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Original Article

Bartonella spp. and hematological changes in privately owned domestic cats from Rio de Janeiro, Brazil

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Abstract

Introduction: *Bartonella* infection in cats can represent a risk to owners, particularly today when considering the increase in cat populations and their role in human bartonellosis epidemiology. In the present study, we aimed to detect *Bartonella* spp. in blood samples from 163 asymptomatic privately-owned cats from the metropolitan area of Rio de Janeiro State, Brazil by using a conventional PCR test and also to evaluate the association between *Bartonella* spp. and hematological changes in positive cats.

Methodology: PCR assays were performed targeting the *Bartonella* spp heat shock protein (*htr*A) gene and complete blood counts were also performed in all samples. Positive PCR samples were confirmed by the presence of two genes, citrate synthase (*gltA*) and RNA polymerase beta-subunit-encoding (*rpoB*).

Results: A total of 74.85% (122/163) of the tested cats were positive for *Bartonella* spp and partial sequencing confirmed to be *B. henselae*. All hematological findings from the 163 cats tested (PCR-positive and negative), presented normal limits.

Conclusions: This study demonstrates that *B. henselae* is present in almost 75% asymptomatic privately-owned domestic cats in the metropolitan region of Rio de Janeiro State, Brazil. Our results also show that hematological findings in *Bartonella* spp. infected cats are uncommon. In this scenario, the use of PCR as a diagnostic tool in feline *Bartonella* infections should be considered. Finally, these results also demonstrate the potential risk of *Bartonella* spp. infection in the human population of the metropolitan area of Rio de Janeiro State, Brazil.

Key words: Bartonellaceae; feline; PCR; complete blood count.

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Introduction

Domestic cats are the main reservoir for *Bartonella henselae, B. clarridgeiae* and *B. koehlerae* and are considered the most important animals involved in the transmission of these microorganisms through scratches, bites or licks [1]. Fleas (*Ctenocephalides felis*) are pointed as vectors of *Bartonella henselae* and transmission occurs mainly through infected flea feces [2]. Yet, *Bartonella* species DNA has also been detected from ticks and biting flies [1-3]. *Bartonella* spp. are the causative agent of many diseases in humans, such as cat scratch disease (CSD), endocarditis, uveitis and meningoencephalitis, and can lead to death, mainly in immunodeficient individuals [1-5].

Two main *B. henselae* genotypes have been reported in domestic cat populations: type I (Houston) and type II (Marseille) [6].

Naturally-infected cats present few clinical signs or are asymptomatic, even though they can develop endocarditis and myocarditis. Case-control studies have not proven an association between *Bartonella* and anemia, gingivostomatitis, neurologic conditions or uveitis [7,8]. Fever, lethargy, anorexia, local lymphadenopathy, myocarditis, neurologic disorders, among others, have been observed in experimental infections [7-11].

Few hematological changes are associated with *Bartonella* spp. infections in cats, although anemia, neutropenia and eosinophilia have been observed in experimental infections [9,10]. However

lymphocytosis and hyperglobulinemia have been reported in cats with antibodies anti-*B. henselae* [10,11].

Domestic cats are becoming one of the most popular pets in the world. Although in Brazil canine population is still bigger than cats, an increase in cat population has been noticed [12]. In Brazil, there is a wide variation in the prevalence of *Bartonella* spp DNA in domestic cats, ranging from 0.5% to 97.3% [13-19]. Hence, the aim of this study was to detect the presence of *Bartonella* spp. in asymptomatic privately-owned domestic cats (*Felis catus*) from the metropolitan area of Rio de Janeiro State (RJ), Brazil and also to evaluate the hematological changes that may occur in positive cats.

