

Molecular characterisation of *Bartonella* species in cats from São Luís, state of Maranhão, north-eastern Brazil

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Bartonella species are fastidious bacteria that predominantly infect mammalian erythrocytes and endothelial cells and cause long-lasting bacteraemia in their reservoir hosts. Reports that describe the epidemiology of bartonellosis in Brazil are limited. This study aimed to detect and characterise *Bartonella* spp DNA from cat blood samples in São Luís, Maranhão, north-eastern Brazil. Among 200 cats tested for multiple genes, nine (4.5%) were positive for *Bartonella* spp: six cats for *Bartonella henselae* and three for *Bartonella clarridgeiae*. Based on the phylogenetic analysis of four genes, the *B. henselae* strain matched strains previously observed in Brazil and was positioned in the same clade as *B. henselae* isolates from the United States of America. Moreover, sequence alignment demonstrated that the *B. clarridgeiae* strain detected in the present study was the same as the one recently detected in cats from southern Brazil.

Key words: *Bartonella henselae* - *Bartonella clarridgeiae* - cats - north-eastern Brazil

Bartonella species are fastidious Gram-negative bacteria that predominantly infect mammalian erythrocytes and endothelial cells and cause long-lasting bacteraemia in their reservoir hosts (Chomel et al. 2004). *Bartonella* spp are usually vector-borne bacteria and the vector varies depending on the bacterial species, host and reservoir (Chomel et al. 2006b).

Domestic cats are the main reservoir for *Bartonella henselae* (the most frequent agent of cat scratch disease), *Bartonella clarridgeiae* (suspected in a few cases of cat scratch disease) (Chomel et al. 2004) and *Bartonella koehlerae* (Avidor et al. 2004).

There are few reports of *Bartonella* infection in Brazil. A low seroprevalence of *Bartonella* spp has been detected among Brazilian dogs (Diniz et al. 2007a, b) and *B. henselae* and *Bartonella vinsonii berkhoffii* DNA has been detected in Brazilian dogs at a very low prevalence (Diniz et al. 2007b). Conversely, a very high prevalence of antibodies to *B. henselae* has been observed among free-ranging wild felids in Brazil (Filoni et al. 2006) and the parasite has been detected in wild felids by molecular techniques (Guimarães et al. 2010). Recently, a seroprevalence for *Bartonella* spp of 35.7% was observed among cats belonging to human immunodeficiency virus (HIV) antibody-positive individuals (Lamas et al. 2010). In addition, *Bartonella* spp have been molecularly detected in cats from the states of Rio Grande do Sul (RS) (Stagge-meier et al. 2010) and Rio de Janeiro (RJ) (Souza et al. 2010, Crissiuma et al. 2011).

There are few studies addressing the occurrence of *Bartonella* spp in humans in Brazil. Antibodies to *B. henselae* and *Bartonella quintana* have been detected in 13.7% and 12.8% of healthy residents of the state of Minas Gerais (MG), respectively (da Costa et al. 2005). A seroprevalence of *Bartonella* spp of 38.4% and 34.4% has been observed in HIV-positive patients and healthy human populations, respectively, in Jacarepaguá, RJ (Lamas et al. 2010).

This study aimed to detect and characterise *Bartonella* spp DNA from Brazilian cat blood samples from São Luís, Maranhão (MA), north-eastern Brazil.

MATERIALS AND METHODS

Sample collection and study area - Between October 2008-January 2009, whole blood samples were collected from 200 domestic cats (102 males, 98 females) that were allowed outside in São Luís (194 cats from suburban areas of São Luís county and six from Raposa county). The study region is an invasion area that has suffered a 70% forest reduction. Cats were selected without specific inclusion criteria and were apparently healthy at the time of sample collection. Sampled cats had contact with other cats and dogs. Blood samples for polymerase chain reaction (PCR) were collected from jugular or cephalic veins, immediately aliquoted into tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant and stored at -20°C until PCR analysis. All procedures were performed according to Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee of the College of Agricultural Sciences and Veterinary, São Paulo State University (UNESP) Jaboticabal, state of São Paulo (SP) (2008/025862-07).

