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Original article

Canine bartonellosis: serological and molecular prevalence in Brazil and evidence of co-infection with *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii*

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Abstract – The purpose of this study was to determine the serological and molecular prevalence of *Bartonella* spp. infection in a sick dog population from Brazil. At the São Paulo State University Veterinary Teaching Hospital in Botucatu, 198 consecutive dogs with clinicopathological abnormalities consistent with tick-borne infections were sampled. Antibodies to *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii* were detected in 2.0% (4/197) and 1.5% (3/197) of the dogs, respectively. Using 16S-23S rRNA intergenic transcribed spacer (ITS) primers, *Bartonella* DNA was amplified from only 1/198 blood samples. *Bartonella* seroreactive and/or PCR positive blood samples (n = 8) were inoculated into a liquid pre-enrichment growth medium (BAPGM) and subsequently sub-inoculated onto BAPGM/blood-agar plates. PCR targeting the ITS region, pap31 and rpoB genes amplified *B. henselae* from the blood and/or isolates of the PCR positive dog (ITS: DQ346666; pap31 gene: DQ351240; rpoB: EF196806). *B. henselae* and *B. vinsonii* subsp. *berkhoffii* (pap31: DQ906160; rpoB: EF196805) co-infection was found in one of the *B. vinsonii* subsp. *berkhoffii* seroreactive dogs. We conclude that dogs in this study population were infrequently exposed to or infected with a *Bartonella* species. The *B. henselae* and *B. vinsonii* subsp. *berkhoffii* strains identified in this study are genetically similar to strains isolated from septicemic cats, dogs, coyotes and human beings from other parts of the world. To our knowledge, these isolates provide the first Brazilian DNA sequences from these *Bartonella* species and the first evidence of *Bartonella* co-infection in dogs.

dogs / *Bartonella* infections / heart disease / culture / Brazil

1. INTRODUCTION

Bartonella are fastidious hemotropic bacteria, which cause long-lasting bacteremia in mammals and are transmitted by animal bites and scratches or by many different vectors including sandflies, body lice, fleas and potentially ticks. The genus *Bartonella* contains numerous recently described species, many

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of which are new and emerging human pathogens [8, 12, 16, 24].

In dogs, infection with five *Bartonella* species has been described: *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* (hereafter *B. v. berkhoffii*) DNA was detected in aortic valves of six dogs with vegetative endocarditis, based upon independent studies [9, 14, 32]; *B. elizabethae* DNA was detected in a dog with weight loss and sudden death [37]; *B. henselae* DNA was sequenced from a dog with peliosis hepatis and from a dog with granulomatous hepatitis [22]; and *B. washoensis* was isolated from a dog with mitral valve endocarditis [15]. A potentially new species closely related to *B. clarridgeiae* (proposed name *B. rochalimaea*) has been isolated from three dogs and from 22 gray foxes in California [32][1].

To date, no studies have reported serological or molecular evidence of *Bartonella* infection in dogs from Brazil or other South American countries. With the notable exception of *B. bacilliformis,* there are only a limited number of reports describing human infection with other *Bartonella* species in South America. In Brazil, *B. quintana* was detected by PCR in an infant [3] and two fatal cases of endocarditis were associated with high *B. henselae* antibody titers [41]. In contrast to humans, *B. henselae* seroreactivity was reported in 46% of 102 cats[2] suggesting that exposure to this organism in Brazil may be more prevalent than was previously recognized.

The objective of this study was to determine the serological and molecular prevalence of *Bartonella* infection in a subset of the sick dog population examined at a Veterinary Teaching Hospital in southeastern Brazil. Successful culture and molecular characterization of *B. henselae* and *B. v. berkhoffii* from two clinically ill dogs is also reported, including the first evidence of simultaneous infection with these two organisms in the same host.

