UNIVERSIDADE ESTADUAL PAULISTA – UNESP

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS (FCAV)

CAMPUS DE JABOTICABAL

ZOONOTIC VARIANTS OF *Bartonella henselae* CIRCULATING AMONG CLINICAL PATIENT AND BLOOD DONOR CATS IN CENTRAL-WESTERN BRAZIL

Lorena Freitas das Neves

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Orientador: Prof. Dr. Marcos Rogério André

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Impacto potencial desta pesquisa

Os gatos têm se tornado uma escolha cada vez mais popular entre os animais de estimação no Brasil. Tal crescimento também aumenta os riscos de transmissão de zoonoses. Gatos são os principais reservatórios de *Bartonella henselae, Bartonella clarridgeiae* e *Bartonella koehlerae*, bactéria zoonóticas de distribuição mundial e de importância em Saúde Pública. Para auxiliar futuras ações de controle das bartoneloses, é fundamental investigar a ocorrência e diversidade de variantes de *B. henselae,* principal agente causador da Doença da Arranhadura do Gato (DAG). Ao expandir o conhecimento acerca da diversidade de variantes genéticas zoonóticas de *B. henselae* (ST1, ST2 e ST5) em gatos no centro-oeste do Brasil, nosso estudo contribui para o entendimento da epidemiologia molecular de infecções por este agente no Brasil. Além disso, ao evidenciar exposição/infecção por *Bartonella* spp., por meio de métodos sorológicos, microbiológicos e moleculares em gatos doadores de sangue, o presente estudo enfatiza o potencial risco de transmissão de *Bartonella* spp. por transfusões sanguíneas.

Potential impact of this research

Cats have become an increasingly popular choice for pets in Brazil. This growth also increases the risks of zoonotic transmission. Cats are the primary reservoirs of *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae*, zoonotic bacteria of global distribution and significance for public health. To assist future control efforts for bartonelloses, it is essential to investigate the occurrence and diversity of *B*. *henselae* variants, the main causative agent of Cat Scratch Disease (CSD). By expanding knowledge about the diversity of zoonotic genetic variants of *B*. *henselae* (ST1, ST2, and ST5) in cats from central-western Brazil, our study contributes to the understanding of the molecular epidemiology of infections caused by this agent in Brazil. Additionally, by demonstrating exposure/infection by *Bartonella* spp. through serological, microbiological, and molecular methods in cat blood donors, the present study highlights the potential risk of *Bartonella* spp. transmission through blood transfusions.

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AUTORA: LORENA FREITAS DAS NEVES
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Prof. Dr. MARCOS ROGÉRIO ANDRÉ (Participaçao Virtual)
Departamento de Patologia Reproducao e Saude Unica / FCAV UNESP Jaboticabal

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Profa. Dra. CRISTIANE DIVAN BALDANI (Participaçao Virtual)
Departamento de Medicina e Cirurgia Veterinária / Universidade Federal Rural do Rio de Janeiro (UFRRJ) - Rio de Janeiro/RJ

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DADOS CURRICULARES DA AUTORA

Lorena Freitas das Neves - Filha de Meiriane Souza Freitas e Marcus Vinicius Carvalho das Neves, nasceu em 22 de março de 1997, na cidade de Barbacena, no Estado de Minas Gerais. Em 2017, ingressou no curso de Medicina Veterinária do Centro Universitário do Planalto Central Apparecido dos Santos (UNICEPLAC), em Brasília (DF), graduando-se em de 2021. Durante a graduação, realizou duas monitorias de Bioquímica no período de 2017 e 2018, além de monitoria voluntária em Imunologia Veterinária. Em 2019, realizou estágio no One Health Veterinary (OHV) como auxiliar de Veterinário e Laboratório. Trabalhou no Medicalvet Laboratório Veterinário em Brasília entre 2021 e 2022 como técnica de laboratório. Em 2022, ingressou no curso de Mestrado em Ciências Veterinárias na Universidade Estadual Paulista “Júlio de Mesquita Filho” – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Campus de Jaboticabal, sob orientação do Prof. Dr. Marcos Rogério André, e foi beneficiada com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Atualmente, também é coordenadora da Liga Acadêmica em Saúde Pública (LASP) da FCAV/UNESP.

EPÍGRAFE

“Bring me the spark in my veins

Gimme the freedom to chase

You know it’s all ours

So don’t you stop make this moment last”

-Twice

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SUMÁRIO

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Tipagem Molecular de *Bartonella henselae* por Multilocus Sequencing Typing (MLST) em amostras de sangue de gatos de um hemocentro e laboratório veterinário do Distrito Federal"**, protocolo nº 3971/22, 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 06 de julho de 2022.

Vigência do Projeto	01/08/2022 a 31/07/2024
Espécie / Linhagem	*Felis silvestris catus*
Nº de animais	200
Peso / Idade	Variável
Sexo	Machos e fêmeas
Origem	Distrito Federal

Jaboticabal, 06 de julho de 2022.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel. 16 3209-7100 - www.fcav.unesp.br

VARIANTES ZOONÓTICAS DE *Bartonella henselae* CIRCULANDO ENTRE PACIENTES CLÍNICOS E GATOS DOADORES DE SANGUE NO CENTRO-OESTE DO BRASIL

RESUMO – *Bartonella* spp. apresenta uma preocupação significativa para a saúde global, sendo que *Bartonella henselae* representa uma das espécies mais prevalentes implicadas em várias doenças humanas. Estudos anteriores exploraram a possível transmissão de *Bartonella* spp. por transfusões de sangue em humanos, ressaltando sua relevância zoonótica. Embora vários estudos tenham mostrado a ocorrência generalizada de *B. henselae* em gatos do Brasil, a diversidade genética deste agente zoonótico permanece em grande parte desconhecida. O presente estudo teve como objetivo investigar a ocorrência e a diversidade genética de *B. henselae* em amostras de sangue de gatos domésticos coletadas em clínicas veterinárias e de doadores de sangue felinos do centro-oeste do Brasil, utilizando técnicas sorológicas, microbiológicas e moleculares. Amostras de soro foram submetidas à Reação de Imunofluorescência Indireta (RIFI) para investigar a frequência de anticorpos IgG anti-*B. henselae* na população de gatos estudada. Um ensaio de PCR em tempo real quantitativa (qPCR) baseado na região do rRNA 16-23S (ITS) foi utilizado para a detecção molecular de *Bartonella* spp. em amostras de sangue, tanto antes quanto após incubação em um meio pré-enriquecido para *Bartonella* Alpha Proteobacteria Growth Medium (BAPGM), assim como para confirmar a identidade das colônias isoladas em ágar de chocolate. Anticorpos IgG anti-*Bartonella* sp. foram detectados em 60,75% (96/158) das amostras de soro (33/67 gatos de clínicas veterinárias; 63/91 doadores de sangue). Na qPCR, 32% (32/100) e 46% (46/100) dos gatos de clínicas veterinárias e doadores de sangue, respectivamente, foram positivos para *Bartonella* spp. Após a incubação das amostras de sangue em meio líquido BAPGM, 61 amostras (78,20%) que mostraram-se previamente negativas na qPCR para sangue, tornaram-se positivas na qPCR para *Bartonella* spp. DNA da *Bartonella* e soropositividade para o antígeno de *B. henselae* foram detectados simultaneamente em 25,3% (40/158) dos gatos. Três cepas de *B. henselae* foram isoladas com sucesso, sendo duas originárias de gatos amostrados em clínicas veterinárias e uma de um doador de sangue felino. *Multi-locus Sequencing Typing* (MLST) revelou a ocorrência das variantes zoonóticas de *B. henselae* Sequencing Typing (ST)1, ST2 e ST5. O presente estudo mostrou,

pela primeira vez, a ocorrência de *Bartonella* spp. em doadores de sangue felinos na América do Sul, destacando os potenciais riscos de transmissão de variantes zoonóticas de *B*. *henselae* por transfusão de sangue para gatos. Foi observada uma maior ocorrência de *Bartonella* spp. entre os doadores de sangue felinos. Como 25.95% (9/41) das amostras de soro de gatos doadores positivos para *Bartonella* spp. na qPCR mostraram-se negativas na RIFI para *B. henselae*, a sorologia não pode ser usada para verificar a exposição/infeção por *Bartonella* em doadores de sangue felinos. Este é o primeiro relato de uma variante zoonótica de *B*. *henselae* (ST5) em doadores de sangue felinos. ST2 é documentado pela primeira vez em gatos no continente americano.

Palavras-chave: Bartonelose, Doença da Arranhadura do Gato, MLST, Sequence Type, RIFI

ZOONOTIC VARIANTS OF *Bartonella henselae* CIRCULATING AMONG CLINICAL PATIENT AND BLOOD DONOR CATS IN CENTRAL-WESTERN BRAZIL

ABSTRACT – *Bartonella* spp. present a significant global health concern, with *Bartonella henselae* emerging as one of the most prevalent species implicated in several human diseases. Previous studies have explored the potential transmission of *Bartonella* via blood transfusions in humans, underscoring the zoonotic relevance. Although several studies have shown the widespread occurrence of *B. henselae* in cats from Brazil, the genetic diversity of this zoonotic agent remains largely unknown. The present study aimed to investigate the occurrence and genetic diversity of *B. henselae* in blood samples from domestic cats sampled in veterinary clinics and cat blood donors from central-western Brazil, using serological, microbiological and molecular techniques. Serum samples were subjected to Indirect Fluorescence Antibody Test (IFAT) to investigate the frequency of IgG antibodies in the cat population studied herein. A real-time quantitative real-time PCR (qPCR) assay for *Bartonella* spp. targeting the 16-23S rRNA (ITS) region was used for molecular detection of *Bartonella* spp. in blood samples both before and after incubation in a pre-enrichment *Bartonella* Alpha Proteobacteria Growth Medium (BAPGM), as well as to confirm the identity of colonies isolated onto chocolate agar. Antibodies anti-*Bartonella* sp. were detected in 60.75% (96/158) of the serum samples (33/67 cats from veterinary clinics; 63/91 blood donors). In qPCR, 32% (32/100) and 46% (46/100) cats from veterinary clinics and cat blood donors were positive for *Bartonella* spp. After incubating blood samples in BAPGM liquid medium, 61 samples (78.20%) previously negative in blood-qPCR turned out positive in the qPCR for *Bartonella* spp.. *Bartonella* spp. DNA and seropositivity for *B. henselae* antigen were concurrently detected in 25.3% (40/158) of the cats. Three *B. henselae* strains were successfully isolated, two originating from cats sampled in veterinary clinics, and one from a cat blood donor. Multi-locus Sequencing Typing (MLST) revealed the occurrence of the *B. henselae* zoonotic variants Sequence Type (ST)1, ST2 and ST5. The present study showed, for the first time, the occurrence of *Bartonella* spp. in cat blood donors in South America, highlighting the potential risks of transmission of zoonotic *B. henselae* variants by blood transfusion to cats. Higher occurrence of *Bartonella* spp. was observed among cat blood donors. Since 25.95% (9/41) of the serum samples from *Bartonella* qPCR-

positive blood donors cat showed to be negative in the IFAT for *B. henselae*, serology cannot be used to check the *Bartonella* exposure/infection in cat blood donors. This is the first report of a zoonotic *B. henselae* variant (ST5) in cat blood donors. ST2 is documented for the first time in cats in the American continent.

Keywords: Bartonellosis, Cat Scratch Disease, MLST, Sequence Type, IFAT

CHAPTER 1 – GENERAL CONSIDERATIONS

1. INTRODUCTION

The evolution of diagnostic methodologies has significantly enhanced the identification and detection of *Bartonella* species. *Bartonella* genus comprises pleomorphic and Gram-negative bacteria, thriving in fastidious and aerobic conditions with preference for capnophilia. These oxidase-negative organisms are members of α2 subgroup of *Proteobacteria*, which primarily infect erythrocytes and endothelial cells.

Cats play a role as the main reservoirs for *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae.* The transmission of these agents among cats occurs through feces of the cat flea *Ctenocephalides felis felis*. Humans can become infected through the inoculation of viable *B. henselae* organisms through scratches or bites containing feces of infected *C. felis*. *Bartonella henselae* is the main causative agent of Cat Scratch Disease (CSD) in humans, often characterized by regional lymphadenopathy and fever. There is a potential risk of *Bartonella* transmission during blood transfusions in humans. When it comes to cats, although there are no reports of natural infection, there is evidence of the transmission of *B. henselae* through blood transfusion via experimental infection.

Despite a plethora of studies on the occurrence of *Bartonella* spp. in cats from several regions of Brazil, which reported prevalence ranging from 1.6 to 90.2%, the genetic diversity of *B. henselae* in Brazil have been scarcely investigated. Multiple Locus Sequence Typing (MLST) has been used as the main tool to genotype *B. henselae* isolates around the world, allowing the discrimination of 38 *B. henselae* Sequence Types (ST) in Argentina, Australia, the United Kingdom, the United States, New Zealand, Turkey, Brazil, China, Japan, Algeria, Israel, and others European countries (Croatia, Spain, Czech Republic, Italy, Switzerland, Sweden, Germany, Netherlands, France, and Denmark). In Brazil, only two studies so far have genotyped *B. henselae* strains isolated from cats in the states of Minas Gerais (ST37), São Paulo (ST9), and Goiás (ST1 and ST5).

