversity of hemoplasmas in small rodents, opossums, capybaras, and associated ectoparasites collected in urban and forest fragments from Campo Grande, MS. The results suggested that the hemoplasmas found in the sampled synantropic mammals seems to be quite species-specific.

Additionally, this body of work contributed to the understanding of the expanding distribution of *B. coopersplainsensis*, a rodent-associated *Bartonella* species. Our results evidenced that sampling sites and the absence of fleas may influence the *Bartonella* spp. prevalence in small mammals communities.

Likewise, the present work reported, for the first time, *Bartonella* spp. DNA in cattle, buffaloes and associated ectoparasites in Brazil. The genetic analyses carried out herein showed a picture slightly different from those presented in previous studies.

Last but not least, analyzing wild buffaloes submitted to a translocation process in Africa, this work showed that these animals were exposed to hemoplasmas and *Bartonella* spp. Since translocated buffaloes were sharing the same area with cattle, and the release site is cattle-free, the bacterial species detected prior to translocation might have been introduced into the new area.

These findings have added new elements in the imbricated and complex network involving hemoplasma and *Bartonella* groups, their mammalian hosts and associated arthropods. The results described in this work may stimulate further

studies aiming to access the still obscure gaps in the biology of these bacterial species.