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PCR - DNA was extracted from 200 µL of EDTA-anticoagulated blood with the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA was eluted in 200 µL of EB Buffer (supplied by the DNA extraction kit).

Bartonella genus screening was performed by PCR in which the intergenic transcribed spacer (ITS) was targeted, as described previously (Diniz et al. 2007a). Amplification was performed by conventional PCR in a 25 µL final reaction volume that contained 1X PCR mix (Premix Ex Taq, Takara Bio Inc, Shiga, Japan), 12.5 pmol each primer (Integrated DNA Technologies Inc, Coralville, IA) and 5.0 µL DNA template. PCR was performed in a thermocycler (Mastercycler Pro Gradient S, Eppendorf, Hamburg, Germany) under the following conditions: a single hot-start cycle at 95°C for 2 min, followed by 55 cycles of denaturing at 94°C for 15 s, annealing at 66°C for 20 s and extension at 72°C for 20 s. Amplification was completed by an additional cycle at 72°C for 1 min and products were resolved in a 1.5% agarose matrix by electrophoresis and analysed under ultraviolet exposure. DNA from a healthy, pathogen-free cat was used as a PCR negative control. DNA extracted from *B. quintana* (similar to GenBank accession L35100) was used as a positive control. To prevent PCR contamination, the sample extraction, reaction setup, PCR amplification and amplicon detection were performed in separate areas.

Additionally, when genomic material was available, samples were evaluated for the presence of five additional genes: the bacteriophage-associated heme-binding protein gene (*pap31*) (Diniz et al. 2007a), the RNA polymerase beta subunit gene (*rpoB*) (Diniz et al. 2007a), the riboflavin synthase gene (*ribC*) (Johnson et al. 2003), the heat shock protein gene (*groEL*) (Barber et al. 2010) and the citrate synthase gene (*gltA*). The *gltA* gene was amplified with primers BhCS.781p 5'-GGGGACCAGCTCATGGTGG-3' (Norman et al. 1995) and BvCS.205p 5'-TT-

TATCGYGGTTATCCTATYG-3' (Winoto et al. 2005). The amplification conditions were similar to those used for the ITS, but different annealing temperatures were used for each primer pair: 62°C for *pap31* and *rpoB*, 55°C for *ribC* and *gltA* and 58°C for *groEL*.

Phylogenetic analysis - Amplicons were gel-purified or PCR-purified (MiniElute kit, Qiagen, Valencia, CA, USA) and sequenced by the Department of Technology - UNESP or by Eurofins MWG Operon (Huntsville, AL, USA). Chromatogram evaluations, primer deletions and sequence alignments were performed with the software Contig-Express and AlignX (Vector NTI Suite 10.1, Invitrogen Corp, Carlsbad, CA, USA). Bacterial species and strain types were defined by comparison with other sequences deposited in the GenBank database with the Basic Local Alignment Search Tool (Altschul et al. 1990). Phylogenetic analysis was performed with concatenated sequences of the ITS, *rpoB*, *ribC* and *gltA* genes with the maximum likelihood method based on the Kimura two-parameter model (Kimura 1980) with MEGA4 software (Tamura et al. 2007). The *pap31* and *groEL* sequences were excluded from the concatenated phylogenetic analysis because the available DNA sequences in GenBank from other *Bartonella* species were limited.

RESULTS

Among 200 cats tested for multiple genes, nine (4.5%) were positive for *Bartonella* spp: six for *B. henselae* (4 males, 2 females) and three for *B. clarridgeiae* (1 male, 2 females) (Table). These isolates were positioned in the same clade as other *B. henselae* isolates from dogs and humans from Brazil and the United States of America (USA) and the *B. clarridgeiae* isolate from a cat from the USA, with high bootstrap support (100/100). Sixty (30%) of the cats were parasitized by fleas (*Ctenocephalides felis*), including cats positive for *Bartonella* spp.