2. MATERIALS AND METHODS

2.1. Study population

One hundred and ninety-eight EDTA-anticoagulated blood and serum samples from dogs with historical, clinical and/or laboratory findings consistent with tick-borne diseases were prospectively selected at a Veterinary Teaching Hospital at the College of Veterinary Medicine and Animal Husbandry (FMVZ – Unesp) in Botucatu, southeast Brazil (latitude 22° 53′ 09″ S, longitude 48° 26′ 42″ W) between October 2002 and November 2003. Dogs included in this study had at least three of the following clinical or laboratory criteria: presence of tick infestation at the time of examination, bleeding disorders (epistaxis, melena, ecchymosis, bleeding gums), neurological signs, inflammatory ocular disease (uveitis, chorioretinitis, hyphema), fever (rectal temperature > 39.4 °C), anemia (PCV < 35%), leukopenia (WBC < 6 000 cells/μL), thrombocytopenia (platelets < 150 000 cells/μL) and hyperproteinemia (total protein > 7.8 g/dL). Dogs treated with tetracycline or imidocarb dipropionate during the previous 30 days were excluded from the study population. This study was approved by the Medical Ethical and Animal Care Committee of São Paulo State University. In

[1] Henn J.B., Koehler J.E., Gabriel M., Kasten R.W., Brown R.N., Papageorgiou S., Chomel B.B., Zoonotic *Bartonella* spp. in domestic dogs and gray foxes from California, in: Proc. 20th Meeting of the American Society for Rickettsiology and 5th International Conference on *Bartonella* as Emerging Pathogens, Pacific Grove, 2006, pp. 70.

[2] Slhessarenko N., Camargo M.C.G.O., D'Auria S.R.N., Soroprevalência de *Bartonella henselae* em gatos do municipio de São Paulo, Rev. Soc. Bras. Med. Trop. (1996) 29:104 (Abstract).

addition, each owner authorized blood collection and enrollment of the dog into the study.

From the selected dogs, two had *Bartonella* species detected by culture (techniques described below). Dog #1 was a 6.3 kg 2-year-old male Poodle that was examined because of anorexia, bleeding gums, cutaneous petechiae, and ecchymoses. The dog was febrile (39.7 °C) and infested with ticks. Hematological abnormalities included severe anemia (PCV = 14%; normal ⩾ 39%), leukopenia (2 692/μL; normal ⩾ 6 000/μL), thrombocytopenia (2 500/μL; normal ⩾ 190 000/μL), and hypoproteinemia (4.6 g/dL; normal ⩾ 6.1 g/dL). Cardiovascular abnormalities included hypotension (systolic blood pressure = 75 mmHg; normal ⩾ 90 mmHg), myocardial dysfunction (fractional shortening = 24%; normal ⩾ 30%) and increased serum cTnI concentration (3.05 ng/mL; normal ⩽ 0.11 ng/μL). Electrocardiographic abnormalities were not detected. *B. henselae* was isolated from the EDTA-anticoagulated blood sample. By PCR testing, this dog was found to be co-infected with *Ehrlichia canis*, but was PCR negative for *Anaplasma platys, Anaplasma phagocytophilum, Ehrlichia chaffeensis, Ehrlichia ewingii* and *Rickettsia* spp. DNA. Antibodies to *B. burgdorferi, L. chagasi*, *L. interrogans, R. rickettsii* and *D. immitis* antigens were not detected.

Dog #2, a 34 kg 6-year-old male German Shepherd, was determined to be co-infected with *B. henselae, B. v. berkhoffii* and *E. canis*. This dog was examined due to bilateral epistaxis and had history of tick attachment. The dog was febrile (42 °C) at the time of examination. Hematological abnormalities included mild anemia (PCV = 34%), monocytosis (1936/μL, normal ⩽ 1100/μL), thrombocytopenia (50 500/μL) and hyperproteinemia (11.6 g/dL, normal ⩽ 7.8 g/dL). ECG abnormalities were not detected and blood pressure was normal; however, echocardiography identified an increased left ventricle wall (14.5 mm) thickness resulting in reduction of left ventricular chamber size (33 mm) during diastole. All other infectious disease diagnostic tests described above for Dog #1 were also negative for Dog #2.

2.2. Serology-based assays

The microimmunofluorescence test (IFA) for the detection of anti-*B. v. berkhoffii* (isolate 93-CO-1, ATCC #51672) and anti-*B. henselae* (strain Houston-1, ATCC #49882) antibodies in canine sera was performed as described [30]. The starting dilution was at 1:16 and the cut-off for seroreactivity was defined at 1:64. Endpoint titers were determined as the last dilution at which brightly stained bacteria could be detected on a fluorescence microscope. Exposure to *Leishmania chagasi* and *Rickettsia rickettsii* was evaluated by IFA as described [42, 45]. The cut-off dilution for seroreactivity was defined at 1:64 for *R. rickettsii*, and 1:80 for *L. chagasi*. An ELISA-based test kit (SNAP 4Dx, IDEXX Laboratories, Inc., Westbrook, ME, USA) was used according to the manufacturer's instructions to detect *Borrelia burgdorferi* antibodies, as well as antigens of *Dirofilaria immitis* from dog serum. A microagglutination test for *Leptospira interrogans* with a cut-off dilution at 1:100 was performed as described [51]. One dog was not tested because of insufficient serum.

