

UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS – (FCAV)
CÂMPUS DE JABOTICABAL

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE
***Bartonella* spp. EM FLEBOTOMÍNEOS (DIPTERA:**
PSYCHODIDAE) DAS REGIÕES NORTE E NORDESTE DO
BRASIL

Daniel Antônio Braga Lee

Médico Veterinário

2024

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Orientador: Prof. Dr. Marcos Rogério André

Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Mestre em Ciências Veterinárias; área: Saúde Única

2024

L477d

Lee, Daniel Antônio Braga

Detecção e caracterização molecular de Bartonella spp. em flebotomíneos (Diptera: Psychodidae) das regiões Norte e Nordeste do Brasil / Daniel Antônio Braga Lee. -- Jaboticabal, 2024

62 p. : tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

Orientador: Marcos Rogério André

1. Bartonella spp.. 2. Flebotomíneos. 3. Vetores. 4. Psychodidae. I.
Título.

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

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Impacto potencial desta pesquisa

Flebotomíneos (Diptera: Psychodidae) são insetos de importância em Saúde Única, visto que atuam como vetores de diferentes agentes patogênicos (*Leishmania* spp., *Bartonella* spp. e Phlebovirus). Em países andinos, são os vetores de *Bartonella bacilliformis*, agente causador da doença de Carrión. No Brasil, apesar da grande diversidade de espécies de flebotomíneos (305 espécies distribuídas por todas as regiões e biomas), e proximidade territorial com áreas endêmicas para a doença de Carrión (Peru, Bolívia e Equador), inexistentes são os estudos acerca da ocorrência de *Bartonella* sp. em tais dípteros no território nacional. O presente estudo relatou pela primeira vez a ocorrência de *Bartonella* spp. em flebotomíneos das regiões Norte e Nordeste do Brasil. Genótipos associados à *Bartonella ancashensis*, outro agente causador de Verruga Peruana no Peru (detectado em *Lutzomyia longipalpis* capturado no Ceará), e *Bartonella* sp. associada a morcegos e ectoparasitos associados (detectado em *Nyssomyia antunesi* capturado no Acre) demonstram a ocorrência de diferentes genótipos de *Bartonella* spp. em flebotomíneos no território nacional. Por meio da detecção e caracterização de agentes do gênero *Bartonella* em flebotomíneos, o presente estudo contribui para a expansão do conhecimento no que diz respeito à conservação da biodiversidade e a proteção dos ecossistemas (ODS 15). Adicionalmente, ao identificar e caracterizar tais bactérias em flebotomíneos, a pesquisa contribui diretamente para a Saúde Pública (ODS 3). Desta forma, a presente pesquisa contribui diretamente na promoção da Saúde Pública, integrando também a conservação ambiental em um contexto de desenvolvimento sustentável, favorecendo o bem-estar social e ambiental nas regiões estudadas.

Potential impact of this research

Phlebotomine sand flies (Diptera: Psychodidae) are insects of significant importance in One Health, as they act as vectors for various pathogens (*Leishmania* spp., *Bartonella* spp., and Phlebovirus). In Andean countries, they are vectors for *Bartonella bacilliformis*, the causative agent of Carrión's disease. In Brazil, despite the high diversity of phlebotomine sand fly species (305 species distributed across all regions and biomes), and the proximity to endemic areas for Carrión's disease (Peru, Bolivia, and Ecuador), there is a lack of studies regarding the occurrence of *Bartonella* spp. in

these dipterans within the country. This study reports, for the first time, the occurrence of *Bartonella* spp. in sand flies from the Northern and Northeastern regions of Brazil. Genotypes associated with *Bartonella ancashensis*, another agent causing Peruvian wart in Peru (detected in *Lutzomyia longipalpis* captured in Ceará), and *Bartonella* spp. associated with bats and their ectoparasites (detected in *Nyssomyia antunesi* captured in Acre) demonstrate the presence of different genotypes of *Bartonella* spp. in sand flies within the country. Based on the detection and characterization of *Bartonella* agents in sand flies, this study contributes to expanding knowledge regarding biodiversity conservation and ecosystem protection (SDG 15). Additionally, by identifying and characterizing these bacteria in phlebotomine sand flies, the research directly contributes to Public Health (SDG 3). Thus, this research directly promotes Public Health while also integrating environmental conservation within a sustainable development context, enhancing social and environmental well-being in the studied regions.

CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE *Bartonella* spp. EM FLEBOTOMÍNEOS (DIPTERA: PSYCHODIDAE) NAS REGIÕES NORTE E NORDESTE DO BRASIL

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Aprovado como parte das exigências para obtenção do Título de Mestre em Ciências Veterinárias, área: Saúde Única pela Comissão Examinadora:



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Jaboticabal, 01 de agosto de 2024

DADOS CURRICULARES DO AUTOR

DANIEL ANTÔNIO BRAGA LEE – Filho de Gabriel Isaías Lee Tuñon e Janaina Mabel Braga Lee, nasceu em 20 de Dezembro de 1996 na cidade de São João del Rei, Minas Gerais. Graduiu-se em Medicina Veterinária pela Universidade Federal de Sergipe em Fevereiro de 2022. Durante a graduação foi bolsista da Fundação de Apoio à Pesquisa e à Inovação Tecnológica do Estado de Sergipe (FAPITEC/SE) (2017-2018) e da Coordenação de Pesquisa (COPES) da Universidade Federal de Sergipe (2018-2020), realizando projetos de Iniciação Científica nas área de Parastiologia Veterinária sob a orientação da Profa. Dra. Patricia Oliveira Meira Santos e do Prof. Dr. Victor Fernando Santana Lima. Em Junho de 2022 ingressou no Mestrado de Pós-Graduação em Ciências Veterinárias na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho”, *campus* Jaboticabal como bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) sob orientação do Prof. Dr. Marcos Rogério André.

DEDICATÓRIA

Ao meu amado vovô Juca, cuja vida foi um exemplo de perseverança, sabedoria e amor incondicional. Sua memória e ensinamentos iluminaram cada página desta dissertação. Dedico este trabalho a você, em gratidão eterna pela sua inspiração e apoio inabalável.

AGRADECIMENTOS

Agradeço em primeiro lugar aos meus pais, Gabriel Isaías Lee Tuñon e Janaina Mabel Braga Lee, e à minha irmã, Isabela Braga Lee, por todo o incentivo, apoio e pelos valores que me ensinaram desde o início da minha vida, sendo sempre minha base e porto seguro. Agradeço também por me incentivarem e por sempre destacarem a importância da educação, que tanto impactou positivamente nossas vidas.

Agradeço profundamente à Letícia Viana e aos nossos gatinhos, Batatinha e Popó, por todo o apoio, paciência, incentivo, carinho e pelas memórias inesquecíveis que construímos ao longo dos últimos anos. Estendo meus agradecimentos a toda a sua família pela receptividade, respeito e compreensão ao longo desse período.

Minha mais sincera gratidão ao Prof. Dr. Marcos Rogério André pela confiança e pelo constante estímulo ao longo da minha jornada acadêmica. Seu profundo conhecimento e orientação precisa foram fundamentais para o sucesso desta dissertação. Agradeço pela consideração em aceitar me orientar e por todas as oportunidades que proporcionou desde o início deste percurso acadêmico. A Profa. Darci Moraes Barros Battesti e Profa. Rosangela Zacarias Machado por todo o incentivo e conselhos.

Agradeço em especial ao Prof. Dr. Victor Fernando Santana Lima pela sua orientação e parceria desde o início da graduação. Serei eternamente grato pela sua amizade e pelo incentivo que me proporcionou para ingressar na vida acadêmica. Também expresso minha sincera gratidão à Profa. Dra. Patrícia Oliveira Meira Santos, que logo após assumiu minha orientação, proporcionando-me liberdade e estímulo para aprender e crescer profissionalmente. Agradeço por sua dedicação, paciência e pelo apoio contínuo ao longo da graduação.

A todos os funcionários da UNESP/FCAV, especialmente do Departamento de Patologia, que sempre estiveram à disposição para ajudar durante todo esse período.

Agradeço a todos os colegas do VBBL por todos os ensinamentos e momentos compartilhados ao longo desses anos. Em especial, expresso minha gratidão à Anna e Victoria, que desde o início me proporcionaram uma grande liberdade para aprender e contribuíram de forma significativa para que eu desenvolvesse confiança, segurança e proatividade no novo laboratório. Suas orientações e apoio foram fundamentais para o meu crescimento profissional durante este período.

Agradeço à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela bolsa de estudos concedida a mim (Processo FAPESP 2022/07008-6) e o auxílio à pesquisa concedido ao meu orientador Prof. Dr. Marcos Rogério André (Processo FAPESP 2022/08543-2), que foram essenciais para a execução do projeto.

A todos, meus sinceros agradecimentos por fazerem parte deste importante capítulo da minha vida acadêmica e pessoal.