TABLE

Species of *Bartonella* identified in five cats by DNA amplification and sequencing from São Luis, state of Maranhão, Brazil

Cat number	Genes targeted for <i>Bartonella</i> detection and characterization					
	ITS	<i>rpoB</i>	<i>pap31</i>	<i>ribC</i>	<i>gltA</i>	<i>groEL</i>
3	B.h.	B.h.	B.h.	B.h.	B.h.	NT
6	B.h.	B.h.	B.h.	B.h.	B.h.	NT
7	-	-	-	B.c.	-	B.c.
8	B.c.	-	-	B.c.	NT	NT
10	B.h.	B.h.	B.h.	B.h.	B.h.	B.h.
109	B.h.	-	B.h.	B.h.	B.h.	NT
120	B.h.	-	B.h.	B.h.	B.h.	NT
134	B.h.	-	-	-	B.h.	NT
187	B.c.	-	-	-	-	NT

B.c.: *Bartonella clarridgeiae*; B.h.: *Bartonella henselae*; *gltA*: citrate synthase gene; *groEL*: heat shock protein gene; ITS: intergenic transcribed spacer; NT: not tested due to insufficient genomic material; *pap31*: bacteriophage-associated heme-binding protein gene; *ribC*: riboflavin synthase gene; *rpoB*: RNA polymerase beta subunit gene.

We deposited several partial *B. henselae* Brazil-1 sequences into the GenBank database, including HQ012581 (ITS), HQ012584 (rpoB), HQ012582 (pap31), HQ012583 (ribC), HQ012580 (gltA) and HQ704721 (groEL). Additionally, several *B. clarridgeiae* sequences were deposited, including HQ012586 (rpoB), HQ012585 (ribC) and HQ704720 (groEL).

