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

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INTRODUCTION

The number of zoonotic Bartonella species, the rapid expansion in mammals reservoir 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\(^5\). Of the many species of mammals, pets may play an important role as source for human infection\(^6\). 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\(^1\). 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\(^2\). 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 faeces occur in superficial scratching and tissue trauma of the skin\(^7\). 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 faeces 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 Immunoblot)\(^8\). 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.
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 against *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.  

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 fetoceus *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. IFA Test is indicated for young cats and for cats before adoption by owners who may have immunocompromising status.  

2. **Enzyme-Linked Immunosorbent Assay (ELISA) The principle of ELISA technique is based on antigen-antibody reaction. **Assaraskorn 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 quadruplicate wells, of a microtite 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 1:300 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.  
   Serologic testing has limited value for the diagnosis, since many cats (especially stray cats) are likely to be seropositive against *B. henselae*. 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.  

   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 reproducible test for detection of *Bartonella* infection is serological detection of antibodies to the bacteria, using the Western blot (WB).  

**Bacteria isolations** The bacteria of genus *Bartonella* grows on fresh blood agar, brain-heart infusion (BHI) agar, rabbit-hear infusion agar, and chocolate-blood agar. 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.  

*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. 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.  

Bacterial isolates are identified using PCR-RFLP method using a gltA and 16S rRNA gene fragment. 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) and DdeI (16S rRNA gene). 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. However, cats can take several blood cultures due to recurrent nature of bacteraemia with *Bartonella* in this species.  

**Molecular diagnostics Bartonella** spp. is usually identified by PCR amplification of organism-specific DNA sequences or/and through serological testing. 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), 16S rRNA
gene, 16S-23S intergenic spacer region ITS, rpoB gene (β-subunit RNA polymerase), groEL (heat shock protein), and ribC (riboflavin citrate synthase). 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 ribC and htrA (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. Even if a negative result from testing by PCR of a sample, cannot exclude the presence of Bartonella DNA. 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 gltA gene in amplification of Bartonella from sample fleas. This study was used PCR-RFLP method for differentiating the two species: B. henselae and B. koehlerae.

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.

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. 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 and 16S-23S internal transcribed spacer (ITS).

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.

<table>
<thead>
<tr>
<th>Identified species</th>
<th>Target gene</th>
<th>Base pairs</th>
<th>Primer(s)</th>
<th>Nucleotide sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bartonella spp.</td>
<td>gltA</td>
<td>379 bp</td>
<td>BhCS.781 7n</td>
<td>5'--GGGGACCAGCTCATGGTGG-3'</td>
<td>25, 30, 31, 32</td>
</tr>
<tr>
<td></td>
<td>gltA</td>
<td>379 bp</td>
<td>BhCS.113</td>
<td>5'--AATGCAAAAAGGACGTTAACA-3'</td>
<td>30, 31, 33</td>
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<td></td>
<td>gltA</td>
<td>915 bp</td>
<td>BvCS.205</td>
<td>5'--TTTATCGGTTATCTCTATYG-3'</td>
<td>32</td>
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<td></td>
<td>gltA</td>
<td>249 bp</td>
<td>strat1</td>
<td>5'--GGGGACCAGCTCATGGTGG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gltA</td>
<td>249 bp</td>
<td>strat2</td>
<td>5'--GCGTGATAGCAATATCAGAAGTGG-3'</td>
<td></td>
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<tr>
<td></td>
<td>ITS</td>
<td>190 bp</td>
<td>321s</td>
<td>5'--AGATGATGATCCCAAGCTCTGG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ITS</td>
<td>190 bp</td>
<td>H493as</td>
<td>5'--TGAACCTCCGACCTCACGCTTATC-3'</td>
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<tr>
<td></td>
<td>rpoB</td>
<td>825 bp</td>
<td>1400F</td>
<td>5'--CGCATTTGCTTACTCTGTATG-3'</td>
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<tr>
<td></td>
<td>rpoB</td>
<td>825 bp</td>
<td>2300R</td>
<td>5'--GTAGACTGATTAGAACGCTG-3'</td>
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<td></td>
<td>16S-23S rRNA</td>
<td>500-800 bp</td>
<td>325 s</td>
<td>5'--CTTCAGATGATGATCCCAAGCTTCTGGGC-3'</td>
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<tr>
<td></td>
<td>16S-23S rRNA</td>
<td>500-800 bp</td>
<td>1100as</td>
<td>5'--GAACCGACGACCCCCTGCTTGCAAAGCA-3'</td>
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<tr>
<td>B. henselae</td>
<td>ribC</td>
<td>588 bp</td>
<td>BARTON-1</td>
<td>5'--TAACCGATATTGGTTGTTGGAAG-3'</td>
<td>36</td>
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<td></td>
<td></td>
<td>585 bp</td>
<td>BARTON-2</td>
<td>5'--TAAAGCTAGAAAGTCTGGCAACATAACG-3'</td>
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<td>B. clarridgeiae</td>
<td>16S-23S rDNA</td>
<td>186 bp</td>
<td>P-bhenfa</td>
<td>5'--TCTTCCGTTTCTTTCTTCA-3'</td>
<td>37, 38</td>
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<td></td>
<td></td>
<td>168 bp</td>
<td>P-benhrl</td>
<td>5'--CAAGCGCGCGCTCTAACC-3'</td>
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<td>B. henselae</td>
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<td>152 bp</td>
<td>N-bhenfla</td>
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<td></td>
<td>134 bp</td>
<td>N-bhenr</td>
<td>5'--AACCAACTGAGCTACAAGGC-3'</td>
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<td>B. clarridgeiae</td>
<td>16S-23S rRNA</td>
<td>145 bp</td>
<td>B1623R</td>
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<td>B. henselae</td>
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<td>163 bp</td>
<td>JEN1F</td>
<td>CTCTTCTCAGTGATGATCC</td>
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REFERENCES