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DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE *Bartonella* spp. EM FLEBOTOMÍNEOS (DIPTERA: PSYCHODIDAE) DAS REGIÕES NORTE E NORDESTE DO BRASIL

RESUMO - A família Bartonellaceae é formada por α -proteobactérias Gram-negativas intracelulares facultativas capazes de infectar diversos tipos celulares, principalmente eritrócitos e células endoteliais, de uma grande variedade de mamíferos. Algumas espécies são agentes causadores das bartoneloses, enfermidades emergentes e re-emergentes com amplo espectro clínico e responsáveis por agravos à saúde humana e animal. A principal forma de transmissão destes agentes é por meio da picada ou excretas de artrópodes hematófagos (pulgas, piolhos, carrapatos e flebotomíneos), tornando a busca por vetores essencial. Flebotomíneos (Diptera, Psychodidae, Phlebotominae) atuam como vetores de *Bartonella bacilliformis*, agente causador da Doença de Carrión nos países andinos. Estão amplamente distribuídos pelo território brasileiro, com um total de 304 espécies diferentes identificadas. O presente estudo teve como objetivo investigar a ocorrência e caracterizar molecularmente *Bartonella* spp. em flebotomíneos capturados nas regiões Norte e Nordeste do Brasil. Para tal, foram analisados 634 flebotomíneos, classificados em 44 espécies pertencentes a 14 gêneros diferentes, capturados nos estados do Acre (Rio Branco, n=206; Xapuri, n=106), Alagoas (Estação Ecológica de Murici, n=72), Bahia (Parque Nacional do Pau Brasil, n=91), Ceará (Parque Nacional do Ubajara, n=27), Pará (Floresta Nacional do Tapajós, n=51), Pernambuco (Parque Estadual de Dois Irmãos, n=14) e Roraima (Parque Nacional do Viruá, n=67). DNA foi extraído de cada espécime individualmente utilizando o TRIzol™. Como resultado, 100% (634/634) das amostras de DNA foram positivas para o ensaio de PCR baseado no gene endógeno de invertebrados *cox-1*. Destas, 8,7% (55/634) foram positivas na PCR em tempo real quantitativa (qPCR) baseada na região intergênica 16S-23S (ITS) de *Bartonella* spp.: 48 amostras do Acre (n=18 *Nyssomyia antunesi*; n=sete *Evandromyia walkeri*; n=cinco *Trichophoromyia* sp.; n=quatro *Lutzomyia sherlocki*; n=três *Nyssomyia shawi*; n=dois *Psychodopygus llanosmartinsi*; n=dois *Psychodopygus davisi*; n=um *Bichromomyia flaviscutellata*; n=1 *Evandromyia saulensis*; n=1 *Nyssomyia* sp.; n=1 *Nyssomyia whitmani*; n=um *Pintomyia nevesi*; n=um *Pintomyia serrana*; n=um *Viannamyia furcata*); duas amostras de Alagoas (n=dois *Trichophoromyia viannamartinsi*); duas amostras de Roraima (n=um *Psychodopygus squamiventris*; n=um *Psychodopygus ayrozai*); uma amostra da Bahia (*Trichopygomyia longispina*); uma amostra do Ceará (*Lutzomyia longipalpis*); e uma amostra do Pará (*Psychodopygus paraensis*). Nos ensaios de PCR convencional utilizados para caracterização molecular, quatro (4/55; 7,3%) foram positivas para o gene *gltA*; quatro amostras (4/55; 7,3%) foram positivas para a região intergênica (ITS) 16-23S rRNA; duas (2/55; 3,6%) foram positivas para o gene *ftsZ*; duas (2/55; 3,6%) foram positivas para o gene *pap31*; uma (1/55; 1,8%) foi positiva para o gene *rpoB*; e uma (1/55; 1,8%) foi positiva para o gene *nuoG*. Dois amplicons puderam ser sequenciados, gerando uma sequência de 377 pb do gene *gltA* (detectada em espécime de *Lutzomyia longipalpis* capturado no Ceará) e uma sequência de 345 pb do gene *nuoG* (detectada em espécime de *Nyssomyia antunesi* capturado no

Acre). A sequência *nuoG* demonstrou uma similaridade de aproximadamente 94% com sequências de *Bartonella* sp. previamente detectadas em morcegos da Guatemala. Já a sequência *gltA* demonstrou uma similaridade >96% com sequências de *B. ancashensis* previamente isoladas de humanos do Peru, além de posicionar-se filogeneticamente no mesmo sub-clado que tais sequências e uma sequência de *Bartonella* sp. previamente detectada em um flebotomíneo (*Dampfomyia beltrani*) do México. O presente estudo forneceu a primeira evidência molecular da ocorrência de *Bartonella* spp. em flebotomíneos do Brasil, em *L. longipalpis* do Ceará e *N. antunesi* do Acre.

Palavras-chave: *Bartonella*; Flebotomíneos; vetores; Psychodidae

MOLECULAR DETECTION AND CHARACTERIZATION OF *Bartonella* spp. IN PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) FROM NORTH AND NORTHEASTERN REGIONS OF BRAZIL

ABSTRACT - The Bartonellaceae family is composed of Gram-negative, facultative intracellular α -proteobacteria capable of infecting various cell types, primarily erythrocytes and endothelial cells, in a wide range of mammals. Some species are the causative agents of bartonellosis, emerging and re-emerging diseases with a broad clinical spectrum, responsible for health issues for both humans and animals. The main route of transmission of these agents is through the bite or excreta of hematophagous arthropods (fleas, lice, ticks, and sand flies), making the search for vectors essential. Sand flies (Diptera, Psychodidae, Phlebotominae) act as vectors for *Bartonella bacilliformis*, the causative agent of Carrion's disease in Andean countries. They are widely distributed throughout Brazil, with a total of 304 different species identified. The present study aimed to investigate the occurrence and molecularly characterize *Bartonella* spp. in sand flies captured in the North and Northeast regions of Brazil. A total of 634 sand flies, classified into 44 species belonging to 14 different genera, were captured and analyzed from the states of Acre (Rio Branco, n=206; Xapuri, n=106), Alagoas (Murici Ecological Station, n=72), Bahia (Pau Brasil National Park, n=91), Ceará (Ubajara National Park, n=27), Pará (Tapajós National Forest, n=51), Pernambuco (Dois Irmãos State Park, n=14), and Roraima (Vuruá National Park, n=67). DNA was extracted from each specimen individually using TRIzolTM. As a result, 100% (634/634) of the DNA samples were positive in the PCR assay based on the endogenous invertebrate gene *cox-1*. Of these, 8.7% (55/634) were positive in the quantitative real-time PCR (qPCR) based on the 16S-23S intergenic region (ITS) of *Bartonella* spp.: 48 samples from Acre (n=18 *Nyssomyia antunesi*; n=seven *Evandromyia walkeri*; n=five *Trichophoromyia* sp.; n=four *Lutzomyia sherlocki*; n=three *Nyssomyia shawi*; n=two *Psychodopygus llanosmartinsi*; n=two *Psychodopygus davisii*; n=one *Bichromomyia flaviscutellata*; n=one *Evandromyia saulensis*; n=one *Nyssomyia* sp.; n=one *Nyssomyia whitmani*; n=one *Pintomyia nevesi*; n=one *Pintomyia serrana*; n=one *Viannamyia furcata*); two samples from Alagoas (n=two *Trichophoromyia viannamartinsi*); two samples from Roraima (n=one *Psychodopygus squamiventris*; n=one *Psychodopygus ayrozai*); one sample from Bahia (*Trichophoromyia longispina*); one sample from Ceará (*Lutzomyia longipalpis*); and one sample from Pará (*Psychodopygus paraensis*). In the conventional PCR assays used for molecular characterization, four (4/55; 7.3%) were positive for the *gltA* gene; four samples (4/55; 7.3%) were positive for the 16-23S rRNA intergenic region (ITS); two (2/55; 3.6%) were positive for the *ftsZ* gene; two (2/55; 3.6%) were positive for the *pap31* gene; one (1/55; 1.8%) was positive for the *rpoB* gene; and one (1/55; 1.8%) was positive for the *nuoG* gene. Two quality sequences were obtained: a 377 bp sequence of the *gltA* gene (detected in a *Lutzomyia longipalpis* specimen captured in Ceará) and a 345 bp sequence of the *nuoG* gene (detected in a *Nyssomyia antunesi* specimen captured in Acre). The *nuoG* sequence showed approximately 94% similarity with *Bartonella* sp. sequences previously detected in bats from Guatemala. The *gltA* sequence showed >96% similarity

with *B. ancashensis* sequences previously isolated from humans in Peru, as well as phylogenetically positioned in the same sub-clade as these sequences and a *Bartonella* sp. sequence previously detected in a sandfly (*Dampfomyia beltrani*) from Mexico. This study provided the first molecular evidence of the occurrence of *Bartonella* spp. in sand flies from Brazil, in *L. longipalpis* from Ceará and *N. antunesi* from Acre.

Keywords: *Bartonella*; phlebotomine sand flies; vectors; Psychodidae

CHAPTER 1 - General Considerations

1. Introduction

Brazil is home to a rich diversity of Phlebotomine sand fly species, with over 300 identified species across all regions of the country. The diversity of sand flies in Brazil is of significant importance, due to their role as vectors of numerous pathogens, including the causative agents of leishmaniasis and bartonellosis.

The *Bartonella* genus comprises opportunistic Gram-negative intracellular bacteria that primarily infect erythrocytes and endothelial cells from various mammalian hosts. The primary route of transmission for these bacteria is through blood-feeding arthropods, such as ticks, lice, fleas, and sand flies. Among the diseases caused by *Bartonella* species, Carrion's Disease is of paramount importance. This disease is caused by *Bartonella bacilliformis* and is transmitted by sand flies in high-altitude regions of the Inter-Andean Valleys in Peru. The two confirmed sand fly vectors responsible for transmitting this *Bartonella* species are *Lutzomyia peruensis* and *Pintomyia verrucarum*.

Although Carrion's disease has a focal occurrence, studies have reported the presence of *B. bacilliformis* and *Bartonella* sp. DNA in sand flies from other countries, including Bolivia, Ecuador, and Mexico. These findings suggest the potential involvement of additional sand fly species in the transmission of Carrion's disease in these regions and indicate that sand flies may play a role in the epidemiological cycles of different *Bartonella* species. Given Brazil's geographic proximity to Carrion's disease endemic countries and its diverse phlebotomine sand fly fauna, it is important to study the occurrence of these

agents in the country. Furthermore, there are no studies in Brazil that have investigated the occurrence of Bartonellaceae agents in phlebotomine sand flies.

The present study investigated the molecular occurrence and molecularly characterized *Bartonella* spp. in sand flies from the North and Northeastern regions of Brazil. This work will contribute to the understanding of Bartonellaceae agents and their potential phlebotomine hosts in Brazil.