Foremost, in order to shed light on the molecular epidemiology of *Bartonella* infection in domestic cats and specially in cat blood donors, studies aiming at investigating genetic variants of *B. henselae* in Brazil are much needed. This work

aimed to investigate the occurrence and genetic diversity of *B. henselae* in cat blood donors and cats associated to clinics accredit to a laboratory from Brasília, Distrito Federal, Midwest of Brazil, using serological, microbiological and molecular techniques.

2. LITERATURE REVIEW

2.1 Genus *Bartonella*

The genus *Bartonella,* which belongs to the family *Bartonella*ceae and the order Hiphomicrobiales, was initially proposed by Alberto Leonardo Barton Thompson in 1909 when describing *Bartonella bacilliformis* as the etiological agent of diseases known as Oroya fever and Peruvian wart (Boulouis et al., 2005; Breitschwerdt, 2014). Thus, just after 1993, other species were added to the genus when species from the genera *Rochalimae* (1993) and *Grahamella* (1995) were reclassified as members of the genus *Bartonella* (Brenner et al., 1993; Birtles et al., 1995).

Bartonella spp. are fastidious Gram-negative aerobic with preference for capnophilia, short, pleomorphic (coccobacillary or bacillary rods, measuring 0.6 μm × 1.0 μm) bacteria included into the alpha-2 subgroup of alpha-proteobacteria (Chomel and Kasten, 2004). *Bartonella* spp. are highly adapted to mammalian reservoirs, which makes the diagnosis of infection by these agents in non-reservoir adapted hosts extremely challenging (Duncan et al., 2007). Although not all diseases caused by *Bartonella* spp. occur in accidental hosts, they are increasingly implicated as a cause of zoonotic infections (Breitschwerdt et al., 2010).

The isolation of *B. henselae* in 1992 from domestic cats favored the subsequent detection and description of new *Bartonella* species or subspecies in a plethora of domestic and wild carnivores (Chomel et al., 2004). *Bartonella henselae*, *Bartonella berkhoffii* and *Bartonella koehlerae* appear to be the most medically important *Bartonella* species that infect cats, dogs, horses and potentially humans. Indeed, *Bartonella henselae* has been documented in cows, dogs, horses, marine mammals, small terrestrial mammals, and sea turtles, complicating the epidemiology of this medically important species (Breitschwerdt et al., 2010; 2014). *Bartonella bacilliformis* and *Bartonella quintana* are the important human pathogens, with primates considered reservoir hosts (Chomel et al., 2009). An increasing number of animal reservoir hosts

have been identified for various *Bartonella* species, indicating a co-evolution. This fact has created a hidden, large and constant source of infection for accidental hosts in natural environments (Breitschwerdt et al., 2012; Ben-Tekaya et al., 2013). Currently, *Bartonella* transmission is associated with various hematophagous arthropods, including sand flies (*Lutzomyia verrucarum*), lice (*Pediculus humanus corporis*), fleas (*Ctenocephalides felis, Ctenophthalmus nobilis, Pulex irritans*), and possibly ticks (*Ixodes ricinus, Rhipicephalus sanguineus*) (Cotté et al., 2008; Chomel et al., 2009; Breitschwerdt et al., 2010; Tsai et al., 2011; Silaghi et al., 2016; Breitschwerdt, 2017; Wechtaisong et al., 2020).

Bartonella henselae was first isolated from humans from cutaneous angioproliferative lesions (bacillary angiomatosis) of individuals infected with the human immunodeficiency virus (HIV) (Koehler et al., 1994). With the improvement of molecular and bacterial isolation techniques, there has been an increase in documenting the occurrence and description of novel *Bartonella* species. Likewise, the number of studies related to emerging and reemerging diseases caused by this genus has also increased in the last years. The diagnosis of the infection relies on a workflow combining polymerase chain reaction (PCR) and bacterial isolation from blood samples. The culture medium requires enriched media with defibrinated horse, sheep, or rabbit blood, with slow growth (around 45 days) and incubation at 35°-37°C with 5% carbon dioxide, depending on the *Bartonella* species (Chomel and Kasten, 2010). The most suitable way to culture *Bartonella* is by using the *Bartonella* Alpha Proteobacteria Growth Medium (BAPGM), a chemically modified pre-enrichment liquid medium for *Bartonella* species (Maggi et al., 2005; Duncan, Maggi and Breitschwerdt, 2007). BAPGM provides the necessary conditions for *Bartonella* growth, facilitating detection through PCR and successful bacterial isolation on blood or chocolate agar plates. Previous studies confirmed that the combination of qPCR assays and pre-enrichment BAPGM liquid medium increase the sensitivity in diagnosing *Bartonella* spp. (Furquim et al., 2021; Do Amaral et al., 2022a; b; Dias et al., 2023).

A quantitative real-time PCR (qPCR) assay based on a fragment of the *nuoG* gene was developed by André et al. (2015) to detect *Bartonella* species DNA with higher sensitivity and specificity compared to conventional PCR assays. A study in the city of Campo Grande, Mato Grosso do Sul state, documented 46 (30.5%) of 151 blood samples from domestic cats tested positive for qPCR assay targeting the *nuoG* gene, while only 18 (39.1%) of the previously positive samples showed to be positive in

conventional PCR assays based on other molecular markers (ITS, *rpoB*, *gltA*, and *pap-31*). The mentioned qPCR assay has proven to be capable of amplifying *Bartonella* DNA in blood samples from cats (Furquim et al., 2021; Dias et al., 2023), dogs (Müller et al., 2018; André et al., 2019), bats (André et al., 2019; Ikeda et al., 2017; 2020), ruminants (Gonçalves et al., 2018; 2020) rodents (Gonçalves et al., 2016; Do Amaral et al., 2022a), mammals of the Superorder Xenarthra (Calchi et al., 2020), mongooses (Mau et al., 2021), martens (Sepúlveda-García et al., 2021), cattle and buffaloes (Gonçalves et al., 2020), marsupials (Do Amaral et al., 2022b; Braga et al., 2023), tapirs (Mongruel et al., 2023), and, more recently, neotropical birds (Alabí Córdova et al., 2024) and dromedaries (Osman et al., 2024).

Recently, a Digital Droplet PCR (ddPCR) assay based on the 16S-23S intergenic region of 16S rRNA (ITS) was developed to detect DNA from at least 16 species of *Bartonella* (Maggi et al., 2020). This new technology was able to detect *Bartonella* DNA in blood samples with DNA concentrations as low as 0.001 pg/µL, and it showed improved performance in detecting *Bartonella* DNA when combined with liquid BAPGM-enriched hemoculture (Breitschwerdt et al., 2020; Lashnits et al., 2021).

2.2 Transmission

The increasing description of new species and subspecies of *Bartonella*, as well as the involvement of a high number of animal hosts and vectors, add complexity to transmission dynamics under natural conditions (Breitschwerdt, 2014).

Bartonella species are mainly transmitted by blood-sucking arthropods (Lins et al., 2019). The main known arthropod vector for *B. henselae* is the cat flea (*Ctenocephalides felis*) (Bouhsira et al., 2013). After ingestion of *B. henselae*- infected cat blood by *C. felis* fleas, the bacterium persists and multiplies in the intestine for up to nine days. Once excreted in feces, it remains viable for up to three days, representing a significant source of environmental contamination (Higgins et al., 1996; Finkelstein et al., 2002). *Ctenocephalides felis* is essential for the maintenance of *B. henselae* infection within the feline population. The transmission among cats occurs naturally through the feces of infected-*C. felis* fleas (Guptill, 2010). These findings are supported by studies that showed that transmission among cats does not occur through scratches, bites, or fomites, in the absence of fleas (Abbott et al., 1997; Guptill

et al., 1997; 1998). Other routes of transmission, such as saliva or regurgitation during *C. felis* feeding, remains unconfirmed (Moore et al., 2024).

In cats, *Bartonella* has been frequently molecularly detected or isolated from clinically healthy cats with flea infestations. Although cats infected can be bacteremic over a period of more than one year, these mammals are typically asymptomatic. Once *Bartonella* spp. infect an animal, they primarily infect erythrocytes and endothelial cells. Members of *Bartonellaceae* possess a unique strategy for bacterial persistence involving intra-endothelial and intra-erythrocytic locations, promoting prolonged intraerythrocytic bacteremia in their specific mammalian reservoir hosts (Chomel et al., 2003). This versatility in cell tropism, along with their ability to promote endothelial cell proliferation and inhibit apoptotic pathways, allow *Bartonella* spp. to establish persistent infections while evading the host immune response (stealth strategy) (Breitschwerdt et al., 2010; Ben-Tekaya et al., 2013). Moreover, these bacteria stand out for their capacity to induce vasoproliferative lesions by invading endothelial cells and triggering their proliferation and migration. In addition to erythrocytic and endotheliotropic cell invasion, *Bartonella* spp. can also infect various other cell types, including dendritic cells, microglial cells, pericytes, monocytes, and bone marrow progenitor cells (Ben-Tekaya et al., 2013). Blood transfusion may comprise an additional route of *Bartonella* transmission. The risk of this sort of transmission has been proved in experimentally infected cats (Kordick and Breitschwerdt, 1997) and mice (Silva et al., 2016).

Transmission of *Bartonella* spp. from cats to humans typically occurs through scratch or bites from cats infested by *B. henselae*-infected *C. felis*, albeit that transmission via flea feces cannot be ruled out (Guptill, 2010). *Bartonella henselae* transmission from cat to humans occurs indirectly by the cat flea, mainly due to cats' grooming habits that favor accumulation of flea feces under their nails or in the oral cavity (Bouhsira et al., 2013). The presence of clinical manifestations is more common in immunocompromised individuals, especially HIV-positive humans, to whom the risk of infection tends to increase when owing cats (Lamas et al., 2010). Microbiological evidence indicates that the most common *B. henselae* genotypes found in bacteremic cats differ from those found in Cat Scratch Disease (CSD) patients, suggesting that only a subset of the more virulent *B. henselae* strains found in cats causes acute CSD In humans (Chaloner et al., 2011).

The possibility of *B. henselae* infection by transfusion of blood units collected from asymptomatic infected blood donors were demonstrated by Magalhães et al. (2008). These authors successfully isolated *B. henselae* using chocolate agar culture after a 35-day storage period of red blood cell (RBC) units. Additionally, electron microscopy confirmed the presence of structures resembling bartonellae within erythrocytes. Later on, Pitassi et al. (2015) reported positivity for *Bartonella* spp. in 16/500 asymptomatic human blood donors: while 15 were infected by *B. henselae,* one individual showed to be positive for *B. clarridgeiae*. These findings highlight the potential risk of *Bartonella* transmission through blood transfusion.

2.3 Feline bartonellosis

Bartonella henselae has been associated with cats for at least 800 years, since DNA from this *Bartonella* species was found in the dental pulp of cats from burial sites in France dating from the 13th to 16th centuries (La et al., 2004). Indeed, domestic cats are the main reservoirs for *B. henselae*, *B. clarridgeiae* and *B. koehlerae* (Boulouis et al., 2005), and can also be infected by *B. quintana* and *B. bovis* (Breitschwerdt et al., 2010). Previous studies have shown that cats harbor various *Bartonella* species or variants. In France, Gurfield et al. (1997) reported the coinfection by *B. henselae* and *B. clarridgeiae* in 5 out of 436 domesticated cats. In Rio de Janeiro, southeastern Brazil, among 51 cat blood samples positive in the PCR assays for *Bartonella* species based on the ITS (16S-23S rRNA) and *gltA* gene, three cats were concurrently infected with *B. henselae* and *B. clarridgeiae* and two cats were coinfected with *B. clarridgeiae* and *B. koehlerae* (Raimundo et al., 2019).

Quite often, most naturally infected cats do not exhibit clinical signs, meaning they are asymptomatic when naturally infected. Nonetheless, these animals seem to be prone to develop lymphadenitis, gingivitis, and stomatitis, and are predisposed to urological diseases when infected by *Bartonella* spp. (Boulouis et al., 2005; Chomel, 2005). Hence, determining the causal agent of these disorders is challenging since clinical conditions may be associated with various factors in association of the high occurrence of Bartonella in cats (Guptill, 2010). The positivity of *B. henselae* have been associated with uveitis and endocarditis in cats (Lappin et al., 2000; Chomel et al., 2003). Although the clinical signs may vary depending on the *Bartonella* strain used, experimentally infected cats are also usually asymptomatic. Nevertheless,

lymphadenopathy, brief periods of fever, lethargy and anorexia, mild neurological signs and cardiopathy have been reported in experimentally infected cats (Guptill et al., 2010; Boulouis et al., 2005; Chomel, 2005). Experimentally infected cats can also develop peripheral lymph node hyperplasia, lymphocytic cholangitis/pericholangitis, splenic follicular hyperplasia, lymphocytic hepatitis, lymphoplasmacytic myocarditis, and interstitial lymphocytic nephritis (Kordick et al., 1999).

