

Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats in the south of Brazil: a molecular study

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Bartonella spp are the causative agent of cat scratch disease in humans. Cats are the natural reservoir of these bacteria and may infect humans through scratches, bites or fleas. Blood samples from 47 cats aged up to 12 months were collected for this study. All animals were lodged in municipal animal shelters in the Vale do Sinos region, Rio Grande do Sul, Brazil. *Bartonella spp* were detected by genus-specific polymerase chain reaction (PCR) and when the PCR was positive, the species were determined by DNA sequencing. A Giemsa-stained blood smear was also examined for the presence of intraerythrocytic elements suggestive of *Bartonella spp* infection. Phylogenetic analysis was also performed for all positive samples. Using molecular detection methods, *Bartonella spp* were detected in 17.02% (8/47) of the samples. In seven out of eight samples confirmed to be positive for *Bartonella spp*, blood smear examination revealed the presence of intraerythrocytic elements suggestive of *Bartonella spp*. Phylogenetic analysis characterized positive samples as *Bartonella henselae* (5) or *Bartonella clarridgeiae* (3). To the best of our knowledge, this is the first molecular study demonstrating the presence of *Bartonella spp* in cats from the Southern Region of Brazil.

Key words: *Bartonella* - cats - cat scratch disease - Rio Grande do Sul

Bartonella spp are small, Gram-negative, pleomorphic, fastidious, hemotropic bacteria that infect humans in addition to domestic and wild mammals (Angulo et al. 1994) and they are considered emergent infectious agents (Spach & Koehler 1998, Dehio & Sander 1999). Human infections by *Bartonella spp* are endemic in some South American countries, such as Peru, Ecuador and Colombia (Maco et al. 2004). Today, more than 23 species are recognized within this genus (Lamas et al. 2008) and, among these, 13 species are related to human diseases (Jacomino et al. 2002), especially *Bartonella bacilliformis*, *Bartonella quintana*, *Bartonella henselae*, *Bartonella elizabethae* (Anderson & Neuman 1997) and *Bartonella clarridgeiae* (Kordick et al. 1997).

Bartonella spp have different arthropod vectors, such as fleas (*Ctenocephalides felis*), lice (*Pediculus humanus corporis*), sandflies (*Lutzomyia verrucarum*, *Lutzomyia peruensis*) (Jacomino et al. 2002) and ticks (*Ixodes pacificus*) (Chang et al. 2001, Cotté et al. 2008). The main animal reservoirs for these bacteria are cats, dogs and rodents (Jacomino et al. 2002) and contact with vectors and cat scratches are the most significant modes of transmission (Carithers 1985).

In both humans and domestic animals, infection with *Bartonella* may be asymptomatic and can resolve spontaneously without any treatment; however, in some cas-

es, the disease may be fatal if left untreated (Jacomino et al. 2002). Historically, the most frequently diagnosed bartonellosis diseases are Carrion's disease (*B. bacilliformis*) (Maguina et al. 2001), trench fever (*B. quintana*) (Spach et al. 1995) and, more recently, cat scratch disease (CSD) (*B. henselae* and *B. clarridgeiae*) (Lamas et al. 2008). *Bartonella spp* infect humans and can cause chronic bacteremia, which may be followed by fever, endocarditis, sepsis and neurological, psychiatric, ophthalmological, bone and hematologic manifestations, among others (Boulouis et al. 2005). The peak incidence of clinical manifestations is seen in children and young adults, aged between 2-14 years old (Anderson & Neuman 1997). The first signs and symptoms of CSD may appear one to two weeks after exposure, though symptoms can take several months to appear. The specific symptoms are directly related to the species involved (Carithers 1985).