2. Literature review

2.1 Brazilian sand fly fauna and distribution

The subfamily Phlebotominae (Diptera: Psychodidae), comprised of arthropods commonly known as phlebotomine sand flies, encompasses over 1,060 species distributed globally, predominantly in tropical and subtropical regions (Shimabukuro et al., 2017). These dipterans are subject of extensive research worldwide due to their role in the transmission of significant infectious agents to both animals and humans (Bates et al., 2015). The female sand flies, which exhibit a blood-feeding habit, can transmit various pathogens, including viruses (e.g., Phleboviruses), protozoa (e.g., *Leishmania* spp.), and bacteria (e.g., *Bartonella* sp.) (Jancarova et al., 2023). The biological characteristics of these insects, including their natural shelters (shaded and humid areas), as well as their remarkable ability to adapt to human-modified environments, such as hen houses, pigsties, and barns, underscore their significance in public health. Moreover, their anthropophilic habits further enhance their potential impact on human health, as they seek

out human hosts for blood meals, thereby increasing the risk of pathogens transmission (Rangel & Shaw, 2018).

Brazil has a rich diversity of phlebotomine sand fly species, with 304 species occurring in the national territory, of which 80 are endemic (Shimabukuro et al., 2024). These species are distributed across all federative regions: 218 species in the North, 155 in the Midwest, 132 in the Southeast, 129 in the Northeast and 49 in the South (Shimabukuro et al., 2024). Furthermore, phlebotomine sand flies are present in all Brazilian biomes: 195 species in the Amazon Rainforest, 121 species in Atlantic Forest, 60 species in Cerrado, 7 species in Pantanal and two species in both Caatinga and Pampa biomes (Shimabukuro et al., 2024). In Brazil, phlebotomine sand flies, especially from the *Bichromomyia*, *Lutzomyia*, *Migonemyia*, *Nyssomyia*, *Pintomyia*, *Psychodopygus* and *Trichophoromyia* genera, are targets of public health policies due to their importance in the maintenance and transmission of *Leishmania* spp. between humans and animals, in urban and wild environments (Rangel & Shaw, 2018).

2.2 *Bartonella* spp.

2.2.1 Etiological agent and hosts

The genus *Bartonella* (Proteobacteria: Hyphomicrobiales), which is the single representant of the Bartonellaceae family, comprises pleomorphic, intracellular, opportunistic, Gram-negative α -proteobacteria (Minnick & Anderson, 2015). These agents have a worldwide distribution and are responsible for causing emergent and re-emergent diseases in a wide range of mammalian hosts, representing pathogens of great concern

for public health (Okaro et al., 2017). Because *Bartonella* bacteria are highly fastidious, the development of molecular tools has become essential for characterizing fully or partially species belonging to this genus. To date, over 37 *Bartonella* species have been confirmed, and an increasing number have been assigned as *Candidatus* species (Reif, 2022; Ruiz et al., 2022).

Bartonella species infect mainly erythrocytes, endothelial cells, pericytes, dendritic cells, progenitor cells and macrophage-type cells, which can explain the wide variety of clinical signs associated with infection with these agents (Gomes & Ruiz, 2018). The clinical manifestations and disease progression is correlated to the *Bartonella* species, vertebrate host immunocompetence, the vector interaction with the agent and the type of cell infected (Gomes e Ruiz, 2018; Zangwill, 2021). Among other clinical signs (including endocarditis, fever, lymphopathies, myocarditis and neuroretinitis), persistent erythrocytic bacteremia is often observed in *Bartonella* spp. infections (Angelakis & Raoult, 2014).

The ability to maintain a persistent chronic infection associated with the diverse set of genes responsible for infection of endothelial and red blood cells allowed these bacteria to establish infections in a broad range of mammalian hosts, including humans (Bisch et al., 2018). Their main hosts/reservoirs include several domestic (e.g. dogs, cats), wild (e.g. foxes, rabbits, rodents, marsupials, bats) and livestock (e.g. cows, sheep) mammals (Minnick & Anderson, 2015). Relationships between multiple hosts, vectors and *Bartonella* species are intrinsically correlated to the transmission and coevolution between bacteria and mammalian hosts (Lei & Olival, 2014).

2.2.2 Routes of transmission

As stated above, cases of persistent intraerythrocytic bacteremia, commonly observed in *Bartonella* spp. infections, not only are associated to the adaptation and evolution of the bacteria within its vertebrate host, but also favors the main route of transmission of these agents: blood-sucking arthropods (Breitschwerdt, 2014; Chomel et al., 2009; Rudolf et al., 2020). The capacity of bacteria to be transmitted by arthropod vectors can be one of the possible explanations to the specificity of *Bartonella* species to their mammalian hosts, since hematophagous arthropods have their own host range (Vayssier-Taussat et al., 2009).

Several species of arthropods, including phlebotomine sand flies, fleas, lice and ticks, have been identified as competent vectors in the transmission cycles of different *Bartonella* species (Billeter et al., 2008; Cotté et al., 2008; Król et al., 2021). Simultaneously, several hematophagous arthropods have been implicated in the transmission of the bacteria, due to molecular detection of *Bartonella* spp. and epidemiological observations, such as mosquitoes (Rudolf et al., 2020), biting midges (Sacristán et al., 2021), triatomine bugs (Laroche et al., 2017) and mites (Melter et al., 2012; Reeves et al., 2006) (**Table 1**).

Table 1. Known and suspected vectors for *Bartonella* species. Adapted from Billeter et al. (2008).

Confirmed Vector	Suspected Vector	<i>Bartonella</i> species	Reference
Sand flies			
<i>Lutzomyia peruensis</i>		<i>B. bacilliformis</i>	Gomes & Ruiz, 2018
<i>Pintomyia verrucarum</i>		<i>B. bacilliformis</i>	
	<i>Lutzomyia pescei</i>	<i>B. bacilliformis</i>	Lydy et al. 2018
	<i>Lutzomyia noguchii</i>	<i>B. bacilliformis</i>	Noguchi et al. 1929
	<i>Lutzomyia ayacuhensis</i>	<i>B. bacilliformis</i>	Minnick et al. 2014
	<i>Pintomyia robusta</i>	<i>B. bacilliformis</i>	Carrasco-Montalvo, 2017
	<i>Pintomyia maranonensis</i>	<i>B. bacilliformis</i>	Ulloa et al. 2018
	<i>Pintomyia colombiana</i>	<i>B. bacilliformis</i>	Alexander et al. 1995
Lice			
<i>Pediculus humanus humanus</i>		<i>B. quintana</i>	Swift, 1920
	<i>Pediculus humanus capitis</i>	<i>B. quintana</i>	Sasaki et al. 2006
Fleas			
<i>Ctenocephalides felis</i>		<i>B. henselae</i>	Koehler et al. 1994
	<i>Ctenocephalides felis</i>	<i>B. clarridgeiae</i> ; <i>B. quintana</i> and <i>B. koehlerae</i>	Rolain et al. 2003
<i>Ctenophthalmus nobilis nobilis</i>		<i>B. grahamii</i> and <i>B. taylorii</i>	Bown et al. 2004
	<i>Ctenocephalides canis</i>	<i>B. henselae</i>	Ishida et al. 2001
Ticks			
<i>Ixodes ricinus</i>		<i>B. henselae</i>	Cotté et al. 2008
	<i>Rhipicephalus sanguineus sensu lato</i>	<i>B. henselae</i>	Wechtaisong et al. 2020; 2021
	<i>Ixodes ricinus</i>	<i>B. birtlesii</i>	Reis et al. 2011
		<i>B. grahamii</i> ; <i>B. schoenbuchensis</i>	Król et al. 2021
Biting midges			

<i>Culicoides</i> sp.	<i>Bartonella</i> sp.	Sacristán et al. 2021
Keds <i>Lipoptena cervi</i>	<i>B. schoenbuchensis</i>	De Bruin et al. 2015
Mites <i>Dermanyssus</i> sp. <i>Steatonyssus</i> sp.	<i>Bartonella</i> sp. <i>Bartonella</i> sp.	Melter et al. 2012 Reeves et al. 2006
Mosquitoes <i>Culex pipiens</i> <i>Aedes vexans</i> <i>Aedes maculipennis</i>	<i>Bartonella</i> sp. <i>Bartonella</i> sp. <i>Bartonella</i> sp.	Rudolf et al. 2020
Triatomine bugs <i>Eratyrus mucronatos</i>	' <i>Candidatus</i> Bartonella rondoniensis'	Laroche et al. 2017

Although the main route of transmission of *Bartonella* species are via blood-feeding of arthropods, some species have other routes of transmission within their epidemiological cycles. In addition to the flea bite, *B. henselae* and *B. clarridgeiae*, can be transmitted by scratching or biting of infected cats (Breitschwerdt & Kordick, 2000; Duncan et al., 2007). Becker et al. (2018) also suggested that saliva (biting) might be an additional route of *Bartonella* transmission among hematophagous bats sampled in Peru and Belize.

2.2.3 *Bartonella* spp. in humans

Among *Bartonella* species, some species stand out due their zoonotic potential. The main species that cause disease in humans are *Bartonella henselae*, the causative agent of Cat Scratch disease (CSD), *Bartonella quintana*, the causative agent of Trench Fever, and *Bartonella bacilliformis*, the causative agent of Carrion's disease (Breitschwerdt, 2014; Garcia-Quintanilla et al., 2019). CSD is an important zoonosis transmitted to humans by cat scratches and bites worldwide (Smolar et al., 2022). The

main clinical sign of the disease is the development of lymphadenopathy in the proximal site of the cat scratch or bite, with potential progression to more severe complications, such as neuroretinitis, endocarditis and encephalitis (Nelson et al., 2016). *Bartonella clarridgeiae* also causes a zoonotic disease with similar clinical course as CSD (Chen et al., 2007; Kordick et al., 1997). Infections by *B. quintana* are usually characterized by fever, headache, joint pain, endocarditis and bacillary angiomatosis in more severe cases (Anstead, 2016). Carrion's disease, which occurs mainly in Peruvian Interandean Valleys and to a lesser extent in Colombia and Ecuador, is a neglected disease due to its focal occurrence and challenging diagnosis (Gomes & Ruiz, 2018). The disease can be manifested in two different syndromes, which can occur independently or subsequently: Oroya Fever, an acute manifestation characterized by hemolytic anemia and high rates of fatality, and Verruga Peruana, a chronic manifestation characterized by the infection of endothelial cells and formation of hemangiomas in the patient skin (Garcia-Quintanilla et al., 2017; Gomes & Ruiz, 2018).