2.3. DNA extraction

DNA was extracted from 300 μL of each dog's frozen EDTA-blood pellet using a commercially available GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Piscataway, NJ, USA).

The final eluted volume was 200 μL per sample. The absence of PCR inhibitors was demonstrated by the amplification of a fragment of the constitutive gene for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein, an enzyme related to the glycolysis pathway that is expressed in all mammal cells [5, 7].

2.4. DNA amplification-based assays

2.4.1. Bartonella *16S-23S ribosomal RNA intergenic spacer (ITS) PCR amplification*

Bartonella genus screening was performed as described previously [34]. Amplification was performed in a 25 μL final volume reaction containing 2mM $MgCl_2$, 0.625 units of Taq DNA polymerase (Takara Ex Taq, Takara Bio Inc, Shiga, Japan), 12.5 pmol of each primer (325s 5'-CTT CAG ATG ATG ATC CCA AGC CTT YTG GCG -3′ and 1100as 5′-GAA CCG ACG ACC CCC TGC TTG CAA AGC A-3′), 200 μM (each) dATP, dTTP, dCTP, and dGTP (Fisher Scientific, Pittsburgh, PA, USA) and 1 to 5 μL of DNA template, according to DNA concentration determined for each sample (5 to 200 ng/reaction). *B. v. berkhoffii* identity was established using specific ITS primers (46s: 5′- CCT CAT TCT TTA AAA AAA GAG GGC TTT TTA AG-3′ and 590as: GAA AGC GCT AAC CCC TAA ACC GAT T-3′) and the same PCR conditions. PCR was performed in a Mastercycler EP (Eppendorf, Hamburg, Germany) under the following conditions: a single hotstart cycle at 95 °C for 2 min followed by 55 cycles of denaturing at 94 °C for 15 s, annealing at 66 °C for 15 s, and extension at 72 °C for 15 s. Amplification was completed by an additional cycle at 72 °C for 1 min, and products were analyzed by 2% agarose gel electrophoresis under UV exposure. DNA from a healthy, specific pathogen-free dog was used as a PCR negative control. Plasmid clones of partial sequences of 16-23S ITS region of *B. henselae* Houston-1 (L35101) and *B. v. berkhoffii* type I (AF167988) were used as positive controls. The detection limit observed in 100% of 10 replicate reactions was 2.5 DNA copies of *B. henselae* and 5 copies of *B. v. berkhoffii* per reaction.

2.4.2. Bartonella *heme-binding protein gene (pap31) PCR amplification*

ITS region PCR positive dogs were subsequently tested for the pap31 bacteriophage associated gene [33]. Oligonucleotides for *Bartonella* genus 1s 5′-ACT TCT GTT ATC GCT TTG ATT TCR RCT-3′ and 688as 5′-CAC CAC CAG CAA AAT AAG GCA TMA Y-3′ were used at the same concentration and amplification conditions as described above, where the annealing temperature was 58 °C. *B. henselae* identity was established using specific pap31 forward primer (pap31 Bhs: 5′-TAA GGT TGA AAT AAC TGA TCC GAA T-3′) in conjunction with the 688as reverse primer. Plasmid clones of partial sequences of pap31 gene of *B. henselae* Houston-1 (BX897699) and *B. v. berkhoffii* type I (AY663045) was used as a positive control. The detection limit observed in 100% of 10 replicate reactions was 5 copies per reaction for both organisms.

2.4.3. Bartonella *RNA polymerase beta subunit (rpoB) gene PCR amplification*

ITS region PCR positive dogs were subsequently tested for the rpoB gene. The following oligonucleotides were manually designed for *Bartonella* genus amplification: rpoB 1615s 5′-ATY ACY CAT AAR CGY CGT CTT TCT GCT CTT GG-3′ and rpoB 2267as 5′-GGA TCT AAA

TCT TCY GTY GCA CGR ATA CG-3′. The PCR conditions were similar as described above, with annealing temperature at 62 °C. As positive controls, DNA extracted from isolates of *B. henselae* Houston-1 (BX897699) and *B. v. berkhoffii* type I (ATCC strain 93-CO-1) were used as genomic equivalent (GE) serially diluted in DNA obtained from a specific pathogen-free dog. The detection limit for both organisms was 2.5 GE per reaction.