Methodology

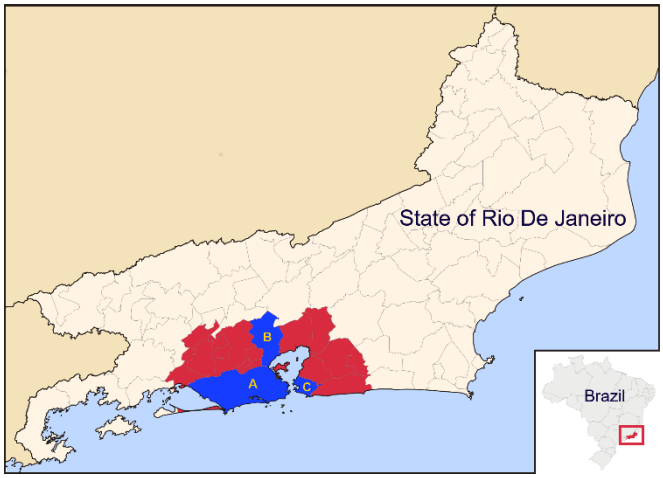
All procedures were performed according to the Ethical Principles of Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee of the Universidade Federal Fluminense (UFF), Rio de Janeiro State, Brazil (0072/09).

Blood samples were collected from 163 privately-owned domestic cats from three cities in the metropolitan region of Rio de Janeiro. The study was conducted in the municipalities of Rio de Janeiro (22º54'10"S 43º12'27"W), Duque de Caxias (22º47'08"S 43º18'42"W), and Niteroi (22º53'00"S 43º06'13"W) (Figure 1), located in the Atlantic Forest, during October and November, 2009.

All samples were collected from jugular or cephalic veins and aliquoted into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. One aliquot was stored at -20ºC until PCR analysis; the other aliquot was processed for complete blood count (CBC) up to 6 hours after sample collection. Blood films from each aliquot were done immediately following sample collection. Only one sample was collected from each animal. The inclusion criteria for selecting cats were that they be from the selected study area and privately-owned. Shelter animals were excluded in this study. The animals had no clinical signs of disease at the time of sample collection.

Complete blood counts were performed with EDTA anti-coagulated samples at Laboratório de Pesquisa Clínica e Diagnóstico Molecular Professor Marcilio Dias do Nascimento of Universidade Federal Fluminense (LAMADIN-UFF) and processed in a Sysmex pocH-100iV Diff automated veterinary hematology counter (Sysmex do Brasil Indústria e Comércio Ltda®, São José dos Pinhais, Paraná, Brasil), whereas differential cell counts were performed manually with Diff-Quik-stained blood smears examined under an optical microscope (E-200 Nikon) by observing 100 random microscopic fields (Magnification: 1000x) to check for the presence of *Bartonella* spp. inclusion.

Figure 1. Distribution of privately-owned domestic cat samples collected for *Bartonella henselae* DNA detection and association with hematological changes from three cities in the metropolitan region of Rio de Janeiro State, Brazil: Rio de Janeiro, Duque de Caxias and Niteroi.



A QIAamp DNA Mini Kit (Qiagen; Hilden, Germany) was used for DNA extraction from whole blood cells according to manufacturer's instructions. For *Bartonella* genus DNA detection, a polymerase chain reaction (PCR) was done using the specific primers CAT1 [5'-GATTCAATTGGTTTGAA(G/A)GAG GCT-3'] and CAT2 [5'-TCACATCACCAGG (A/G)CGTATTC-3'] targeting the heat shock gene *htr*A. PCR reactions were essentially performed as previously described [20].

All samples were retested with other primers in order to evaluate the performance of the *htr*A assay. The citrate synthase gene (*gltA*) was targeted with primers BhCS781p (5'-GGGGaCCaGCTCAtGGtGG-3') and BhCS1137n (5'-AaTGCAAAAAGaACAGTaAA CA-3') as described [21]. The RNA polymerase beta-subunit-encoding gene (*rpoB*) was targeted with primers 1400F (5'-GCATTGGCTTACTTCGTATG-3') and 2300R (5'-GTAGACTGATTAGAACGCTG-3') used as described [22]. DNA extracted from a *Bartonella henselae* infected HEp2 cells and sterile distilled water was used as positive and negative controls, respectively. Reagents and samples were handled with disposable gloves, DNA/RNase-free microtubes and

pipetting tips in safety cabinets equipped with UV lights. Molecular techniques were performed in four separate rooms to avoid DNA contamination. An unidirectional workflow was strictly imposed between pre-PCR areas (sample handling, PCR set up and DNA extraction) and post-PCR areas (DNA amplification, gel analysis, and amplicon purification).

