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Case report

THE FIRST CLINICAL CASE OF *BABESIA VOGELI* INFECTION IN A DOG FROM SERBIA

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The city of Belgrade, Serbia, with its continental climate and suburban green spaces, is an endemic region for canine babesiosis. Molecular analyses showed that the clinical manifestation of the disease is dominantly caused by *Babesia canis*, transmitted by a winter tick *Dermacentor reticulatus*. Thus, the occurrence of canine babesiosis is typically seen in winter and springtime. The presented case demonstrates for the first time an active infection with *Babesia vogeli* during the summer, in an elderly dog from Belgrade, without a previous history of travel. The patient was presented at the Faculty of Veterinary Medicine, Belgrade, with respiratory problems and thrombocytopenia. The Romanowsky-stained blood smears revealed unusually large babesia merozoites and microfilaria presence, and the dog tested positive for *Dirofilaria immitis* antigens. The patient was treated against both infections, with positive outcomes. After performing DNA extraction and sequencing, the singular *B. vogeli* infection was demonstrated. Although being one of the least pathogenic babesia species, the data regarding an active infection with *B. vogeli* in the Belgrade region is medically and epidemiologically significant. This report shows that canine babesiosis due to infection with *B. vogeli* during the summer months, when the tick vector *Rhipicephalus sanguineus* is active, should be considered in a differential diagnostic plan. Also, it is important to perform molecular diagnostics to *B. vogeli* in dogs that don't have the typical acute phase response, seen in *B. canis* infection, but have thrombocytopenia.

Keywords: blood smear, large babesia, microfilaria, molecular identification, *Rhipicephalus sanguineus*, thrombocytopenia

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INTRODUCTION

The Belgrade region, Serbia, has been identified as endemic for canine babesiosis [1,2]. Molecular analysis revealed that *Babesia canis* is the dominant babesia species, causing vigorous acute clinical disease with marked thrombocytopenia, or even pancytopenia in mono-infections [3], and co-infections with *Dirofilaria immitis* [4]. The majority of cases are diagnosed in winter and springtime [5]. Opposite to *B. canis*, only several clinical cases of infection with *Babesia gibsoni* have been detected in the Belgrade region [3]. In Europe, other *Babesia* spp. causing infections in dogs are *Babesia vogeli*, and *Babesia vulpes* [6]. In Mediterranean countries, *B. vogeli* has been identified in asymptomatic dogs and dogs showing signs consistent with canine babesiosis [7]. In all studies, the prevalence of infection with *B. vogeli* is lower than the prevalence of infection with *B. canis* [8], but these results might not reflect the true situation due to the cryptic/invisible nature of the infection. With low pathogenicity, *B. vogeli* causes asymptomatic infection in immunocompetent adult dogs and leads to overt clinical signs only in young and immunocompromised older dogs [9]. Noteworthy, clinical cases of canine babesiosis due to infection with *B. vogeli* were not reported in Belgrade and/or Serbia, until the present. However, epidemiological studies showed that only a small number (1.9%) of asymptomatic owner dogs from the northern Serbia have *B. vogeli* DNA material [10], and that a substantial number (13.5%) of dogs are seroreactive against *B. vogeli* [1].

Examination of Romanowsky-stained blood smears is frequently used to diagnose overt canine babesiosis, without a possibility to distinguish *B. vogeli* from *B. canis*. However, the presence of these two babesia species in the continental Belgrade climate, and the investigation of the potential pathogenic effect of *B. vogeli* is of clinical importance. The recorded case focuses on a geriatric dog from Belgrade suburb, which presented at the veterinary clinic during the summer, with mild clinical signs and thrombocytopenia, and a blood smear positive for large *Babesia* spp.

CASE PRESENTATION

Clinical examination

In July 2022, an eleven-year-old mix-breed, female outdoor dog, without prior history of travel, was presented at the Faculty of Veterinary Medicine, University of Belgrade, Serbia. The owner reported that the dog first had decreased appetite, and the day before the presentation, finally ceased eating and drinking water, while breathing heavily. The dog had a history of recurrent vomiting during the previous month, but after changing the diet, the problems were solved. The owner noted the left eye being "reddish". The patient was regularly vaccinated and treated against ecto- and endoparasites, with an unknown formulation, and ticks were never found on the dog.