2.4.4. Other tick-borne pathogen DNA amplification-based assays

Because of the risk of polymicrobial infection, other tick-borne pathogens were evaluated. *Anaplasma* and *Ehrlichia* 16S rRNA gene was targeted by conventional PCR [10, 30] with a detection limit of 10 copies per reaction in 100% of 10 replicates. *Rickettsia* spotted-fever species were screened by real-time PCR targeting the ompA gene [44] with a detection limit of 5 copies per reaction in 96% of 22 replicates.

2.5. *Bartonella* species culture

Two hundred microliters of blood from *Bartonella* ITS region-PCR positive dogs and/or IFA seroreactive dogs were inoculated into liquid *Bartonella* alpha-Proteobacteria growth medium (BAPGM) [35] and incubated at 35 °C in 5% CO_2, water-saturated atmosphere. After a 14-day incubation period, 1 mL from liquid culture was sub-inoculated onto BAPGM/blood-agar plates and incubated for 14 days. For maximization of *Bartonella* DNA recovery, multiple bacterial colonies were collectively swabbed from the surface of each BAPGM/blood-agar plate, resuspended in sucrose-phosphate-glutamate (SPG) buffer and stored at −80 °C until processed for DNA extraction. DNA from 200 mL of BAPGM liquid culture and from frozen SPG bacterial colonies was extracted using a commercially available MagAttract DNA blood kit (BioRobot M48, Qiagen, Chatsworth, CA, USA).

2.6. Cloning and sequencing of ITS region and pap31 gene amplicons

PCR products obtained from EDTA-anti-coagulated blood, BAPGM liquid culture and/or the plate colonies were cloned into plasmid pGEM-T Easy Vector System (Promega, Madison, WI, USA) and the recombinants selected based on white/blue screening, EcoR I digestion and 2% agarose gel electrophoresis. After plasmid purification using commercial kit (QIAprep Spin Miniprep, Qiagen, Valencia, CA, USA), clones were sequenced by Davis Sequencing (Davis, CA, USA). Chromatogram evaluation and sequence alignment were performed using ContigExpress and AlignX softwares (Vector NTI Suite 10.1, Invitrogen Corp., Carlsbad, CA, USA). Bacteria species and strain were defined by comparing similarities with other sequences deposited in the GenBank database prior to April 2007 using the Basic Local Alignment Search Tool (Blast version 2.0) [1].

2.7. Cardiac evaluation

Bartonella infected and/or seroreactive dogs were evaluated for cardiac disturbances. Non-invasive blood pressure was established using a Doppler ultrasonographic method (DV-10, Microem, Brazil) [46]. An electrocardiogram (ECG) was recorded with a 12-lead computer-based system (ECG-PC, TEB, Brazil) [49]. Echocardiography was performed with a 5 MHz electronic ultrasound probe (EUB-405, Hitachi, Norcross, GA, USA) [13].

Table I. Serology and PCR results from 8 seroreactive or PCR positive sick dogs enrolled in this study. *Bartonella* titers ⩾ 64 were considered seroreactive.

Case #	IFA reciprocal titer		*Bartonella* genus ITS region PCR		
	B. henselae	*B. v. berkhoffii*	Blood	Liquid culture	Plate colonies
1	< 16	< 16	+	+	+*
2	32	64	-	-	+*
3	2048	< 16	-	-	-
4	256	< 16	-	-	-
5	256	< 16	-	-	-
6	128	< 16	-	-	-
7	< 16	64	-	-	-
8	< 16	64	-	-	-

* By DNA sequencing, Dog #1 was infected with *B. henselae* and Dog #2 was co-infected with *B. henselae* and *B. vinsonii* subsp. *berkhoffii*.

Serum cardiac troponin I (cTnI) was measured using human cTnI kits (Dimension RxL clinical chemistry analyzer, Dade Behring, Deerfield, IL, USA), previously validated for testing dog serum [38].