Other species, including *B. alsatica*, *B. clarridgeiae*, *B. elizabethae*, *B. grahamii*, *B. koehlerae*, *B. melophagi*, *B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *berkhoffii* have already been detected in humans, usually in cases of fever of unknown origin and culture-negative endocarditis cases, with a wide range of mammals acting as their main hosts (cats, dogs, rodents, rabbits and sheep) (Breitschwerdt et al., 2007; Okaro et al., 2017; Regier et al., 2016; Raoult et al., 2006; Shapira et al., 2004).

Among the continually emerging new species and Candidatus within the *Bartonella* genus, some of them have recently been associated to disease in humans. *Bartonella*

ancashensis has been isolated from patients under Carrion's disease treatment in the rural region of Ancash, Peru (Mullins et al. 2015; 2017). This species is closely related to the causative agent of Carrion's disease, *B. bacilliformis*, but has only been isolated from patients suffering from "Verruga Peruana", the chronic presentation of the disease. It is considered to be less pathogenic, although coinfections of both species can occur, since their geographic distribution overlaps (Mullins et al., 2015; Minnick et al., 2023).

A bat-associated *Bartonella* species, '*Candidatus Bartonella mayotimonensis*', was isolated from a patient suffering of fatigue, weight loss, shortness of breath and endocarditis from a rural area in Iowa, United States (Lin et al., 2010). Later studies identified closely related sequences in blood samples from non-hematophagous bats from the United States (Lilley et al., 2017) and Finland (Veikkolainen et al., 2014). More recently, another case of human infection by '*Candidatus Bartonella mayotimonensis*' was detected in a patient with similar clinical signs, including endocarditis, from the United States (McCormick et al., 2023). '*Candidatus Bartonella rousetii*', another bat-associated species, was isolated from Egyptian fruit bats (*Rousettus aegyptiacus*) and molecularly detected in bat-associated Nycteribiidae flies (*Eucampsipoda africana*) in Nigeria (Bai et al., 2018). Local residents, which had close contact with these animals and the caves they roosted, were seropositive to '*Candidatus Bartonella rousetii*', without sero-reactivity to other *Bartonella* species.

2.2.4 *Bartonella* spp. in sand flies

The causative agent of Carrion's disease, *B. bacilliformis*, is the main *Bartonella* species associated with phlebotomine sand flies. Only two sand fly species have been

confirmed as vectors of this agent: *P. verrucarum* and *L. peruensis* (Gomes & Ruiz, 2018). These sand fly species are found in high altitudes (between 500 and 3,200 meters) in the Peruvian Interandean Valleys, where most cases of Verruga Peruana and Oroya Fever have been reported (Garcia-Quintanilla et al., 2019). Although Ulloa et al. (2018) detected the presence of *B. bacilliformis* DNA in *Lutzomyia maranonensis* sand flies from the northern region of Peru, further studies are needed to elucidate the role of this species in the transmission cycle of Carrion's disease. Despite the fact that sand flies are suspected vectors for *B. ancashensis*, no studies have yet reported the occurrence of this agent in blood-sucking arthropods (Minnick et al., 2023).

Although the vectors for Carrion's disease have a very restricted area of occurrence, outbreaks of the disease in non-endemic areas suggest a possible involvement of other sand fly species in the epidemiological cycle of the disease (Lydy et al., 2018). A study conducted in three different ecological regions of Peru (High Jungle, Low Jungle and Andean Region) detected the occurrence of *Bartonella* sp. phylogenetically associated to *B. bacilliformis* in individual females of *Pintomyia nevesi* and *Lutzomyia sherlocki* and pooled females of *Psychodopygus hirsutus* and *Nyssomya whitmani*, based on sequences from the *gltA* gene (Zorilla et al., 2021). Furthermore, the authors also detected *Bartonella* sp. related to '*Candidatus Bartonella rondoniensis*' in individual *P. nevesi* and *L. maranonensis*. DNA of *Bartonella* sp. phylogenetically related to *B. bacilliformis* was also detected in *Pintomyia robusta* in the border between Peru and Ecuador (Montalvo et al., 2017).

Based on epidemiological observations, *L. gomezi*, *Psychodopygus panamensis*, *Pintomyia serrana* and *Pintomyia columbiana* have been pointed out as possible vectors of Carrion's Disease in Colombia. Although no molecular studies were performed, the presence of these sand fly species in outbreaks of the disease, and the lack of presence of the competent vectors (*P. verrucarum* and *L. peruensis*), suggest a possible involvement of such species in the transmission of *B. bacilliformis* in that country (Alexander, 1995; Minnick et al., 2014).

For the first time in Mexico, Lozano-Sardaneta et al. (2019) detected the presence of *Bartonella* DNA in female *Lutzomyia* sp. sand flies (8.7%; 2/23), using a conventional PCR (cPCR) based on the *gltA* gene. The BLASTn analysis of the obtained sequences demonstrated a high degree of similarity (100%) between them and a similarity of 96% with *Bartonella* sp. sequences detected in rodents from Thailand and China. Later molecular studies in the country reinforced the occurrence of the bacteria in different species of sand flies. In 2021, the authors detected a putative novel lineage of *Bartonella* sp., associated with *Psathyromyia shannoni* and *L. cruciata*. Based on phylogenetic inferences based on the *gltA* gene, the obtained sequences were positioned in a sister clade to the sequences detected previously in Mexico and *Bartonella* sp. detected in rodents from China (Lozano-Sardaneta et al., 2021). Recently, the authors detected *Bartonella* sp. sequences in *Pintomyia ovallesi* grouping in the same clade as the sequences detected in the previous years, reinforcing the occurrence of a new *Bartonella* lineage in sand flies from Mexico (Lozano-Sardaneta et al., 2023).

Furthermore, studies conducted by Battisti et al. (2015) and Minnick et al. (2023) demonstrated experimental colonization of *Lutzomyia longipalpis* by *B. bacilliformis* and *B. ancashensis*, respectively. Both studies demonstrated that in experimental infections both *Bartonella* species remained viable in the anterior midgut of sand flies for up to 7 days. Although the authors were not able to confirm the vectorial competence of the sand flies, they suggested that *L. longipalpis* can play a short-term role in the epidemiological cycle and maintenance of *Bartonella* sp. and potentially serve as a vector during that time.

Although Brazil has great richness of sand fly species and bordering countries where Carrion's disease is endemic or occurs, no studies have sought to detect the occurrence of *Bartonella* sp. in these dipterans. Despite that, as stated above, many studies from other countries have reported the presence of *Bartonella* sp. DNA in sand fly species that also occur in Brazil, including *Pintomyia nevesi*, *Lutzomyia sherlocki*, *Nyssomyia whitmani*, *Psychodopygus hirsutus*, *Lutzomyia peruensis* and *Lutzomyia sherlocki* in Peru (Zorilla et al., 2021) and *Lutzomyia cruciata* and *Psathyromyia shannoni* from Mexico (Lozano-Sardaneta et al., 2019; 2021). Furthermore, studies regarding experimental infections with *B. bacilliformis* (Angkasekwinai et al., 2014; Battisti et al., 2015) and *B. ancashensis* (Minnick et al., 2023) suggests that *Lutzomyia longipalpis*, an important vector of *Leishmania infantum* in Brazil (occurring in all regions and biomes), can be a potential vector for *Bartonella* species (Lainson & Rangel, 2005).

3. Objectives

3.1 General objective

The present study aimed to investigate the occurrence and phylogenetic positioning of *Bartonella* spp. in phlebotomine sand flies (Diptera, Psychodidae) captured in the North (Acre, Pará and Roraima states) and Northeastern (Alagoas, Bahia, Ceará and Pernambuco states) regions of Brazil

3.2 Specific objectives

- To investigate, through molecular assays, the occurrence of *Bartonella* spp. in phlebotomine sand flies sampled in the states of Acre, Alagoas, Bahia, Ceará, Pará, Pernambuco and Roraima states;
- To molecularly characterize the detected *Bartonella* genotypes using conventional PCR assays based on the 16-23S rRNA intergenic region (ITS) and the *gltA*, *rpoB*, *ftsZ*, *ribC*, *pap31*, *groEL* and *nuoG* genes;
- To infer the phylogenetic positioning of the detected *Bartonella* spp. sequences in relation to sequences obtained in other parts of the world.

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CHAPTER 2 – *Bartonella* spp. in sand flies (Diptera, Psychodidae, Phlebotominae) from Brazil

Abstract

We investigated the molecular occurrence of *Bartonella* spp. in 634 phlebotomine sand flies specimens, belonging to 44 different sand fly species, sampled between 2017-2021 from North and Northeastern Brazil. Using a quantitative real-time polymerase chain reaction (qPCR) targeting the 16S-23S ITS intergenic region, *Bartonella* sp. DNA was detected in 8.7% (55/634) of the specimens. Phylogenetic analysis positioned the *Lutzomyia longipalpis*-associated *Bartonella* *gltA* sequence in the same sub-clade as *Bartonella* *ancashensis* sequences, and with a *Bartonella* sp. sequence detected in a *Dampfomyia beltrani* sand fly from Mexico. A bat-associated *Bartonella* *nuoG* sequence was amplified from a specimen of *Nyssomyia antunesi*. Our findings document the presence of *Bartonella* DNA in sand flies from Brazil, which suggests possible involvement of these insects in the epidemiological cycle of these two *Bartonella* spp.