Clinical examination revealed a body temperature of 39°C, without dehydration (< 5%). Lymph nodes were unchanged. Capillary refill time was 2.5 seconds (reference interval: 1-2 seconds), and a pale buccal mucosa was noted. The dog had a normal heart beat (less than 140 beats/min) and a slightly increased respiratory rate (above 40 breaths/min). Additionally, ectropion of the lower left eyelid was diagnosed.

Laboratory analysis

A whole blood sample was collected from the cephalic vein, and the complete blood count was assessed using Mindray BC-5000 Vet analyzer (Mindray, China). Results showed mild monocytosis and moderate thrombocytopenia (Table 1).

Table 1. Hematology results, units and reference range of the tested parameters (Mindray BC-5000 Vet, Mindray, China).

Analyte	Result	Unit	Reference Range
White blood cell count	10.57	10^9/L	5.05 – 16.76
Neutrophils	6.67	10^9/L	2.95 – 11.64
Lymphocytes	2.22	10^9/L	1.05 – 5.10
Monocytes	**1.54 ↑**	10^9/L	0.16 – 1.12
Eosinophils	0.09	10^9/L	0.06 – 1.23
Basophils	0.05	10^9/L	0.00 – 0.10
Neutrophils	63.1	%	
Lymphocytes	21.0	%	
Monocytes	14.6	%	
Eosinophils	0.8	%	
Basophils	0.5	%	
Red blood cell count	5.99	10^{12}/L	5.65 – 8.87
Hemoglobin concentration	141	g/L	131 – 205
Hematocrit	39.0	%	37.0 – 61.7
MCV	65.1	fL	61.6 – 73.5
MCH	23.5	pg	21.2 – 25.9
MCHC	361	g/dL	320 – 379
RDW-CV	**13.3 ↓**	%	13.6 – 21.0
RDW-SD	35.4	fL	
Platelet count	**55 ↓**	10^9/L	148 – 484
MPV	9.1	fL	8.7 – 13.2
PDW	18.5		9.1 – 19.4
Plateletcrit	**0.050 ↓**	%	0.140 – 0.460

Abbreviations: **MCV**, mean cellular volume; **MCH**, mean cellular hemoglobin; **MCHC**, mean cellular hemoglobin concentration; **RDW-CV**, red cell distribution width – coefficient of variation; **RDW-SD**, red cell distribution width – standard deviation; **MPV**, mean platelet volume; **PDW**, platelet distribution width.

Duplicate Romanowsky-stained blood smears (Bio-Diff, Biognost, Croatia), were analyzed microscopically (Olympus CX21, Olympus Life Science, Japan), at 100× magnification. The smears confirmed decreased platelet count, and revealed the presence of activated monocytes and erythrophagocytosis, without polychromatophilia. Unusually large babesia merozoites (1-4) were found in the red blood cells (Figure 1).

