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FIRST EVIDENCE OF TICK-BORNE PROTOZOAN PATHOGENS, *BABESIA* SP. AND *HEPATOZOON CANIS,* IN RED FOXES (*VULPES VULPES*) IN SERBIA

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Tick-borne haematozoans cause severe diseases in domestic animals, and some of them have zoonotic potential. The results of previous studies in Europe point to the important role of foxes in natural endemic cycles of several tick-borne pathogens, including protozoa. The aim of the present research was to acquire information on the prevalence and distribution of tick-borne protozoan parasites among foxes in Serbia. Legally hunted foxes from 14 localities throughout Serbia were analysed. Spleen samples were collected from 129 animals and tested for the presence of *Babesia* spp. and *Hepatozoon* spp. by PCR. In total, 79/129 (61.2%) of the tested foxes were positive for *H. canis,* while the presence of two *Babesia* species was confirmed: *B. vulpes* (37/129, 28.7%) and *B. canis* (1/129, 0.8%). Co-infection with *B. vulpes* and *H. canis* was present in 26/129 (20.2%) foxes and one animal (1/129, 0.8%) was co-infected by *B. canis* and *H. canis*. The results of this study indicate the important role of foxes in the epizootiology of *B. vulpes* and *H. canis* in the Republic of Serbia and stress the need for further research to clarify all elements of the enzootic cycle of the detected pathogens, including other reservoirs, vectors, and transmission routes.

Key words: *Babesia canis*, *Babesia vulpes*, *Hepatozoon canis*, PCR, red fox, Serbia

Tick-borne protozoan pathogens are the causative agents of severe diseases in domestic animals, and some of them have zoonotic potential as well (Solano-Gallego et al., 2016). They are maintained in nature in enzootic cycles that include ticks and different vertebrates as hosts. Thus, from the epizootiological point of view, knowledge about elements of the enzootic cycles of a particular

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pathogen in a given area is of great importance. The diseases caused by protozoa of the genus *Babesia* are highly important in domestic animals. Canine babesiosis in Europe is associated with four *Babesia* species: *B. canis* and *B. vogeli* (traditionally referred as 'large' babesial species); and *B. gibsoni* and *B. microti*-like ('small' babesial species) (Irwin, 2009). For *B. microti*-like, there is no formal valid description in accordance with the International Code of Zoological Nomenclature (James Harris, 2016), and numerous synonyms for this species exist in the literature, i.e., *Theileria annae*, *Babesia* 'Spanish dog isolate', *Babesia annae*, *Babesia* cf. *microti*, and the most recently suggested name *Babesia vulpes* sp. nov. (Baneth et al., 2015), which will be used below in order to avoid confusion. The occurrence of canine babesiosis in Europe is closely linked with the presence of tick species that parasitise domestic dogs, like *Dermacentor reticulatus*, *Rhipicephalus sanguineus* sensu lato (s.l.), *Haemaphysalis* sp., and *Ixodes ricinus*. The most common cause of canine babesiosis in Europe is *B. canis* and its presence in many European countries is related to the broad geographical distribution of the main tick vector, *D. reticulatus* (Petra et al., 2018). The second 'large' babesial species with clinical importance in European dogs, *B. vogeli*, is globally distributed (Irwin, 2009) and *R. sanguineus* s.l. is recognised as the main vector (Dantas-Torres and Otranto, 2015). Infection with *B. vogeli* can be subclinical but may also cause serious illness in infected dogs (Baneth, 2018). *Babesia gibsoni* is the most prevalent small *Babesia* species, with global distribution. It is associated with vectors from the genus *Haemaphysalis*, while *H. bispinosa* and *R. sanguineus* s.l. have been proposed as potential vectors (Baneth, 2018). Besides being vector-borne, for *B. gibsoni* vertical transmission and the possibility of direct infection by infected blood (e.g., through transfusion, fighting of dogs) has been proven (Birkenheuer et al., 2005). *Babesia vulpes* has been detected as a cause of canine babesiosis in several European countries (Miró et al., 2015). Epizootiological studies indicate that *I. hexagonus*, *I. canisuga*, *I. ricinus*, and *D. reticulatus* are candidates as vectors for *B. vulpes* (Najm et al., 2014). Babesiosis caused by *B. canis* and *B. gibsoni* has been described in Serbian dogs (Davitkov et al., 2015), while *B. vogeli*, *B. vulpes*, and the zoonotic but not canine pathogen *B. microti* were detected in healthy dogs using PCR (Gabrielli et al., 2015). Recently the DNA of *B. canis* (4.2%) has been detected in golden jackals (*Canis aureus*) from Serbia (Sukara et al., 2018).