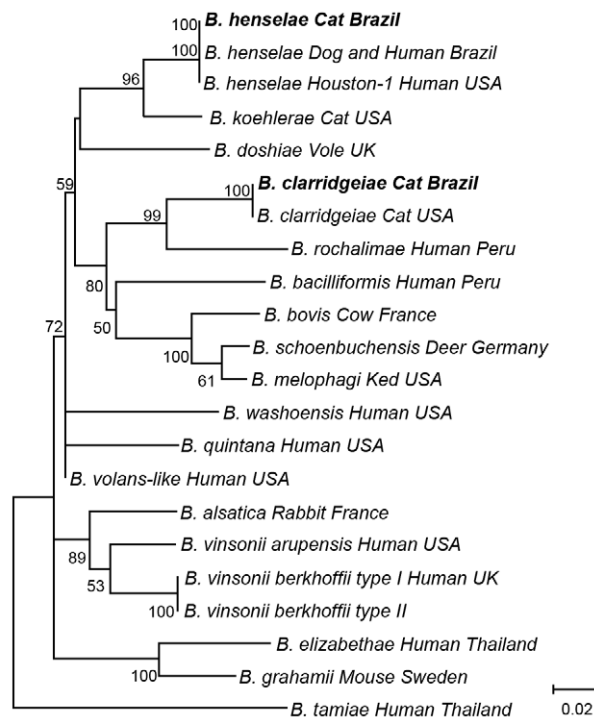
DISCUSSION

The ITS and *rpoB* sequences obtained from three cats infected with *B. henselae* were 100% homologous (ITS = 546/546 bp, rpoB = 585/585 bp) to sequences of the *B. henselae* Brazil-1 strains previously detected from a 36-year-old, HIV antibody-positive Brazilian male with severe anaemia, panseritis, ascitis and cryptogenic hepatitis (Velho et al. 2007) and from a dog in Brazil with fever, severe anaemia, thrombocytopenia and myocarditis (Diniz et al. 2007a). In addition, the partial *pap31* sequence obtained from these three cats was 100% homologous (490/490 bp) to the *B. henselae* Brazil-1 strain detected from the sick dog (Diniz et al. 2007a), but could not be compared to the human case because *pap31* sequences were not obtained in that reported case (Velho et al. 2007). This is the first description of this *B. henselae* strain infecting cats in Brazil and these results may represent an important aspect of the epidemiology of this organism. The entire genome sequence of the *B. henselae* Brazil-1 strain is not available, but based on the phylogenetic analysis of the four genes (Figure), this strain is positioned in the same clade as other *B. henselae* isolates. Because the cats evaluated in this study are located in a distinct region of Brazil, approximately 2.880 km (1.789 miles) from the region in which the human and canine cases were originally reported, the *B. henselae* Brazil-1 strain may represent the endogenous *B. henselae* strain of Brazil. A recent study in the Southern region of Brazil detected two *B. henselae*-infected cats with *ribC* sequences similar to the Brazil-1 strain (HM588661 and HM588662); however, other genes were not amplified and sequenced (Staggemeier et al. 2010). Our results expand the molecular characterisation of the *B. henselae* Brazil-1 strain, with partial *ribC* (536 bp), *gltA* (915 bp) and *groEL* (565 bp) gene sequences that were not available for the human or canine cases previously reported. These new DNA sequences provide important data for future multilocus sequence analysis and phylogenetic studies of *B. henselae* strains.

Three cats were infected with *B. clarridgeiae* and the sequences of the *rpoB*, *ribC* and *groEL* genes from two of these cats revealed that this species was 100% homologous to a strain of *B. clarridgeiae* (ATCC 51734) that was isolated from a kitten in Texas, USA (Clarridge et al. 1995). Moreover, it was also similar to a strain of *B. clarridgeiae* (CIP 104772) that was isolated from a cat in Strasbourg, France (Lawson & Collins 1996). Multiple sequence alignments of the *ribC* gene demonstrated that these two cats from the north-eastern region of Brazil were infected with the same strain recently detected from one of three cats infected with *B. clarridgeiae* in the southern region of Brazil (HM588660) (Staggemeier et al. 2010). In addition, unpublished DNA sequences de-

posited in GenBank indicate that the same sequence was identified in China (EU571943 and EU836705), although the host species was not indicated.

Fleas are responsible for the transmission of *B. henselae* and *B. clarridgeiae* among cats (Chomel et al. 1996, Foil et al. 1998). Thirty percent of the cats sampled in this study were infested with *C. felis* (including those positive for *Bartonella* spp by PCR), but were apparently healthy at the moment of blood collection. An increasing number of arthropod vectors, including keds, lice, biting flies, sandflies and ticks, have been observed or suspected to be associated with the transmission of *Bartonella* spp among animal populations (Breitschwerdt et al. 2010a). Although numerous molecular surveys have detected *Bartonella* DNA in ticks, predominantly in dogs and humans, there is little evidence that *Bartonella* spp can replicate within ticks and no definitive evidence of transmission by a tick to a vertebrate host (Angelakis et al. 2010). Although vertical transmission of *Bartonella* spp among natural rodent hosts has been confirmed (Kosoy et al. 1998), *B. henselae* is not transmitted transplacentally, via colostrum or milk, or venereally, among



Phylogenetic tree based on concatenated partial sequences of intergenic transcriber spacer, RNA polymerase beta subunit gene, riboflavin synthase gene and citrate synthase gene of *Bartonella henselae* and *Bartonella clarridgeiae* detected from cats in Brazil (herein indicated in boldface) compared to other *Bartonella* species described infecting human beings by using maximum likelihood method based on the Kimura two-parameter model. The number at nodes indicates percentages of bootstrap support based on 1,000 replicates. Percentages corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Scale bar indicates 0.02 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA4 software (Tamura et al. 2007).

cats (Abbott et al. 1997, Guptill et al. 1998). Blood transfusion is another route of *Bartonella* spp transmission among cats (Kordick et al. 1999).