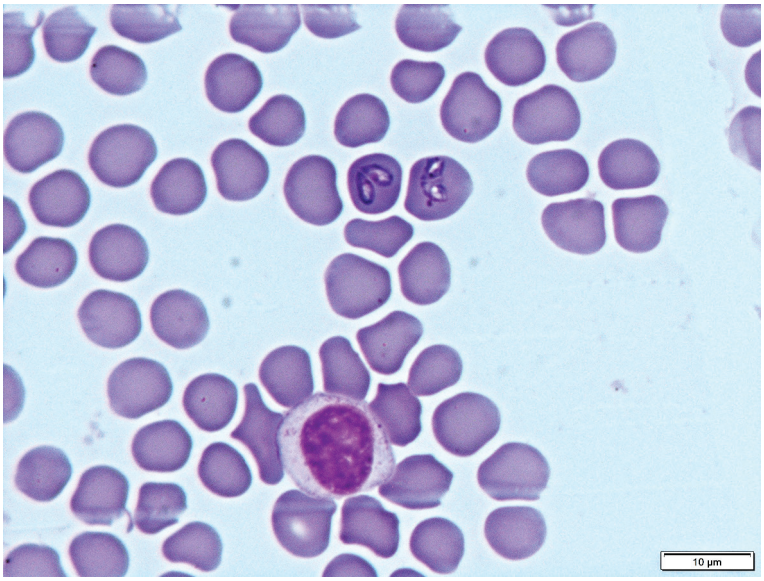

Figure 1. Two red blood cells with large babesia merozoites (up), and a lymphocyte (down). Bio-Diff stain (Biognost, Croatia), Olympus CX21 (Olympus Life Science, Japan), 100× magnification.

As microfilaria was also detected on the blood smear (Figure 2), *D. immitis* antigens were confirmed by rapid CaniV-4 Test Kit 2.0 (Bio-Note, Korea). The same rapid test resulted as antibody negative for *Anaplasma* spp., *Ehrlichia canis* and *Borrelia burgdorferi.*

After confirming the infection with *Babesia* spp., the patient was treated with the initial dose of imidocarb dipropionate (6.6 mg/kg, s.c, ImoChem, Interchemiewerken "De Adelaar", Holland), clavulanate-potentiated amoxicillin (8.75 mg/kg, s.c., Synolux, Zoetis, UK), and metoclopramide (0.3 mg/kg, i.v., Klometol, Galenika, Serbia).

When completing the therapy against babesiosis, the patient was further examined to determine the severity of the diagnosed heartworm infection. The dog was categorized between mild (class 1) and moderate form (class 2), and underwent a "slow-kill" treatment against *D. immitis* infection, under American Heartworm Society Guidelines [11]. A two-year follow-up of the patient confirmed the resolution of the disease.

The dog owner signed an informed consent that allows the use of the residual blood sample and scientific publication of obtained results.

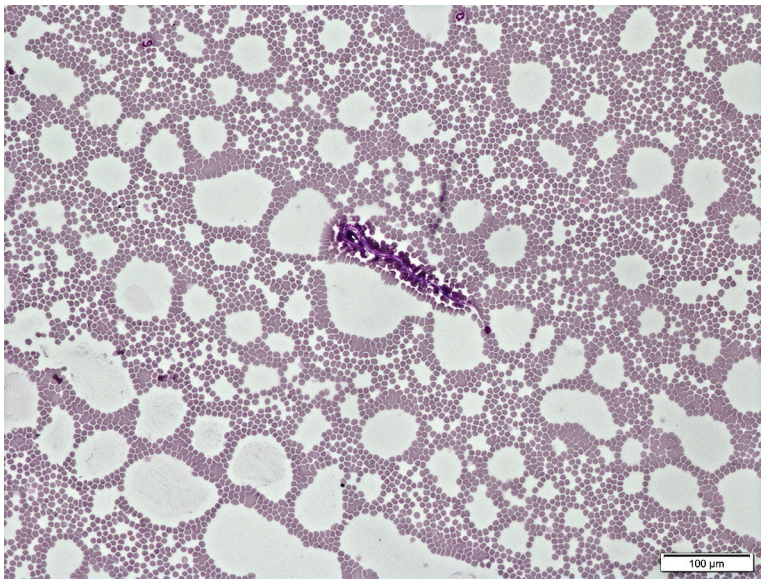

Figure 2. Microfilaria. Bio-Diff stain (Biognost, Croatia), Olympus CX21 (Olympus Life Science, Japan), 10× magnification.