Hepatozoon canis is a haematozoan parasite widespread among domestic and wild carnivores in Europe. Infection with *H. canis* in dogs is commonly subclinical, but in predisposed animals it can lead to severe disease (Baneth, 2011). Autochthonous dog hepatozoonosis is present in many European countries with a Mediterranean climate (e.g. Turkey, Bulgaria, Greece, Croatia, Italy, Portugal) (Baneth, 2011), while high prevalence of *H. canis* has been detected in foxes all over Europe (Hodžić et al., 2015). In Europe, *H. canis* is transmitted mainly by *R. sanguineus* s.l. (Dantas-Torres and Otranto, 2015). The presence of *H. canis*

in regions considered free of *R. sanguineus* s.l. suggests other (e.g. transplacental) transmission routes (Hodžić et al., 2018). So far in Serbia, the presence of *H. canis* has only been confirmed in a single healthy dog showing no clinical signs from the southern part of the country (Gabrielli et al., 2015).

Commonly, the same species of *Hepatozoon* and *Babesia* are found to infect wild canids and domestic dogs. Based on results obtained using an evolutionary approach, Penzhorn (2011) suggested that these haematozoan pathogens had been transmitted to dogs from wild canids. It therefore seems possible that these protozoan pathogens are transmitted by arthropod vectors from wild canids to domestic dogs in regions where ecological niches of wild and domestic canids overlap (Margalit Levi et al., 2018). In recent years, researchers across Europe have indicated the potential role of red foxes (*Vulpes vulpes*) as a reservoir for several tick-borne agents (Hodžić et al., 2015; Millán et al., 2016). Red foxes are the most widespread carnivore species in the world, widely distributed in the Northern Hemisphere and introduced elsewhere. They are highly adapted to living in anthropogenically modified habitats and appear to be closely associated with people. Today, increased numbers and densities of fox populations are documented in all European countries involved in rabies control programmes (including Serbia), since rabies virus has a great impact on the population dynamics of this species. With the recovery of European populations in rural regions, foxes established populations in major cities of countries in the continental part of Europe after the 1980s (Plumer et al., 2014). In Serbia, the range of foxes covers the whole territory of the country (Ćirović, 2000). In the countries bordering on Serbia, a relatively high prevalence of the haemoprotozoan parasites *Babesia* spp. and *H. canis* was confirmed by molecular methods in fox populations (Farkas et al., 2014, 2015; Hodžić et al., 2015). Research on the role of foxes in the epizootiology of *H. canis* and *Babesia* spp. has not been conducted so far in Serbia. Furthermore, there are no data on the prevalence of *H. canis* in other autochthonous wild canids (grey wolves, *Canis lupus*, and golden jackals, *C. aureus*) in Serbia. Therefore, the objective of the present study was to acquire information on the prevalence and distribution of *Babesia* spp. and *H. canis* among foxes in Serbia and evaluate the obtained results from the epizootiological point of view.