B. henselae is known to be the causative agent of CSD, which is characterized by benign chronic lymphadenopathy (Lamas et al. 2008) and, in some cases, Parinaud oculoglandular syndrome, encephalopathy, convulsions, endocarditis, hepatosplenomegaly, glomerulonephritis, pleurisy, mediastinal adenopathy, nodules in the head of the pancreas, osteomyelitis, atypical pneumonia, mammary tumours, haemolytic anaemia and eosinophilic purpura (Boulouis et al. 2005). In addition to CSD, this pathogen can cause bacillary angiomatosis, which is observed mainly in patients with immunodeficiencies (Boulouis et al. 2005). Several studies have demonstrated that cats are the main reservoir of *B. henselae* (Jacomino et al. 2002) and this species has the ability to infect the erythrocytes of felines (Kordick & Breitschwerdt 1995). Transmission of these bacteria to humans occurs through bites or scratches of infected cats or by

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their fleas (*C. felis*) (Chomel 2000). It has been reported that most patients suffering from CSD were scratched or bitten by kittens or cats younger than 12 months of age (Zangwill et al. 1993). Similarly to *B. henselae*, *B. clarridgeiae* has been reported as a possible cause of CSD (Kordick et al. 1997) and it is also found in and transmitted by infected cats (Breitschwerdt & Kordick 2000).

Laboratory diagnosis of bartonellosis may be difficult. The currently available techniques include blood smears (Billeter et al. 2008), culturing (Brenner et al. 1997, La Scola & Raoult 1999), serology (Boulouis et al. 2005) and molecular biology (Jensen et al. 2000). Samples commonly used for analysis are blood, tissue fragments and secretions collected from lesions (Billeter et al. 2008).

The main objective of this study was to investigate the presence of *Bartonella* spp in cats, using molecular analysis of blood samples.

MATERIALS AND METHODS

Blood samples of 47 cats from two municipal animal shelters [Centro Municipal de Proteção aos Animais, Novo Hamburgo (29°40'42"S 51°07'50"W), and Canil Municipal de São Leopoldo (29°45'37"S 51°08'50"W)] were collected during the period from August-October in 2009. The animals' ages varied between 6-12 months old, as determined by the size and the dentition of the cats. Young cats were chosen because infection by *Bartonella* spp is more common in young animals (Zangwill et al. 1993). Blood samples (5 mL) were collected by puncturing the internal jugular vein after application of a combination of xylazine and ketamine (0.2 mg/kg each) as an anaesthetic. Blood samples were transferred to sample tubes containing ethylenediamine tetraacetic acid as the anticoagulant agent. Then, the samples were aliquoted into small plastic vials (500 µL) and then kept frozen at -20°C until needed for the molecular analyses.

Giemsa blood smears - Blood smears and Giemsa staining were performed essentially as described by Bentzel et al. (2008). After smearing and drying, the glass slides were covered with 96% ethyl alcohol for 2 min and then they were stained with Giemsa for 8 min. After staining, the slides were washed briefly with distilled water and then dried at room temperature. The slides were analyzed by optical microscopy (1000X).

Molecular detection - DNA was extracted from 200 µL of total blood using the QIAamp Kit (Qiagen®) as recommended by the manufacturer. To monitor the extraction process for any foreign DNA contamination, a negative DNA extraction control, consisting of 200 µL of sterile phosphate buffered saline (pH 7.2), was included in each batch of samples that was processed. The primers used for the polymerase chain reaction (PCR) were BARTON-1 (5'-TAACCGATATTGGTTGTGTGAAG-3') and BARTON-2 (5'-TAAAGCTAGAAAGTCTGGCAACATAACG-3'), which are specific for the *Bartonella* genus and target a fragment of the riboflavin synthase C gene of the bacteria. The PCR reactions were essentially performed as described previously (Johnson et al. 2003) with the following modifications: an initial denaturation of 5 min at 95°C, followed by 37 cycles consist-

ing of denaturation at 96°C for 20 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min and a final incubation for 3 min at 72°C. A negative control, consisting of distilled water and positive control, consisting of extracted DNA from a blood sample known to contain *B. bacilliformis* (kindly provided by Alexander von Humboldt, Tropical Medicine Institute, Universidad Peruana Cayetano Heredia, Peru), were used in each PCR run. Following amplification, 5 µL of each PCR reaction was subjected to electrophoresis on a 3% agarose gel containing ethidium bromide. The presence of DNA fragments, indicating a positive reaction, was detected under a ultraviolet transilluminator (*B. henselae* 588 bp, *B. clarridgeiae* 585 bp). Positive samples were submitted to direct sequencing after the PCR products were purified with an AccuPrep(R) PCR Purification Kit (Bioneer), as directed by the package insert. Direct sequencing was performed using an ABI PRISM Dye TM Terminator Cycle Sequencing Ready Reaction kit (Big Dye - Applied Biosystems, Inc, USA), following the manufacturer's instructions, and electrophoresis was performed using an ABI 3110 automatic sequencer (Applied Biosystems, Inc, USA). The nucleotide sequences were submitted to analysis by BLAST at the DDBJ website (DNA Databank of Japan) and the results were used to confirm the genus and species of *Bartonella*.

