# *Survey of* Bartonella *species in cats from Abruzzo region, Italy*

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**Summary**
Cat scratch disease (CSD) is a zoonotic disease, caused predominantly by *Bartonella henselae* and transmitted to humans through a scratch or bite of the cat. Cat represents the principal reservoir and healthy carrier of *Bartonella*, which is mainly transmitted, among cats, by the flea *Ctenocephalides felis*. During 2014, fifty-two samples of whole blood and sera were collected randomly from cats in Abruzzo region and were examined by real-time PCR and IFAT tests, respectively. Seven samples out of fifty-two (13.5%) resulted positive for *Bartonella* spp. in both tests, while six specimens (11.5%) resulted real-time PCR negative but IgG positive; thirty-nine were instead both real-time PCR and IFAT negative (75%). Sequence analysis of a fragment of DNA identified *B. henselae* and *B. clarridgeiae* in four and in two real-time PCR positive samples, respectively.

Cat scratch disease (CSD) is a zoonotic disease mainly caused by an aerobic and pleomorphic Gram-negative bacterium, *Bartonella henselae* belonging to α sub-group of Proteobacteria, family *Bartonellaceae*. Cat acts as the principal reservoir of *B. henselae* which is transmitted among cats mainly by the flea *Ctenocephalides felis* (Bouhsira *et al.* 2013, Fabbi *et al.* 2004, Greco *et al.* 2019, Iannino *et al.* 2018, Pinna Parpaglia *et al.* 2007). *Bartonella clarridgeiae* is another agent of CSD, though rare (Capitta *et al.* 2010, Greco *et al.* 2019).

Humans mainly acquire infection through an infected cat scratch or bite and can exhibit an acute febrile lymphadenopathy. Immunosuppressed individuals may show hepatic and/or splenic peliosis and also bacillary angiomatosis (Breitschwerdt 2008, Breitschwerdt *et al.* 2010, Capitta *et al.* 2010, Fabbi *et al.* 2004a, Iannino *et al.* 2018, Pinna Parpaglia *et al.* 2007, Zobba *et al.* 2009).

Naturally-infected cats with *B. henselae* are usually healthy carrier and can be bacteremic for weeks to months/years, representing an important reservoir for *Bartonella* (Breitschwerdt 2008, Chomel *et al.* 2006, Fabbi *et al.* 2004b). Young cats (< 1 year) can develop a stronger bacteremia than older cats, as well as the street cats compared to pet cats (Chomel *et al.* 2006).

Thus, infected cats may represent a risk to human health. Moreover, positive cats are considered the most important vehicle for the spread of the disease (Ebani *et al.* 2012, Fabbi *et al.* 2004 a, b, Pinna Parpaglia *et al.* 2007, Zobba *et al.* 2009).

*Bartonella* infections in symptomatic cats should be confirmed by culturing the organism from blood or tissues but some molecular diagnostic approaches have been developed during the last years. In particular, real-time PCR assays are commonly used to detect *Bartonella* spp. Since *Bartonella* genus shows high genetic variability, a universal method is difficult to develop, so different conventional PCR and real-time PCR methods, targeting different portions of the genome, have been proposed. For example, gene gltA is a common genetic target for *Bartonella* detection and is considered a reliable tool for distinguishing genotypes (La Scola *et al.* 2003, Tapp *et al.* 2003). However, this test has showed high cross-reactivity with other bacteria, such as *Ehrlichia* spp. (Colborn *et al.* 2010).

The immunofluorescence antibodiy test (IFAT) is the most used serological diagnostic tool for detecting *Bartonella* exposure.

This study aimed at detecting *Bartonella* infection in cats from Abruzzo region. During 2014, a total of fifty-two samples of whole blood and sera were

collected randomly from cats in Abruzzo region and were examined at IZSAM by IFAT and real-time PCR tests.

*B. henselae* Houston1 (ATCC 49882) was cultivated in *Brucella* broth (BBL Microbiology System Cockeysville, MD, USA) supplemented with haemin (250 µg/ml) and 8% Fildes solution, and incubated at 37 °C in 5% $CO_2$. Log -phase cultures were centrifuged (4,500 *g*, 30 min), washed three times in PBS, pH 7.2 and inoculated into a 25-$cm^2$ flask (Corning, NY, USA) containing L929 cells (ECACC 85011425) and Dulbecco minimal essential medium (Gibco, Introvigen, Grand Island, NY, USA) supplemented with 2 mM L-glutamine (200 mM) and 10% (v/v) fetal bovine serum (Gibco) and maintained at 37 °C with 5% $CO_2$. L929 cells infected with *B. henselae* were harvested by centrifugation at 4,500 *g* for 30 min and washed twice with PBS. The final pellets were resuspended in PBS and 10 µl were added onto each well of 18-well slides (GSG Robotix Italy). Slides were air-dried for at least 1 h, fixed with cold acetone for 10 min and used immediately or stored at - 20 °C until use (Capitta *et al.* 2010, Zobba *et al.* 2009).

*Bartonella* spp. IgM and IgG antibodies were determined by IFAT: 10 µl of serum diluited (1:40 to 1:640) in PBS was placed on the *B. henselae* L929 slide and incubated at 37 °C in a humidified chamber for 30 min. After two washes in PBS (10 min) and one rinse with distilled water the slides were incubated for 30 min at 37 °C with fluorescein isothiocyanate-labelled goat anti cat IgM (Bethyl Laboratories Inc- Montgomery, Texas) and IgG immunoglobuline (Sigma-Aldrich, St. Louis, MO, USA), diluted in PBS 1% Evans Blue (Sigma-Aldrich, St. Louis, MO, USA). The slides washed and dried as described above, were examined with a fluorescence microscope. The titre of < 1:40 was considered negative, a titre of ≥ 1:40 was considered positive for both classes of antibodies (Capitta *et al.* 2010, Zobba *et al.* 2009).