Extraction and PCR detection

From the blood sample, DNA was extracted using Thermo Scientific™ GeneJET Whole Blood Genomic DNA Purification Mini Kit, according to the manufacturer's recommendations. To identify babesia parasites, PCR was conducted with specific primers PIRO–A (5'–AATACCCAATCCTGACACAGGG–3') and PIRO–B (5'–TTAAATACGAAT GCCCCCAAC–3'), targeting a 410 bp fragment of the 18S–rRNA gene of *Babesia* spp. PCR reactions were conducted in 25 µL volumes, comprising 1 × PCR buffer (Kapa Biosystems), 1.5 mM $MgCl_2$ (Kapa Biosystems), 100 µM dNTP (Kapa Biosystems), 2 µM of each primer, 0.5 U of Taq polymerase (Kapa Biosystems), and 5 µL of template DNA. Amplification conditions consisted of an initial DNA denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Amplification products were separated on a 2% agarose gel stained with ethidium bromide and visualized under UV light, with a commercial O'RangeRuler™ 100 bp DNA Ladder (Fermentas) serving as a size marker. PCR results showed positive for *Babesia* spp., with a length of approximately 405 bp.

To exclude other infectious agents, *Anaplasma platys*, *A. phagocitophylum* and *E. canis*, PCR analyses were performed, yielding negative results.

Sanger sequencing

After confirmation to be positive, the sample underwent additional purification utilizing the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, this sample was subjected to bidirectional sequencing through a sequencing service (Macrogen Europe, Amsterdam, Netherlands). Quality checks were carried out on the obtained sequences, with those achieving quality scores above 30 considered suitable for further analysis. The initial steps included removing primer sequences through the 'Trim Ends' functionality found in the Geneious Prime software (Dotmatics, Boston, MA, USA). A high-quality consensus sequence was derived using the software's 'Generate consensus sequence' feature, setting the quality threshold at 75%. The high-quality sequence was then submitted to the National Center for Biotechnology Information's GenBank database (NCBI). In this research, the sequence generated was compared against 67 sequences of different *Babesia* spp. 18s rRNA available in the NCBI database. This was followed by the alignment of the sequences, utilizing the MAFFT algorithm. Phylogenetic evaluation was conducted using the Molecular Evolutionary Genetic Analysis software, MEGA X. The analysis utilized the Maximum Likelihood approach and the Jukes-Cantor, selected through the "Find Best DNA/Protein model" function available in MEGA X (Figure 3).

The sequence was submitted to GenBank (NCBI) under accession number PP504866 (*Babesia_vogeli*_isolate_SRB_Mona_3137). The sequence was identified using a BLAST search provided by NCBI. The highest percentage of similarity was found with sequences MN625891.1 (Egypt), OP604259.1 (Egypt), OP604258.1 (Egypt), HQ662635.1 (Romania), AM183215.1 (Turkey), and AY371197.1 (Egypt), exhibiting 100% query coverage and a 99.47% identity match. When comparing our sequence to those from the neighboring countries, a 99.47% identity was recorded with sequences from Slovenia and Croatia (AY072925, FJ200218) and a 99.36% identity with sequences from Romania (JF461252.1) with a 100% query coverage. The phylogenetic analysis of the 18S rRNA sequence segment grouped the isolate *Babesia_vogeli*_isolate_SRB_Mona_3137 with other representatives of *B. vogeli* species, forming a distinct branch separate from *B. canis* and *B. gibsoni* species.