To estimate the size of fragments a 100 bp ladder of DNA (invitrogen, Carlsbad, CA, USA) in each gel was used. The presence of a 414 bp band was considered positive for *htrA* gene. For *gltA* the presence of a 380 bp band was considered positive and for *rpoB* gene the presence of an 825 bp band was considered positive. Amplicons obtained from PCR were column-purified using the Pure Link Quick Gel extraction purification kit (Invitrogen). The presence of PCR inhibitors in DNA samples that tested negative in our PCR test was excluded by the amplification of a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Two PCR-amplified fragments were then cloned. The amplicons obtained from PCR by using primers designed for the *htrA* gene were column-purified using the Pure Link™ Quick Gel extraction purification kit (InvitrogenTM) and then the PCR-amplified fragments were cloned into the pGEM-T easy vector (Promega), in accordance with the manufacturer's instructions. The fragments were ligated into the pGEM-T easy vector and transformed by means of electroporation into *Escherichia coli* Bl21 electrocompetent cells. The transconjugants were selected in LB agar containing X-Gal (50 µg/mL) and ampicillin (100 µg/mL) after incubation overnight at 37 °C. White colonies were chosen and grown in LB broth with 100 µg/mL ampicillin under agitation (250 rpm) at 37 °C, as previously described [23]. The plasmid vector containing the insert was extracted using the alkaline lysis extraction method [24] and was sequenced with primer pairs targeting the *htrA* gene to confirm the cloned DNA fragment.

For sequencing analyses, two clones and three other amplicons were purified using GFXPCR DNA and a Gel Band purification kit (GE Healthcare) and subjected to direct sequencing using the BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems, Los Angeles, CA, USA). Multiple sequence alignments were done with sequences obtained from this study and compared with sequences from GenBank using the MUSCLE algorithm implemented in the MEGA 6.0 software [25]. The *Bartonella* phylogenetic tree was built using the maximum likelihood method (ML) and the Hasegawa, Kishino and Yano + Gamma (HKY + G) model of sequence evolution as determined by the molecular evolutionary genetics analysis (MEGA) 6.0 software. Support for tree nodes was calculated using 1,000 bootstrap replicates.

Statistical analysis (chi-square test) was applied to assess the effect on treatment, on frequency's dispersion of responses with a 5% significance. Descriptive statistics were performed in order to evaluate analysis and results are expressed as Mean and Standard Deviation (SD) in Table 1.

Table 1. Mean and standard deviation of hematological parameters in *Bartonella* spp. negative and positive cats from the Metropolitan Region of Rio de Janeiro State.

	***Bartonella* spp. negative cats**		***Bartonella* spp. positive cats**		**Reference values**
Test (units)	**Mean**	**SD**	**Mean**	**SD**	
Hematocrit (%) *	32.36	8.75	35.98	6.65	24 - 45
RBC (× 10^6/ µL) *	6.73	1.85	7.52	1.51	5 - 10
Hemoglobin (g/dL) *	10.62	2.85	11.91	2.24	8 - 15
MCV (fL)	48.68	4.87	48.18	2.88	39 - 55
MCHC (%)	32.82	1.49	33.09	1.47	30 - 36
WBC (/ µL) *	13756	8063.48	13613.56	6884.0	5500 - 19500
Basophils (/ µL)	36.6	145.68	33.01	96.75	Rare
Eosinophils (/ µL)	714.72	1238.95	676.27	659.94	0 - 1500
Bands (/ µL)	182.72	656.06	97.41	312.60	0 - 300
Neutrophils (/ µL) *	9737.4	6751.34	9468.41	5940.96	2500 - 12500
Lymphocytes (/ µL)	2715.9	2047.43	2973.8	1561.53	1500 - 7000
Monocytes (/ µL)	268.54	290.91	291.26	316.38	0 - 850
Platelets (/ µL)	368660	215440	336330	163300	300000 - 800000
PP (g/dL) *	7.25	0.75	7.66	1.00	6 - 8

SD = standard deviation; RBC = red blood cell count; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; WBC = White blood cell count; PP = plasma protein and * = $p < 0.05$ by chi square test [30].

Results

Among the 163 sampled cats, 149 were from Rio de Janeiro municipality (84 females and 65 males), 3 from Duque de Caxias municipality (3 females) and 11 (6 females and 5 males) from Niteroi municipality. Of the 29 young cats under one year of age, 26 and 3 were from Rio de Janeiro and Duque de Caxias, respectively. Of the 134 were adult cats over one year of age, 123 and 11 were from Rio de Janeiro and Niteroi, respectively.