3. RESULTS

3.1. Serological and molecular prevalence

B. henselae and *B. v. berkhoffii* antibodies were detected in 2.0% (4/197) and 1.5% (3/197) of the 198 dogs in this study, respectively (Tab. I). ITS region amplification for *Bartonella* genus was not obtained from the blood of any of the 7 *Bartonella* seroreactive dogs. However, *B. henselae* DNA was detected from 1/191 non-seroreactive dog blood samples (Dog #1, Tab. I). After blood culture (described in detail below), one additional dog (#2) was detected as co-infected with *B. henselae* and *B. v. berkhoffii*. This dog had a reciprocal titer of 64 for *B. v. berkhoffii* but no *Bartonella* DNA was amplified from the blood sample. Based upon PCR testing of blood and blood culture of seroreactive dogs, the *B. henselae* and *B. v. berkhoffii* molecular prevalence in this population was 1% (2/198) and 0.5% (1/198), respectively.

Six of seven *Bartonella* seroreactive dogs detected in this study were co-infected with *E. canis*. Dog #1, *Bartonella* PCR positive but seronegative, also was infected with *E. canis*. The only *E. canis* PCR negative dog (#5, Tab. I) was co-infected with *A. platys*, and had a reciprocal *B. henselae* titer of 256. DNA of *A. phagocytophilum*, *E. chaffeensis, E. ewingii*, *Rickettsia* spp. or exposure to *B. burgdorferi, L. chagasi*, *L. interrogans, R. rickettsii* or antigens of *D. immitis* were not detected in any *Bartonella* seroreactive or PCR positive dogs.

3.2. Culture and molecular characterization of *B. henselae* and *B. vinsonii* subsp. *berkhoffii*

From the only PCR-positive blood sample (Dog #1), several colonies were obtained from the BAPGM/blood-agar culture, from which DNA was collectively extracted. The ITS region and pap31 gene amplicons from these colonies were of

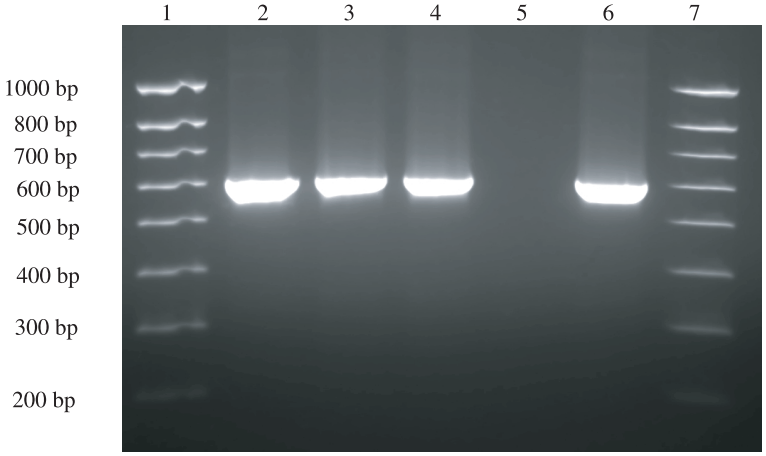

Figure 1. PCR results of 16S-23S rRNA intergenic spacer region for *Bartonella* genus from Dog #1. Lane 2, blood sample; lane 3, BAPGM liquid pre-enrichment medium; lane 4, BAPGM/blood-agar plate colonies; lane 5, negative dog DNA; lane 6, *B. henselae* positive control; lanes 1 and 7, 1-Kb DNA ladder.

the expected size for *B. henselae* (Fig. 1). DNA sequences from blood, BAPGM liquid culture, and the plate isolates were 100% homologous. Sequences were deposited into the GenBank database under accession numbers DQ346666 (ITS) and DQ351240 (pap31 gene).

Blood from each of the seven *Bartonella* seroreactive dogs was also cultured, and from a second patient (Dog #2) several colonies were obtained. After extracting DNA of these colonies conjointly, ITS region PCR amplicons were 100% homologous (604/604 bp) with *B. henselae* strain Houston-1 (BX897699). However, cloned pap31 sequences derived from the pooled colonies were 99.5% homologous (545/548 bp) with *B. v. berkhoffii* type I (Tab. II). This sequence was deposited under accession number DQ906160. The co-infection was confirmed using species-specific primers targeting the *B. henselae* pap31 gene and the *B. v. berkhoffii* ITS region. Consensus of multiple sequences of pap31 gene was 100% homologous (398/398 bp) to *B. henselae* Houston-1 (Tab. III) and of consensus of ITS region was 99.8% homologous (553/552 bp) to *B. v. berkhoffii* type I (AF167988). Dog #2 was *B. v. berkhoffii* seroreactive (reciprocal titer 64), but was not *B. henselae* seroreactive.