Keywords: Bartonellaceae; Phlebotominae; vector; Natural infections

Introduction

The genus *Bartonella* (Alphaproteobacteria: Bartonellaceae) comprises emergent and re-emergent opportunistic bacteria classified in 39 validated species (<https://psn.dsmz.de/genus/bartonella>), that are able to cause disease in both animals and humans (1). Mammals (e.g. rodents, bats, cats, dogs, ruminants, and others), including humans, are the main reservoirs for bartonellae. The *Bartonella* species that are most often associated with diseases in humans are *Bartonella henselae* (the causative

agent of the Cat Scratch Disease), *Bartonella quintana* (the causative agent of Trench Fever), and *Bartonella bacilliformis* and *Bartonella ancashensis* (the causative agents of Carrion's disease and "Verruga Peruana") (2, 3, 4). Other species, including *Bartonella clarridgeiae*, *Bartonella koehlerae*, *Bartonella vinsonii* subsp. *berkhoffii*, *Bartonella elizabethae* and 'Candidatus *Bartonella mayotimonensis*' have been associated to disease in humans, especially in fever of unknown origin and culture-negative endocarditis cases (5, 6). *Bartonella* spp. infect a variety of cells, including erythrocytes, pericytes, endothelial, dendritic and macrophage cells, and are associated to the persistent intraerythrocytic bacteremia, which indicates a possible coevolution between these bacteria and their hosts, which may explain their remarkable adaptability to one or more mammalian species (2, 7, 8). Furthermore, the ability to maintain a persistent bacteremia over time favors vector transmission by blood-sucking arthropods (9). Based on molecular epidemiological surveys and clinical observations, many hematophagous arthropods have been implicated in the transmission cycles of *Bartonella* spp., such as mosquitoes (9), biting midges (10), triatomine bugs (11), mites (12, 13) and flies (14) besides those already identified as competent vectors (fleas, lice, phlebotomine sand flies, and ticks) (15, 16).

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) comprises over 1,060 species, distributed worldwide, especially in tropical and subtropical regions (17). Due to their hematophagous feeding habit, female sand flies are insects of significant public health concern, since they act as vectors in the transmission of different pathogenic

agents (bacteria, protozoa and virus), such as *Bartonella* sp., *Leishmania* sp. and Phleboviruses (18).

Within the Bartonellaceae family, *B. bacilliformis* is notably the most important agent transmitted by phlebotomine sand flies. This species is the causative agent of Carrion's disease, which can manifest as two different syndromes (that can occur sequentially or independently): Oroya Fever, characterized by an acute hemolytic anemia with an untreated fatality rate of up to 90%, and Verruga Peruana, characterized by a widespread formation of hemangiomas (verrugas) on the skin, along with a persistent bacteremia (3, 7). The primary vectors of *B. bacilliformis* are *Pintomyia verrucarum* and *Lutzomyia peruensis*, which can be found in the Interandean Valleys of Peru, at altitudes ranging from 500 to 3,200 meters (7). Carrion's Disease is a neglected disease, due to its focal occurrence (Andean valleys in Peru, and to a lesser extent in Colombia and Ecuador) and challenging diagnosis (lack of resources and difficult access to endemic areas). The occurrence of the disease in non-endemic areas and the detection of *B. bacilliformis* DNA in other sand fly species, suggests that other sand flies may be involved in the epidemiological cycle of Carrion's Disease (19). Although their role as vectors has not yet been elucidated, *B. bacilliformis* DNA has been detected in wild captured *Pintomyia robusta* in the border between Ecuador and Peru (20) and in *Pintomyia maranonensis* in the northern part of Peru (21). In Colombia, *Lutzomyia gomezi*, *Psychodopygus panamensis*, *Pintomyia serrana* and most notably *Pintomyia columbiana* have been proposed as possible vectors of Carrion's Disease due to their presence in areas of the disease outbreaks (22, 23), albeit without molecular confirmation of the presence of

Bartonella sp. DNA in those sandfly specimens. Other suggested vectors for transmission of *B. bacilliformis* include *Lutzomyia pescei*, *Lutzomyia noguchii* and *Lutzomyia ayacuchensis* (19, 23, 24).

Bartonella ancashensis, a species closely related to *B. bacilliformis*, has been isolated from patients undergoing treatment for verruga peruana in the rural region of Ancash, Peru (25, 26). This species has not been isolated from Oroya Fever patients and seems to be less pathogenic than *B. bacilliformis*, although coinfections can occur since the geographic distribution of *B. ancashensis* overlaps with *B. bacilliformis* (4, 26). The involvement of sand flies in the transmission cycle of *B. ancashensis* has not yet been elucidated.

In Brazil, there is a rich diversity of 304 phlebotomine sand fly species (89 endemic), classified within 19 genera, distributed across all 5 federative regions of Brazil: 218 species in the North, 155 in the Midwest, 132 in the Southeast, 129 in the Northeast, and 49 in the South (27). Despite the diverse phlebotomine sand fly fauna present in Brazil, and the proximity to regions endemic or reporting cases of Carrion's Disease, previous studies have not investigated the occurrence of *Bartonella* spp. in these dipterans. However, studies from other countries have detected the presence of *Bartonella* sp. DNA in sand fly species that also inhabit Brazil. In Peru, individual females of *Pintomyia nevesi* and *Lutzomyia sherlocki* and pooled females of *Nyssomyia whitmani* and *Psychodopygus hirsutus* tested positive for *Bartonella* sp. DNA, phylogenetically associated to *B. bacilliformis* and 'Candidate *Bartonella rondoni*ensis' (28). In Mexico, *Bartonella gltA* genotypes associated with a putative new lineage of *Bartonella* in sand

flies were detected in females of *Lutzomyia cruciata* and *Psathyromyia shannoni* (29). In this study, we aimed to investigate the occurrence and molecular identity of *Bartonella* species in sand flies collected in seven states from the North and Northeastern regions of Brazil.

Material and methods

Sand fly specimens and studied areas

We analyzed sand fly specimens collected from November 2017 to December 2021. Specimens were captured using CDC or Shannon traps in ecological reserves and parks from Brazil: preserved forest areas in the cities of Xapuri and Rio Branco (Acre); Murici Ecological Station (Alagoas); Pau Brasil National Park (Bahia); Ubajara National Park (Ceará); Tapajós National Forest (Pará); Dois Irmãos State Park (Pernambuco); Viruá National Park (Roraima). DNA was extracted from dissected sand flies (without heads and three last abdominal segments, which were used for morphological identification following previously described taxonomic keys) (30) using the TRIzol™ (Invitrogen®, Thermo Fisher Scientific, <https://www.thermofisher.com/>), following the manufacturer instructions. DNA concentration and quality (260/280 ratio) were evaluated using a spectrophotometer (Nanodrop®, Thermo Fisher Scientific, <https://www.thermofisher.com/>). The presence of potential PCR inhibitors was assessed using a conventional PCR based on the cytochrome c oxidase subunit-1 (*cox-1*), an endogenous gene among invertebrates.

In total, the occurrence of *Bartonella* sp. DNA was investigated in 634 individual sand fly DNA samples that were classified into 44 species, belonging to 14 genera, and sampled from seven different states from North and Northeastern Brazil (**Table 2**).

Table 2. Species and number of sand flies after PCR screening for amplification of the endogenous (house keeping) gene *cox-1*. All sandfly samples were used for PCR amplification and phylogenetic characterization of *Bartonella* spp.

Genera	Species	State of Sampling
<i>Bichromomyia</i> (4)	<i>flaviscutellata</i> (4)	Acre (AC)
<i>Brumptomyia</i> (12)	sp. (12)	Acre (AC)
<i>Evandromyia</i> (60)	<i>begonae</i> (1)	Acre (AC)
	<i>infraspinoso</i> (1)	Acre (AC)
	<i>saulensis</i> (14)	Acre (AC)
	<i>termitophila</i> (1)	Acre (AC)
	<i>walkeri</i> (43)	Acre (AC)
	<i>longipalpis</i> (27)	Ceará (CE)
<i>Lutzomyia</i> (46)	<i>sherlocki</i> (19)	Acre (AC)
	<i>trinidanensis</i> (1)	Acre (AC)
<i>Micropygomyia</i> (2)	sp. (1)	Pará (PA)
	<i>antunesi</i> (76)	Acre (AC)
<i>Nyssomyia</i> (132)	<i>shawii</i> (15)	Acre (AC)
	<i>umbratilis</i> (28)	Pará (PA) n=14; Pernambuco (PE) n=14
	<i>whitmani</i> (12)	Acre (AC)
	sp. (1)	Acre (AC)
	<i>nevesi</i> (5)	Acre (AC)
	<i>serrana</i> (6)	Acre (AC)
<i>Pintomyia</i> (13)	sp. (2)	Bahia (BA)
	<i>choti</i> (15)	Bahia (BA)
	sp. (13)	Acre (AC) n=5; Bahia (BA) n=8
<i>Pressatia</i> (28)	<i>elizabethdorvalae</i> (2)	Acre (AC)
	sp. (1)	Acre (AC)
<i>Psychodopygus</i> (163)	<i>amazonensis</i> (3)	Acre (AC)
	<i>ayrozai</i> (40)	Alagoas (AL) n=2; Bahia (BA) n=8; Roraima (RR) n=30
	<i>carreirai</i> (25)	Acre (AC) n=22; Roraima (RR) n=3
	<i>chagasi</i> (26)	Alagoas (AL) n=2; Pará (PA) n=6; Roraima (RR) n=18
	<i>complexus</i> (3)	Alagoas (AL) n=2; Pará (PA) n=1
	<i>davisi</i> (30)	Acre (AC) n=24; Pará (PA) n=6
	<i>guyanensis</i> (1)	Pará (PA)
	<i>hirsutus</i> (2)	Alagoas (AL) n=1; Bahia (BA) n=1
	<i>lainsoni</i> (2)	Acre (AC)
	<i>llanosmartinsi</i> (11)	Acre (AC)
	<i>paraensis</i> (17)	Pará (PA) n=5; Roraima (RR) n=12

	<i>squamiventris</i> (1)	Roraima (RR)
	sp. (2)	Acre (AC) n=1; Roraima (RR) n=1
<i>Sciopemyia</i> (2)	<i>sordelli</i> (2)	Acre (AC)
<i>Trichophoromyia</i> (106)	<i>ubiquitalis</i> (1)	Pará (PA)
	<i>viannamartins</i> (65)	Alagoas (AL)
	sp. (40)	Acre (AC) n=24; Pará (PA) n=16
<i>Trichopygomyia</i> (61)	<i>dasypodogeton</i> (2)	Acre (AC)
	<i>longispina</i> (55)	Bahia (BA)
	sp. (4)	Bahia (BA) n=2; Roraima (RR) n=2
<i>Viannamyia</i> (2)	<i>furcata</i> (2)	Acre (AC)

The dataset is publicly available in the Sistema de Informação sobre a Biodiversidade Brasileira (SiBBR) and the Global Biodiversity Facility Information (GBIF) (<https://doi.org/10.15468/3cnmuw>).