Phylogenetic analysis was performed and the evolutionary relationships for the eight obtained sequences were inferred by comparing the obtained sequences with 54 nucleotide sequences from different *Bartonella* species found in GenBank, using the neighbor-joining method (Saitou & Nei 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are expressed in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analysis was conducted using the computer program MEGA4 (Tamura et al. 2007). The nucleotide sequences obtained were submitted to GenBank and deposited with the accesses HM588655-HM588662.

This study was approved by the Ethical Committee of Feevale University, protocol 2.12.03.09.1391, and cats were handled according to the recommendations and laws regarding the maintenance of animal welfare.

RESULTS

In this study, a PCR reaction using *Bartonella* genus-specific primers was used to evaluate the presence of DNA from *Bartonella* spp in blood samples from 47 cats. Using this method, eight samples were positive for *Bartonella* DNA, representing a 17.02% (8/47) positivity rate (Fig. 1) and all positive samples were confirmed to be *Bartonella* spp by direct sequencing of the PCR products.

Phylogenetic analysis of the sequences obtained from these positive samples clearly demonstrated that five sequences belonged to *B. henselae* and three corresponded to *B. clarridgeiae*, representing prevalences of 10.63% (5/47) and 6.38% (3/47), respectively (Fig. 2).

Using Giemsa-stained blood smears, the presence of intraerythrocytic corpuscles suggesting *Bartonella* in-

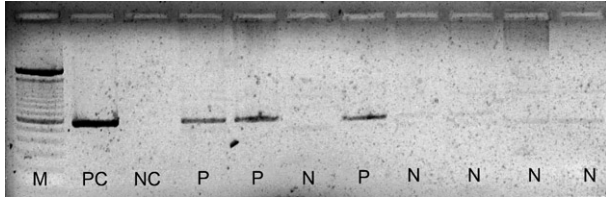


Fig. 1: photo produced polymerase chain reaction for identification of the genus *Bartonella*. M: molecular mass marker, 100 bp (Invitrogen); N: negative sample; NC: negative control; P: positive sample for the genus *Bartonella*; PC: positive control, *Bartonella bacilliformis* strain.

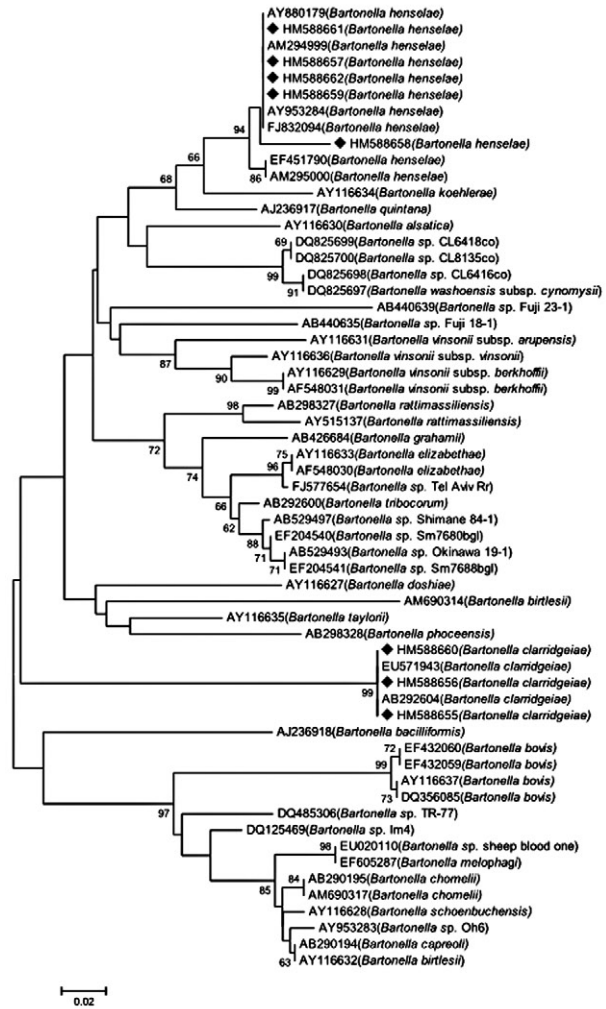


Fig. 2: evolutionary relationships of *Bartonella* isolated from stray cats from Vale dos Sinos, Rio Grande do Sul (diamonds), to other world-wide distributed strains. Bootstrap values are shown on branches.