PCR for rpoB gene was positive only from blood from Dog #1 and agar plate colonies from Dog #2. Alignment of multiple sequences confirmed previous results: Dog #1 was infected with *B. henselae* Houston-1 (655/656 bp – 99.8% homologous to BX897699) and Dog #2 was infected with *B. v. berkhoffii* (656/656 bp – 100% homologous to AF165989). *B. henselae* and *B. v. berkhoffii* rpoB sequences were deposited under accession numbers EF196806 and EF196805, respectively.

3.3. Cardiac evaluation

In addition to the two cases reported in detail above, four other *Bartonella* spp. seroreactive dogs had evidence of cardiac disease. Dogs #3 and #4 (Tab. I) were seroreactive to *B. henselae*. Dog #3

Table II. *Bartonella vinsonii* subsp. *berkhoffii* heme-binding protein gene (pap31) nucleotide differences of the sequence from this study (Iso-756) compared to GenBank database sequences available on April 2007.

B. vinsonii berkhoffii (Bvb)	Nucleotide difference at position[a]															Accession number
	32	55-56	70	97	173	245	251	268	286	316	385	417	435	501	536	
Iso-756	G	CG	A	G	A	A	G	A	A	C	C	A	C	T	G	DQ906160
Coyote (Y-12)	•	••	•	•	•	•	•	•	•	T	•	•	•	•	•	[35]
Dog - Bvb Type I	•	•A	•	•	•	•	•	•	•	T	•	•	•	•	T	AY663045
Dog - Bvb Type II	A	••	•	•	•	•	A	•	•	T	T	G	T	•	•	DQ059762
Fox - Bvb Type III	•	A•	T	A	•	G	A	T	•	T	•	•	•	•	•	DQ071677
Dog - Bvb Type IV	A	••	•	•	G	•	A	•	G	T	T	G	T	C	•	DQ112677

[a] The number represents the nucleotide position of *B. vinsonii* subsp. *berkhoffii* Iso-756; • same base compared with Brazilian isolate.

had diastolic left ventricular enlargement and increased cTnI of 0.28 ng/mL (normal ⩽ 0.11 ng/mL). Dog #4 had increased cTnI (0.51 ng/mL). Dogs #7 and #8 were *B. v. berkhoffii* seroreactive (Tab. I) and, despite cTnI levels within normal range, Dog #7 had evidence of myocardial dysfunction (fractional shortening = 22.3%; normal ⩾ 30%) and Dog #8 had left ventricle enlargement. No dog had echocardiographic findings consistent with infective endocarditis or was hypertensive. In total, 6 of 8 IFA and/or PCR *Bartonella* spp. positive dogs had cardiac abnormalities.

4. DISCUSSION

To our knowledge, this study provides the first data relative to *B. henselae* and *B. v. berkhoffii* serological and molecular prevalence in dogs from Brazil. In addition, this is the first report of *Bartonella* species isolation from dogs from South America and the first documentation of co-infection with two *Bartonella* species in a dog. In 2004, *B. henselae* DNA was reported for the first time in a blood culture isolate obtained from a dog from Gabon [23]; however, no information about amplicon size, strain and genetic sequence was provided. The *Bartonella* seroprevalences detected in this study were lower than expected for a country with tropical and sub-tropical climates. *B. henselae* seroreactivity was detected from 3.0% (3/100) of dogs in the United Kingdom [6], 7.7% (4/52) of dogs in Japan [50] and 27.2% (82/301) of sick dogs in North Carolina [43]. *Bartonella vinsonii* subsp. *berkhoffii* antibodies were detected in 10% (4/40) of dogs from Israel [4] and in 38% (19/49) of dogs from Thailand [48]. Bartonellosis may predispose the host to insidious nonspecific manifestations; therefore, we can not exclude a selection bias, based upon narrowly defined entry criteria of this study.