DNA concentration measurement of samples showed a range of 15.5 ng/μL and the purity ratio at 260/280 nm was 1.734 (+- 0.256). Amplified target DNA for *Bartonella* spp. was detected in 122 of the 163 (74.85%) privately-owned domestic cat samples. The presence of PCR inhibitors in DNA samples was excluded by the amplification of a fragment of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene in all 41 (25.15%) negative samples.

Sequences obtained from five PCR products were 100% homologous to *B. henselae* Houston-1 strain *htr*A gene located in GenBank (accession number: BX897699). Sequences generated during this work were deposited in the Genbank database with the following accession numbers: KU179418; KU179419; KU179420; KU179421; e KU194201 (Figure 2).

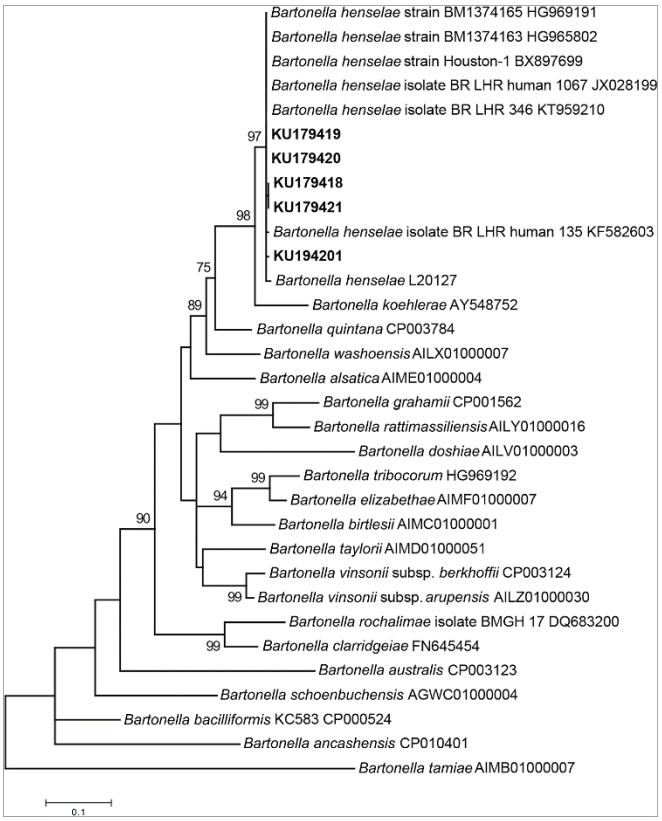
Regarding CBC results from negative and positive cats for *Bartonella* spp., they were similar and within reference values. Despite this, significant variation ($p<0.05$) was observed between groups in hematocrit, red blood cell count (RBC), hemoglobin, white blood cell count, neutrophils and plasma protein results by chi square tests. Hematological findings between each group are demonstrated on Table 1.

Discussion

In the present study, a high prevalence for *Bartonella* spp. DNA was found among privately-owned domestic cat samples from Rio de Janeiro State, Brazil (74.85%). A similar prevalence was found in domestic cats in Alabama (USA) for *B. henselae* and *B. clarridgeiae* (60.9%). In the same study, *Bartonella* spp. DNA was found in 65.2% of the fleas (*C. felis*) from cats. The authors recommended flea control as a preventive measure for infection from this pathogen in endemic areas [26].

In Brazil, a high frequency (97.3%; 36/37) of *Bartonella* spp. DNA in sheltered domestic cats in Vassouras municipality (RJ) was detected by conventional PCR targeting the *htr*A gene. Apparently, the only explanation for this event was probably the proximal contact between cats [13], increasing the risk of direct (by scratches or bites) or indirect (by vectors)

Figure 2. Phylogenetic relationships within the *Bartonella* genus based on a 378 bp fragment of the *htr*A gene. The phylogenetic tree was inferred by using maximum likelihood (ML) and HKY + G model of sequence evolution. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 70% accessed with 1,000 replicates.



transmission [27,28]. Cats from that study underwent flea and tick control, but all had a prior flea/tick infestation history, which also increased the risk for infection.