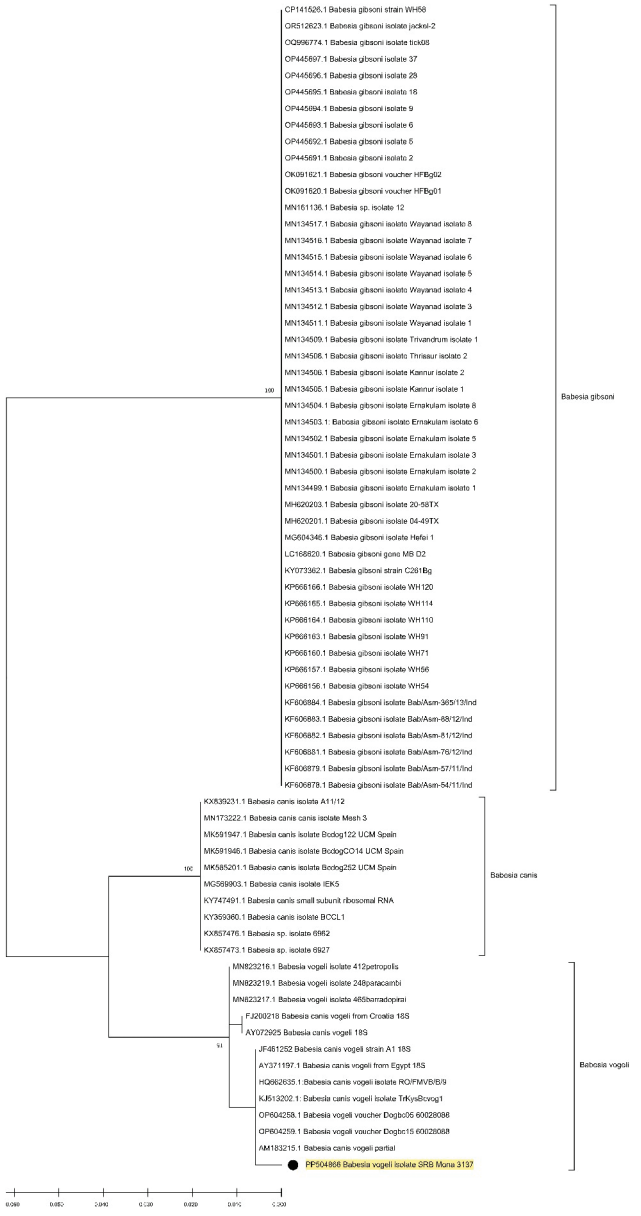

Figure 3. A phylogenetic tree showing the genetic relationships between the Serbian strain (PP504866) and strains from the NCBI representing other strains of *Babesia* spp. MEGA X software was utilized for this investigation, employing the Maximum Likelihood Method along with the Jukes-Cantor model. To enhance precision, the study incorporated 1000 bootstrap replicates, incorporated a Gamma distribution to accommodate variable rates across sites (+G) within five rate categories. Branches lacking support in at least 70% of the bootstrap replicates were condensed. The software's "Find best DNA/Protein Models" feature was employed to determine the most appropriate analysis model. Sequences originating from Serbia were marked with a black dot and highlighted.

DISCUSSION

The case of a senior dog with thrombocytopenia and ectropion that presented in the summer month, and was diagnosed to have co-infection of *B. vogeli* and *D. immitis*, underlines the importance of recognizing the pathogenic effect of babesia species other than *B. canis*. This is also the first case of symptomatic *B. vogeli* infection described in Serbia, which has a continental climate. The *Babesia vogeli* isolate SRB_Mona_3137 exhibits a sequence that is distinct from other sequences reported for this pathogen and is unique to Serbia, having not been previously described. Moreover, the phylogenetic analysis of the sequenced region does not provide sufficient resolution for further classification within the *B. vogeli* species.

Epidemiologically, this case complements other *B. vogeli* isolated clinical cases described in dogs in the neighboring countries: Croatia [12], Slovenia [13], and Turkey [14], pointing to a low pathogenicity of the parasite in this region.

Seasonality investigation of canine babesiosis in a study of 872 cases confirmed by blood smear examination, demonstrated that only several cases are documented during the summer, whereas the majority were recorded between mid-February and mid-May [5]. It is possible that those several cases were consequences of infection with *B. vogeli*, as its tick vector *Rhipicephalus sanguineus*, is active at a temperature range typical for the summer season [15]. Frequently found in the Mediterranean region [16], *R. sanguineus* is spreading into countries with continental climates such as Serbia [15], Romania [17], and Hungary [18], reflecting the emerging nature of this tick as a possible consequence of climate change, global warming, and intensive dog traffic specifically related to the adoption of outdoor dogs from the Mediterranean region.

