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Molecular detection of *Bartonella henselae* and *Bartonella clarridgeiae* in clinical samples of pet cats from Southern Italy

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ABSTRACT

Bartonella henselae is considered an emerging pathogen of veterinary and medical interest that can be occasionally transmitted to humans. Cats are considered to be the only reservoir host for *B. henselae*. In this study, we used a nested-PCR assay to investigate the prevalence of *B. henselae* and *Bartonella clarridgeiae* DNA in peripheral blood samples, fine needle lymph node aspirate specimens and oral swabs from 85 cats in order to develop an easy diagnostic strategy for the selection of infection-free cats that are being considered as pets, especially for immunocompromised patients. Overall, molecular analysis showed that 71 cats (83.5%) tested PCR positive for the presence of *B. henselae* DNA. PCR amplification of DNA *B. henselae* produced positive products from lymph node aspirate specimens (62/85; 72.9%) similar to those obtained from blood samples (60/85; 70.6%) and higher than those from oral swabs (51/85; 60%) of cats. No PCR product was obtained for *B. clarridgeiae*. The simultaneous analysis of three different clinical samples in our study increased the diagnostic possibilities for *B. henselae* infection in the examined cats from 60–72.9% to 83.5%. Lymph node aspirates were found to be the most effective clinical samples for the detection of *B. henselae* and blood samples were the next best. Oral swab samples were used in this study with good results when considered in combination with blood and/or lymph node aspiration. The use of nested-PCR assay on these three clinical samples may enhance the diagnostic sensitivity for bartonellosis in cats irrespective of the clinical status of animals.

1. Introduction

Bartonella henselae is a gram-negative, intraerythrocytic, feline-adapted fastidious bacterium prevalent among cats throughout most temperate regions of the world. Transmission among cats is mediated by the cat flea, *Ctenocephalides felis* (Chomel et al., 1996) and human infection occurs through contamination of cat scratches with flea excrement or cat bites, if cat blood or flea excrement contaminates the wound. Substantial evidence has linked *B. henselae* to various human infectious diseases and it is considered a zoonotic agent (Chomel et al., 2004). Human infections include vasoproliferative illness-bacillary angiomatosis, hepatosplenic granulomatosis, peliosis hepatitis, fever, central nervous disorders and, more commonly, cat scratch disease (Welch et al., 1992; Kordick et al., 1995; Chomel et al., 2006a). Exposure to cats has been proven to be an important acquisition factor for *B. henselae* infections. According to the immune status of the host and to the bacterial species and strain, naturally infected cats can develop a chronically recurrent bacteraemia, thus playing a major role as a reservoir for the bacterium (Chomel, 2000; Chomel et al., 2006b). The clinical spectrum of the natural infection in cats is not adequately known because of the high prevalence of *B. henselae* infection in the cat population. Naturally infected cats seem primarily to be asymptomatic carriers of the bacterium (Chomel et al., 2004; Boulouis et al., 2005; Breitschwerdt and Kordick, 2000). However, sporadic cases of uveitis (Lappin et al., 2000) and two rare cases of valvular endocarditis (Chomel et al., 2003) have been associated with infection caused by *B. henselae*. According to retrospective studies, seropositive sick cats were more likely to have a variety of kidney and urinary tract diseases and stomatitis. Co-infection of cats with *B. henselae* and Feline Immunodeficiency Virus (FIV) was significantly associated with gingivitis or lymphoadenomegaly than was either infection alone (Ueno et al., 1996). In experimentally infected cats asymptomatic infection or transient fever, lethargy, anorexia, lymphadenomegaly, mild neurologic signs, myalgia and reproductive disorders have been reported and their severity varied with the strain used for inoculation (Regnery et al., 1996; Guptill et al., 1997; Yamamoto et al., 2002). Co-infection of cats

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with *B. henselae* and *Bartonella* clarridgeiae has been reported (Gurfield et al., 2001). Although *B. clarridgeiae* has also been linked to cases of cat scratch disease (Kordick et al., 1997), the role of this organism in causing human disease in unclear. Veterinarians are increasingly being asked to test pets belonging to owners who have, or are considered most susceptible to *Bartonella*-related diseases. Currently, the laboratory diagnosis of bartonellosis in cats is based on bacterial culture, serology or polymerase chain reaction (PCR) from blood and tissue specimens. Isolation is the gold standard for proving the infection but the need of specialized media limit to the research field this technique. Serologic testing usually overestimates active infection and is of limited diagnostic value for bacteraemia because the positive predictive value is only 46.4% and the negative predictive value is 89.7% (Glaus et al., 1997). Nested PCR on DNA extracted from blood has been considered a sensitive method for diagnosis of *B. henselae* infection (Roy et al., 2001).

As no data claiming the molecular evidence of *B. henselae* and *B. clarridgeiae* in cats are available in Southern Italy, the aim of this study was to determine the carriage rate and the distribution of these bacteria in different biological samples (oral swabs, blood, fine needle lymph node aspirates) from 85 pet cats recruited in Sicily by using a nested-PCR assay and thus to develop an easy diagnostic strategy for the selection of infection-free cats that are being considered as pets, especially for immunocompromised patients. Evaluation of oral swabs for *Bartonella* DNA detection could represent an additional, non invasive diagnostic tool useful for enhancing the sensitivity of the test results.