Moreover, Kordick et al. (1999) incriminated *Bartonella* species as the causative agents of several idiopathic diseases in cats. *Bartonella* DNA was detected in several tissues, such as blood, lymph nodes, myocardium, liver and kidneys. Hematological and biochemical evaluation of these cats showed no significant alterations, although there were rare cases of mild anemia, neutropenia, eosinophilia and thrombocytopenia (Guptill, 2010; Souza et al., 2017) or decreased levels of alkaline phosphatase and neutrophilia (Williams et al., 2022). Thus, hyperproteinemia and hyperglobulinemia have been detected in sick animals (Silva et al., 2019; Whittemore et al., 2012; Álvares-Fernández et al., 2018). Although *Bartonella* transmission through blood transfusion has been demonstrated in cats through experimentally induced bacteremia (Kordick and Breitschwerdt, 1997), there are limited studies on the occurrence of *Bartonella* spp. in cat blood donors. In the United States, among 146 healthy cat blood donors, two (1.4%) cats were positive in the PCR for *Bartonella henselae* (Hackett et al., 2006).

In Brazil, the presence of *Bartonella* spp. in cats was reported in the states of Maranhão (Braga et al., 2012), Pernambuco (Fontalvo et al., 2017), Mato Grosso (Miceli et al., 2013; Braga et al., 2015), Mato Grosso do Sul (André et al., 2015), Rio Grande do Sul (Staggemeier et al., 2010; 2014; Malheiros et al., 2016), Santa Catarina (Pedrassani et al., 2019), São Paulo (Bortoli et al., 2012; André et al., 2014; Drummond et al., 2018; Furquim et al., 2021), Rio de Janeiro (Souza et al., 2010; Crissiuma et al., 2011; Souza et al., 2017; Silva et al., 2019; Raimundo et al., 2019), Minas Gerais (Furquim et al., 2021) and Goiás (Dias et al., 2023), with occurrence ranging from 1.6 to 90.7% (Braga et al., 2015; Drummond et al., 2018) (**Table 1**). The variability in *Bartonella* spp. positivity rates depends on the sort of cat population sampled, sample size, geographic region, level of flea infestation, and the laboratorial techniques used in the diagnosis (Guptill et al., 2010; Furquim et al., 2021).

As demonstrated in **Table 1**, diagnostic sensitivity increases when different methodologies are combined, resulting in higher percentages of animals identified as positive for *Bartonella* spp. Most studies on molecular occurrence of *Bartonella* spp. in

cats performed in Brazil are concentrated in the Southeast region, with positivity rates ranging from 4.3% to 90.2% (Crissiuma et al., 2011; Bortoli et al., 2012; André et al., 2014; Souza et al., 2017; Raimundo et al., 2019; Silva et al., 2019; Drummond et al., 2018; Furquim et al., 2021). In São Paulo state, when Drummond et al. (2018) and Furquim et al. (2021) employed molecular assays associated with BAPGM pre-enrichment liquid medium, followed by bacterial isolation on blood or chocolate agar, occurrence of 90.2% and 33.1%, respectively, for *Bartonella* spp. were found among domestic cats.

In the Southern region, the occurrence of *Bartonella* spp. among cats ranged from 13.3% to 25.5% (Staggemeier et al., 2010, 2014; Malheiros et al., 2016; Pedrassani et al., 2019). In the Northeast region of the country, it ranged from 4.5% to 20.7% (Braga et al., 2012; Fontalvo et al., 2017; Cruz et al., 2022). In the Brazilian Midwest, the occurrence of *Bartonella* spp. among cats ranged from 1.6% to 42% (Braga et al., 2015; Miceli et al., 2013; André et al., 2015; Dias et al., 2023).

Table 1. Occurrence of *Bartonella* spp. among cats in Brazil, according to the studied region, sampled cat population, applied diagnostic method and detected species by molecular techniques.

REGION	STATE	DIAGNOSTIC METHODS			*BARTONELLA* SPECIE	REFERENCE
		Culture	Sorology	PCR		
Northeast	Maranhão	-	-	PCR- ITS, *rpoB*, *pap-31*, *ribC*, *gltA*, *groEL* - 4,5% (9/200)	*B. henselae* and *B. clarridgeiae*	Braga et al. (2012)*
	Pernambuco	-	IFAT 15% (6/40)	*ribC* cPCR - 0/40	*Bartonella* spp.	Fontalvo et al. (2017)
	Bahia	-	-	*nuoG* qPCR *gltA*, *rpoB*, ITS, ftsZ cPCR 20,7% (39/188)	*B. henselae* and *B. koehlerae*	Cruz et al. (2022)*
Central-West	Goiás	BAPGM + Solid Culture = 12 isolates	-	*nuoG* qPCR – 42% (42/100)	*B. henselae*	Dias et al. (2023)*
	Mato Grosso	-	-	cPCR - ITS, *rpoB*, *pap-31*, *ribC*, *gltA* 2.24% (4/178)	*B. henselae* and *B. clarridgeiae*	Miceli et al. (2013)*
		-	-	cPCR - *ribC* - 1.6% (3/182)	*B. clarridgeiae*	Braga et al. (2015)
	Mato Grosso do Sul	-	-	qPCR – *nuoG* cPCR- ITS, *rpoB*, *pap-31*, *ribC*, *gltA* 30.46% (46/151)	*B. henselae* and *B. clarridgeiae*	André et al. (2015)*
South	Rio Grande do Sul	-	-	cPCR – *ribC* - 17% (8/47)	*B. henselae* and *B. clarridgeiae*	Staggemeier et al. (2010)

REGION	STATE	DIAGNOSTIC METHODS			*BARTONELLA* SPECIE	REFERENCE
		Culture	Sorology	PCR		
SOUTH	Rio Grande do Sul	-	-	qPCR – *gltA* 25.5% (12/47)	*B. henselae* and *B. clarridgeiae*	Staggemeier et al. (2014)
		-	-	qPCR – *nuoG* 20% (6/30)	-	Malheiros et al. (2016)*
	Santa Catarina	-	-	qPCR – *nuoG*, *gltA*, *rpoB* 13.3% (4/30)	*B. henselae* *B. clarridgeiae* *B. koehlerae*	Pedrassani et al. (2019)*
SOUTHEAST	São Paulo	-	-	cPCR - ITS, *rpoB*, *pap-31*, *ribC*, *gltA* 4.3% (2/23)	*B. henselae*	Bortoli et al. (2012)*
		-	-	cPCR - ITS, *rpoB*, *pap-31*, *ribC*, *gltA* 30% (11/37)	*B. henselae* *B. clarridgeiae*	André et al. (2014)*
		BAPGM + Solid Culture = 11 isolates	-	nPCR (blood) – *fstZ* 76.8% (86/112) nPCR (BAPGM) – *fstZ* 45.5% (51/112) cPCR (BAPGM) – ITS 27.7% (31/112) Total 90.2% (101/112)	*B. henselae*	Drummond et al. (2018)
	Minas Gerais	BAPGM + Solid Culture = 4 isolates	-	qPCR *nuoG* (BAPGM + blood) 33,1% (50/151)	*B. henselae*	Furquim et al. (2021)*
		BAPGM + Solid Culture = 13 isolates	-	qPCR *nuoG* (BAPGM + blood) 57,4% (89/155)	*B. henselae*	Furquim et al. (2021)*
	Rio de Janeiro	-	IFAT - 47% (19/40)	cPCR – *hrtA* 42% (17/40)	-	Crissiuma et al. (2011)
		-	-	cPCR – *gltA*, *rpoB*	*B. henselae*	Souza et al. (2017)

		-	-	cPCR – *gltA* 24.7% (22/89)	*B. henselae* *B. clarridgeiae*	Silva et al. (2019)
		-	-	cPCR – *gltA* 39.9% (93/208)	*B. henselae* *B. clarridgeiae* *B. koehlerae*	Raimundo et al. (2019)

nuoG: nicotinamide adenine dinucleotide dehydrogenase gamma subunit; *gltA*: citrate synthase gene; *groEL*: heat shock protein gene; ITS: intergenic spacer region; *pap-31*: bacteriophage-associated heme-binding protein gene; *ribC*: riboflavin synthase gene; *rpoB*: RNA polymerase beta subunit gene; *htrA*: 60kDa heat shock protein gene; *ftsZ*: Cell division protein *ftsZ*

*Research developed by our research group

2.4 Human bartonellosis

The majority of cases of human bartonellosis have been diagnosed by PCR testing of tissue biopsies or serology using specific antigens for certain *Bartonella species*. *Bartonella* isolation from the blood is relatively rare in most cases (Jacomo et al., 2002). The primary pathogenic species associated with human bartonellosis are *Bartonella bacilliformis*, *Bartonella henselae*, and *Bartonella* quintana (Dehhaghi et al., 2019). These *Bartonella* species are associated with various illnesses, including Carrion's disease, Oroya fever (*B. bacilliformis*) bacillary angiomatosis and peliosis, CSD (*B. henselae*), endocarditis and trench fever (*B. henselae*) (Boulouis et al., 2005; Breitschwerdt et al., 2019). *Bartonella henselae* has been incriminated as the main causative agent of Cat Scratch Disease (CSD), which was first reported in 1950 (Chomel; Kasten, 2010). Indeed, *B. henselae* has been found to be the main etiological associated with CSD. Regnery et al. (1992) detected seroreactivity to *B. henselae* antigens in 88% of human patients with suspected CSD in USA. Anderson et al. (1994) detected the presence of *Bartonella* DNA in lymph nodes from 84% of patients with suspected CSD in the USA.

Usually characterized as a self-limiting disease, CSD manifestation can lead to fever and lymphadenopathy. In the site of *Bartonella* inoculation by cat scratch or bite, an erythematous, non-pruritic papule develop after 3 to 12 days after (Boulouis et al., 2005; Breitschwerdt et al., 2010; Lins et al., 2019). Moreover, annular granuloma, cutaneous vasculitis, and striae have also been described (Lins et al., 2019; Breitschwerdt et al., 2019). These dermatological lesions are observed in approximately 90% of cases, making them important clinical signs to be searched for during the anamnesis (Lins et al., 2019).

Nonetheless, dermatologic manifestations are not the only clinical signs associated with CSD. Malaise, anorexia, headache have also been reported (Boulouis et al., 2005). Additionally, atypical manifestations (bacillary angiomatosis and peliosis, endocarditis, Parinaud's oculoglandular syndrome, neuroretinitis, glomerulonephritis, osteomyelitis and hepatosplenomegaly) have also been observed in 20% of immunocompromised individuals (mainly those infected with the HIV virus) (Canneti et al., 2019; Koehler, 1994; Vermeulen et al., 2006; Pinto-Junior et al., 2008; Chomel et al., 2009; Mazur-Melewska et al., 2015). Although immune status has been associated

with the severity of *Bartonella* infections (Angelakis and Raoult, 2014), only 8.6% of the non-neurological CSD patients were in fact immunocompromised (Canneti et al., 2019).

Recent studies have suggested that *B. henselae* and *B. berkhoffii* can induce persistent intravascular infection in immunocompetent humans, favoring the occurrence of symptoms such as fatigue, arthritis, and neurological or neurocognitive abnormalities (Breitschwerdt et al., 2010). The association between neurological abnormalities and *Bartonella* infection were seem in a cross-sectional study involving 29 patients with neurological symptoms who tested positive for *Bartonella* spp. (Breitschwerdt et al., 2020). However, 83% of these patients developed cutaneous lesions, and the elicit cutaneous lesions or neuropsychiatric symptoms remains unclear. Lashnits et al. (2021) proposed a potential link between these processes and the development of schizophrenia, since *Bartonella* sp. infection can lead to encephalitis and potentially trigger autoimmune responses.

2.5 Multi-Locus Sequence Typing (MSLT) genotyping

The widespread prevalence and diverse host range of *Bartonella* species result in a variety of *Bartonella* species and genotypes present in animal tissues. Epidemiological investigations relying on genotyping approaches are needed to shed light on the genetic diversity and the ecology of *Bartonella* infecting animals, while also measure the risks posed by these bacteria to public health (Kosoy et al., 2017). For instance, Harrus et al. (2009) provided genetic characterization of *Bartonella* strains from synanthropic rodents in Israel, shedding light on the link between human lymphadenopathy in Tbilisi, Georgia, and rat-associated *Bartonella* (Kandelaki et al., 2016). Similarly, connections have been established between human cases of myocarditis and meningitis in the USA and *Bartonella* genotypes found in ground squirrels (Kosoy et al., 2003; Osikowicz et al., 2016).

In order to distinguish and detect *Bartonella* species and subspecies, as well as to explore their phylogenetic relatedness, several methods have been employed, such as Pulsed-Field Gel Electrophoresis (PFGE) (Sander et al., 1998), Multispacer Typing (MST) (Foucault et al., 2005), Multi-Locus Sequence Typing (MLST) (Iredell et al., 2003), and Multi-Locus Variable Number Tandem Repeats Analysis (MLVA) (Monteil et al., 2007).

MLST, developed in 1998, compares alleles among isolates, indexing six to nine genes with well-characterized maintenance functions (Jolley and Maiden, 2014). Each gene fragment sequence is assigned to an allele number, which is then incorporated into an allelic profile or a Sequence Type (ST) (Urwin and Maiden, 2003). These designations can be used to define strains, group them into clonal complexes or lineages, as well as to associate them with biological properties such as zoonotic potential or drug resistance (Maiden et al., 2013).