Cats may be more likely to exhibit clinical symptoms upon infection with a non-reservoir-adapted *Bartonella* species (Breitschwerdt et al. 2010a). *Bartonella* spp induces asymptomatic infection in preferred hosts, behaving like a stealth pathogen (Kordick & Breitschwerdt 1998). However, *Bartonella* infections in cats have been associated in some cases with fever, lethargy, anorexia, reproductive failure, lymphadenopathy, stomatitis, uveitis, endocarditis and neurological dysfunction (Glaus et al. 1997, Guptill et al. 1998, Lappin & Black 1999, Breitschwerdt et al. 2010a, Dowers et al. 2010).

Several *Bartonella* species are considered zoonotic, including *B. henselae* and *B. clarridgeiae*. Cats are the primary reservoir for *B. henselae* and likely *B. clarridgeiae* and are the transmission vector for human infection. The most frequent infection route to humans is through scratches contaminated with flea faeces (Chomel et al. 1996). Cat bites are a less likely route, as shedding of *B. henselae* in cat saliva has not been clearly documented (Chomel et al. 2006a). Recently, evidence for *B. vinsonii* subsp. *berkhoffii* and *B. henselae* transmission to children *in utero* or during caesarean section has been reported (Breitschwerdt et al. 2010b). In addition, the ability of *B. henselae* to adhere and invade mature human erythrocytes (Pitassi et al. 2007) and remain viable in red blood cells after 35 days of storage at 4°C (Magalhães et al. 2008) underscore the possibility that these organisms may be transmitted by blood transfusion (Magalhães et al. 2009, Velho 2009). Although immunocompromised patients may present with a systemic and fatal disease, immunocompetent individuals infected with *Bartonella* spp may develop non-specific and usually non-life-threatening clinical symptoms that have a significant impact on quality of life (Breitschwerdt et al. 2008, 2010a, b, Kaiser et al. 2011, Kalogeropoulos et al. 2011). Epidemiologic data on human bartonellosis in Brazil is very limited. Among 457 healthy adults in the countryside of MG, 13.7% and 12.8% harboured *B. henselae* and *B. quintana* (cut-off titre of 64), respectively (da Costa et al. 2005). Another study in a semi-rural area of RJ detected *Bartonella* spp seroprevalences of 38.4% and 34.4% (cut-off titre of 20) among 125 asymptomatic HIV-positive patients and 125 blood donors (Lamas et al. 2010). In this study, contact with cats was identified as a risk factor for *Bartonella* exposure. Clinical cases associated with high IgG titres against *Bartonella* spp have been reported in Brazil, including two fatal cases of culture-negative endocarditis (Siciliano et al. 2006) and one asymptomatic case (Lamas et al. 2007). Additionally, a 40-year-old man tested seropositive for *Bartonella* spp, likely from a cat scratch, which was complicated with aseptic meningitis and neuroretinitis (Pinto Júnior et al. 2008). Ophthalmic findings, including retinal infiltrates, angiomatous lesions, neuroretinitis and visual loss, were associated with general symptoms (fever, lymphadenopathy, spleen and liver enlargement and rash) in cat scratch disease cases diagnosed in human patients who were seropositive for *Bartonella* spp (Curi et al. 2010).

The percentage of *Bartonella* spp-positive cats identified by PCR in the present study was lower than that in other regions of Brazil. For example, a molecular study of cats from RS observed that 17% of 47 feral cats were infected with *Bartonella* spp; five (10.6%) were infected with *B. henselae* and three (6.4%) were infected with *B. clarridgeiae* (Staggemeier et al. 2010). Most cats from an animal shelter in city of Vassouras, RJ were infected (97.3% PCR-positive) (Souza et al. 2010). Recently, *B. henselae* DNA and antibodies to *Bartonella* spp were detected in 42.5% and 47.5% of cats, respectively, undergoing neutering or spaying in RJ (Crissiuma et al. 2011).

Further detection and genetic sequencing of *Bartonella* species from multiple areas in Brazil is needed to more precisely characterise their geographic distribution, prevalence, new hosts and reservoirs and zoonotic impact.

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