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# ADVANCED DIAGNOSTIC METHODS AS TOOLS TO INVESTIGATE THE EXPOSURE TO *BARTONELLA* INFECTIONS IN CATS

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*Domestic cats represent the primary reservoir for zoonotic Bartonella henselae, Bartonella clarridgeiae and Bartonella koehlerae, and is transmitted by their fleas. B. henselae is the most frequent causative agent of the cat scratch disease (CSD) in humans. The confirmatory diagnosis in cats, can not be determined based on clinical signs, since most cats with chronic infection are often asymptomatic. Therefore, currently, diagnosis of Bartonella spp. infection is established by direct methods (bacterial isolation, molecular biological methods, Polymerase Chain Reaction-based) and indirect methods (serological tests: IFA, ELISA, Western Immunobloot). For serological testing, to detect antibodies to Bartonella spp., blood samples collected from animals are used, while for the identification of Bartonella species through molecular methods, the samples for analysis are: blood, lymph node aspirate, tissue aspirate, saliva, articular liquid, ocular exudate, or biopsy samples. Considering the high exposure to Bartonella infections in both animals and humans, epidemiological studies based on advanced diagnostic methods emphasized the higher sensitivity and specificity of these modern techniques for species-specific identification of Bartonella organisms.*

*Key words: Bartonella* spp., cats, epidemiology, diagnostic methods

## INTRODUCTION

The number of zoonotic *Bartonella* species, the rapid expansion in mammals reservor infections, but also the large number of arthropods that were involved in the transmission of *Bartonella* species identified in the last 15 years has increased considerably[1,2]. Of the many species of mammals, pets may play an important role as source for human infection[2]. Domestic cats represent the primary reservoir for the zoonotic *Bartonella* species: *Bartonella henselae* (*B. henselae*), *Bartonella clarridgeiae* (*B. clarridgeiae*) and *Bartonella koehlerae* (*B. koehlerae*). All the *Bartonella* species belong to the group of the small intracellular bacteria, Gram-negative organisms, and vector-borne[3]. The transmission of *Bartonella* spp. in mammals and humans involves blood-sucking arthropods, amongst them cat fleas (*Ctenocephalides felis*) playing a major role. Nevertheless, other potential vectors (ticks, haematophagous insects) were identified as carriers of *Bartonella* spp.[1]

The confirmatory diagnosis in cats cannot be determined based on clinical signs. Infected cats with *Bartonella* spp. are usually asymptomatic, but can still present recurrent bacteraemia, which may last from months to years[4]. When an infected arthropod comes into contact with an uninfected host reservoir, it is possible intra-/subcutaneous inoculation or direct blood contact of the bacterium through arthropod bite, but it is considered that the highest number of bacteria is inoculated via arthropod faeces[5,6]. Most probably, the intra-/subcutaneous inoculation of bacteria through feces occur in superficial scratching and tissue trauma of the skin[6].

Experimental studies have shown that *Bartonella* spp. can multiply in the digestive system of the cat flea, and that survive a few days in the feces of fleas. The bacteria were present in the gut fleas three hours after feeding with blood and persisted until nine days after the flea was fed with blood infected with *Bartonella* spp.[7]

Therefore, currently, diagnosis of *Bartonella* spp. infection is established by direct methods (bacterial isolation, molecular biological methods, Polymerase Chain Reaction-based) and indirect methods (serological tests: IFA, ELISA, Western Immunobloot)[8]. For serological testing, to detect antibodies to *Bartonella* spp., blood samples are collected from animals, while for the identification of *Bartonella* species through molecular methods, the samples for analysis are: blood, lymph node aspirate, tissue aspirate, saliva, articular liquid, ocular exudate, or biopsy samples.

The aim of these paper was to detailed some literature data on various ways to investigate the exposure to *Bartonella* infections in cats, these being useful in disease management.

**Serological diagnosis** In the specialty literature, serological methods for detection of antibodies anti-*Bartonella* have been described and used more than any other technical methods, especially for detecting *Bartonella* infections in cats. The antibody titers against-*Bartonella* is determined by IFA (Indirect Fluorescent Antibody) and ELISA (Enzyme-Linked Immunosorbent assay) technique, using the other membrane proteins (OMP) of *Bartonella* spp. Although IFA test is the technique used most frequently, it lasts more time than ELISA test, and interpretation would be less objective[9].

1. Indirect Fluorescent Antibody Test (IFA)

For this test, serum is diluted in phosphate buffer saline (PBS) and incubated on slides containing the cells infected with *B. henselae* (and possibly with *B. clarridgeiae*) from fetuses *Felis catus*. Slides are washed and impregnated with fluorescein isothiocyanate (FITC) goat anti-cat IgG. Any serum with a titer of ≥ 64 is considered to be positive[10]. IFA Test is indicated for young cats and for cats before adoption by owners who may have immunocompromising status[11].