Materials and methods

Tissue samples were collected in co-operation with local hunters from legally hunted foxes over a period of seven years (2010–2016). In total, 129 animals were dissected and spleen samples were collected. The date of hunting, sex information, and location data were recorded for each fox sampled. All collected samples originated from 14 localities throughout Serbia (Fig. 1). The collected samples were transported at 4 °C to the Institute for Medical Research and stored

in a freezer (–80 °C) up to DNA extraction. The DNA extraction was performed using the Gene Jet Genomic DNA Purification Kit (Fermentas, Thermo Scientific). A small portion of deep-frozen spleen tissue (up to 10 mg) was homogenised by micropestles (Eppendorf™), and the extraction was carried out according to the manufacturer's instructions. The extracted DNA was stored at –20 °C until PCR analysis.

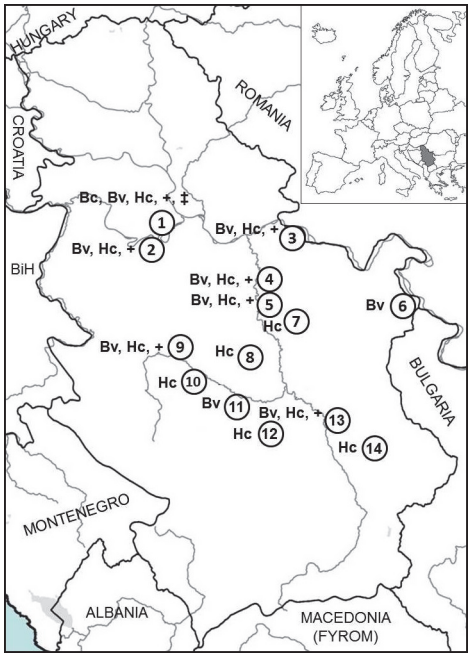

Fig. 1. Geographical distribution of foxes positive for, and co-infected with, *Babesia vulpes*, *Babesia canis* and *Hepatozoon canis* in the Republic of Serbia. 1 – Surčin, 2 – Obrenovac, 3 – Veliko Gradište, 4 – Velika Plana, 5 – Svilajnac, 6 – Negotin, 7 – Despotovac, 8 – Rekovac, 9 – Kraljevo, 10 – Vrnjačka Banja, 11 – Trstenik, 12 – Blace, 13 – Niš, 14 – Bela Palanka. Bc – *Babesia canis*, Bv – *Babesia vulpes*, Hc – *Hepatozoon canis*; +: co-infection with *Babesia vulpis* and *Hepatozoon canis*; ‡: co-infection with *Babesia canis* and *Hepatozoon canis*

PCR assay

Five individual randomly selected samples of the extracted DNA were combined into one pool. Whenever the pools were positive, the individual DNA samples were subjected to analysis. For initial detection of *Babesia* spp., we used the BabF (5'–GCGATGGCCCATTCAAGTTT–3') and BabR (5'–CGCCTG CTGCCTTCCTTAGA–3') primers to amplify a 146-bp fragment of the 18S ssrRNA gene (Theodoropoulos et al., 2006). For all positive samples, further PCR assays were performed with the BJ1 (5'–GTCTTGTAATTGGAATGA TGG–3') and BN2 (5'–TAGTTTATGGTTAGGACTACG–3') primers for amplification of a larger fragment (408 bp) of babesial 18S rRNA gene (Casati et

al., 2006). Detection of the DNA of *Hepatozoon* spp. in the tested samples was performed using the HepF_for (5'-ATACATGAGCAAAATCTCAAC–3') and HepR_rev (5'-CTTATTATTCCATGCTGCAG–3') primers, which amplify the 666-bp fragment of the 18S ssrRNA gene (Inokuma et al., 2002). The PCR reactions used in screening spleens for *Babesia* spp. and *Hepatozoon* spp. were prepared using PCR Master Mix (2X) (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Amplification of PCR products was performed in a Veriti Thermal Cycler device (Applied Biosystems).