Molecular Assays

We conducted the molecular screening for *Bartonella* spp. using a qPCR (quantitative real-time polymerase chain reaction) based on a 243 bp fragment of the 16S-23S ribosomal DNA internal transcribed spacer (ITS).

All reactions were performed in a final volume of 10 µL containing 2x qPCRBIO Probe Master Mix Buffer (PCR Biosystems, Wayne, PA, USA), 1.2 µM of each primer and probe, 1 µL of DNA sample and ultra-purified sterilized water q.s.p. The primers and probe and thermal conditions used for the reactions are showed in Appendix Table 1. For the construction of the standard curve of each reaction, serial dilutions were performed at different concentrations (1×10^7 to 1×10^1 copies) of a G-BLOCK encoding a 243 bp fragment of the ITS genic region of *Bartonella henselae* (Accession number: L35101) (Integrated DNA Technologies, Coralville, IA, USA). These G-BLOCKS were also used as positive controls. The number of gene copies was determined by the formula (XG/µL DNA/

$[\text{Gene Block Length (BP)} \times 660]) \times 6.22 \times 10^{23} \times \text{gene copies}/\mu\text{L}$. The amplification efficiency (E) was calculated according to the slope of the standard curve using the formula $E = 10^{-1/\text{slope}}$. Each DNA sample was evaluated in duplicate. Samples that presented differences in Cq values higher than 0,5 were retested in triplicate. We considered a Cq value cut off of 42 for negative results. Ultra-purified sterilized water was used as a negative control for each reaction, which was carried out in a C1000-CFX96 thermocycler (BIORAD, Hercules, CA, USA).

Samples positive in the screening qPCR were then characterized using conventional PCR assays based on eight different molecular markers: *gltA* (380-400 bp); (767 bp); *ftsZ* (515 bp); *groEL* (752 bp); *nuoG* (346 bp); *pap31* (564 bp); *rpoB* (825 bp); *ribC* (585-588 bp) and 16S-23S ITS (453-717 bp). The primers/hydrolysis probes and thermocycler conditions used for each conventional (including the endogenous gene *cox-1*) and quantitative real-time PCR are described in Appendix Table 1.

Purification and Phylogenetic Analyzes

The amplicons obtained in the PCR assays were purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, <https://www.promega.com/>). Purified amplicons were submitted for Sanger sequencing in both directions (forward and reverse) at the Centro de Estudos do Genoma Humano e Células Tronco (University of São Paulo – USP) using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, <https://www.thermofisher.com/>). A consensus sequence for each sample was assembled using Geneious Prime 2023.2 (<https://www.geneious.com/>) and BioEdit 7.2 (31) softwares.

An alignment was produced for each genetic region, using the obtained sequences, closely related sequences from BLASTn analyzes (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and reference sequences previously deposited on GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). The alignments were made using the MAFFT version 7 software (<https://mafft.cbrc.jp/alignment/server/index.html>) and trimmed using the BioEdit 7.2 (31) software. For phylogenetic inferences, a Maximum Likelihood (ML) analysis, with 10³ UltraFast Bootstrap Replicates for each alignment was performed using the IQTREE2 1.6.12 software (<http://www.iqtree.org/>). The best-fitting evolutionary model for each alignment was chosen using the MrModeltest2 2.4 (MrModeltest 2.4 by Johan A. A. Nylander. Original code by David Posada, U. Vigo, Spain; <https://github.com/nylander/MrModeltest2>) through the PAUP4* Version 4c software (<https://paup.phylosolutions.com/>). The resulting phylogenetic trees were rooted (via outgroups) and edited using FigTree 1.4.4 (<https://tree.bio.ed.ac.uk/software/figtree/>) and iTOL version 5 (<https://itol.embl.de/>) softwares.

Results

The DNA extraction of individual specimens of sand flies using the TRIzol was satisfactory, yielding DNA concentrations ranging from 1 to 15 ng/μL. We were able to obtain positive samples in the *cox-1* conventional PCR for all the 634 (100%) specimens.

A total of 55/634 (8.67%) sand flies were positive in the molecular screening for *Bartonella* spp. using the qPCR assay targeting the 16S-23S ITS region: 48 from Acre (n=18 *Nyssomyia antunesi*; n=seven *Evandromyia walkeri*; n=five *Trichophoromyia* sp.; n=four *Lutzomyia sherlocki*; n=three *Nyssomyia shawi*; n=two *Psychodopygus*

Ilanosmartinsi; n=two *Psychodopygus davisi*; n=one *Bichromyia flaviscutellata*; n=one *Envandromyia saulensis*; n=one *Nyssomyia* sp.; n=one *Nyssomyia whitmani*; n=one *Pintomyia nevesi*; n=one *Pintomyia serrana*; n=one *Viannamyia furcata*); two from Alagoas (n=two *Trichophoromyia viannamartinsi*); two from Roraima (n=one *Psychodopygus squamiventris*; n=one *Psychodopygus ayrozai*); one from Bahia (*Trichopygomyia longispina*); n=one from Ceará (*Lutzomyia longipalpis*); and n=one from Pará (*Psychodopygus paraensis*) (**Figure 1**).

The Cq values of positive samples ranged from 30.1 to 41.8. We selected 16 of these samples (based on the lowest PCR Cq values) and were able to obtain 7 readable sequences. Based on BLASTn analysis, we confirmed that all seven sequences corresponded to a *Bartonella* sp. (Appendix Table 2). These sequences were too short (from 179 to 222 base pairs) to be used for phylogenetic inferences. The values of the qPCR efficiency, R^2 , Y-intercept and slope ranged from 98.7% to 104.8% (mean= 102.3; SD= 2.29), 0.834 to 0.986 (mean= 0.978; SD= 0.05), 34.429 to 42.318 (mean= 37.97; SD= 2.75) and -3.22 to -3.35 (mean= 3.27; SD= 0.05), respectively. We were unable to measure the DNA load of positive samples, since the Cq difference between replicates was higher than 0,5, possibly due the Monte Carlo effect (32).

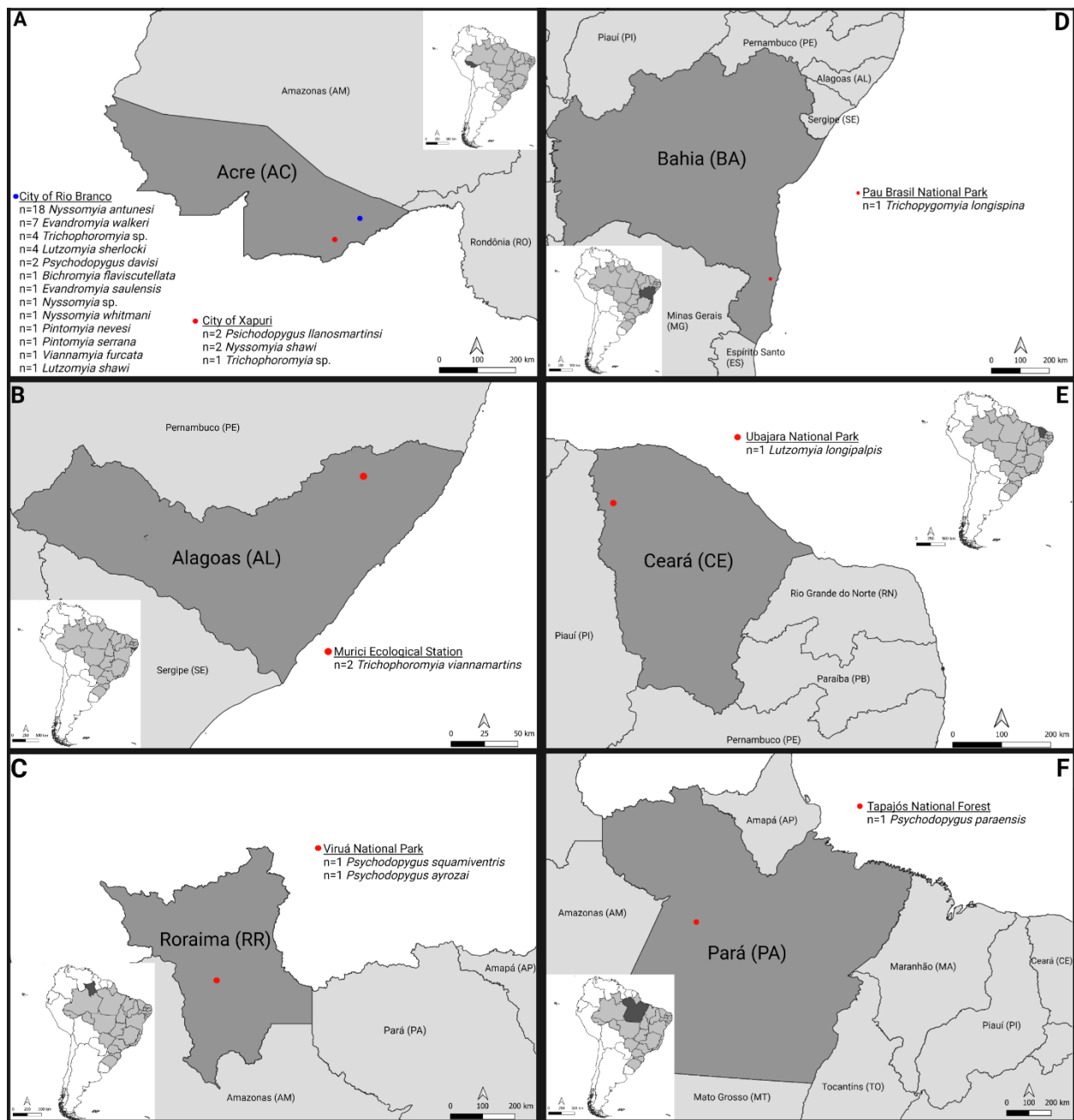


Figure 1. Sampling locations for sand flies that were qPCR positive in the screening for *Bartonella* spp. DNA. Each state with positive specimens is represented in darker grey, with red and blue dots representing the geographical location or city for the sampling site. A) State of Acre, Northern Brazil; B) State of Alagoas, Northeastern Brazil; C) State of Roraima, Northern Brazil; D) State of Bahia, Northeastern Brazil; E) State of Ceará, Northeastern Brazil; F) State of Pará, Northern Brazil.