2. Materials and methods

2.1. Cats

A total of 85 pet cats (39 males and 46 females) were recruited in this study between November 2003 and June 2006 at the Small Animal Clinic of the Faculty of Veterinary Medicine of the University of Messina. Data concerning age, breed, gender and neutering status, environmental history (indoor/outdoor, single cat household/multi-cat household) of the cat, history or presence of flea and tick infestation were recorded together with physical examination findings and laboratory investigations. Both healthy (admitted for vaccination or neutering) and unhealthy cats were enrolled as specified in item 3.1; cats affected by lymphadenomegaly and/or oral pathologies were specifically targeted because of a concomitant cytological study on the same cats (data not shown).

2.2. Clinical samples

2.2.1. Blood samples

Blood samples (1 ml) obtained by aseptic procedure from the jugular veins of cats were placed in serum separator and ethylene-diamine-tetraacetate (EDTA) treated tubes.

2.2.2. Lymph node aspirate

A lymph node fine needle aspirate from a submandibular lymph node was taken for molecular analysis and for a cytological evaluation from each cat (not reported here).

2.2.3. Oral swab

A dry cotton swab was rolled over the vestibular area (normal cats) or over lesions (cats affected by gingivo-stomatitis). Cytological samples were also carried out from the same mucosal area (data not shown).

All clinical samples were stored at −20 °C until tested.

2.3. DNA extraction

DNA was extracted from all materials using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, clinical samples were added to an appropriate volume of phosphate buffered saline (PBS) and homogenized by vortexing. Twenty microlitres of a proteinase K solution (20 mg/ml) and 200 µl of buffer AL provided in the kit were then added, followed by incubation at 56 °C for 10 min. Next, 200 µl of ethanol (96%) were added. The mixture was then loaded on the QIAamp spin column and centrifuged at 6000*g* for 1 min. The QIAamp spin column was placed in a 2 ml collection microtube, and the tube containing the mixture was discarded. The column material was washed (500 µl each) with the first washing buffer (Buffer AW1) and with the second washing buffer (Buffer AW2) provided in the kit. Finally, the DNA was eluted with 150 µl of a third buffer (Buffer AE) provided in the kit.

2.4. Oligonucleotide primers

Two pairs of primers targeting species-specific size differences in the 16S–23S rDNA intergenic regions were used in nested-PCR for the detection of *B. henselae* and *B. clarridgeiae*. The outer primer pairs P-bhenfa (5′-TCTTCGTTTCTCTTTCTTCA-3′) and P-benr1 (5′-CAAGCGCGCGCTCTAACC-3′) gave a 186 bp fragment for *B. henselae* and 168 bp fragment for *B. clarridgeiae*. Nested inner primers N-bhenf1a (5′-GATGATCCCAAGCCTTCTGGC-3′) and N-bhenr (5′- AACCAACTGAGCTACAAGCC-3′) amplified a 152 bp fragment for *B. henselae* and a 134 bp for *B. clarridgeiae* (Rampersad et al., 2005).

Additionally, a second primer pair for the *pap31* gene, PAPn1 (5′-TTCTAGGAGTTGAAACCGAT-3′) and PAPn2 (5′-GAAACACCACC-AGCAACATA-3′), as described by Zeaiter et al. (2002), was used to compare all *B. henselae* positive results of the first primer sets, obtained from oral swabs samples. The expected size of the product generated with PAPn1- PAPn2 is a 275 bp. All primers were purchased from MWG-Biotech AG.

2.5. PCR protocols

Nested PCR protocol for amplification of the *B. henselae and B. clarridgeiae* DNA was applied to all DNA extracted.

The reaction mixture of the first step, which was made up to 25 µl with sterile water, contained 0.5 pmoles/ml of each primer (P-bhenfa and P-benr1), 0.2 mM of each deoxyribonucleotide triphosphate, 1× PCR buffer (50 mM KCL, 10 mM Tris–HCL pH 8.8, 0.1% Triton X-100), 3 mM $MgCl_2$, 0.5 U Taq DNA polymerase and 5 µl extracted DNA.

The amplification conditions for the first step were 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s for 40 cycles, followed by a final extension step of 72 °C for 5 min. The reaction mixture of the second step (25 µl) contained 0.5 pmoles/ml of each primer (N-bhenf1a and N-benr), 0.2 mM of each deoxyribonucleotide triphosphate, 1× PCR buffer (50 mM KCL, 10 mM Tris–HCL pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase and 1 µl of the primary reaction mixture. The amplification conditions for the second step were 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s for 40 cycles; the second step was finalized by 72 °C for 5 min. In each experiment, purified DNA obtained from a cultured *B. henselae* Houston-1 (ATCC 49882), *B. clarridgeiae* (ATCC 51734) and DNA-free water were used as positive and negative controls, respectively.

PCR protocol for amplification of the *Bartonella pap31* gene was applied to DNA extracted from oral swabs samples.

Pap31 PCR amplification gene was carried out under the following conditions: an initial 3 min of denaturation at 94 °C was followed by 44 cycles of denaturation for 30 s at 94 °C, annealing