The website PubMLST.org hosts a collection of open-access, curated databases containing population sequence data along with origin and phenotype information for over 100 microbial species and genera. The *B. henselae* PubMLST database is built upon a scheme outlined by Iredell et al. (2003), who characterized 37 previously described *B. henselae* isolates from humans and cats originating from Australia and other countries. In that study, loci encompassing fragments of genes responsible for essential housekeeping functions, including 16S rDNA, *eno, ftsZ, gltA, groEL, ribC, and rpoB*, along with other less-defined proteins, such as *batR* and *nlpD* were selected. Each gene fragment sequence is assigned to an allele number, which is then incorporated into an allelic profile or a Sequence Type (ST). The *eno* gene showed no variation and was thus excluded from the analysis due to zero nucleotide variation within the loci. MLST has distinguished 497 *B. henselae* isolates into 38 Sequence Types (STs) worldwide (https://pubmlst.org/organisms/*Bartonella-henselae*).

Studies using MLST have characterized *B. henselae* variants from human biological samples and cat blood samples from several countries, including the United States of America (USA), United Kingdom (UK), Germany, Croatia, Japan, Spain, Italy, France, China, Japan, Algeria, Argentina, Brazil, and Turkey (Iredell et al., 2003; Arvand et al., 2007; Yanagihara et al., 2010; Chaloner et al., 2011; Mietze et al., 2011; Yuan et al., 2011; Azzag et al., 2012; Gil et al., 2013; Cicuttin et al., 2014; Stepanić et al., 2019; Furquim et al., 2021; Dias et al., 2023; Can et al., 2023; Stepanić et al., 2024).

Based on MLST analysis, Iredell et al. (2003) characterized *Bartonella henselae* strains isolated from cats in Australia and New Zealand, as well as from humans in Australasia, the USA, and France. In that study, seven STs (ST1 to ST7) were identified, with ST1, ST3 to ST7 detected in cats, and ST1 and ST6 in humans. In France, ST8 was identified in human biological samples (Arvand and Viezens, 2007).

ST1, ST5, ST7 and ST6 were detected in humans and cats from Germany, France, Switzerland, USA, Australia and New Zealand (Arvand and Viezens, 2007). Arvand et al. (2007) identified six STs (ST9 to ST14) in cats and humans from the Americas, Australia, Israel and Europe (United Kingdom, Czech Republic, Italy, Switzerland, Sweden, Germany, Netherlands, France, Denmark).

Four STs were detected in cats in China, namely ST1, ST16, ST17, and ST18 (Yuan et al., 2011). Yanagihara et al. (2010) found ST1, ST6 and ST15 in domestic cats from Japan. Eleven STs (ST16 to ST26) were identified in cats from Germany (Mietze et al., 2011). ST27 to ST29 were found among cats in the UK (Chaloner et al., 2011). In Turkey, Can et al. (2023) identified seven STs (ST1, ST5, ST9, ST35 and ST36) in cats, with one (ST38) representing a novel variant. A recent study by Stepanić et al. (2024) reported ST1, ST5, ST6, ST24, and ST33 in domestic cats from Croatia.

In South America, the first MLST genotyping of *B. henselae* variants (ST1, ST5, ST6, and ST8) from domestic cats' blood samples was performed in Argentina (Cicuttin et al., 2014) described. In Brazil, Furquim et al. (2021) reported ST9 and a new ST (ST37) in cats' blood samples from the states of São Paulo and Minas Gerais, respectively. ST1 and ST5 were described among cats from the state of Goiás (Dias et al., 2023).

While ST1 and ST5 are the most commonly detected zoonotic variants (Gil et al., 2013; Stepanić et al., 2019), ST6 and ST7 appear to be more adapted to domestic cats (Arvand et al., 2007; Mietze et al., 2011). *Bartonella henselae* STs may be associated with zoonotic strains (ST2, ST5 and ST6) or clinical manifestations in humans, such as endocarditis (ST4 and ST7) (Chaloner et al., 2011). The ST5 strain stands out as the most prevalent variant affecting both cats and humans (Gil et al., 2013; Stèpanic et al., 2019). Indeed, the occurrences of ST1, ST5, and ST8 have been reported in human patients with Cat Scratch Disease (Iredell et al., 2003; Arvand et al., 2007; Chaloner et al., 2011; Gil et al., 2013; Stèpanic et al., 2019).

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CHAPTER 2 - Zoonotic variants of *Bartonella henselae* in domesticated cats, including blood donors, in central-western Brazil

Lorena Freitas das Neves[1]; Clara Morato Dias[1]; Anna Claudia Baumel Mongruel[1]; Gabrielly de Oliveira Lopes[1]; Liliane Maria do Rosario Batista; Francisco Anilton Alves Araujo; Gener Tadeu Pereira[2]; Rosangela Zacarias Machado[1]; Marcos Rogério André[1]*

[1]Vector-Borne Bioagents Laboratory (VBBL), Departamento de Patologia, Reprodução e Saúde Única, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual "Júlio de Mesquita Filho" (FCAV/UNESP), Jaboticabal, SP, Brazil
[2]Departamento de Ciências Exatas (DCE), Universidade Estadual "Júlio de Mesquita Filho" (FCAV/UNESP), Jaboticabal, SP, Brazil

***Corresponding author:**

Prof. Dr. Marcos Rogério André, Vector-Borne Bioagents Laboratory (VBBL), Departamento de Patologia, Reprodução e Saúde Única, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual "Júlio de Mesquita Filho" (FCAV/UNESP), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP: 14884-900, Jaboticabal, São Paulo, Brazil. Phone: +55 (16) 3209-7302. e-mail: mr.andre@unesp.br

Abstract: *Bartonella* presents a significant global health concern, with *Bartonella henselae* emerging as one of the most prevalent species implicated in several human diseases. Previous studies have explored the potential transmission of *Bartonella* via blood transfusions in humans, underscoring the zoonotic relevance. Although several studies have shown the widespread occurrence of *B. henselae* in cats from Brazil, the genetic diversity of this zoonotic agent remains largely unknown. The present study aimed to investigate the occurrence and genetic diversity of *B. henselae* in blood samples from domestic cats sampled in veterinary clinics and cat blood donors from central-western Brazil, using serological, microbiological and molecular techniques. Serum samples were subjected to Indirect Fluorescence Antibody Test (IFAT) to investigate the frequency of IgG antibodies in the cat population studied herein. A real-time quantitative real-time PCR (qPCR) assay for *Bartonella* targeting the 16-23S

rRNA (ITS) region was used for molecular detection of *Bartonella* spp. in blood samples both before and after incubation in a pre-enrichment *Bartonella* Alpha Proteobacteria Growth Medium (BAPGM), as well as to confirm the identity of colonies isolated onto chocolate agar. Antibodies anti-*Bartonella* sp. were detected in 60.75% (96/158) of the serum samples (33/67 cats from veterinary clinics; 63/91 blood donors). In qPCR, 32% (32/100) and 46% (46/100) cats from veterinary clinics and cat blood donors were positive for *Bartonella* spp. After incubating blood samples in BAPGM liquid medium, 61 samples (78.20%) previously negative in blood-qPCR turned out positive in the qPCR for *Bartonella* spp.. *Bartonella* DNA and seropositivity for *B. henselae* antigen were concurrently detected in 25.3% (40/158) of the cats. Three *B. henselae* strains were successfully isolated, two originating from cats sampled in veterinary clinics, and one from a cat blood donor. Multi-locus Sequencing Typing (MLST) revealed the occurrence of the *B. henselae* zoonotic variants Sequence Type (ST)1, ST2 and ST5. The present study showed, for the first time, the occurrence of *Bartonella* in cat blood donors in South America, highlighting the potential risks of transmission of zoonotic *B. henselae* variants by blood transfusion to cats. Higher occurrence of *Bartonella* spp. was observed among cat blood donors. Since 25.95% (9/41) of the serum samples from *Bartonella* qPCR-positive blood donors cat showed to be negative in the IFAT for *B. henselae*, serology cannot be used to check the *Bartonella* exposure/infection in cat blood donors. This is the first report of a zoonotic *B. henselae* variant (ST5) in cat blood donors. ST2 is documented for the first time in cats in the American continent.

Keywords: Bartonellosis, Cat Scratch Disease, MLST, Sequence Type, IFAT

1. Introduction

Alphaproteobacteria from the genus *Bartonella* poses significant concern to healthcare professionals due to their zoonotic potential, which has been associated with emerging and re-emerging human diseases (Breitschwerdt et al., 2010; Angelakis and Raoult, 2014). The *Bartonella* genus encompasses facultative intracellular Gram-negative bacteria, belonging to the alpha subgroup of the Proteobacteria class, that is phylogenetically associated with the genera *Brucella*, *Agrobacterium*, and *Rhizobium* (Chomel et al., 2004).

Cats act as the main reservoirs for *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae*, playing a crucial role in transmitting these agents to humans by scratches or bites contaminated with flea feces containing bartonellae (Chomel et al., 2004; Avidor et al., 2004). The vector responsible for transmitting *B. henselae* - and likely the other abovementioned *Bartonella* species - among cats is the cat flea *Ctenocephalides felis felis* (Breitschwerdt, 2017; Bouhsira et al., 2013; Moore et al., 2024).

Bartonella henselae stands out as one of the most significant zoonotic *Bartonella* species that can cause Cat Scratch Disease (CSD) in humans, along with other clinical manifestations, such as endocarditis, Parinaud's Oculoglandular Syndrome, psychiatric disorders, bacillary angiomatosis, and peliosis hepatis (Boulouis et al., 2005; Breitschwerdt et al., 2010, 2019, 2020; Lashnits et al., 2021). These two latter manifestations are typically associated with patients infected with the Human Immunodeficiency Virus (HIV), to whom the risk of infection tends to increase when owing cats (Lamas et al., 2010).

The potential transmission of *B. henselae* and *B. clarridgeiae* through blood transfusion were reported in human blood donors in southeastern Brazil (Pitassi et al., 2015). When it comes to cats, there is an urgent need of screening of potential blood donors for infectious agents. Although *Bartonella* is one of the recommended agents to be investigated (Pennisi et al., 2015; Wardrop et al., 2016), to the best of the authors' knowledge, there is no report of transmission of *Bartonella* spp. by blood transfusion between cats. Nonetheless, *Bartonella* bacteremia has been experimentally induced in cats through injection of infected blood and therefore evidenced the possible transmission of *B*. *henselae* through blood transfusion (Kordick and Breitschwerdt, 1997).

Although the molecular occurrence of *Bartonella* spp. in cats from Brazil has been extensively reported (Braga et al., 2012; André et al., 2014; André et al., 2016; Pedrassani et al., 2019), ranging from 1.6% (Braga et al., 2012) to 90.2% (Drummond et al., 2018), studies exploring the genetic diversity of this pathogen are scant. For instance, Multi-locus Sequence Typing (MLST) of *Bartonella henselae* strains isolated from cats showed the circulation of ST (Sequence Type) 37 in the state of Minas Gerais, ST9 in São Paulo (Furquim et al., 2021), and ST1 and ST5 in the state of Goiás (Dias et al., 2023). Additional studies on the genetic diversity and evolution of this

group of bacteria are needed aiming at achieving a better comprehension of the epidemiology and distribution of *Bartonella* spp. worldwide (Azzag et al., 2012).

In order to expand the knowledge on *Bartonella* population structure, diversity, evolution, and alternative routes of transmission, this work aimed to investigate the occurrence and genetic diversity of *B. henselae* in blood samples from domestic cats, including felines registered as blood donors in a private clinical pathology laboratory in central-western Brazil, using serological, microbiological and molecular techniques.

2. Material and methods

2.1 Ethics Statement

The protocols and handling procedures concerning domestic cats received approval from the "Comissão de Ética e Uso de Animais" of the Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP), under the protocol number 3971/22.

2.2 Sampling

Between January/2023 and March/2023, blood samples from domestic cats arriving in a laboratory and blood center located in the Distrito Federal (central-western Brazil) through accredited veterinary clinics and from registered blood donor cats were selected without any exclusion criteria. In total, 100 cat blood samples (54 males; 46 females) from accredited clinics and 100 cats (67 males; 33 females) registered as blood donors were collected. While part of the blood samples was obtained from veterinary clinics that were partners of the laboratory, the samples from blood donors were collected directly at the blood center. Aiming to perform DNA extraction, molecular analyses, and culturing, approximately 1 mL was collected directly from the EDTA tubes and blood bags. Additionally, out of the 200 blood samples collected from cats, 158 blood serum samples were separated from these same cats (67/100 from accredited clinics; 91/100 from blood donors). Subsequently, the samples were transported in liquid nitrogen containers to the Vector-Borne Bioagents Laboratory (VBBL), Department de Pathology, Reproduction and One Health, Faculty of Agrarian and Veterinary Sciences, São Paulo State University (FCAV/UNESP, Jaboticabal, São Paulo), and stored at -70°C.

2.3 DNA extraction and conventional PCR (cPCR) for mammal endogenous gene from cat blood samples

DNA was extracted from each cat blood sample using a commercial kit (Biopur Kit Extração Fast DNA/RNA - Mobius), according to manufacturers' instructions. DNA concentration and the absorbance rates (260/280) were measured in a NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA). PCR assay targeting the mammal-endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh)* was performed aiming to verifying the success of the extraction and the lack of PCR inhibitors (Birkenheuer et al., 2003). The PCR amplified products were subjected to horizontal electrophoresis on a 1.0% agarose gel stained with ethidium bromide.