Further molecular characterization by conventional PCR of those samples positive in the ITS screening qPCR assay, generated amplicons for the following genes: 4 for the *gltA*; 4 for the ITS; 2 for the *ftsZ*; 2 for the *pap31*; 1 for the *rpoB* and 1 for the *nuoG*. Of these, two readable sequences were obtained: one 377 bp *gltA* sequence (GenBank accession number PP421218) from a *Lutzomyia longipalpis* captured in the state of Ceará, and one 345 bp *nuoG* sequence from a *Nyssomyia antunesi* from Acre.

The BLASTn analysis demonstrated that the *gltA* sequence obtained from *Lutzomyia longipalpis* demonstrated >96% identity with *B. ancashensis* sequences previously obtained from infected humans ([CP010401.1](#); [KC886736.1](#); [KC178618.1](#)). Phylogenetic analyses positioned this sequence in the same sub-clade as *B. ancashensis* sequences, and with a *Bartonella* sp. sequence detected in a *Dampfomyia beltrani* sand fly from Mexico ([OQ343492.1](#)), with a bootstrap clade support value of 95 (**Figure 2**).

The BLASTn analysis of the *nuoG* sequence from *Nyssomyia antunesi* indicated a 94.04%-94.47% identity with two *Bartonella* sp. sequences obtained from *Pteronotus davyi* bats from Guatemala ([MN270091.1](#); [MN270098.1](#)). The few *Bartonella* *nuoG* sequences in GenBank and low values of bootstrap clades hampered robust phylogenetic inference using this molecular marker.

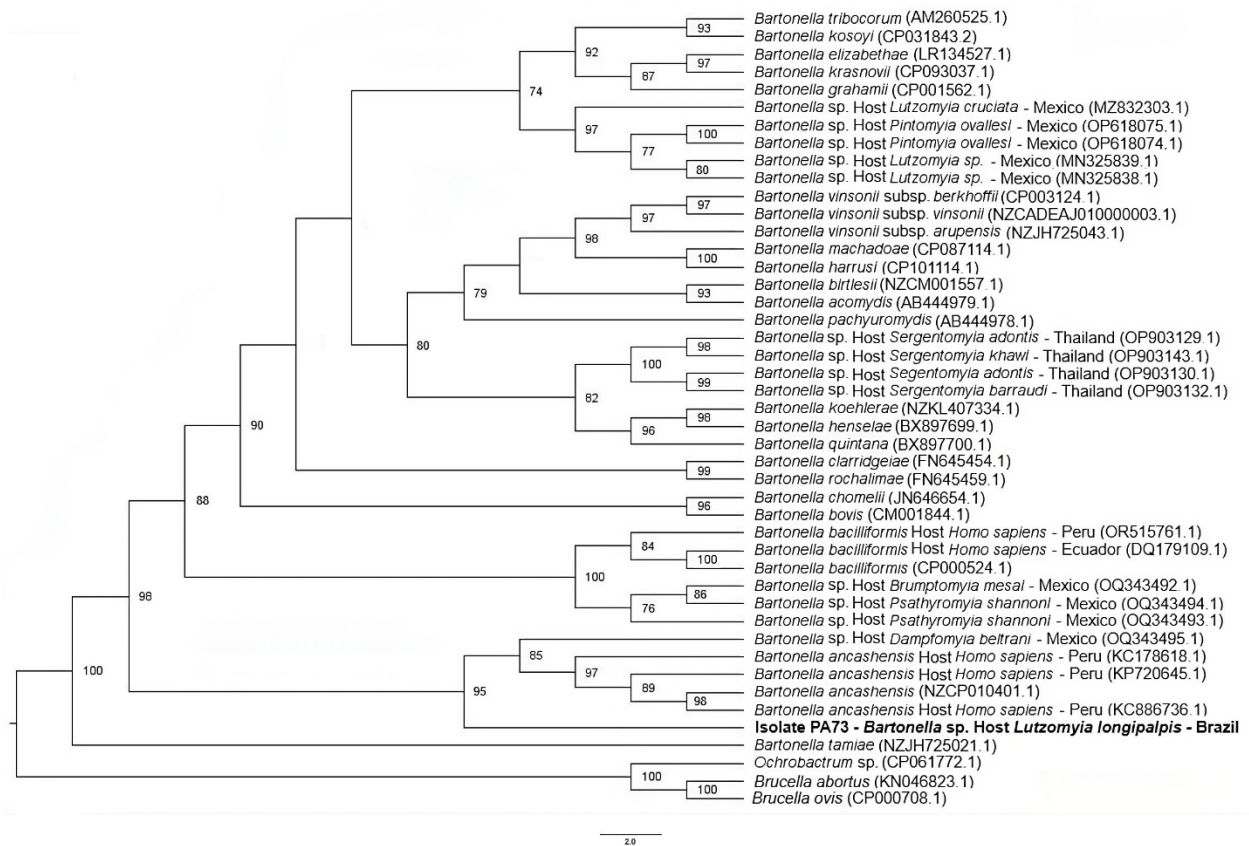


Figure 2. Phylogenetic tree based on an alignment of 380 bp-length of the *gltA* sequences using Maximum Likelihood (ML) method and GTR+I+G as the evolutionary model. Sequences detected in the present study are highlighted in bold. *Ochrobactrum* sp., *Brucella ovis* and *Brucella abortus* were used as outgroups. Only bootstrap values >70 are shown.

Discussion

We documented the presence of *Bartonella* spp. DNA in phlebotomine sand flies from Brazil. The occurrence rate observed in the present study (55/634 specimens; 8.67%) is similar to that reported from Southern Mexico (2/23 specimens; 8.69%) (33),

Peru (17/228 pools; 6.02%) (28), (2/76 pools; 2.63%) (21) and Mexico (27/531 specimens; 5.08%) (34), (11/532 specimens; 2.06%) (29). Differences in low occurrence rates can be explained by the wide diversity of sand fly species present in different countries, the method of molecular analysis employed for DNA amplification, and as illustrated in this study, technical limitations in obtaining phylogenetically relevant *Bartonella* DNA sequences from these small insects. As stated previously, the phlebotomine vectors of *Bartonella* spp. are very restricted to defined geographical areas; however, there have been minimal efforts to investigate the prevalence of this bacterial genus in sand flies from regions other than Peru. In this study, the selection of a broad diversity of sand fly species for *Bartonella* detection can be misleading, since most of the species are not confirmed to be carriers of these bacteria. In this context, we can assume that sand flies that were negative for the *Bartonella* sp. detection are either unable to host the bacteria or can be considered infrequent vectors. Further studies are necessary to elucidate the role of different sand fly species in the *Bartonella* epidemiological cycles.

Although pooling specimens for analysis may have yielded a higher quantity of DNA (ng/μL), we would not have been able to accurately quantify the number of specimens that contained *Bartonella* sp. DNA, potentially leading to an underrepresentation of PCR positive sand flies. Therefore, we opted to individually extract the DNA from the specimens using the TRIzol™ (Invitrogen®, Thermo Fisher Scientific, <https://www.thermofisher.com/>) reagent, which resulted in satisfactory DNA quality, with concentrations ranging from 1 to 15 ng/μL, and provided enough volume to perform the

molecular detection and characterization. The absence of PCR inhibitors was confirmed by the successful amplification of the invertebrate *cox-1* gene in all samples.

The 55 samples positive in the qPCR for *Bartonella* spp. belonged to 9 genera classified in 21 different species. Our findings include the first report of the presence of *Bartonella* spp. in *Bichromyia*, *Evandromyia*, *Psychodopygus*, *Trichophoromyia*, *Trichopygomyia* and *Viannamyia* phlebotomine sand flies. Additionally, we detected *Bartonella* DNA in sand fly species that have been previously associated to *Bartonella* sp. in Peru, including *L. sherlocki* (n=4), *P. nevesi* (n=1) and *N. whitmani* (n=1) (28). These findings reinforce that these phlebotomine sand fly species serve as hosts for *Bartonella* sp. and might be involved in the epidemiological cycles of Bartonellaceae agents in Brazil and Peru.

Herein, we reported, the detection of *Bartonella* sp. in *L. longipalpis* from the Ceará state, Northeastern Brazil. The obtained 377 bp *Bartonella gltA* sequence clustered in the same sub-clade as *B. ancashensis* sequences obtained from humans with verruga peruana and a genotype recently detected in pools of *Dampfomyia beltrani* sand flies from Mexico (35). The results described herein corroborate previous findings reported in Mexico (35), with description of putative novel *Bartonella* genotypes closely related to *B. ancashensis* in sand flies from non-endemic areas for “Verruga Peruana”. Interestingly, genotypes closely related to *B. bacilliformis* were previously detected in *Psathyromyia* sand flies from Mexico, a non-endemic country for Carrion Disease (29). Collectively, findings to date highlight the occurrence of putative novel genotypes belonging to ancient

Bartonella lineages in sand flies from Brazil and Mexico, whose zoonotic potential remains unknown.