Considering how a high prevalence of *B. henselae* antibodies in cats in warmer climate regions has been previously described [26,27], we can hypothesize that tropical climate areas of the conducted study, could facilitate fleas (*C. felis*) infestation and, consequently, the transmission of *Bartonella* spp. among cats population is higher, making the studied area endemic.

In another study also conducted in the south of Brazil, a lower positivity rate (17.02%; 8/47) was found in sheltered cats in Rio Grande do Sul State (RS), where 10.63% (5/47) were positive for *B. henselae* and 6.38% (3/47) for *B. clarridgeiae* by conventional PCR targeting a fragment of the riboflavin synthase C (*rib*C) specific for the *Bartonella* genus. The authors reinforced that the crowded conditions found in shelters may increase chances of bacterial spread due to the

close contact between healthy and previously infected animals [14].

Recently, authors demonstrated a multiplex SYBR green qPCR in those samples and improved the detection of *B. henselae* and *B. clarridgeiae* DNA to 25.5% (12/47) of the blood samples. [29].

Household cats from a spaying/neutering program in Rio de Janeiro State (RJ) and in São Paulo State (SP) screened for *Bartonella* spp. by conventional PCR targeting the *htr*A and the intergenic transcribed spacer (ITS), respectively. It was found a prevalence rate of 42.5% (17/40) in Rio de Janeiro and 4.3% (2/46) in Sao Paulo [15,16], which is even lower than the prevalence found in the conducted study.

Similar prevalence was found among apparently healthy cats in the state of Maranhão (4.5% - 9/200) in the northeast state of Brazil, of which 3% (6/200) were of *B. henselae* and 1.5% (3/200) of *B. clarridgeiae,* by conventional PCR targeting the ITS gene [17]. A low prevalence was also detected among shelter cat populations in Cuiaba City in the state of Mato Grosso (central-west of Brazil), also targeting the ITS gene of *Bartonella* spp., where 1.7% (3/178) was positive for *B. henselae* and 0.5% (1/178) for *B. clarridgeiae* [18].

Recently, 30.46% (46/151) sampled cats (86 domiciled and 65 stray) showed positive results in qPCR targeting the nictonamide adenine dinucleotide dehydrogenase gamma subunit (*nuo*G) for *Bartonella* species in Midwestern Brazil. Eighteen (39.1%) of the 46 positive samples tested in the qPCR were also positive in conventional PCR assays, targeting intergenic transcribed spacer, *rib*C, *glt*A, *pap*31 and *rpo*B, followed by sequencing and basic local alignment search tool analysis [19].

In this study, *Bartonella* spp. negative cats were asymptomatic; however we cannot exclude subclinical diseases such as feline immunodeficiency virus (FIV), feline leukemia virus (FeLV) or mycosis such as sporotrichosis. Results from recent study that searched the association between such diseases showed no associations [29].

Significant variations ($p<0.05$) were found in the hematological values of hematocrit, RBC, hemoglobin, WBC, neutrophils and plasma protein parameters among groups. Hematological findings in *Bartonella* spp. infected cats are uncommon [9-11], although anemia, neutropenia and eosinophilia have been described in experimental infections [9,10]. Lymphocythosis has been strongly associated with the presence of *B. henselae* antibodies among naturally infected cats, probably due to induced polyclonal gammopathy [10].

Despite the significant variations found in this study, all results were within reference values [29], even plasma proteins. Naturally-acquired bartonellosis in cats seems to cause mild or no clinical signs or hematological changes. Hypergamaglobulinemia has been found in *Bartonella*-seropositive cats. [11].

Intraerythrocytic corpuscles suggesting *B. henselae* were not found in blood films of positive cats in this study. Other authors considered these corpuscles presumptive of infection, but showed that only 1% of red blood cells were infected [14].

Conclusions

The results obtained in this study support a few theories: (i) naturally-acquired *Bartonella* spp. is present in high frequencies in privately-owned domestic cats in the metropolitan region of Rio de Janeiro State, Brazil; (ii) confirm that hematological findings in *Bartonella* spp. infected cats are uncommon and (iii) reinforce that exposure to cats in these municipalities should alert physicians to the possibility of *Bartonella* infections, especially in patients with risk factors for severe diseases, such as valve surgery, immunosuppression or advanced age, among other factors.

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