Cats are the main reservoir of *Bartonella* spp. infection for humans [16]. However, based upon serological evidence, human cases of bartonellosis have also implicated dogs as a source of *B. henselae* infection [28, 52]. *B. v. berkhoffii* has been detected by PCR in dogs, coyotes and foxes [29, 36]; however, the extent to which dogs contribute to the transmission of *Bartonella* to humans is unknown [24]. In Brazil, *Rhipicephalus sanguineus,* the most prevalent tick that infests dogs [18] also parasitizes humans [17, 21]. Epidemiological evidences suggest that *R. sanguineus* may be involved in the transmission of *Bartonella* species in dogs [39].

Table III. *Bartonella henselae* heme-binding protein gene (pap31) nucleotide differences of the strains Brazil-1 and Brazil-2 compared to GenBank database sequences available on April 2007.

B. henselae	Nucleotide difference at position[a]																					Accession number
	16	81	244-245	325	329	334	337	350	353-354	359	375	386	390	394	398	407-408	412	418-419	421-422	425-426	429	
Brazil-1	G	G	GA	A	T	A	G	T	AC	A	C	T	C	A	A	TG	G	AA	CA	AT	A	DQ351240
Fizz	S	•	••	•	•	•	•	•	••	•	•	•	•	•	•	••	•	••	••	••	•	AF308167
CAL-1	/	•	••	•	•	•	•	•	••	•	•	•	•	•	•	••	•	••	••	••	•	AF308166
Marseille	C	•	••	•	•	•	•	C	••	•	•	•	T	•	•	••	•	••	••	••	•	AF308169
U4	•	•	AG	•	•	•	•	C	••	•	•	•	T	•	•	••	•	••	••	••	•	AB091503
URLIE 9	•	•	AG	•	•	•	•	C	••	•	•	•	T	•	•	GT	•	••	••	••	•	AF308170
Brazil-2	/	/	••	G	C	G	C	A	CA	G	A	C	T	C	G	GT	A	GC	GC	TG	T	*
Houston-1	•	C	••	G	C	G	C	A	CA	G	A	C	T	C	G	GT	A	GC	GC	TG	T	BX897699
San Ant 2	•	C	••	G	C	G	C	A	CA	G	A	C	T	C	G	GT	A	GC	GC	TG	T	AF308168
90-615	•	C	••	G	C	G	C	A	CA	G	A	C	T	C	G	GT	A	GC	GC	TG	T	AF308165
Dolphin	•	C	••	G	C	G	C	A	CA	G	A	C	T	C	G	GT	A	GC	GC	TG	T	DQ529248

[a] The number represents the nucleotide position of *B. henselae* Brazil-1; • same base as the Brazilian isolate; / sequence not available; S, degenerate oligonucleotide (C or G).

* Sequence not deposited at GenBank database because it is 100% homologous to other sequences.

Table IV. *Bartonella henselae* 16S-23S ribosomal RNA intergenic spacer (ITS) nucleotide differences for the strains Brazil-1 and Brazil-2 compared to GenBank database sequences available on April 2007.

B. henselae	Nucleotide difference at position[a]										Accession
	1	6	7	12	20	61	82	253-285	322	542	number
Brazil-1	A	G	–	A	C	–	G	–	A	T	DQ346666
CAL-1	•	•	–	•	•	–	•	–	•	•	AF369527
URBHLLY 8	•	•	–	•	•	–	•	–	•	C	AF312495
URBHLIE 9	•	•	–	•	•	–	•	–	•	C	AF312496
Brazil-2	•	•	–	•	•	–	C	–	•	C	*
Houston-1	•	•	–	•	•	–	C	–	•	C	L35101
90-615	•	•	–	C	•	–	•	–	C	C	AF369528
Fizz	W	A	T	•	•	C	•	–	•	•	AF369526
San Ant 2	•	•	–	•	–	–	•	Insertion	•	C	AF369529
From Dolphin	•	•	–	•	•	–	•	Insertion	•	C	DQ529247

[a] The number represents the nucleotide position of *B. henselae* Brazil-1; • same base as the Brazilian isolate; –, deletion; W, degenerate oligonucleotide (A or T); insertion consisting of: ATTGCTTC-TAAAAAGATTGCTTCTAAAAAG.

* Sequence not deposited in GenBank because homology was 100% with *B. henselae* Houston-1.

Thus, this tick potentially could pose a risk for human infection.