Although natural *Bartonella* sp. infections have not been previously reported in *L. longipalpis* sand flies, experimental studies using this species demonstrated infection with *B. ancashensis*, which remained viable in the anterior midgut for up to 7 days (4). Furthermore, experimental infections with *B. bacilliformis* in *L. longipalpis* sand flies, reported similar bacterial viability results to experimental infection with *B. ancashensis* (36). Although *L. longipalpis* has been used as a model for sand fly infection with *B. bacilliformis*, there are no reports of this species in Peru, where Carrion's Disease is endemic (37). Prior investigators have suggested *L. longipalpis* may play a short-term role in the maintenance of *Bartonella* and potentially serve as a vector during this time (4, 36). Once more, we reinforce the need for further investigations regarding the potential role of various sand flies for transmission of *Bartonella* spp. to human patients and sick animals. Future research focusing on *L. longipalpis* is of particular importance, as this sand fly species is the main vector of *Leishmania infantum* and it is widely distributed in Brazil (occurring in all regions and biomes), besides being found throughout Central and South America (38). Although absent from Peru, *L. (Lutzomyia) longipalpis* belongs to the same genus, albeit from a different subgenus, as the primary vector of *Bartonella bacilliformis* in Peru, namely *Lutzomyia (Helcocyrtomyia) peruensis*. Besides that, *L. longipalpis* is related to species in which *Bartonella* DNA have already been detected, namely *Lutzomyia (Tricholateralis) gomezi*, *Lutzomyia (Tricholateralis) cruciata*, *Lutzomyia (Tricholateralis) sherlocki*, or to species that have been incriminated as additional putative

vectors for *B. bacilliformis*, namely *Lutzomyia (Helcocyrtomyia) pescei*, *Lutzomyia (Helcocyrtomyia) noguchii* and *Lutzomyia (Helcocyrtomyia) ayacuchensis* (7, 19, 20, 23, 24, 30). These findings highlight the importance of the genus *Lutzomyia sensu stricto* in the transmission cycles of *Bartonella* in South America.

A *Bartonella* sp. *nuoG* sequence with ~94% identity to sequences previously detected in insectivorous *Pteronotus davyi* bats from Guatemala was also amplified in this study. The obtained genotype also shared 88% to 91% identity to other *Bartonella* sp. sequences previously detected in bats and their associated ectoparasites from Brazil (data not shown), including sequences amplified from vampire bats *Diphylla ecaudata* and *Desmodus rotundus* bats (39) and *Trichobius dugesii* flies (40). Despite the diverse phlebotomine sand fly fauna found across many Brazilian biomes, such as the Amazon Rainforest containing 193 species, Atlantic Forest (120 species) and Cerrado (58 species), no previous study has reported the occurrence of *Bartonella* in these dipterans in Brazil. Additionally, despite the geographical proximity to endemic regions or non-endemic regions reporting cases of Carrion's Disease and *Bartonella* sp. in sand flies, this occurrence remains undocumented to our knowledge (27). However, based upon phlebotomine sand fly feeding habits (41), many studies have reported the occurrence of *Bartonella* sp. in vertebrates that act as hosts for sand fly blood meals, including rodents (42, 43), marsupials (44), bats (39, 40, 43), and xenarthrans (45). Although Streblidae and Nycteribiidae flies act as the main putative vectors of *Bartonella* species transmission among bats (40, 46), many sandfly species that feed on bats may acquire *Bartonella* spp. infections during blood-feeding. Although the role of numerous sand fly species in the

transmission and maintenance of *Bartonella* spp. in Brazil remains undefined, sand fly feeding habits and the high prevalence of *Bartonella* infection in many reservoir mammalian hosts indicates a potential relationship and involvement of sand flies in the epidemiological cycles of these bacteria.

Conclusion

In summary, *Bartonella* spp. DNA was amplified and successfully sequenced from *Lutomyia longipalpis* and *Nyssomyia antunesi*, indicating possible involvement of these phlebotomine sand fly species in the maintenance and/or transmission cycle of *Bartonella* sp. The *Bartonella gltA* genotype was closely related to *B. ancashensis* and the *nuoG* genotype was most closely related to bat-associated *Bartonella* sp.

Acknowledgments

The present study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Process numbers 2022/07008-6 and 2022/08543-2), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Productivity Grant for MRA, CNPq Process No. 303701/2021-8). PHFS would like to thank Fundação de Amparo à Pesquisa do Estado de Minas Gerais for financial support (PPM-00676-18).

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<https://doi.org/10.1371/journal.pone.0198629>

APPENDIX

***Bartonella* spp. in sand flies (Diptera, Psychodidae, Phlebotominae) from Brazil**

Appendix

Appendix Table 1. Description of molecular assays (target gene, primers/probe identification and sequence, thermal conditions, amplicon size and reference) used for sand flies endogenous gene, screening and characterization of *Bartonella* spp. in the present study.

Molecular Marker	Primers/Probe	Thermal conditions	Amplicon size (bp)	Reference
<i>cox-1</i>	LCO1490 (5' -GGTCAACAAATCATAAAGATATTGG- 3') HCO2198 (5' -TAAACTTCAGGGTGACCAAAAAATCA- 3')	95°C for 1min; 35 cycles of 95°C for 1min, 40°C for 1min and 72°C for 1.5min; 72°C for 10min	710	(1)
16S-23S ITS	BsppITS325S Forward (5' – CTTCAGATGATGATCCCAAGCCTTCTGGCG – 3') 543as Reverse (5' – AATTGGTGGGCCTGGGAGGACTTG – 3') BsppITS500 (5' – [6FAM]GTTAGAGCGCGCGCTTGATAAG[BHQ1] – 3')	95°C for 3min; 45 cycles of 94°C for 10s, 68°C for 10s, 72°C for 10s and plate read; 72°C for 30s	243	(2)
<i>gltA</i>	BhCS.781p (<i>gltA</i> F) (5' GGGGACCAGCTCATGGTGG- 3') BhCS.1137n (<i>gltA</i> R)	95°C for 5min; 35 cycles of 95°C for 30s, 51°C for 30s	380-400	(3)

	(5' AATGCAAAAAGAACAGTAAACA- 3')	and 72°C for 2min; 72°C for 5min		
	CS443f (5' -GCTATGTCTGCATTCTATCA- 3')	94°C for 2min; 45 cycles of 94°C for 30s, 48°C for 1min and 72°C for 1min; 72°C for 7min	767	(4)
	CS1210r (5' -GATCYTCAATCATTTCTTTCCA- 3')			
ftsZ	ftsZ F (5' -CATATGGTTTTTCATTACTGCYGGTATGG- 3')	94°C for 2min; 40 cycles of 94°C for 2min, 61°C for 45s and 72°C for 45s; 72°C for 7min	515	(5)
	ftsZ R (5' -TTCTTCGCGAATACGATTAGCAGCTTC- 3')			
groEL	GroEL F (5' -GGAAAAAGTGGGCAATGAAG- 3')	94°C for 2min; 40 cycles of 94°C for 2min, 47°C for 45s and 72°C for 45s; 72°C for 7min	752	(5)
	GroEL R (5' -TCCTTTAACGGTCAACGCATT- 3')			
nuoG	nuoG F (5' -GGCGTGATTGTTCTCGTTA- 3')	94°C for 5min; 35 cycles of 94°C for 30s, 53°C for 30s and 72°C for 5min; 72°C for 5min	346	(6)
	nuoG R (5' -CACGACCACGGCTATCAAT -3')			
Pap31	165s (5' -GACTTCTGTTATCGCTTTGATTT- 3')	95°C for 5min; 45 cycles of 94°C for 30s, 56°C for 30s and 72°C for 45s; 72°C for 5min	564	(7)
	688as (5' -CACCACCAGCAAMATAAGGCAT- 3')			
rpoB	1400F (5' -CGCATTGGCTTACTTCGTATG- 3')	94°C for 2min; 35 cycles of 94°C for 30s, 53°C for 30s and 72°C for 1min; 72°C for 2min	825	(8)
	2300R (5' -GTAGACTGATTAGAACGCTG- 3')			
ribC	Barton-1 (5' -TAACCGATATTGGTTGTGTTGAAG- 3')	95°C for 10min; 37 cycles of 95°C for 1min, 51°C for 1min and 72°C for 1min; 72°C for 3min	585-588	(9)
	Barton-2 (5' -TAAAGCTAGAAAGTCTGGCAACATAACG- 3')			

16S-23S ITS	321s (5' -AGATGATGATCCCAAGCCTTCTGGCG- 3')	95°C for 5min; 45 cycles of 94°C for 30s, 54°C for 30s and 72°C for 45s; 72°C for 5min	453-717 (10)
	938as (5' -TGTTCTYACAACAATGATGATG- 3')		

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Appendix Table 2. Sequence identity of the 16S-23S ITS positive samples to the most closely related *Bartonella* species.

ID	Sand fly species	Sequence size (bp)	BLASTn result	Host	Query Cover	E value	Identity	Country	Accession Number
AB121	<i>Trichophoromyia</i> sp.	222	<i>Bartonella</i> <i>ancashensis</i>	<i>Homo sapiens</i>	98%	9e-63	87.56%	Peru	KC886740.1
AB410	<i>Nyssomyia antunesi</i>	197	<i>Bartonella</i> sp.	<i>Mimon cozumelae</i>	81%	4e-71	98.16%	Guatemala	MN504740.1
			<i>Bartonella</i> sp.	<i>Micronycteris microtis</i>	81%	4e-71	98.16%	Guatemala	MN258132.1
AB594	<i>Psychodopygus davisi</i>	189	<i>Bartonella</i> sp.	<i>Mimon cozumelae</i>	87%	2e-78	100%	Guatemala	MN504740.1
			<i>Bartonella</i> sp.	<i>Micronycteris microtis</i>	87%	2e-78	100%	Guatemala	MN258132.1
AB164	<i>Psychodopygus llanosmartinsi</i>	189	<i>Bartonella</i> sp.	<i>Apodemus sylvaticus</i>	87%	1e-60	93.49%	United Kingdom	AJ269793.1
AB537	<i>Evandromyia walker</i>	179	<i>Bartonella</i> sp.	<i>Mimon cozumelae</i>	89%	4e-75	99.38%	Guatemala	MN504740.1
			<i>Bartonella</i> sp.	<i>Micronycteris microtis</i>	89%	4e-75	99.38%	Guatemala	MN258132.1

AB540	<i>Pintomyia serrana</i>	175	<i>Bartonella</i> sp.	<i>Apodemus</i> <i>sylvaticus</i>	100%	1e-60	92.09%	United Kingdom	AJ269793.1
AB12	<i>Nyssomyia</i> <i>antunesi</i>	197	<i>Bartonella</i> sp.	<i>Apodemus</i> <i>sylvaticus</i>	89%	1e-65	93.3%	United Kingdom	AJ269793.1