2.4 Quantitative real-time PCR (qPCR) for *Bartonella* spp.

Cat blood DNA samples positive for the endogenous mammalian gene were subjected to a quantitative real-time (q)PCR assay for *Bartonella* based on the 16-23S rRNA (ITS) region, using primers BsppITS325s: 5′- CTTCAGATGATG ATCCCAAGCCTTCTGGCG 3′ (Forward), 543as: 5′- AATTGGTGGGC CTGGGAGGACTTG 3′ (Reverse), and hydrolysis probe BsppITS500probe: 5′- FAM-GTTAGAGCGCGCGCTTGATAAG -IABkFQ 3′ (Maggi et al., 2020). Amplification reactions were performed in a CFX96 Thermal Cycler (BioRad®, Hercules, California, United States). UltraPure™ DNase/RNase-Free Distilled Water (Thermo Scientific, Waltham, MA, USA) was used as a negative control. Serial dilutions (2.0×10^7 to $2.0 \times 10^{\circ}$ copies) of a G-BLOCK containing a 226 bp fragment of the ITS intergenic region of *B*. *henselae* (Integrated DNA Technologies, Coralville, Iowa, United States) were used as positive controls as well as to construct the standard curve. DNA samples were evaluated in duplicates and when the Cq differences between replicates were higher than 0.5, the samples were repeated in triplicates. The number of copies was determined by following formula: (XG / µL DNA / [Plasmid size (bp) x 660]) x 6,22 x 10^{23} x plasmid copies / µL. All analyses were performed according to the standards stablished by MIQE ("Minimum Information for Publication of Quantitative real-time PCR Experiments"). The amplification efficiency (E) was calculated according to the slope of the standard curve using the formula $E=10^{-1/slope}$ (Bustin et al., 2009).

The cat blood and cat blood + BAPGM (*Bartonella*-Alphaproteobacteria Growth Medium) DNA samples positive in the qPCR for *Bartonella* spp. were subjected to a PCR targeting the *pap31* gene (564 bp) (Maggi and Breitschwerdt, 2005).

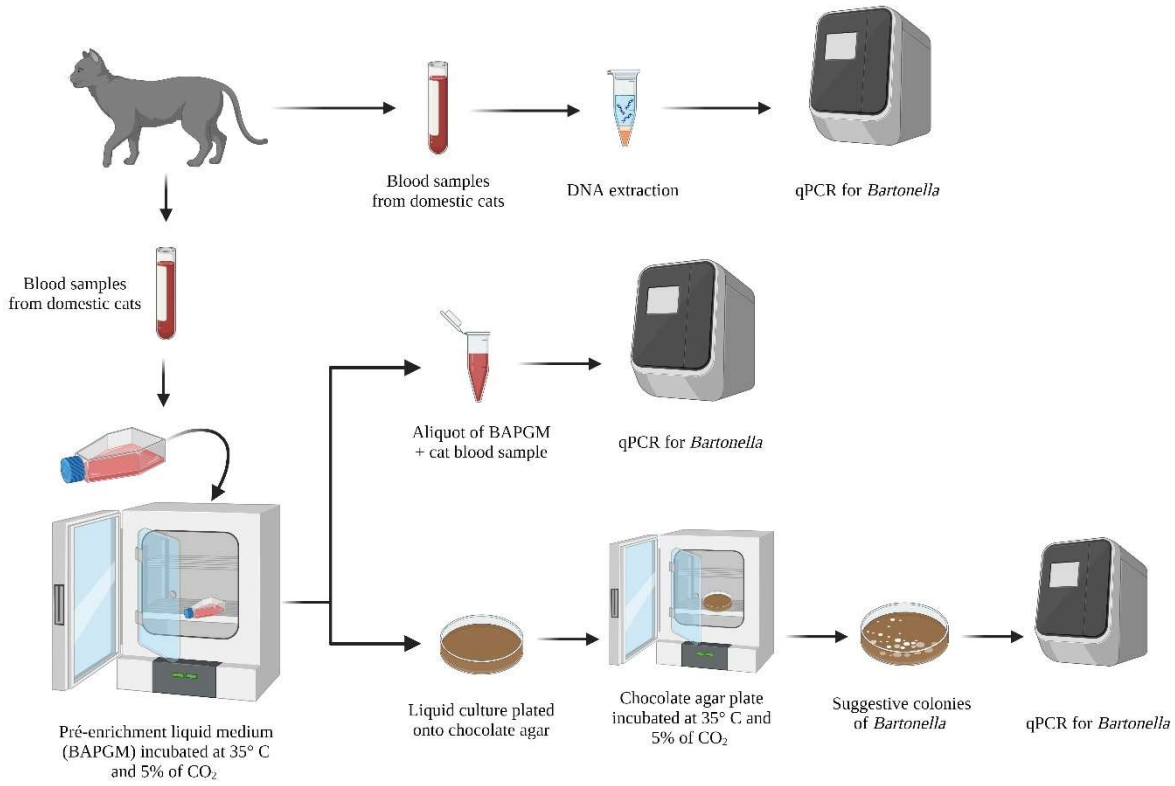

Figure 1. Flowchart of the methodologies applied in this study for *Bartonella* genotyping. Created with BioRender.com.

2.5 *Bartonella* spp. isolation

The pre-enrichment liquid medium used in this study was developed based on previous protocols described by Maggi et al. (2005) and Duncan et al. (2007), with adaptations made to suit *Bartonella*-Alphaproteobacteria (BAPGM) medium (Furquim et al., 2021). To prepare this medium, 500 mL of IPL-41 Insect Medium (Sigma-Aldrich, St. Louis, Missouri, USA) was supplemented with the following components: 0.055 mg of NAD, 0.69 mg of NADP, 1.1 mg of ATP, and 1.1 mg of sodium pyruvate (all sourced from Sigma-Aldrich, St. Louis, Missouri, USA). Amino acid supplementation was achieved by adding 35.1 mg of L-arginine HCl, 8.66 mg of L-cystine HCl, 11.64 mg of L-histidine, 14.6 mg each of L-isoleucine and L-leucine, 20.14 mg of L-lysine, 4.16 mg

of L-methionine, 9.02 mg of L-phenylalanine, 13.22 mg of L-threonine, 2.7 mg of L-tryptophan, 12 mg of L-tyrosine 2Na 2H2O, and 13 mg of L-valine (Dinâmica, Indaiatuba, São Paulo, Brazil).

Following supplementation, the pH of the medium was adjusted to 6.2 and sterilized using a 0.2 µM filter (Corning, Corning, New York, United States). Once sterilization was complete, the medium was combined with 55 mL of defibrinated sheep blood, previously confirmed negative for *Bartonella* sp. (via qPCR), and adjusted to a final concentration of 11% vol/vol. Subsequently, 200 µL of each cat blood sample was inoculated into culture bottles (20 cm^2, model 430639, Corning, Corning, New York, United States) containing 2 mL of the liquid medium supplemented with sheep's blood. Two flasks of negative controls were included, one flask contained only the pre-enrichment medium, while the other flask contained both the pre-enrichment medium and defibrinated sheep blood. These bottles were then incubated for seven days at 35°C and 5% CO_2 in a Water Jacketed CO_2/O_2 incubator (NuAire, Plymouth, Massachusetts, USA) with continuous agitation (Maggi et al., 2005; Duncan et al., 2007; Furquim et al., 2021).

After a seven-day incubation period, 200 µL of the liquid culture was subjected to DNA extraction using the Biopur Kit Extraction Fast DNA/RNA (Mobius), following the manufacturer's instructions. Subsequently, these extracted DNA samples were subjected to qPCR for *Bartonella* spp. Additionally, 200 µL of the contents from each bottle were plated onto enriched chocolate agar (Laborclin, Pinhais, Paraná, Brazil) and subjected to the same incubation conditions, as described above, for a minimum period of 30 days with daily observation. For each batch of tested samples, three negative controls were included: two corresponding to the negative controls previously used in the pre-enrichment liquid culture, and another one represented by a chocolate agar plate without any sample. Smooth and circular colonies suggestive of *Bartonella* were collected and subjected to DNA extraction using the boiling method (Keim et al., 2000) and qPCR assay for *Bartonella* spp. based on the intergenic region of the 16-23S rRNA (ITS) (Maggi et al., 2020). Colonies confirmed to be *Bartonella* spp. were then sub-cultured through three passages until pure isolates were obtained.

2.6 Conventional PCR assays for Multi- Locus Sequence Typing (MLST) for *Bartonella* spp.

DNA samples extracted from *Bartonella* pure isolates were subjected to PCR assays for Multi-Locus Sequence Typing (MLST) targeting eight molecular markers (*gltA*, *rpoB*, *ftsZ*, 16S rDNA, *groEL*, *bartR*, *nlpD* and *ribC*), as previously described by Iredell et al. (2003).

2.7 Amplicons purification and sequencing

The obtained amplicons were purified using ExoSap IT PCR Product Cleanup Reagent (Applied Biosystems TM), following the manufacturer's recommendations. The sequencing of the amplified products was carried out by the chain termination method with dideoxynucleotide (Sanger et al., 1977) and performed at the "Centro de Estudos do Genoma Humano e Células-Tronco" (Genoma USP) at USP, São Paulo, SP. The obtained sequences were subjected to a screening test using Geneious 11.1.3 software to assess the quality of the peaks corresponding to each nitrogenous base. Furthermore, the consensus sequence obtained from each sample was subjected to BLASTn analysis (Altschul et al., 1990), aiming to compare the obtained sequences with those deposited in the Genbank database (http://www.ncbi.nlm.nih.gov/genbank).

2.8 MLST analyses

The identification of *B. henselae* Sequence Types (STs) was assigned according to the *B. henselae* MLST online database (http://b*henselae*. mlst.net) (Iredell et al., 2003). The STs found corresponding to the *B. henselae* isolates were used in the analysis of Minimum Spanning Trees generated from the GrapeTree program (https://pubmlst.org/bigsdb?db=pubmlst_b*henselae*_isolates&page=plugin&name=GrapeTree) (Zhou et al., 2018). Additionally, the sequences were aligned and used in the analysis of polymorphism using the software DnaSP v5, aiming at calculating the nucleotide diversity (π), level of polymorphism (genotype diversity [gh], number of genotypes [h] and mean number of nucleotide differences [K]) (Librado and Rozas, 2009).

2.9 Indirect Fluorescent Antibody Test (IFAT)

In order to check for the presence of IgG antibodies to *Bartonella* sp. in cat serum samples, IFAT was performed using slides containing the *B. henselae* ST9 antigen (Furquim et al., 2021). Immunofluorescence reactions were performed as previously established (Henn et al., 2007). Serum samples from cats were used as negative and positive controls (Furquim et al., 2021). Briefly, cat serum samples were diluted (1:64) in PBS (pH 7.2) and 10 µL were added onto individual wells of the slide containing the *B. henselae* antigen. After incubation of the slide in a humid chamber for 30 minutes at 37 °C, three washes of 5 minutes were performed with PBS. After drying at room temperature, the slides were incubated for 30 minutes at 37 °C with the anti-cat IgG conjugate (Sigma®, St. Louis, USA) (1:32) coupled to fluorescein isothiocyanate and diluted with PBS and Evans Blue (10%). Subsequently, three washes were performed with PBS + 0.01% Tween 20. Finally, the slides were mounted with coverslips, with the addition of buffered glycerin (pH 8.7), covered with glass coverslips, and analyzed using a UV microscope at 40X objective (Olympus®, BX-60). Samples showing strong fluorescence intensity responses, after a screening of 1:64, were progressively diluted at 1:64, 1:128, 1:256, 1:512, and 1:1024 in PBS + 0.5% powdered milk, until the “end point”.

2.10 Statistical analyses

The Fisher-exact test was used to determine associations between positive or negative results in the qPCR for *Bartonella* spp. Odds ratio (OR), 95% confidence interval and p values were calculated for each variable. Also, statistical software (https://www.R-project.org/) with a specified significance level (*P*) of <0.05 to test the difference in the percentage of *Bartonella* spp. positivity between both sample groups (undergoing clinical-pathological laboratory routine exams and blood donors). Data were compiled and analyzed in R Foundation. The analyses based on 95% confidence interval and p-values were calculated for each variable.

Furthermore, data from both cat groups (from accredited clinics and blood donors) were combined, resulting in the analysis of 158 cats for *Bartonella* spp. using four different methods: Blood qPCR, Liquid culture qPCR, Isolate and IFAT. Cochran's Q Test (Cochran, 1950) was performed to assess differences in the proportions of positive results among all methods. As post-hoc analysis, pairwise comparison was

conducted using McNemar's Test. Additionally, pairwise comparison of correlation was measured by calculating Phi coefficient. Phi coefficient results were interpreted according: <0.1 = negligible; 0.1-0.3 = weak association; 0.3-0.5 = moderate association; 0.5-1 = strong association (Agresti, 1996; Smithson, 2003; Akoglu, 2018). To evaluate the agreement between the four different methods, Fleiss' Kappa (Fleiss, 1971; Fleiss et al., 2003) was calculated. All statistical analyses were performed using RStudio 2021.09.02 software (http://www.rstudio.com/) and packages DescTools and irr.

3 Results

3.1 DNA quality and amplification of the *gapdh* endogenous gene

All 200 cat blood DNA samples from domestic cats were positive in the PCR based on the mammal endogenous *gapdh* gene, confirming the absence of PCR inhibitors. Cat blood DNA samples presented a mean concentration of 7.20 ng/µL (ranging from 1.0 to 24.9 ng/µL, standard deviation [SD] ± 4.89), and a mean absorbance ratio (260/280 nm) of 4.25 with a standard deviation of ± 10.95.