In order to prepare *Babesia* spp. positive samples for sequencing, the BJ1 and BN2 primers were used in the present study. Amplifications were performed in a reaction mixture composed of 24.75 µl of nuclease-free water, 10 µl of 5X Reaction Buffer (7.5 mM MgCl2; pH 8.5), 1 µl of dNTPs (10 mM), 0.250 µl of Taq polymerase (5 u/µl, GoTaq G2 DNA Polymerase, Promega Corporation, USA), 4 µl of each primer (10 pmol/µl), and 6 µl of tested DNA. The amplification conditions were: initial denaturation step of 2 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for 1 min. Final extension was at 72 °C for 5 min. Samples positive for *Hepatozoon* spp. were prepared for sequencing using the primers HepF_for and HepR_rev, while the PCR reaction was set up in the same way as for *Babesia* spp. (GoTaq G2 DNA Polymerase, Promega Corporation, USA). Cycling conditions were 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 5 min.

Sequencing and sequence analysis

The purification and Sanger sequencing of obtained amplicons were performed by a commercial laboratory (Macrogen, Amsterdam, The Netherlands). The resulting sequences were processed in the FinchTV software (ver. 1.5.0) and compared with available sequences using BLAST in GenBank (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST). Representative sequences were deposited in the GenBank database.

Statistical analysis

Statistical analyses were performed using SigmaPlot version 12 for Windows (Systat. Software Inc., San Jose, CA, USA). The Shapiro–Wilk test was used to test for normal distribution of the data. The Mann–Whitney U test was used to test pathogen distribution according to the sex of foxes. P values less than 0.05 were considered statistically significant. Confidence intervals for proportion were calculated using an online calculator available at http://vassarstats.net/prop1.html.

Results

The conducted PCR assay revealed that 79/129 (61.2%) of the tested spleen samples were positive for *Hepatozoon* spp. DNA, while 38/129 (29.5%) samples were found to be positive for the presence of babesial DNA. After analysing the obtained sequences, the presence of two *Babesia* species was confirmed: *B. vulpes* in 37/129 samples [28.7%, 95% confidence interval (CI): 21.6–37%]; and *B. canis* in 1/129 samples (0.8%, 95% CI: 0.1–4.3%). No statistically significant differences in the prevalence of *B. vulpes* infections were detected between females (10.2%, 54/128) and males (18.8%, 74/128) (P = 0.900). All samples positive for the presence of DNA of *Hepatozoon* spp. were identified as *H. canis*, 79/129 (61.2%, 95% CI: 52.6%–69.2%). Also, no statistically significant differences in the prevalence of *H. canis* infections were detected between females (25.6%, 55/129) and males (35.65%, 74/129) (P = 0.760). Co-infections with *B. vulpes* and *H. canis* were present in 26/129 (20.2%) foxes, and their prevalence varied from 15.4% to 42.9% at different sampling sites. One animal was co-infected with *B. canis* and *H. canis* (1; 0.8%). Samples found to be positive for *B. vulpes* DNA originated from nine out of 14 localities, while animals positive for the presence of DNA of *H. canis* originated from a total of 12 of the 14 locations. The only fox positive for the presence of *B. canis* was shot at the Surčin locality (GenBank accession number: MH702200). Detailed results are presented in Table 1 and Fig. 1.

Table 1

Distribution of foxes positive for, and co-infected with, *Hepatozoon canis*, *Babesia canis* and *Babesia vulpes* by sampling site

No.	Locality	No. of collected samples	No. of positive samples				
			Hepatozoon canis (%)*	*Babesia canis* (%)	*Babesia vulpes* (%)	Co-infection *H. canis*–*B. canis* (%)	Co-infection *H. canis*–*B. vulpes* (%)
1	Surčin	29	15 (51.7)	1 (3.4)	15 (51.7)	1 (3.4)	10 (34.5)
2	Obrenovac	6	5 (83.3)		2 (33.3)		2 (33.3)
3	Veliko Gradište	45	28 (62.2)		9 (20)		7 (15.6)
4	Velika Plana	7	6 (85.7)		3 (42.9)		3 (42.9)
5	Svilajnac	13	11 (84.6)		3 (23.1)		2 (15.4)
6	Negotin	2			1 (50)		
7	Despotovac	1	1 (100)				
8	Rekovac	5	3 (60)				
9	Kraljevo	3	3 (100)		1 (33.3)		1 (33.3)
10	Vrnjačka Banja	5	2 (40)				
11	Trstenik	3			1 (33.3)		
12	Blace	3	1 (33.3)				
13	Niš	5	3 (60)		2 (40)		1 (20)
14	Bela Palanka	2	1 (50)				
Total number		129	79 (61.2)	1 (0.8)	37 (28.7)	1 (0.8)	26 (20.2)