fection were seen in seven out of the eight PCR-confirmed samples of *Bartonella* spp-containing blood (Fig. 3A, B). Only one PCR-positive sample had no visible corpuscles inside the erythrocytes. Overall, the sensitivity of the direct smear observation was considered to be 87.5% (7/8).

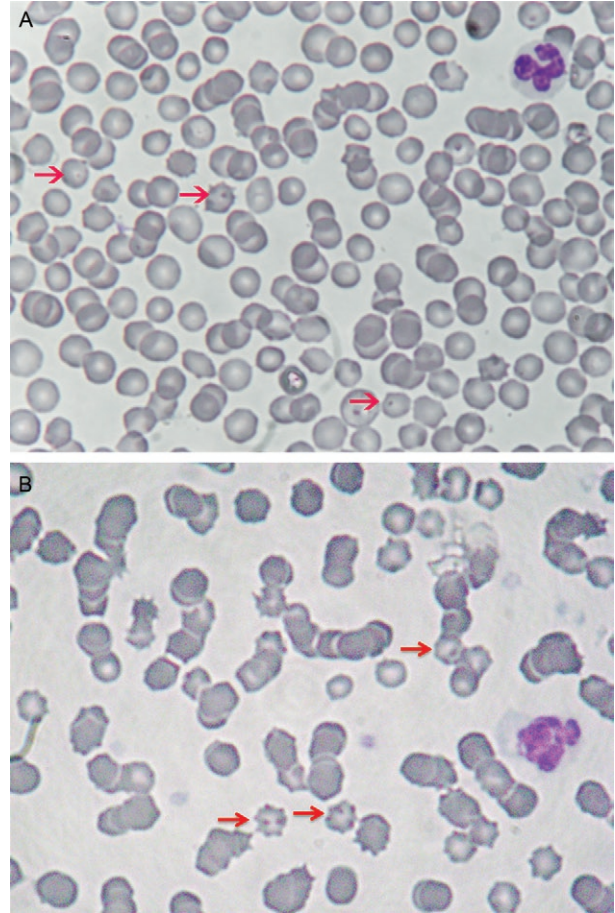


Fig. 3A: intraerythrocytic corpuscles compatible with *Bartonella* with coloration ranging from lilac-blue; B: erythrocytes housing multiple bacteria characterized by the presence of intraerythrocytic corpuscles.

DISCUSSION

To the best of our knowledge, this is the first molecular study of *Bartonella* in cats from the Southern Region of Brazil. Cats are considered the main zoonotic reservoir of *B. henselae* and *B. clarridgeiae* (Anderson & Neuman 1997, Kordick et al. 1997) and are responsible for CSD as well as other *Bartonella*-associated diseases. Other authors have already demonstrated the occurrence of CSD (Amaro et al. 1996, Curi et al. 2006) and endocarditis (Lamas et al. 2007) caused by *Bartonella* spp in Brazil. In addition, the presence of these microorganisms has been reported in Minas Gerais (Costa et al. 2005), Rio de Janeiro (RJ) (Souza 2009, Lamas et al. 2010) and São Paulo (SP) (Filoni et al. 2006).