3.2 Positivity for *Bartonella* spp.

Among the DNA samples extracted directly from cat blood, 8/100 (8%) samples from accredited veterinary clinics tested positive in the qPCR for *Bartonella* spp., with Cq values ranging from 28.44 to 38.35. When each sample was subjected to qPCR in duplicates, quantification was possible to be achieved in 4/8 samples; after repeating the remaining four samples in triplicates, quantification was possible in 6/8. The quantification of the number of copies of a fragment of the intergenic region of the 16-23S rRNA (ITS) per microliter of blood DNA sample from cats originated from accredited veterinary clinics ranged from 5.10×10^0 to 2.05×10^2.

On the other hand, 9/100 (9%) of blood samples from cat blood donors tested positive in the qPCR for *Bartonella* spp., with Cq values ranging from 20.62 to 40.10. All positive DNA samples from cat blood donors cat needed to be repeated in triplicates, as the difference of Cq values between replicates was greater than 0.5,

resulting in quantification of 1/9 samples (7.62 x 10^1 copies of a fragment of the 16-23S rRNA intergenic region (ITS) per microliter of DNA).

Regarding qPCR results from liquid culture + cat blood samples, 30 (30%) DNA blood samples from cats from accredited veterinary clinics were positive for *Bartonella* spp., with Cq values ranging from 20.12 to 42.03. Out of the 30 positive samples, quantification was possible in 8/30 when each DNA sample was subjected to qPCR in duplicates. When the remaining samples were repeated in triplicates, quantification was possible to be achieved in another 3/30, totaling 11/30. The quantification of copies of a fragment of the 16-23S rRNA intergenic region (ITS) per microliter of DNA from liquid culture + blood samples from cats accredited veterinary clinics ranged from 1.21 x 10^0 to 1.99 x 10^6.

On the other hand, 40 (40%) blood samples from cat blood donors subjected to liquid culture showed positivity in the qPCR for *Bartonella* spp., with Cq values ranging from 20.17 to 48.94. After repeating positive samples in triplicates, quantification was possible to be achieved in 6/40 samples. The quantification of the number of copies of a fragment of the 16-23S rRNA intergenic region (ITS) per microliter of DNA from liquid culture samples added to blood from cat blood donors ranged from 1.72 x 10^0 to 3.77 x 10^4.

The qPCR assays presented efficiency, R^2, slope, and Y-intercept values ranging from 96.7% to 101.8%, 0.726 to 0.998, -3.280 to -3.498, and 34.048 to 37.345, respectively, following MIQE recommendations.

After passage through liquid culture, 2 (2/8) DNA blood samples from cats accredited veterinary clinics that were previously positive in the qPCR *Bartonella* spp. were negative, and 24 previously negative samples tested positive. Additionally, while 6 (6/9) positive samples from cat blood donors were negative after incubation in pre-enriched liquid medium, 37 cat blood samples previously negative for *Bartonella* spp. tested positive after enrichment in BAPGM medium (**Table 1**). Although pre-enrichment liquid medium for *Bartonella* associated with molecular methods has been shown to increase diagnostic sensitivity for detecting bartonellae (Maggi et al., 2005), DNA positive samples extracted directly from blood that tested negative in the qPCR for *Bartonella* after enrichment in BAPGM medium might indicate a possible "dilution effect," as well as bacteria's failure to grow properly in liquid medium (Duncan et al., 2007).

Table 1. Cat blood samples and cat blood samples incubated in pre-enrichment liquid culture positive in the qPCR assays for *Bartonella* spp. based on the 16-23S rRNA (ITS) region according to gender of sampled animals.

	***Bartonella* spp. positive samples**				
Cat blood samples	**Blood**	**Blood + liquid culture**	**Total**	**Female**	**Male**
From accredited veterinary clinics	8/100 (8%)	30/100 (30%)	32/100 * (32%)	10/32 [a] (31.25%)	22/32 [a] (68.75%)
From blood donors	9/100 (9%)	40/100 (40%)	46/100 * (46%)	19/46 [b] (41.30%)	27/46 [b] (58.70%)

*values with statistically significant differences.
Similar letters indicate no statistically significant difference between females and males.

Out of the 200 blood samples subjected to pre-enrichment liquid culture (BAPGM) and plating onto chocolate agar, three (1.5%) showed smooth and circular suggestive colonies of *Bartonella* after 7-12 days of plating, whose identity was confirmed in the qPCR for *Bartonella* spp. Among the positive blood samples, two cats (FL45 and FL97) originated from accredited veterinary clinics (Cq values obtained in liquid culture samples ranging from 27.47 to 27.57 and 37.15 to 37.17, respectively), and one from a cat blood donor (FB99) (Cq values obtained in liquid culture samples ranging from 20.42 to 20.12).

Considering the positive results in the qPCR for *Bartonella* spp. obtained from all three methods (DNA extraction from blood samples and after passage through liquid culture, as well as isolation on chocolate agar), a total of 32/100 (32%) cats from veterinary accredited clinics tested positive: 10/32 (31.25%) females and 22/32 (68.75%) males. Among cat blood donors, 46/100 (46%) teste positive: 19/46 (41.30%) females and 27/46 (58.70%) males (**Supplementary Table 1**). The number of cat blood donors positive for *Bartonella* spp. was statistically higher when compared to cats accredited veterinary clinics (p-value 0.04239). The positivity according to the gender of the sampled cats did not exhibit statistical significance in both groups: p-value = 0.06789 for undergoing routine laboratory exams (**Supplementary Table 2**) and p-value = 0.1566 for cat blood donors (**Supplementary Table 3**).

3.3 Basic Local Alignment Search Tool for nucleotides analysis

In the Basic Local Alignment Search Tool for nucleotides (BLASTn) analysis of two ITS sequences obtained from blood samples from cats originated from accredited veterinary clinics, while one ITS sequence obtained (FL100; Cq = 29.07) showed 100% identity (E-value = 2e-16; query coverage = 100%) to *Bartonella henselae* (MT095054.1) previously detected in *Ctenocephalides felis* fleas from Rio de Janeiro, the sequence detected in cat FL101 (Cq = 28.58) showed 100% identity (E-value = 2e-17; query coverage = 100%) to *Bartonella clarridgeiae* (MN809212.1) previously detected in a cat from Paraguay.

Additionally, 18 samples (1 blood sample and 7 BAPGM + blood samples from cats accredited veterinary clinics, and 10 BAPGM + blood samples from blood donor cats) were selected based on *Bartonella* qPCR quantification results (ranging from 5.18 x 10^1 to 3.77 x 10^4) to perform cPCR targeting the *pap-31* gene. Out of the 10 positives samples for cPCR based on *pap-31* positive, five sequences were obtained. Specifically, one from blood samples (FL91) and others two sequences were obtained from BAPGM + blood samples from cats accredited veterinary clinics (FL45 and FL101). Furthermore, two sequences were obtained from BAPGM + blood samples from donor cats (FB45 and FB71). The five sequences showed 100% identity (E-value = 0.0; query coverage = 100%) with *B. henselae* sequences (CP020742.1; CP072898.1; MH350813.1) previously detected in humans and cats from Germany and cats from Italy.

3.4 MLST analyses

The samples FL45, FL97, and FB99 showed allelic profiles corresponded to ST1, ST2 and ST5, respectively, based on *B. henselae* MLST online database (**Table 2)**. While the ST1 (FL45) and ST2 (FL97) were found among cats from accredited veterinary clinics, ST5 (FB99) was detected in a cat blood donor. All three ST's (#1, #2 and #5) have been associated with human diseases. While ST1 and ST5 are the most frequently found Sequence Types (**Figure 2**), ST2 has only been reported in cats from Spain and United Kingdom (**Figure 3**), according to the isolates submitted at PubMLST database (https://pubmlst.org/organisms/Bartonella-henselae).

Table 2. Allelic profiles of *B. henselae* isolates obtained from blood samples from cats sampled in Distrito Federal (Central-Western Brazil) and their respective Sequence Types (STs).

Isolate	*Sample*	*Host*	*gltA*	*groEL*	*ribC*	*batR*	*nlpD*	*ftsZ*	*rpoB*	*16S*	ST
FL45	Blood	Cat	1	1	1	1	1	1	1	1	**1**
FL97	Blood	Cat	1	2	1	1	1	1	1	1	**2**
FB99	Blood	Cat	1	2	1	1	1	1	1	2	**5**

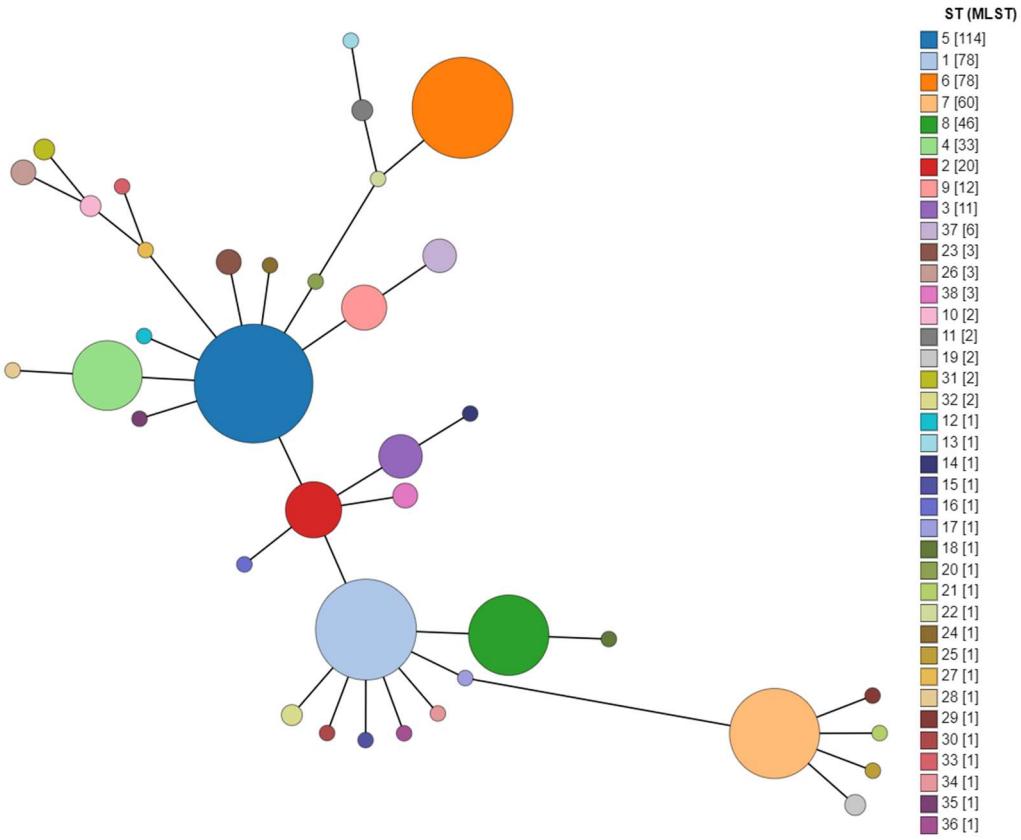

Figure 2. Cluster analysis performed by GrapeTree of the 37 *Bartonella henselae* STs available in the PubMLST database, showing by the colors the most to the least STs detected.

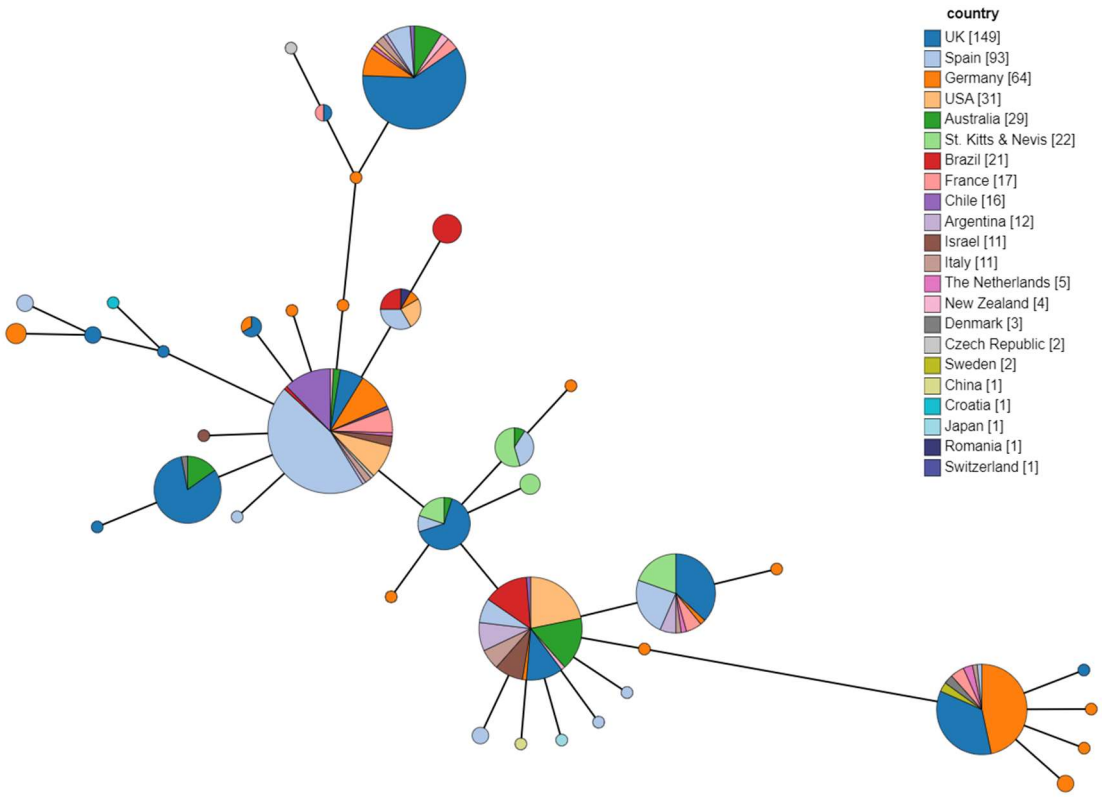

Figure 3. GrapeTree clustering of 37 STs of *Bartonella henselae* available in the PubMLST database, showing by the colors the STs detected according to the countries.