*Prevalence

When the 18S rRNA sequences of *H. canis* obtained in this study were compared with the sequences available in GenBank, they showed 100% similarity to the sequences obtained in several European countries from different hosts, e.g. from foxes (AY150067, HM212626, GU371448, KM096414, KX887327), from golden jackals (KJ572976, KX712129, KX712123), from dogs (FJ497022, KY247115), and also from *R. sanguineus* (KY197000, KY196999). Representative sequences of *H. canis* obtained in the present study were deposited under accession numbers MH699884–MH699892.

The 18S rRNA sequences of *B. vulpes* obtained in this study (representative sequences were deposited under accession numbers MH699381–MH699396) showed complete mutual similarity, and 100% identity was evident when compared with previously deposited sequences available in GenBank from foxes living in different countries (e.g. Italy MG451839, Austria KY693667, Slovakia KX761397, Spain KT223483).

Discussion

In recent years, an increasing number of studies in Europe have pointed out the potential role of foxes as reservoirs for tick-borne pathogens (Tolnai et al., 2015; Hodžić et al., 2018). The present study reports for the first time the occurrence of *B. canis*, *B. vulpes* and *H. canis* in foxes in the Republic of Serbia. Co-infections of *H. canis* with *B. vulpes* or *B. canis* were also detected and the relatively high prevalence of *H. canis*/*B. vulpes* co-infection could be explained by similarities in the enzootic cycles of these protozoan agents.

Hepatozoon canis has been detected in many European countries in foxes with a wide range of prevalence (e.g. Portugal 75.6%, Austria 58.3%, Croatia 23%, Hungary 22.2%, Bosnia and Herzegovina 38.6%) (Deždek et al., 2010; Cardoso et al., 2014; Duscher et al., 2014; Hodžić et al., 2015). The prevalence of *H. canis* detected in our study (61.2%) is higher than in most of the other regions with the exception of Portugal. The high prevalence of *H. canis* among tested foxes and its wide geographical distribution in Serbia point to the important role of foxes in the epizootiology of this pathogen.

The tick *R. sanguineus* s.l., suggested as the main vector of *H. canis* (Baneth, 2011), is present and has been found to parasitise foxes in Serbia (Tomanović et al., 2013). However, infection of foxes with *H. canis* can be found in regions that are considered free of *R. sanguineus* s.l. (Duscher et al., 2014; Farkas et al., 2014), suggesting a potential role for other tick species in the transmission of *H. canis*. Fairly recently, Giannelli et al. (2013) have suggested that *I. ricinus* is not a competent vector of *H. canis*, while further research is needed to elucidate whether other tick species could be competent vectors of *H. canis* (Najm et al., 2014). Moreover, vertical and oral routes of infection by ingestion

of infected prey have been proposed as additional transmission pathways that need to be evaluated through further studies (Baneth, 2011).