Dog #1 in this study, co-infected with *B. henselae* and *E. canis,* had a clinical diagnosis compatible with myocarditis, based upon cardiac dysfunction and high cTnI level, a cardiac biomarker highly specific and sensitive for myocardial injury [38]. Moreover, 6 of 8 (75%) *Bartonella* seroreactive or PCR positive dogs had evidence of cardiac disease. Myocardial injury has been reported in infective endocarditis in dogs [11, 32] and in human bartonellosis [25, 47]. Surprisingly, there was not echocardiographic evidence of endocarditis in any dog in this study. Seven of eight *Bartonella* seropositive or PCR positive dogs were infected with *E. canis*, a tick-transmitted, obligatory intracellular bacterium that is also associated with myocardial injury[3]. Hence, abnormalities detected in this study could have been due to infection with either *E. canis* or *E. canis, B. v. berkhoffii* and *B. henselae* co-infection, and/or due to other undetermined factors.

Three genes were targeted in this study to confirm *Bartonella* co-infection in Dog #2 and to better characterize the *B. henselae* isolate from Dog #1. Partial ITS region sequences obtained from Dog #1 were 100% homologous to *B. henselae* CAL-1 (546 bp/546 bp) (Tab. IV), which was isolated from a septicemic human being in the USA [27]. However, partial pap31 sequences were 99.8% homologous to strain Fizz (514 bp/514 bp) (Tab. III), which was isolated from a bacteremic cat in Switzerland [26]. These two strains (CAL-1 and Fizz) are 98% homologous within the ITS region (971/983 bp) and 100% homologous within pap31 gene. This suggests that *B. henselae* strains CAL-1 and Fizz may belong to the same genetic variant, as suggested previously [2]. The rpoB gene sequences from Dog #1 were 99.8% homologous (655/656 bp) to *B. henselae* Houston-1. Potentially, the discrepancy

[3] Diniz P.P.V.P., De Morais H.S.A., Schwartz D.S., Cardiac troponin I in dogs naturally infected by *Ehrlichia canis*, J. Vet. Intern. Med. (2004) 18:454-455 (Abstract).

among sequenced genes from Dog #1 can be explained by the absence of CAL-1 and Fizz rpoB sequences in the GenBank database and by the high homology of rpoB gene among *Bartonella* species. Sequences of longer length and targeting additional genes would be necessary for a more complete phylogenetic classification of this *B. henselae* isolate [31].

In this study, unanticipated co-infection with *B. henselae* and *B. v. berkhoffii* was successfully documented in a dog for the first time (Dog #2). By independently targeting the ITS region and pap31 gene with species-specific primers, Dog #2 was found to be co-infected with *B. henselae* Houston-1 and *B. v. berkhoffii* type I. The *B. v. berkhoffii* pap31 sequence from Dog #2 differed in 1 bp among 548 bp from a *B. v. berkhoffii* type I isolate (Y-12) obtained from a coyote [36] and 3 bp from *B. v. berkhoffii* type I (strain 93-CO-1, ATCC 51672) isolated from a dog with endocarditis [29]. Targeting a third gene (rpoB), infection with *B. v. berkhoffii* was confirmed (100% homology (656/656 bp) with strain 93-CO-1). Because colonies containing both organisms were unknowingly pooled and extracted together, molecular confirmation of co-infection by targeting multiple genes was required. This observation indicates that dogs, as is well recognized for cats, can be co-infected with more than one *Bartonella* species.

Bartonella PCR and IFA test results were discrepant among many of the dogs in this study (Tab. I). Different antigenic expression among *Bartonella* strains could result in false negative serology results, as previously shown in humans [19] and documented in our laboratory [20]. Gene expression and pathogenicity of *B. henselae* strains are based upon incompletely characterized mechanisms and do not directly correlate with genetic differences [40]. Furthermore, since clinically ill dogs were selected for this study, acute infection cases might have been preferentially included, resulting in PCR positivity prior to development of a detectable humoral immune response, as found in Dog #1.

In conclusion, the low prevalence of amplifiable *Bartonella* DNA in conjunction with low *B. henselae* and *B. v. berkhoffii* seroreactivity detected in this study indicates that these pathogens are uncommonly transmitted to dogs with a high risk of tick infestation in southeastern Brazil. The two species of *Bartonella* detected from sick dogs in Brazil were homologous to other species previously isolated from septicemic human beings, coyotes or dogs from the USA. *Bartonella* co-infection with *E. canis* or *A. platys* might increase disease severity and contribute to myocardial disease.

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