3.5 IFAT results

Out of 158 cat serum samples subjected to serological detection IgG antibodies to *Bartonella* sp., 60.75% (96/158) were positive, represented by 33/67 (49.25%) cats from accredited veterinary clinics and 63/91 (69.23%) cat blood donors. Among the seroreactive cats from accredited veterinary clinics, the following titers were found: 64 (6/33; 18.2 %), 128 (2/33; 6.1 %), 256 (6/33; 18.2 %), 512 (6/33; 18.2%), 1024 (8/33, 24.2%), and 2048 (5/33, 15.1%). Among the seroreactive blood cat donors, the following titers were found: 64 (23/63; 36.5%), 128 (1/63; 1.6%), 256 (5/63; 7.9%), 512 (17/63; 27%), 1024 (10/63; 15.9%), and 2048 (7/63; 11.1%).

The Cochran's Q Test was performed to evaluate differences between the four diagnostic methods (**Supplementary Table 4**). The test revealed a statistically significant difference ($Q = 166.8$, $df = 3$, p-value < 2.2e-16), indicating that the methods do not perform equally in detecting positivity for *Bartonella* spp. The low *p*-value suggests that at least one method differs significantly from the others. Post-hoc analysis using McNemar's Test demonstrated that all methods present significant

differences in their outcomes. Results corroborated with calculation of Phi coefficient, once all methods comparisons presented negligible or weak correlation. Fleiss' Kappa was used to assess the agreement between the four methods. Kappa value obtained was -0.0621 (z-score: 1.91) indicating disagreement among the methods with no statistical significance among agreements (*p*-value = 0.0559). The Venn Diagram displays overlapping results among all methods (**Figure 4**). In summary, our results demonstrated a high rate of disagreement among the four diagnostic methods tested herein.

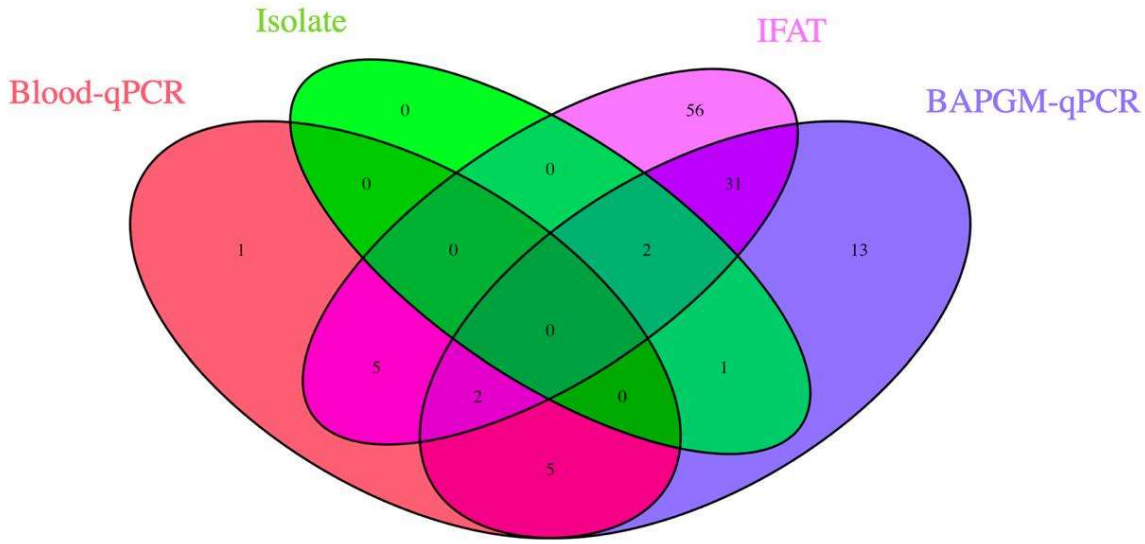

Figure 4. A Venn Diagram constructed to visualize overlapping results among the four methods for *Bartonella* spp. diagnosis tested herein. Constructed using RStudio 2021.09.02 software and VennDiagram package.

4. Discussion

The present work expanded the knowledge regarding the occurrence and genotyping of *B. henselae* in blood samples originated from two groups of cats: i.) from accredited veterinary clinics and ii.) registered as blood donors in central-western Brazil.

Despite several reports of *Bartonella* spp. in domestic cats across different regions of Brazil (Braga et al., 2012; André et al., 2014; Drummond et al., 2018; Pedrassani et al., 2019; Furquim et al., 2021; Cruz et al., 2022; Dias et al., 2023), the real prevalence of these agents remains underestimated, mainly due to

challenging diagnosis (Guptill et al., 2010; Gonçalves et al., 2014; Furquim et al., 2021; Dias et al., 2023). Misdiagnosis often occurs due to the challenging nature of isolating *B. henselae*. The prolonged incubation period frequently leads to false-negative culture results, making the identification of new strains difficult (Chenoweth et al., 2004). Maggi et al. (2005) development a novel insect-based liquid culture medium (*Bartonella* Alpha-Proteobacteria Growth Medium - BAPGM). This innovative medium has successfully supported the growth of seven different *Bartonella* species and enables co-culturing of diverse *Bartonella* species. Moreover, BAPGM has been effective in isolating *B. henselae* from blood samples obtained from naturally infected cats. In order to enhance diagnostic sensitivity, molecular methods have been combined with pre-enrichment liquid medium (BAPGM) and isolation of these fastidious bacteria using blood/chocolate agar (Maggi et al., 2005; Drummond et al., 2018; Furquim et al., 2021; Dias et al., 2023). Indeed, previous studies using cat blood samples and blood samples added to pre-enrichment medium have reported molecular detection rates ranging from 33.1% to 42% (Furquim et al., 2021; Dias et al., 2023). These findings mirror those reported in the present study, where 39% (78/200) of the sampled cats were positive for *Bartonella* spp. After incubation of blood samples in BAPGM liquid medium, 61 (78.20%) liquid culture samples originated from blood samples previously showing negative results in the qPCR assays, turned out positive for *Bartonella* spp. This enhanced sensitivity of molecular assays performed after submitting blood samples to pre-enrichment liquid culture has already been observed in previous studies (Furquim et al., 2021; Dias et al., 2023; do Amaral et al., 2022a). Noteworthy, *B. henselae* isolates in the present study were obtained from cat blood samples that showed to be negative in the initial molecular screening, emphasizing that a negative qPCR result from DNA blood samples does not rule out the infection by *Bartonella* spp.

Nonetheless, eight positive samples originated from DNA extracted directly from blood samples were negative after incubation in pre-enriched liquid medium. This result might be due to a possible "dilution effect" associated with bacteria's failure to proliferate properly in liquid medium (Duncan et al., 2007). Besides that, pre-analytical treatment interferences (such as antimicrobials, medications, and additives) might have hindered the proper growth of *B. henselae* in those eight samples. Therefore, in order to achieve an improved molecular detection of *Bartonella* spp., qPCR should be performed from DNA samples extracted from both blood and blood + pre-enrichment

liquid culture followed by attempts of isolation in blood/chocolate agar (Furquim et al., 2021; Dias et al., 2023; do Amaral et al., 2022a; b).

In this study, 32% of cats from accredited veterinary clinics were found to be positive for *Bartonella* spp.. This finding corroborates previous studies from several Brazilian regions, such as 30.5% - 36% in Midwest region (André et al., 2016; Dias et al., 2023), 13.3% in South region (Pedrassani et al., 2019), 24.7 - 39.9% in Southeast region (André et al., 2014; Silva et al., 2019; Furquim et al., 2021; Raimundo et al., 2019), and 20.7% in the Northeast region (Cruz et al., 2022). Among accredited veterinary clinics, both *B. henselae* and *B. clarridgeiae* were identified in the same cat, indicating coinfection between these two species, as previously described (Gurfield et al., 1997). Cats are the primary reservoirs for both *Bartonella* species (Anderson and Neuman, 1997; Kordick et al., 1997), which comprise causative agents of Cat Scratch Disease (CSD) (Lamas et al., 2008).

The risk of *Bartonella* transmission during blood transfusion has been previously suggested (Silva et al., 2016; Kordick and Breitschwerdt, 1997). *Bartonella* species exhibit tropism for erythrocytes and are also capable of infecting microglial cells, dendritic cells, pericytes, monocytes, and CD34+ bone marrow progenitor cells (Chomel et al., 2009; Breitschwerdt, 2017). Studies have demonstrated that *B. henselae* can survive in human red blood cell (RBC) units for up to 35 days after storage (Magalhães et al., 2008). In humans, *B. henselae* have already been isolated from 6 out of 500 human blood donors sampled from southeastern Brazil (Pitassi et al., 2015). Studies on cat blood donors are scarce. *Bartonella henselae* has been detected, among cat blood donors in the USA, where one cat was infected by only *B. henselae*, and another one was co-infected by both *'Candidatus Mycoplasma haemominutum*' and *B. henselae* (Hackett et al., 2006). Herein, out of 100 cat blood donors sampled, 46% tested positive for *Bartonella* spp.. Comparing these data, the *Bartonella* prevalence among cat blood donors was statistically higher (*p*-value =0.04239) when compared to that found among cats from accredited veterinary clinics (32%). These findings emphasizes that asymptomatic cat blood donors may harbor *Bartonella* spp. within their red blood cells, presenting a genuine risk of spread of zoonotic bartonellae. According to Wardrop et al. (2016), it is highly recommended that cat blood donors are seronegative and test negative for *Bartonella* by both PCR and culture.

The detection of *Bartonella* spp. DNA by qPCR or antibodies to *Bartonella* spp. by IFAT showed that most cats in this study have been exposed to bacteria of the genus *Bartonella*. Specifically, *Bartonella* DNA was detected in the blood of 39% (78/200) of cats, while 60.75% (96/158) of the serum samples exhibited reactivity to *Bartonella* sp.. Previous studies in Brazil have reported seropositivity rates to *Bartonella* spp. ranging from 15% to 47.5% among cats (Fontalvo et al., 2017; Kitada et al., 2014; Crissiuma et al., 2011). On the other hand, the seroprevalence of *Bartonella* spp. ranged from 4% to 81% among cats sampled in the USA (Chomel et al., 1995; Osikowicz et al., 2021). qPCR-positive / IFAT-negative results may suggest recent infection, where the IgG antibody level is not detectable by the test. While seropositivity does not prove current infection, seronegativity cat does not exclude the infection (Pretorius et al., 1999). In the current study, DNA and antibodies to *Bartonella* spp. were detected concurrently in 25.3% (40/158) of samples. Likewise, IG antibodies to *B. henselae* and *Bartonella* DNA (*htr*A gene-based PCR) were both detected in 22.5% (9/40) clinically healthy cats from Rio de Janeiro, (Crissiuma et al., 2011). These findings collectively underscore the variable and significant prevalence of *Bartonella* spp. exposure among cats from central-western Brazil. As demonstrated by our statistical analyses, when comparing diagnostic methods for *Bartonella*, no single test proves superior, highlighting the importance of using a combination of methods to improve accuracy. This also emphasizes the complexity of diagnosing infections caused by the *Bartonella* genus, as no single approach can fully capture the diversity of exposure and infection status.

Herein, ST1 and ST2 were identified among the sampled cats from accredited veterinary clinics and ST5 from a blood donor. Although ST1 and ST5 have already reported among cats from Goiás state (Dias et al., 2023), midwestern Brazil, this is the first report of ST2 in cats from the American continent. Previous studies reported ST2 in humans from Australia and United Kingdom (Iredell et al., 2003; Chaloner et al., 2011). While ST1 and ST5 represent the most frequently detected zoonotic variants (Gil et al., 2013; Stepanić et al., 2019), ST2 *B. henselae* strains have also been associated with zoonotic reports (Chaloner et al., 2011). Additionally, the ST5 strain identified in a single cat blood donor in our study stands out as the most prevalent variant affecting both cats and humans (Gil et al., 2013; Stèpanic et al., 2019). These findings highlight the potential transmission by blood transfusion of the *B. henselae* ST5 variant among cats in the Distrito Federal. Although the variants identified in this

study have been previously associated with to human disease, there are no documented cases of *Bartonella* prevalence in humans in the Distrito Federal. Also in Brazil, ST9 has been detected in cats from São Paulo state (Furquim et al., 2021). Regarding *B. henselae* STs reported worldwide, while ST1, ST5, and ST6 have been found in North America, Australia, Japan, and Europe, ST7 has been frequently reported in Europe (Arvand et al., 2007; Yanagihara et al., 2010). ST1 is considered the main variant associated with human infection (Arvand et al., 2007). In the United Kingdom, while ST2, ST5, and ST8 have been frequently associated with humans showing clinical signs of bartonellosis, ST4, ST6, and ST7 have mostly been detected in domestic cats (Chaloner et al., 2011). Among cats, ST5 and ST7 have been detected in Germany, whereas ST5, ST6, and ST9 were reported Spain (Mietze et al., 2011; Gil et al., 2013). ST1, ST5, ST9, ST35, ST36 were reported among cats from Turkey (Can et al., 2023) and ST1 and ST8 in Argentina (Cicuttin et al., 2014). Recently, Stepanić et al. (2024) identified ST1, ST5, ST6, ST24, and ST33 in domestic cats from Croatia. Novel ST have been reported in cats from Brazil (ST37) (Furquim et al., 2021) and Turkey (ST38) (Can et al., 2023), highlighting that the genetic diversity of *B. henselae* is higher than expected. Future studies on different geographical regions and cat populations should be performed, aiming at unraveling the real genetic diversity of *B. henselae* and shedding light on the possible association between specific STs and risk of bartonellosis-associated pathologies (e.g. psychiatric disorders, endocarditis, hepatic peliosis, etc.).