2. Enzyme-Linked Immunosorbent Assay (ELISA)

The principle of ELISA technique is based on antigen-antibody reaction.

Assarasakorn *et al*. (2012) describes this technique used for highlighting antibodies IgG against-*Bartonella* spp., using cut off 1:64 for each serum sample, a positive serum control and a negative serum control, were pipetted into qvadruplicate wells, of a microtitre plate coated with *B. henselae* antigen. The conjugates were incubated separately for each plate for 30 minutes at 37 ° C and then washed three times with 200 ml of phosphate buffer solution (PBS) containing 0.05% Tween-20. In appropriate wells was pipetted one hundred milliliters of a 1:3000 dilution of peroxidase-labeled goat anti-cat IgG in PBS-Tween solution. The plate was incubated for 30 min. at 37°C, and after another washing step, 100 µl of substrate were pipette into each well. The enzymatic reaction was stopped after 10 minutes. The optical density (OD) of each well was read at 450 nm with an automatic micro-ELISA reader. A sample is considered positive for *Bartonella* spp. IgG antibodies if the average OD value is greater than the average OD value plus 3SD (standard deviation) of samples negative titer ≥ 64[12].

Serologic testing has limited value for the diagnosis, since many cats (especially stray cats) are likely to be seropositive against-*B. henselae*[13]. Compared to the bacterial isolation, which lasts between 4 and 6 weeks, the serological tests have the advantage that they are easier to use, and have duration of 1-2 days, while being economic. Cats infected produce specific antibodies against bacterial protein, which indicates the presence of bacteria[11].

After several years of research, Hardy *et al.* (1995) have compared the bacterial isolation with serology tests, their data showing that the most accurate and reproductible test for detection of *Bartonella* infection is serological detection of antibodies to the bacteria, using the Western blot (WB)[14].

**Bacteria isolations** The bacteria of genus *Bartonella* grows on fresh blood agar, brain-heart infusion (BHI) agar[4], rabbit-heart infusion agar[15,16], and chocolate-blood agar[3,17]. The culture conditions requiring prolonged periods (at least 21 days) of 5% CO2 up to 10% and high humidity. After staining and microscopic examination, the colonies with different morphology are subcultured harvested and frozen at -70 ° C in 100% fetal calf serum[11,18].

*Bartonella* organisms grow very slowly and are fastidious, requiring special growth medium. Some practitioners recommend blood culture as a reliable test, but it is necessary more consecutive cultures, since these organisms *Bartonella* circulate intermittently[19]. The cultures can last up to several weeks and are more expensive than serological tests, which making this technique impractical and not very accurate for practitioners. Positive cultures should be confirmed as infected with *Bartonella* by PCR or antigen analysis which significantly increases costs[20].

Bacterial isolates are identified using PCR-RFLP method using a gltA and 16S rRNA gene fragment[10,21]. The strains isolated were confirmed by gel electrophoresis and then subjected to enzymatic digestion using the restriction endonucleases: Taq I and Hha I (gltA gene)[13] and DdeI (16S rRNA gene)[18].

Isolation species of *Bartonella* in cats with bacillary angiomatosis is much easier (where serologic testing is not useful, because they often do not show detectable antibody) than isolating these organisms from other animals or people non-immunocompromised.

A positive blood culture and culture of other tissues, represents the relevant test for the definitive diagnosis of infection with *Bartonella* spp.[22]. However, cats can take several blood cultures due to recurrent nature of bacteraemia with *Bartonella* in this species[23].

**Molecular diagnostics** *Bartonella* spp. is usually identified by PCR amplification of organism-specific DNA sequences or/and through serological testing[24]. For detection *Bartonella* species are used these techniques: PCR conventional, PCR-RFLP (Polymerase Chain Reaction/Restriction Fragment Length Polymorphism), nested-PCR, Multiplex SYBR Green-Real Time PCR (qPCR), MLST (Multilocus Sequence Typing), AFLP (Amplified Fragment Length Polymorphism).

Sequencing and analysis of bacterial DNA by PCR has been shown to be effective in the diagnosis and identification of different species of *Bartonella*. Molecular techniques using genetic markers (target gene), such as: *gltA* gene (citrate synthase)[25], 16S rRNA

gene[26], 16S-23S intergenic spacer region ITS[27], *rpoB* gene (β-subunit RNA polymerase)[28], *fts*Z (cell division-associated protein), *gro*EL (heat shock protein) and *rib*C (riboflavin citrate synthase)[29,30]. **Table 1** provides information from specialized literature regarding the identification of *Bartonella* spp. through the use of genetic markers, nucleotide sequences, base pairs, and various primers.