In this study, two techniques were performed in parallel for the detection of bacteria in the blood samples collected from cats in the south of Brazil. Using molecular methods, a prevalence of 17.02% for *Bartonella* spp was found, which can be considered high and correspond to a theoretical presence of *Bartonella* spp in about two out of 10 cats kept in shelters in our region. This result is similar to those of other studies performed in others parts of the world using similar molecular

methods (PCR), including Italy (18%) (Fabbi et al. 2004), Holland (22%) (Bergmans et al. 1997), France (16.5%) (Gurfield et al. 2001) and Thailand (16.3%) (Inoue et al. 2009). However, the prevalence described here is lower than that shown in other reports, including reports from South Korea (33.3%) (Kim et al. 2009) and Brazil (RJ, 90%) (Souza 2009), both of which used molecular studies similar to ours, as well as reports from the Philippines (61%) (Chomel et al. 1999) or even in other regions of Brazil (SP, 46%) (Slhessarenko et al. 1996). These other studies were based on serological methods, which were used to detect anti-*Bartonella* antibodies, thus detecting not only active infection but also any past infection that resulted in antibody production. It is noteworthy that our data reflect the prevalence of *Bartonella* spp in cats, kept in animal shelters, where crowded conditions may increase the chance of bacterial spread due to close contact between healthy and previously infected animals. Therefore, these results cannot be extrapolated to household cats or pets. In Brazil, *Bartonella* spp were also found to be associated with dogs, although the prevalence (3.6%) was found to be lower than that found in cats (Diniz et al. 2007). Whether dogs represent a risk for in terms of spreading the infection to humans or other animals remains to be determined.

In our study, *B. henselae* was detected in 10.63% of the blood samples, a prevalence rate similar to those found in molecular studies in other countries, such as Switzerland (8.3%) (Glaus et al. 1997), the Czech Republic (8%) (Melter et al. 2003), Turkey (8.2%) (Celebi et al. 2009), Japan (9.1%) (Maruyama et al. 1998), the United Kingdom (9.4%) (Birtles et al. 2002) and Germany (13%) (Sander et al. 1997). On the other hand, this prevalence rate was higher than those observed in Sweden (2.2%) (Engvall et al. 2003) and Portugal (6.7%) (Childs et al. 1995); the reason for these discrepancies is not clear. Compared with the results of molecular methods, seroprevalence results for *B. henselae* are usually higher: United States (39.5%) (Chomel et al. 1995), Canada (17.8%) (Leighton et al. 2001), Denmark (22.6%) (Chomel et al. 2002), Poland (86%) (Podsiadly et al. 2003), Indonesia (54%) (Marston et al. 1999) and Singapore (47.5%) (Nasirudeen & Thong 1999).

B. clarridgeiae was detected in 6.38% of our samples and, to the best of our knowledge, this is the first description of this species in cats in Brazil. Our detection rate was higher than that reported in Germany (1%) (Arvand et al. 2001), Turkey (1.2%) (Celebi et al. 2009) and France (2%) (Rolain et al. 2004) but lower than that of other countries, such as France (16%) (Heller et al. 1997) and Indonesia (61%) (Chomel et al. 1999), both of which used serology methods.

Giemsa-stained smears suggested the presence of an intraerythrocytic infection in seven out of eight PCR-confirmed positive samples, as judged by the presence of corpuscles inside the red blood cells, suggestive of the presence of *Bartonella* spp (Fig. 3A). Microscopic analysis (1000X) suggested a low level of intracellular infection, with approximately 1% of the red blood cells being infected, which is similar to the value published by Mehock et al. (1998). However, the presence of intraeryth-

rocytic corpuscles can only be considered presumptive for *Bartonella* infection, because false-positive results due to dye precipitates or artefacts cannot be excluded and, hence, further confirmation with other methods is imperative. Because no culture method is currently recommended as a gold standard for *Bartonella* isolation, in cases where infection by these agents is suspected, molecular detection methods, such as PCR, may be used to confirm or rule out the presence of these agents. For patients, especially those with some degree of immunosuppression, molecular methods could provide rapid diagnosis of bartonellosis and allow faster initiation of treatment, thus preventing other consequences of the disease.

In summary, our results showed that *B. henselae* and *B. clarridgeiae* are prevalent in cats in our region, representing 10.63% and 6.38% of the infectious cases, respectively, which is similar to the rates described in other countries. The species found here are those associated with CSD and, therefore, they may pose some risk to public health, mainly for HIV-positive individuals and for patients with rheumatic X (Lamas et al. 2007). The demonstration of the existence of this pathogen in cats in our region reinforces the importance of considering CSD in the differential diagnosis of patients with lymphadenopathy, abdominal pain, prolonged fever or other characteristic symptoms.

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