A quantitative PCR method was developed to detect *Bartonella* spp. DNA in blood cat samples. The highly conserved region of *nuoG* gene (André *et al.* 2016, Colborn *et al.* 2010, Diaz *et al.* 2012) was selected to design primers and probe by Primer Express software (ThermoFisher Scientific).

Total genomic DNA was extracted from 300 µl of whole blood samples or from *B. henselae* strain by Maxwell® 16 Instrument using Maxwell R 16 Blood DNA Purification kit (Promega), according to the manufacturer's instructions.

The limit of detection (LOD) of real-time PCR assay was determined using 10-fold serial dilutions of DNA extracted from *B. henselae* (ATCC-49882D-5). Standard curve was generated and used to calculate the amount of DNA that could be detected by Probit Analysis with 95% of sensitivity. Amplification efficiency was calculated from slope of the standard curve using the following formula: $E = (10^{-1/slope} - 1) \times 100$ (Vaerman *et al.* 2004).

Specificity was assessed by testing nucleic acid from closely genetically related bacterial species, or different species that may infect cats including *Anaplasma phagocytophilum, Borrelia afzelii, Rickettsia helvetica, Rickettsia monacentis, Rickettsia slovaca* and *Coxiella burnetii*.

The intra-assay variance (repeatability or short-term precision) was determined by testing three replicates of the 10-fold serial DNA dilutions (equal to $3.07^4$ to $3.07^2$ copy of DNA/reaction) in the same run. Similarly, the inter-assay variance (reproducibility or long-term precision) was determined by running triplicates of the DNA dilutions ($3.07^2$ to $3.07^1$ copy of DNA/reaction) in three different runs and on separate days. These replicates were used to determine the mean and standard deviation of CT values and the coefficient of variation.

The reaction mix (20 µl) contained 2 µl of extracted nucleic acid, 10 µl Go Taq Probe qPCR MMix 2x (Promega), primers and probe at a final concentration of 800 nM and 250 nM, respectively (Table I). Real-time PCR was performed on Quantstudio 7 Flex Systems Istrument (Life Technologies) with the thermal condition showed in Table I.

In order to confirm and characterize the results of real-time PCR a partial region of the 23S gene was sequenced (Parra *et al.* 2016).

Obtained sequences were aligned using Clustal V (DNAStar, Madison, WI). Each sequence was compared to other homologous regions present in Genbank by Blast search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Seven blood samples out of fifty-two (13.5%) tested positive for *Bartonella* spp. IFAT revealed IgG for *Bartonella* spp. in all real-time PCR positive cats, while IgM were identified in two cats. Six specimens were real-time PCR negative, IgM negative and IgG positive (11.5%) with values ranging from 1:40 to 1:160, whereas thirty-nine

**Table I.** *Primers and probe sequences and thermal condition.*

| | | |
|---|---|---|
| nuoG_60_F | 5'-GCGGCATAATTCGCATAACC-3' | |
| nuoG_60_R | 5'-CACTTGGCAGTGCTATCCGTATT-3' | |
| nuoG_P | 5'-FAM-ACGACCCCGGCTAT-3'- MGB | |
| Temperature | Time | |
| 95°C | 2 min | 1 cycle |
| 95°C | 15 sec | 45 cycles |
| 60°C | 1 min | |

**Table II.** *Serological and real-time PCR results.*

| No. of Cats | IgG | IgM | Real-time PCR |
|---|---|---|---|
| 1 | 1/160 | Neg | POS |
| 2 | 1/320 | Neg | POS |
| 3 | 1/160 | Neg | POS |
| 4 | 1/320 | Neg | POS |
| 5 | 1/80 | 1/40 | POS |
| 6 | 1/40 | 1/40 | POS |
| 7 | 1/160 | Neg | POS |
| Total positive | 7/7 | 2/7 | 7/7 |

POS = positive; Neg = negative; No = number.

samples resulted negative by both assays (75%). Results are summarized in Table II.

The amplification efficiency was 100.4% (slope = - 3.312; $R^2 = 0.997$). The LOD, determined as the lowest dilution of DNA that could be detected with 95% sensitivity, was equivalent to 0.32 genome copies.

The coefficient of variation, determined for intra- and inter-assay repeatability, showed mean values of 0.33% and 2.16%, respectively.

No amplification signal was obtained from a panel of different bacterial species tested to evaluate the specificity of the molecular assay.

The sequence analysis showed the presence of two different *Bartonella* species: four samples were identified as *B. henselae* and two as *B. clarridgeiae*.

One positive sample was not identified as probably due to the lower concetration of DNA.

This survey demonstrated correlation between serological and virological results. Reasonably, serological positive individuals without evidence of bacterial DNA in the bloodstream might be related with the presence of past infections. However non bacteremic cats may still represent a risk for humans given that reinfections might occurr (Fabbi *et al.* 2004a).

It is generally accepted that the IFAT test should be performed standalone only when the prevalence of bacteric cats is high (40-60%). However, when the transmission to humans (for example immune-depressed individuals) is a risk, virological and serological tests should be performed in order to get a complete health status of a cat (Fabbi *et al.* 2004b). In this perspective, the novel molecular method which has been developed in this study, is useful to detect *Bartonella* infected cats and thus for prevalence studies in feline populations.

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