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First molecular detection of *Babesia canis* in dogs from Bosnia and Herzegovina

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ABSTRACT

Babesia spp. are tick-transmitted protozoan haemoparasites of great economic, veterinary and medical impact worldwide. Herein we reported the very high prevalence of autochthonous babesiosis in symptomatic dogs from Bosnia and Herzegovina in the period from 2014 to 2016. Eighty dogs that did not leave the country were examined using parasitological and molecular analyses and babesiosis was diagnosed in 82.5% and 85.0% of them, respectively ($p < 0.001$). One species, *Babesia canis* was identified using molecular methodology (PCR and sequence analysis). Statistical analyses showed that epizootiological characteristics have no influence on the possibility of infection. Agglomerative hierarchical clustering (AHC) analyses used for comparing the symptoms and clinical signs of infection in dogs pointed out that a high degree of anemia, followed by thrombocytopenia (89%), lethargy (100%), loss of appetite (95%), fever (66%) and icterus (61%) was dominant. In addition, results of the statistical analysis performed showed that more dogs with no data of tick prophylaxis (70%) were found *Babesia* infected. Those results point to further intensified epizootic surveys in the territory of Bosnia and Herzegovina.

1. Introduction

Babesiosis is one of the worldwide-distributed dog infections caused by intracellular haemoprotozoan parasites of the genus *Babesia* (Lobetti, 1998; Boozer and Macintire, 2003; Solano-Gallego and Baneth, 2011). The disease can be caused by large (4–5 µm) or small (1–2.5 µm) *Babesia* species transmitted by ixodid ticks. According to the genetic characteristics, severity of clinical manifestations, different tick vectors and geographic distribution, large *Babesia* spp., previously considered to be *B. canis*, are divided into three separated species: *Babesia canis, Babesia vogeli* and *Babesia rossi* (Zahler et al., 1998; Carret et al., 1999; Caccío et al., 2002; Solano-Gallego and Baneth, 2011; Solano-Gallego et al., 2016.). *B. canis,* transmitted by *Dermacentor reticulatus,* is the most common *Babesia* species in Europe. In tropical and subtropical regions, *B. vogeli's* vector is *Rhipicephalus sanguineus* sensu lato, whereas in South Africa, the canine babesiosis caused by *B. rossi* is transmitted by *Haemaphysalis elliptica* (formerly *H. leachi*). *B. gibsoni, B. conradae* and *B. microti*-like are small piroplasms, which can also cause babesiosis in dogs (Conrad et al., 1991; Zahler et al., 2000; Camacho et al., 2001; Kjemtrup et al., 2006; Apanaskevich et al., 2007).

Canine babesiosis is classified as uncomplicated or complicated (Jacobson and Clark, 1994), based on severity of the infection and clinical manifestations. Parasites cause red blood cell lysis, leading to hemolytic anemia. However, there are several clinical forms with a panel of different symptoms and complications affecting numerous organ systems. Also, it can cause two syndromes - systemic inflammatory respond syndrome and/or multiple organ dysfunction syndrome, which could include acute renal failure, coagulopathy, cerebral babesiosis, hepatopathy, acute respiratory distress syndrome, hypotension, hemoconcentration, acute pancreatitis, acid-base balance disturbance, and consequential cardio vascular system dysfunction (Conrad et al., 1991; Wozniak et al., 1997; Jacobson and Clark, 1994; Lobetti, 1998; Welzl et al., 2001; Leisewitz et al., 2002; Máthé et al., 2006; Schetters et al., 2009; Matijatko et al., 2009). Due to the diversity of clinical findings in the diagnosis of babesiosis, it is important

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to identify the intraerythrocytic parasites in blood smears using Giemsa or Wright staining (Irwin and Hutchinson, 1991). However, microscopic tests could give false results, especially when the parasitemia level is low (Scurrell, 2006). Serological analysis is a very useful diagnostic method, but has its limitations, due to a cross-reactivity between different Babesia species (Birkenheuer et al., 2003; Scurrell, 2006) and it cannot differentiate the acute infection from a prior exposure to the parasite (Shaw et al., 2001). Molecular diagnostic methods (e.g. PCR) are sensitive, specific and the most reliable ones for babesial DNA detection in blood (Caccío et al., 2002; Inokuma et al., 2003; Oyamada et al., 2005; Földvári et al., 2005).

Data related to *Babesia* infections in Bosnia and Herzegovina were provided by Hodžić et al. (2015), on the basis of molecular characterization and detection of natural *Babesia* infection (*B. canis* and *B.cf microti*) in the spleen of red foxes. There are no genetic research data on babesiosis, despite a high number of clinical cases diagnosed in dogs from Bosnia and Herzegovina. Given those facts, the aim of this study was to identify the agent of clinical canine babesiosis in the Sarajevo area, Bosnia and Herzegovina, thereby contributing to knowledge about their distribution in Europe, but also to the better characterization of symptoms and clinical signs of canine babesiosis caused by *B. canis*.

2. Materials and methods

2.1. Study area

The survey was conducted in Sarajevo Canton, Bosnia and Herzegovina (43°51′N18°15′E), during the two-year period, from 2014 to 2016.