Although *Bartonella* infection has rarely been associated with clinical disease in cats, the detection of *Bartonella* in apparently healthy cat blood donors emphasizes the potential transmission of zoonotic strains of *B. henselae* by blood transfusion to cats without outdoor access. Immunocompromised cat owners should be aware of the potential risk of householding flea-infested cats that received blood transfusion in the past in transmitting *Bartonella* through bites and scratches. Finally, considering that 25.95% (9/41) of *Bartonella*-qPCR positive cat blood donors sampled herein showed to seronegative to *B. henselae*, serology should not be used as a screening test to check the *Bartonella* infection/exposure in this group of animals.

5. Conclusions

The present study showed, for the first time, the occurrence of *Bartonella* in cat blood donors in South America and the potential risks of transmission of zoonotic *B. henselae* variants by blood transfusion to cats. A higher occurrence of *Bartonella* spp. DNA was observed among cat blood donors when compared to cats accredited veterinary clinics. Similarly, IFAT showed a high level of exposure to *Bartonella* spp. among the studied population. The lack of agreement among the methods evaluated in this study, with significant differences among proportions of positive outcomes, indicate that a single test is not sufficient to show evidence of *Bartonella* exposure/infection. The combination of different methods is necessary to improve *Bartonella* spp. diagnostic accuracy in cats. IFAT cannot be used as a screening method to check the *Bartonella* infection/exposure in cat blood donors. Three *B. henselae* variants were detected among cats sampled in Federal District, midwestern Brazil, namely ST1, ST2 and ST5. This is the first report of a zoonotic *B. henselae* variant (ST5) in cat blood donors. ST2 is documented for the first time in cats in the American continent.

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CRediT authorship contribution statement

Lorena Freitas das Neves: Data curation, Formal analysis, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Clara Morato Dias:** Formal analysis, Methodology, Investigation, Writing – review & editing. **Anna Claudia Baumel Mongruel:** Methodology, Writing – review & editing. **Gabrielly de Oliveira Lopes:** Methodology, Writing – review & editing. **Liliane Maria do Rosario Batista:** Methodology, Writing – review & editing. **Francisco Anilton Alves Araujo:** Methodology, Writing – review & editing. **Gener Tadeu Pereira:** Methodology, Writing – review & editing. **Rosangela Zacarias Machado:** Methodology, Investigation, Writing – review & editing**. Marcos Rogério André:** Writing – original draft, Writing – review & editing, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Investigation.

Declaration of competing interest

All authors confirm that there are no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary Table 1. qPCR results, quantification (number of copies of a fragment of the intergenic region of the 16-23S rRNA (ITS) / µL DNA), isolation and IFAT (antibody titers) of *Bartonella* spp. from blood samples of cats from accredited veterinary clinics and blood donors.

Group of cats	Sample ID	qPCR quantification (number of copies of a fragment of *Bartonella* ITS / µL)		Isolation onto chocolate agar	IFAT titer
		Blood sample	Liquid culture (BAPGM)		
From accredited veterinary clinics	FL1	Negative	Not-quantified	Negative	Not-collected
	FL6	Negative	Negative	Negative	128
	FL7	Negative	Negative	Negative	1024
	FL9	Negative	Negative	Negative	64
	FL11	Negative	1.35×10^{1}	Negative	Not-collected

	FL13	1.84×10^2	1.46×10^2	Negative	Negative
	FL14	Negative	Negative	Negative	64
	FL15	Negative	Not-quantified	Negative	1024
	FL16	Negative	5.17×10^1	Negative	Not-collected
	FL17	Negative	Negative	Negative	256
	FL18	Negative	Not-quantified	Negative	Not-collected
	FL22	5.53×10^1	5.66×10^0	Negative	Negative
	FL24	Negative	Not-quantified	Negative	1024
	FL26	Negative	Not-quantified	Negative	Not-collected
	FL28	Negative	Negative	Negative	256
	FL29	Negative	Negative	Negative	512
	FL34	5.10×10^0	4.62×10^0	Negative	Not-collected
	FL36	Negative	Negative	Negative	256
	FL38	Negative	Negative	Negative	2048
	FL40	Negative	Negative	Negative	1024
	FL41	Negative	Negative	Negative	2048
	FL42	Negative	Negative	Negative	1024
	FL44	Negative	Not-quantified	Negative	Negative
	FL45	Negative	2.88×10^4	**Positive**	Negative
	FL47	Negative	Negative	Negative	512
	FL49	Negative	Negative	Negative	1024
	FL53	Negative	Not-quantified	Negative	Not-collected
	FL55	Negative	Negative	Negative	2048
	FL56	Negative	Negative	Negative	128
	FL62	Negative	Not-quantified	Negative	Negative
	FL63	Negative	Not-quantified	Negative	Negative
	FL64	Negative	2.39×10^4	Negative	Not-collected
	FL65	Negative	Negative	Negative	64
	FL66	Negative	Negative	Negative	1024
	FL67	Negative	Not-quantified	Negative	2048
	FL69	Negative	Negative	Negative	64
	FL71	Negative	Not-quantified	Negative	256

	FL73	Not-quantified	Not-quantified	Negative	2048
	FL75	Negative	Negative	Negative	256
	FL76	Negative	Negative	Negative	512
	FL77	Negative	Negative	Negative	256
	FL78	Negative	1.99×10^6	Negative	Not-collected
	FL80	Negative	Negative	Negative	512
	FL81	Negative	Negative	Negative	1024
	FL86	Negative	Not-quantified	Negative	Not-collected
	FL89	Negative	Not-quantified	Negative	Negative
	FL91	2.05×10^2	Negative	Negative	Not-collected
	FL92	Negative	Not-quantified	Negative	Negative
	FL93	Negative	Not-quantified	Negative	Negative
	FL96	Negative	Not-quantified	Negative	64
	FL97	Negative	1.21×10^0	**Positive**	512
	FL98	Negative	Negative	Negative	512
	FL99	Negative	Not-quantified	Negative	Not-collected
	FL100	5.44×10^1	2.51×10^1	Negative	Negative
	FL101	9.58×10^1	5.18×10^1	Negative	Negative
	FL102	Negative	Not-quantified	Negative	64
	FL106	Not-quantified	Negative	Negative	Not-collected
Blood donors	FB6	Negative	Negative	Negative	2048
	FB8	Negative	Not-quantified	Negative	2048
	FB9	Negative	Negative	Negative	64
	FB10	Negative	Negative	Negative	256
	FB12	Negative	2.35×10^1	Negative	Negative
	FB13	Negative	Negative	Negative	64
	FB14	Negative	Negative	Negative	512
	FB15	Negative	Negative	Negative	512
	FB16	Negative	Negative	Negative	64
	FB17	Negative	Negative	Negative	1024
	FB18	Negative	Not-quantified	Negative	64
	FB19	Negative	Negative	Negative	128

	FB21	7.62×10^1	Negative	Negative	512
	FB23	Negative	Negative	Negative	512
	FB25	Negative	Not-quantified	Negative	Negative
	FB26	Negative	Negative	Negative	512
	FB28	Negative	Negative	Negative	1024
	FB30	Negative	Negative	Negative	1024
	FB32	Negative	Not-quantified	Negative	256
	FB33	Negative	Negative	Negative	64
	FB34	Negative	Not-quantified	Negative	64
	FB35	Negative	Negative	Negative	64
	FB36	Negative	Not-quantified	Negative	Not-collected
	FB37	Negative	Not-quantified	Negative	64
	FB38	Negative	Not-quantified	Negative	Negative
	FB39	Negative	Not-quantified	Negative	Negative
	FB41	Negative	Negative	Negative	64
	FB42	Negative	Not-quantified	Negative	512
	FB43	Not-quantified	Negative	Negative	512
	FB44	Negative	Negative	Negative	1024
	FB45	Negative	7.57×10^3	Negative	Negative
	FB47	Negative	Not-quantified	Negative	512
	FB48	Negative	Negative	Negative	1024
	FB49	Negative	Negative	Negative	1024
	FB50	Negative	Not-quantified	Negative	64
	FB51	Negative	Negative	Negative	2048
	FB52	Negative	Negative	Negative	64
	FB54	Negative	Not-quantified	Negative	64
	FB55	Negative	Not-quantified	Negative	512
	FB57	Negative	Not-quantified	Negative	Negative
	FB58	Negative	2.79×10^0	Negative	512
	FB59	Negative	Not-quantified	Negative	512
	FB60	Negative	Not-quantified	Negative	64
	FB61	Negative	Negative	Negative	64

	FB62	Not-quantified	Negative	Negative	256
	FB63	Negative	Not-quantified	Negative	2048
	FB64	Negative	Not-quantified	Negative	256
	FB65	Negative	Not-quantified	Negative	Not-collected
	FB66	Negative	Not-quantified	Negative	Not-collected
	FB68	Negative	Negative	Negative	512
	FB69	Negative	Not-quantified	Negative	2048
	FB71	Negative	Not-quantified	Negative	64
	FB72	Negative	Not-quantified	Negative	1024
	FB73	Negative	Negative	Negative	64
	FB74	Negative	Not-quantified	Negative	64
	FB75	Negative	Negative	Negative	2048
	FB76	Negative	Not-quantified	Negative	256
	FB77	Negative	Not-quantified	Negative	Not-collected
	FB78	Negative	Not-quantified	Negative	64
	FB79	Negative	Negative	Negative	64
	FB80	Not-quantified	Not-quantified	Negative	Negative
	FB83	Negative	Negative	Negative	512
	FB85	Negative	Negative	Negative	512
	FB87	Not-quantified	Negative	Negative	64
	FB89	Negative	Not-quantified	Negative	Negative
	FB90	Negative	Negative	Negative	2048
	FB91	Not-quantified	Negative	Negative	Negative
	FB92	Not-quantified	1.72×10^{0}	Negative	512
	FB93	Not-quantified	Negative	Negative	1024
	FB95	Not-quantified	Not-quantified	Negative	Not-collected
	FB96	Negative	Negative	Negative	64
	FB97	Negative	Negative	Negative	512
	FB98	Negative	4.13×10^{1}	Negative	512
	FB99	Negative	3.77×10^{4}	**Positive**	1024
	FB100	Negative	Not-quantified	Negative	64
	FB101	Negative	Not-quantified	Negative	64

FB102	Negative	Not-quantified	Negative	1024

Supplementary Table 2. Statistical analysis comparing the *Bartonella* occurrence using molecular/microbiological methods within male and female cats from accredited veterinary clinics.

		Positivity for *Bartonella* spp.				
		+/c	**(%)**	**95% CI**	**OR**	***p*-value**
Sex	**Male**	22/54	40.74	28.66-54.04	2.475	0.06789
	Female	10/46	21.74	12.08-35.76		

+, Number of positive animals for *Bartonella* sp.; c, number of tested cats; 95% CI, 95% confidence interval; OR, odd ratio.
p-values <0.05 were considered statically significant and were highlighted in bold.

Supplementary Table 3. Statistical analysis comparing the *Bartonella* occurrence using molecular/microbiological methods within male and female cat blood donors.

		Positivity for *Bartonella* spp.				
		+/c	**(%)**	**95% CI**	**OR**	***p*-value**
Sex	**Male**	27/67	40.3	29.38-52.27	0.4979	0.1566
	Female	19/33	57.58	40.79-72.78		

+, Number of positive animals for *Bartonella* sp.; c, number of tested cats; 95% CI, 95% confidence interval; OR, odd ratio.
p-values <0.05 were considered statically significant and were highlighted in bold.

Supplementary Table 4. Pairwise comparison of the four diagnostics methods for *Bartonella* sp. employed in the present study using McNemar's Test and correlation analysis. Statistical significance was considered when *p*-value < 0.05.

Comparison of Methods		**McNemar's Test**			**Correlation Analysis**
		Statistical test (χ^2)	**Degree of freedom**	***p*-value**	**Phi coefficient**
Blood-qPCR	BAPGM-qPCR	30.189	1	3.92e-08	0.124
	Isolation	5.0625	1	0.02445	0.041
	IFAT	70.779	1	2.2e-16	0.042
BAPGM-qPCR	Isolation	49.02	1	2.534e-12	0.193
	IFAT	21.012	1	4.563e-06	0.060
Isolation	IFAT	89.095	1	2.2e-16	0.017

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