PCR is a sensitive test for DNA amplification, being utilized to amplify *Bartonella spp.* DNA; because the bacteria circulates only intermittently, the test does not offer many advantages over culture. Fragments of specific genes for both *rib*C and *htr*A (heat shock protein), are highlight by PCR analysis in the majority of *Bartonella* infections. DNA of these organisms is extracted from the blood and/or tissue, followed by real time PCR[29]. Even if a negative result from testing by PCR of a sample, cannot exclude the presence of *Bartonella* DNA[20]. These markers used to detect *Bartonella* species, having the ability to distinguish morphologically similar species, such as *B. henselae* and *B. koehlerae*.

Nasereddin *et al*. conducted a study by molecular techniques (conventional PCR), in which have detected *Bartonella* species encountered to fleas. *ITS* gene has a higher sensitivity and specificity compared to *glt*A gene in amplication of *Bartonella* from sample fleas. This study was used PCR-RFLP method for differentiating the two species: *B. henselae* and *B. koehlerae*[30].

A study carried by Mietze *et al*. highlights *Bartonella* species identification in cats by real time PCR. For this, *B. henselae* isolates were genetically characterized by AFLP (amplified fragment length polymorphism) and MLST (multilocus sequence typing); each method has confirmed genetic diversity of *B. henselae* on the strain level. By combining MLST analysis and AFLP typing proved that *B. henselae* of the same AFLP subgroup belongs to the same clonal complex. The techniques used in the study emphasize that *B. henselae* may evolve clonally[34].

Following a study conducted by Pennisi *et al*., (2010), they used the nested-PCR technique for the detection of *B. henselae* and *B. clarridgeiae* (from blood samples, lymph node aspirate and oral swab), targeting species-specific differences in 16S-23S rDNA ITS and *pap*31[38].

In another study, the DNA of *B. henselae* and *B. quintana* was isolated from dental pulp from two domestic cats (from France) by PCR, using fragments of gene *pap*31 and 16S-23S internal transcribed spacer (ITS)[40].

## CONCLUSIONS

Considering the high exposure to *Bartonella* infections in both animals and humans, epidemiological studies based on advanced diagnostic methods emphasized the higher sensitivity and specificity of these modern techniques for species-specific identification of *Bartonella* organisms.

**Table 1.** Data on genetic markers, sequences nucleotide, base pairs and primers, used in the identification of *Bartonella* spp.

| Identified species | Target gene | Base pairs | Primer(s) | Nucleotide sequence | References |
|---|---|---|---|---|---|
| *Bartonella* spp. | *gltA* | 379 bp | BhCS.781 p | 5'-GGGGACCAGCTCATGGTGG-3' | 25, 30, 31, 32 |
| | *gltA* | 379 bp | BhCS.113 7n | 5'-AATGCAAAAAGAACAGTAAACA-3' | 30, 31, 33 |
| | *gltA* | 915 bp | BvCS.205 p | 5'-TTTATCGYGGTTATCCTATYG-3' | 32 |
| | *gltA* | 249 bp | strat1 | 5'-GGGGACCAGCTCATGGTGG-3' | 34 |
| | *gltA* | 249 bp | strat2 | 5'-GCGTGATAGCAATATCAAGAAGTGG-3' | |
| | *ITS* | 190 bp | 321s | 5'-AGATGATGATCCCAAGCCTTCTGG-3' | 30 |
| | *ITS* | 190 bp | H493as | 5'-TGAACCTCCGACCTCACGCTTATC-3' | |
| | *rpoB* | 825 bp | 1400F | 5'-CGCATTGGCTTACTTCGTATG-3' | |
| | *rpoB* | 825 bp | 2300R | 5'-GTAGACTGATTAGAACGCTG-3' | |
| | 16S-23S rRNA | 500-800 bp | 325 s | 5'-CTTCAGATGATGATCCCAAGCCTTCTGGCG-3' | 35 |
| | 16S-23S rRNA | 500-800 bp | 1100as | 5'-GAACCGACGACCCCCTGCTTGCAAAGCA-3' | |
| *B. henselae* | *ribC* | 588 bp | BARTON-1 BARTON-2 | 5'-TAACCGATATTGGTTGTGTTGAAG-3' 5'-TAAAGCTAGAAAGTCTGGCAACATAACG-3' | 36 |
| *B. clarridgeiae* | | 585 bp | | | |
| *B. henselae* | 16S-23S rDNA | 186 bp | P-bhenfa P-benr1 | 5'-TCTTCGTTTCTCTTTCTTCA-3' 5'-CAAGCGCGCGCTCTAACC-3' | 37, 38 |
| *B. clarridgeiae* | | 168 bp | | | |
| *B. henselae* | | 152 bp | N-bhenf1a N-bhenr | 5'-GATGATCCCAAGCCTTCTGGC-3' 5'-AACCAACTGAGCTACAAGCC-3' | |
| *B. clarridgeiae* | | 134 bp | | | |
| *B. clarridgeiae* | 16S-23S rRNA | 145 bp | B1623R JEN1F | AACCAACTGAGCTACAAGCC CTCTTTCTTCAGATGATGATCC | 39 |
| *B. henselae* | | 163 bp | | | |

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