2.1.1. Clinical examination and samples collection

Blood samples were collected from 80 dogs (53 males and 27 females), aged 2 months to 13 years, with typical clinical signs of babesiosis (fever, loss of appetite, dark urine, lethargy). The dogs were presented to the Clinic for Internal Diseases of the Veterinary Faculty in Sarajevo, for clinical examination. Basic information, including breed (Table 1), gender, age, tick infestation, tick prophylaxis and travel history, were obtained from their owners.

Samples for hematological analysis were collected from the cephalic vein and placed in tubes. EDTA and hematological values were determined using a LaserCyte® Hematology Analyzer (IDEXX Laboratories). Blood samples for parasitological and molecular analyses were collected from the earlobe of each animal, whilst 300–500 μL of the blood were used to impregnate filter papers for molecular analyses.

Table 1
Dog breeds included in the study.

Breed	N
Mixed	21
Golden retriever	9
Pekinese	6
Belgian shepherd	6
German shepherd	4
Poodle	3
English cocker spaniel	3
Rottweiler	2
West highland white terrier	2
Labrador retriever	2
Puli	2
Shih tzu	2
Bosnian and Herzegovinian-Croatian shepherd dog	2
German boxer, Miniature schnauzer, Samoyed, Kangal, Yorkshire terrier, Lhasa apso, Tibetan spaniel, Doberman, Great dane, Bernese Mountain Dog, English setter, Staffordshire bullterrier, American pit bull terrier, Shar pei, Bichon Frise, Central Asian Shepherd Dog	16[a]
Total number	**80**

[a] One dog per breed.

2.1.2. Parasitological analysis

Two thin blood smears were prepared for each dog and those were air-dried, stained with Diff Sem Quik Cit (Semikem, Sarajevo, Bosnia and Herzegovina), modified with Giemsa-May-Grünwald staining and examined under the light microscopy with $\times 1000$ magnification (100 fields per smear).

2.1.3. Molecular analysis

DNA was extracted from dried blood spots on a filter paper using the protocol reported by Plowe et al., 1995. Diameter circles (3 × 3 mm) of each dried blood spot were placed briefly in 1 mL of 0.5% saponin, in phosphate-buffered saline (PBS), inverted 2–3 times and incubated overnight at 4 °C. The brown solution was aspirated and replaced with 1 mL of PBS, and the tube was inverted again and incubated for 15–30 min at 4 °C. During this step, 50 μL of a stock solution of 20% Chelex-l00 (Bio Rad, Richmond, CA) in water was added to 150 μL of water in a 0.65-mL tube and heated to 100 °C in a thermal cycler. After aspiration of the PBS, the strips were placed in the hot Chelex, vortexed at high speed for 30 s, and replaced in the cycler with a brief vortex again, at 100 °C for 10 min, once during and once after the incubation. After centrifugation at 10.000 × *g* for 2 min, the supernatant was recovered, centrifuged again under the same conditions, and collected into a new tube. Supernatants were used for the PCR procedure, which was performed using the primers CRYPTO F (5′-AACCTGGTTGATCCT GCCAGT-3′) (Herwaldt et al., 2003) and RLB-R2 (5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) (Georges et al., 2001) that amplify a fragment of approximately 800 bp of the piroplasm 18S ribosomal RNA.

Amplifications were performed in a volume of 25 μL containing: 0.2 μL DNA Polymerase (Bioline, Italy), 0.5 μL of each primer (50 mM), 3 μL of template (25 ng/μL), 2.5 μL of dNTP (10 mM), 2 μL of $MgCl_2$ (50 mM), 2.5 μL PCR Buffer (10X) and 13.8 μL of ddH2O. All PCR reactions included positive controls (identified by our group in previous works; Cassini et al., 2009) and negative controls (sterile deionized water).

PCR conditions included 95 °C for 10 min and were followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 1.5 min. The final extension was done at 72 °C for 7 min, followed by a hold step at 4 °C.

Amplification products were electrophoresed in 1.5% agarose gel stained with ethidium bromide 5 μg/mL. Amplicons were purified using the SureClean kit (Aurogene, Rome, Italy), following the manufacturer's instructions, and directly sequenced with PCR primers in both directions by an external sequencing core service (Eurofins Genomics, Anzinger, DE). Sequences obtained were corrected by visual analysis of the electropherograms, aligned using ClustalW program (Larkin et al., 2007) and compared both mutually and with those available in GenBank™ dataset by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.1.4. Statistical analysis

General and clinical data collected from 80 dogs' medical history, were statically analyzed using SPSS (version 21.0, IBM Corp, 2012) and GraphPad Prism (version 5.03, San Diego, CA, USA). They were further compared using Fishers exact (in cases where 0 is present in one of the cells) and Chi-square tests. The performed analyses were carried out in order to determine whether there are differences in epizootiological characteristics between PCR positive and negative dogs. Agglomerative hierarchical clustering (AHC) and the method utilized the general and clinical data as the original variables [the values were taken to be 1 if positive and 0 if negative; in the case of anemia the positivity was taken to be 3 (severe), 2 (moderate) and 1 (mild); in terms of dogs age the values were 4 (> 10 years), 3 (5–10 years), 2 (1–5 years) and 1 (0–12 months)]. AHC was determined using Pearson dissimilarity – where the aggregation criterion was a simple linkage, unweighted pair-group average and complete linkage – and Euclidean distance – where the aggregation criterion were weighted pair-group average, unweighted

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