Clinically, the case is presented as lack of appetite and acute breathing problems, without fever. Breathing problems could be ascribed to respiratory or cardiovascular pathology due to *D. immitis* infection, as the clinical examination after *B. vogeli* resolution confirmed a mild to moderate form of heartworm disease. However, thrombocytopenia is most probably the consequence of *B. vogeli* infection. Other cases of *B. vogeli* infection report regenerative anemia, leukocytosis and thrombocytopenia [7,19]. In cases of puppies and juvenile dogs, severe to fatal hemolytic anemia occurs [9]. The dog presented in this case had the total white blood cell count within the reference interval, but mild monocytosis, with activated and vacuolized monocytes, and erythrophagocytosis indicating an ongoing process of mild hemolysis, without anemia. Erythrophagocytosis is a rare phenomenon in the peripheral blood and usually indicates auto-immune hemolytic disease [20]. In contrast to the presented case, acute *B. canis* and asymptomatic *D. immitis* co-infection led to a more severe form of anemia than singular *B. canis* infection [4].

The DNA of *Anaplasma* spp. and *E. canis* was not detected in the blood of the patient, thus excluding acute infection with these two rickettsial pathogens. Namely, *R. sanguineus* also transmits *E. canis*, a pathogen that could lead to similar clinical and

laboratory findings [21]. However, both natural and *in vivo* experimental infections of dogs with *B. vogeli* indicate the pathogen's tendency towards the subclinical form [7,19].

CONCLUSION

Clinicopathological data on canine babesiosis is medically and epidemiologically significant. This report shows that canine babesiosis due to infection with *B. vogeli* during the summer months, when the tick vector *R. sanguineus* is active, should be considered in a differential diagnostic plan. Also, it is important to perform molecular diagnostics of *B. vogeli* in dogs that don't have the typical acute phase response, seen in *B. canis* infection, but have thrombocytopenia.

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The sequences were generated through the Sequencing Service of the Animal Production and Health Sub–Programme of the Joint FAO-IAEA Centre in Vienna, Austria.

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Authors' contributions

KS performed the complete hematology analysis, DD, DG and FJ performed PCR analysis, PS was the lead clinician, KS, DD and DG wrote the manuscript, and MKF revised and edited the manuscript. All authors have read and approved the manuscript.

Declaration of conflicting interests

The authors do not have any financial or personal conflicts of interest that could bias the study.

Statement of informed consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria Beograd.

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PRVI SLUČAJ KLINIČKE MANIFESTACIJE *BABESIA VOGELI* INFEKCIJE KOD PSA IZ SRBIJE

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Kontinentalna klima i zelenilo predgrađa čine grad Beograd endemskim područjem za babeziozu pasa. Molekularne analize su pokazale da kliničku sliku bolesti dominantno izaziva *Babesia canis*, koju prenosi zimski krpelj *Dermacentor reticulatus*. Prema tome, babezioza pasa se najčešće dijagnostikuje tokom zime i u rano proleće. Prikazani slučaj po prvi put opisuje aktivnu infekciju vrstom *Babesia vogeli,* tokom leta u Beogradu, kod starijeg psa, koji prethodno nije nigde putovao. Pacijent je pregledan na Fakultetu veterinarske medicine u Beogradu, gde je ustanovljeno otežano disanje, kao i trombocitopenija. Neobični merozoiti velike babezije i mikrofilarije su utvrđeni na krvnim razmazima obojenim *Romanowsky* tipom bojenja, a brzi dijagnostički test bio je pozitivan na prisustvo antigena *Dirofilaria immitis*. Pacijent je uspešno terapiran protiv obe infekcije. Ekstrakcija DNK i sekvenciranje su potvrdili infekciju uzročnikom *B. vogeli*. Premda je reč o vrsti babezije koja spada u najmanje patogene, podaci o aktivnoj *B. vogeli* infekciji u Beogradu imaju medicinski i epidemiološki značaj. Prikaz datog slučaja

upućuje da babezioza, prouzrokovana vrstom *B. vogeli* kod psa, tokom letnjih meseci, kada je vektor *Rhipicephalus sanguineus* aktivan, treba biti uključena u diferencijalni dijagnostički plan. Takođe, važno je sprovoditi molekularnu dijagnostiku *B. vogeli* kod pasa koji nemaju tipičan odgovor akutne faze, karakterističan za *B. canis* infekciju, a imaju trombocitopeniju.