Rhipicephalus sanguineus s.l. is an ectoparasite of dogs in Serbia (Potkonjak et al., 2016). The presence of *H. canis* has been confirmed by PCR in a healthy dog from the southern part of the country (Gabrielli et al., 2015); however, hepatozoonosis in domestic dogs has not been confirmed so far. The high prevalence of *H. canis* in foxes observed in this study, and the presence of the main vector *R. sanguineus* s.l. which is a mutual ectoparasite of dogs and foxes, suggest the possibility that *H. canis* could also be expected to occur with a higher prevalence in dogs in Serbia. Taking into account the current epizootiological situation regarding *H. canis*, and the fact that *H. canis* is often present in co-infection in ticks with other canine vector-borne pathogens that cause similar clinical signs, veterinarians should pay particular attention to dogs with signs such as fever, anaemia, cachexia, and lethargy which should raise the suspicion of autochthonous canine hepatozoonosis.

In our study, the DNA of *B. canis* was detected only in a single fox (1/129, 0.8%) shot at the Surčin locality. To the best of our knowledge, this is the third molecular confirmation of the presence of *B. canis* in a population of red foxes so far worldwide (Cardoso et al., 2013; Hodžić et al., 2015). Recently, the presence of *B. canis* (4.2%) has been confirmed in golden jackals in Serbia. Moreover, *D. reticulatus*, the main vector of *B. canis*, was found to parasitise foxes and jackals in Serbia (Tomanović et al., 2013; Sukara et al., 2018). Since the natural habitats of red foxes and golden jackals overlap (Penezić and Ćirović, 2015), their mutual exposure to the ectoparasite fauna is highly probable. This hypothesis could explain the presence of *B. canis* in a fox in Serbia. However, further studies are needed to elucidate whether foxes have a relevant role in the epizootiology of *B. canis* or whether the molecular confirmation of its presence was a coincidence.

A relatively high prevalence of *B. vulpes* (37/129, 28.7%) was confirmed among the tested foxes in the present study. Previous research efforts have shown that *B. vulpes* is commonly found in fox populations in Europe (Duscher et al., 2014; Checa et al., 2018). Also, different research groups have reported a relatively high prevalence of *B. vulpes* in foxes from neighbouring countries, e.g. Croatia (5.2%; Deždek et al., 2010), Bosnia and Herzegovina (31.9%; Hodžić et al., 2015), Hungary (20%; Farkas et al., 2015), and Romania (20.1%; Daskalaki et al., 2018). Although high prevalence of this pathogen has been detected in foxes across Europe, only one clinical case of the disease caused by *B. vulpes* has been described so far in a fox from Canada (Clancey et al., 2010), indicating the role of foxes as a reservoir for *B. vulpes.*

The geographical distribution of positive samples obtained in the present study indicates that *B. vulpes* is widespread among the fox populations in Serbia and potential vectors of *B. vulpes* (i.e., *I. hexagonus*, *I. canisuga*, *I. ricinus*, *D.*

reticulatus) are found to parasitise foxes in that country (Tomanović et al., 2013). Although the DNA of *B. vulpes* has been confirmed by PCR only in clinically healthy dogs from Serbia (Gabrielli et al., 2015), the pathogenicity of *B. vulpes* in dogs has been previously confirmed (Solano-Gallego et al. 2016).

Thus, canine babesiosis caused by *B. vulpes* may be a fact in Serbia. Since the treatment of canine babesiosis caused by *B. vulpes* (Baneth, 2018) is different from that used against the most frequent causative pathogen (*B. canis*) of dog babesiosis in Serbia (Davitkov et al., 2015), we alert veterinarians to the need of identifying the pathogenic species accurately (Solano-Gallego et al., 2016).

In conclusion, the present study is the first report on the occurrence of *B. canis*, *B. vulpes*, and *H. canis* in red foxes from the Republic of Serbia. Our findings indicate the importance of foxes in the epizootiology of these blood protozoans and their role as reservoirs. The fact that very often the same species of *Babesia* and *Hepatozoon* infect dogs and wild canids, the diseases caused by these pathogens in dogs in Serbia could dangerously emerge. Further research efforts are needed to identify the tick species that are competent vectors of *H. canis* and *B. vulpes*, and to clarify the exact role of other autochthonous wild canids (wolves and jackals) in the epizootiology of these protozoan parasites.

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